Editorial

The Editors of Food Microstructure are pleased to announce the appointment of Dr. I. Heertje of Unilever Research Laboratory in Vlaardingen, The Netherlands as an additional editor of the journal. He will represent the journal in Europe where great progress has been achieved in all aspects of food structure.

Dr. Heertje's interests in food structure are very wide and include the studies of fats, oils, proteins, and milk products. He has introduced modern techniques into structural studies of foods, for example, confocal scanning laser microscopy (Food Microstructure, 6(2), 115, 1987) and immunoelectron microscopy (presented at the 1989 Food Microstructure meeting in Salt Lake City. Dr. Heertje has provided enthusiastic support to the publication as well as the conference activities of Food Microstructure for past several years. His appointment has been made in order make the contact of European authors and other contributors with Food Microstructure more convenient and to provide them with an easy-to-reach assistance. Manuscripts originating in Europe may now be sent directly to Dr. I. Heertje, Unilever Research Laboratory, P.O. Box 114, 3130 Ac Vlaardingen, The Netherlands. The new editor may also be contacted by telephone at 31-10-450 5513, and / or facsimile at the number 31-10-460 5800.

Milos Kalab, on behalf of The Editors
An International Conference on

FOOD MICROSTRUCTURE

will be held at the Hyatt Regency Hotel in Bethesda, Maryland (just outside of Washington DC) during the Scanning Microscopy 1990 meeting scheduled May 5 to 10, 1989. Scanning Microscopy International is the sponsor of this event.

The aim of the meeting is to present latest advances in the study of the structure (as determined by optical and electron microscopies, microanalysis, and other relevant instrumental methods) of foods, feeds, and their ingredients, with special emphasis on relations between processing, molecular properties, microstructure, and macroscopic behavior. The sessions currently planned and their organizers are:

Tutorials on techniques to study food structure: D.N. Holcomb, Glenview, IL; C.M. Lee, Kingston, RI
New techniques for studying food structure: S.H. Cohen, Natick, MA; V.E. Colombo, Switzerland; and D.F. Lewis, U.K.
Meats and meat products: R.G. Cassens, Madison, WI; and H.J. Swatland, Guelph, Canada
Milk and dairy products: B.E. Brooker, U.K.; and M. Kalab, Ottawa, Canada
Plant foods (cereals, fruits and vegetables etc.): W.J. Wolf, Peoria, IL; S.H. Yiu, Ottawa, Canada; and D.J. Gallant, France
Fats and oils: I. Heertje, Netherlands; K. Sato, Japan; and A.E. Waltking, Union, NJ
Food contamination and packaging: J.E. Charbonneau, Washington, DC

Papers can be offered for oral presentation at Food Microstructure meeting or for publication in FOOD MICROSTRUCTURE or both. A Letter of Intent form (see page ii) is to be completed only when offering a paper for oral presentation. A brief summary of up to 100 words must be provided. The deadline for submission of the Letter of Intent is March 5, 1989. Papers for publication may be submitted at any time.

The following types of papers may be offered:

A. Original research papers which present new unpublished findings as in a paper or note submitted to any scientific journal.
B. Review papers which contain an extended literature review and bibliography (including author's own work), emphasize author's unpublished findings and in an extended discussion put the topic of review in proper perspective.
C. Tutorial papers which present the topic in a teaching manner and contain an organized comprehensive review and bibliography of all relevant published materials.

Original research papers are usually presented within a 20 min time period but longer times may be assigned for the presentation of review and tutorial papers. Please indicate your desired time for the presentation on the Letter of Intent form.

AUTHORS MUST NOT COMMIT THEMSELVES UNLESS THEY ARE CONFIDENT THAT THEY CAN MAKE THE PRESENTATION. In case of an unexpected situation, authors are requested to write as soon as possible if they are withdrawing their participation at the meeting.

Authors of oral presentations made at the meetings are invited to publish their papers in FOOD MICROSTRUCTURE. They may submit complete papers for publication, per the Instructions for Authors included in this issue, before or after the meeting.

TRAVEL SUPPORT FOR FOOD MICROSTRUCTURE MEETINGS

Authors offering papers for oral presentation may apply for travel support by attaching a separate application (to the Letter of Intent form) indicating the extent of subsidy desired (limited to $300 for travel within North America and $500 from elsewhere). The decision to support the travel is made in consultation with the organizers of the specific programs following established guidelines. This support can only be considered for speakers submitting their complete papers for publication in Food Microstructure. Please apply as soon as possible. Each applicant will be notified in writing and awarded funds will be provided only after the paper has been accepted for publication (following the receipt of the revised paper after reviewing).

Please send Letters of Intent as well as papers to:

FOOD MICROSTRUCTURE, c/o Scanning Microscopy International, P.O. Box 66507, AMF O'Hare (Chicago), IL 60668-0507, U.S.A. (phone: 312 529 6677; FAX 312 980 6698).
ELECTRON MICROSCOPIC LOCALIZATION OF CHOLESTEROL IN BOVINE MILK FAT GLOBULES

Abstract

An electron microscopic method designed for the detection of cholesterol in milk fat was evaluated for reliability. This method is based on the incubation of cream from raw milk with filipin (a polyene antibiotic) which has a specific affinity for cholesterol followed by freeze fracturing and electron microscopic examination of fat globules. Cholesterol was localized within the membrane and the triglyceride core of milk fat globules. Cholesterol was highly organized within membrane portions and less organized within triglyceride portions of milk fat globules. The configuration of localized cholesterol was similar to configurations reported for plasma membranes.

Introduction

The origin of cholesterol in milk products is partially from milk serum and to a greater extent from the milk fat globule (MFG). Bovine MFGs are about 1% protein and 99% lipid of which 96-99% is triacylglycerol (Timmen and Patton, 1988). MFGs possess a central core of lipid surrounded by a thin protein inner coat and enclosed by the outer milk fat globule membrane bilayer (Mather, et al., 1977; Freundenstein, et al., 1979; Franke, et al., 1981; Keenan, et al. 1982; Buchheim, 1982, 1986). There currently exist no reports of cholesterol localization in milk fat globules using electron microscopic techniques.

Cholesterol is a major constituent of animal plasma membranes, plasma lipoproteins, and animal fat. Several recent studies have utilized electron microscopic techniques to localize cholesterol in animal cells and tissues (Elias, et al., 1979; Andrews and Cohen, 1979; Friend and Bearer, 1981; Behnke, et al., 1984; Miller, 1984; Harris, 1988). A technique used in cellular localization of cholesterol using electron microscopy has been freeze fracture following incubation with filipin. Filipin is a polyene antibiotic that has a specific affinity to the 6-hydroxysterol molecule. The specific binding of cholesterol with filipin creates a complex that is observable by freeze fracture electron microscopy and enables localization information.

There exist several hypotheses for the appearance of filipin/cholesterol complexes within biological membranes. Dekruyff and Demel (1974) suggested that a polymer with equal molar fractions of filipin and cholesterol was formed in the hydrophobic core of the membrane bilayer. Kitajima, et al. (1976) proposed that the location of the filipin/cholesterol complexes may not specify the exact location of cholesterol. Filipin was assumed to reorient cholesterol from a vertical orientation in the bilayer to a horizontal position at the interfaces of the hydrophobic core with inter- and extracellular environments. Elias, et al. (1979) theorized that filipin induced membrane buckling could be caused by the formation of two groups of four filipin/cholesterol complexes that would account for hemispherical as well as circular complexes.

Miller (1984) used the published diffusion coefficients of cholesterol and calculated that the binding of cholesterol and filipin occurred in...
less than 10 sec. He suggested a resolution of localization ranging from 200 nm to 4 \mu m. Miller (1986) further suggested that filipin may serve as a nucleation site for non-membrane cholesterol, thus yielding polymorphic images when viewed with freeze fracture.

Behnke, et al. (1984) stated that reports of membrane perturbation or fragmentation referring to the physico-chemical effects of filipin may mislead researchers to believe that filipin destroys plasma membranes. Their studies of red blood cells showed that approximately 10% of the cholesterol of the total cell mass was removed during filipin incubation. They further showed that cholesterol-rich microdomains are dependent on filipin concentrations and the number of cells present in the system as well as on the extent of membrane shedding.

The purpose of the present investigation is to evaluate the feasibility of freeze fracture techniques to localize cholesterol in bovine milk fat globules and compare the results with existing studies regarding biological membranes. The data will be used to theorize possible configurations of cholesterol within MFGs.

Materials and Methods

Raw milk, from Holstein cattle, was received within 24 h after expression and cooled to 4°C in a bulk holding tank. Raw milk samples (100 g) were placed in centrifuge tubes and centrifuged at 1900 rpm for 5 min. on a Clay Adams #0011 centrifuge. Experimental cream samples (35 - 0.25 ml replicates) were removed and placed in separate depressions of ceramic spot plates with 0.25 ml filipin solution (10 ng-filipin-Sigma/1.0 ml dimethylformamide) and incubated at 4°C for 1.0 h. Control samples (0.25 ml cream) were incubated at 40°C in 1.0 ml dimethylformamide. Samples were placed in Balzers specimen supports, cryofixed in Freon 22 cooled in liquid nitrogen, and coated with 20 nm platinum. The fracture plane just below the protein inner coat and clearly within the triglyceride core. The spatial arrangement was identical to the arrangement observed in the MFGs.

Figure 6 shows an experimental cream replica with patches of MFGM over the triglyceride core of a MFG. The spatial arrangement of filipin/cholesterol complexes in similar to those presented earlier. Figure 7 represents a fracture through a similarly treated MFG sample. The fracture plane was just below the protein inner coat and clearly within the triglyceride core. The spatial arrangement was identical to the arrangement observed in the MFGs. Figure 8 shows filipin/cholesterol complexes localized within the triglyceride core of experimental MFGs. Complexes within these vicinityes were arranged in a less organized manner.

Discussion

Although there are pros and cons to the use of filipin as a cholesterol probe many researchers from different laboratories have obtained very similar results with variations of this technique. Miller (1984) points out that the consistency between individual researchers utilizing this technique cannot be ignored and may signify properties of plasma membranes not yet understood. The following discussion should be weighed carefully due to the controversy that exists using filipin methods.

It is difficult to compare existing accounts of cholesterol localization in cells and tissues to the present investigation concerning bovine MFGs. This is due to the origin of the MFG. It is generally accepted that milk fat is expressed via the Golgi apparatus of secretory cells in mammary epithelia (Mather and Keenan, 1983; Wooding, 1971). The complex secretion mechanism results in the milk fat being surrounded by protein components (inner protein coat) as well as the outer MPCM (Keenan and Dylewski, 1985; Keenan et al., 1982). This is in contrast with most plasma membranes that can be replenished by organelles found in living cells.

The highly organized pattern of filipin/cholesterol complexes within MFGM are strikingly similar to reports for plasma membranes of animal cells and tissues (Ilia, et al., 1979; Friend and Bearer, 1981; Behnke, et al., 1984; Andrews and Cohen, 1979). The main morphological difference between the filipin/cholesterol pattern in MFGMs and animal cells is the higher density of filipin/cholesterol complexes within plasma membranes of living cells. This is not unexpected due to the origin of MFGMs. The cholesterol configuration observed within MFGMs is possibly similar to the configuration within the apical plasma membrane of the mammary secretory cell or the secretory vesicles.

The difference in cholesterol localization from the triglyceride core to the outer membrane suggests an organization of cholesterol in MFGMs.
**Cholesterol localization**

**Figure 1.** Freeze fracture replica of control milk fat globule incubated at 40°C. The triglyceride (tg) exhibits solid (S) and liquid (L) regions within the milk fat. Scale bar equals 0.5 μm.

**Figure 2.** Freeze fracture replica of control milk fat globule with fracture plane through outer membrane (me). Note smooth appearance of membrane. Triglyceride (tg). Scale bar equals 0.5 μm.

**Figure 3.** Freeze fracture replica of milk fat globule incubated with filipin. The depressions within the outer membrane represent filipin/cholesterol complexes (fc). Casein micelles (m) are apparent on the outer membrane. Scale bar equals 0.2 μm.
It is inviting to propose that cholesterol has a random arrangement within the lipid core and becomes highly organized in the surrounding membrane. However, the random nature of fracture planes through triglyceride regions makes it difficult if not impossible to visualize the arrangement of filipin/cholesterol complexes. The distance between complexes within the core is such that a judgment concerning the looseness of the complexes can be made. Timmen and Patton (1988) state that bovine triglyceride is primarily liquid at bovine body temperature. Since milk samples were incubated at \(40^\circ C\), a majority of the milk fat was considered liquid. This, in turn, increases molecular motion and may increase the probability of a random arrangement of cholesterol within the core. Hence, filipin/cholesterol complexes of the triglyceride core appear to be less organized than those observed in the surrounding membrane. The closer the complexes to the inner coat and outer membrane the more organized they become. This is supported by the data shown in Figures 6 and 7 when compared to Figure 8.

In conclusion, it is apparent that cholesterol within MFGMs has a very different arrangement from cholesterol within the triglyceride core. Immunocytochemical localization with TEM could improve localization resolution and may contribute a quantitative microscopic method for measuring the amount of cholesterol in milk fat.

Acknowledgments

The author would like to thank Drs. Allen Blaurock and Shu-Guang Cheng for their help in understanding the physical and chemical properties of cholesterol in milk fat. Mr. Roger S. Unger for his photographic assistance and Ms. Samantha Verzek for her word processing expertise.

References


Cholesterol localization

Figure 5. Freeze fracture replica of filipin incubated milk fat with fracture plane within triglyceride (tg). Filipin/cholesterol complexes (fc). Note arrays of crystalline lipid at small arrowheads. Scale bar equals 0.5µm.

Figure 6. Freeze fracture replica of filipin incubated milk fat with fracture plane revealing membrane patches (me) adhering to triglyceride (tg). Note pattern of filipin/cholesterol complexes (fc). Scale bar equals 0.5µm.

Figure 7. Freeze fracture replica near outer membrane region of filipin incubated milk fat globule. Note pattern of filipin/cholesterol complexes (fc) embedded within the triglyceride (tg). Scale bar equals 0.1µm.

Figure 8. Freeze fracture replica of filipin incubated milk fat globule showing arrangement of filipin/cholesterol complexes (fc) within triglyceride (tg). Scale bar equals 0.2µm.


Freudenstein C, Keenan TW, Eigel WN, Sasaki M,


Discussion with Reviewers

B E Brooker: In several figures of both control and experimental samples, casein micelles are present at the surface of globules. Is this an effect of the DMF? If so, is it not possible that there are changes induced in the MFGM by this treatment that affect the nature and/or distribution of filipin/cholesterol complexes?

R.G. Miller: As I understand it, experimental samples (i.e. filipin-treated) contained 50% DMF as a cryoprotectant, whereas control samples contained 80% DMF. Is there a reason for this difference in DMF treatment? Since a portion of the MFG sample is non-aqueous, the final concentration of DMF within the aqueous phase might be substantially higher than these values. Do you feel that incubation in this high concentration of an organic solvent have an effect upon the morphology of the MFG? Might this have an effect on filipin cholesterol binding?

Author: It is certainly possible DMF may cause a biochemical perturbation not detectable by freeze fracture methodology. DMF was used primarily to solubilize filipin not as a cryoprotectant for freeze fracture methodology. The difference in DMF concentration between control and experimental treatments was used to determine possible artefacts caused by the DMF treatments. It is very important to confirm the data regarding the behavior of cholesterol with filipin using other methods such as computer imaging or immunocytochemistry. Work in progress shows the presence of milk protein components on the surface of MFGs a common feature of raw milk from bulk storage. Of course a quantification of MFG surface protein from bulk storage versus DMF treatments may indicate characteristics of solvent treatments pertinent to the adherence of milk proteins and other possible artefacts.

B E Brooker: Filipin/cholesterol complexes appear at the end of the focus of several 'triglyceride arrays'. What is the significance of this clear spatial relationship?

Author: There are several possible interpretations for the location of filipin/cholesterol complexes at the focus of crystalline lipid arrays. The configuration may be indicative of cholesterol associated lipids that are highly organized with milk fat. Another possibility is that these are fixation artefacts introduced by too low freezing velocities. These possibilities are purely speculative and should be studied by controlled experimentation.

M Kalab: In this paper, the localization of cholesterol in the fat globule membrane is based on the assumption that cholesterol is present in the membrane and reacts with filipin. Evidently, it would be useful to confirm the findings using an experimental system under controlled conditions. Such a system would consist of an oil/water emulsion which would contain protein and in which the concentration of cholesterol would be controlled. Was an attempt made to study such a system?

I Heerdtje: Have controlled experiments with filipin ever been performed on membrane systems containing no cholesterol?

Author: The idea of using model emulsion systems to confirm the binding of filipin and cholesterol is a good one. Preliminary results from freeze fracture studies of synthetic liposomes exhibit similar results as presented in the current paper.

I Heerdtje: May the filipin technique be considered a localization technique in the strict sense, considering the mobility of cholesterol and the aggregation of filipin?

D Holcomb: It is thought that filipin causes reorientation of the cholesterol. Could the author give more explanation of why this is not a serious problem in this work?

Author: Due to the apparent mobility of cholesterol, filipin localization with freeze fracture methodology should be used only as a qualitative measure in conjunction with biochemical analyses. Hence, the presence of cholesterol would first be determined by freeze fracture and then confirmed by further analyses.

I Heerdtje: Are proper antibodies available for localization of cholesterol by immuno EM? If so, are such studies envisaged?
R.G. Miller: Indeed, there are many problems with localization of a mobile substance such as cholesterol. The solution which you propose in the last paragraph is raising antibodies which are specific to cholesterol. First, I don’t see that the use of antibodies is going to help in localization of highly mobile species such as cholesterol. Do you feel that an antibody probe would fare any better than filipin?

Secondly, in order to make such an antibody, an antibody-producing animal must be found which does not have a substantial amount of endogenous cholesterol. Have you found such a beast?

Author: Currently there are no commercial antibodies available for cholesterol localization using immuno EM. Immuno fracture labeling using such an antibody would be very important to our overall understanding of cholesterol localization in membranes. The advantage to antibody labeling is that cholesterol may not be reoriented by the antibody during binding as has been proposed for filipin/cholesterol binding. A number of laboratories are presently attempting to produce polyclonal and monoclonal antibodies to cholesterol.

I Heertje: Do other sterols present in milk give rise to the same reaction?
Author: To my knowledge the filipin molecule reacts only with 5-hydroxyysterol (cholesterol) which composes more than 99% of the sterols in milk fat.

D Holcomb: How do you know where the fracture plane is? Maybe the author could explain how those locations were determined?
Author: The interpretation of fracture planes through membranes and triglyceride portions of MFGs are based upon repeatable morphological characteristics found during the course of this study, relevant published micrographs, and relevant theories of freeze fracture methodology.

R.G. Miller: Although many have used filipin in order to provide some information concerning the localization of cholesterol in membranes, to my knowledge, this is the first report which strives to localize cholesterol in the bulk phase of a liquid. A priori, there’s no reason to expect that filipin cholesterol complexes in bulk phase should have any morphological resemblance to a filipin cholesterol complex in a bilayer membrane. How sure are you that you are indeed picking up filipin cholesterol complexes within the tri-acyl glyceride core of the milk fat globule? What is the measured concentration of cholesterol in the milk fat globule membrane compared to the concentration within the tri-acyl glyceride core? does this correlate in any way with the number of filipin cholesterol complexes that are in the two regions?

Author: The morphological characteristics of filipin cholesterol complexes in membranes versus triglyceride regions are strikingly different. There really is no reason to believe they would be the same based upon the structural differences of the two regions. Work in progress on aqueous systems shows the filipin cholesterol complexes may take on a variety of morphological characteristics dependant upon the nature of the system. Hence, I am relatively confident that the structures isolated within triglyceride regions of MFGs are indeed filipin cholesterol complexes. The concentration of cholesterol in MFGs is approximately 2.5 mg/g fat. The total cholesterol within the core ranges between 97-83% and 1.5-7.8% in the membrane portion dependant upon the season the milk was collected. It is difficult to correlate the number of filipin cholesterol complexes detected by freeze fracture to these concentration values as the current method is strictly a qualitative measure.
Lipid vesicles have become of considerable importance as model membranes and drug delivery systems. Recently, applications in the food industry have been suggested for microencapsulation and immobilization of enzymes.

A number of methods for the preparation and characterization of liposomes have long been available. For the production of small unilamellar vesicles we have used a microfluidization technique. Microfluidization is based on a submerged jet principle in which two fluidized streams collide at extremely high velocities in a precisely designed interaction chamber.

Advantages of this technique include the absence of organic solvents or detergents, the high lipid concentrations that can be employed and the high encapsulation efficiencies that can be achieved.

Electron photomicrography was used to characterize the liposome preparation. Laser light scattering spectrometry proved to be the most reliable method for determination of mean size and size distribution of small unilamellar vesicles.

**Materials and Methods**

23 g of purified lecithin (OVOTHIN 170, Lucas Meyer, Hamburg, Germany) was dispersed in 200 ml of buffer K-phosphate solution 0.025 M at pH 6.88. The solution corresponded to about 150 μmol phospholipids per ml of solution. The dispersion was heated to 30°C and stirred for 1 hour under a light stream of nitrogen. The hydrated lipids were then passed through the Microfluidizer. Fig. 1 shows a schematic representation of the Microfluidizer M110 which was used (Microfluidics, Newton, MA, USA).

The crude suspension of phospholipids was placed in the reservoir and the air regulator adjusted to the selected operating pressure (860 bars). With such a setting, when the air valve is open, the liquid dispersion flows through a filter (5 μm) into the interaction chamber where it is separated into two streams which interact at extremely high velocities in
dimensionally defined microchannels. The suspension can be recycled through the machine and, in this eventuality, the suspension must be cooled because of the temperature increase in the interaction chamber at high operating pressure. Flow rates in the order of 100 ml/min were employed, and volumes of 200 ml were processed.

A Malvern Photo Correlation Spectrometer (Malvern Instruments) was used to measure the mean size and the size distribution of the liposomes by light scattering. The spectrometer was equipped with a 64-log-channels Malvern Autocorrelator 7032 and a Spectra-Physics 15 mw He-Ne laser (wavelength 632.8 nm).

Freeze-fracture electron microscope photographs of liposomes were obtained by use of a Cryofract (Reichert-Jung, France) and an electron microscope EM 300 (Philips, Holland).

Characterization of Liposomes

Vesicle formation can be modified by varying the pressure or the number of passes through the interaction chamber. Fig. 2 shows the reduction of the mean size of liposomes during successive passes through the Microfluidizer.

Conjointly with the size reduction, a progressive decrease of the spread of the size distribution which corresponds - in Fig. 2 - to the contraction of the bars magnitude as a function of the number of passes, was observed.

Increasing the number of microfluidization cycles beyond 20-25 did not result in any further reduction in liposome size.

Fig. 3 shows a typical liposome size distribution obtained after the 20th pass in the Microfluidizer. This distribution was obtained from light scattering data. The mean size of the particles was 31 nm and about 86% of the liposomes population was situated in the range 5 nm - 50 nm.

A common example of electron micrographs of liposomes produced in this way is shown in Fig. 4. These images illustrate the smooth spherical shape of the liposomes obtained by microfluidization, and the trapped volume compartment. The smooth fracture faces are representative.
Fig. 4. Freeze-fracture electron micrographs.
of the entire field, and the absence of multistep surfaces unambiguously demonstrates the unilamellar nature of the liposomes. Moreover, the vesicles are seen to be fairly homogeneous in size. This confirms the results obtained by light scattering. The few larger vesicles, and also the material which does not produce vesicles, can easily be removed by centrifugation or by gel chromatography.

Discussion

Microfluidization provides a practical and convenient means of preparing research or commercial quantities of small unilamellar vesicles. According to our own experience, the best characterized unilamellar liposomes were obtained by microfluidization of aqueous suspensions of egg phospholipids. As confirmed by light scattering measurements, the main advantages of the use of Microfluidizer technique are the uniformity of the size distribution and that the liposomes formed are smaller than the smallest unilamellar vesicles prepared by more conventional means such as sonication.

The composition of lipids may have great importance on the size of the unilamellar vesicles that can be produced (Mayhew et al., 1984). However, the Microfluidizer can operate at considerably higher lipid concentrations than are possible with other techniques.

The Microfluidizer process does not involve the use of organic solvents and the liposomes can be prepared by a continuous process rather than a batch process normally required for other preparations.

Electron microscope pictures of the vesicles obtained by microfluidization show smooth spherical shapes and give clear evidence of the unilamellarity of the liposomes.

References


Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.
SOLUBLE AND INSOLUBLE DIETARY FIBER IN COOKED COMMON BEAN (PHASEOLUS VULGARIS) SEEDS

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Abstract

The common bean (Phaseolus vulgaris) requires cooking for extended periods of time prior to consumption. In this investigation both quantitative and microstructural changes in common bean dietary fiber as a result of cooking were examined. Cooking resulted in a slight decrease in soluble dietary fiber and a marked increase in insoluble dietary fiber. The increase in insoluble dietary fiber was responsible for a 15-30 percent increase in total dietary fiber.

Scanning electron microscopy was used to examine the microstructure of uncooked and cooked bean flours and the insoluble and soluble dietary fiber fractions of these two flours. In uncooked whole bean flour large (10-30 μm) spherical starch granules and small (1-5 μm) protein bodies characteristic of the common bean were observed. However, after cooking, only amorphous material containing gelatinized starch and denatured proteins was visible.

Few microstructural differences were observed between uncooked and cooked insoluble dietary fiber fractions. Both fractions consisted primarily of cell wall remnants from which starch and protein storage bodies had been removed. Also present in both insoluble fiber fractions were partially digested fragments of the seed coat palisade cell layer, and long, thin fibers which appear to be remnants of the nutrient transporting phloem. The cooked and uncooked soluble dietary fiber fractions were microstructurally similar consisting of thin, irregularly shaped sheets and long, thin rods.

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Key words: common bean, insoluble dietary fiber, soluble dietary fiber, total dietary fiber, scanning electron microscopy, seed microstructure, autoclaving, cell walls, seed coat, palisade cells, phloem.

Introduction

The importance of dietary fiber in human nutrition has received a great deal of attention in recent years from scientists and consumers alike (Olson et al., 1987). Numerous health benefits have been associated with consuming adequate amounts of dietary fiber including lower blood cholesterol, reduced risk of heart disease, increased fecal bulk, decreased intestinal transit time, reduced risk of colon cancer, and improved glucose tolerance which is especially beneficial for diabetics (Schneeman, 1986; Toma and Curtis, 1986). Special interest has been focused on common bean dietary fiber because of its high content of metabolically active soluble dietary fiber and its effectiveness in lowering blood cholesterol (Anderson et al., 1984). Fig. 1 shows the insoluble and soluble dietary fiber profiles of the common bean and several commonly consumed cereal products (Dreher, 1987).

Although substantial research has been done on the chemical composition of dietary fiber, considerably less is known about the structural characteristics of dietary fiber. Scanning electron microscopy (SEM) has been used previously to examine the structure of dietary fiber, but this research has focused almost exclusively on insoluble dietary fiber. Insoluble dietary fiber from wheat (Mares and Stone, 1973), wheat bran (Moss and Mugford, 1986; Saunders et al., 1972), rice (Shibuya et al., 1985), corn and soy hull bran (Dintzis et al., 1979), and oat bran (Cadden, 1987) have all been examined with SEM. In contrast, little is known about the structural characteristics of naturally occurring soluble dietary fiber is lacking. Also, with the exception of some work on soybeans, research on the structural characteristics of dietary fiber has focused primarily on cereals rather than legumes (Wisler et al., 1985). Legume seeds typically contain more dietary fiber than cereals and are a better source of metabolically active soluble dietary fiber.

One area of current research interest is the effect of various forms of processing, including thermal processing, on the dietary fiber content of foods. The effect of various types of cooking on the dietary fiber content of wheat flour (Björck et al., 1984; Siljestrom et al., 1986; Varo et al., 1983), whole wheat flour (Björck et al., 1984; Varo et al., 1983), whole grain wheat (Siljestrom et al. 1986), and potatoes (Dreher et al., 1983; Varo et al., 1983) has been reported. Varo et al. (1983) reported the results of an interlaboratory study where six
different laboratories analyzed wheat flour, and whole wheat flour which received one of three treatments: no
cooking, normal cooking and cooking under severe
conditions. The dietary fiber content of potatoes which
were boiled, pressure cooked or French fried was also
studied (Varo et al., 1983). Several laboratories
participating in the study reported that total dietary fiber
in both wheat and whole wheat flour increased as a result
of cooking under severe conditions, while other
laboratories reported decreases in total dietary fiber
using the same samples and cooking conditions. Varo et
al. (1983) concluded that measuring changes in the
dietary fiber content of foods during cooking is
complicated by variability between different analytical
methods. Bjoerck et al. (1984) reported significant
increases in the total dietary fiber content of extruded
whole wheat flour as well as slight increases in the total
dietary fiber content of wheat flour when it was extruded
under severe conditions. Additionally, a redistribution
of insoluble to soluble dietary fiber was observed by
Bjoerck et al. (1984) in all extruded wheat flour samples.
Sjilström et al. (1986) examined changes in dietary fiber
and starch in white wheat flour that had been drum-dried
or extruded and whole grain wheat which had been
autoclaved, popped or steam flaked. Significant changes
in total dietary fiber as a result of cooking were observed
only in heat treated whole grain wheat. The total dietary
fiber content of whole grain wheat decreased when the
grains were either extruded or popped under severe
conditions (Sjilström et al., 1986).

Both Varo et al. (1983) and Dreher et al. (1983)
examined the effects of different cooking processes on
potato dietary fiber. Total dietary fiber increased in
potatoes with all forms of cooking investigated including
baking, boiling, pressure cooking and the commercial
preparation of French fries and potato chips. Soluble
dietary fiber content in potatoes was largely unaffected by
cooking, with the increase in total dietary fiber resulting
from increases in insoluble dietary fiber (Varo et al., 1983;
Dreher et al., 1983).

No data are available on the effect of cooking on
bean dietary fiber. Cooking is particularly important in
the preparation of beans because they contain several
heat labile antinutrients (Lien er, 1962) and slow swelling
starch granules (Thorne et al., 1983; Wursch et al., 1986).

Cooking times longer than those needed for cereals are
generally required by beans in order to inactive the heat
labile antinutrients and allow for adequate swelling of the
starch.

The purpose of this research was to measure
changes in common bean dietary fiber content with
cooking and to use SEM to observe microstructural
changes in the insoluble and soluble dietary fiber
fractions as a result of cooking.

Materials and Methods

Common bean (Phaseolus vulgaris) seeds with a
black seed coat (cv. Tamazulapa) were obtained directly
from the producer in the State (Department) of Jutiapa,
Guatemala shortly after the 1988 harvest. White beans
from the 1988 harvest were purchased shortly after
harvest from a seed warehouse in Guatemala City,
Guatemala. Both black and white beans were analyzed
for changes in dietary fiber content as a result of cooking.
However, only black beans were examined with SEM.

Beans were cooked in water (1:3) by autoclaving for 20
minutes at 15 psi and 121°C, and dried overnight in a
circulating air oven at 60-70°C. Bean flours were
obtained by separately milling uncooked and cooked
beans to pass through a 60 mesh screen. The insoluble
and soluble dietary fiber content of the resulting flours
was determined using the procedure of Asp et al. (1983).

Figs. 3 & 4. Microstructure of whole bean flours. Fig. 3 shows uncooked whole bean flour with both starch
granules (S) and protein bodies (P) being present. Fig. 4
shows autoclaved white bean flours in which the starch
granules and protein bodies are no longer distinguishable.
Fig. 3, bar = 20 μm; Fig. 4, bar = 50 μm.

Figs. 5-8. Microstructure of uncooked and cooked
common bean insoluble dietary fiber. Fig. 5 shows the
cell wall remnants typically found in both cooked and
uncooked insoluble dietary fiber. Figs. 6, 7 & 8 show the
seed coat and palisade cell layers present in the insoluble
dietary fiber fraction. Fig. 6 shows an uncooked seed coat
which remains largely intact, Fig. 7 shows a cooked
palisade cell layer and Fig. 8 shows the interior or lower
surface of a cooked palisade cell layer. Figs. 5 and 6,
bar = 50 μm; Figs. 7 and 8, bar = 10 μm.
Soluble and Insoluble Common Bean Dietary Fiber
Bean flour was initially gelatinized with termamyl (100 °C, 20 min), a heat stable alpha-amylose, and then digested with pepsin (40 °C, 60 min) and pancreatin (40 °C, 60 min) to remove protein and starch (Prosky et al., 1984). The digestion mixture was filtered to obtain insoluble dietary fiber. Four volumes of 95% ethanol at 60 °C was added to the supernatant to precipitate soluble dietary fiber. After one hour soluble dietary fiber was also separated by filtration.

Black bean flour for analysis by SEM was obtained by modification of the procedure of Asp et al (1983). Insoluble and soluble dietary fiber residues for viewing by SEM were obtained by centrifugation instead of filtration. Insoluble dietary fiber was separated by centrifuging for 30 min at 4000 rpm and freeze dried. Soluble dietary fiber was obtained by centrifuging for 40 minutes at 4000 rpm after ethanol precipitation. Precipitated soluble dietary fiber was initially dried overnight in a vacuum oven at 20 °C to remove residual ethanol, and redissolved in distilled water and freeze dried. Dried whole bean flour, and insoluble and soluble dietary fiber samples for examination by SEM were mounted on aluminum stubs and sputter coated with gold (Hummer-Technics). All samples were viewed and photographed at 20 kV with a Hitachi S-570 Scanning Electron Microscope.

Results and Discussion

Cooking and dietary fiber content.

The effect of cooking on the soluble, insoluble and total dietary fiber content of the two bean cultivars examined is shown in Fig. 2. In both black and white beans, cooking resulted in a slight decrease in soluble dietary fiber and a marked increase in insoluble dietary fiber. The increase in insoluble fiber resulted in an increase in total dietary fiber of approximately 15 percent in white beans and 30 percent in black beans (Fig. 2). Other researchers looking at the effect of thermal processing on changes in dietary fiber content of wheat and potatoes have reported either no change (Varo et al., 1983) or increases in insoluble fiber with no change in soluble dietary fiber (Björck et al., 1984; Dreher et al., 1983; Varo et al., 1983). Changes in common bean dietary fiber with cooking most closely resemble changes observed previously in potatoes where insoluble dietary fiber increased and soluble fiber remained largely unchanged regardless of the type of cooking (Dreher et al., 1983; Varo et al., 1983).

The chemical basis for changes in the dietary fiber content of foods during cooking remain unclear. The formation of resistant starch (Björck et al., 1986), amylose-lipid complexes and Maillard-reaction products (Björck et al., 1984) have been hypothesized as contributing to observed increases in dietary fiber. However, amylose-lipid complexes appear to be digestible in vivo (Holm et al., 1983), and in a low lipid food like the common bean, formation of amylose-lipid complexes is not likely to contribute significantly to changes in dietary fiber as a result of cooking. Additional research on the formation of resistant starch and Maillard-reaction products during cooking is needed to determine their contribution to changes in dietary fiber content.

Microstructure of whole bean flour.

Structural differences between uncooked and cooked common bean flours are shown in Figs. 3 and 4. Present in the uncooked flour are large (10-30 μm), spherical starch granules and smaller (1-5 μm) protein bodies similar to those previously observed in intact seeds (Hughes and Swanson, 1985; Swanson et al., 1985). In the cooked flour, starch granules have been gelatinized and protein bodies denatured leaving primarily amorphous material that is irregular in size and shape (Fig. 4).

Microstructure of insoluble dietary fiber.

The microstructure of cooked and uncooked common bean insoluble dietary fiber is shown in Figs. 5-9. Common bean insoluble dietary fiber is composed primarily of cellulose and hemicelluloses (Selvendran, 1984). Few differences were observed between the uncooked and cooked insoluble dietary fiber fractions. Structural differences may have been minimized by gelatinization of the uncooked sample for 20 min at 100 °C during the first stage of digestion (Asp et al., 1983). Both uncooked and cooked insoluble fiber consisted primarily of cell wall remnants from which all starch and protein storage bodies had been removed (Fig. 5). Also visible were undigested portions of the seed coat, with the long cylindrical cells of the seed coat palisade cell layer (Fig. 6). The outer surface of the palisade cell layer in uncooked fiber was relatively flat (Fig. 6) and similar to exterior surfaces previously observed in unimbibed whole seeds (Swanson et al., 1983). However, examination of the outer surface of the palisade cell layer of cooked insoluble dietary fiber revealed a rolling, uneven surface (Fig. 7). The uneven surface appears to have been caused by differential swelling of the palisade cells during cooking.

When viewed from the exterior surfaced or in cross-section, palisade cells in both uncooked and cooked insoluble dietary fiber appear intact. However, examination of the interior or lower surface reveals that the lower surface of the palisade cells was removed, presumably by enzymatic digestion during preparation of the dietary fiber (Fig. 8). Removal of the lower surface of the palisade layer allowed the internal contents of the cells to be digested and removed. Thus, while palisade cells in insoluble dietary fiber appear unaffected by digestive enzymes, these cells are actually empty chambers or lumens from which all internal contents have been removed. Similar palisade cell chambers were observed by Dintzis et al. (1979) when examining digested soy bean hulls.

Long, thin fibers (Fig. 9) were an unusual feature of insoluble dietary fiber not observed in other fractions. These fibers were approximately 10-15 μm wide and coarse in appearance (Fig. 9). Though the exact origin of the fibers is uncertain, they appear morphologically...
Soluble and Insoluble Common Bean Dietary Fiber
similar to the nutrient transporting phloem previously observed in whole seeds (Hughes and Swanson, 1985).

**Microstructure of soluble dietary fiber.**

The microstructure of the cooked and uncooked soluble dietary fiber fractions is shown in Figs. 10-14. Common bean soluble dietary fiber is composed primarily of pectic substances (Selvendran, 1984). Microstructurally, common bean soluble dietary fiber appears as thin, irregularly shaped sheets (Figs. 10 and 11) attached to a framework of long thin rods (Figs. 11, 12 and 13) in both uncooked and cooked samples. Closer examination of the rods observed in the soluble fiber (Fig. 14) revealed that they were thinner (2-4 µm) and less fibrous in nature than the fibers observed in the insoluble fiber (Fig. 9). Many of the rods also exhibited pods or bulges at one end (Figs. 12 and 13). The soluble dietary fiber structures observed in this investigation are very different from the structures observed by Cadden (1987) in samples of commercially available soluble dietary fiber. The structures reported by Cadden (1987) are similar to structures observed previously (Hughes and Swanson, unpublished data) of soluble dietary fiber after ethanol precipitation and before being redissolved in water and freeze dried. Differences between our observations and those reported by Cadden (1987) probably result from different preparation procedures and should not be interpreted as significant structural differences in soluble dietary fibers from different sources. SEM examination provides useful information on the structural characteristics of soluble dietary fiber. However, it is important to remember that in its natural state the fiber is solubilized in water and structural artifacts may result from the extensive dehydration required.

**Conclusions**

The cooking of common bean flours resulted in marked increases in insoluble dietary fiber and total dietary fiber while soluble dietary fiber content decreased slightly. In uncooked whole bean flour, SEM revealed starch granules and protein bodies characteristic of the common bean while cooked flour contained amorphous material consisting of gelatinized starch and denatured proteins. Microstructural examination revealed common bean insoluble dietary fiber consisting primarily of cell wall remnants and portions of the seed coat palisade cell layer. Long thin fibers believed to be remnants of the nutrient transporting phloem were also observed. The common bean soluble dietary fiber, in contrast, consists of thin, irregularly shaped sheets and long, thin rods. No significant microstructural changes in either the insoluble or soluble dietary fiber fractions were attributed to cooking. Structural differences between uncooked and cooked may have been minimized by the need to digest uncooked samples for 20 min at 100°C.

Changes in the dietary fiber content of foods with cooking is a complex and poorly understood phenomenon influenced by the analytical method used, the type of food studied, and the type, duration and severity of cooking utilized. Additional research on the chemical processes responsible for quantitative changes in dietary fiber as a result of cooking will clarify the relative contribution of each of these variables.

**Acknowledgements**

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**References**


Soluble and Insoluble Common Bean Dietary Fiber


Discussion with Reviewers

W.L. Wolf: Since the soluble fiber fraction was freeze-dried, one must be concerned about possible artifacts, particularly the sheet-like materials. If the sheet-like materials pre-existed, is it likely that the fiber would have been soluble?

Authors: As indicated in the paper, the examination of a water-soluble material in its dehydrated state has numerous drawbacks. It would seem reasonable to assume that rods would be more water soluble than sheet-like material, and that the sheets were formed during dehydration as a result of the agglomeration of rods. However, we have no evidence to support or contradict such a hypothesis.

F.R. Dintzis: Were both soluble and insoluble dietary fiber fractions starch and/or protein free?

Authors: The residual protein content of all dietary fiber fractions was determined according to the method of Asp et al. (1983) and protein was found in all fractions in widely varying quantities (3-25% of total protein). We did not examine the bean dietary fiber fractions for starch, but resistant starch has been reported in cereal dietary fiber (Björck et al., 1986) and could logically be assumed to also be present in common bean dietary fiber.

G.L. Hosfield: How many seeds were examined before a particular photomicrograph was chosen to represent the corresponding SEM observations as a figure?

Authors: Three to five samples of each of the flours or dietary fiber fractions were examined prior to selecting a representative micrograph. Our experience has been that thorough examination of a few samples is more fruitful in obtaining representative micrographs than cursory examination of a large number of samples.

G.L. Hosfield: From a human nutritional viewpoint, what is the significance of the dietary fiber research results and conclusions?

Authors: Two nutritional implications seem readily apparent. First, in foods such as beans which are rarely if ever consumed raw, data on the dietary fiber of cooked beans is more valuable than data on raw beans. Second, though both insoluble and soluble dietary fiber are desirable in the diet, their metabolic effects are different. Therefore, any shifts in soluble and insoluble dietary fiber as a result of cooking should be included in food databases in order to more accurately represent the expected metabolic effects of the dietary fiber present in the food.

G.L. Hosfield: What role does the seed coat play in fiber quantity and quality?

Authors: The seed coat of the common bean typically comprises 8% of the whole seed by weight. The seed coat is also typically high in insoluble dietary fiber and low in soluble dietary fiber.

A.C. Olson: The SEM was only done on the black bean which is not a major bean of commerce in the United States. How widely is this bean an item of commerce?

Authors: Though black beans are not widely cultivated or consumed in the United States, they are widely produced and consumed throughout Latin America. In several Latin American countries including Guatemala, black beans are the preferred bean and are consumed on a daily basis. The particular cultivar (Tamazulapa) investigated in this study, however, is not to our knowledge grown and consumed outside of Guatemala.

A.C. Olson: What effect (if any) do you think the heat treatment during digestion with termamyl and the milling had on your results?

Authors: The need to digest the uncooked flour for 20 min at 100°C probably minimized microstructural differences between cooked and uncooked dietary fiber fractions and may have also reduced quantitative differences. Foods are routinely milled prior to determination of dietary fiber content. However, very finely milled samples of a food have been shown to have a lower insoluble dietary fiber content than coarsely milled samples. For this reason it is important to report the size to which the food was milled prior to dietary fiber determination.
HEAT-SET GELS BASED ON OIL/WATER EMULSIONS: AN APPLICATION OF WHEY PROTEIN FUNCTIONALITY

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Abstract

The microstructure of protein/lipid gels produced by heat treatment of whey protein stabilized oil-in-water (O/W) emulsions was studied. Scanning electron microscopy as well as transmission electron microscopy was performed on glutaraldehyde/osmium tetroxide fixed, and critical point-dried samples. Microstructure analysis showed that in the case of homogenized O/W emulsions, extensive coating of the fat globule surface with coagulated protein led to a "cauli-flower-like" structure. In such gels, uncoated fat globules having smooth surfaces were not present. This specific microstructure was not obtained with protein/lipid gels in which whey protein was added to the continuous phase and mixed with a O/W emulsion stabilized by lecithin.

Introduction

The most important functional property of whole egg is its ability to form heat-set gels. This "functionality" is exploited to form textural properties in culinary applications of egg. To design a functional egg substitute from whey protein, we developed a process in which whey protein concentrates are combined with selected lipids of vegetable or animal origin. High pressure homogenization of such compositions leads to stable O/W emulsions in which whey protein acts as emulsifier and, at a subsequent process stage, as the gelling agent (Jost et al., 1986).

A remarkable feature of the gelling O/W emulsion is the fact that lipids, although having no gelation capacity on their own, contribute to the firmness of the gelled texture, under the condition that they are present as a homogeneous population of small droplets. Critical droplet size and size distribution parameters were, e.g., 700 nm diameter for the mode of the distribution as an upper limit and a polydispersity index of < 0.35 (Baechler et al., 1986).

Another feature of gels produced from such O/W emulsions is their smooth texture and lipid-like "mouth-feel". Microstructure studies of such protein/lipid gels were aimed at finding characteristics in their fine structure which might explain textural properties and elucidate the role of high pressure homogenization.

Materials and Methods

O/W emulsions were produced from whey protein concentrates (aqueous phase with 5-10% w/v of WPC solids at pH ranging from 5.5 to 8.0) and from sunflower oil as previously described (Jost et al., 1986). The composition used for microstructure studies was an emulsion with 15% (v/v) of sunflower oil and 7.5% WPC solids, corresponding to 6% of protein. The pH of our emulsions was adjusted to 7.0 by addition of KOH(1 N) or NaOH(1 N), prior to gelation. Heat-induced gelation of the degassed O/W emulsions was performed in hermetically closed glass beakers (25 ml total volume, 27 mm inner diameter) placed in a metal rack for the incubation in a water bath at 90°C.
for 30 min. Following cooling to room temperature, the gels were aged 24 h at 4°C prior to further processing.

Measurement of particle size distribution in emulsion was done by dynamic light-scattering, using a Malvern photon correlation spectrometer (Masson and Jost, 1986).

Scanning electron microscopy (SEM)

Gel slices (1 mm x 2 mm x 6 mm) were fixed in phosphate (0.1 M, pH 7.2) buffered 3 % glutaraldehyde. Fixation was performed for about 12 h and was followed by post-fixation in 2.5 % osmium tetroxide for 2 h. Glutaraldehyde fixation had been shown to preserve particularly well the original structure of protein gels (Heertje and van Kleef, 1986). Critical point-drying in CO2 following the dehydration in alcohol was preferred to freeze-drying, thus minimizing the risk of ice crystal formation (Woodward and Cotterill, 1985).

Particles which had been freshly dry-fractured were mounted on SEM stubs with a conductive carbon cement and coated with a 20 nanometer-layer of gold in a Polaron sputter coating unit. The preparations were observed in a Philips 505 SEM at an accelerating voltage of 30 kV.

For fixation of the (liquid) O/W emulsion, "deep-well" stubs were used. A drop of the emulsion was placed in the cavity of the stubs and covered with a Nuclepore filter membrane (0.4 μm), and sealed with a metal ring. At this stage, the stubs were processed in an analogous manner as the gels.

Transmission electron microscopy (TEM)

Small cubes of gel (1 mm³) were fixed as described for SEM. Dehydration was accomplished in a graded alcohol series up to 95 % ethanol concentration. Infiltration was with mixtures of 95 % alcohol and LR White (2/1 v/v overnight and 1/2 v/v for another 12 h). Subsequent infiltration of pure LR White in gelatin capsules was performed overnight at 4°C. Followed by polymerization at 52°C. Thin sections (60 nm) stained with uranyl acetate and lead citrate were examined in a Philips 300 TEM at an accelerating voltage of 80 kV.

Results and Discussion

SEM of an unheated O/W emulsion (15 % v/v of dispersed oil and 6 % w/v of whey protein, pH = 7) shows primarily the fat globules in a perfectly spherical shape. The mean diameter established by light scattering analysis on the liquid parent emulsion, roughly 400 nm, corresponds well with the projected globule diameters as revealed by SEM (Fig. 1a). The protein which is essentially present in a soluble state is of rather discrete appearance or not visible at all.

Following heat-induced gelation of the emulsion, SEM shows an entirely different structure. Again, the lipid droplets are very clearly visible but, in the gel, their surface is rough and structured (Fig. 1b) and at a higher magnification, a "cauliflower-like" structure is evident (Fig. 1c). The granulated surface structure of the fat globules is thought to result from extensive protein coagulation on the lipid surface. Smooth lipid globules are not seen nor can we distinguish protein coagulum not associated with the lipid globules, at least not in considerable amounts.
Whey protein gels based on O/W emulsions

It is very instructive to compare the fine structure of the gels based on the emulsion with a whey protein gel produced under the same heating conditions using a mere aqueous dispersion of whey protein concentrate (12% w/v of total solids or 9.6% of protein). Such a protein gel appears in SEM as a dense but porous structure composed of strands of protein granules (Fig. 2). The diameter of the protein granules which are roughly spherical, is near 100 nm. We can imagine that the same kind of protein granules melted together on the fat surface, leads to a microstructure similar to that shown in Figs. 1b and 1c.

We learn from this comparison that in an O/W emulsion stabilized by whey protein and possibly other proteins, the dispersed lipids when coated with the protein, act as a matrix on which further protein will preferentially coagulate during the heating step. TEM of the gelled emulsions brings additional strong evidence in favour of a massive interfacial protein coagulation, resulting from the preceding homogenization step during and after which protein "migrated" from the continuous phase to the interface. Thin sections show the protein coating of the lipid globules (Fig. 3a) and the higher magnification clearly shows how gelled protein crosslinks the coated fat globules (Fig. 3b). Additional experiments were done to highlight the particularity of the gelled emulsions and the role of homogenization in the presence of the protein for the formation of this structure. It appeared that the formation of a uniform and integrated protein/lipid microstructure could not be obtained unless homogenization at relatively high pressures (20-35 MPa, 2-5 passages) in the presence of the protein, preceded the gelation step. Stabilization of the emulsion resulting from protein migration to the interface is necessary to favour protein coagulation at the lipid surface. Varying the proportions of lipids and proteins will certainly modify the fine structure of the gel. As we increased the protein content of the emulsion and consequently the protein/lipid ratio, more protein coagulation took place in the continuous phase, while at protein/lipid ratios < 0.5, most of the protein was associated with the fat globules.

A good demonstration that interfacial protein coagulation depends on the presence of the protein during the homogenization step was obtained from SEM analysis of gels produced from a lecithin-stabilized O/W emulsion, to which whey protein was added. The protein was simply well
dispersed in the emulsion and the combined emulsion subjected to heat treatment. Gels obtained according to such a procedure were considerably weaker than their counterparts produced with protein stabilized emulsions. Weak gels were formed provided that the lecithin concentration was < 1 %, whereas at higher lecithin concentrations, the emulsion remained completely liquid after heating. SEM of lecithin-based gels show smooth lipid spheres, devoid of adsorbed protein, randomly distributed in the gel matrix made of whey protein (Fig. 4).

**Conclusions**

The particular texture and microstructure observed in whey protein/lipid gels reflects the sequence of operations during processing (Fig. 5). High pressure homogenization in the presence of protein is, in view of the desired gelation properties, to be preferred to the use of an emulsifier such as lecithin during the homogenization step required to produce the parent O/W emulsion. Subsequent heat-induced gelation then results in a particular "integrated" microstructure characterized by an extensive protein coagulation at the surface of the fat globules. Repeated homogenization at pressures of 20-40 MPa increases the fat surface available for protein adsorption and coating and thus favours the subsequent deposition of heat-coagulated protein at the interphase. With an increasing homogeneity of the dispersed lipids and a reduction of the fat globule size, the heat-set gel increases in its strength. Apparently, under these conditions, the fat globules assume the role of filler particles as proposed by Dickinson et al. (1985) who showed this effect with gelatin-based O/W emulsions.

Due to the volume fraction which is occupied by the fat globules in emulsion, the protein concentration is also increased in the continuous phase. This explains why in an emulsion, firm gels can be obtained at comparatively lower protein concentrations than in the case of aqueous dispersions of the protein.

In gelled O/W emulsions, proteins and lipids form an integrated particular microstructure which is macroscopically smooth and gives a pleasant "mouth-feel". This may be favourably exploited in different food applications.

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**References**


**Discussion with Reviewers**

**Sheng-Chin Yang:** Can you explain why you use 50°C as the emulsification temperature? What if the temperature is lower than 50°C?

**Authors:** By raising the temperature during homogenization, the reduction of fat globule size is facilitated. However, an upper limit is given by the risk of protein denaturation, and, in the case of whey proteins, this limit temperature is 60°C at neutral pH. Homogenization at 50°C is therefore a compromise between a good homogenization effect and the risk of protein denaturation. Homogenization at 50°C avoids unwanted denaturation at the stage of the emulsion.

**D. Holcomb:** Does whey protein serve as an emulsifier in the unheated emulsion? If so, what happens to the emulsifier whey protein when the system is heated? Is that protein also denatured/coagulated? Does the heating alter the protein's emulsifying capacity and the emulsion stability?

**Authors:** The whey protein is the only emulsifier present in the system. Following homogenization, a stable emulsion is obtained in which no coalescence nor creaming is observed, nor does the fat globule size distribution change significantly over several days. The experimental conditions selected for gelling the emulsion (pH near 7, temperature 90°C, holding time 30 min), suggest that practically all the protein, adsorbed or not, is fully denatured during the heat treatment. As a result of heat denaturation, rearrangement of protein molecules adsorbed to the fat globule surface, may have occurred. In practice it is no more possible to distinguish between adsorbed and "non-adsorbed" protein once the gel is formed. Following heat-induced gelation, the initial emulsion is "frozen" in a gelled structure. It cannot change for this reason, unless the gel is dehydrated or destroyed by other means. Microscopic analysis of the gels indicated projected fat globule diameters which are close to the diameters established by light-scattering on the liquid parent emulsion.

**D. Holcomb:** It is mentioned that protein coagulation is seen in the outer phase when the protein concentration is increased. What protein concentration is such coagulation visible?

**Authors:** The decisive factor is the protein/lipid ratio, not the absolute protein concentration. If this ratio exceeds 0.5, there is an increasing amount of agglomerated protein observed in the space between the protein-covered fat globules (Fig. 6). This agglomerated protein shows a structure resembling the one seen with protein gels produced from aqueous dispersions.
Whey protein gels based on O/W emulsions

Fig. 4. Gel obtained from a lecithin-stabilized emulsion with whey protein used as the gelling agent in the continuous phase.

Fig. 5. Scheme illustrating steps in the formation of a lipid-rich gel based on a protein-stabilized O/W emulsion.

D. Holcomb: Please provide an illustration of the "deep-well" stub.
Authors: The stub in which we perform the processing of the liquid emulsion is shown in Fig. 7. The (inner) diameter of the well is 7 mm.

Sheng-Chin Yang: If the pH and homogenization conditions remain constant, would the heated (90°C/30 min) whey protein/lipid emulsion be affected by different heating temperature and time?
Authors: Concerning the effect of temperature, variations within the range of 85-95°C have little effect on the gel-strength, but at temperatures below 85°C the gels become markedly weaker. 70°C is the lower limit to obtain gelation. Higher temperatures (e.g. by autoclaving the emulsion in hermetically sealed tins), can lead to higher gel-strength. The appropriate holding time at a given temperature depends on the heat transfer in the emulsion. Thus, the 30 minutes specified in this work are adequate for 25 ml of emulsion heated in glass beakers of the size specified in the Materials and Methods section.
Based on the levels of \( \kappa \)-lactalbumin and \( \beta \)-lactoglobulin in whey, and the area of the oil/water interface produced in this example, is there the possibility of selective adsorption of protein to the surface, thus influencing the composition of the heat-set protein film?

Authors: \( \beta \)-lactoglobulin accounted for about 50\%, \( \alpha \)-lactalbumin for 20\% of the total protein in our whey protein concentrate. These proteins, together with minor whey proteins, will compete for the fat surface which in our example may be near 500 m\(^2\)/100 ml of emulsion (oil volume fraction 0.4, mean fat globule diameter 450 nm). We didn't study the selectivity of protein adsorption in our system but may mention the findings of Shimizu et al. (1981) who showed that at neutral pH, the relative abundance of individual whey proteins at the fat surface of an O/W emulsion well reflected their mass proportion in the whey protein concentrate. Thus, \( \beta \)-lactoglobulin was the most abundant protein in the film at neutral and alkaline pH, but at acidic pH, \( \alpha \)-lactalbumin was more abundant. We deduce that protein adsorption to fat surfaces is strongly dependent on the pH and the isionic pH of a given protein. Under the conditions of our emulsions, it is likely that \( \beta \)-lactoglobulin is a major fraction in the primary protein film.

What is believed to be the effect of the added lecithin, and is the order of addition significant? Proteins can displace each other at the interface (Dickinson et al., 1988) and sodium dodecyl sulfate can interpenetrate protein films or possibly displace them (Jaynes and Flood, 1985). Is this what occurs?

Authors: Once the emulsion was produced with lecithin instead of protein, whey protein cannot efficiently adsorb to the fat globules (see fig. 4). This may be due to charge interaction between the lecithin's polar head and ionic sites on the protein, which will keep the protein off the fat surface. In such a case we may still get a stable emulsion, but no gel or only a very weak one. We cannot fully explain this poor gelation behaviour but assume that lecithin also interacts directly with protein and interferes with its gelation properties. Lecithin introduced after homogenization in the presence of whey protein, likewise interfered with gelation. This could indicate that in the unheated emulsion, lecithin is able to displace protein from the fat surface.

Is the high pressure (> 150 bar) required in order to obtain a fine and homogenous emulsion or in order to deposit protein at the interface?

Authors: Although protein migration to the interface takes place at lower homogenization pressure, high homogenization pressure allows, with a limited number of passages, to achieve a low mean lipid globule size and therefore a large fat surface.

Would other proteins show a similar behaviour?

Authors: We have a limited experience with egg white showing that in similar O/W emulsions high pressure homogenization under adequate temperature and pH conditions leads to improved gelation properties.

Additional References

MICROSTRUCTURAL CHANGES IN WHEAT STARCH DISPERSIONS DURING HEATING AND COOLING

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Abstract

Microstructural changes in 8-11% wheat starch dispersions during heating, cooling and cold storage have been evaluated by light microscopy and scanning electron microscopy.

Heat treatment of wheat starch dispersions gives rise to two stages of swelling and solubilization. During the first phase of swelling, solubilized amylose was observed in the centre of the granules and, to some extent, outside the granules. Further swelling deformed the granules and more amylose was released. When the temperature treatment took place under shear, the outer layer of the swollen granules fractured at 94°C and above, and amylopectin fragments were dispersed into the continuous amylose phase. Fragmentation of amylopectin was not observed when samples were heated with a minimum of mechanical action. There were also differences in the final gel structure between samples due to the mechanical treatment during preparation.

Aggregation of amylose took place on cooling and could be observed as irregularities in the gel structure. When the amount of released amylose was limited during the initial phase of swelling and below the critical concentration for gel formation, cooling resulted in deposition of amylose at the surface of the granules. This was expected to have an impact on the behaviour of the granules on further processing.

Introduction

Wheat starch is commonly used because of its thickening and gel forming properties in viscous foods and gels. Numerous studies have been made to characterize the rheological behaviour of heat-treated wheat starch dispersions in the concentration range 5-14% (Doublier, 1981; Wong and Lelievre, 1981; Bagley and Christianson, 1983; Eliassen, 1986 and Doublier et al., 1987). Similar studies of the microstructure of wheat starch dispersions in this concentration range are surprisingly scarce. Heated starch systems can be described as colloidal systems in which the particles are dispersed in a macromolecular solution, and wheat starch dispersions in this concentration range have the ability to form gels on cooling.

Most studies of the microstructure have dealt with low concentrations (0.25-1.5% w/w) and have mainly concerned changes in the shape of lenticular wheat starch granules during heating (Ghiasi et al., 1982; Varriano-Marston et al., 1985; Bowler et al., 1980, 1987 and Eliassen, 1985). In these studies, wheat starch granules at low concentrations were washed in distilled water before being examined under the microscope. The washing procedure removed exuded amylose as well as other components which may contribute both to the rheological properties and the microstructure of starch dispersions. The heating was usually done in tubes with a minimum amount of mechanical treatment. In all studies, the swelling and deformation of lenticular wheat starch granules followed the same pattern.

Bowler et al. (1980) used both light microscopy (LM) and scanning electron microscopy (SEM) in order to follow the heat-induced changes in the granules. On the basis of their own as well as previously published results; they proposed the following model for the swelling of lenticular wheat starch granules. The granule started to swell radially to about three times its original diameter at approximately 50°C. In the next stage the granules started to swell tangentially in the xy-plane. Little or no swelling took place in the z-direction, which meant that the disc started to pucker out of the xy-plane and the extent of puckering increased with temperature. The first type of change occurred relatively slowly and was well defined, but the second type took place rapidly and the resulting granules appeared flexible. They also noted that the equatorial groove prevails during the whole heating process up to 97°C. Later Bowler et al. (1987)
investigated the effect of different preparation techniques for SEM, such as dehydration and critical point drying, freeze drying and cryo stage techniques. Even if differences were observed, the preparation techniques did not affect the above described swelling patterns of the granules. The effects of surfactants on the swelling of wheat starch granules have been investigated by Ghiasi et al. (1982) and by Eliasson (1985). They found that surfactants restricted the first but not the second stage of the swelling of wheat starch granules.

The amount of amylose released from wheat starch granules during heating has been investigated by means of iodine-binding at low concentrations in the range 0.25-2.5% (Doublier, 1981; Ghiasi et al., 1982; Eliasson, 1986). Here again two stages were observed. In the lower temperature range of about 55-80°C the release of amylose was slow, but it increased considerably at higher temperatures.

Light microscopy was used on all samples, and the few micrographs presented here have been chosen out of several hundred.

All samples were stained with iodine in a diluted 1:1 Lugol's solution for one minute, whereafter the sample was covered and sealed. Preliminary studies were also made with other stains, such as safranin, Congo red, gentian violet and aniline blue with Orange G, in combination with different illumination techniques. The prepared samples were examined with a Nikon macrophot FX microscope.

Smears. The starch dispersion was quickly and gently smeared out onto an object glass and stained directly. The advantage of this technique is that the hydrated sample can be observed without freezing or dehydration. It is also possible to observe intact swollen granules and obtain a three-dimensional impression by focusing through the depth of the sample. The disadvantage is that a sample with swollen granules gets rather thick, which decreases the resolution and results in a loss of detail.

Cryosections. Samples were frozen in liquid nitrogen and sectioned frozen. A Leitz cryostat was used and 7-8 μm thick sections were cut. The advantage of cryosections is that very quick changes in the microstructure are detectable as the structure is "frozen". A better resolution is obtained with this technique than with the smearing technique. The disadvantage is that the freezing can induce ice crystals that can damage the microstructure. This damage is more severe in the gel than in the fluid, viscous starch systems, where the dissolved macromolecules act as cryoprotectants.

Embedded sections. Samples were chemically fixed in 3% glutaraldehyde with 0.1% ruthenium red, dehydrated and embedded in historesin, LR White, as recommended by the manufacturer. A Reichert-Jung Ultracut E, with glass knives, was used to section the samples. Thin sections, 1 μm, were cut to obtain the best possible resolution, and thicker sections, 3-4 μm, were cut to achieve better contrast staining. This technique gives the best resolution for light microscopy, and no freeze damage or mechanical redistribution is induced in the sample. The samples were stained after sectioning, which means that microstructural changes due to the presence of iodine in the staining solution can be ruled out.

The disadvantage is that it may be difficult to interpret such thin sections with regard to dispersions with small and large irregular particles. The chemical fixation and dehydration used in this technique may also affect the macromolecular phase, but such effects cannot be seen from the resolution given by light microscopy.

Scanning electron microscopy was used in a Cambridge Stereoscan 200 equipped with a Hexland cold stage using accelerating voltage between 2 kV-10 kV.

### Experimental

**Materials**

In this study commercial wheat starch from CPC-Cerestar was used. Concentrations in the range of 2 to 11% w/v starch were investigated, but in the main study 8% w/v was used if not otherwise stated. In a preliminary study different batches from three producers were tested to get some idea of the effects of the wheat quality, and of environmental and processing conditions. Samples were purchased from Raisio Tehtaat OY, Kröner and CPC. All wheat starch samples showed the same type of general behaviour even if there were some discrepancies in the temperatures at which different phenomena occurred. Sample preparation

Samples were prepared by mixing 36 g wheat starch with 414 ml distilled water.

The starch suspension was heated at the rate of 1.5°C/min to a required temperature, held at that temperature for 30 minutes and cooled to 25°C at a rate of 1.5°C/min in a Brabender Amylograph. The temperature dependency was studied by performing experiments at the following max. temperatures 60, 70, 75, 80, 85, 90, 94, 95, 97 and 98°C. This temperature treatment was combined with mechanical treatment consisting of continuous stirring at 75 rpm. Mechanical treatment was kept to a minimum for two samples by preheating them in the Brabender, as described above. One sample was preheated to 85°C and then heated for 30 min at 95°C in a water bath without any stirring. The other sample was preheated to 85°C, and then heated for 30 min at 95°C in a water bath without any stirring. After cooling, samples were stored at -4°C.
Microstructural changes in wheat starch dispersions during heating and cooling

Figures 1 and 2 are color micrographs at page 33.

Fig. 3. Scanning electron micrograph of a critical point dried 8% starch dispersion heated to 90°C and cooled to room temperature.

Critical point drying.
Small samples were chemically fixed in 1% glutaraldehyde with 0.1% ruthenium red, dehydrated, through graded alcohol, and critical point dried. The dried samples were fractured and mounted on SEM with "Leit-C", carbon glow. The samples were sputter coated, Au/Pd in a Polaron E5000 before examination in the SEM.

Cryo-stage. Very small samples about 1 mm³ were placed in copper sandwich holders and extra rapidly fast frozen in a propane jet freezer, Balzer. Frozen samples were transferred to the cold stage in the microscope, fractured and etched at -90 to -100°C. Samples were examined at -150 to -176°C, both uncoated and gold coated at low accelerating voltage 2-3 kV.

Results and Discussion
Heat-induced changes in starch dispersions
Microstructural changes in wheat starch have been studied as a function of temperature combined with mechanical treatment. Samples were taken at various stages of heat treatment from 60 to 120°C for microstructural evaluation. Results from some heating temperatures were chosen to illustrate the most significant heat-induced changes. Wheat starch granules heated to 75°C are representative of the first stage of swelling, where the release of amylose is limited and the concentration of amylose is not high enough for gel formation. Heating temperatures of 90°C and above were chosen to illustrate phenomena occurring during the second stage of swelling.

The effect of heating an 8% wheat starch dispersion to 75°C is illustrated by the iodine-stained smear shown in Figure 1. There is a variation in the degree of swelling of the starch granules, and it is important to realize that starch consists of a population of granules which vary with regard to factors such as shape, deformation, release of amylose during heating, etc. From Figure 1 it can be seen that the majority of the granules heated to 75°C have retained their original shape and that they are stained dark blue which means that they are amylose-rich. These granules are in the first stage of swelling according to the swelling pattern described by Bowler et al. (1980). There is an exuded blue phase surrounding these granules, which means that the restricted leakage of amylose out of granules in the first stage of swelling can be detected by light microscopy. A number of granules in the second stage of swelling can also be seen from Figure 1. These are more brown and less blue in colour and have a more irregular shape. Iodine stains pure amylose blue and amylopectin beige-brown with varying intensity.

Figure 2 shows the structure of the 8% starch dispersion at 90°C. More amylose leaks out at this temperature, and the continuous phase surrounding the swelled granules is stained blue. There are also some blue-stained granules in the first stage of swelling at this temperature, but the majority of the granules are in the second stage of swelling. They are mainly beige in colour, and especially the outer layers are very weakly stained, indicating that amylose has leaked out. From Figures 1 and 2 it is also evident that the starch dispersion contains small granules of B-starch as well as lenticular shaped A-starch granules.

From the smears it is not possible to characterize the exact shapes of the lenticular A-starch granules at different stages of swelling. Corroborative SEM studies of heated dispersion show that the changes found in the shape of the granules in 4-8% dispersions were similar to those found in studies of
dilute suspensions (Bowler et al., 1980; Ghiasi et al., 1982; Eliasson, 1985; Varriano-Marston et al., 1985). Figure 3 shows a SEM micrograph of a granule in an 8% dispersion after cooling from 90°C. The granule has a shape typical of the second stage of swelling and is surrounded by a network structure of amyllose. This sample was prepared by chemical fixation, dehydration and critical point drying. Figure 4 shows a fractured granule in a 4% dispersion after cooling from 90°C, preparation by rapid freezing and examination on a cryostage. The use of low-temperature techniques for isolated granules has been discussed by Bowler et al. (1987). The advantage of this technique is that the sample can be investigated fully hydrated without additions of chemicals, provided that the freezing rate is high enough to prevent ice crystal formation, which can damage the structure. Some difficulties attached to working with highly aqueous samples of dispersions and gels are worth pointing out. Figure 4 shows a sample which should have been deeply etched under the etching conditions of 15 min at -90°C. However, the water is firmly held by the swollen granule and by the biopolymer-rich continuous phase. Therefore, it is very difficult to sublimate water from the surface, which makes the contrast low and makes it difficult to reveal fine details of the structure.

**Fragmentation of amylopectin**

When the starch dispersion was heated in the Brabender to 95°C, the outer layer of the swollen granules fractured and fragments were released into the blue stained amyllose solution. These fragments are weakly stained and believed to be composed mainly of hydrated amylopectin and not any amyllose with an affinity for iodine. Figures 5 and 6 show 8% wheat starch dispersion heated to 95°C. Amylopectin fragments of varying size, are dispersed in the exudated blue amyllose phase as seen in Figure 5. The micrograph at a higher magnification shown in Figure 6 illustrates a granule where the outer layer has been partly sheared off. In three dimensions the outer layer that fractures would correspond to the outer part of the puckered granule as described by Bowler et al. (1980). The mechanism of fragmentation is schematically illustrated in Figure 7, showing the swelling of the granule, the formation of an outer layer and the fracture of the outer layer into fragments.

Fragmentation started at 94-95°C for samples prepared in the Brabender Amylograph at 75 rpm, and the amount of dispersed fragments increased with elevated temperatures and holding times at 94°C or above. This phenomenon has not previously been demonstrated by microscopy. The smear technique was chosen to introduce heat-induced changes and the presence of small fragments in the continuous amyllose phase. With any of the sectioning techniques, a small particle as seen under the microscope may have been part of a bigger particle that has been sectioned close to its outer surface.

**Fragmentation in dispersions and gels**

The amylopectin fragments seemed to be fractured from the outer layer of the swollen granules, and it was considered of interest to study the effect of the mechanical treatment of the dispersion during preparation in the Brabender with regard to amylopectin fragmentation.

Figure 8 shows a cryosection of an 11% wheat starch dispersion heated at 94°C for 30 min and subjected to mechanical treatment. Figure 9 shows a cryosection of an 11% wheat starch dispersion heated at 94°C for 30 min and not subjected to mechanical treatment. Figure 10 shows a cryosection of an 8% gel, formed after heating at 95°C, cooling and storage at room temperature for 5 h.

Figure 11 shows a smear of an 8% gel formed after heat treatment at 120°C for 30 min.

**Fig. 1.** Smear of an 8% wheat starch dispersion heated to 75°C.

**Fig. 2.** Smear of an 8% wheat starch dispersion heated to 90°C.

**Fig. 3.** Smear of an 8% wheat starch dispersion heated to 95°C.

**Fig. 4.** Smear of an 8% wheat starch dispersion heated to 95°C.

**Fig. 5.** Smear of an 8% wheat starch dispersion heated to 95°C.

**Fig. 6.** Smear of an 8% wheat starch dispersion heated to 95°C.

**Fig. 7.** Schematic illustration of fragmentation of the outer layer of swollen granules.
Microstructural changes in wheat starch dispersions during heating and cooling

Fig. 14. Cryosection of an 8% wheat starch dispersion heated at 95°C for 30 min.

Fig. 15. Cryosection of an 8% wheat starch dispersion heated at 95°C for 30 min and cooled to room temperature.

Fig. 16. Smear of an 8% wheat starch dispersion heated at 75°C for 30 min.

Fig. 17. Smear of an 8% wheat starch dispersion heated at 75°C for 30 min and cooled to room temperature.

Fig. 18. Smear of an 8% wheat starch dispersion heated at 75°C for 30 min, cooled to room temperature and stored 15 days in a refrigerator.

Fig. 19. Embedded thin section of an 8% wheat starch dispersion heated at 75°C for 30 min cooled to room temperature and stored 4 days in a refrigerator.

Fig. 20. Embedded thin section of an 8% wheat starch dispersion heated at 75°C for 30 min, cooled to room temperature and stored for 24 h.

total and only a small part of the amylopectin was found in the soluble fraction. These are interesting observations, but direct comparisons are difficult to make, since the rotation speeds were considerably higher, the equipment different and the concentrations of wheat starch used in the solubility experiments lower than in this study.

Figures 8 and 9 show cryosections that are 7-8 μm thick, which means that swollen lenticular granules are sectioned and only parts of the granules can be seen. Taking this into account, there is a good agreement between smears and cryosections. The cryosections are not thin enough to provide fine details of the granules. The appearance depends on how the granules have been sectioned and how structural components are super imposed. By embedding and sectioning 1 μm sections, details of the interior of the swollen granules can be seen. The thin sections in Figures 10 and 11 show clearly that there is a blue stained amylose phase inside the swollen granules.

Figure 10 shows an embedded thin section of an 8% gel formed after cooling a dispersion heated to 95°C by continuously stirring in the Brabender. The amylopectin rich fragments in the thin sections stained very poorly and appear almost colourless in Figures 10 and 11. A large number of amylopectin fragments are dispersed in the amylose phase of the gel shown in Figure 10, and it is reasonable to assume that the degree of fragmentation increases due to the shearing during cooling. The large number of amylopectin fragments in the amylose phase will probably interfere with the gelation of amylose. Shearing will also disrupt the amylose network, and it can be noted that it took five h for this sample to form a gel after it had been cooled to room temperature. There is an unstained amylopectin-rich outer layer surrounding the granules in Figure 10.

Additional studies showed that there was a tendency towards phase separation of the small amylopectin fragments from the amylose phase when the gel was stored at +4°C.

Figure 11 shows a thin section of an 8% gel formed after cooling from 120°C without mechanical treatment. Even after heat treatment at 120°C for 30 min, swollen granules still exist and their inner parts are larger than those shown in Figure 10. As in the dispersion shown in Figure 9, there are hardly any
visible amylopectin fragments in the gel formed without mechanical treatment. Instead the outer amylopectin-rich layer of the granules occupies a much larger volume than was the case in the gel shown in Figure 10, where these layers have been partly sheared off. The gel formed from 95°C without mechanical treatment seems to have two continuous structures separated from each other; one consisting of a network of released amylose and one consisting of granules with a highly swollen amylopectin-rich layer connecting them together. The network of released amylose formed from heat treatment at 120°C differed from that formed at 95°C, but evaluation of amylose network structures requires the resolution given by transmission electron microscopy (TEM) and will be the subject of a separate study.

The amylopectin-rich areas of the thin sections stained very poorly, and it can be questioned whether these areas really contain highly swollen amylopectin and not just water-filled voids caused by phase separation. Corroborative studies of these regions were made by differential interference contrast of cryosections (LM) and cryo-SEM. The results showed that these regions had a structure which cannot have been caused by low molecular components in a water solution. Figure 12 is a SEM micrograph obtained by the low temperature technique, showing the smooth flaky structure of the amylopectin-rich outer layer and the small aggregates of released amylose formed by heat treatment at 120°C.

The structure of the gel formed by heat treatment at 120°C and not subjected to mechanical treatment is summarized by the schematic drawing in Figure 13. The swollen granules with amylose in the centre and the outer aqueous amylopectin-rich region form a continuum, and the network of aggregated released amylose forms another continuous phase. This gel was considered firmer than that formed by heat treatment at 95°C and mechanical treatment, and no storage at room temperature was necessary for a firm gel to form.

Apart from providing fine details of the structure, the embedding and thin-sectioning technique was more suitable for gels than the cryotechnique. The amylose network was sensitive to freezing, and freeze artifacts were often observed after cryosectioning of gels. The results are often fragmented at the interface between the amylose and the amylopectin-rich regions. Before gel formation amylose acted as a cryoprotectant, and cryosectioning gave the best results for hot and cooled dispersions.

Aggregation of amylose during cooling from the second stage of swelling and solubilization

Figure 14 shows a cryosection of an 8% dispersion heated and treated mechanically at 95°C for 30 min. This dispersion was prepared in the same way as the 11% dispersion shown in Figure 8. The staining of amylopectin fragments was better at the higher concentration and the high water content may be one factor contributing to the lack of staining intensity. The degree of fragmentation was somewhat higher at the higher concentration due to the difference in shear force. Otherwise the two structures are similar in character.

When the dispersion was cooled to 25°C no gel was formed directly but amylose started to aggregate. Aggregates can be seen in Figure 15 as dark blue areas. The presence of amylose aggregation can also be seen from the thin section of the gel in Figure 10 even if the differences in colour intensity are not as striking in the 1 μm section as in the 8 μm cryosection shown in Figure 15. The same type of amylose aggregation cannot be observed in Figure 11, and one possibility is that amylose is deposited on amylopectin fragments. From the cryosections it is not possible to say whether the amylose aggregates are solid or whether they cover an amylopectin fragment. The size of a fragment is about 10 μm in diameter which corresponds approximately to the thickness of the section. Despite the lack of resolution, cryosectioning is the best technique for dispersion due to the possibility of freezing the structure at any given state of aggregation.

Aggregation of amylose during the first stage of swelling and solubilization

Interesting observations were made from dispersions heated to 75°C. At this temperature the amount of amylose released from the granules is limited, gels do not form, and there is no amylopectin fragmentation. Figure 16 shows a smear of a dispersion kept at 75°C for 30 min. More amylose has leaked out due to the holding time than observed in Figure 1 when the dispersion had just reached 75°C and the majority of the granules are in the first stage of swelling. When this dispersion was cooled to room temperature, shown in Figure 17, the interesting observation was made that there was less amylose outside the granules than at 75°C. Thus, the released amylose seemed to have been readorsbed by the swollen granules. After 15 days of storage at 4°C this effect was striking and no amylose was visible outside the granules, which can be seen from Figure 18. Some of the granules in this sample had a hollow appearance.

Preparation by embedding and thin-sectioning gave information about the structural states of granules after heat treatment at 75°C, cooling and storage at 4°C. Figure 19 shows granules stored for 24 h and Figure 20 granules stored for 4 days. The granules already have an amylose-rich zone in the centre after heating at 75°C. There seems to be a passage through the equatorial groove facilitating transport of amylose from the interior zone out of the granules and vice versa. It may then be possible that released amylose can be readorsbed to the central zone on cooling from 75°C.

It can also be seen from Figures 19 and 20 that a thin layer of amylose has been deposited on the surface of the granules. It is possible that such a layer of amylose can form a film around the granules, especially if the dispersion is dried. This amylose film may have a similar effect as hardening of the granules and results in a delay of the swelling of the granules on rehydration and reheating.

Iodine has been added at different stages of preparation, so possible effects of iodine on the state of aggregation of amylose as seen by the light microscope can be excluded. In the thin sections iodine was added after the structure was completely fixed by chemical fixation, dehydration and embedding and no rearrangements were possible.

New aspects of the release of amylose

As described above, amylose is already released from the structure and concentrated in the centre of the granules at 75°C when the solubilization of amylose determined by analysis of the supernatant is still limited (Doublier, 1981; Ghiasi et al., 1982; Eliasson, 1986). The reason for this can be the adsorption of water and the concentration of solubilized amylose in the amorphous central part of the
Microstructural changes in wheat starch dispersions during heating and cooling

granules. It is postulated that there are openings in the equatorial groove allowing for transport of amylose out of and into the central zone without diffusion through the granular structure; as depicted by the schematic drawing in Figure 21. Amylose is probably squeezed out of the central zone during mechanical shear or centrifugation during preparation of samples. A comparison of Figures 10 and 11 shows that the sheared granules are more deformed and elongated and have a smaller amylose zone in the centre than the unsheared sample. The effect of processing conditions on the amount of amylose in the centre may be a reason for the differences in the amount of amylose in the sediment as described by Doublier (1981) even if it is very difficult to compare experimental conditions.

Amylose also diffuses through the granular structure and very little amylose is left in the outer layer of the granules in the second stage of swelling and solubilization as shown by Figures 6, 10, 11, 14 and 15.

There seems to be a difference in the state of aggregation between amylose in the centre and amylose outside the granules, as well as differences in the network due to processing conditions, but transmission electron microscopy is needed to reveal differences at this dimensional level. It is impossible to say whether a difference between the amylose structure inside the granules and that outside the granules is due to concentration, fractionation of amylose molecules of different molecular weights and degree of branching or if such a difference is due to environmental factors during processing.

Conclusions

By a combination of microscopy techniques the following observations have been made on heating, cooling and cold storage of 8-11% starch dispersions.

* The presence of an amylose-rich phase in the centre of the granules already in the first stage of swelling when the amount of amylose released from the granules is limited.
* The fragmentation of the outer amylpectin layer induced by shear during the heating process.
* The deformation of swollen granules and release of amylose from the central zone on mechanical treatment.
* Aggregation of amylose on cooling. Amylose was deposited on the surface of the granules and was expected to affect their behaviour when they were reheated.

Acknowledgement

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References


Bowler P, Evers AD, Sargent J. (1987). Dehydro-

Fig. 21. Schematic drawing of a swollen granule with an enriched amylose centre with a passage through the granule at the equatorial groove.

Condition artefacts in gelatinized starches. Starch 39, 46-49.


Discussion with Reviewers

E.A. Davis: When smears were made on slides of hydrated material did this result in dehydration by air drying? What care was taken to ensure that the sample was hydrated when viewed?

Authors: Samples were applied on the object glass and stained immediately. Excess of stain solution was gently removed when the cover glass was adapted. The
cover glass was sealed with entellan to prevent evaporation and the sample was examined in the hydrated state. This is a very important point since only fully hydrated smears provide evidence for the fragmentation of granules and the distribution of fragments in the continuous amylose phase. With any of the sectioning techniques, a small particle as seen under the microscope may have been part of a bigger particle as stated in the text. Techniques were combined made and compared in order to make sure that the preparation technique did not induce any structural changes that could be misleading. The drawback of smears is the lack of resolution due to the sample thickness.

E.A. Davis: When cryosections were made, were they viewed on a cryostage for a light microscope or were they freeze-dried?

J. Grider: To fully understand the findings from the light microscopy cryosections, it is necessary to know whether the cryosections were examined at low temperatures or air dried and viewed at room temperature. When was the staining procedure done in the cryosection method?

Authors: Cryosectioning was used as a routine method for screening a large number of wheat starch samples. The cryosections were picked up on slides at room temperature and kept in a desiccator before staining. Thereafter the staining solution was added and the sections were rehydrated before examination under the light microscope. In preliminary experiments staining was done directly, before drying. No structural differences could be observed in the wheat starch samples between sections stained before and after drying.

E.M. Varriano-Marston: Liquid nitrogen is not necessarily quick-freezing because the boiling vapor causes insulation and reduced cooling. It would have been better to freeze rapidly in isopentane cooled in liquid nitrogen.

Authors: We are aware of this and small samples were rapidly frozen in a propane jet freezer for cryo-SEM. However, in our experience, rapid freezing often results in cracking of the larger samples (8 x 8 mm) prepared for light microscopy. Furthermore, rapid freezing is only possible over a very small distance and not relevant for larger samples. In order to prevent the sample from cracking, it was repeatedly dipped in liquid nitrogen.

As stated in the text, freeze artifacts were observed for wheat starch gels and no cryosections of gels are presented in the paper. Freeze artifacts were not observed in hot or cooled dispersions. This indicates that solubilized starch has a cryoprotective effect and that this effect is lost when the gel network is formed.

E.M. Varriano-Marston: Figure 4 is more like the real structure than Figure 3, where the polymers have been obviously disorganized by the fixation and critical point drying techniques. For Figure 3, you cannot tell if the particles surrounding the starch are amylose when using the SEM technique. Chemical fixation and CPD techniques have been shown to alter the polymer structure. Carbohydrate chemists have long known that polymer morphology and functionality are dramatically affected by drying techniques. These problems should be mentioned with the CPD technique.

Authors: There is a lot of documentation about drawbacks of chemical fixation, dehydration and critical point drying of biological specimens (see e.g. Boyle and Wood, 1969. But in this in mind, the cryotechnique for SEM is a very attractive approach. However, we have encountered practical problems with highly aqueous samples and we thought it would be of general interest to mention some of the difficulties. The crucial question is whether it is possible to control the sublimation of amorphous ice during preparation of highly aqueous biopolymer network structures. Despite the documented disadvantages of the CPD technique, we have obtained good results with this technique for biopolymer network structures and we have also compared this technique with other EM techniques (see e.g. Hermansson and Buchheim, 1981; Hermansson and Langton, 1988; Hermansson, 1988). In this paper we mainly wanted to illustrate the shape of the swollen granules by SEM. For detailed studies of amylose aggregates and network structures, it would be better to prepare amylose samples for TEM, for example, by the sandwich mica technique (Hermansson, 1989).

D.D. Christianson: Is the retrograded amylose in beads? It looks like this from the structures at the surface of the granules. Is there a temperature dependence with regard to the structure of amylose aggregates?

Authors: A separate study is planned to evaluate the fine structure of the amylose phase. As indicated above, we will use TEM rather than SEM for this study.

D.D. Christianson: You make a very intriguing point about phase separation of small amylopectin fragments from amylose. The kinetics should be studied of separation of both small and large fragments.

J. Grider: You make reference to additional studies showing the tendency towards phase separation of small amylopectin fragments from the amylose phase when the gel was stored at +40°C. What evidence from these studies support this conclusion?

Authors: The number of colour micrographs presented here is too limited. The separation of amylose on cooling is illustrated by a cryosection of a cooled dispersion. The effect of cooling and cold storage of gels was also studied by embedding and thin sectioning. From these micrographs we observed a tendency towards phase separation with storage. Amylopectin fragments clustered together and the amylose phase became more even. The kinetics of this process needs to be studied further.

E.A. Davis: Do you think that as amylopectin is solubilized it no longer looks "brownish" in the presence of iodine or is it mainly a concentration effect?

Authors: In this case we think that the concentration is an important factor. However, other factors may also contribute to the ability of amylopectin to stain. We have observed differences in staining intensity between wheat starch from different types of wheat and differences between potato starch subjected to different degrees of chemical modification and native potato starch.

D.D. Christianson: Is redsorption of amylose evidence for the annealing process in starch? What correlations can be expected with DSC measurements?

Authors: The annealing process of starch is not fully understood. Generally, annealing refers to rearrangements of molecules inside the starch granule to a more
ordered structure. An alternative is reabsorption of amylose and the formation of an amylose film on the surface of the granule. This may have a similar effect on physical properties in heating as changes inside the granules, and it is possible that both phenomena are involved in the, so called annealing process.

K. Ghiasi: The authors suggest the possibility of amylose coming out of the granules and going back in. This is a very interesting hypothesis. Do the authors have any other data on this?

Authors: This is a hypothesis based on observations of differences between the size and shape of the amylose zone in the centre of the granules. The thin sections obtained by the plastic embedding technique have made it possible to observe the inner zone of the granules, which is not the case for smears or cryo-sections. The size and shape of the central amylose zone of mechanically treated granules are smaller and narrower than for granules heated under static conditions, indicating that amylose has been squeezed out of the granules. The thin sections show that amylose is adsorbed on the granule surface on cooling. The possibility of amylose going back into the granule is a speculation and further studies are needed in order to elucidate the transport mechanisms of amylose.
MICROSTRUCTURE AND TEXTURE OF MEAT EMULSIONS SUPPLEMENTED WITH PLANT PROTEINS

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Abstract

A model meat emulsion system was used to evaluate the effects of cooking time and temperature on texture, microstructure and cook stability of meat emulsions containing soy or canola protein isolate. The plant proteins were incorporated either dry or rehydrated at replacement levels of 33.3% and 66.7% of the meat protein, and the emulsions were cooked at 70 or 95°C for 25 or 50 min. Texture of the cooked emulsions was assessed by an instrumental texture profile analysis (TPA) using an Instron tester. Analysis of TPA and stability data revealed several complex interactions between experimental variables; however, level of protein replacement was the predominant factor, with decreased firmness and increased yield resulting from increased replacement of meat protein.

Microstructure of the cooked emulsions was examined by light microscopy and scanning electron microscopy. Although there were slight differences in the fat particle distributions of emulsions containing plant proteins, the distributions were similar, where particles with diameters larger than 50 μm approximated a normal distribution and were thought to be relatively intact fat cells, while the number of particles with diameters of 10-50 μm increased in an essentially logarithmic manner as size decreased. Microstructure of the proteinaceous matrix was affected primarily by protein source, replacement level and cooking conditions.

Introduction

Finely comminuted meat products such as frankfurters or bologna are commonly prepared by chopping lean meat in a brine solution to form a proteinaceous slurry in which animal fat is then finely divided and dispersed. The resulting batter, resembling an oil in water emulsion (Hansen, 1960), is subsequently cooked to form a product in which fat globules are entrapped within a rigid protein matrix. Previous work has documented the role of the salt-soluble meat proteins myosin and actomyosin in emulsion formation and stabilization through coating the fat droplets with a stable membrane (Hansen, 1960; Hegarty et al., 1963; Swift et al., 1961). Reviews by Saffle (1968) and Webb (1974) considered emulsification to be the primary factor responsible for stability of such products. Other workers have suggested that too much emphasis has been placed on the importance of emulsification (van den Oord and Visser, 1973). Theno and Schmidt (1978) examined the microstructure of three commercially acceptable frankfurters and found that only one could be called a true meat emulsion. While these products may not be emulsions in the strictest sense, the term "meat emulsion" has been in common use for many years and is retained in the present study.

Attention has shifted from the emulsification properties of meat proteins to their involvement in matrix formation through thermally induced gelation, with the entrapment of fat and development of the characteristic product texture. Several recent reviews have examined this aspect (Schmidt et al., 1981; Acton et al., 1983; Schmidt, 1984). Ziegler and Acton (1984) and Asghar et al. (1985) detailed the denaturation, aggregation and gelation reactions of muscle proteins. Lee (1985) surveyed the microstructural aspects of meat emulsion formation and stabilization. He reviewed the evidence for emulsion and nonemulsion theories of fat stabilization and concluded that although both theories should be considered, from photomicrographic data and physical analysis, the nonemulsion theory should receive more consideration.

The use of plant proteins as extenders or replacements for meat protein in frankfurter-type comminuted meat products has been the subject of

Key Words: Meat emulsions, soy protein, canola protein, microstructure, texture, profile analysis, protein matrix, food lipids.

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much investigation. At high levels of replacement, the texture of these products usually becomes soft and mushy. Frankfurter processing schedules do not usually employ temperatures greater than approximately 75°C but higher temperatures are often required for denaturation and gelation of many globular proteins (Catsimpoolas and Meyer, 1970; Hermansson, 1979). The objectives of this research were to investigate the effects of processing conditions on texture, cookability, and microstructure of a model meat emulsion system in which large amounts of meat protein have been replaced with soy or canola protein isolate. Soy protein is used in a wide variety of foods including bakery products, cereals, dairy foods and comminuted meats (Kinsella, 1979). Canola is the major oilseed crop grown in Canada and northern Europe, and canola protein possesses numerous attractive functional properties (Sosulski et al., 1976; Thompson et al., 1982; Gill and Tung, 1978).

Materials and Methods

Experimental design

A model meat emulsion formulation containing 10.5% beef protein, 29% pork fat, 57.5% water, and 2.5% sodium chloride was used as a control. Beef protein was replaced with either soy or canola protein isolate at either 33.3 or 66.7% (w/w). Lauck (1975) found that the hydration state of a whey protein product influenced the stability of frankfurters. To test this effect, the isolates were added either dry or rehydrated overnight in distilled water [3:1 (w/w), H2O:isolate]. The emulsions were then cooked at 70 or 95°C for 25 or 50 min. The effects of (i) replacement, (ii) protein source, (iii) pretreatment (i.e., state of hydration), (iv) cook temperature and (v) cook time on texture and cook stability of the finished products were tested using a 2^4 fractional factorial experimental design and analysis of variance (Taguchi, 1957; Tables 1 and 2). Product microstructure was examined using light microscopy (LM) and scanning electron microscopy (SEM).

Emulsion preparation

Boneless beef chuck and pork backfat were purchased from a local abattoir, trimmed of visible fat and meat traces, respectively, minced and vacuum packaged separately in 450 g lots, then frozen at -35°C. Prior to use, beef and backfat were allowed to thaw at 4°C, then kept on ice when taken from the coldroom. Soy protein isolate [%N(d.b.)=14.56] and canola protein isolate [%N(d.b.)=14.42] were purchased from the POS Pilot Plant Corp. (Saskatoon, SK). The isolates were prepared by alkaline extraction followed by acid precipitation, neutralization, and recovery of the protein by spray drying (POS Corp., personal communication). Differential scanning calorimetry of the isolates indicated little protein denaturation.

Laboratory scale emulsion batches were prepared with a Sorvall Omnimixer (Ivan Sorvall, Inc., Norwalk, CT). The Omnimixer was modified to allow the jar to be moved up and down relative to the blades to give improved chopping of the entire sample (Morrison et al., 1971). Ground beef, salt, distilled water and plant protein were blended for 25 s at half speed, pork backfat was added, and the emulsion formed by chopping at full speed for 2 x 30 s with intermediate scraping and hand mixing. Final emulsion temperatures after chopping ranged from 16-18°C. The emulsions were then cooked at 70 or 95°C overnight in distilled water [3:1 (w/w), H2O:isolate]. The emulsions were then cooked at 70 or 95°C for 25 or 50 min. The effects of (i) replacement, (ii) protein source, (iii) pretreatment (i.e., state of hydration), (iv) cook temperature and (v) cook time on texture and cook stability of the finished products were tested using a 2^4 fractional factorial experimental design and analysis of variance (Taguchi, 1957; Tables 1 and 2). Product microstructure was examined using light microscopy (LM) and scanning electron microscopy (SEM).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Level (%)</th>
<th>Pretreatment**</th>
<th>Cook Temp. (°C)</th>
<th>Cook Time (min)</th>
<th>Rupture Force (N)</th>
<th>First Bite Hardness (N)</th>
<th>Second Bite Hardness (N)</th>
<th>Springiness (%)</th>
<th>Cohesiveness (%)</th>
<th>Cook Stab. (%)</th>
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<tbody>
<tr>
<td>Soy</td>
<td>66.7</td>
<td>r</td>
<td>70</td>
<td>25</td>
<td>15.2</td>
<td>27.4</td>
<td>19.0</td>
<td>46.9</td>
<td>0.138</td>
<td>99.0</td>
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<tr>
<td></td>
<td>66.7</td>
<td>d</td>
<td>70</td>
<td>50</td>
<td>13.3</td>
<td>25.5</td>
<td>19.0</td>
<td>39.9</td>
<td>0.148</td>
<td>98.2</td>
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<tr>
<td></td>
<td>66.7</td>
<td>r</td>
<td>95</td>
<td>25</td>
<td>17.0</td>
<td>30.1</td>
<td>22.2</td>
<td>60.8</td>
<td>0.167</td>
<td>98.6</td>
</tr>
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<td></td>
<td>66.7</td>
<td>r</td>
<td>95</td>
<td>50</td>
<td>17.5</td>
<td>30.9</td>
<td>22.5</td>
<td>49.0</td>
<td>0.152</td>
<td>97.5</td>
</tr>
<tr>
<td>Canola</td>
<td>66.7</td>
<td>d</td>
<td>70</td>
<td>25</td>
<td>35.1</td>
<td>48.2</td>
<td>36.1</td>
<td>60.1</td>
<td>0.149</td>
<td>95.9</td>
</tr>
<tr>
<td></td>
<td>66.7</td>
<td>d</td>
<td>70</td>
<td>50</td>
<td>35.1</td>
<td>48.2</td>
<td>36.1</td>
<td>60.1</td>
<td>0.149</td>
<td>95.9</td>
</tr>
<tr>
<td>All-meat</td>
<td>-</td>
<td>-</td>
<td>70</td>
<td>25</td>
<td>59.9</td>
<td>60.9</td>
<td>46.8</td>
<td>53.6</td>
<td>0.136</td>
<td>98.4</td>
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<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>70</td>
<td>50</td>
<td>62.6</td>
<td>65.6</td>
<td>50.3</td>
<td>64.6</td>
<td>0.135</td>
<td>95.5</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>95</td>
<td>25</td>
<td>56.6</td>
<td>64.1</td>
<td>52.2</td>
<td>52.2</td>
<td>0.135</td>
<td>87.3</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>95</td>
<td>50</td>
<td>66.5</td>
<td>64.9</td>
<td>52.3</td>
<td>57.8</td>
<td>0.141</td>
<td>82.0</td>
</tr>
</tbody>
</table>

*Mean of duplicate samples; **r = rehydrated, d = dry
Table 2. Instrumental texture profile analysis of protein-replaced meat emulsions; significant experimental factors

<table>
<thead>
<tr>
<th>Texture Profile Component</th>
<th>Significant Experimental Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Rupture Force (N)</td>
<td></td>
</tr>
<tr>
<td>(i) Replacement Level**</td>
<td>Control: 61.9 ± 3.5 66.7%</td>
</tr>
<tr>
<td></td>
<td>33.3%</td>
</tr>
<tr>
<td></td>
<td>37.0 ± 2.3 16.7 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>(ii) Level x Cook Temp.*</td>
</tr>
<tr>
<td></td>
<td>70°C:38.6 ± 1.9 15.4 ± 1.6 18.0 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>95°C:35.3 ± 1.2</td>
</tr>
<tr>
<td>First Bite Hardness (N)</td>
<td></td>
</tr>
<tr>
<td>(i) Replacement Level**</td>
<td>Control: 63.8 ± 2.2 66.7%</td>
</tr>
<tr>
<td></td>
<td>33.3%</td>
</tr>
<tr>
<td></td>
<td>48.3 ± 2.7 31.4 ± 4.1</td>
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<tr>
<td></td>
<td>(ii) Protein Source**</td>
</tr>
<tr>
<td></td>
<td>Soy 42.0 ± 8.7</td>
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<tr>
<td></td>
<td>Canola</td>
</tr>
<tr>
<td></td>
<td>(iii) Pretreatment x Cook Temperature*</td>
</tr>
<tr>
<td></td>
<td>70°C:37.3 ± 11.6 40.5 ± 10.9 39.4 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>95°C:42.4 ± 10.4</td>
</tr>
<tr>
<td>Second Bite Hardness (N)</td>
<td></td>
</tr>
<tr>
<td>(i) Replacement Level**</td>
<td>Control: 50.4 ± 2.5 66.7%</td>
</tr>
<tr>
<td></td>
<td>33.3%</td>
</tr>
<tr>
<td></td>
<td>36.6 ± 8.1 22.9 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>(ii) Protein Source**</td>
</tr>
<tr>
<td></td>
<td>Soy 31.4 ± 7.1</td>
</tr>
<tr>
<td></td>
<td>Canola</td>
</tr>
<tr>
<td></td>
<td>(iii) Pretreatment x Cook Temperature*</td>
</tr>
<tr>
<td></td>
<td>70°C:27.7 ± 8.6 30.0 ± 9.0 29.5 ± 6.6</td>
</tr>
<tr>
<td></td>
<td>95°C:31.8 ± 8.6</td>
</tr>
<tr>
<td>Springiness (%)</td>
<td></td>
</tr>
<tr>
<td>(i) Replacement Level**</td>
<td>Control: 57.1 ± 5.6 66.7%</td>
</tr>
<tr>
<td></td>
<td>33.3%</td>
</tr>
<tr>
<td></td>
<td>51.5 ± 5.3 45.8 ± 7.2</td>
</tr>
<tr>
<td></td>
<td>(ii) Cook Temperature**</td>
</tr>
<tr>
<td></td>
<td>70°C 52.5 ± 6.3</td>
</tr>
<tr>
<td></td>
<td>95°C</td>
</tr>
</tbody>
</table>

* p<0.05; ** p<0.01
for 6 min, stained in 0.5% Sudan Black B in propylene glycol for 10 min, then differentiated in 85% propylene glycol for 6 min and 50% propylene glycol for 2 min. These sections were not counterstained for protein as fine detail tended to be obscured. To examine the protein matrix independently, separate sections were stained with 0.17% Light Green in 0.33% acetic acid for 1 min, rinsed in distilled water, dehydrated with 90%, then 100% ethanol, then delipidated with xylene. The samples were examined and photographed under brightfield illumination with 5X and 10X objectives, using a Wild M20 microscope and a Pentax 35 mm camera. Micrographs representative of those from each treatment are shown in Figures 3 to 7. Although there have been several reports where differential staining has been used to successfully identify soy proteins (e.g., Coomaraswamy and Flint, 1973) or wheat gluten (Flint and Johnson, 1979) in meat products, in this study attempts to distinguish between canola and meat proteins by differential staining with periodic acid-Schiff reagent and Light Green (Coomaraswamy and Flint, 1973) or Toluidine Blue (Flint and Johnson, 1979) were unsuccessful.

Fat particle distributions of the emulsions containing plant proteins heated at 95°C were obtained from 18 cm x 24 cm light micrographs of sections stained for lipid (actual specimen area = 1.89 x 106 square micrometers). One micrograph was used for each treatment. As not all fat particles were spherical, the equivalent area diameter of the particles [the diameter of a circle having the same area as the particle (Davies, 1962; Silverman et al., 1971)] was obtained using a circle template, and these were classed into 32 size categories between 10 and 126 µm in diameter. As the number of fat particles within each category increased rapidly as size decreased and a manual counting method was employed, the following procedure was used to obtain an unbiased estimation of the size distributions while reducing counting time. Each micrograph was partitioned into 48 squares (3 cm x 3 cm) to facilitate counting. Fat particles with diameters of 34-126 µm (23 size categories) were counted in all 48 squares of each micrograph. Particles with diameters of 24-34 µm (4 size categories) were counted in 36 squares selected at random and the counts were projected to an estimated count for the entire field. Similarly, fat particles with diameters of 18-24 µm (2 size categories) were counted in 14 randomly selected squares, while particles of 10-18 µm in diameter (3 size categories) were counted in 5 squares. The individual fat particle distributions were then combined and averaged to give the following contrasts: (1) 66.7% soy vs. 66.7% canola protein substitution, (2) 33.3% soy vs. 33.3% canola, (3) 33.3% soy vs. 66.7% soy, (4) 33.3% canola vs. 66.7% canola, (5) soy vs. canola, and (6) 33.3% vs. 66.7% substitution. Each fat particle distribution in the contrasts was the mean of either 2 (contrasts 1, 2, 3 and 4) or 4 (contrasts 5 and 6) fields. As the distributions could not be represented by a single mathematical relationship, no statistical comparative procedures were performed.

Scanning electron microscopy

Small cubes of cooked emulsions approximately (4 mm)3 were cryofractured in liquid nitrogen by placing the cubes between 2 flat surfaces and administering a sharp blow (Theno et al., 1978). Small fragments approximately (1-1.5 mm)3 were fixed in 4% glutaraldehyde in 0.07 M phosphate buffer (pH 7.0) for 12-24 h at 4°C. After rinsing three times in phosphate buffer, secondary fixation was accomplished with 1% osmium tetroxide in phosphate buffer for 4 h. After a second set of phosphate buffer rinses, the samples were dehydrated through a graded ethanol series followed by exchange of ethanol with a graded series of amyl acetate in 100% ethanol, then 100% amyl acetate (to more easily detect completion of drying) for 1 h. The samples were dried in a Parr critical point drying bomb (Parr Instrument Co., Moline, IL) using liquid CO2, mounted on aluminum stubs, gold coated in a Technics sputter coating unit (Technics Inc., Alexandria, VA), and observed with a Cambridge Stereoscan 250 SEM (Cambridge Instruments Inc., Montreal, PQ) at an accelerating voltage of 20 kV.

Results and Discussion

Texture profile analysis

Analysis of variance of TPA data revealed a complex interaction between experimental factors and texture profile components, although replacement level was most predominant (Tables 1 & 2). For rupture force, replacement level was the only significant main effect (p<0.01) where rupture force values decreased as replacement level increased. An interaction was also found between replacement level and cook temperature (p<0.05); at 66.7% replacement of meat protein, the 95°C cook produced higher rupture force values than at 70°C, whereas at 33.3% replacement the opposite was true.

For first bite hardness and second bite hardness, replacement level was again significant (p<0.01) with decreased hardness values at increased substitution levels. Canola protein isolate produced significantly greater hardness values.
Fig. 2. Fat particle distributions of meat emulsions containing soy or canola protein: (A) 66.7% soy vs. 66.7% canola; (B) 33.3% vs. 66.7% substitution; (C) 33.3% soy vs. 33.3% canola; (D) 33.3% vs. 66.7% soy; (E) 33.3% vs. 66.7% canola; (F) soy vs. canola substitution.

than soy protein isolate (p<0.01). An interaction was found between cook temperature and state of hydration (p<0.05) with greater hardness at 70°C if the isolates were rehydrated prior to addition, whereas the 95°C cook produced the opposite effect. As replacement level increased, rupture force decreased at a faster rate than hardness.

Springiness, expressed as percent recovery from the original deformation, was influenced by
Fig. 3. 66.7% soy protein meat emulsion cooked at 95°C for 50 min: (A) and (B), light micrographs of lipid and protein staining, respectively, (bar=200μm); (C) SEM micrograph (bar=100μm). F and P indicate fat and protein, respectively. Arrows indicate deformed fat particles (Fig. 3C).

Fig. 4. 66.7% canola protein meat emulsion cooked at 95°C for 50 min: (A) and (B), light micrographs of lipid and protein staining, respectively, (bar=200μm); (C) SEM micrograph (bar=100μm). Arrows (Fig. 4C) indicate pores.

Fig. 5. 33.3% soy protein meat emulsion cooked at 95°C for 25 min: (A) and (B), light micrographs of lipid and protein staining, respectively, (bar=200μm); (C) SEM micrograph (bar=100μm).

two factors; replacement level (p<0.01), where greater springiness was found with 33.3% than 66.7% replacement, and cook temperature (p<0.01), where 95°C produced springier products than 70°C. For the control emulsions, cook time appeared to be more important for springiness than temperature, with a 50 min cook producing
Cook stability

The treatments had small but statistically significant effects on cook stability. Stability was affected by replacement level (98.5% yield at 66.7% replacement vs. 97.2% at 33.3% replacement; p<0.01), cooking temperature (98.6% at 70°C vs. 97.1% at 95°C; p<0.01), cooking time (98.1% at 25 min vs. 97.7% at 50 min; p<0.05) and protein source (canola, 98.1% vs. soy, 97.8%, p<0.05), as well as interactions between protein source and pretreatment (p<0.05), protein source and cooking time (p<0.05), and cook temperature and replacement level (p<0.01). These small variations are probably unimportant from a practical standpoint when compared to the all-meat control emulsions, in which yield varied from 82.0% with a 95°C, 50 min cook to 98.4% with a 70°C, 25 min cook. These data are consistent with the work of Randall et al. (1976) and Sosulski et al. (1977).
who attributed improved cook stability of frankfurters containing plant proteins to increased water holding capacity, and Schut (1976) who described decreased water holding capacity of meat proteins with increased severity of thermal treatment as being due in part to protein denaturation, coagulation and shrinkage.

Microstructure

Fat particles ranged in size from less than 1 μm to 130 μm in diameter. The distributions of fat particles with diameters of 10-128 μm were obtained for emulsions containing plant proteins (Fig. 2).

Fig. 3A shows the fat particles in a 66.7% soy-substituted emulsion cooked at 95°C for 50 min. The proteinaceous matrix (Fig. 3B) had an open, lacy appearance with regularly spaced areas of more densely staining protein material. An SEM micrograph (Fig. 3C) showed fat particles embedded in the proteinaceous matrix, several of which appeared to be deformed perhaps due to coalescence during cooking. The 66.7% canola-substituted emulsions had a greater number of fat particles with diameters of 10-50 μm and fewer particles with diameters greater than 50 μm (Fig. 2A and 4A) and the protein matrix had a more compact and less lacy appearance than the 66.7% soy emulsion (Fig. 4B and C). Also seen were a number of pores and openings in the fat particles (Fig. 4C, arrows) which are similar to those reported by Jones and Mandigo (1982) who suggested that the pores may serve as a pressure release mechanism for the fat globules during cooking. These were seen in other samples as well. At this replacement level the canola emulsions had firmer texture but were less springy than those containing soy protein. It was noted that in addition to having poorer textual attributes, emulsions containing plant protein that were cooked at 70°C were very difficult to section for LM and tore easily during staining so were not used for determination of fat particle distributions. These effects may be attributable to greater thermal denaturation and enhanced gelation of plant proteins under the more severe cooking conditions.

All fat droplet distributions (Fig. 2) had similar shapes; the large droplets (greater than approximately 50 μm in diameter) approximated a normal distribution, while below 50 μm the particle number increased in an essentially logarithmic manner. The size range of the large droplets was very similar to that reported by van den Oord and Visser (1973) and Lee (1985) for the cell diameter of adipose tissue. Thus it appears as though the fat particle distributions were the result of relatively intact fat cells as well as a wide range of finely dispersed particles that resulted from fat disintegration during the comminution process. The microstructure of meat emulsions is influenced by such factors as the types of meat and fat, the levels of fat, moisture and salt, the comminution process (e.g., chopping speed, emulsion viscosity), and cooking conditions (Ackerman et al., 1971; Lee, 1985).

The microstructure of a 33.3% soy-substituted emulsion is shown in Fig. 5. At this replacement level, the protein matrix of both soy and canola-substituted emulsions had a tighter, less lacy appearance than at 66.7% replacement, whereas the matrix of the canola emulsion (not shown) again appeared somewhat more compact than the soy emulsion. The fat particle distribution of the 33.3% substituted emulsions showed fewer particles with diameters greater than 15 μm than the 66.7% substituted emulsions (Fig. 2A), but a greater number of particles with smaller diameters. As with the 66.7% replacement level, the 33.3% canola emulsions had a greater number of fat particles with diameters less than 50 μm as compared to the 33.3% soy emulsions, while the 33.3% soy emulsions had more particles larger than 50 μm in diameter (Fig. 2C). The 66.7% soy emulsions had more fat particles between 10 and 100 μm in diameter than were found in the 33% soy emulsions (Fig. 2D) while 66.7% canola emulsions had more particles between 20 and 50 μm and greater than 90 μm than 33% canola emulsions (Fig. 2E). Overall, both soy and canola emulsions showed similar numbers of large particles (Fig. 2F) but canola emulsions had more particles smaller than 50 μm in diameter. As the fat particle distributions were estimated by a manual counting technique, only a small number of fields could be examined, which limited the reliability of the data. Recent developments in Image analysis, which allow for rapid collection and processing of this type of data, should greatly increase the efficacy of analyzing not only the fat particle distribution but also the matrix structure of meat emulsions (Kempton et al., 1982; Kempton and Trupp, 1983). The significance of fat particle size distribution on texture is unclear; Cassens and Schmidt (1979) observed that commercial processed meats tended to have an inverse relationship between fat particle mean size, diameter and resistance to penetration, but in the present investigation the effect of particle size would appear to be secondary to factors influencing strength of the protein matrix.

It is interesting to note that greater rupture force was obtained with a 95°C cook at 66.7% replacement, whereas the opposite tendency was found at 33.3% replacement, especially with soy protein. It would appear that at 66.7% replacement the functional behavior of the non-meat proteins was more pronounced. While at 33.3% replacement, the meat protein predominated. Siegel et al. (1979) suggested that isolated soy protein interferes with gel-forming interactions between myosin molecules. King (1977) found an interaction between the 75 fraction of soy protein and myosin when exposed to temperatures in the 75-100°C range, while Peng et al. (1982a,b) reported an interaction between the 11S fraction of soy protein and myosin at temperatures greater than 85°C. Since these interactions take place at temperatures which are greater than those generally used in comminuted meat products, high levels of non-meat proteins probably act only as a diluent, decreasing meat protein interactions and gelation, and resulting in softer product texture when cooked at normal processing temperatures.

Microstructure of all-meat control emulsions varied with processing conditions. With a 70°C, 25 min cook (Fig. 6) the distribution of fat particles appeared fairly similar to that of the
Plant Proteins in Meat Emulsions

33.3% replacement emulsions, but with a slightly more open matrix. The large lipid droplets were also more oblong than in the samples containing plant protein, perhaps reflecting the increased viscosity of the system and an orientation effect during stuffing. In the sample cooked at 95°C for 50 min (Fig. 7) there were more large and intermediate size droplets, perhaps as a result of shrinkage of the matrix and droplet coalescence (Fig. 7C, arrow). This sample had greater firmness and springiness than that shown in Fig. 6 but also had much lower yield, which was probably a contributing factor to the textural and microstructural differences.

Several authors have attempted to relate functional properties of non-meat proteins to their performance as ingredients in comminuted meat products. Thomas et al. (1973) and Lauck (1975) suggested a relationship between apparent solubility and hydrophobicity, meat proteins but also as a result of coagulation and gelation of the added proteins. Torgersen and Toledo (1977), working with novel proteins in a comminuted meat system, found a significant negative correlation between protein solubility and fat binding, as well as solubility at 100°C and textural mechanical properties. Comer (1979) indicated that the performance of fillers in comminuted meat products was better correlated to the matrix. Cassens et al. (1984) related the stability and firmness of frankfurters to protein source, plant proteins. Therefore, by the time the plant proteins were able to contribute to structure formation, the matrix had already set. The observed differences in fat droplet distribution and matrix appearance may have resulted from the thermal processing conditions as well as decreased apparent viscosity of the emulsions as replacement levels increased (Volsey and Randall, 1972), which would affect the mobility of the fat droplets during comminution and their resistance to the cutting action of the Omnimerx blades. As noted by Froning and Neelakantan (1971), for chicken frankfurters showing greater tensile strength there was greater uniformity in the appearance of the fat globules and a heavier matrix of protein surrounding the fat globules. This may also be a contributing factor to the textural differences found between protein replacement levels in the present study.

Acknowledgements

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References


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Siegel DG, Church KE and Schmidt GR. (1979). Gel structure of non-meat proteins as related to their ability to bind meat pieces. J. Food Sci. 44, 1276-1279,1284.


Discussion with Reviewers

P. Allan-Wojtas: What is the origin of the methods you used for staining fat and protein?
Authors: Staining meat emulsions with Light Green for protein or with Sudan Black B for lipid have been used routinely in a number of laboratories (e.g., Kempton and Trupp, 1983 (Light Green); Ackerman et al., 1971 (Sudan Black B)). The specific methods we used were modifications that we found to be suitable for our studies.

A.-M. Hermansson: To what extent is the fat extracted and recondensed in the structure during the dehydration and critical point procedure and how has the result been checked for artifacts due to redistribution of fat particles during preparation?
Authors: Conventional fixation techniques (glutaraldehyde and osmium tetroxide) were used to preserve protein and lipid structure. If fat is extracted and recondensed or redistributed, this should be readily apparent by the presence of numerous fat globules that appear "out of place", i.e., not associated with a corresponding void in the matrix or just sitting on the surface. We did not observe features such as these in our micrographs.

P. Allan-Wojtas: Why did you analyze the light micrographs by the methods you described?
Authors: As we did not have access to an image analyzer, we had to rely on a manual counting technique to obtain fat particle size data. We decided to determine particle size distribution rather than an arithmetic mean and standard deviation because there appeared to be a lack of distribution data in the meat emulsion literature. We were limited to the number of fields that could be examined, however, because of the laborious nature of manual counting methods. The "equal area diameter" is a rapid and reasonably accurate method for obtaining a single measurement for non-spherical particles, and a circle template was used with photomicrographs rather than direct observation and measurement with an eyepiece graticule because more size categories could be obtained with less fatigue using the template method. Although we would have preferred to examine more than one field per treatment, we felt that since different micrographs within each treatment were very similar and the treatment contrasts were based on mean values from at least two fields, the particle size distributions would provide sufficient complementary information to the textural and stability data and would be superior to data based on mean particle size. In light of the bimodal nature of the distributions, we feel that our choice was justified, as merely obtaining a mean particle size and standard deviation would be misleading and statistically incorrect.

C.J. Randall: Would the meat industry use isolates in the manufacture of meat emulsion type products, given their cost in relation to flours and concentrates?
Authors: Yes, provided that the isolates, because of their greater functionality, provide benefits in product quality (by either enhancing or maintaining quality while replacing other components) that outweigh their cost. Non-meat fillers have been used mainly at low levels to improve water and fat binding, and for this application there is little real advantage in using protein isolates. We are seeing work now, though, where plant proteins are being modified in various ways to improve their functionality, especially the ability to form rigid gels at temperatures used in meat processing. If these improved isolates can be provided at a reasonable cost, we can expect to see their increased use in the future.

P. Allan Wojtas: Why wasn't transmission microscopy used? Plant and muscle proteins can be distinguished using this technique.
Authors: The high resolution and histochemical opportunities afforded by TEM have indeed been valuable in previous studies on meat emulsions. As our study was in many respects a screening experiment where we examined the effects of a relatively large number of treatments on texture and cook stability, we felt that sufficient complementary microstructural information would be provided by LM and SEM. In future studies where we wish to study fewer variables in greater depth, we will certainly consider using TEM.
EFFECTS OF PROTEIN CONCENTRATION IN ULTRAFILTRATION MILK RETENTATES AND THE TYPE OF PROTEASE USED FOR COAGULATION ON THE MICROSTRUCTURE OF RESULTING GELS

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Abstract

Milk retentates (35% total solids, 13% protein) obtained by ultrafiltration were diluted with the permeate to 3.2, 5.0, 6.5, 10.0, and 13.0% protein and coagulated using commercial proteases. Rennet or one of microbial proteases isolated from Bacillus polymyxa, Endothia parasitica, Mucor miehei, or Mucor pusillus were used. Coagulation times were decreased with the Mucor proteases as the protein concentrations in the retentates were increased but the coagulation times were increased with B. polymyxa and E. parasitica proteases under similar conditions. Firmness was higher in gels made from homogenized retentates than from nonhomogenized retentates of the same protein concentrations. Scanning as well as transmission electron microscopy showed increasing densities of the protein matrices in the gels as the protein concentrations were increased. Large fat globules and their clusters were noticeable in gels made from nonhomogenized retentates. Gelation of homogenized retentates resulted in uniform matrices with the dimensions of the dis-integrated fat particles similar to those of casein micelles. These minute fat particles were closely associated with the protein matrix. Firm gels made by coagulating the retentates with rennet consisted of extensively branching casein particle chains whereas softer gels made with B. polymyxa protease consisted of small casein particle clusters.

Introduction

Ultrafiltration (UF) of milk is rapidly becoming one of the most important processes in the dairy industry, particularly because it reduces the volume of the milk by removing a substantial amount of water from it. A part of lactose and mineral salts present in the milk is also removed. Thus, the retentate has a higher protein content than the original milk whereas the lactose and mineral contents remain at about the same levels as in the milk.

The use of milk retentates in cheese manufacture has, therefore, numerous advantages which are of both technological and economical nature. The most important advantages are higher yields (5-15%), continuity of processing following the introduction of automation, decrease of up to 50% in the requirement for starter and rennet, increased nutritive value because whey proteins are retained in the product, a lower biological oxygen demand for the breakdown of the waste materials, and a closed sanitary system. However, complications may arise from differences in the chemical composition and physical properties of the retentates and the milk, e.g., the changed ratios of the individual components resulting in an increased buffering capacity of the retentate, a higher calcium concentration in it, and a higher viscosity of the retentate. The effects of proteolytic enzymes other than calf rennet (which is in short supply on the global scale) on UF retentates may also differ from the effects of this enzyme on milk. Only proteases isolated from a few species of fungi, such as Mucor miehei, M. pusillus, and Endothia parasitica, and bacteria, such as Bacillus subtilis, B. cereus, and B. polymyxa meet the criteria for good quality cheese production.

The objective of the study was to examine the effects of commercial proteolytic enzymes of various origins on the coagulation of UF retentates having varying protein concentrations, to test the resulting coagula for firmness, and to correlate firmness and the microstructure of the protein matrices as seen under the electron microscope.

KEY WORDS: Bacillus polymyxa protease, Electron microscopy, Endothia parasitica protease, Gelation of milk, Homogenization, Microbial proteases, Milk retentates, Mucor miehei protease, Mucor pusillus protease, Proteolytic enzymes, Ultrafiltration of milk.
Preparation of milk retentates and which contained 3.2% protein fat. The milk was concentrated at 55°C by UF to
ed into two aliquots, one of which was repasteur­
tate outlet were
the final concentration of 36% total sol ids using
pared.

Table 1. Effect of ultrafiltration on chemical composition of milk retentates

<table>
<thead>
<tr>
<th>Component</th>
<th>Milk</th>
<th>Total solids (%)</th>
<th>Fat (%)</th>
<th>Lactose (%)</th>
<th>Casein (%)</th>
<th>Whey proteins (%)</th>
<th>Total nitrogen (%)</th>
<th>Nonprotein nitrogen (%)</th>
<th>Ash (%)</th>
<th>Calcium (mM/L)</th>
<th>Phosphorus (mM/L)</th>
<th>Acidity (°SH)</th>
<th>Acidity (pH)</th>
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<tr>
<td></td>
<td></td>
<td>12.13 ± 0.21</td>
<td>3.24 ± 0.13</td>
<td>3.87 ± 0.10</td>
<td>4.30 ± 0.18</td>
<td>2.74 ± 0.12</td>
<td>0.37 ± 0.04</td>
<td>0.51 ± 0.02</td>
<td>0.021 ± 0.0041</td>
<td>0.71 ± 0.03</td>
<td>27.88 ± 0.66</td>
<td>30.47 ± 1.58</td>
<td>6.61 ± 0.03</td>
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<tr>
<td></td>
<td>total solids (%)</td>
<td>34.77 ± 0.85</td>
<td>13.14 ± 0.13</td>
<td>16.44 ± 0.59</td>
<td>3.65 ± 0.60</td>
<td>11.75 ± 0.15</td>
<td>1.28 ± 0.19</td>
<td>2.06 ± 0.21</td>
<td>0.017 ± 0.0021</td>
<td>1.46 ± 0.07</td>
<td>93.80 ± 5.02</td>
<td>84.46 ± 4.68</td>
<td>16.79 ± 1.04</td>
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<tr>
<td></td>
<td>Retentates</td>
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<tr>
<td></td>
<td>Nonhomogenized</td>
<td>35.13 ± 0.58</td>
<td>13.07 ± 0.03</td>
<td>16.57 ± 0.41</td>
<td>4.00 ± 0.24</td>
<td>11.73 ± 0.13</td>
<td>1.23 ± 0.08</td>
<td>2.05 ± 0.20</td>
<td>0.017 ± 0.0002</td>
<td>1.42 ± 0.02</td>
<td>96.19 ± 3.60</td>
<td>84.15 ± 1.25</td>
<td>16.75 ± 1.32</td>
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<tr>
<td></td>
<td>Homogenized</td>
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A total of 15 milk batches and 15 retentate batches were analyzed. Relatively large standard deviations are
the result of milk batches obtained from various sources over a period of time.

Materials and Methods

Preparation of milk retentates and their analysis

High-temperature short-time (HTST) pasteur­
ized commercial milk was standardized to 3.9% milk
fat. The milk was concentrated at 55°C by UF to
the final concentration of 38% total solids using
an industrial line Pasilac DDS Model 35 equipment
(Nakskov, Denmark) furnished with GR 61PP mem­
branes. Pressures at the milk inlet and the reten­
tate outlet were 3.0 bar (300 kPa) and 1.5 bar
(150 kPa), respectively. Each retentate was divid­
ed into two aliquots, one of which was repasteur­
ed at 81°C for 10 s and homogenized at 70 bar
(7 MPA). The nonhomogenized and homogenized reten­
tates were diluted with the permeate to obtain
solutions containing 5.0, 6.5, 10.0, and 13.0% protein. Their mean fat contents were 6.0, 7.9,
12.3, and 16.2%, respectively. The original milk
which contained 3.2% protein (3.9% fat) was used
for control. A total of 15 retentates were pre­
pared.

The following parameters were determined in
the retentates and retentate gels:

Dry matter was determined by drying at
102 ± 2°C according to the AOAC methods [4]. Milk
fat content was determined by the method of Gerber
[4]. Total and nonprotein nitrogen was measured
according to the AOAC methods [4]. Lactose was
determined according to the method recommended by
the International Dairy Federation [22]. Ash was
determined by mineralization at 550°C [4]. Calcium
was determined using a complexometry technique
[26] while phosphorus was determined by spectro­
photometry [4].

Preparation of retentate gels

Protease solutions (a) to (e), 100 mL each,
were prepared by dissolving the following: (a)
Rennet (Astra, Nova Pazova, Yugoslavia), 1.00 g.
(b) Mucor miehei protease (Novo Industry, Copen­
hagen, Denmark), 0.25 g, (c) Mucor pusillus prote­
ase (Meito Sanyo Co., Tokyo, Japan), 0.15 g, (d)
Endothia parasitica protease (Pfizer Chemie, Wies­
baden, Federal Republic of Germany), 1.00 mL,

Fig. 1. Effect of protein concentrations in UF nonhomo­
genized retentates on their coagulation times using
rennet (10, 20, 30, 40, and 50 mg/50 mL) as the
coagulant.

Fig. 2. Effect of protein concentrations in UF nonhomo­
genized retentates on their coagulation times using
Bacillus polymyxa protease (4, 8, 12, 16, and
20 mg/50 mL) as the coagulant.
measurements were carried out using a consistometer (VEB Praefgeräte-Werk, Medingen, Dresden, German Democratic Republic). Gels were established using the method by Follman [11].

Determination of coagulation time

The onset of the primary and secondary coagulation phases, induced by fungal proteases [3], were established using the method by Foltman [11] developed for rennet. Coagulation curves were obtained by plotting reciprocal values of the enzyme concentrations (1/c) against coagulation time (t). The slope of the resulting line indicated the duration of the primary phase, and the intercept for t = 0, indicated the duration of the secondary phase.

Firmness measurement

Firmness was measured in gels made from homogenized as well as nonhomogenized retentates containing 5.0, 6.5, 10.0, and 13.0% protein. The measurements were carried out using a Höppler consistometer (VEB Prüfgeräte-Werk, Medingen, Dresden, German Democratic Republic). Gels containing 3.2 and 5.0% protein were tested with a spherical weight of 40.2 g (diameter of 85 mm, 0.71 g/cm²) and gels containing 6.5, 10.0, and 13.0% were tested with a weight of 99.0 g (diameter of 60 mm, 3.51 g/cm²). Firmness was expressed in the depth (mm) to which the weights penetrated the gels 30 or 45 min after gelation.

Electron microscopy

The gels were sampled for electron microscopy according to a formula: Sampling Time = 3 x Coagulation Time. Gel particles, approximately 10 mm in diameter, were fixed in a 2.85 glutaraldehyde solution at 20°C for 24 h and mailed to Ottawa for electron microscopy [1].

For scanning electron microscopy (SEM), the particles were trimmed to form prisms, approximately 10 mm long, 1 mm² in cross section. In order to visualize fat globules, selected samples were postfixed in an imidazole-buffered 2% osmium tetroxide solution [2]. All samples were then dehydrated in a graded ethanol series. Fat was removed by extraction with chloroform from samples which had not been postfixed with osmium tetroxide. All samples were impregnated with absolute ethanol, frozen in Freon 12 at -150°C, and freeze-fractured under liquid nitrogen. The fragments were thawed in ethanol, critical-point dried from carbon dioxide, mounted on SEM stubs, sputter-coated with gold, and examined at 20 kV in an ISI O5-130 scanning electron microscope equipped with an external oscilloscope [5]. Micrographs were taken on 35 mm film.

For TEM, the samples fixed in glutaraldehyde were cut into particles approximately 1 mm in diameter and postfixed in a 2% osmium tetroxide solution in a 0.05 M veronal-acetate buffer, pH 6.75. The postfixed samples were embedded in a Spurr's low-viscosity medium (J. B. EM Service, Ultrafiltration Milk Retentate Gels

<table>
<thead>
<tr>
<th>Table 2. Effect of protein concentration in homogenized (H) and nonhomogenized (NH) UF retentates on gel firmness</th>
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<tr>
<td>Protease used</td>
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<td>----------------</td>
</tr>
<tr>
<td>Rennet</td>
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<td></td>
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<tr>
<td>M. nichel protease</td>
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<td>M. pusillus protease</td>
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<td>E. parasitica protease</td>
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<td>B. polymixa protease</td>
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* Gels containing 3.2% and 5.0% protein were measured using a probe with a force of 0.71 g/cm² and gels having higher protein concentrations were measured using a probe at 3.51 g/cm².

Firmness values which are marked in each column with identical superscript (a to f) do not significantly (P<0.05) differ from each other.

(e) Bacillus polymixa protease (Godo Shusei Co., Tokyo, Japan), 0.45 g.

Coagulating activities of the individual enzymatic preparations were not determined but the enzyme concentrations were adjusted in such a way that similar coagulation times were obtained. The need to use working enzyme solutions which varied in concentration indicates that specific activities of the enzyme preparations differed from each other. Standardization of the coagulation time was introduced in order to compare other characteristic properties of the retentates [14].

Retentate aliquots (50 mL) were coagulated in glass beakers using the diluted protease solutions (1 mL). The temperatures of coagulation were 29.0, 32.0, and 35.0°C for gels made at pH 6.65; the temperature of 32°C was also used for gels made at pH 6.5 and 6.5. The retentates were adjusted to 2 M NaOH or 2 M HCl to obtain the predetermined pH values.

Determination of coagulation time

The onset of the primary and secondary coagulation phases, induced by fungal proteases [3], were established using the method by Foltman [11] developed for rennet. Coagulation curves were obtained by plotting reciprocal values of the enzyme concentrations (1/c) against coagulation time (t). The slope of the resulting line indicated the duration of the primary phase, and the intercept for t = 0, indicated the duration of the secondary phase.
Pointe Claire, Dorval, Quebec, Canada). Thin sections were stained with uranyl acetate and lead citrate solutions [29] and examined in a Philips EM-300 electron microscope operated at 60 kV [23].

Results and Discussion

Chemical composition

Ultrafiltration of milk resulted in considerable changes in its chemical composition. Individual analyses of the 15 retentates prepared were reported elsewhere [14]. Mean values have been summarized in Table 1. The total solids content of 12.1% in the initial milk was increased by ultrafiltration to 35%. In a similar way, protein was increased from 3.2% to 13.1% and fat was increased from 3.9% to 16.5%. Lactose and nonprotein nitrogen contents were slightly lower in the retentates. This is to be expected as they are likely to be at the same concentration in the aqueous phase, which is a smaller proportion of the total in the retentates. The relatively high standard deviations were the result of milk batches obtained from various suppliers. Calcium and phosphorus, which in the form of calcium phosphate are part of the casein micelles, were retained in the retentates and their concentrations were increased from 27.9 and 30.5 mM/L, respectively, in the milk to 93.8 and 84.4 mM/L, respectively, in the retentates.

Coagulation time

In order to study the effect of protein concentration on the gelation of the retentate, the final retentates were diluted with the permeate in varying proportions. As the protein concentrations in the retentate solutions were increased, the times needed to coagulate them with rennet, and the W. miehei and W. pusillus proteases decreased...
but increased when the *E. parasitica* and the *B. polymyxa* proteases were used. Results on this subject have been published in detail elsewhere [14]. *Mucor* proteases and proteases obtained from *E. parasitica* and *B. polymyxa* have shown, in general, a higher proteolytic activity than rennet. *E. parasitica* protease, e.g., hydrolyzed 25% of the peptide bonds in casein compared to only 10.2% of the peptide bonds hydrolyzed by rennin [32]. Because the *E. parasitica* protease hydrolyzes whey proteins in addition to the caseins (κ-casein as well as αs- and β-casein), this action has to be taken into consideration when selecting a protease for the manufacture of a particular cheese variety [15].

Characteristic examples obtained with nonhomogenized retentates are shown in Figs. 1 and 2 using rennet and *B. polymyxa* protease, respectively, to coagulate the milk proteins. Coagulation times with homogenized retentates were shorter than the coagulation times with nonhomogenized retentates.

The reduction in the coagulation times, as the protein concentrations of the retentates coagulated with rennet were increased, may be attributed to a number of factors such as an increased number of effective collisions between the enzyme and the casein molecules [21], a lower number of indispensable hydrolyzed casein micelles to start coagulation [13], and a high concentration of calcium although most of it was in colloidal form. The properties of the *M. miehei* and *U. pusillus* proteases were anticipated to be similar because both enzymes belong to so-called aspartic proteases [12]. In contrast, the *B. polymyxa* protease belongs to metallotripeases, i.e., a group of enzymes considerably different from the previous group as far as their structure and mechanism of splitting protein molecules are concerned.

These differences may be responsible for the rate of the proteolysis of κ-casein and the final value of coagulation time.

The behaviour of the *E. parasitica* protease in the retentates having higher protein concentrations differed from the anticipated behaviour. This enzyme possesses an aspartate group in the active centre and, thus, should follow the proteolytic pattern of the other proteases of fungal origin. However, the absence of the carbohydrate moiety in the molecule of this enzyme, which is in contrast to the *Mucor* proteases [10], a different structure of this enzyme and a different pH of optimal activity [28] considerably affected the behaviour of this enzyme in the presence of high-protein substrates.

However, M. L. Green (personal communication) has suggested that the differences in the behaviour of the proteases relate to the differences in specificity rather than to differences in structure. The Michaelis-Menten equation predicts that the subsidiary substrates will compete better with the main substrate as the total substrate concentration increases. This will have a considerably greater effect on the relatively nonspecific enzymes. Data in Table 2 also suggest that nonspecific proteolysis is favoured in the more concentrated milk retentates.
TEM of gels made from nonhomogenized milk (Fig. 8A: 3.2% protein) and from a nonhomogenized diluted UF retentate (Fig. 8B: 6.5% protein; Fig. 8C: 13% protein) using rennet. Fat globules (F) are relatively evenly distributed in the protein matrix (C) composed of casein micelle chains in the low-protein gels (Figs. 8A and 8B) but appear to be in the form of clusters in the 13% protein gel (Fig. 8C). There are void spaces (V) around most fat globules with only a few points of contact (arrows) between the fat globules and the protein matrix.

Fig. 9. Details of the protein matrices in gels made by rennet from a nonhomogenized UF retentate at varying protein concentrations (Figs. 9A: 5%; Fig. 9B: 6.5%; Fig. 9C: 10%, and Fig. 9D: 13%). Branching of the casein micelle chains (arrows) is increased with the increased protein concentration and may give the impression of casein micelle cluster formation.
Gel firmness

Gel firmness was another property of the gels where correlations with the protein concentration and the type of enzyme used have been clearly established. These characteristics have been presented and discussed in detail elsewhere [14] in an attempt to analyze the relationships between the nature of the proteolytic enzymes of various origins used and the rheological properties of the retentate gels formed.

It is evident from Table 2 that gel firmness was affected by the time when it was measured. The firmness was found to be higher, i.e., the depth of the penetrating probe was decreased, when the gels were measured 45 min after gelation as compared to measurements carried out 30 min after gelation. This difference may be explained by proteolysis extended for another 15 min leading to a more advanced aggregation of casein micelles and formation of a higher number of linkages between them. In comparison to milk, interactions of casein micelles were facilitated in high-protein retentates. According to Storry and Ford [31], gel formation in milk starts about 2.5 min after coagulation time but Mocquot [25] reported that in retentates, gelation started considerably sooner. Although the observed start of gel formation depends on the measurement method [18], accelerated gelation in the retentates may be explained by shorter distances between the micelles. Whereas in milk the mean distance between 2 casein micelles is approximately 3 micelle diameters, in milk retentates the casein micelles are, on an average, only about 1 micelle diameter apart [18]. The higher curd firmness in the retentates than in the milk is related to the increased solids content in the retentates rather than to the rate of casein aggregation [17].

In the experiments summarized in Table 2, the firmest gels were obtained using rennet and the softest gels were produced by the B. polymixa protease. The differences were relatively smaller in gels made from nonhomogenized retentates containing 3.2% protein (1.19 mm penetration depth for rennet vs. 1.68 mm for B. polymixa protease) than in gels containing 13% protein (0.72 mm vs. 1.88 mm, respectively).

Effects of protein concentrations and homogenization on firmness of the resulting gels are closely related to their structures and will be discussed below.

Gel structure

In general, there are two different microstructures of the milk retentate gels under study depending on whether the retentates had been or had not been homogenized prior to coagulation. In gels made from nonhomogenized retentates, large fat globules and their aggregates were embedded in a protein matrix which was formed by casein micelle chains and clusters. The heterogeneity of these gels is noticeable even at a low magnification. In contrast, gels made from homogenized retentates consisted of protein matrices in which minute fat globules were evenly distributed. In both kinds of gel, the densities of the protein matrices varied depending on the protein content of the gels. This is shown by both scanning electron microscopy (SEM) and transmission electron

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Fig. 10. Gels made by rennet from homogenized milk and UF retentates from which fat had been removed prior to the SEM examination. Fig. 10A: 3.2% protein; Fig. 10B: 5% protein; Fig. 10C: 13% protein. Void spaces (V) are considerably smaller than in the gels made from nonhomogenized milk and UF retentates.
microscopy (TEM) using gels made from nonhomogenized retentates. In the SEM micrographs, void spaces in the protein matrices were noticeable (Figs. 3 - 7), The largest void spaces were present in gels made from the initial milk (3.2% protein) but it was difficult to assess as to whether all the void spaces had initially been occupied with fat (Fig. 3A) prior to the extraction of the fat during the preparation of the samples for SEM. Fixation of the fat globules with imidazole-buffered osmium tetroxide [2] revealed (Fig. 3B) that fat had occupied only some of the void spaces whereas others had been filled with whey. The fat globules and their aggregates were not associated with protein. They were surrounded with void spaces and only a few points of contact with the protein matrix could be seen (Fig. 4B). As the protein content of the gels was increased, the protein matrices became denser (Figs. 4 - 7). In gels containing 10.0 or 13.0% protein (Figs. 6 and 7, respectively), the presence of fat globules and their clusters was clearly evident from the void spaces in the protein matrices. Most such void spaces contained residues of the fat globule membranes.

These findings were confirmed by TEM. Large fat globules were present in all gels made from nonhomogenized retentates (Fig. 8). Only a small proportion of the fat globules was associated with protein and void spaces surrounded individual fat globules in the low-protein gels (Figs. 8A and 8B). Thus, no imprints by the fat globules have been left in the protein matrix following their removal in contrast to the high-protein gels, where the casein particles were tightly packed with no free space left around the fat globules (Fig. 8C). Individual fat globules having diameters of up to 5 μm prevailed in low-protein gels but were mostly clustered in high-protein gels.

A great proportion of the protein was in the
Ultrafiltration Milk Retentate Gels

Homogenization of the retentates considerably reduced the dimensions of the fat globules. Coagulation of homogenized retentates resulted in gels having more uniform structures (Fig. 10) than the gels made from nonhomogenized retentates. Although the differences were small in the gels made from the initial milk (3.2% protein) as follows from a comparison of Figs. 3A and 10A, they were quite noticeable as the protein concentrations were increased, as may be seen by comparing Figs. 4A and 10B (5% protein) and Figs. 7 and 10C (13% protein). Because of the increased homogeneity of the gels made from homogenized milk and UF retentates, higher magnifications than those used with gels made from nonhomogenized retentates had to be used to show the characteristic features. The disintegrated fat particles were relatively evenly distributed throughout the protein matrices (Figs. 11 and 12). SEM of a gel made from homogenized milk (3.2% protein) from which fat had been extracted (Fig. 11A) shows that protein had been attached to the fat particles. This finding was confirmed by retaining the fat particles in the gels (Figs. 11B-11D) and also by TEM. The latter technique showed the association between the small fat globules and protein particularly well (Figs. 12 and 13). The protein attached to the fat globules is part of the matrix, thus linking the neighbouring fat globules and making them part of the entire gel structure.

The micrographs shown above are in agreement with the findings that homogenization results in the disintegration of the original fat globules into smaller droplets, and that the combined surface of the fat particles, which is increased 5 to 6 fold [7], becomes rapidly covered with milk proteins [20]. This coating facilitates the integration of the minute fat particles with the protein matrix and contributes to a higher firmness in the gels made from homogenized retentates (Table 2). The participation of homogenized fat particles in the formation of the gel structure is particularly clearly noticeable in TEM micrographs (Figs. 12 and 13). The micrographs in Fig. 12 are of the same magnification as Fig. 9 to make a comparison of the structures possible. The close association of the homogenized fat particles with the protein component of the gels is particularly well evident at a higher magnification in Fig. 13, where a gel obtained using the M. miehei protease is shown. In contrast, the large fat globules and their clusters in gels made from nonhomogenized retentates are not in close contact with the protein component, as was shown in Figs. 3B, 4B, 8A, and 8B) and probably act as weak areas in the gel matrices making the gels softer. Apparently, these findings

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**Fig. 12.** TEM of a gel made from homogenized milk (3.2% protein, Fig. 12A) and a gel made from a homogenized UF retentate (13% protein, Fig. 12B). Both gels were made by rennet. Homogenized fat particles (F) are coated with protein particles (arrows) and are integrated in the protein matrices.

**Fig. 13.** Detail of protein (arrowheads) adhering to homogenized fat particles (F) in a gel made from a UF retentate (13% protein) using M. miehei protease. Casein micelle chains (large arrows) connect the fat particles with each other. Minute black particles (small arrows) are an artefact [27].
Details of the protein matrices (fat removed) of gels made from a homogenized UF retentate (13% protein) using rennet (Fig. 14A), M. pusillus protease (Fig. 14C), and E. para-silica protease (Fig. 14D).

may be related to studies in which glass particles and oil droplets were incorporated in protein gels [6, 24]. SEM showed that particles with a hydrophilic surface became an integral part of the gel whereas hydrophobized particles incorporated in a similar gel failed to develop any strong links with the protein matrix and easily separated from it when the gel was fractured. The presence of particles with hydrophilic surfaces in the gels provided a greater strength in compression than did particles with hydrophobic surfaces [24].

Based on the micrographs presented, the differences in gel firmness related to protein concentrations or to homogenization of the retentates may be understood from the microstructures of the gel matrices as viewed at low-magnification SEM and/or TEM. In some respects, similar results were obtained with yoghurts made at different concentrations of total solids [19]. The differences in gel firmness caused by the nature of the proteases used, however, are more difficult to correlate to the microstructure as they may be more subtle. At low-magnification SEM, the gels made from nonhomogenized retentates (13.0% protein) using rennet and proteases of microbial origin appeared to have similar microstructures and, likewise, the microstructures of the gels made from homogenized retentates at the same protein level also resembled each other (Figs. 14A - 14D). In order to facilitate the comparison of the protein matrices, fat had been extracted from the samples shown. Extraction of fat particles about 1 μm in diameter resulted in the development of noticeable void spaces, but the extraction of smaller fat particles made the gel structures appear to be more porous than the gel (13% protein, coagulation by rennet) with the fat retained which is shown in Fig. 11D at the same magnification. However, differences in the microstructures are apparent when high-magnification TEM micrographs of a firm gel produced by coagulating a nonhomogenized retentate (13% protein) with rennet are compared with micrographs of a soft gel produced by coagulating the same retentate using the B. polymixa protease...
Pfizer Chemie, Wiesbaden, Federal Republic of Germany, for the donation of proteases used in this study, and the United States Department of Agriculture and SIZN R Vojvodina for financial assistance. Assistance provided by the staff members of the Somboled Dairy Plant in Sombor, where experiments on an industrial scale were carried out, is acknowledged. Electron Microscope Unit, Agriculture Canada in Ottawa provided facilities for structural studies. The authors thank Dr. H. W. Modler for useful suggestions. Contribution 808 from the Food Research Centre.

References


Ultrafiltration Milk Retentate Gels

Y. Kakuda: Were the coagulation times with homogenized retentates shorter than with nonhomogenized retentates at all levels of solids and with all proteases? Can you suggest reasons why homogenization decreases the coagulation time? Was this a common effect seen with all proteases and for all protein concentrations tested?

Authors: A separate study has been prepared which answers these questions: in all cases, the coagulation times were shorter with homogenized retentates than with nonhomogenized retentates. The differences in the coagulation times were, however, smaller at high protein concentrations than at low protein concentrations.

The reasons for this phenomenon were studied by Robson and Dalgleish [30]. They observed reduced concentration of casein micelles in the milk serum due to their adsorption on the homogenized fat globules.

R. Cartwright: You explained differences in casein micelle chain structure between rennet and B. polymixa protease. What differences did you notice between rennet and the other enzymes used?

Authors: The differences between the effects of rennet and the B. polymixa protease were most clearly noticeable: the protein matrices of gels made using rennet consisted mostly of branching casein micelle chains whereas casein micelle clusters were predominant in gels made using the B. polymixa protease. The ratios of chains to clusters varied in the other gels which were made using the other microbial proteases (Figs. 16 - 20) and the ratios were to some degree related to the gel firmness. In addition, firm gels had the casein particle aggregates more robust (Figs. 15A, 16, and 17) than the soft gels (Figs. 15B and 18-20).

Y. Kakuda: Is it possible that increased proteolysis and nonspecific proteolysis may be interfering with the coagulation process at high protein concentrations (in the case with bacterial proteases) resulting in increased coagulation times?

Authors: Yes, it is possible.

R. Cartwright: How would you expect homogenization of the fat to affect fat loss in the whey?

Authors: This aspect was not investigated in this study but according to Davis [8], homogenization leads to less fat loss in the whey.

D. P. Dylewski: My question concerns the degree of whey protein denaturation and its possible role in textural properties and gel strength. Was an attempt ever made, through the use of electrophoresis, to determine the degree of denaturation?

Authors: Polyacrylamide gel electrophoresis showed very low concentrations of serum proteins in the Figs. 16 - 20. Details of protein matrices in gels made from a non-homogenized retentate (13% protein) using rennet (Fig. 16), M. miehei protease (Fig. 17), M. pusillus protease (Fig. 18), E. parasitica protease (Fig. 19), and B. polymixa protease (Fig. 20). The occurrence of casein micelle chains (arrows) is decreasing and the occurrence of casein micelle clusters is increasing from Fig. 16 to Fig. 20.
aqueous phase of the retentate gels but the results were not evaluated quantitatively.

R. Cartwright: What role would you expect the liberated fat globule membrane material to play in the gel formation process with regard to water retention and gel strength?
Authors: Since the total surface area of the fat globules is considerably (5 to 6-fold) increased as the result of homogenization, a part of the membrane fragments may be adsorbed on the newly formed surface. A potential exists for the fat globule membrane fragments to participate in emulsion stability through interactions with the phospholipid component of the membrane fragments.

R. Cartwright: What effect does homogenization have on the flavour and structure of the finished cheese?
Authors: Cheese made from homogenized milk has a smoother texture and an enhanced flavour compared to cheese made from nonhomogenized milk. Cheddar cheese made from homogenized low-fat milk was found by Emmons et al. [9] to be firmer than Cheddar cheese made from whole nonhomogenized milk although both cheeses had the same moisture levels in the nonfat matter. Curd granule junctions and milled curd junctions in the cheese made from homogenized milk were less apparent than in the other cheese because the protein-dense areas constituting the junctions (which in regular cheese result from the loss of fat globules during the cutting of curd) were considerably thinner. Microstructure of cheese made from nonhomogenized whole milk (3.6% fat) and from low-fat (1.4%) milk which had not been homogenized or had been homogenized at 5,200 and 1,700 kPa or 13,800 and 3,400 kPa was shown by SEM and TEM.

D. P. Dylewski: If and when do you think microbial proteases will replace traditional rennet on a significant and commercial basis?
Authors: Microbial proteases with specificity resembling that of rennet are already being used on a commercial scale. However, they are used mostly in soft and semi-hard cheeses which have relatively shorter ripening times. A more intensive proteolysis, which takes place in hard cheeses, may produce bitter-tasting peptides and off-flavours. An objective for genetic engineering has arisen: to tailor microbial proteases for use in cheese manufacture.
THE EFFECT OF IRRADIATION ON STARCH CONTENT IN GOLDEN DELICIOUS APPLES

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Abstract

Starch content in apple (Golden Delicious) skin and flesh was studied as a function of radiation dose (0, 0.5, 1, 2 and 5 kGy) after 8 weeks shelf life (16°C, 80-90% RH).

Starch was generally not found in the flesh with the exception of the 5 kGy sample. Starch grains were observed, however, in plastids of the epidermis and especially in the hypodermis in correlation with the radiation dose. 1 kGy dose caused a significant effect (P<99.98%) on retardation of starch breakdown measured by electron microscopic morphometry and chemical analysis.

Analysis showed that increasing radiation dose increased glucose concentration in the flesh. In the skin the concentration of all three sugars, glucose, fructose and sucrose, increased with irradiation up to 2 kGy, but they decreased with higher doses.

Introduction

Starch begins to accumulate in a developing apple fruit 3 to 4 weeks after bloom. Over the next 2 months it accumulates to a maximum value and subsequently declines as the fruit matures. Starch synthesis and decomposition are modified by different circumstances. Starch is a reserve carbohydrate that is eventually quantitatively converted to soluble sugars, that are stored in the vacuole or metabolized by the cell through respiration.

Simons and Chu (1982; 1983) investigated corking, a physiological disorder of apples. Cytoplasmic membrane breakdown occurred in cortical cells adjacent to the vascular bundles at least 45 and 60 days prior to fruit maturation. Cell walls were thick in fruit exhibiting corking. Starch accumulation occurred within localized areas and was attached to the cell walls. Starch grain accumulation on cortical cell walls and proliferation of starch grains in cells having thick walls was noticed 30 days before maturation (Simons and Chu, 1982; 1983). Profiles of tissue development and subsequent breakdown indicate that this physiological disorder has developed in formative and developmental stages of growth, with extensive tissue breakdown. Electron microprobe studies provided evidence that both potassium and calcium concentrations were low in all affected tissues, although the potassium level was higher than calcium in most of the sampled areas (Simons and Chu, 1982).

Mahanty and Fineran (1975) investigated the cellular ultrastructure of control and calcium sprayed apples which were cool-stored for 3 months. Cells of the control sample were less well preserved compared to those of calcium sprayed apples. Chromoplasts of the epidermis had prominent plastoglobuli and thylakoids which were scattered or sometimes alveolar rather than compacted. Cells from hypodermis showed increased lysosomal activity. Mitochondria and

KEY WORDS: apple, shelf-life, irradiation, Identification of irradiation treatment, transmission electron microscopy, ultrastructure, chloroplast, amyloplast, starch, prolamellar body-like structures, sugar-starch interconversion.
plastids containing starch appeared normal. In the chromoplasts of the calcium sprayed apples, compacted thylakoids and plastoglobuli were present. The inner cortical cells had a large central vacuole, and the cytoplasm surrounding this was usually granular and contained numerous plastids with starch. Gamma-irradiation has been reported to increase the sucrose content of potato tubers and sweet potato roots (Hayashi and Kawashima, 1982a, b). Sucrose accumulation was dependent upon irradiation dose, and the preferable dose range was between 1-2 kGy for roots of sweet potato. The sucrose content of irradiated vegetables and fruits increased for a long period after irradiation. It was suggested that an enhanced activity of sucrose synthase and sucrose-phosphate synthase played an important role in the sucrose accumulation in irradiated potatoes (Hayashi and Kawashima, 1983; Hayashi and Aoki, 1985).

Kovács and co-workers (1988) investigated the microstructure of calcium treated and irradiated apples, a short time after treatment and after 3 months subsequent cool storage. All treatments (calcium, irradiation, calcium treatment combined with irradiation) maintained the basic compartmentation of the cells into cytoplasm, plastids and vacuoles. By contrast, and mainly in the epidermis, the integrity of vacuoles had been lost in the control by the end of storage. Calcium treatment either alone or in combination with irradiation resulted in a somewhat better preservation than irradiation alone. Irradiation and combined treatment preserved starch. The difference was evident already 4 days after the treatment, and the effect lasted for at least 3 months.

It is known that metabolic disorders in apples always cause a decreased starch breakdown. Early theories on cause of bitter pit all were based on starch that remained in the tissue reviewed by Faust and Shear (1968).

The aim of the present work was to study the effect of irradiation on the ultrastructure of plastids and the starch-sugar interconversion in the apple. An identification method of irradiation treatment in apple has also been sought.

Materials and Methods

Raw materials and treatments

Apples (cv. Golden Delicious) were harvested in an orchard of Dánzsantmiklós Horticultural Station near Budapest, Hungary. Harvest time was 103-110 days following full bloom apples are harvested in Hungary in this stage for winter-storage. After picking at the green skinned stage fruits were irradiated by a Co-60 radiation source (0, 0.5, 1, 2 and 5 kGy doses, the dose rate was 1 kGy. Rl) using facilities at the Institute of Isotopes of Hungarian Academy of Sciences. After irradiation, samples were stored for 8 weeks (shelf life at 16°C, 80-90% relative humidity [RH]). This temperature was chosen to hasten the physiological and biochemical changes. The ripening stages of apple and the short time effect (5 days) of irradiation was monitored by the starch iodine pattern index (Reid et al., 1982) (Fig. 1a.). All ultrastructural and chemical investigations were carried out 8 weeks after treatments when the apple skin was yellow and the starch disappeared from the fruit flesh (except 5 kGy) (Fig. 1b).
The effect of radiation on starch

Fig. 2. Ultrastructure of plastids in the epidermis of samples stored for 8 weeks (Golden Delicious apple) (a: control; b: 1kGy; c: 2kGy; d: 5kGy), s = starch grain. Bars = 1 μm.

Electron microscopy and morphometry

Fixation was carried out in 6% (v/v) glutaraldehyde (in 0.035 M K-Na phosphate buffer, pH 7.2) for 2 h at 4°C. After thorough washing in the buffer, samples were postfixed in 1% (w/v) OsO₄ for 1.5 h, dehydrated in an acetone series and embedded in Spurr’s resin. Using flat molds, samples from the skin were oriented for subsequent transverse sectioning.

Sections were cut with a Porter-Blum ultramicrotome equipped with a diamond knife, post-stained with uranyl acetate and lead citrate and examined in a Tesla BS 500 electron microscope operated at 60 kV.

Starch grains were measured with a square lattice grid superimposed on enlarged prints, in 24-27 plastids from the outer hypodermis (h₁-h₃). The volume of starch grains was expressed as a percentage of plastid volume.

Determination of sugar

10 g of the skin or flesh sample was homogenized with 50 ml of 80% ethanol for 5 minutes. Each filtrate was evaporated to dryness to remove ethanol, then redissolved in 100 ml of water. Each solution was analyzed for soluble sugars by high pressure liquid chromatography (HPLC) using a LABOR MIM (Hungary) apparatus combined with a Beckman pump (USA) and RI detector (Beckman), having sensitivity of 2x10⁻⁵. The column was Chromsil-NH₂ (6 μm, 250x4.6 mm), operated at 250°C with
acetonitrile-water (75: 25) as mobile phase at 1 ml.min\(^{-1}\) flow rate.

**Determination of starch**

5 g of skin or flesh of apple was homogenized with 100 ml of 1 N HCl for 5 minutes. The homogenate was refluxed on a boiling water bath for 3 hours, made up to 1000 ml, glucose determined by HPLC and expressed as starch.

**Results and Discussion**

The skin of stored apples examined after different radiation doses showed several ultrastructural changes. Data concerning the epidermis and hypodermis will be evaluated separately. Plastids in the epidermis of the control sample were roundish with many electron-translucent vesicles. They generally contained dense inclusions but not starch grains (Fig. 2a). Unevenly distributed starch grains occurred in the epidermis of samples irradiated with 1 kGy or higher doses (Fig. 2b-c). The normal organization of the cell was broken down by an irradiation dose of 5 kGy (Fig. 2d).

Plastids in hypodermal cells of the control sample contained small and large grana. In the outer hypodermis plastoglobuli were electron translucent, and starch grains were rare and small. In deeper hypodermal layer plastoglobuli are more electron dense, and plastids are free of starch (Fig. 3a). In the 0.5 kGy sample the lumina of thylakoids in hypodermal plastids were often electrondense (Fig. 3b). Structures similar to prolamellar bodies could be seen in inner layers of hypodermis (Fig. 3c).

Earlier this structure was considered as a characteristic feature of the etioplasts; later it was established that prolamellar bodies also appear to differentiate following exposure to continuous red light (Boardman et al., 1971) or white light (Rascio et al., 1980). They are formed when the equilibrium of the membrane components (or their synthesis) is disturbed. We suppose, that in our case the prolamellar body-like structure is the result of disturbance of the membrane equilibrium caused by ionizing radiation.

Fig. 3. Ultrastructure of plastids in the hypodermis of samples stored for 8 weeks (Golden Delicious apple) (a: control; b: 0.5 kGy; t = thylakoid, g = granum; pb = prolamellar body-like structures. Bars = 1 \(\mu\)m.)
The effect of radiation on starch

Table 1
Starch content determined by morphometry and chemically in hypodermis of Golden Delicious apples stored for 8 weeks after irradiation

<table>
<thead>
<tr>
<th>Dose (kGy)</th>
<th>Starch content as % of plastid</th>
<th>Starch content as % of glucose after hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.38 ± 0.21</td>
<td>2.83 ± 0.92</td>
</tr>
<tr>
<td>0.5</td>
<td>1.52 ± 0.69</td>
<td>2.85 ± 0.91</td>
</tr>
<tr>
<td>1</td>
<td>15.82 ± 2.39 XXX</td>
<td>6.32 ± 0.90 XXX</td>
</tr>
<tr>
<td>2</td>
<td>28.53 ± 3.16 XXX</td>
<td>6.56 ± 0.85 XXX</td>
</tr>
<tr>
<td>5</td>
<td>38.21 ± 3.72 XXX</td>
<td>15.06 ± 2.40 XXX</td>
</tr>
</tbody>
</table>

\( \bar{x} \) = average; \( s \) = standard deviation; 
\( xx \) significantly differed from the control, at level 95% 
\( xxx \) significantly differed from the control, at level 99%

Plastids of the hypodermis of 1 kGy apple cortex contained small and large starch grains (Fig. 4a) in addition to occasional prolamellar body-like structures. Apples irradiated with 2 kGy contained conspicuous starch grains, mainly in the outer hypodermal cells (Fig. 4b), but cells with damaged membranes also occurred. Large starch grains remained in the outer layer of the hypodermis (h.) of the 5 kGy irradiated apples. In the inner layer chloroplasts with large amounts of starch were observed (Fig. 4c). According to the results of morphometry, the starch content of the hypodermal cells had already differed significantly at an irradiation dose of 1 kGy from the control (Table 1). According to the chemical analysis more starch remained in the skin at a dose 1-5 kGy. The data of morphometry and chemical analysis correlate well.

Fruit flesh was also examined for starch grains. Both in the control and irradiated (0.5-2 kGy) flesh, the small plastids were free of starch (Fig. 5a-b). Starch grains were present in cells of 5 kGy irradiated apple (Fig. 5e). This was also evident from the iodine test (Fig. 1b).

Fig. 4. Ultrastructure of plastids in the hypodermis of samples stored for 8 weeks (Golden Delicious apple) (a: 1 kGy, b: 2 kGy, c: 5 kGy), s = starch. Bars: 1 μm.
The analysis of sugars indicated that glucose concentration increased in the flesh with the increasing radiation doses, which means that most of the decomposed starch was converted into glucose during storage after irradiation (Fig. 6). At the same time in the skin of apple the glucose, fructose and sucrose concentration increased only up to 1 and 2 kGy, then decreased (Fig. 7). Both phenomena have also been observed in sweet potato roots as a function of radiation dose (Hayashi and Kawashima, 1982a, b.).

Several factors may have influence on preserving starch in the irradiated fruits. These include Ca mobilization from the flesh to the skin (Kovács et al., 1985; Kovács and Zackel, 1987) and effects involving the integrity of membranes (Romani et al., 1968; Isherwood, 1976). The question is further complicated by the fact that Ca also has an effect on the state of the membranes. Further investigations are needed for understanding of the interaction of these factors.

Fig. 5. Ultrastructure of plastids in the flesh of samples stored for 8 weeks (a: control; b: 0.5 kGy; c: 1 kGy, d: 2 kGy, e: 5 kGy), s = starch. Bars: 1 μm.
The effect of radiation on starch

Fig. 6. The effect of irradiation on the sugar content in the flesh of apple stored for 8 weeks

Fig. 7. The effect of irradiation on the sugar content in the skin of apple stored for 8 weeks

Acknowledgements

Mr. B. Bóka, Ms. J. Bencze Bócs, Ms. K. Lehofer and Ms. G. Gácsi are thanked for their participation in the technical work.

References


Discussion with Reviewers

T. Hayashi: What is the purpose of irradiating apple? If it is disinfestation, the dose required is in the range of 0.1 to 1 kGy, which is lower than that used in your study. Why did you choose the dose range in your study?

Authors: The 0-5 kGy dose range was chosen to follow up the effect of irradiation on starch. The aim of apple irradiation is either disinfestation (0.1-1 kGy) or to extend shelf life using irradiation (1 kGy) in combination with Ca-treatment.

Reviewer IV: Could you give some information on the location of the samples with respect to the cross-section of the fruit?

Authors: Samples for both the skin and flesh were collected from the region of the largest diameter, the flesh samples were cut out halfway between the carpels and the skin.
ULTRASTRUCTURAL EFFECTS OF POSTHARVEST TREATMENTS ON THE VACUOLAR INCLUSIONS IN PEAR (PYRUS COMMUNIS L. CV. HARDENPONT) PEEL

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Abstract

In the course of our investigations on the ultrastructural effects of postharvest treatments applied for shelf life extension of Hardenpont pear, we have observed specific changes in the vacuolar content of peel. In the hypodermal cells of the untreated control compact dense vacuolar inclusions developed during storage for two months. CaCl₂ solution applied after picking changed the structure of the inclusions by the end of storage, while gamma-irradiation (1 kGy) reduced their size. We regard these changes as possible ultrastructural markers of the treatments. We have not found evidence by X-ray microanalysis for the accumulation of Ca in the inclusions after CaCl₂ treatment, while Ca accumulated in the vacuolar granules of the fruit flesh.

Introduction

In a series of papers we have presented results on the ultrastructural effects of postharvest manipulations applied for shelf life extension including gamma-irradiation (mushrooms), or CaCl₂ treatment, gamma-irradiation and both in combination (apple and pear) (Keresztes et al. 1985, Keresztes and Kovács 1987, Kovács et al. 1988).

The specific response varied with the different foodstuffs. In mushrooms irradiation generally preserved the cyto- or mictoplasm while causing cell necrosis in the hymenium. With apple peel the CaCl₂ treatment seemed somewhat more beneficial than irradiation in respect to the integrity of the membranes, while the combined treatment stimulated starch resynthesis in pear flesh the combined treatment somewhat retarded the dissolution of the middle lamellae.

In this paper we report on the effects of CaCl₂ treatment and gamma-irradiation on Hardenpont pear peel, with special regards to the changes of the electron dense vacuolar content.

Material and Methods

Fruits of Pyrus communis L. cv. Hardenpont were treated with a 4% (w/v) CaCl₂ solution for 10 minutes, or 1 kGy gamma-irradiation, immediately after picking. Samples were irradiated in the Institute of Isotopes of Hungarian Academy of Sciences by a 60Co radiation source (total activity 3.7 pCi), at a dose rate 1 kGy·h⁻¹. The effects were investigated after a 2 month storage at 16°C, 95 - 97% RH.

Tissue pieces from the peel and fruit flesh were fixed in 6% (v/v) glutaraldehyde (in 0.035 M K-Na phosphate buffer, pH 7.2) for 2 hours at 4°C. After thorough washing in the above buffer, samples were postfixed in 1% (w/v) OsO₄ for 1.5 hours, dehydrated in an acetone series and embedded in Spurr's resin. Using flat molds, samples from the skin could be oriented so that they were always sectioned transversely. After sectioning with a Porter-Blum ultramicrotome, thin sections were stained with uranyl acetate and lead citrate, and then investigated in a Tesla BS 500 electron microscope. Thicker sections without staining were used for X-ray microanalysis in a JEOL TEMSCAN 100 CX analytical electron microscope operated at...
Results and Discussion

Shortly after picking, a finely or coarsely granulated vacuolar content could be seen in the hypodermal cells (Fig. 1). During storage of two months, compact dense vacuolar inclusions developed in these cells (Fig. 2). If CaCl₂ treatment was applied right after picking, two months later the vacuolar inclusions had a reticular structure (Fig. 3). It seems as if numerous droplets of the electron transparent phase have been entrapped by the aggregating dense phase. If the fruits were irradiated immediately after picking, then after storage the inclusions were definitely smaller than those in either the untreated or Ca-treated samples, and had a variable shape and structure (Fig. 4).

As the changes in size and structure of the vacuolar inclusions seem to be specific to the treatment, we regard these as possible ultrastructural markers of the different postharvest manipulations. This test, however, cannot be applied to apple, as the dense precipitations seen in freshly harvested Mutsu and Gloster apple peel invariably disappeared during storage after the treatments (Kovács et al. 1986).

Although there are no direct data regarding the chemical nature of the inclusions, on the basis of the phytochemical investigations by Williams (1960) on apple and pear fruits, Bain and Mercer (1963) inferred that the precipitation consisted of polyphenols. We wanted to know whether Ca acted on the structure of the inclusion directly or indirectly. In the first case we may expect an elevated Ca-level in the inclusion. In an attempt to answer this question, we carried out X-ray microanalysis on semi-thin sections. We found no significant change in Ca-content of the inclusion after CaCl₂ treatment as compared to the stored control (Figs. 5 and 6, Ca-peak: background ratios being 87 : 41 and 69 : 28, respectively). In order to check the penetration of the CaCl₂ solution into the fruit, we recorded X-ray spectra of the vacuolar content in the fruit flesh. After CaCl₂ treatment this granular precipitation was dense and contained Ca abundantly, while that of the stored control was faint and no characteristic peaks could be identified in its X-ray spectra (Figs. 7 and 8, in Fig. 8 the Ca-peak: background ratio is 127 : 24). Considering that other elements besides Ca accumulate in the

Figs. 1-4. Parts of the hypodermis from the fresh control (Fig. 1), stored control (Fig. 2), CaCl₂ treated (Fig. 3) and irradiated (Fig. 4) samples, i = inclusion, v = vacuole. Bars equal 5 µm.

80 kV in the STEM mode, by tilting the specimen 37°.
vaccules of Ca-treated fruit flesh, it is doubtful whether the high Ca-peak here represents entirely exogenous Ca. It is probable that the Ca-treatment enhanced the sequestering of different ions, including Ca, into the vacuole. In any event, this also proves the penetration of the exogenous Ca.

Acknowledgements

Mrs. P. Petrovits and Mrs. G. Gácsi are thanked for their technical assistance.

References


Keresztes Á, Kovács E. (1987) Effect of ionizing irradiation and storage on mushroom ultrastructure. II. The stipe and the upper part of the cap of Agaricus bisporus (Lge.) Imbach. Food Microstruct. 6, 75-79.


Figs. 7-8. Probed areas (marked) and their X-ray spectra in vacuoles of the stored control (Fig. 7a,b) and CaCl$_2$ treated (Fig. 8a,b) fruit flesh. Characteristic peaks in the spectrum of Fig. 8: Na (at 1.0 keV), S (at 2.3 keV), K (at 3.3 keV) and Fe (at 6.4 keV). Counts for Ca and background were collected at 3.64-3.76 or 4.22-4.34 keV, respectively. Bars equal 1 μm.


Discussion with Reviewers

M. Faust: Could you give information on the degree of maturity at which the pears were harvested?

Authors: The degree of penetration was 0.638 ± 0.09 mm in 5 seconds measured with a LABOR MIM penetrometer.

J.H. Moy: Why were not more than one CaCl$_2$ concentration or irradiation dose used to have a more comprehensive comparison?

Authors: The CaCl$_2$ concentration was optimized in respect to Ca uptake. 1 kGy was choosen on the basis of preliminary experiments and practice.

J.H. Moy: Why was the examination done after two months of storage and not sooner? Does this have any commercial significance or relationship to marketing?

Authors: Storage was shorter than that usual in practice, because temperature was higher. An earlier sampling for EM did not seem advisable for the first run, considering the probability of visible changes.
Ultrastructure of the vacuolar inclusions in pear

B.A. Fineran: How much material was examined during the course of your experiments from which you obtained your results? For instance, approximately how many samples were examined for each treatment and what number of thin sections were viewed for each sample?

Authors: Two fruits were used from each group, 10-12 samples cut out from both the peel and the flesh and about 20-30 sections examined for each treatment.

B.A. Fineran: In the flesh of the fruit, if the high concentration of calcium in the vacuole does not come from the treatment, as you seem to imply, where then does it come from?

Authors: We cannot exclude the possibility that the Ca-induced sequestration of different ions into the vacuole might also have involved endogenous Ca, e.g., from the cytoplasm or mitochondria.

B.A. Fineran: What precautions did you take to prevent the loss of calcium during processing of the tissue for TEM? In this connection, have you considered examining your material for X-ray microanalyses using SEM? With this approach, specimens can be prepared physically without loss of contents by freeze/fracture methods, with sample then being either freeze-dried or examined directly in the frozen state.

Authors: No such precautions were taken against a loss of diffusible ions. Therefore we can demonstrate bound Ca only, without quantitative evaluation. Still the comparison of the different samples may be meaningful.

M. Faust: What may be the significance of the difference in time of scans? Some were scanned for a T (time) of 000100; others, 000200; marked on the photographs. Similarly the scanned area is also different. Figures 7 and 8 are compared yet the scanned area in Fig. 7 is only one tenth (0.12 um²) of that in Fig. 8 (1.2 um²).

Authors: Within certain limits the time of scans is not critical if the peaks are compared to their backgrounds rather than to each other. The scanned area was selected arbitrarily, so that it was morphologically as homogeneous as possible. When analysing in the scanning mode, count rate is proportional to concentration independently of the area scanned.
IMAGE ANALYSIS OF THE FAT DISPERSION IN A COMMINUTED MEAT SYSTEM

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Abstract

In a series of experiments on a comminuted meat system, image analyses were conducted to quantify changes in structure as affected by polyphosphate, fat level, and chopping time and temperature. Four batches of comminuted meat were investigated which contained neck beef, pig head meat, rind, ice, salt (2%), fat at a level of either 27 or 40%, and polyphosphate at a level of either 0 or 0.5%. Structural changes in different stages of comminution coincided with a shift in size distribution, shape factor, density and area percentage of fat particles. The density of the protein matrix (matrix defined as all disintegrated tissues, without intact pieces of muscle fibres, connective tissue and fat cell clusters) was markedly affected by polyphosphate. Polyphosphate led to a fine dispersion of the fat, as reflected by the formation of a larger proportion of fat droplets smaller than 3.5 μm². Results indicate that in meat batters physical entrapment of larger fat particles as well as emulsification of smaller droplets occur simultaneously in the course of fat stabilization.

Introduction

The mechanisms of fat and water binding have been the subject of many studies which were recently reviewed by Hermansson (1986). In addition to physico-chemical studies, microscopy is increasingly applied to investigate the relationship between microstructure and functional properties of comminuted meat products (Lee, 1985; Schmidt et al., 1985; Cassens et al., 1987; Hermansson, 1987; Oelker and Dehmlow, 1988). Based on microscopical observations, two different theories have been postulated about fat stabilization in comminuted meats, namely, fat emulsification (Hansen, 1960) and physical entrapment of fat in the protein matrix (Van den Oord and Visser, 1973). Additional research supported both these theories (Jones and Mandigo, 1982; Lee, 1985). However, more and more it became clear that the microstructure of a comminuted meat system is very complicated and cannot be explained by one phenomenon only (Swasee et al., 1982; Schmidt, 1984). Moreover, the importance of fat emulsification versus physical restriction of fat coalescence may vary with different processing conditions (Smith, 1988). It is the current view that a comminuted meat product represents a complex multiphase system consisting of a solution, a suspension, a gel and an emulsion (Wirth, 1985; Hermansson, 1988).

Until recently image analysis was only applied to meat quality control, i.e., the quantification of the tissue composition of comminuted meat products (Hildebrandt and Hirst, 1985). The application of image analysis for meat technology research remained limited (Kempton and Trupp, 1983; Lee, 1985) in spite of its broad application possibilities. Not only in microstructural studies, but also in the examination of the macrostructure of meat and meat products, video image analysis has an increasingly important role (Newman, 1987). The additional information about microstructural and thus functional properties provided by image analysis can be useful in process control as well as product development.

In our study image analysis was used in an attempt to relate microstructural changes with morphological variables. Particular attention was paid to the dispersion of fat particles in the...
protein matrix of raw meat batters with a different formulation.

Materials and Methods

Experiments were conducted on four different batches of comminuted meat. The batches were prepared according to the recipe given in Table 1. Beef neck meat, ice, salt (containing 0.6% NaNO2) and polyphosphate (Lutwix, commercial mixture 8f tetrasodium diphosphate and potassium polyphosphate at a 1/2 ratio, containing 60% P2O5, Degens, Vlaardingen, The Netherlands) were chopped in a bowl chopper (Laska, model KT 60-3 MV, Linz, Austria) until a temperature of 9°C was reached. Subsequently, the other ingredients were added and further comminuted until a final temperature of approximately 30°C was reached. During the comminution process, 200 g cans were filled with the meat batters. Subsequently, these were heated for 75 min at 115°C simulating a retort process at a F0 value of 2.0. After cooling to 23°C, fat and water loss was determined as described by Tinbergen and Olsman (1979). The chemical composition of the unheated meat batters was determined by International Organization for Standardization (ISO/R597 (1969); ISO 1442 (1973); ISO 1444 (1973)) procedures described in Table 1.

Sampling for microscopical examination was done in the course of the comminution process at two or 3 min intervals depending on the temperature rise. This means that four samples were taken, each measuring 1.5 x 1.5 x 0.5 cm, which were frozen in isopentane cooled with liquid nitrogen. The samples were stored at -80°C. Simultaneously, eight similar samples were collected and fixed in a buffered neutral formalin solution for 24 h.

To investigate structural changes at different stages of the comminution process, microscopical examination was conducted at the following time intervals: batch 1 at 9, 11 and 14 min; batch 2 at 8 and 16 min; batch 3 at 8, 10 and 13 min and batch 4 at 4 and 15 min. Samples at these time intervals represented stability or instability of the raw batter microstructure. The frozen samples were cryosectioned (cryostat, type HR, Slee, London, UK) whereafter 8 µm thick sections were stained for fat with Oil red O (Cassens et al., 1977). The formalin-fixed samples were embedded in paraffin and sectioned at 5 µm thickness using an American Optical 820 microscope. Subsequently, the sections were stained with toluidine blue (Swasdee et al., 1982).

All sections were examined by light microscopy at different magnifications to observe structural changes in the batters. To quantify these changes the following morphological variables were determined by image analysis: total area (%) and density of fat particles below and above shape factor 0.45 and the total area (%) of the protein matrix. Shape factor, indicating the degree of roundness of particles (e.g. shape factor = 1.0 describes a circle), was calculated by the formula:

\[ \text{shape factor} = \frac{4 \times \pi \times A}{U^2} \]  

\[ \text{where } \pi = \text{pi}, \ A = \text{area}, \ U = \text{perimeter.} \]  

The level of 0.45 was chosen after random-measuring of 200 differently shaped fat particles. Intact fat cells and most protein-covered particles had a shape factor well above the 0.45 level. Shape factors below 0.45 were found for coalesced fat and conglomerates which exhibited the random shape. The degree of fat stabilizability and the amount of fat stabilized by released muscle protein.

In a pilot experiment, all morphological variables were measured in a number of fields of view in different sections. Data were subjected to an analysis of variance to determine the optimum number of fields of view and sections, required to obtain reliable results.

Image analysis was done with an IBA system (Kontron Bildanalyse GmbH, Eching b. München, FRG) and a television camera (Plumbicon Video 50, type LDH 0500/01, Philips) connected directly to the microscope. The measurements were conducted at two magnifications, viz. 40x and 250x. At the 250x magnification, all fat particles ranging from 3.5 to 200 µm² were measured. Image analysis at 40x magnification included all particles larger than 200 µm². In addition, size distributions of all the fat particles from each sample with a shape factor larger than 0.45 were plotted in histograms of 25 class ranges. The average area of 325 intact fat cells from adipose tissue was determined in both paraffin and frozen sections to evaluate the contribution of intact fat cells in terms of size range in the total size distribution of fat particles.

Data from the different morphological variables were subjected to Student t-tests.

Results and Discussion

The effect of fat content and the addition of polyphosphate on water and fat holding as affected by chopping time and temperature is shown in Figure 1. Instability in batches 1 and 3 increased rapidly after 9 min of chopping, whereas in batches 2 and 4 water loss remained low and fat loss negligible. Examination by light microscopy revealed structural changes at the different stages of the comminution process. In batch 1, the microstructure of the meat batter after 9 min of comminution was characterized by a continuous protein matrix of disintegrated muscle and collagenous tissue in which intact adipose tissue, individual fat cells and many smaller fat particles were distributed (Figure 2). At 11 min chopping time, fat was partly coalesced into larger fat particles (Figure 3). At 14 min chopping time, fat had run together into large channels which disturbed the integrity of the protein matrix (Figure 4). Similar features were observed in batch 3 in which the formation of large fat
Image analysis of fat in a meat batter

Figure 1. Fat and water loss percentages as a function of chopping time and temperature. There was no fat loss in batch 2.

Table 1. Recipe and chemical composition of the meat batters in percentages.

<table>
<thead>
<tr>
<th></th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
<th>Batch 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef neck meat</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Pork rind</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Added ice/water</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Salt</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Pig head meat</td>
<td>31</td>
<td>31</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>Pork fat</td>
<td>20.5</td>
<td>20</td>
<td>40.5</td>
<td>40</td>
</tr>
<tr>
<td>Polyphosphates</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

channels and the discontinuity of the protein matrix were even more pronounced due to the higher fat content of the meat batter. Scattered between the discontinuous protein matrix intact fat cell membranes were observed. Batches 2 (Figures 5 and 6) and 4 (Figure 7) revealed a different microstructure. The protein matrix was more dense and the large number of finely distributed small fat particles, especially in batch 4, was striking. Extended chopping times did not affect the integrity of the protein matrix and all fat particles remained surrounded by protein.

By image analysis the area percentage, density and shape factor of all fat particles were measured simultaneously. Through analysis of
Figure 2. Micrograph of the meat batter microstructure (batch 1) after 9 min of chopping showing a continuous protein matrix. (A) Toluidine blue staining, (B) Oil Red O staining, P = protein, L = lipid.

Figure 3. Micrograph of the meat batter microstructure (batch 1) after 11 min of chopping showing the formation of fat channels.

Figure 4. Micrograph of the meat batter microstructure (batch 1) after 14 min of comminution showing the discontinuity of the protein matrix and coalescence of fat.

variance, it was verified in a pilot experiment that the examination of four fields of view per section in four different sections, i.e., a total of 16 fields of view for each sample, yielded reliable results at both magnifications for all morphological variables measured. The results of the image analysis are shown in Figure 8. In batch 1, the optimum fat stability was reached after a chopping time of 9 min. This stability was reflected by an area percentage of 15 for fat, mainly covered by spherical particles at both magnifications. The protein matrix covered an area of approximately 80%. At 11 min chopping time, instability started to occur. This coincided with a shift towards more large fat particles with a shape factor lower than 0.45 (p<0.001).
Figure 5. Micrograph of the meat batter microstructure (batch 2) after 8 min of chopping showing a continuous protein matrix and a uniform dispersion of fat particles. (A) Toluidine blue staining, (B) Oil Red O staining, P = protein, L = lipid.

After 14 min of chopping, the increased instability was marked by a distinct increase in the area for fat, a decrease in the particle density for both magnifications and a decrease in the area for protein matrix (p<0.001).

Batch 3 revealed a similar pattern as observed in batch 1. Stability was associated with a large area of protein matrix, a high fat particle density and a large area of fat that mainly consisted of particles with a shape factor larger than 0.45. Here also instability was associated with a decreased density of small fat particles and an increase in the density of larger fat particles with a lower shape factor. "Overchopping" of this batch with a high fat content, which was observed at 13 min chopping time, caused phase separation and large fat channels occupied 50% of
the area. Due to the discontinuous microstructure, it was impossible to properly measure the protein matrix area and the particle density at the 250x magnification.

The microstructure of batches 2 and 4 differed considerably from that of batches 1 and 3. The addition of polyphosphate resulted in a higher density of the protein matrix which occupied 85-90% of the area. As compared with batches 1 and 3, the fat particle density and area of fat in batch 2 at the 40x magnification were lower (p<0.05). The area and density of small fat particles, measured at the 250x magnification, remained at the same level even after extended chopping. Most fat particles did not coalesce and held a shape factor larger than 0.45. The same trend was visible in batch 4. In contrast with batch 3, there was a clear shift towards smaller...
Image analysis of fat in a meat batter

**BATCH 1**

- **9 min**: n = 1191
- **11 min**: n = 900
- **14 min**: n = 439

*40 X magn.*

**BATCH 2**

- **8 min**: n = 899
- **16 min**: n = 363

*40 X magn.*

**BATCH 4**

- **4 min**: n = 1795
- **15 min**: n = 800

*40 X magn.*

- **4 min**: n = 1973
- **15 min**: n = 2367

*250 X magn.*

---

**Figure 9.** Size distribution of fat particles determined at 40x and 250x magnification (batches 1, 2 and 4): Only 10 of the 25 particle size classes are represented.

Fat particles. This was shown by a decrease in particle density and area for fat measured at the 40x magnification, as well as by an increase in particle density measured at the 250x magnification after 15 min of chopping. However, this increase in particle density of small fat droplets was not associated with an increase in the fat area. It is likely that this shift to smaller particles went beyond the level of 3.5 \( \mu m^2 \) which represents particles with a diameter of 2.11 \( \mu m \).

The latter conclusion is confirmed by the results of the particle size distribution of fat particles with a shape factor greater than 0.45 (Figure 9). At the low magnification of 40x a smaller proportion of smaller particles was observed for batches 1 and 2 at increased chopping times. The distribution pattern of particles with a size varying from 3.5 to 200 \( \mu m^2 \) from batch 1 shows the same tendency. This shift to larger particles was caused by coalescence into larger fat pools with a shape factor below 0.45. In batch 2, the distribution pattern, area for fat and particle density determined after 8 and 16 min chopping time (magnification 250x) did not change. This can be explained by the fact that in batch 2 with salt, polyphosphate and a relatively low fat content muscle protein was abundantly available to stabilize the fat droplets. However, in batch 4 a shift towards smaller fat particles, determined at the 250x magnification occurred, at
extended chopping time, while the particle density increased and the area for fat remained unchanged. In this meat batter with a higher fat content, an extended chopping resulted in the formation of numerous small fat droplets that required a large proportion of the available muscle protein to be covered. Figure 1 clearly shows that enough protein was available to stabilize these droplets. Both the increased particle density and the shift to smaller particles indicate that fat was partly comminuted into droplets smaller than 3.5 μm². This was confirmed by the fact that measuring particles from 3.5 μm² onwards did not increase the total area % for fat which we expected after examining the same sections by light microscopy.

The formation of numerous fat droplets smaller than 3.5 μm² which were stabilized by the polyphosphate-induced improved protein functionality suggests that emulsification of smaller droplets occurred in addition to fat dispersion of larger particles. Our study afforded limited information on size distribution due to section thickness and magnifications used. Amongst others, Jones and Mandigo (1982) described an emulsification mechanism of fat droplets by protein membranes. All of these studies involved scanning or transmission electron microscopy to qualitatively evaluate the microstructure. More research on quantitative microscopy at this magnification level is essential to elucidate the size distribution and binding of fat particles smaller than 3.5 μm².

The average fat-cell shape factor determined from 325 individual cells was 0.75 with a standard deviation of 0.07. The average size of these fat cells was 3400 μm² with a standard deviation of 1100 μm². This area coincided with largest diameters of 66 to 105 μm depending on where the slicing plane intersected the fat cells. However, these diameters are quite arbitrary, in order to refine on measurements of particle size, we preferred to express particle size not only in μm² (Lee, 1985), but both in μm² and shape factor. Particle size of all fat particles with a shape factor greater than 0.45 ranged from 3.5 to 8200 μm². In all four meat batters, we observed fat particles in the 2300-4500 μm² range with shape factors greater than 0.65. Our data strongly indicate that particles within this size range mainly consisted of intact fat cells. This agrees with observations of Lee (1985) who also stressed the importance of particles in the range from 20-80 μm diameter in the fat holding mechanism. The observed size distribution and the intact fat cell membranes in the disrupted protein matrix of batch 3 indicate that integrity of the fat cell membranes represents an important factor in the mechanism of fat stabilization. Schut (1978) reported likewise and Timbergen and Olsman (1979) came to the same conclusion by their fat extraction technique.

Adipose tissue fragments, intact fat cells and larger droplets of membrane-free fat were physically entrapped in the protein matrix and between other components of the protein matrix. This could clearly be observed in the 9 and 8 min samples of batches 1 and 3. The dispersion of larger fat particles throughout the continuous phase to a large extent contributed to fat-holding. Fat stabilization by emulsification is likely to occur in finely comminuted batters with very small particles and at ample protein availability (Hermansson, 1986; Smith, 1988). Our experiments indicate that emulsification took place to a greater extent in batches 2 and 4 with polyphosphate addition, than in batches 1 and 3.

In addition, the results show that shape factor as a morphological variable can be used to classify fat particles in terms of stability. Shape factor in combination with particle density and area percentage can be effectively employed to distinguish between stabilized and non-stabilized fat particles.

Conclusions

Differences in chopping time, phosphated addition and fat content resulted in microstructural changes of the meat batters. These changes were quantified in morphological data determined by image analysis. With the aid of polyphosphate a dense protein matrix was formed in which numerous small fat droplets were stabilized, even after extended chopping time and increased fat content. Without polyphosphate, meat batters became less stable to increased chopping time and fat content. The predominant way of fat stabilization in the absence of polyphosphate was effected through physical entrapment of the larger fat particles in a coarser matrix. Both physical entrapment and emulsification contributed to fat-binding in all meat batters investigated, but varied with different formulations. The shape factor proved to be a useful morphological tool in discriminating between stabilized and coalesced fat particles.

Acknowledgements

This work was supported by the Commodity Board for Livestock and Meat at Rijswijk, The Netherlands.

References


Hermansson A-M (1988). The structure and
Image analysis of fat in a meat batter


Discussion with Reviewers

C.M. Lee: On what basis can you conclude that emulsification occurred in batches 2 and 4 with polyphosphate? According to your micrographs, fat was simply finely dispersed. Without a fine micrograph such as TEM, it would be presumptuous to state such.

Authors: We agree that without micrographs at higher magnifications, it is impossible to demonstrate emulsification of small fat particles. Our results show that in batches 2 and 4, in contrast with batches 1 and 3, a distinct shift to numerous smaller fat particles occurred. However, this increased particle density in batches 2 and 4, as measured at 250x magnification, was not associated with an increase of the fat area. Chopping fat cells into many smaller particles involves a tremendous surface area increase (Barbut, 1988). Since no increase in fat area in batches 2 and 4 was found for particles of 3.5 \( \mu m^2 \) or larger, the area increase must be attributed to many particles smaller than 3.5 \( \mu m^2 \). As a result of phosphate addition, enough muscle protein was available to stabilize these droplets. It is likely, that under these circumstances emulsification occurred.

A.-M. Hermansson: Fat staining was made with unified sections, probably at room temperature. From the micrographs it seems as if the fat phase has been affected by the staining solution and not only by, e.g., overchopping in the bowl chopper. Have the authors any comments on the fat staining procedure?

Authors: Fixation of the sections was done in a formal-calcium solution as described by Cassens et al. (1977). Fat staining by Oil red O was performed at room temperature. It is true that the staining procedure somewhat affected the morphological and location of the fat phase. Any fat-staining solution, as well as the mounting procedure, will cause some dislocation of the non-protein- covered small fat droplets at the surface of the sections. Further, it was noticed that during sectioning of the batters with phase separation, some disruption occurred, which was not the case in sections from batters without phase separation. However, this limitation did not detract from the fact that an accurate comparison between the batters could be made. This was verified by polariscopy of unstained cryosections, through which it became clear that the staining procedure had affected the overall picture only to a limited extent.

A.-M. Hermansson: There is no discussion of the presence of pores in the meat batters, e.g. the correlation between the fat-stained and the protein-stained sections with regard to the discontinuous phase. Can the authors please make some comments?

Authors: In order to evaluate the presence of air pockets, pores, cracks, etc. in the sections, we applied dark-ground microscopy and polariscopy to unstained cryosections. In this way it was possible to distinguish between fat, protein and empty areas in the sections. Empty areas were only observed in the batters with phase separation. This was caused by some disruption during sectioning, resulting in slippage of fat globules. From this observation we concluded that the contribution of...
pores to the total surface area, measured in the protein stained sections, was negligible. Hence, the presence of only a few pores could not have had a significant effect on the fat dispersion pattern determined.

A.-M. Hermansson: Did image analysis at the lower magnification not include any particles less than 200 μm², or was there any overlap between the two magnifications?
Authors: There was no overlap between the two magnifications used. The IBAS system offers the possibility of putting lower and upper size limits (μm²) to particles to be measured. In this way one is able to define size ranges in which particles can be measured. We chose for the lower limit of 200 μm² for the 40x magnification. Below this limit particles could be measured more accurately at higher magnifications.

A.-M. Hermansson: How did the measured fat area correspond to the fat content of the batters?
Authors: The measured fat area corresponded well with the fat content of the different batches. This can be seen from figure 8. A higher fat level was associated with a higher fat area and a lower protein area; see batch 1 versus batch 3, and batch 2 versus batch 4.

Additional Reference

Abstract

In this study, transmission electron microscopy was used to visualize the adherence of Lactobacillus acidophilus to human intestinal tissue cells (HITC) in vitro. There appeared to be a layer of electron dense material on the bacterial cell and on the intestinal cell which may mediate adherence. When L. acidophilus attached to intestinal tissue cells after a short contact period, it did not appear to disrupt the integrity of the intestinal cell. Treatment of the bacterium with sodium periodate and Concanavalin A reduced the adherence to HITC, suggesting that a carbohydrate was involved. Electron micrographs of periodate-treated cells revealed that the layer appeared to be partially removed after 1 hour and was almost completely absent after 3 hours of treatment.

Introduction

Lactobacillus species are used in the production of numerous foods including yogurt, acidophilus milk, kefir, Italian cheese, sourdough bread, distillery mash, pickles, olives, and some cured meats. Milk is the substrate for several lactic acid fermentations involving the lactobacilli. One such product is acidophilus milk. It, however, has not received a great deal of consumer acceptance as it has a very sour flavor and high lactic acid content. Metchnikoff (1908) speculated that consuming "sour milk" could terminate the unhealthy fermentation going on in the gut and lead to a longer life. Several investigators have suggested that L. acidophilus may have other beneficial effects for humans. These would include cholesterol assimilation, cancer suppression, and antibiotic production (Sandine, 1979; Gilliland et al., 1985; Goldin and Gorbach, 1984).

Methods have been proposed to make products containing high numbers of L. acidophilus cells without the undesirable taste. One of these products is "Sweet Acidophilus Milk." One method of producing this product is by adding high numbers of freeze-dried viable cells to cold (5°C) pasteurized milk (Speck, 1978). The cells remain viable in the cold milk but do not grow until the milk is consumed. A novel product consisting of acidophilus milk blended with banana, tomato juice, and sugar and then spray-dried has also been suggested (Prajapati et al., 1986).

Brownlee and Moss (1961) first demonstrated that lactobacilli adhere to stomach epithelial cells in the rat stomach. Scheidler et al. (1965) noticed that lactobacilli isolated from normal mice immediately established themselves throughout the gastrointestinal tract of germ-free mice. In another example, Fuller and Turvey (1971) found that the main sites of attachment of lactobacilli in the chicken gastrointestinal tract were the crop, ileum, and caecum. The adherence of the lactobacilli was unaffected by diet, and they suggested that there was a great degree of specificity between the microorganisms and host because only avian lactobacilli would adhere to chicken crop epithelial cells.

Other investigators have suggested species-specificity in the adherence of the lactobacilli. Barrow et al. (1980) found that only strains of Lactobacillus fermentum and Streptococcus salivari isolated from pigs and wild boars would adhere to pig epithelial cells. In another example, lactobacilli isolated from pigs and fowl did not adhere to keratinized squamous epithelium cells of the mouse stomach, while a rat...
isolate did adhere (Wesney and Tannock, 1979). Conaway et al. (1987), in a comprehensive study, determined the survival of four strains of lactic acid bacteria in human gastric juice, in vivo and in vitro, and in pH 1 to 5 buffered saline. In addition, they studied the adherence of two strains of Lactobacillus acidophilus, L. bulgaricus, and Streptococcus thermophilus to freshly collected human and pig small intestinal cells and to pig large intestinal cells. They demonstrated that the ability of these four microbes to survive in gastric juices and adhere varied significantly. In their study, survival and adherence was enhanced by milk, and all strains tested adhered to some extent to both human and pig intestinal cells. They suggest that the adhesion mechanism is probably nonspecific, which is in conflict with other reported specific adhesion of lactobacillus (Barrow et al., 1980; Kotarski and Savage, 1979; Mayra-Makinen et al., 1983). Therefore, it is important to study the adherence of organisms to the species of interest.

The objectives of this study were to use electron microscopy to 1) examine the adherence of L. acidophilus to human intestinal cells, and 2) to investigate mechanisms by which L. acidophilus adheres to human intestinal cells.

Materials and Methods

Bacterial strains and media

L. acidophilus BG2F04 (obtained from T. Kaehammer, North Carolina State University) was grown by daily transfer in MRS broth (Difco) incubated at 37°C. The identity of the L. acidophilus was confirmed by determining carbohydrate fermentation patterns using the Rapid CH Strip (dms/laboratories; ordered from API, Plainview, NY).

Tissue culture

Human intestinal tissue cells (HITC) F110074 (ATCC CCL 241) were cultured in a monolayer on Falcon plastic tissue flasks (25 cm² surface area). For adherence work, the cells were used at passage levels of 21 to 25. Growth medium consisted of Dulbecco's Modified Eagle Medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 0.2 units/ml insulin (Sigma), 1 mM oxalacetic acid (Sigma), 0.5 mM sodium pyruvate (Sigma), 0.1 mM non-essential amino acids (GIBCO), and 30 ng/ml epidermal growth factor (GIBCO). The cells were incubated at 37°C in a 5% CO₂ atmosphere and were allowed to grow confluent (approximately 1 week of growth) before use. Spent medium was removed and replaced with new medium every 2-3 days.

To determine relative adherence, the cells were cultured in plastic Leighton tubes (Costar Labs., Cambridge, MA). For electron microscopy, the tissue cells were grown in the Falcon plastic culture flasks.

Adherence study

The method of Kleeman and Kaehammer (1982) was followed. Bacteria were incubated with the tissue cells for 20 min. Earle's balanced salt solution (EBBS) without calcium and magnesium (GIBCO) was used as the rinse and suspending medium. Preparations were examined using a Nikon Labphot microscope at a magnification of 400X.

Effect of chemical treatments on adherence

The effect of several treatments on the adherence of L. acidophilus BG2F04 was investigated by suspending the organism in solutions prior to adherence testing. The treatments included sodium periodeate (10 mg/ml) (Sigma), Concanavalin A (0.2 mg/ml) (Sigma), EDTA (4.5%) and ruthenium red (RR) (0.05%) (EMS, Port Washington, PA). All treatments except EDTA were carried out at 37°C for 1 h. The EDTA treatment was done just prior to adding the bacterial suspension to the human intestinal tissue cells. Relative adherence was measured by counting the number of bacteria per tissue cell and dividing that number by the number of bacteria adhered to in an untreated control.

Transmission electron microscopy

To examine the cellular morphology of cells growing in broth, the bacterial cells were grown as previously described in 30 ml MRS broth, collected by centrifugation (3000 x g for 10 min), and rinsed once by resuspending the pellet in 0.1 M sodium cacodylate buffer (pH 7.2). The cells were collected by centrifugation (3000 x g for 10 min) and prepared for electron microscopy as described below. The effect of sodium periodate was studied by suspending the cells in a sodium periodate solution and incubating 1 and 3 h at 37°C.

Fixation

Primary fixation was done with 2.5% glutaraldehyde (EMS) in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h at room temperature. Secondary fixation was done with 2% OsO₄ in 0.1 M sodium cacodylate buffer for 1 h at room temperature.

Ruthenium red

For staining, 0.1% RR (EMS) was mixed with double strength primary fixative in a ratio of 1:1. The final concentration of RR was 0.05%.

Colloidal iron

After primary fixation the samples were treated with a mixture of distilled water, glacial acetic acid, and stock iron solution (Mowry, 1963) in the ratio 9:6:5 for 1 h (Brooker and Fuller, 1975). The pH of the solution was 1.8.

Dehydration, embedding, and post stains

The cells were collected, dehydrated in graded acetone series (3 min each in 25%, 50%, 75%, 95%, and 3 times in 100%) and infiltrated with 4:1 acetone to Spurr's resin (cured for 20 h at 60°C), sectioned with glass knives, and collected on Formvar-coated, copper grids. Post staining was done with lead citrate (3 min) and uranyl acetate (20 min). All samples were viewed on a Philips 300 transmission electron microscope.

To examine bacterial adherence to the tissue cells, the bacteria were prepared as described for adherence testing. The suspension was allowed to contact the tissue cells for 20 min, at which time the monolayer was rinsed twice with EBBS and once with sodium cacodylate buffer. The monolayer was fixed, dehydrated in a graded ethanol series, infiltrated, and embedded in situ. After the resin was cured, blocks were cut with an electric jigg saw and sectioned and stained as previously described.

Scanning electron microscopy

Sample tissue cells with adhered bacteria were prepared by placing a sterile, round glass coverslip in the tissue culture flask. The cells were allowed to grow confluent over the coverslip, and then the method of Kleeman and Kaehammer (1982) was followed to allow the bacteria to adhere to the tissue cells. The monolayer was rinsed twice with EBBS and once with 0.1 M sodium cacodylate buffer. Samples were fixed in 2.5% glutaraldehyde containing 0.05% RR for 1 h at room temperature.
Adherence of L. acidophilus to human intestinal cells

The monolayer was rinsed again and then fixed in 2% OsO4, the coverslip was removed with a forceps and placed in a holder. Primary dehydration was done at room temperature using a graded acetone series (5 min each in 25%, 50%, 75%, 95%, and 3 times in 100%). Secondary dehydration was done by critical point drying using CO2 as the transition medium. The coverslips were mounted on aluminum stubs and coated with gold-palladium in a Kinney vacuum evaporator (model KSE2-AM). The samples were viewed in a Philips 500X scanning electron microscope at 12 kV.

Results and Discussion

Adherence testing

When selecting lactobacilli to be used in food for their possible therapeutic value, factors concerning the capabilities to perform desired roles should be considered (Speck, 1980). One of those factors may be the ability of the organism to associate with and colonize in the intestinal tract. Methods using tissue culture have been suggested as a means for examining the adherence of bacteria to eukaryotic cells (Hartley et al., 1978). However, it should be noted that the environment of the intestine differs greatly from tissue culture medium used in vitro.

L. acidophilus BG2F04 has been shown to strongly adhere to human intestinal and tissue cells (Kleeman and Klaenhammer, 1982). Previous research in our laboratory revealed that strain BG2F04, when stained with RR, showed an electron dense layer exterior to the cell wall (Hood and Zottola, 1987). RR is commonly used to stain carbohydrate material but may also react with lipids (Luft, 1971). When L. acidophilus BG2F04 was allowed to adhere to HITC, electron microscopy showed a dark layer of material which could be seen on the bacterial cell as it attached to the intestinal cell (Fig. 1). There also appeared to be an electron dense layer on the HITC. It is common to find an acid mucopolysaccharide on the surface of mammalian cells (Behnke, 1968). It may be the association of the two surface layers that mediates adherence.

While the microorganism appeared to be very closely associated with the HITC, it did not appear to disrupt the integrity of the HITC. In Fig. 2, it appeared that the HITC had conformed to the shape of the bacterial cell. This may be an attachment site on the HITC, or it may just have been an artifact of the preparation procedure. Ward and Watt (1972) described similar "cushion-like" structures in electron micrographs of Neisseria gonorrhoeae adhering to urethral epithelial cells.

There is substantial evidence to indicate that the adherence of lactobacilli to mucosal surfaces is mediated by a polysaccharide on the surface of the cell. In a study using electron microscopy to observe bacterial adherence, Brooker and Fuller (1975) employed ruthenium red, colloidal iron, and alcian blue stains to determine the composition of the material associated with the adherence of lactobacilli to chicken crop epithelium. The material stained dark or were electron dense with these dyes. Curren et al. (1965) and Luft (1971) have shown that these stains are specific for polysaccharides and consequently can be used for identifying complex carbohydrates as cell components utilizing electron microscopy. When colloidal iron was used to stain the cells in this study, an electron dense layer was seen exterior to the cell wall (Fig. 3). The colloidal iron method is another technique which has been used to...
identify acidic polysaccharides. This stain is specific for carboxyl groups that would be found in such a carbohydrate. It will also stain other molecules which contain carboxyl groups. The results presented here suggest the involvement of a polysaccharide layer and agrees with the results shown by Brooker and Fuller (1975).

Conway et al. (1987) in their study of the adherence of four different strains of lactic acid bacteria to both human and pig intestinal cells in vitro utilized radioactively labelled bacterial suspensions to determine adherence. They estimated the number of bacteria bound to the intestinal cells from the bacterial specific activity. No attempt was made by these authors to visualize adherence by using electron microscopic techniques. The amount of manipulation done with the bacterial suspensions and the intestinal cells from both pigs and humans makes one wonder if what they observed was indeed adherence and not an artifact created by the techniques used. Their data are contrary to other investigators (Barrow et al., 1980; Kotarski and Savage, 1973; Maya-Makinen et al., 1983) who clearly demonstrated the specificity of adherence of lactobacilli to mammals other than humans. Although only one strain of *L. acidophilus* was used in the results presented here, it is also in disagreement with the findings of Conway et al. (1987), as adherence appeared to be specific in this study.

**Scanning electron microscopy**

Many gram-negative organisms, such as Salmonella, produce extracellular fibrils to aid in their attachment to solid surfaces (Schwach and Zottola, 1982). This phenomenon was also observed when organisms thought to be lactobacilli adhered to chicken crop cells (Brooker and Fuller, 1975). When *L. acidophilus* BGF204 was viewed on HITC, attachment fibrils were not detected (Fig. 4). The surface of the HITC is not smooth, so some bacterial cells may be trapped by microvilli and foldings on the surface of the HITC. From these results, it appeared unlikely that fibrils were involved in the adherence of *L. acidophilus* to the human gastrointestinal tract. The effect of chemical treatments on adherence is given in Table 1. Sodium periodate greatly reduced the ability of *L. acidophilus* BGF204 to adhere to HITC. Fuller (1975) reported that periodate had a similar effect on the adherence of lactobacilli to chicken crop cells. Periode is a strong oxidizing agent and has the capacity to cleave C-C bond of 1,2 diols that occur in sugars and which may be constituents of a polysaccharide (Hay et al., 1965). It has also been used in light microscopy in combination with Schiff's reagent to stain polysaccharides. Lipoteichoic acids (LTAs) also have been indicated as a component of the cell that may be involved in adherence (Sherman and Savage, 1986). Since LTAs contain glucose side groups, it is possible that periodate is disrupting the LTAs. In either case, this evidence points to a carbohydrate component on the cell surface that is responsible for the ability of the organism to adhere.

Electron micrographs of *L. acidophilus* treated with sodium periodate revealed that, after 1 h of exposure (Fig. 5A), the exterior layer was still present, although it was not as dense as the layer seen in Fig. 1. When treated for 3 h, it appeared that the layer was almost completely absent (Fig. 5B). It would seem that after 1 h, the component of the cell responsible for adherence was changed sufficiently to inhibit adherence, although the putative polysaccharide layer is still partially present. This suggested that the chemical structure of this layer was important for adherence.

Con A also appeared to reduce the ability of *L. acidophilus* to adhere to HITC, although the reduction was not as great as that by periodate (Table 1). Steinberg and Gepner (1973) suggested the use of Con A as a means to identify sites of adhesion which involve carbohydrates. Con A specifically binds to polysaccharides containing alpha-D-glucopyranosyl, alpha-D-mannopyranosyl, or certain 5-carbon rings and thus may be blocking sites of adherence on the bacterial cell. Ruthenium red did not appear to inhibit the adherence of *L. acidophilus* to HITC. As previously stated, RR is used to stain acidic polysaccharides, so it might be expected to reduce adherence. The fact that adherence was not reduced may be an indication that the carboxyl groups involved in ionic binding with RR were not important in the adherence of *L. acidophilus* to HITC.

### Table 1. Effect of various chemical treatments on the relative adherence of *L. acidophilus* to human intestinal cells in tissue culture.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative Adherence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0</td>
</tr>
<tr>
<td>Sodium Periodate</td>
<td>0.03</td>
</tr>
<tr>
<td>Ruthenium Red</td>
<td>0.99</td>
</tr>
<tr>
<td>Con A</td>
<td>0.74</td>
</tr>
</tbody>
</table>

*Relative adherence = no. bacteria per HITC / no. bacteria control HITC

**Summary**

There appeared to be a layer of electron dense material on the intestinal cell, and the bacterium may associate with this layer when adhering. When *L. acidophilus* attached to intestinal tissue cells after a short contact period, it did not appear to disrupt the integrity of the intestinal cell. Treatment of the organism with sodium periodate and Con A reduced the adherence to HITC, also indicating that a carbohydrate was involved. Although RR did not inhibit adherence, it is possible that RR did not bind to the functional groups involved in adherence. Electron micrographs of periodate-treated cells revealed that the layer appeared to be partially removed after 1 h and was almost completely absent after 3 h of treatment.
Adherence of L. acidophilus to human intestinal cells

Figure 4. SEM of L. acidophilus BG2F04 on HITC. Attachment fibrils are not produced by the bacterial cell. Note the rough texture of the intestinal cell.

Acknowledgements

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References


Figure 5. TEM of L. acidophilus BG2F04 treated with sodium periodate for A) 1 h, and B) 2 h.


Mowry RW. (1963). The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins. With revised directions for the colloidal iron stain, the use of alcian blue G8X and their combinations with the periodic acid-Schiff reaction. Ann. N.Y. Acad. Sci. 106, 402-423.


Discussion with Reviewers

T.A. McMeekin: What is the nature of the fibrils observed in earlier work with gram-negative bacteria? Are these simple extensions of the outer membrane?

Authors: The nature of the fibrils of gram-negative bacteria is addressed in another manuscript soon to be published in the Journal of Food Science (Herald and Zottola).

T.A. McMeekin: Is there species specificity involved in the adherence of Lactobacillus acidophilus to HITC? Do the results of your previous publication (Hood and Zottola, 1987, text reference), in which L. acidophilus adhered to glass, suggest that the carbohydrate layer mediates non-specific adhesion?

Authors: We feel that species specificity is involved in the adherence of L. acidophilus to HITC. In our previous study several different strains of L. acidophilus were examined for adherence properties. Several were able to adhere to glass that lacked the carbohydrate layer as defined in the paper here. This suggests that there is another non-specific mechanism for adherence to inert surfaces. Only one strain was used in the results given in this paper and in this instance species specificity seems to be involved.

T.A. McMeekin: Figure 2, and possibly Figure 1, indicate polar adhesion of L. acidophilus to HITC. Was this orientation seen consistently? Is there a specific adhesion site at the pole, or is it more hydrophobic than rest of the cell? Figure 2 is reminiscent of micrographs by Marshall and Cruickshank (1973), Arch. Microbiol. 91:29, showing polar orientation of bacterial cells at surfaces.

Authors: Polar adhesion was observed frequently but, at this time, it is not known if specific adhesion sites exist at the poles.

P. Allan-Wojtas, M. Kalab: The list of references should be expanded. JW Costerons is well known in the field of bacterial attachment and his work, in particular following two of his papers should be included and discussed. (1) Costerons JW, Geesey GG, Cheng KJ. (1978). How bacteria stick. Scientific American 238, 86-95; and (2) Costerons JW, Irvin RT. (1981). The bacterial glycocalyx in nature and disease. Ann. Rev. Microbiol. 35, 299-324. The former paper is important because it discusses the fact that "in nature (but not in laboratory cultures), bacteria are covered by a "glycocalyx" of fibers that adhere to the surfaces and to other cells." This may explain why the authors failed to see fibrils on their cultured bacteria. Many books published on the subject of the manuscript have not been mentioned e.g., Boedeker EC. (1984). Attachment of organisms to gut mucosa, 1 and 2, CRC Press, Inc.

The effect of various types of preparation on the appearance of bacteria have been studied by others e.g., Fraser TW, Gilmour A. (1986). Scanning electron microscopy preparation methods: their influence on the morphology and fibril formation in Pseudomonas fragi (ATCC 4972), J. Applied Bacteriol. 60, 527-533.

Authors: We are well aware of these references. The manuscripts from Costerons' group deal with primarily gram-negative marine organisms and gram-negative pathogenic bacteria. We did not feel these were pertinent to the study reported here and thus did not include them. Boedeker's book is a compilation of review papers from many authors, which did not appear to be applicable to the topic. Several of the authors in the book are referenced in other ways in this paper. The paper by Fraser and Gilmore is concerned with a gram-negative organism and its apparent attachment on inert material and does not appear to be relevant to this study.

P. Allan-Wojtas, M. Kalab: Wasn't there a chance that the washing of the bacteria in solutions used might have killed them and, consequently, they may not have adhered because they were dead rather than because their surface has been changed?

Authors: It is quite likely that the bacteria were killed in the washing solutions; however, in another study (Hood and Zottola, J. Food Science, in press) we report that dead L. acidophilus were able to adhere.

P. Allan-Wojtas, M. Kalab: Whose TEM techniques (fixation, dehydration, etc., ruthenium red and colloidal iron staining techniques, etc.) were followed? Dehydration is done in both acetone and ethanol, why the difference?

Authors: References are given in the methods section where appropriate. Where none are given the procedure used was developed by the authors. Acetone is used for dehydration of the bacterial cells alone. Ethanol was used when dehydrating the HITC because acetone solubilized the plastic tissue culture flasks and HITC support was lost.

P. Allan-Wojtas, M. Kalab: Why were blocks cut with an electric jigsaw rather than trimmed with a...
Adherence of *L. acidophilus* to human intestinal cells

glass knife or steel blade?
Authors: The embedding resin was poured into the tissue culture for trimming with a steel blade, the flasks had to be cut apart into small blocks. This was accomplished very efficiently with an electric jigsaw.

P. Conway: From Figure 4, the authors detected no fibrils. Their assumption is therefore that fibrils may not be involved in the human GI tract. Their sample preparation may not have preserved fine fibrils and the direct extrapolation from their cell line to the human gut is a little large.

Authors: Other work carried out in our laboratory has dealt with many different gram-negative and gram-positive bacteria. The preparation methods used with these organisms were similar. Gram-negative bacteria usually show fibrils, whereas gram-positive organisms do not. We stand by our assumption that no fibrils are produced by *L. acidophilus*.

P. Conway: The authors must also test protease treatments. While they have shown that periodate and concanavalin A inhibit, how can they be sure the adhesin is not sterically masked by the large concanavalin A molecule or that the oxidation of carbohydrates by the periodate does not destroy a nearby component which may be the adhesin? The inhibition for the concanavalin A is quite poor and as no statistics are presented, the question must be raised as to how significant the value of Con A is. If it is not significant, then the inhibition is only by the periodate. In the absence of the use also of sodium iodate as a control, the action of the periodate is questionable. Sodium periodate can contain sodium iodate which can be inhibitory without oxidizing carbohydrate groups.

Authors: The comments related to the use of chemical treatments are appreciated. They simply have not yet been done with this organism. In another study in our laboratory using the gram-negative organism, *Pseudomonas fragi*, this has been done and has been reported (Herald and Zottola, The effect of various agents upon the attachment of *Pseudomonas fragi* to stainless steel. J. Food Science, In press). In the above noted study, we did as was suggested by Dr. Conway and concluded that attachment by *P. fragi* is mediated by a complex polysaccharide. Eventually a similar study will be done with *L. acidophilus*.

P. Conway: Figure 5 shows that the electron dense material is removed by periodate, but without analysis of this released material, the authors cannot be sure they have not also released other material e.g. the adhesin. This problem was discussed by Fuller (1975) who showed that the proteolytic enzyme, pepsin, released material from the bacterial cell surface that had an affinity for concanavalin A. Material released by periodate may contain protein.

Authors: Our response to this comment is the same as given above. What is suggested has been done with *P. fragi* and is reported in the above noted paper. The results given in the paper by Herald and Zottola imply that a lipoprotein-polysaccharide complex is involved in the attachment of *P. fragi* to inert surfaces. A similar study will be done with *L. acidophilus* and adherence to human intestinal cells in tissue culture to see if a similar compound is involved.
Abstract

Factors that determine the digestibility of carbohydrates and minerals in cereals are examined. Most carbohydrates and minerals in cereals are structurally bound, either surrounded by or associated with cell wall components not easily digested by non-ruminant animals and humans. Treatments such as mechanical grinding and heat improve the digestibility of nutrients. Further processing and cooking result in structural and physicochemical changes of cereal starch, phytate, and dietary fiber. Such changes greatly influence the physiological and metabolic effects in animals and humans. The digestive breakdown of most nutrient components is also dependent on the activities of enzymes in cereals and in the mammalian digestive system. However, starch, phytate, and dietary fiber are not entirely and readily degraded by enzymes. Undegraded components reduce both the caloric value of the food and the availability of other nutrients by interacting with them in the gastrointestinal tract. Studies on availabilities of carbohydrates and minerals in cereal foods are conducted in humans and rats or under in vitro conditions, using various analytical methods including microscopy. The advantage of applying light microscopy and scanning electron microscopy coupled with energy dispersive X-ray microanalysis to study the digestive breakdown of structural components in cereal foods is highlighted by demonstrating the capabilities of the techniques to reveal both structural and microchemical information.

Introduction

Cereals are good sources of carbohydrates and minerals important for sustaining the energy and growth requirements of humans and animals. Cereals also contain dietary fiber. Increased consumption of dietary fiber has been associated with various health benefits (Trowell, 1976; Anderson and Chen, 1979).

Information concerning factors that affect the nutritional quality of cereal food derives from studies conducted in humans, or animals such as rats, fed diets containing various cereal products (McCance and Widdowson, 1935; Jenkins et al., 1975; Ismail-Beigi et al., 1977; Simpson et al., 1981; Navert et al., 1985; Heaton et al., 1988). In vitro conditions, simulating gastrointestinal environments, have also been used (Snow and O'Dea, 1981; Holm et al., 1985, 1988; Platt and Clydesdale, 1984, 1987). Oat and wheat brans are commercially available products and most frequently studied because of interests in the physiological and metabolic effects of phytic acid and dietary fiber (Promare and Heaton, 1973; Reinhold et al., 1975, 1981; Davies et al., 1977; Anderson et al., 1984; Moak et al., 1987; Shinick et al., 1988). Results obtained from many nutritional studies indicate that the structures and physical forms of cereal food components greatly affect the availability and utilization of the nutrients (Snow and O'Dea, 1981; Van Soest, 1984; Jenkins et al., 1986; Heaton et al., 1988; Holm et al., 1988).

Most cereal carbohydrates and minerals are associated with microscopically distinct structures (McMasters et al., 1971; Fulcher and Wong, 1980). Microscopic studies reveal much detail concerning the morphological organization and nutrient composition of oat and wheat grains before and after cereal processing (Pomeranz and Shellenberger, 1961; Buttrose, 1978; Fulcher and Wong, 1980; Fulcher, 1986; Lookhart et al., 1986; Yiu, 1986; Yiu et al., 1987). Using oat and wheat products as examples, the present review demonstrates how microscopy, particularly fluorescence and other light microscopy, has contributed to the understanding of relationships between cereal structures and the availability of carbohydrates and minerals in cereal foods. More specifically, the review examines factors such as...
processing, cooking, and enzymes that influence the structures and digestibilities of starch, phytate, and dietary fiber. Some of the effects of undigested fiber and phytate on the absorption of other nutrients in the mammalian gastrointestinal tract are also discussed.

Starch

Structure and Distribution

Starch is located inside the cells of the endosperm and is rarely found in the germ and aleurone tissues in mature grains. Starch occurs in the form of colorless translucent bodies, identified as starch granules. Reviews by Evers (1979), Hood and Liboff (1982), and Fulcher (1986) gave detailed descriptions of the structures of starch granules. Cereal starch granules vary in size and morphological appearance, depending on the species of the cereal grain. For example, wheat starch consists of both small (2 - 10 μm) spherical and large (20 - 40 μm) lenticular granules (Fig. 1). Unlike wheat starch, oat starch occurs chiefly as compound granules which are aggregates of sub-granules (Fig. 2). The oat starch granules range from 20 to 100 μm in size. The various techniques of light microscopy are suitable for studying the distribution of starch in cereal grains. For instance, staining procedures using iodine - potassium iodide, the periodic acid-Schiff reagent, or fluorescein-coupled plant lectins such as Lens culinaris agglutinin and Concanavalin A, are appropriate for revealing the location and structural organization of starch in a variety of cereals and cereal foods (Jensen, 1962; Fulcher and Wong, 1980; Miller et al., 1984; Yiu, 1986). The staining procedures provide both convenience and speed for detecting starch content in cereals. Scanning and transmission electron microscopy are also useful for investigating the complex structure of starch (Gallant and Guilbot, 1969; Yamaguchi et al., 1979). Most cereal starches exhibit birefringence under polarized light (Wivinis and Maywald, 1967; Greenwood, 1979). Both oat and wheat starches show the characteristic 'maltese cross' pattern under a polarized light microscope (Fig. 3). Birefringence is lost when a starch granule undergoes physical changes associated with gelatinization (Sandstedt, 1961; Lineback and Wongrikasem, 1960; Varriano-Marston, 1982).

Processing and Cooking

During the milling of wheat, endospermic cells of the subaleurone layer are more resistant to the force of grinding, and are reduced in size less readily than cells of the inner starchy endosperm (Kent, 1966; Pomeranz, 1982). The coarse fraction of wheat flour, which derives primarily from the centre of the endosperm, has higher starch content than the finer flour fraction, which contains more fragments of the protein-rich subaleurone layer (Pomeranz, 1982). Endosperm cells in soft wheat varieties contain starch granules embedded in a friable protein matrix which is susceptible to the grinding force, resulting in the release of intact starch granules with little damage (Kent, 1969). On the other hand, endosperm cells of hard wheat varieties tend to shatter rather than powder due to the continuous protein matrix, resulting in breakage of both starch and the protein matrix (Moss et al., 1980; Pomeranz, 1982). Starch damage in the flour increases the water binding capacity and susceptibility to α-amylase degradation (Jones, 1940; Pomeranz, 1982).
Figure Captions

Unless otherwise stated, all micrographs show 3% glutaraldehyde-fixed, glycol methacrylate-embedded sections of oat or wheat grain tissues. Numbers at scale bars are in μm. Photographed using fluorescence exciter/barrier filters set for maximum transmission at 355 nm/418 nm (FCI) or 490 nm/520 nm (FCII).

Fig. 10 A section of wheat bran stained with 0.1% Acridine Orange, showing the presence of phytin globoids (arrows). FCI.

Fig. 11 A section of puffed wheat stained with 0.1% Acriflavin HCl, demonstrating changes in the aleurone cell structure (arrows). FCII.

Fig. 12 A section of ileo digesta removed from a rat fed a diet containing oat bran, and stained with 0.1% Acridine Orange, showing the presence of phytin globoids (arrows) within the aleurone cells. FCII. (Yiu and Mongeau, 1987).

Fig. 13 A section of rat colonic digesta stained with 0.1% Acriflavin HCl to show the presence of undigested phytin globoids (small arrows) and cell wall fragments of high phenolic contents (large arrows). FCI. (Yiu and Mongeau, 1987).

Fig. 14 An elemental profile of an undigested phytin globoid. A 2 μm thick, glycol methacrylate-embedded, carbon-coated section of rat colonic digesta examined under a scanning electron microscope at 20 kV, and analyzed with an energy dispersive X-ray microprobe for 100 s/site. Probe current: 5 × 10⁻⁵ A. Probe size: 180 nm. Ca: calcium, K: potassium, Mg: magnesium, P: phosphorus.

Fig. 15 A section of oat kernel stained with 0.01% Calcofluor White in 50% ethanol, showing the distribution of β-glucan-rich aleurone (A) and sub-aleurone (*) cell walls. FCI.

Fig. 16 A section of instant rolled oats stained with fluorescein-labelled Lens culinaris agglutinin (1.2 mg/ml in 0.01M sodium phosphate buffer, pH 7) and 0.01% Congo Red to show the extent of cell fracture (arrows) after processing. FCII.

Fig. 17 A section of regular rolled oats stained and photographed the same way as in Fig. 16 to demonstrate the intact cell wall structures (arrows).

Fig. 18 A section of rat ileo digesta stained with 0.01% Cellulofluor in 50% ethanol, showing the partially digested sub-aleurone (large arrows) and relatively intact aleurone (small arrows) layers. FCI. (Yiu and Mongeau, 1987).

Fig. 19 A section of rat colonic digesta stained with 0.1% Acridine Orange to show the partially digested aleurone cell walls (arrows). FCII. (Yiu and Mongeau, 1987).

Mechanical grinding, e.g., rolling and flaking, tends to induce the breakdown of compound starch granules in oats (Lookhart et al., 1986; Yiu, 1986). The thinner the oat flake, such as those of quick-cooking rolled oats, the more the breakdown occurs (Fig. 4). However, the structural integrity of starch sub-granules remains unchanged (Yiu, 1986).

When starch is heated in the presence of water, the granule swells as a result of water absorption. The swelling is initially hampered by the rigidity of the cell wall, resulting in many distorted and convoluted starch structures (Fig. 5). The integrity of the granule is lost when starch becomes completely gelatinized. The expanded structure of starch provides greater accessibility to enzymes, resulting in an increased rate of starch digestion (Wursh et al., 1986; Yiu et al., 1987). Furthermore, the rate of increase of glucose and insulin concentrations in blood is directly related to the percentage of starch gelatinized (Holm et al., 1985, 1988; Ross et al., 1987).

Processing methods such as extrusion cooking, explosion puffing, and instantization hydrate the starch granules or disrupt the native structures, similar to but surpassing the result of conventional heating (Brand et al., 1985). Such processing conditions appear to change starch digestibility and elicit different glycemic responses (Holm et al., 1985; Jenkins et al., 1986; Ross et al., 1987).

Digestibility

Cereal starches are readily digested by humans and animals. Starch-specific hydrolytic enzymes are abundant in most cereal grains, microorganisms, and mammalian salivary and pancreatic secretions (Jones, 1940; Marshall and Whelan, 1979). Detailed mechanisms involving the enzymatic hydrolysis of starch are described by Manners (1985). Briefly, α-amylase randomly hydrolyzes amylose and amylpectin to maltosaccharides which are degraded by α-glucosidases to glucose. The present review mainly examines factors that influence the digestive breakdown of cereal starch.

Particle size reduction of the starch-bearing matrix increases the digestibility of starch. Greater accessibility to enzymatic reactions, makes starch of finely ground flour more readily digested than starch of unprocessed cereals (Snow and O'Dea, 1981; Heaton et al., 1988). Damaged starch is more susceptible to amylase degradation than intact granules (Jones, 1940).

The presence of α-amylase inhibitors in cereals reduces starch digestibility (Shainkin and Birk, 1970; Rea et al., 1985). Alpha-amylase inhibitors can be removed through milling and cooking (Snow and O'Dea, 1981; Rea et al., 1985). Results of in vitro studies indicate that interactions can take place between starch and other food components like lipids (Larsson and Miezis, 1979; Holm et al., 1983), proteins (Anderson et al., 1981; Jenkins et al., 1987), polyphenols (Thompson et al., 1984; Bjorck and Nyman, 1987; Knudsen et al., 1988), or phytic acid (Voon et al., 1983; Thompson, 1986).
resulting in the formation of complexes that resist enzymatic degradation. A fraction of starch extracted from processed cereals has been identified as resistant to breakdown by α-amylase both in vitro and in the small intestine of man (Levine and Levitt, 1981; Englyst and Cummings, 1985). Processing procedures, particularly freezing and thawing, cause retrogradation of the starch and increase resistance to amylolytic action (Englyst et al., 1983). Starches that resist digestion are available for microbial fermentation in the lower gut, but the generated energy is reduced (Waslien, 1988). Resistant starches can cause inaccuracy in quantifying the amount of dietary fiber in food products. Methods which rely on gelatinization in water and enzymatic removal of starch prior to the quantification of dietary fiber are affected by the presence of resistant starches. A method has been developed to determine the amount of starch in processed cereals resistant to amylolytic enzymes used for dietary fiber determination (Englyst et al., 1983). However resistant starches constitute only a small fraction of starch that escapes in vivo digestion (Englyst and Cummings, 1985). Hence, alternative additional methods are required to assess the content and digestibility of starch in cereal foods.

Microscopy serves as a practical tool for detecting and analyzing the digestive breakdown of starch in cereal foods. For example, light microscopy using iodine – potassium iodide as a staining reagent, can be used to detect starch in the small intestine of the rat (Fig. 6). The structural appearance of starch present in rat digesta reflects the extent of starch breakdown (Fig. 7). According to Sandstedt (1955) and Evers et al. (1971), α-amylase-digested starch has a hollow centre linked to the surface by a few radial channels, whereas amyloglucosidase-digested starch has a surface covered with shallow pits.

Phytate

Structure and Distribution
Phytate (myo-inositol hexaphosphate) accounts for 70-90% of the total phosphorus reserve in most mature cereal grains (Ashton and Williams, 1958; O'Dell et al., 1972; Lolas et al., 1976; Frolich and Nyman, 1988). Chemical data (O'Dell et al., 1972) indicate that the majority of the phosphorus reserve is contained in the bran and germ fractions of cereal grains like wheat. Microscopic studies contribute to knowledge of the occurrence and distribution of phytate in cereal grains. Phytate-containing particles can be identified and located in the aleurone and scutellum tissues of most cereal grain kernels by electron microscopy coupled with scanning electron microscopy (Tanaka et al., 1974), transmission electron microscopy (Ogawa et al., 1975), and electron dispersive X-ray (EDX) microanalysis (Liu and Pomeranz, 1975; Buttrose, 1978). The phytate-containing particles are electron-dense inclusions embedded in the protein matrix of the aleurone grains, and are referred to as phytin globoid crystals or phytin globoids (Lott and Spitzer, 1980). Ranging from 1 to 2 μm in diameter, phytin globoid crystals are mostly spherical in shape and contain high concentrations of phosphorus (P), potassium (K), and magnesium (Mg) (Lott and Ockenden, 1986). When subjected to EDX microanalysis, the globoid crystals emit X-rays characteristic of their elemental composition. A typical EDX spectral profile of oat phytin globoids is composed of three major element peaks, P, K, and Mg (Fig. 8). A small quantity of other elements is also present (Buttrose, 1978). The concentrations vary depending on grain varieties and locations of growth (Buttrose, 1978; Batten and Lott, 1986).

Rapid detection of the distribution of phytin globoids in cereals and cereal foods can be achieved using optical light microscopy. Polarized light microscopy effectively locates the birefringent structures of phytin globoids in hand-prepared or glycol-methacrylate embedded materials without any staining (Fulcher, 1982; Yiu et al., 1982). For confirmation, other types of light microscopy are often used. Cathodoluminescent stains such as Acriflavine HCl, Acridine Orange, and Toluidine Blue are suitable microscopic markers for phytin inclusions (Yiu et al., 1982; Fulcher, 1962; Yiu, 1986; Yiu and Mongeau, 1987). Fig. 9 illustrates the distribution of phytin globoids in the aleurone cells of wheat as revealed by fluorescence microscopy.

Processing and Cooking
Milling reduces the phytate content in wheat (Nayini and Markakis, 1983) by removing the bran and germ fractions from the flour, but milling does not dissociate the structural attachment of phytin globoids from bran and germ fractions (Fig. 10). Vigorous processing methods like extrusion cooking and puffing induce structural changes in cereals to such an extent that components within the aleurone cells are no longer identifiable by phytin-specific staining (Fig. 11). Extrusion cooking alters the physicochemical properties of phytate, reduces phytate degradation in the intestine (Sandberg et al., 1986) and eliminates endogenous activities of phytate-specific enzymes (phytase) in cereals (Sandberg et al., 1987). The decrease in phytate degradation is associated with decreased absorption of zinc, phosphorus, and magnesium in the human small intestine (Kivisto et al., 1986).

Milder heat treatment like domestic cooking reduces the phytate content in cereals such as wheat and rye, but not oats (Sandstrom et al., 1987). During bread making, the presence of additional phytases from yeast and the baking process significantly reduce the phytate content in bread (de Lange et al., 1961; Nayini and Markakis, 1983). Phytate-reduced bread has less effect on in vitro and in vivo absorption of minerals than phytate-containing bread (Reinhold et al., 1974; Navert et al., 1989).

Digestibility of Cereal Phytate and Nutritional Implications
Early metabolic studies indicated that phytate phosphorus is not readily available for digestive absorption by humans and animals (McCance and Widdowson, 1935; Mellanby, 1949). Dietary
deficiency of phosphorus is unlikely since phosphorus is readily available from other dietary sources. However, when cereals constitute a large portion of the diet, the degree to which humans can utilize phytate may become important.

The degradation of dietary phytate chiefly depends on the hydrolytic activities of phytases (Nayini and Markakis, 1986). Phytases, or myo-inositol hexakisphosphate phosphohydrolases, are enzymes that break down phytic acid to myo-inositol and inorganic phosphate via intermediate myo-inositol phosphates (penta- to mono-phosphates).

Phytase activities exist in the endosperm of wheat (Peers, 1953), and in the aleurone cells of rice (Yoshida et al., 1978), barley (Tronier et al., 1971), sorghum (Adams and Novelle, 1975), and corn (Chang, 1967). Phytase activity increases during germination (McCance and Widdowson, 1944; Bartnik and Szafranska, 1987) resulting in a decrease in the phytate content of the grain (Nayini and Markakis, 1986). By comparison with wheat and rye, oats have lower phytase activities both before and after germination (McCance and Widdowson, 1944; Bartnik and Szafranska, 1987). Recent results based on "31P-nuclear magnetic resonance spectroscopy confirm that oat phytase is inactivated by the heat treatment received during commercial oat processing (Frolich et al., 1986). Other studies conclude that processing such as extrusion cooking impairs phytase activity in wheat bran (Sandberg et al., 1986 & 1987). Hence, low or reduced activities of phytases account for the relatively high phytate contents and low phytate digestibilities in certain oat and extruded wheat products (Mellanby, 1949; Sandberg, et al., 1987; Yiu and Mongeau, 1987).

Phytase activities are located in the mucosal tissues of most mammalian intestines (Nayini and Markakis, 1986). Some of the phytate present in the small intestine is likely hydrolyzed by mucosal phytase. The extent of the hydrolytic action depends on the presence and concentration of dietary minerals like Ca and Zn (Wise, 1986). The significance of the involvement of mucosal phytase in phytate degradation is not clear. One feeding study shows that mucosal phytases and enzymes such as alkaline phosphatase do not play important roles in phytate digestion, as close to 95% of the ingested phytate from phytate-deactivated wheat bran can be recovered in human ileostomy contents (Sandberg and Andersson, 1988). Another study reports that much of the ingested phytate, present in oat bran fed to rats, remains undigested in the small intestine (Yiu and Mongeau, 1987). Microscopic examination of the rat digesta shows that many of the ingested phytin globoids are structurally associated with the aleurone tissues (Fig. 12). Microscopic observations also provide direct evidence that the majority of the phytate is not taken up by the lower gut of the animal (Yiu and Mongeau, 1987). Phytases originating from the microflora, which usually populate the large intestines of animals and humans, seem to play a key role in phytate degradation. However, despite the presence of microbial phytase, phytate degradation is significantly influenced by phytase activities endogenous to most cereals (Bartnik and Szafranska, 1987; Sandberg and Andersson, 1988), and certain processings and baking (de Lange et al., 1961; Reinhold et al., 1974; Nayini and Markakis, 1983; Navert et al., 1986).

Microscopic examination of dietary phytate in colonic contents of rats revealed intact oat bran phytin globoids (Yiu and Mongeau, 1987). The undigested phytin globoids not only retained morphological and staining characteristics (Fig. 13) but also the elemental contents as revealed by EDX-microanalysis (Fig. 14). Undigested phytin is a cause of concern since the cation-binding activities may impair mineral bioavailability (Mellanby, 1949; Erdman, 1979; Oberleas and Harland, 1981).

Mineral deficiencies have been noted in humans and monogastric animals whose diets consist predominantly of whole grains of high phytate content (Mellanby, 1949; Erdman, 1979). The presence of six ortho-phosphate moieties in the phytic acid molecule provides the compound with a potential for complexing cations such as zinc, iron, magnesium, and calcium (O'Dell, 1969; Morris, 1986). Interactions between phytic acid and cations result in the formation of insoluble complexes, thereby reducing the availability of minerals (Erdman, 1979; Platt and Clydesdale, 1987). In addition, interactions can occur between protein and phytic acid, protein-cation and phytic acid, or starch and phytic acid (Cheryan, 1980; O'Dell and de Boland, 1976; Thompson, 1986; Wise, 1986). However, the deleterious effect of phytate on mineral metabolism in humans and animals is avoidable when diets are well balanced, especially in mineral contents (Morris, 1986; Moak et al., 1987).

Dietary Fiber

The definition of dietary fiber is still a debatable subject (Trowell, 1976; Cummings, 1976; Southgate, 1978; Selvendran, 1983; Englyst et al., 1987; Asp et al., 1988). However, it is generally accepted that indigestible plant materials are the main constituents (Trowell, 1988). This review examines only cereal brans that are known to be associated with the major physiological effects of dietary fiber and are of major commercial interest (Anderson and Chen, 1979; Anderson, 1985; Schneeman, 1987). Cereal bran is a product of commercial processing, the outer part of a grain kernel isolated through mechanical grinding and sieving (Deane and Connors, 1986). Bran is composed of several layers of fibrous tissues, including the pericarp and seed coat, and parts of the endosperm which include the aleurone and subaleurone cells (Figs. 10 & 15).

Physiological and Metabolic Effects of Oat and Wheat Brans

Oat and wheat brans attract public interest because of known physiological and metabolic effects believed to be beneficial. Oat and wheat brans can bind water, bile salts, and other substances in the intestinal tract (Promare and Heaton, 1973; Eastwood and Mowbray, 1976; Eastwood et al., 1980; Spiller et al., 1986; Anderson and Chen, 1986), resulting in various potential health benefits (Trowell, 1976; Anderson and 105
Chen, 1979; Anderson, 1985; Anderson and Tietjen-
Clark, 1986; Schneeman, 1987; Burkitt, 1988).
However, oat and wheat brans differ in colonic
and metabolic functions. Oat bran is effective in
reducing serum cholesterol levels and slowing
glycemic responses, and wheat bran in increasing
fecal weight and decreasing transit time, thereby
decreasing the incidence of diverticulosis and
colorectal cancer (Kritchevsky et al., 1984;

Fiber Composition of Oat and Wheat Brans

Dietary fiber has been divided into two ca-
tegories, soluble and insoluble (Anderson and
Chen, 1979). The rationale for this division is
based on solubility in hot water. Soluble cellular
fiber includes polysaccharides referred to as
gums and some hemicelluloses, whereas insoluble
fiber includes cellulose, some hemicellulosic
polysaccharides and lignin (Southgate, 1978;
Anderson and Chen, 1979; Southgate and Kritchevsky,
1981). Physiologically based definitions, such as
adopted by the Canadian Expert Committee on
Dietary Fiber (Health and Welfare Canada, 1979),
refer to plant materials not digestible by man.
Such materials consist of nonstarch polysaccha-
drides and lignin, and may include associated
substances.

Oat and wheat brans differ in their fiber
contents and compositions. According to Frolich
and Nyman (1988), commercial oat bran has less
than half the amount of total dietary fiber of
wheat bran, but its soluble fiber content is
greater, approximately 35%, as compared to wheat
bran which has about 2%. Furthermore, the major-
ity of the soluble oat fiber is present in the
form of (1-3)(1-4)-β-D-glucan, generally known as
oat gum (Wood, 1966), whereas arabinoxylans are
the major soluble fibers in wheat (Selvendran,
1983).

Structures of Oat and Wheat Brans

Cereal cell walls, particularly cell walls
present in the bran, are major sources of dietary
fiber (Cummings, 1976; Southgate, 1978; Selven-
dran, 1983). Chemical studies of isolated cell
wall fractions provide detailed information on
the chemical composition of cereal cell walls.
Most cereal cell walls are composed of cellulose
microfibrils embedded in a matrix of hemicellu-
oses, some of which are cross-linked by lignin
and phenolic esters, and/or proteins (Mares and
Stone, 1973; Bacic and Stone, 1981; Selvendran,
1983).

Differences in fiber composition between oat
and wheat brans are best revealed by fluorescence
microscopy which provides both structural and
microchemical information. When stained with
dyes such as Congo Red or Calcofluor White (Wood
et al., 1983), oat bran is characterized by its
β-D-glucan content located chiefly in the inner
aleurone and subaleurone cell walls (Fig. 11). Wheat
bran, on the other hand, does not have the
same histochemistry: its aleurone cell walls are
dominated by the relatively high phenolic content
best revealed by fluorescence microscopy when
viewed under short wavelength (365nm) excitation
(Fig. 9). Intense autofluorescence is detected in
the subaleurone cell wall because of its
ferulic and p-coumaric acid contents (Frolich
et al., 1972; Fulcher, 1982). Other structural
components of the bran, including the outer peri-
carp and seed coat layers, which have high lignin
and cutin contents (Ring and Selvendran, 1980;
Schwarz et al., 1988) as well as the subaleurone
starch granules, can also be revealed with fluo-
rescence microscopy (Fig. 9).

Processing and Cooking

Mechanical processing breaks down the endo-
sperm cell walls of oats and wheat, reducing par-
ticle size (Schultze and MacMasters, 1962; Moss
et al., 1980; Yiu, 1986). The extent of process-
ing affects the degree of cell wall breakdown.
For example, instant rolled oats have consider-
ably more cell wall fractures (Fig. 16) than
regular rolled oats (Fig. 17), as the former are
subjected to more processing steps than the lat-
ter (Deane and Commers, 1986). Particle size
reduction through grinding collapses the physical
structure of wheat bran and alters its physico-
chemical properties to such an extent that the
water-holding (Van Soest, 1984; Cadden, 1987) and
bile salt-binding capacities of wheat bran (Mon-
geau and Brassard, 1982) are reduced. On the
other hand, processing wheat bran of low moisture
content by extrusion cooking at high temperature
and pressure and short duration of time increases
not only the soluble fiber content but also the
digestibility of wheat bran in the rat (Bjorck
et al., 1984).

Domestic cooking reduces the water-holding
capacity of wheat bran (Nyman et al., 1976). Extensive heating and chemical treatments, such as isolation and purification of fiber components like delignified cellulose, decrease the hydra-
tion capacity of the fibers, slow down the rate
of degradation in the colon (Van Soest, 1984),
and modify mineral-binding activity (Frolich
et al., 1984). In rolled oats, cooking facilitates
the release of β-D-glucan from the cell wall (Yiu
et al., 1987). The amount of the β-D-glucan re-
leased is greater in porridge prepared by cooking
rolled oats gradually from room temperature than
by cooking rolled oats rapidly in boiling water
(Fig. 20). Microscopic examination indicated

![Fig. 20](https://example.com/fig20)

Effect of different preparation methods
on β-glucan release from rolled oats. Gradual
cooking: (●●●), Rapid cooking: (■■■), soaking:
(○○○). Cooking time = simmering time. (Yiu et
al., 1987).
that gradually cooked rolled oats have considerably more cell wall disruption (Fig. 21) than rapidly cooked rolled oats (Fig. 22).

Figs. 21 & 22 Glycol methacrylate-embedded sections of rolled oats prepared by gradual cooking and rapid cooking, respectively, and stained with 0.01% Calcofluor in 50% ethanol to reveal differences in cell wall breakdown in the inner oat endosperm. (Yiu et al., 1987).

Degradation and Nutritional Implications

The degradation of cereal fibers is dependent on the enzymatic activities provided by the microflora normally present in the colon where absorption of the products of fiber fermentation occurs (Hungate, 1976; Cummings, 1976; Cummings and Englyst, 1987). The products are mostly volatile fatty acids including acetic, butyric, and propionic acids and carbon dioxide, hydrogen, and methane gases. Activities of cellulases, β-glucosidases, cellobiase, and β-glucanases are reported in the human colonic microflora (Salyers et al., 1976; Bacon, 1978). Chemical and metabolic studies of individual fiber components reveal that about 56%-87% of hemicelluloses are digestible, and about 40% of ingested cellulose is degradable (Cummings, 1976; Anderson and Chen, 1979; Nyman et al., 1986). However, highly lignified cell walls and the presence of phenolics, as well as substances such as cutin and silica greatly reduce the digestibility of cereal fibers (Van Soest and Jones, 1968; Cummings, 1976).

Many cell walls remain structurally intact after passing through the colons of humans or rats (Dintzis et al., 1979; Yiu and Mongeau, 1987).

Fluorescence microscopy is a useful tool to study the digestive breakdown of cereal cell walls by animals (Fulcher and Wood, 1983; Yiu and Mongeau, 1987). Microscopic observation provides direct evidence of differences in digestibility among the various structural components of cereal bran subjected to digestive processes (Yiu and Mongeau, 1987). For example, the β-D-glucan-rich subaleurone cell wall of oat bran is susceptible to the digestive environment of the small intestine of the rat (Fig. 18), whereas degradation of the aleurone cell wall does not take place until the bran reaches the colon (Fig. 19). On the other hand, most of the pericarp and seed coat layers (Fig. 13) as well as the trichomes remained undigested (Yiu and Mongeau, 1987). Trichomes, which are hair-like tubular structures found on the surface of most oat grains, have a high silica content.

The detection of undigested cell wall components in the colonic digesta indicates unavailable food materials. Not only are carbohydrates of cereal cell walls unavailable, but structurally associated trace minerals, such as silicon, chromium, manganese, and cobalt likewise are not absorbed (Jones, 1978). Furthermore, certain fiber components of oat and wheat brans, such as lignin, cellulose, and hemicellulose, have affinities for minerals including calcium, iron, zinc, copper, and magnesium (Reinhold et al., 1975; Jones, 1978; James et al., 1978; James, 1980; Gillooly et al., 1984; Platt and Clydesdale, 1984; Moak et al., 1987). The main functional groups in the organic components of cereal cell walls that may be involved in mineral binding include the carboxyl and hydroxyl groups of phenolic compounds, lignin, and certain polysaccharides (Jones, 1978; James et al., 1978). Results of several metabolic studies indicate that long-term intake of high-fiber food increases fecal mineral excretion. However, the excretion has no deleterious effect on mineral balance in humans due to abilities of the human body to adapt to changes in dietary conditions (Ismail-Beigi et al., 1977; Van Dokkum et al., 1982; Morris and Ellis, 1985).

Both phytate and fiber present in the bran are in close proximity to one another. Hence, it is often difficult to assess the individual effect of the bran components on mineral binding by analytical methods involving chemical extraction and determination (Davies et al., 1977; Platt and Clydesdale, 1987). Microscopy, however, can provide such information. Microscopic evidence suggests that indigestible remnants of wheat bran, mostly pericarp tissues, are associated with increases in the excretion of calcium and iron in humans (Dintzis et al., 1985). The structural (Fig. 23) and elemental (Fig. 24) contents of some of the minerals defecated by rats fed a diet rich in oat bran can be analyzed using fluorescence microscopy and scanning electron microscopy coupled with EDX-microanalysis. Structural association between the minerals and oat bran components is not evident. However, the microscopic
Examples given in this review demonstrate how microscopy can be used to study cereal microstructures. The nutritional quality of cereal food is affected by the organization of structural components associated with starch, phytate, and dietary fiber. Processing and cooking as well as specific enzymes are factors which can alter cereal structures to such an extent that nutrient availabilities are affected.

The availabilities of most cereal carbohydrates and minerals for digestive absorption are affected by biological structures associated with cereal foods. Some structures are not readily accessible to the digestive enzymes present in the gastrointestinal tract. Mechanical grinding and heat are used to improve their digestibilities by reducing particle size, breaking down cell walls, inducing starch gelatinization, destroying α-amylase inhibitors and activating phytase in cereals. Excessive processing and high heat, on the other hand, may alter the morphological and physicochemical properties of starch, phytate, or cereal fiber to such an extent that digestibility is reduced. Undigested phytate and dietary fiber have the potential to adversely influence the bioavailability of minerals in humans and animals. However, repeated metabolic studies demonstrate that, with sufficient intake of dietary minerals, mineral balance can be maintained on diets high in phytate and fiber contents.

Used in conjunction with other analytical methods, particularly EDX-microanalysis, microscopy is an important tool which has the ability to obtain not only structural but also microchemical information pertinent to the nutrient composition of cereal foods.

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References


study on excreted materials demonstrates the analytical capability of microscopy combined with other detection methods such as EDX-microanalysis. EDX-microanalysis in conjunction with microscopy may serve as useful techniques for studying direct association between minerals and individual bran components.

Figs. 23 & 24 A glycol methacrylate-embedded, carbon-coated section of rat colonic digesta examined under a scanning electron microscope at 20 kV, and analyzed with an energy dispersive X-ray microprobe for 100 s/site, showing (Fig. 23) the structures of unabsorbed minerals and (Fig. 24) an elemental profile of one of the structures (identified by an * in Fig. 23). Probe current: 5 x 10⁻⁹ A. Probe size: 180 nm. Ca: calcium, P: phosphorus.

S. H. Yiu
Cereal Structure And Nutritional Quality


Jones CR. (1940). The production of mechanically damaged starch in milling is a governing factor in the diastatic activity of flour. Cereal Chem. 17:133-164.


Mares DJ, Stone BA. (1973). Studies on wheat endosperm. I. Chemical composition and ultra-


Cereal Structure And Nutritional Quality


Discussion with Reviewers

B.G. Swanson: What evidence can you provide to suggest that microscopy can serve as an accurate analytical tool?

Author: With the aid of specific staining reagents such as iodine - potassium iodide and acid Fuchsin, microscopy can be used to accurately differentiate starch granules from protein bodies in most cereal grains.

B.G. Swanson: How do you perceive microscopy can quantitatively determine starch digestibility? Author: Microscopic observation can reveal structural changes of starch granules subjected to enzymatic digestion, but cannot quantify how much starch is digested.

B.G. Swanson: What is your conclusion regarding importance of phytate to digestibility, mineral absorption and the nutritional quality of cereals?

Author: The major concern of phytate in relation to the nutritional quality of cereals is the mineral-binding property. However, phytate contents in most cereal grains are reduced as a result of processing, baking, and mild heat treatments. Feeding studies indicated that the deleterious effect of phytate on mineral metabolism can be avoided by maintaining diets that are well balanced in mineral contents.

B.G. Swanson: Can you compare observation of dietary fiber by fluorescence and scanning electron microscopy?

Author: While scanning electron microscopy has better resolving power than fluorescence microscopy, it does not reveal any chemical information of a fiber structure. The distributions of fiber-associated substances, such as phenolic acids and components such as β-D-glucans in cereal cell walls, can be easily detected using fluorescence microscopy.

L.U. Thompson: Will you please further clarify how the appearance and composition of the crystalline minerals illustrated in Figures 23 and 24 may reveal nutrient interactions?

Author: Figures 23 and 24 are included to demonstrate the analytical capability of SEM and EDX microanalysis. Such techniques have the ability to provide both structural and elemental information. Hence, any changes in the elemental composition of phytin globoids after they have passed through the gastrointestinal tract can be detected using the above techniques. Differences in the composition should reflect the mineral-binding activity of the globoids, provided that artifacts such as the migration of soluble elements in and out of the globoid structures during sample preparation are taken into account or eliminated.

L.U. Thompson: Since phytase in cereal foods may affect the breakdown of phytic acid in the gastrointestinal tract, have you or others tried estimating its location and concentration by microscopic techniques? How?

Author: Tronier et al. (1971) localized phytase activity in the aleurone cell of barley using transmission electron microscopy. Other microscopic techniques such as immunofluorescence or enzyme-linked immuno-staining are suitable for detecting and quantitating enzyme activities in animal or plant tissues.

Contribution No. 804

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Ultrastructural changes in meat incubated with different concentrations of acetic acid were investigated by transmission electron microscopy. Discs of bovine M. longissimus dorsi (48 h postmortem) were incubated at 4°C for 48 h in various acetic acid solutions, giving discs with pH values ranging from 5.1 to 3.9. Below pH 4.5, the volume of the discs increased markedly due to water absorption. Discs incubated in 0.01M acetic acid had a pH of 5.1, lower swelling ratios and shorter sarcomeres than control samples at pH 5.5. Structural studies of the samples at pH 5.1 revealed that A-band length decreased whereas I-band length increased. There was fragmentation of thin filaments in the Z-line region and disorganisation of Z-line structure.

In discs treated with 0.05M acetic acid (meat pH 4.5), complete loss of M-lines was observed. Noticeable swelling also occurred across the muscle fibre axis, mainly in the A-band region, and myosin filaments were partially extracted. Z-lines were swollen, disorganised and slightly fragmented. Below pH 4.5, swelling became much more pronounced with further extraction of myosin filaments. Most of the actin filaments were extracted and myofibrils fused together giving an amorphous, coagulated appearance. Z-lines were partially extracted.

In general, the greatest amount of swelling occurred across the muscle fibre axis although significant increases in sarcomere length were observed at pH 4.5 and below. Cooking of control and 0.01M acid treated meat discs at 80°C increased the space between myofibrils whereas minimal shrinkage was noticed in samples below pH 4.5.
M. longissimus dorsi (Voyle et al., 1984) revealed that meat incubated in a salt and pyrophosphate solution at pH 8.0 showed complete extraction of the A-bands. However, in meat incubated in the same solution at pH 5.5, extraction of the A-bands was incomplete and there was no extraction of the A-bands at pH 5.5 in salt solution alone.

The ultrastructure of meat at pH values below the isoelectric point (IEP) has not been examined although further acidification of meat is a common feature of the marinating process (Gault, 1984; Kotula and Heath, 1986) and of the manufacture of some varieties of dry sausages (Terrel, 1977). The objective of this study was to investigate and document ultrastructural changes in acidified discs of bovine M. longissimus dorsi covering the pH range 5.1 to 3.9. It was over this pH range that Gault (1985) observed the major changes in meat tenderness in his studies on the relationship between WHC and cooked meat tenderness in acid treated beef muscles.

Materials and Methods

Preliminary treatment of muscles

One M. longissimus dorsi from each of two 18 month old Simmental heifers was obtained 48 h postmortem from a local abattoir. Methods described by Gault (1985) were used for the preparation and acidification of meat discs. Muscles were cut transversely on a gravity feed slicer into steaks approximately 1.0 cm thick, from which discs of meat 3.0 cm in diameter were prepared using a cork borer. Muscles and blocks were tempered at -25°C until the surfaces were rigid in order to facilitate slicing and preparation of discs.

Adjustment of muscle pH

Acetic acid solutions of 0.01, 0.05, 0.10 and 0.25M were used to adjust the pH of the meat discs from pH 5.1 to 3.9. Eight meat disc was accurately weighed and placed in a 200 ml capacity screw-cap polystyrene jar to which were adjusted with 1M acetic acid to give an identical pH range to that found in each of the acidified meat discs.

Samples measuring approximately 5 x 2 x 2 mm were dissected from the interior of four raw and four cooked meat discs selected from each treatment. These samples were then fixed in acidified 2.5% glutaraldehyde solution for 2 h. They were then transferred to acidified 5% sucrose solution and stored at 4°C for further processing. The pHs of these glutaraldehyde and sucrose solutions were initially adjusted with 1M acetic acid to give an identical pH range to that found in each of the acidified meat discs.

Samples were post-fixed in 1% osmium tetroxide for 2 h. Dehydration through graded alcohols was followed by impregnation in propylene oxide and Durcupan medium resin. The tissues were then embedded in fresh Durcupan resin in coffin moulds and cured in an oven at 60°C for 18 h.

Thick sections (1 μm) were cut from each block and stained with toluidine blue for light microscopy examination. Selected areas from each block were then raised on a LKB pyramitome and ultrathin sections (50 nm) were cut with glass knives on a LKB ultramicrotome. These sections were stained with uranyl acetate for 15 min followed by lead citrate for 5 min.

The sections were examined using a JEOL 1200 EX electron microscope and representative areas were photographed. Approximately 50 sarcomere lengths were measured from the photographic negatives of longitudinal muscle sections from each treatment using the macro-system of a LEITZ TAS PLUS computerised image analyser. Unfortunately, the measuring system of the image analyser was not programmed to give a standard deviation and so it was only possible to measure the mean sarcomere length for each treatment. This was possible throughout the entire pH range studied because of the relative stability of the Z-lines to the various treatments. However, it was not possible to measure all myofibril diameters accurately from either transverse or longitudinal muscle sections since adjacent myofibrils fused together to give an overall amorphous appearance in those samples treated with 0.10 and 0.25M acetic acid. Consequently, no attempt was made to measure changes in myofibril diameters.

Results and Discussion

Table 1 summarises the effects of different acetic acid treatments on the pH, swelling ratios and sarcomere lengths of the meat discs. The mean pH of the raw meat discs decreased progressively with increasing acid concentration. Mean swelling ratios (WHC) increased considerably with decreasing pH except in those meat discs closest to the IEP.
Ultrastructure of Beef under Acidic Conditions

Table 1. Influence of acetic acid concentration (M) on the pH, raw meat swelling ratios (RMS), cooked meat swelling ratios (CMS) and sarcomere lengths (SL) of discs of beef M. longissimus dorsi.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.01</th>
<th>0.05</th>
<th>0.10</th>
<th>0.25</th>
<th>s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.54</td>
<td>5.10</td>
<td>4.48</td>
<td>4.30</td>
<td>3.92</td>
<td>0.014</td>
</tr>
<tr>
<td>RMS</td>
<td>0.94</td>
<td>0.90</td>
<td>1.39</td>
<td>1.77</td>
<td>2.04</td>
<td>0.026</td>
</tr>
<tr>
<td>CMS</td>
<td>0.60</td>
<td>0.48</td>
<td>1.07</td>
<td>1.45</td>
<td>1.74</td>
<td>0.021</td>
</tr>
<tr>
<td>SL (um):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>raw</td>
<td>1.69</td>
<td>1.56</td>
<td>1.98</td>
<td>2.41</td>
<td>2.56</td>
<td>-</td>
</tr>
<tr>
<td>cooked</td>
<td>1.51</td>
<td>1.38</td>
<td>1.76</td>
<td>2.32</td>
<td>2.43</td>
<td>-</td>
</tr>
</tbody>
</table>

of the myofibrillar proteins at pH 5.1, where decreased swelling was observed. These results support the findings of Hamm (1960) and Gault (1985).

Fig. 1a. Control, raw muscle (pH 5.54). A: A-band; I: I-band; Z: Z-line; m: M-line; S: sarcomere length. (Bar = 0.5um).

Fig. 1b. Control, cooked muscle. Note the loss of M-lines (small arrow), fragmentation of Z-lines (big arrow) and shrinkage of myofibrils compared to raw muscle (Fig. 1a). (Bar = 0.5um).

Fig. 1c. Control, raw muscle (pH 5.54). (Bar = 1.0um).

Fig. 1d. Control, cooked muscle. (Bar = 1.0um).

Table 1 also indicates that the mean sarcomere lengths varied in a similar manner with changing pH, the shortest sarcomeres being found at pH 5.1. It is interesting that Harrel et al. (1978) also found that beef muscle at pH 4.9 had shorter sarcomere lengths than control samples of normal pH. At all pHs cooking resulted in a decrease in both WHC and sarcomere length.
Qualitative details of the accompanying changes in the ultrastructure of these acidified muscle samples are illustrated in Figs. 1-5.

The ultrastructure of the longitudinal and transverse sections of the control samples of raw meat at pH 5.5 are shown in Figs. 1a and 1c, respectively. In Fig. 1a, the very short I-band length compared to that of the A-band is indicative of the short sarcomere length of these muscle samples. Cooking nevertheless resulted in a slight decrease in sarcomere lengths (Fig. 1b), whereas myofibril diameters were greatly reduced (Fig. 1d). Similar changes have also been observed by Paul (1965) and Giles (1969) using light microscopy, and by Schmidt and Parrish (1971) using electron microscopy.

From Fig. 1b it is also clear that cooking of these normal pH samples resulted in fragmentation of Z-lines and a complete loss of actin filaments, although pseudo H-zones or bare zones remained visible. A-band filaments were coagulated and there was a partial loss of structure of the I-band filaments. Such observations confirm the results of other studies on cooked beef muscle. Giles (1969), for example, found a complete loss of actin filaments in different beef muscles cooked at 60°C for 20 min and suggested that actin filaments were more heat labile than myosin filaments. Schmidt and Parrish (1971) reported that A-bands became amorphous, detail was lost from Z-lines and the structure of the I-bands was disrupted when beef M. longissimus dorsi was cooked at 90°C.
Ultrastructure of Beef under Acidic Conditions

Muscle samples at pH 5.10 show shrinkage whereas muscle samples at pH 4.48 and below show increased swelling compared to the control muscle samples at pH 5.54.

In the raw samples at pH 5.1 (Fig. 2a), Z-lines were partially disorganised and fragmentation of the thin filaments was observed in the region of the Z-lines in comparison to the corresponding samples at pH 5.5. There was also some loss of M-line structure. It is also interesting that the length of the A-bands appeared to be smaller, while that of the I-bands appeared to be longer than in the corresponding samples at pH 5.5. These latter changes could have been due to a partial loss of myosin at the A/I band junctions. However, this explanation is most unlikely since solubility studies of beef muscle samples around the IEP showed no dissolution of the major myofibrillar proteins (Gault and Tolland, unpublished work). The most likely explanation, therefore, is that longitudinal as well as transverse shrinkage of the actomyosin filaments occurred at the IEP as they attained their most compact structure, thereby pulling the thin filaments away from the Z-lines. The tension generated by this shrinkage in adjacent sarcomeres may have been sufficient to bring about the observed fragmentation of the thin filaments in the region of the Z-lines as well as the partial disorganisation of the Z-lines discussed above. When these muscle samples were cooked (Fig. 2b), there was a complete loss of M-line...
structure and the A-band and I-band filaments became amorphous in appearance.

Over the pH range 4.5 to 3.9, raw meat samples showed a progressive increase in both sarcomere length and myofibril diameter (Figs. 3a, c; 4a, c; 5a, c) compared to both the control samples at pH 5.5 and those at pH 6.1. Such was the extent of swelling across adjacent myofibrils at pH 4.3 and 3.9 that they fused together giving an overall amorphous appearance in both longitudinal (Figs. 4a, 5a) and transverse (Figs. 4c, 5c) sections. Cooking of these samples resulted in a slight decrease in both sarcomere length and myofibril diameter (Figs. 3b, d; 4b, d; 5b, d). However, the shrinkage brought about by cooking in this pH range was not as extensive as that brought about by cooking those samples at pH 5.5 and pH 5.1 as seen by the absence of gaps between the myofibrils of cooked samples at pH 4.5 and below (Figs. 3d, 4d, 5d).

A more detailed examination of the ultrastructure of raw meat samples at pH 4.5 (Fig. 3a) shows quite clearly that M-line structure has been completely lost. It would also appear that much of the myofibrillar

Fig. 4a. Acidified muscle at pH 4.30 - raw. Adjacent myofibrils have fused together and extraction of A-band and I-band filaments has occurred giving an amorphous appearance. Z-lines partially extracted (Z). (Bar = 0.5μm).

Fig. 4b. Acidified muscle at pH 4.30 - cooked. Similar to raw muscle at same pH (Fig. 4a). (Bar = 0.5μm).

Fig. 4c. Acidified muscle at pH 4.30 - raw. (Bar = 1.0μm).

Fig. 4d. Acidified muscle at pH 4.30 - cooked. (Bar = 1.0μm).

swelling occurred in the A-band region where there was also some evidence of partial extraction of myosin filaments as seen by the partial loss of filamentous structure in the A-band region (Fig. 3a). In contrast, there was comparatively less swelling observed in the I-band region. Z-lines, however, were much more swollen and fragmented than in the higher pH samples. It is likely that the hydrogen ion concentration at pH 4.5 caused a sufficient increase in the net positive charge of the M-line proteins to render them completely
Ultrastructure of Beef under Acidic Conditions

Fig. 5a. Acidified muscle at pH 3.92 - raw. Note further loss of myofibrillar structure and Z-line material compared with raw muscle at pH 4.30 (Fig. 4a). (Bar = 0.5μm).

Fig. 5b. Acidified muscle at pH 3.92 - cooked. Note further loss of Z-line structure (Z) compared to raw muscle at same pH (Fig. 5a). (Bar = 0.5μm).

Fig. 5c. Acidified muscle at pH 3.92 - raw. (Bar = 1.0μm).

Fig. 5d. Acidified muscle at pH 3.92 - cooked. (Bar = 1.0μm).

soluble, thereby removing the structural restraint to myofibrillar swelling from the centre of the A-band. It is also likely that depolymerisation of myosin filaments had begun at the same pH while the actin filaments and Z-line material remained relatively insoluble, showing only restricted swelling. On cooking

Note the fusion of adjacent myofibrils in samples at pH 4.30 and pH 3.92.

(Fig. 3b), further fragmentation of Z-lines was observed with little effect on the other myofibrillar ultrastructural details.

In those raw meat samples at pH 4.3, further myofibrillar swelling was observed and adjacent myofibrils were seen to fuse together (Fig. 4a). H-zones were completely lost for the first time, indicating complete depolymerisation of actomyosin and dispersion of myosin filaments. There was also further extraction of A-band and I-band filaments giving an amorphous appearance throughout each sarcomere. Z-lines were partially extracted and the remaining Z-line structures were seen to fuse together to form a longitudinal band across adjacent myofibrils. Cooked samples had a similar appearance except in the proximity of the Z-lines which were comparatively shrunken (Fig. 4b).
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There was evidence of extensive swelling and further loss of structure in the raw meat samples at pH 3.9 (Fig. 5a) with only faint traces of Z-line material remaining. These alterations were more severe in cooked samples (Fig. 5b) where the Z-lines became barely visible.

The ultrastructural changes brought about by the action of these solutions over the pH range 4.5 to 3.9 clearly indicate the severe disruptive nature of low pH on the myofibrillar components of meat. Nevertheless, most of these changes, which involved myofibrillar swelling and dissolution, are similar to those observed in individual myofibrils by Offer and Trinick (1983) and by Voyle et al. (1984) in meat blocks treated with various combinations of salt and pyrophosphate solutions in the pH range 5.5 to 8.0.

Offer and Trinick (1983) hypothesized that the removal of one or more transverse structural constraints, such as the actin/myosin cross-bridges, the M-lines or the Z-lines, by salt and pyrophosphate would be an important role in myofibrillar swelling by allowing the filament lattices to enlarge. The loss of M-line structure was observed by Voyle et al. (1984) in pork muscles incubated with salt and pyrophosphate solutions for 24 h. However, in samples incubated for 5 h, M-lines were still present, and observations on the variable appearance of the M-lines in meat treated with polyphosphates has been attributed by Lewis et al. (1986) to calcium availability, since these authors have shown that the addition of calcium resulted in a complete loss of M-line structure. Porzio et al. (1979), who studied the extraction and isolation characteristics of different myofibrillar proteins, have also indicated that the M-line proteins are the weakest link in the structural strength of myofibrils in raw muscle. In our studies, we have observed a complete loss of M-line structure at pH 4.5, indicating that the M-lines are also the most vulnerable structural components to the acid treatment of raw muscle. This loss of M-line structure could explain why swelling was much more extensive in the A-band region than in the I-band region at pH 4.5, particularly since there was only a partial loss and slight fragmentation of Z-line structure at the same pH.

It is also worth emphasising that in the studies of Offer and Trinick (1983), certain myofibrils showed a complete loss of Z-lines only when a critical concentration of salt or salt and pyrophosphate had been reached. However, in other myofibrils subjected to similar treatments (Offer and Trinick, 1983) there was no apparent loss of Z-line material, and in meat treated with salt alone (Lewis et al., 1986), Z-lines could still be seen. In our experiments, we found that Z-lines were the most resistant of all myofibrillar components to acetic acid treatment, and the loss of Z-line structure with increasing acidity was a gradual process. This would have contributed to the greater swelling observed in the I-band region at pH 4.3 and general loss of all structural integrity within the myofibrils, particularly at pH 3.9, where only faint traces of Z-line structure remained.

In conclusion, the extensive swelling observed within the myofibrils of the more acidified samples of beef muscle would appear to be related to the solubility characteristics of the various structural proteins retained within the myofibril. Clearly, some structural components were more resistant to depolymerisation and solubilisation than others. It is, therefore, very likely that the extent of swelling observed was related primarily to the osmotic pressures generated by the presence of a highly charged and concentrated dispersion of myofibrillar proteins. The limited myofibrillar swelling at the higher pHs was a function of the relative integrity of the transverse constraints, the strongest of which were the Z-lines, with the weakest being the M-lines. At pH 5.1, shrinkage of the myofibrils occurred as the constituent myofilament lattices attained their most compact structure at the IEP.

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References


Ultrastructure of Beef under Acidic Conditions


Discussion with Reviewers

A.-M. Hermansson: How did tempering to -25°C affect the microstructure?

Authors: Previous studies have indicated that freezing of meat causes the formation of ice crystals between muscle fibres and that this brings about fragmentation of myofibres (Voyle, 1979; Martino and Zaritzky, 1986). These studies have also indicated that freezing results in protein denaturation and a loss of water-holding capacity. However, we would like to emphasize that in our studies the muscles were tempered at -25°C in a chill cabinet for 1 h, rather than blast frozen, to facilitate the slicing procedure. Consequently, it was only the peripheral areas of these muscles which were frozen, and the cores of meat which were subsequently used as controls did not show any damage to muscle components when examined either by electron microscopy, as in the present study, or by light microscopy (Rao et al., 1989). We would therefore conclude that the tempering of muscles to -25°C for short periods of time did not have any significant effect on the microstructure of our meat samples.

G.R. Schmidt: Could the addition of from 0 to 0.25M acetic acid have altered the ionic strength and/or the osmolality to produce the swelling observed? Could the varying level of acetate have been standardized by a different experimental design?

Authors: Yes. The addition of these increasing amounts of acetic acid would certainly have increased both the ionic strength and osmolality of the meat discs. We nevertheless feel that the increased swelling was primarily due to the influence of H ions increasing the net positive charge of the myofibrillar proteins below the isoelectric point. However, it is also likely that an osmotic swelling effect may predominate as the pH approaches 4.0, where the dissociation of acetic acid (pKa = 4.7) becomes more suppressed.

The varying level of acetate could have been standardized by using different buffer systems made up with acetic acid and sodium acetate. However, this would alter the ionic balance with respect to sodium and hydrogen ion concentration, particularly at the higher pH values close to the isoelectric point. What effect, if any, this would have on the pH-hydration curves of beef discs is perhaps an area of study worthy of further investigation.

G.R. Schmidt: Were the untreated meat discs which were used as controls also swirled in the orbital incubator?
Author: The untreated meat discs were placed in the orbital incubator as dry controls, i.e. without the addition of distilled water.

D.F. Lewis: How does your process relate to domestic/commercial cooking procedures and how does the structure vary from the inside to the outside of the pieces?

Author: The cooking procedures were identical to those used by Gault (1985) in his studies on the tenderness of marinated meat. The procedures used ensured that the internal temperature of the meat discs was maintained at 80°C for 15 min., a satisfactory time/temperature combination to ensure the thermal denaturation of native myofibrillar proteins and collagen.

All our samples for microscopy were dissected from the interior of the meat discs where leaching of myofibrillar proteins into the equilibrating solution would be minimal at the lower pH values. We would, however, expect considerable protein extraction below pH 4.5 from the outer regions of the meat discs. Consequently, we would expect that the structural appearance of the peripheral regions of these meat discs, both before and after cooking, would be extensively disrupted.

D.F. Lewis: What is the effect of fixing control meat in acidified glutaraldehyde, or for that matter, of fixing acidified meat in pH 7.0 glutaraldehyde?

Author: The rationale behind our approach to the fixation process was to minimise changes in tissue pH brought about by the fixative. Hayat (1970) has also recommended this approach since inadvertent changes in tissue pH brought about by any fixative would undoubtedly alter the structure and behaviour of tissue proteins. Since we were generally looking at changes in muscle structure below the iso-electric point, we felt that it was essential to keep the pH of the glutaraldehyde solutions as close to that of the meat discs as possible.

With regard to the efficiency of our fixation process, it is generally recognised that condensation reactions between aldehyde and amino groups are favoured under mildly acidic conditions. Under the pH conditions used in our work, the imino links formed between bi-functional glutaraldehyde molecules and the amino groups of different protein molecules would be expected to remain stable.

D.F. Lewis: What effect does pH treatment of meat have on the staining characteristics of myofibrillar proteins with uranyl acetate and lead citrate?

Author: As we appeared to get good contrast in all our sections, we were quite happy with the staining procedure we used.

The staining technique we used was based on post-fixation of samples with osmium tetroxide followed by several dehydration stages before the samples were embedded. Ultra thin sections of embedded tissue were then stained with uranyl acetate followed by lead citrate to enhance the contrast of the osmium post-fixed material. Therefore, we would not expect the original pH of the meat samples to have any effect on the efficiency of the uranyl acetate and lead citrate counterstains.

P.J. Knight: The small change from pH 4.5 to 4.3 appears to cause a dramatic change from filamentous to granular in the appearance of longitudinal sections of raw meat (Figs. 3a and 4a). Is this a consistent effect or is there variation within or between samples?

Author: Yes. This granular appearance of myofibrils in meat samples at pH 4.3 was consistent in all of the samples we obtained although there were small variations in the appearance of the granular structure between samples.

P.J. Knight: Is the 50% increase in sarcomere length of raw samples after acid treatment reflected in an increase in the thickness of the meat discs?

Author: We observed a gradual increase in the thickness of raw meat discs treated with 0.05M and higher concentrations of acetic acid. We believe that this increase was certainly due to the lengthening of sarcomeres at pH values below the IEP. However, we did not measure the actual physical dimensions of either the control or the acid treated meat discs.

P.J. Knight: Do the changes in dimensions of the myofibrils on cooking account for the changes in weight of the meat pieces?

Author: Yes. The extent of longitudinal and transverse shrinkage observed in myofibrils due to cooking was closely associated with the extent of swelling ratio reduction (weight loss) measured for the various meat discs at each particular pH.

We have also carried out detailed light microscopy studies on six different types of beef muscle treated with similar concentrations of acetic acid. These studies showed that both raw and cooked meat swelling ratios were positively correlated (r=0.001) with both muscle fibre diameter and sarcomere length, although these correlations were much stronger with fibre diameter than with sarcomere length (Rao et al., 1989).

Additional References


RHEOLOGY AND MICROSTRUCTURE OF STRAINED YOGHURT (LABNEH) MADE FROM COW’S MILK BY THREE DIFFERENT METHODS

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Abstract

Labneh is the name for strained yoghurt, i.e., yoghurt made with an elevated solids content, which has originated in the Middle East. For this study, three types of Labneh were made from cow’s milk: (a) “Traditional Labneh” was produced by straining yoghurt in a cloth bag, (b) “UF Labneh” was made by ultrafiltration of warm yoghurt, and, (c) “UF Retentate Labneh” was obtained by culturing homogenised ultrafiltration (UF) milk retentate. All products were passed through a lactric curd homogeniser to smoothen the Labneh curd. Total solids contents of the products were within the range of 21.0 to 24.2%, protein was 6.8 to 8.7%, and fat was 9.2 to 10.5%.

Rheological properties such as consistency of traditional Labneh and UF Labneh were similar and no syneresis was observed after breaking the coagulum. The coagulum of the UF Retentate Labneh was very firm and its texture was crumbly. Syneresis was noticeable after the coagulum had broken. The best sensory attributes were found with the UF Labneh followed by the traditional Labneh whereas the UF Retentate Labneh appeared not to be satisfactory.

Electron microscopy revealed that the microstructures of all three Labnehs were similar and consisted of casein micelle chains and clusters. Minute fat particles which originated from the homogenisation of milk or retentate were embedded in the casein micelle clusters. Smoothening somewhat reduced the dimensions of the casein particle chains and clusters in all Labnehs.

Introduction

The origin of yoghurt-making in the Middle East dates back several thousand years. The nomads tending their herds prepared yoghurt in earthenware vessels or in containers made from animal skins. The product was kept in these containers until it was all consumed. It is probable that during this time, the liquid phase (whey) seeped through the container or evaporated. The strained yoghurt thus obtained, which has been called Labneh, would keep for a longer time than the original yoghurt, partly due to an increased lactic acid concentration which preserved it.

At present, modern manufacture of yoghurt is carried out under controlled conditions using low-fat or whole milk which is inoculated with mixed starter cultures of Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus (Rasic and Kurmann, 1978; Tamime and Robinson, 1985, 1988a, 1988b; Marshall, 1987). In the Middle East, however, Labneh is still produced in the traditional manner with the cloth bag used universally for the straining of the yoghurt in spite of the centralized processing of milk in dairy plants.

Similar cultured milk products have been popular in various countries. Such products are known as Tan or Than in Armenia, Torba, Kurut, or Tulum in Turkey, Lebneh in Lebanon, and Labneh or Lebneh in most Arab countries (Tamime and Robinson, 1978; El-Gendy, 1983; Abou-Donia, 1984). Other products closely related to Labneh are known as Chakka and Shirhand in India, Skyr in Iceland, and Ymer in Denmark (Tamime and Robinson, 1988a).

The compositional standard of Labneh in Lebanon contains 26% total solids, 10% fat, and 1% salt, where as in Saudi Arabia, Labneh contains 22% total solids and 7% fat (Tamime and Robinson, 1988a). As a dish, Labneh is garnished with dried herbs and olive oil and is eaten with pita bread.

The traditional manufacture of Labneh is labour-intensive and unhygienic. Losses of the product due to its adherence to the cloth bags are quite high. Over the past ten years, attempts have been made to mechanize the process for creamery-scale operations. Several systems of manufacturing have been developed. In one of them, Labneh is produced from heated yoghurt by centrifugation (Dagher and Ali-Ghariebeh, 1985).
Sensory attributes of the product are similar to the traditional Labneh, i.e., Labneh obtained by draining yoghurt in a cloth bag. Alternatively, Labneh may be made by concentrating a mixture of yoghurt and brine (Kharrazi, 1984) using a centrifugal separator. In another method, warm skim-milk yoghurt is concentrated to the desired level of solids using a nozzle or quarg separator and cream is later blended with the product using a mixed strain yoghurt starter culture (Kharrazi et al., 1984; Robinson and Tamime, 1986, Rasic, 1987). Labneh may also be made from ultrafiltration (UF) milk retentate by culturing using a mixed strain yoghurt starter culture (Tamime et al., 1989) in this study.

Labneh is a smooth cultured milk product, the production of which may be further improved by passing it through a lactic curd homogeniser.

The objective of this study was to examine the individual Labnehs by electron microscopy and to assess their microstructures with respect to the manufacturing procedures and rheological properties of the products.

**Materials and Methods**

**Preparation of the milk**

Whole cow’s milk was obtained from the West of Scotland College Farm in January and March, 1988. In each trial the milk was divided into two portions for the production of Labneh using three different methods.

**Production of Labneh**

Three different types of Labneh were manufactured as described by Tamime et al. (1989), and in brief they could be described as follows:

**Process I (UF Retentate Labneh)** - The milk was pre-warmed to 50°C before concentration by UF to 22% total solids (TS) using an Alfa-Laval pilot scale plant (Fig. 1). The retentate was homogenised at 17.2 MPa, heated to 90°C for 5 min in a water bath (steam was used as heating medium), cooled to 42°C and inoculated with a yoghurt starter culture. The retentate was incubated (in bulk and in 150 ml plastic cups) until the acidity reached pH 4.6. After incubation, the product was refrigerated overnight, the bulk portion of it was passed through the lactic curd homogeniser, dispensed into 150 ml plastic cups, and refrigerated at 5-7°C overnight.

**Process II (UF Labneh)** - Yoghurt was prepared according to the method described by Tamime et al. (1984), but without fortification of the milk solids. At the end of the incubation period, the warm yoghurt was concentrated by UF using the same Alfa-Laval plant (Fig. 1) and refrigerated in bulk and in 150 ml plastic cups overnight. The following day the bulk Labneh was passed through the lactic curd homogeniser, dispensed into 150 ml plastic cups and refrigerated at 5-7°C.

**Process III (Traditional Labneh)** - The yoghurt (in bulk) was produced as described in Process II above. The refrigerated yoghurt was mixed, emoted into a polyester cloth bag (Ets. Henri Bastien, 59157 Beauvais-en-Cambresis, France) and pressed overnight in a refrigerated room (Fig. 2). For every 10-12 kg of yoghurt, 4.5 kg weight was used to press the product during the drainage period. It was observed that some Labneh was lost, i.e. adhered to the bag, when collecting it. The Labneh was then processed and packaged as described above.

**Ultrafiltration Plant**

The milk and yoghurt were concentrated by using the same Alfa-Laval UF pilot scale plant reported by Tamime et al. (1986), but it was slightly modified. The specifications of the membrane were: type PM-50, series No. 6 PL 1256 S, surface area 1.3 m², fibre internal diameter 1.5 mm, membrane material polysulfone and molecular weight cut-off 50,000 dalton.

**Starter Culture**

A commercial mixed strain of concentrated freeze-dried yoghurt starter culture MOY - 87 (Eurozyme Ltd., London, UK) was used to ferment the milk. This starter (i.e., direct to vat inoculation) was used at a rate of 16 units/100 L, and was incubated at 42°C.

**Lactic Curd Homogeniser**

The homogeniser type ALM (Pierre Guerin S.A., Mauze, France) was used to smoothen the Labneh at 2°C using a pressure of 8 MPa, and the homogeniser head employed was No. D-170 (Tamime and Crawford, 1994).

**Rheological Analysis**

A Stevens LFRA Texture Analyser (C. Stevens & Son Ltd., Hertfordshire, UK) was used to assess the consistency of Labneh. The operating conditions were: cone type TA3-TFE 105-504 (25 x 25 mm cylinder), penetration distance 15 mm, speed of probe 0.5 mm/s and chart recorder (C. Stevens & Son Ltd.) operating at 200 mV and 30 mm/min chart speed.

The packaged Labneh in the plastic container was squeezed gently between the thumb and forefinger to visually assess the texture and elasticity characteristics of the product.

**Microscopic Analysis**

Labneh was sampled using a glass tube, 7.0 mm in diameter. Sample columns, approximately 10 mm long, were fixed in a 2.8% aqueous glutaraldehyde solution and mailed to Ottawa for electron microscopy (Allan-Wojtas, 1984). After arrival, the samples were prepared for scanning electron microscopy and for transmission electron microscopy similar to other milk gels (Kalab et al., 1983).

Scanning electron microscopy (SEM). The Labneh columns were cut into prisms, 1 x 1 x 10 mm, and the prisms were dehydrated in a graded ethanol series (20, 40, 60, 80, 96, and 100% ethanol). Dehydrated samples were defatted in chloroform, returned into absolute ethanol, rapidly frozen in Freon 12 at -150°C, and freeze-
Rheology and Microstructure of Strained Yoghurt (Labneh)

fractured under liquid nitrogen. The fragments were melted in absolute ethanol, critical-point dried, mounted on SEM stubs, sputter-coated with gold, and examined in an ISI DS-130 scanning electron microscope equipped with an external oscilloscope (Bond and Kalab, 1988).

Transmission electron microscopy (TEM). The Labneh samples were cut into approximately 0.5 x 0.5 mm cubes, washed with a 0.05 M veronal-acetate buffer, pH 6.8, postfixed for 2 h in a 2% osmium tetroxide solution in the same veronal-acetate buffer, dehydrated in ethanol, embedded in medium hard Spurr's low-viscosity resin (J.B. EM Service, Inc., Pointe Claire-Dorval, Quebec, Canada), and sectioned. Sections, approximately 90 nm thick, were stained with uranyl acetate and lead citrate solutions (Reynolds, 1963) and examined in a Philips EM-300 electron microscope operated at 60 kV. Micrographs were taken on 35-mm film.

Results and Discussion

The Labnehs under study were made in January and March, 1988 from two batches of whole cow's milk. The milk contained 12.5% total solids, 4.8% lactose, 3.9% fat, 3.1% total protein, and 0.7% minerals (Fig. 3). The composition of the Labnehs is summarised in Fig. 3. It is evident that the total solids, fat, and protein contents were proportionally increased in the products compared to the milk, whereas the lactose content was reduced in all Labnehs to a level that ranged between 4.0 - 4.4%. The highest total solids, fat, and protein contents were found in the traditional Labneh (24.2, 10.5, and 8.2%, respectively) and the lowest values were found in
attributed to the extent of draining and the resulting increased concentration of the solids contents. Protein losses in the permeates and in the cloth bag filtrate were approximately the same, i.e., within the range of 0.2 to 0.3%. No fat losses were observed in any of the manufacturing processes used and, thus, the solids contents in the permeates and the filtrate consisted almost exclusively of lactose and minerals (Fig. 3).

Rheological properties of the Labnehs produced were to a great extent affected by the manufacturing procedure and, in particular, by the application of the lactic curd homogeniser to smooth the products (Fig. 4). Similar results were obtained with Labnehs made in January as well as in March.

Unsmoothened UF Retentate Labneh was firmest (766 g) despite its lowest total solids and protein contents. Following the passage through the lactic curd homogeniser (shaded peak), the firmness of this Labneh dropped markedly to below 50 g. In the UF Labneh, the change in consistency (from 183 to 105 g) is due to the homogenisation which was not as extensive. Interestingly, traditional Labneh had the highest firmness (134 g) of all three homogenised Labnehs. It may be hypothesized that the differences in consistency are related to the way in which the coagulum was formed during the culturing of milk. Thus, the UF Retentate Labneh would resemble a set-style yoghurt whereas the other two Labnehs would be similar to the stirred-type yoghurt.

The absence of data on the consistency of unsmoothened traditional Labneh in Fig. 4 was caused by the heterogeneous nature of the product which resulted from draining the Labneh in a cloth bag. The layer of curd adhering to the cloth had a considerably higher total solids content than the central portion of the product and was lumpy. Consequently, a wide range of consistency readings was obtained when sampled for analysis. However, reproducible readings of 134 g were obtained after the Labneh had been smoothened by passage through the lactic curd homogeniser. Provided that the difference in the protein content between the traditional Labneh and the UF Labneh was taken into consideration, the consistency readings of both Labnehs were comparable (see Fig. 3 - black column).

Elasticity of Labnehs made by the various procedures and whey separation was monitored with the products stored in plastic containers. The UF Labneh and traditional Labneh behaved in a similar way both before and after smoothening. UF Retentate Labneh had a tendency to crack and crumble and was considerably less elastic than the UF Labneh. After the coagulum of the UF Retentate Labneh had been broken with a spoon, syneresis was immediately noticeable in contrast to UF Labneh which was free from this defect (Fig. 5).

It may be concluded on the basis of consistency measurements that UF Labneh and traditional Labneh are similar to each other whereas the UF Retentate Labneh is somewhat different (Fig. 4).

Concerning the microstructure as examined by electron microscopy, however, all Labneh samples were, in general, similar to each other irrespective of whether they had been or had not been smoothened. SEM at a low magnification showed, for example, that there were no noticeable differences in the microstructures of the UF Retentate Labneh before and after passage through the lactic curd homogeniser (Figs. 6 a and 6 b, respectively). The unsmoothened UF Labneh had a similar structure consisting of a relatively uniform matrix in which, occasionally, small lumps of fluffy protein aggregates were found to be hollow (Fig. 7a). Such lumps were not found after the Labneh had been passed through the lactic curd homogeniser (Fig. 7b). In the traditional Labneh, the difference between the unsmoothened and smoothened structure has also been subtle. The smoothened product, however, was found to be separated into fluffy areas, each less than 0.2 mm in diameter (Fig. 8). The apparent fluffiness of these areas did not interfere with the smooth perception of the product. It is probable that these areas were formed following the passage of the Labneh through the lactic curd homogeniser or during its slow cooling afterwards.
SEM examination of all Labneh at higher magnification suggests that the protein matrices are composed of casein particle chains and clusters; however, the lactic acid bacteria became apparent. The micellar chains were short to medium and the clusters were relatively small. The protein matrices appeared to be slightly influenced by the effect of smoothening of the product and the level of protein in Labneh should not be overlooked (Fig. 3). The resulting matrices of the UF Retentate Labneh, UF Labneh and traditional Labneh that were smoothened were slightly less compact and more open than the matrices of the same types of Labneh before the smoothening stage. An illustration is shown in Fig. 9. The more open matrices were due to the formation of larger "pores" possibly as a result of the mechanical action of the lactic curd homogeniser resulting in re-clustering of the casein micelles. The most compact matrix appeared in traditional Labneh which was not smoothened and contained the highest level of protein.

The relationship between the total solids content and the density of the protein matrix was difficult to assess by visual examination of the micrographs presented, because the differences in the densities of the matrices were not as great as those found by Harwalkar and Kalab (1983) in yoghurts which were made from reconstituted nonfat dry milk and contained 10 to 30% total solids. The differences in the total solids contents
Fig. 6. UF Retentate Labneh before (a) and after passage through a lactic curd homogeniser (b). The uniform structures are the result of using homogenised milk retentate to produce the Labneh.

Fig. 7. UF Labneh before (a) and after (b) the homogenisation stage. Small hollow protein lumps (L) were occasionally seen in the UF Labneh before homogenisation. Passing the same product through the lactic curd homogeniser led to separation (arrows) of fluffy areas.

Fig. 8. Microstructure (SEM) of traditional Labneh that has been homogenised. Separation of fluffy areas (arrows) is clearly noticeable.
between the UF Retentate Labneh and the traditional Labneh were insufficient to make the differences in the densities of the protein matrices apparent. Digital image analysis of many micrographs of the Labnehs under study would be necessary to establish such a correlation.

There was evidence of cavities present in the protein particles both before and after the homogenisation stage (Figs. 9a and 9b, respectively). The reason for the development of such cavities may be understood from the examination of the samples by TEM. In thin sections, minute fat particles produced from original fat globules as the result of the homogenisation of the milk or retentate are shown to be embedded in the protein matrix (Fig. 10). Occasionally, some fat globules as large as 5 μm in diameter passed through the curd homogeniser intact as is evident in Fig. 11. Fig. 12 shows a fat globule, 0.5 μm in diameter, with the fat globule membrane intact whereas in Fig. 13, a fat globule of a similar dimension has been ruptured.

TEM examination of all the Labnehs showed chains of agglomerated casein particles and fat globules. Several samples of each Labneh suggest that the casein particle chains were shorter and the clusters were smaller in Labnehs which were smoothened by passage through a lactic curd homogeniser. This difference may be evident from Figs. 10a and 10b which are shown as an example, although one must be aware of the possibility that structures which appear to be small clusters, may in fact be cross sections of long chains (Kalab et al., 1976). In addition all the Labnehs showed some evidence of micelle fusion before smoothening (Fig. 10a).
The microstructure of Labneh markedly differs by the presence of fat and the distribution of the fat particles incorporated in the protein matrices, from the microstructure of yoghurt (Kalab et al., 1983; Harwalkar and Kalab, 1983) which is made from nonfat or low-fat milk. Another difference is the density of the protein matrix which reflects the higher protein content in Labneh than in yoghurt.

In conclusion, the differences in the microstructure of Labneh made by three different procedures were subtle. Irrespective of whether the Labneh obtained by culturing whole milk or homogenised milk retentate were smoothened, the protein matrices were relatively uniform and consisted of casein particle chains and clusters. However, the casein particle chains in Labneh smoothened by the passage through a lactic curd homogeniser appeared to be somewhat shorter than in the unsmoothened Labneh.

It is recommended that the lactic curd homogeniser should be employed to smoothen the traditional Labneh. In view of the shearing effect of such processing and to minimise the rheological changes, for example in UF Labneh, the homogeniser head No. D-280 should be used rather than No. D-170. Further work is still required to establish the effect of such processing on the structure of Labneh. The process of smoothening may not be necessary if the UF Labneh was cooled quickly after concentration by using a scraped surface cooler rather than cooling slowly in bulk.

Acknowledgements

The authors thank Dr. H.E. Medler for useful comments, Mr. A.S. Chehade, Mr. M.A. Mahdi, Mrs. Isabel Hamilton, Mrs. Paula Allan-Wojtas and Miss Gisele Larocque for skillful technical assistance. Electron Microscope Centre, Research Branch, Agriculture Canada in Ottawa provided facilities. Contribution 815 from the Food Research Centre in Ottawa.

Fig. 11. Microstructure (SEM) of fat globule membrane residue in Labneh. Occasionally, a medium-size fat globule (F) is left intact following the passage of the Labneh through the lactic curd homogeniser. Extraction of fat reveals the fat globule membrane residue (small arrow), large arrow points to streptococci.

Fig. 12. A small fat globule (F) with the fat globule membrane intact (arrow) passed through the lactic curd homogeniser.

Fig. 13. Passage through the lactic curd homogeniser ruptured the fat globule membrane (arrow) of a fat globule (F).
Rheology and Microstructure of Strained Yoghurt (Labneh)

References


Discussion with Reviewers

D. Holcomb: Are the authors confident that the relatively large samples (7 x 10 mm cylinders) were completely fixed, i.e., that glutaraldehyde had penetrated the interior of the samples? Authors: As the SEM micrographs show, the samples were quite porous and, therefore, easily penetrated by aqueous glutaraldehyde. In addition, extended fixation taking several days before the samples were received for electron microscopy was taken into consideration. On arrival, the samples appeared to have uniform colouration in cross sections while they were trimmed into smaller prisms. (Allan-Wojtas and Kalab, 1984).
A.Y. Tamime, M. Kalab and G. Davies

D. Holcomb: A goal of this sort of research might be to establish relationships between rheology and microstructure. However, the authors find that "on the basis of consistency, elasticity and susceptibility to syneresis" UF Retentate Labneh is somewhat different from the other Labnehs, while, on the basis of electron microscopy "all Labneh samples were, in general, similar ...." In light of these observations, do the authors feel that rheology-microstructure correlation is an attainable goal?

Authors: In our opinion, the inability to find a correlation between rheology and microstructure in Labneh does not mean that there is a total absence of any relationship. Gavaric et al. (1989), for example, reported that a correlation between firmness and microstructure of milk retentate gels obtained by using proteases of various origins was noticeable only when thin sections of the gels were examined at a high magnification. Gels consisting mostly of casein micelle chains were firmer than gels consisting mostly of casein micelle clusters. Only persistent structural and rheological studies of various milk products will reveal whether correlations between these two parameters exist.

E. Parnell-Clunies: Homogenisation (smoothening) appeared to create a more open protein matrix (Fig. 9). Was this a temporary effect or did the authors observe the re-appearance of the more compact network with time (e.g., weeks post manufacture)? If so, was this re-aggregation accompanied by syneresis?

Authors: Passage of curd through a homogeniser breaks the curd granules into minute particles. Although the finished product appears to be smoother by sensory evaluation than the original curd, electron microscopy reveals the existence of the minute particles composed of casein micelle chains and clusters. At the same magnification, the microstructure of the larger curd grains appears to be more uniform than the homogenised curd. This phenomenon is similar to that observed in stirred yoghurt (Kalab et al., 1975) or homogenised cream cheese (H.W. Modler, personal communication). The structure of Labneh was not studied again after a prolonged period of time.

D.G. Pechak: How common were the protein aggregates? You state "occasionally" in the text but your micrograph shows three in one field of view? If such structures are as common as the micrograph implies then they would definitely affect the texture and water binding characteristics.

Authors: There is no discrepancy between the statement that the aggregates were seen only occasionally and the micrographs showing 3 aggregates within one field of view. A large area of the freeze-fractured planes was examined and the aggregates were found to be quite rare but similar to each other. A micrograph, in which 3 such aggregates are featured (a rare occasion) was selected to show their nature.

D.G. Pechak: Is it possible that the cracks or separations that you describe as fluffy areas are a result of one odd preparation during the cryo-fracture step? How many preparations of this sample showed similar structures and were the spacings or cracks also seen at the light microscope level in "thick sections" of the Spurr's embedded material, which did not receive the freezing step?

Authors: Dehydration of samples fixed in glutaraldehyde and their impregnation with absolute ethanol prior to freeze-fracturing prevents the development of artefacts which are associated with ice crystal formation in hydrated samples. This report is not based on the observation of one odd preparation. The separations were a common feature unlike the occasional occurrence of protein aggregates dealt with in the previous question. No sections of the embedded samples were examined by light microscopy.
Rheology and Microstructure of Strained Yoghurt (Labneh)

Y. Kakuda: Were the hollow aggregates seen only in the UF Labneh? Any ideas on how these structures were formed?

Authors: Yes, the hollow aggregates were seen only in the UF Labneh. We have no idea about how they were formed but because of their low occurrence, they probably do not contribute to the structure of the smoothened Labneh.

Y. Kakuda: Were there any pumping or fouling problems during the UF treatment of the milk or yoghurt?

Authors: No, the problem of fouling during UF treatment of the milk was not observed because we were only handling small quantities; however, during the UF of yoghurt the permeate flux rate was reduced as the product became thick and towards the final stages of concentration the outlet valve had to be fully open to avoid any blockages of the membrane.

Y. Kakuda: Is it possible that the heat treatment of the retentate (compared to unconcentrated milks) was insufficient to produce the desired textural properties in the UF Retentate Labneh?

Authors: No, the heat treatment of both types of milks was sufficient, i.e. 90°C for 5 min, and the use of higher temperatures may cause other problems that can affect the texture of the product (Tamime and Robinson, 1985, 1988a).

L. Krsev: Why is the lactic curd homogeniser recommended to be used in Labneh manufacture?

Authors: It is essential that the lactic curd homogeniser is used to smoothen the traditional Labneh in order to remove the evidence of lumps in the product. As mentioned in the text such process may not be required if the UF Labneh is cooled directly after ultrafiltration.

L. Krsev: The obtained results show negative effect of the lactic curd homogeniser when using the homogeniser head No D-170. What is the reason you think that the homogeniser head No. D-280 would be better?

Authors: The homogeniser head No. D-280 consists of fewer number of grooves as compared with D-170 and hence the sheering effect on the Labneh will be reduced. As a result, the drop in the consistency measurement after the homogenisation stage will not be great and the structure of the ALM homogeniser head has been published elsewhere (Tamime and Crawford, 1984). Despite the slight reduction in the firmness of the Labneh after it was passed through the lactic curd homogeniser, all the different types of Labneh appeared smoother and improved the overall shine of the product.

Additional References


Abstract

Fluorescence, bright field and scanning electron microscopy were used to characterize the structure of selected mature pearl millet caryopses from the World Germplasm Collection. Kernel shape (globose, lanceolate, obovate and hexagonal), kernel endosperm color (white, yellow and grey) and external appearance (color) of the samples were documented for 96 varieties. Color of the pearl millet kernel was due to the combined effects of pigmentation in the pericarp, aleurone and endosperm, as well as the pericarp thickness. White kernels had few pigmented areas, yellow kernels had pigments primarily in the epicarp and endosperm, and brown kernels had pigments in the epicarp, aleurone and endosperm. The majority of white, yellow and brown kernels had a thick pericarp. Purple kernels also had pigments in the epicarp, aleurone and endosperm, but had a thin pericarp. Grey kernels had pigments in the aleurone and endosperm, and had a thin pericarp. The pericarp was different from that found in sorghum in that the epicarp cells could be large, round, multilayered and full of pigments, or flat, single-layered and empty. The seed coat and aleurone layer were similar to those found in sorghum. Phytin and nicotinic acid were present in the germ. β-D-glucans were present in the cell walls in the endosperm.

Introduction

The physical and structural properties of pearl millet [Pennisetum americanum (L.) Leeke] vary significantly among varieties (Appa Rao et al., 1985; Rachie and Majmudar, 1980). There are more than 15,000 pearl millet lines in the World Germplasm Collection. While size, shape, germ to endosperm ratio, endosperm texture, pericarp thickness and appearance of the kernel affect processing properties, little information is available to define the variation in structure. In addition, factors affecting pearl millet color are not understood. In contrast, kernel characteristics affecting the color of sorghums and their genetics are clearly understood (Rooney and Miller, 1982). Information on kernel characteristics and its relationship to structure and processing properties of millets would help to improve pearl millet processing quality through breeding and selection.

The structure of pearl millet has been evaluated with scanning and transmission electron microscopy, and bright field microscopy (Badi et al., 1976; Sullins and Rooney, 1977; Adams et al., 1976; Angold, 1979; Zeleznak and Varriano Marston, 1982). In these studies, the research was conducted on a few samples that did not represent the wide variation in kernel properties that exists within the world collection of pearl millet. In general, these studies have suggested that pearl millet structure was similar to that of sorghum kernel structure with two exceptions: pearl millet had no starch in the pericarp, and it had a higher germ to endosperm ratio. Fussell and Dwarte (1980) monitored the development of phenolic compounds in pearl millet with autofluorescence, and found that most of the phenolic compounds were fully developed in the pericarp by 18 days after anthesis. Sullins and Rooney (1977) mentioned that membranous tissue was present between the tube cells and the aleurone layer, but did not classify it as a seed coat. Zeleznak and Varriano-Marston (1982) did not report the presence of a seed coat in pearl millet. Other research has reported that there was a seed coat...
Table 1

<table>
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a) Data are presented as % of n varieties for each location displaying each characteristic; sample size for each variety was 20 kernels.

The objectives of our research were 1) to describe the structural characteristics found in the majority of pearl millet kernels, 2) to describe the relationship between pearl millet structure and its processing properties, and 3) to provide information on the location of pigmented materials in the kernel that relate to its appearance.

**Materials and Methods**

**Samples**

The pearl millet terminology used in this paper is based on that used in Mali, West Africa. A Souna millet is one that matures early in the growing season (less than 100 days), while a Sanio millet is one that matures late (up to 150 days; Bilquez, 1963). The market samples collected in Mali are designated only as Souna or Sanio millets, along with the location of the market, as in Souna Banamba. There are no specific variety names available for these samples as they are composites of many locally grown varieties. Souna and Sanio are also used to define the shapes of kernel millets. In this sense, Souna millets have elongated kernels, while Sanio millets are more globular (round).

Samples of pearl millet were obtained from Pearl Millet Nurseries in Cinzana, Mali (1983, 1984, 1985), Niger (1984) and India (1985). A total of 96 pearl millet varieties and lines were evaluated for pericarp thickness and color, pigmentation in the seed coat, aleurone and endosperm, and the relative proportions of corneous to floury endosperm (texture). The selections were made from the World Collection of Germplasm to represent the widest and most obvious extremes in pearl millet characteristics. Twelve of the most diverse samples were selected for detailed structural evaluation using scanning electron, bright field and fluorescence microscopy. The descriptions in this paper are based on the microscopic examination of these 12 varieties, and unless otherwise noted, these...
Microstructure of Pearl Millet

LANCEOLATE  OBOVATE  HEXAGONAL  GLOBOSE

Figure 1: Four primary pearl millet kernel shapes found in 96 samples (IBPGR, 1981).

descriptions are deemed applicable to the entire sample population. Thus no distinctions are made between specific varieties.

Fluorescence Microscopy
The pearl millet samples (6 randomly chosen kernels per variety) were cut in half with a razor blade, fixed in 3.0% gluteraldehyde in 0.025M phosphate buffer (pH 6.8) for 48 hrs, dehydrated in an alcohol series and embedded in glycol methacrylate (Feder and O’Brien, 1968). All samples were sectioned on a rotary microtome (1-2 μm thick) with a glass knife and were viewed on a Zeiss Universal microscope equipped with a IIIRS epi-illuminating system and Zeiss Neofluor objectives.

All sections were stained for fluorescence characterization following the methods outlined by Earp and Rooney (1986), some of which were based on those in Fulcher and Wong (1980). Unstained samples (autofluorescence of ferulic acid and lignin), samples stained with Calcofluor (β-D-(1-3)(1-4) glucans), and those with ANS (8-anilino-1-naphthyl sulfonic acid; protein) were viewed under filter combination (FC) I (exciter filter 365nm, barrier filter > 418nm). Sections stained with acid fuchsin (protein), acriflavine hydrochloride (phytin) and congo red (β-D-(1-3)(1-4) glucans) were viewed under FC I and III (exciter filter 546nm, barrier filter > 590nm). Sections stained with nile blue A (lipids), diphenyliboronic acid (flavonoids) and cyanogen bromide (nicotinic acid) were viewed under FC II (exciter filter 450-490nm, barrier filter > 520nm).

Micrographs were taken with Fujichrome 400 film with exposures ranging from 10 sec to 2.5 min.

Bright Field Microscopy
Toluidine blue was used to stain lignin and polyphenolic compounds (O’Brien and McCully, 1981) in the samples (6 randomly chosen kernels per variety) and viewed with a Zeiss Universal microscope equipped with a 100W tungsten light source and Zeiss Neofluor objectives.

Scanning Electron Microscopy
The pearl millet kernels (6 randomly chosen kernels per variety) were cut in half longitudinally with a blunt razor blade, mounted on aluminum stubs with carbon paint, coated with gold-palladium (200Å) and viewed with a JEOL JSM25 scanning electron microscope with an accelerating voltage of 25KV. The dimensions of various kernel structures (starch granules, protein bodies, etc.) were measured using SEM negatives (of known magnification) and a vernier caliper.

Physical and Chemical Analyses
Seventeen samples were analyzed for polyphenol content with the Folin-Ciocalteu assay (Kaluza et al., 1980) and the automated vanillin /HCl method (Maxson and Rooney, 1972; McDonough et al., 1983). Density was determined with a Beckman air comparison pycnometer. Thousand kernel weight was also recorded. Moistures were determined and data were presented on a dry weight basis. Samples were(decorticated for 4 min in a TADD mill (Mwasaru, 1985) and the amount of cleaned decorticated sample remaining was considered to be the yield. Three samples, one each of grey (Inade), yellow (CMM411) and purple (Soua) were prepared for High Performance Liquid Chromatography (HPLC) phenolic acid analysis using the base hydrolysis method of Hahn et al. (1983) and were separated using a 10μm i.d. C-18 column in a Beckman HPLC system.

Results and Discussion

Gross Morphology
The 96 pearl millet varieties in this study represented maximum variation in kernel characteristics (Table 1). The millets exhibited many different shapes (Fig. 1) and colors (IBPGR, 1981). The average profile of a pearl millet variety, based upon observations of all 96 varieties, was an obovate kernel with a thick or thin pericarp, intermediate texture, grey exter-
Figure 2: Endosperm texture rating of longitudinal cross sections of pearl millet kernels; 1 is very corneous, 5 is very floury and 3 is intermediate.

<table>
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<td>Physical characteristics of 12 pearl millet varieties chosen for microscopic analysis</td>
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<th>Kernel Appearance</th>
<th>Continuous Seed coat&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Pigments in Aleurone</th>
<th>Pigments in Endosperm</th>
<th>Pericarp Thickness</th>
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<td>yes</td>
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<tr>
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<tr>
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<td>yes</td>
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<tr>
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a) Ratings: 1- lanceolate; 2- obovate; 3- hexagonal; 4- globose.
b) No grey kernels had a continuous seed coat.

Corneal appearance, and a pigmented aleurone and starchy endosperm. More specifically, most obovate kernels were yellow or grey, had no pigments in the aleurone, had a pigmented endosperm and mostly corneous texture. Lanceolate kernels were primarily grey, with a pigmented aleurone, grey or yellow endosperm and a variety of pericarp thicknesses and endosperm textures. Hexagonal kernels were similar to the lanceolate ones, except that most had thick pericarps and intermediate to floury texture. Globose kernels were grey with a thick pericarp, pigmented aleurone and a floury white endosperm.

The endosperm texture (Fig. 2) was rated from 1 (very corneous) to 5 (very floury). The majority of the samples had an intermediate texture (rated 2-4). The density of the samples ranged from 1.28 to 1.42 g/cc, with the more corneous kernels having the highest density.
Figure 3: A. Overall structure of the endosperm, germ and pericarp of a pearl millet kernel; B. Cross-section of the pericarp and peripheral endosperm of a Souna pearl millet. E: epicarp cell, C: cross cell, t: tube cell, s: seed coat, A: aleurone cell, p: peripheral endosperm.

Figure 5: A. Cutin layer and epicarp cell in the pericarp of a purple Souna variety; B. Double epicarp layer with pigmentation in the epicarp cells of a purple Souna variety. Cu: cutin layer, E: epicarp cell, C: cross cell.

values. The 1000 kernel weight ranged from 2.5 to 20.0 g. The very corneous varieties generally had small kernels.
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...varieties of pearl millet (varieties unknown) with thin epicarp layers that illustrate the difference in pericarp thickness that can occur due to a difference in mesocarp thickness. A. Thick pericarp variety; B. Thin pericarp variety. P: Overall thickness of the pericarp (all structures included), a: aleurone cell, pe: peripheral endosperm cell.

Pericarp

The structural descriptions presented here are based on the detailed study of the 12 diverse varieties previously mentioned (Table 2). The descriptions are consistent, and can be applied to the vast majority of the 96 varieties. Exceptions to this are noted.

The tissues in the pericarp are shown in Figs. 3 and 4A. The pericarp was composed of the epicarp, mesocarp and endocarp layers. The epicarp was usually one to two layers thick, with large blocky cells that contained concentric layers of pigmented tissue (Figs. 4A, 5). However, some varieties of pearl millet had long, narrow, flat epicarp cells with no apparent cell contents (Fig. 6). This demonstrates the varietal differences that occur, since all of the varieties pictured in Fig. 5 and 6 are Souna types. Racchi and Majmudar (1980) reported that some pearl millet varieties (unspecified) had pericarps with flat, empty epicarp cells. A cutin layer covered the outside of the epicarp layer; the cutin stained positive with nile blue A (Fig. 4B).

The mesocarp layer was directly beneath the epicarp and contained several layers of compressed cells that were often indistinguishable from the cross and tube cells (Fig. 6). Frequently it was not possible to distinguish individual cell walls in this layer, due primarily to compression of the cells during grain maturation. The overall thickness of the pericarp could be due to the number of cell layers present in the mesocarp or to the presence of a thick or thin epicarp. There were no starch granules present in the pericarp in any pearl millet variety, contrary to what has been reported in sorghum (Earp, 1984).

Beneath the mesocarp were the cross and tube cells, or endocarp (Fig. 4A), which may be responsible for nutrient and moisture transport around the developing kernel (Rooney and McDonough, 1987). Cross cells were oriented perpendicular to the long axis of the kernel. The tube cells were perpendicular to the cross cells.

There was a seed coat present beneath the endocarp that was observed in all 12 varieties studied; it measured 0.4μm in thickness (Fig. 7). The seed coat appeared to be lightly pigmented, but no distinct cells containing pigments were observed, and it bore little resemblance to the heavily pigmented seed coats found in some sorghum varieties (Earp and Rooney, 1986). The seed coat was continuous in most varieties, with the exception of approximately half of the grey varieties, in which its presence corresponded with the areas that were grey in color. These varieties included both Souna and Sanio type millets. A possible explanation for this may be that pearl millet can have a partial testa similar to that reported in sorghum by Blakely et al. (1979).

When several varieties of pearl millet were decorticated in a TADD mill, the pericarp split from the kernel just beneath the endocarp, leaving the aleurone intact. This agreed with the results of Sullins and Rooney (1977); however, de Francisco et al. (1982) reported that the pericarp split away from the kernel below the aleurone layer. The differences could be attributed to decortication time or method. Decortication characteristics are important in food processing, since the aleurone contains protein, vitamins and minerals that enhance the nutrient value of prepared food. Globose kernel shapes (Sanio-type millets) are more useful under average traditional decortication conditions; if the kernels have a thick pericarp, they can be decorticated with a minimum loss of starchy...
endosperm (Coulibal and Kante, 1983). Globose kernels were decorticated more effectively in this study than hexagonal or lanceolate kernels; the more elongated kernels tended to break in half during decortication and yield was very low.

Aleurone Layer

The aleurone layer was beneath the seed coat, and was one cell layer thick (Fig. 3B). The cell walls were very thick, and fluoresced a deep royal blue under FC I (Fig. 4A); the blue color appeared darker than sorghum aleurone examined under the same conditions Earp (1984). Lipids were visible as small yellow bodies when stained with nile blue A (Fig. 4B) and were found throughout all aleurone cells. Rachie and Majmudar (1980) indicated that the aleurone contained primarily lipids, protein, phytin and occasionally pigments, which added to the overall color perception of the kernel. Lai and Varriano-Marston (1980) reported that there were high levels of lipids in the aleurone.

Starchy Endosperm

The starchy endosperm of pearl millet was composed of peripheral (or subaleurone), corneous and floury areas (Fig. 8). These three areas have already been documented in sorghum (Earp, 1984), corn (Wolf et al., 1952) and pearl millet (Sullins and Rooney, 1977). The cells were small in the peripheral endosperm (21 x 40μm) and larger in the corneous and floury endosperm (73 x 83μm).

The peripheral endosperm was 1-3 cell layers thick, had polygonal starch granules embedded in a thick protein matrix, and contained a large number of protein bodies. The peripheral endosperm cell contents were
Table 3

Factors that contribute to the external appearance of pearl millet kernels

<table>
<thead>
<tr>
<th>Seed color</th>
<th>Pigments in Epicarp</th>
<th>Pericarp Thicknessb</th>
<th>Pigments in Aleuroneb</th>
<th>Pigments in Starchy Endospermb</th>
</tr>
</thead>
<tbody>
<tr>
<td>White</td>
<td>13</td>
<td>no</td>
<td>thick (69.2)c</td>
<td>none (38.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>thin (30.8)</td>
<td>grey (38.5)</td>
</tr>
<tr>
<td>Yellow</td>
<td>15</td>
<td>yes</td>
<td>thick (66.7)</td>
<td>yellow (80.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>thin (33.3)</td>
<td>grey (13.3)</td>
</tr>
<tr>
<td>Brown</td>
<td>18</td>
<td>yes</td>
<td>thick (77.8)</td>
<td>none (44.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>thin (22.2)</td>
<td>yellow (33.3)</td>
</tr>
<tr>
<td>Purple</td>
<td>4</td>
<td>yes</td>
<td>thin (100.0)</td>
<td>grey (100.0)</td>
</tr>
<tr>
<td>Grey</td>
<td>46</td>
<td>no</td>
<td>thin (56.5)</td>
<td>grey (65.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>thick (43.5)</td>
<td>none (30.4)</td>
</tr>
</tbody>
</table>

a) Number of cultivars within each color group.
b) 20 seeds of each variety were hand-dissected; varieties were categorized according to the characteristics observed in the majority of the 20 seeds.
c) Percent of the cultivars in that color group displaying each attribute.

packed tightly together and the protein bodies left distinct indentations in the starch granules. The average sizes of a starch granule and a protein body were 6.4 and 0.7μm, respectively. The protein to starch ratio was highest in the peripheral endosperm layers (Fig. 4C).

The corneous and flouxy endosperm comprised the bulk of the starchy endosperm; the relative amount of each depended on the genotype. The corneous endosperm was composed of cells that were packed with starch granules and a thin, semi-continuous protein matrix. Protein bodies were also present, but in fewer numbers than in the peripheral endosperm. Generally, the corneous endosperm cell contents were not packed tightly enough for the protein bodies to leave indentations in the starch granules. There were no air voids between the granules, which were less polygonal than those found in the peripheral areas; this gave the corneous endosperm a glossy appearance. Starch granules and protein bodies averaged 6.4 and 0.7μm in diameter, respectively.

The flouxy endosperm was composed of cells with loosely packed, larger, round starch granules with a small amount of discontinuous protein matrix. There were many air voids between the starch granules, which gave the flouxy endosperm a chalky appearance. There were few protein bodies present, and thus no indentations in the starch granules. The sizes of the starch granules and protein bodies averaged 7.6 and 0.6μm, respectively.

Protein

The highest amount of protein was found in the peripheral endosperm and decreased from the exterior to the interior of the kernel (blue fluorescence in Fig. 4C). Hoseney and Varriano-Marston (1980) reported that there were no protein bodies found in the flouxy endosperm of pearl millet; however, there were protein bodies present in the flouxy endosperm of all of the varieties observed in this study. The protein bodies were spherical and roughly uniform in size, regardless of their location in the endosperm. Adams et al., (1976) reported that the protein bodies contained invaginations and protuberances, and were not uniform in shape. The protein bodies seen in this study were somewhat smaller than those reported by Sullins and Rooney (1977) and Zeleznak and Varriano-Marston (1982).

A considerable amount of the protein in the pearl millet kernel was found in the protein bodies of the germ, as has been reported previously in many studies. All of the millets examined contained phytin in the germ. Phytein is important due to its interference in the bioavailability of minerals. Simivemba et al., (1984) reported that phytic acid was present in the germ and pericarp, but that the content varied greatly between environmental locations. Nicotinic acid inclusions were present in the protein bodies of the germ, but none were found in the protein bodies of the aleurone cells.
Microstructure of Pearl Millet

Lipids

Pearl millet has a lower endosperm to germ ratio than sorghum (Abdelrahman et al., 1984; Hoseney and Varriano-Marston, 1980). The germ contained a large proportion of the lipids found in the kernel. As previously reported, there was also a high concentration of lipids in the aleurone cells. Small globules of lipid were distributed throughout the endosperm cells of fresh hand sectioned material stained with Nile blue A (not shown); no lipids were visible in samples that had been fixed and dehydrated.

β-Glucans

The aleurone cell walls in pearl millet autofluoresced bright blue (Fig. 4A). The endosperm cell walls exhibited weak autofluorescence (not shown). Earp et al. (1983) reported that ferulic acid was responsible for bright blue fluorescence in the pericarp, aleurone and endosperm cell walls of sorghum. Fussell and Dwarte (1980) used autofluorescence to find that pericarp cells associated with black region development in pearl millet were composed of lignin.

When stained with congo red and viewed under FC I, mixed linkage β-glucan material was located in the cell walls of the scutellar epithelium; the β-glucan material appeared red along the inside of the cell walls around each cell (Fig. 4D). Fulcher and Wood (1983) reported that the red fluorescence under FC I was due to mixed linkage β-D-glucans. Congo red induced red fluorescence in β-D-(1-3)(1-4) glucans in cell walls of the pericarp, endosperm and germ, when viewed under FC III, but no differentiation between ferulic acid and β-glucans was possible using this filter combination.

Role of Pigmentation in External Kernel Color

Pigmentation imparts positive or negative attributes to food products, and in many areas of Africa, foods with a light color are preferred. Thus, it is important to know where the pigments are, what they are, and if they can be removed. In the 96 pearl millet samples studied, the external color perceived for each kernel was due to the interaction of several factors: pericarp thickness, pigmentation in the epicarp, slight pigmentation in the aleurone, and the existence of unidentified pigmentation in the peripheral endosperm (Table 3).

Pericarp: The epicarp cells contain a considerable amount of pigmentation in some varieties; the structure has been described previously. A thick pericarp can mask the presence of pigments in the aleurone or endosperm, which was observed in several white varieties. However, when the pericarp is thin, the pigmentation in the aleurone and endosperm is visible, and the external color of the seed can be yellow, brown, purple or grey. If there are no pigments present in the kernel, and the pericarp is thin, then the color of the kernel is white.

If pigmentation is present in the seed coat, it does not have a great deal of effect on the external color perception of the kernel because the layer is so thin. In contrast, the seed coat (testa) found in sorghum with B1-B2- genes can be heavily pigmented in discreet cells, which definitely influences the external color per-
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Figure 8: Starchy endosperm areas of an intermediate texture grey Souna variety. A. Peripheral endosperm area with dense protein matrix and a large number of protein bodies; B. Corneous endosperm with fewer protein bodies and thin protein matrix; C. Floury endosperm with little protein matrix and a few scattered protein bodies. Al: aleurone cell, M: protein matrix, P: protein body, SG: starch granule, CW: cell wall.

received in the kernel (Earp, 1984). It is easier to obtain an acceptable product color in foods when the pigmentation is primarily in the pericarp, where it will be removed during decortication.

Aleurone: Polyphenols were found in the aleurone cells after staining with toluidine blue; a pale green color resulted (not shown). Pigmentation in the aleurone is dark; it has the greatest effect on external color in varieties with a thin pericarp. This is demonstrated most clearly in the grey and purple millets. The aleurone did not stain positive for flavonoids (diphenylborinic acid), but the compounds may have been extracted during the dehydration process.

Endosperm: Yellow endosperm was observed most frequently in the yellow varieties, and to a lesser extent in brown and white varieties. A very small percentage of grey varieties had a yellow endosperm. The yellow color was most evident in the corneous endosperm. Rachie and Majmudar (1980) reported that $\beta$-carotene in the endosperm can cause the external color of the kernel to appear yellow in seeds with a colorless seed coat and a thin unpigmented pericarp.

Grey pigments were observed in the peripheral and corneous endosperm of all purple, and most grey varieties, and in a small percentage of yellow and brown varieties. There were an equal number of white varieties that contained grey pigments and those that contained no pigments, but usually these varieties had a thick pericarp. When endosperm sections were viewed under FC 1 (autofluorescence), there were often small patches of dark pigmentation located within the peripheral and corneous endosperm cells (not shown).

External Color: It is difficult to say if a specific kernel characteristic produces a specific kernel color. Rather, it is the combination of kernel characteristics that result in specific colors. However, there are a number of possible combinations. In varieties with a pigmented aleurone, the color could be dark with a thin pericarp, or light with a thick pericarp. Likewise, a dark endosperm could produce a grey tint in a white or yellow millet with a thick peri-
Microstructure of Pearl Millet

carp. A dark endosperm in a thin pericarp variety can result in grey or purple color. A yellow endosperm can enhance the yellow color in some varieties with a thin pericarp, or have no effect on the external color in varieties with a pigmented aleurone and pericarp. If pigments are in the epicarp, the external color is most likely going to be the color of those pigments. However, there are often cases where the varieties have bicolored kernels, i.e., grey and brown, or grey and yellow, where there appear to be concentrations of pigmentation in the base and tip of the kernels, with grey color in the midrange. There was a uniform set of kernel characteristics only in the purple millet varieties; a pigmented aleurone and endosperm, a thin pericarp, and dark pigments in the epicarp resulted in the purple color.

Polynphenol Analyses

The phenol analyses of 17 pearl millet varieties revealed that there were levels of polyphenols present in all samples tested. A purple variety had 0.33 mg/100mg polyphenols, while grey and yellow varieties averaged 0.22 and 0.19 mg/100mg, respectively. None of the samples contained tannins, which agreed with results previously reported by Reichert (1979). Reichert (1979) reported that the pigments in the grey pearl millet varieties were composed of C-glycosylflavonoids.

The HPLC analyses of three varieties of pearl millet revealed high levels of ferulic, coumaric, cinnamic and gentisic acids (Table 4). There were differences in phenolic acid content evident between pericarp colors. Total phenolic acid levels were highest in the yellow millet, followed by the grey and purple millets.

Conclusions

The data presented in this study provide some insight into the relationship between kernel characteristics, kernel structure, and processing properties of pearl millet. The diverse array of external characteristics and the lack of genetic information makes it difficult to predict how individual varieties of pearl millet will behave during processing. More knowledge of the kernel structure and kernel characteristics affecting processing properties is required. This study provides a starting point for more definitive work in the future.

Acknowledgements

Appreciation is expressed to the Electron Microscopy Center, TAMU, for the use of their equipment and expertise, and to Dr. Cheryl Earp and Ms. Julie Poe for their assistance in this project. Samples from Drs. Appa Rao, ICRISAT, India; O. Niangado, IER, Mali, and J. Clark, INRAN, Niger were appreciated. Dr. Dave Andrews, U. of Nebraska, reviewed the manuscript during its preparation. This research was partially supported by the

<p>| Table 4 |
| Phenolic acid analyses of 3 pearl milletsa |</p>
<table>
<thead>
<tr>
<th>Phenolic Acids</th>
<th>Purple (Souna)</th>
<th>Grey (Iniade)</th>
<th>Yellow (CMM411)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferulic</td>
<td>624.7</td>
<td>786.3</td>
<td>628.2</td>
</tr>
<tr>
<td>Coumaric</td>
<td>247.8</td>
<td>211.4</td>
<td>346.6</td>
</tr>
<tr>
<td>Genticic</td>
<td>144.2</td>
<td>79.0</td>
<td>96.0</td>
</tr>
<tr>
<td>Cinnamic</td>
<td>271.4</td>
<td>350.1</td>
<td>415.1</td>
</tr>
<tr>
<td>Caffeic</td>
<td>11.3</td>
<td>37.5</td>
<td>15.1</td>
</tr>
<tr>
<td>Vanillic</td>
<td>16.3</td>
<td>26.1</td>
<td>6.5</td>
</tr>
<tr>
<td>p-OH Benzoic</td>
<td>24.1</td>
<td>15.8</td>
<td>26.0</td>
</tr>
<tr>
<td>Syringe</td>
<td>17.8</td>
<td>10.5</td>
<td>23.7</td>
</tr>
<tr>
<td>Sinapic</td>
<td>18.4</td>
<td>15.4</td>
<td>27.7</td>
</tr>
<tr>
<td>Unknowns</td>
<td>669.0</td>
<td>646.5</td>
<td>892.8</td>
</tr>
<tr>
<td>Total Acids</td>
<td>2037.7</td>
<td>2182.4</td>
<td>2486.6</td>
</tr>
</tbody>
</table>

a) values expressed as µg/mg phenolic acids/ gm sample, dry weight basis; seed from locations in Mali, West Africa; values are the averages of two replicates each.

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References


Bilquez AF. (1963). Determinisme genetique des differences de sensibilite a la longueur du jour existant entre les mils du groupe sano et ceux du groupe souan. (Genetic determination of the sensory differences that exist between millets from the groups Sano and Souan.) L’Agronomie Trop. 18:1249-1253.


Microstructure of Pearl Millet

Pearl millet (Pennisetum americanum (L.) Leke) and grain sorghum (Sorghum bicolor (L.) Moench) ultrastructure. Am. J. Bot. 69:1306-1310.

Discussion with Reviewers:

SH Yu: Is the textural difference of the starchy endosperm, i.e. corneous v. floury, dependant on the variety or the maturity of the kernel of pearl millet?

Authors: The texture of the endosperm depends upon both variety and environmental factors. A corneous variety can develop a floury endosperm when it is affected by insects, grain molds and weathering. A floury endosperm variety never develops a corneous texture.

SH Yu: The negative results obtained from staining with diphenylborinic acid may suggest a low concentration of flavonoid compounds or removal of these compounds by alcohols (F.W. Collins, 1986. In "Oats: Chemistry and Technology, AACC, St. Paul, Minnesota) that can occur at the dehydration step during preparation of the glycol methacrylate embedded sections. Did you try staining using hand-prepared or frozen sections?

Authors: Upon this suggestion, fresh sections were prepared by hand and stained with diphenylborinic acid. Some very weak fluorescence was evident in a purple Soula millet, but none appeared in the grey or yellow millets. The dehydration process does seem to be inhibiting the response to diphenylborinic acid, but to what extent is unknown.
THE STRUCTURAL BASIS OF THE WATER-HOLDING, APPEARANCE AND TOUGHNESS OF MEAT AND MEAT PRODUCTS

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John Elsey, Nick Parsons, Alan Sharp, Roger Starr and Peter Purslow

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Abstract

A structural approach greatly clarifies which components of meat are responsible for its tenderness, water-holding and appearance, and the events occurring during processing.

In living muscle, water is held in the spaces between the thick and thin filaments. Changes in the content and distribution of water within meat originate from changes in this spacing. Myofibrils shrink laterally post mortem. The fluid expelled accumulates between fibre bundles and between fibres and is drained by gravity forming drip. In pale, soft and exudative meat, shrinkage of the myosin heads on denaturation increases myofibrillar shrinkage. In salt solutions used in meat processing, myofibrils swell laterally taking up water, probably by an entropic mechanism.

The colour of meat is determined by light scattering as well as by absorption. The light scatterers in meat are observed by scanning confocal light microscopy to be smaller than 1 μm and concentrated in the A-band regions. It is proposed that light scattering arises from the gaps between myofibrils.

The structures responsible for the toughness of cooked meat have been studied by observing its fracture behaviour. The perimysial connective tissue determines the breaking strength when the meat is pulled apart transversely. In longitudinal tensile tests, the initial event is the debonding of fibre bundles from the perimysium so that they contribute independently to load-bearing. At greater extensions, it seems likely that fibre bundles progressively fail leaving perimysial strands as the last structure to break. With aged muscle, the muscle fibres probably fail at smaller extensions and therefore contribute less to the breaking strength.

Key Words: Muscle, meat, light microscopy, confocal microscopy, electron microscopy, water-holding, appearance, colour, toughness, texture.

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Introduction

In Western societies a larger amount is spent on meat and meat products than on any other food, and meat foods present a particularly wide variety of problems and challenges. Structural investigations on meat are very helpful because by identifying the spatial arrangement of components, they can identify the component responsible for a problem affecting meat quality and greatly clarify the mechanism responsible. These problems are caused by rather subtle changes in the colloidal character of meat and, although much is known about the factors producing them, the exact nature of these changes has until recently eluded a precise description; only now are meat scientists beginning to explain them. We are fortunate in being able to draw on a very large amount of structural and biochemical information provided by basic biological research on muscle and connective tissue. We are also fortunate in that muscle cells are very regular and this facilitates both the study of their structure and thinking about mechanisms.

In this brief review we have selected three topics, water-holding, appearance and texture. All levels of structure are informative, from single protein molecules up to coarser features that can be seen by eye. Correspondingly, a number of complementary structural techniques is proving valuable, including X-ray diffraction, scanning and transmission electron microscopy, phase-contrast, fluorescence, polarising, dark-field and scanning confocal light microscopy.

The Structure of Muscle

Before considering these three quality attributes, we will first briefly review the structure of muscle (for detailed recent reviews see Squire and Vibert, 1987). Surrounding each muscle is a thick connective tissue sheath, the epimysium, which is continuous with the tendon (Figure 1). The muscle is divided into muscle fibre bundles by the perimysial connective tissue network. The coarse or primary perimysial network, which is very obvious to the eye, is further sub-divided by thinner sheets of perimysial connective tissue, typically defining a bundle of muscle fibres of the order of 1 mm...
across. The collagen fibres in the perimysium are crimped and arranged in a criss-cross lattice (Rowe, 1974). In muscle at rest length, the collagen fibres in the perimysium are arranged at an angle of about + 55° to the muscle fibre axis but assume larger or smaller angles as the muscle shortens or lengthens. Individual muscle fibres are separated from one another by the endomysial connective tissue network (Figure 1). The muscle fibres are multinucleated cells, typically 10 to 100 \( \mu \text{m} \) wide and as much as several centimetres long. They are filled with myofibrils, the contractile apparatus of muscle. These are long, thin (1 to 2 \( \mu \text{m} \)), roughly cylindrical organelles packed side by side (Figure 2). In white fibres, which are used for bursts of activity, few mitochondria are present and the myofibrils are separated only by the calcium-storing membrane-lined channels of the sarcoplasmic reticulum. In red fibres, which are used for sustained or repetitive activity, mitochondria also intrude between adjacent myofibrils.

Myofibrils have a regular striated appearance and, because they are packed in register, the whole muscle fibre appears striated (Figure 3). The wide protein-dense bands are called A-bands and each is bisected by a M-line. The striations of the myofibril arise because the myofibril is made up of overlapping arrays of two kinds of filaments, thick and thin, which form a repeating pattern (Figure 3). In transverse sections through the region of overlap, the filaments may be seen to lie in a well-ordered hexagonal lattice (see Figure 4). Each thick filament, which is 1.6 \( \mu \text{m} \) long, is made up principally of about 300 molecules of the protein myosin. The myosin molecule has a long tail (156 nm x 2 nm wide) at one end of which two curved pear-shaped heads (19 nm long) are flexibly attached (see Figure 6). In the thick filament the tails pack together to form the

**Figure 1.** Coarse structural organisation in skeletal muscle showing the connective tissue tracts of muscle. Top, whole muscle; bottom, a fibre bundle. (Adapted from Purslow (1987) by permission of von Nostrand Reinhold Company).

**Figure 2.** Scanning electron micrograph of an obliquely cut surface of beef sternomandibularis muscle. The fibre direction is from left to right. In the two fibres near the surface the enclosing endomysial sheaths have been largely removed revealing the closely packed arrangement of myofibrils within them. Bar = 20 \( \mu \text{m} \).

**Figure 3.** Transmission electron micrograph of a longitudinal section through frog skeletal muscle showing the striated appearance. The lower diagram schematically shows how the striations of the myofibrils are caused by the orderly packing of thick and thin filaments. The A-band is 1.6 \( \mu \text{m} \) long. (Reproduced by permission of Dr Craig and the Longman Group).
shaft, the tails in the two halves pointing in opposite directions. This leaves the heads on the surface where they can interact with the thin filament (see Figure 6c). The thin filament is made up principally of about 400 molecules of actin, the precise number depending on the species. Each actin molecule is dumb-bell-shaped and can bind one myosin head. The actin molecules pack in a helical manner with the long axis of each dumb-bell perpendicular to the filament axis. Other proteins, tropomyosin and troponin, bind to the actin filament and confer calcium-sensitivity on the filament.

Muscle contraction is triggered by the release of calcium ions from the sarcoplasmic reticulum in response to a nervous impulse. The calcium ions bind to the thin filament switching it on in such a way that myosin heads can attach to the thin filament and generate force, probably by undergoing a change of shape. Shortening of the muscle occurs by successive cyclic interactions of the myosin heads resulting in increased overlap of thick and thin filaments. The energy for contraction comes from the hydrolysis of ATP catalysed by the myosin heads.

After the death of an animal, the muscle continues to hydrolyse ATP at a slow rate. For a time, the levels of ATP are maintained by the breakdown of glycogen to lactic acid, resulting in a fall in pH from 7 to around 5.6. Eventually the glycogen stores are depleted and the ATP concentration falls to zero. At this time the heads attach strongly to the thin filaments (see Figure 6b) causing the muscle to be rigid and stiff. This condition is known as the rigor state.

Water-Holding of Meat

Lean meat immediately after slaughter contains about 75% water. But, depending on the properties of the meat and how it is treated, it may gain or lose water, (see reviews by Hamm, 1960, 1986; Offer and Knight, 1988a,b). This is important economically since meat is sold by weight. The content of water and its distribution within the meat also determines its quality. For example, it is possible to have two pieces of meat with initially the same water content, one dark and dry in appearance, and the other pale and rapidly exuding water (Wismer-Pedersen, 1959).

Water can be lost from raw meat by evaporation from the surface, or by the exudation from the cut surfaces of drip. This is a concentrated solution of intracellular muscle proteins, including myoglobin, which gives it its red colour. The amount of drip is increased in pale, soft and exudative (PSE) meat. The PSE condition results from the denaturation of proteins caused by the attainment of a low pH while the carcass is still warm (Wismer-Pedersen, 1959). This can occur if the rate of breakdown post mortem of glycogen to lactic acid is fast, the final pH is particularly low, or chilling is slow. Meat loses even more water (up to 40% of the weight of the meat) on cooking and there is a correspondingly large shrinkage.

Figure 4. Hypothesis to explain the origin of changes in the water-holding of raw meat. A myofibril is seen in transverse section in a shrunken state on the left-hand side and a swollen state on the right-hand side. The diagram is simplified and does not depict the depolymerisation of the thick filament that occurs in high salt concentrations. (Reproduced from Offer et al. (1984), by permission of the Royal Society of Chemistry).

Conversely, when meat is treated during processing with NaCl, or NaCl together with polyphosphates, water is taken up. Polyphosphates are added for three reasons. Firstly, they reduce the concentration of NaCl required for water uptake. Secondly, they help solubilize myosin which forms a sticky coat on the surface of meat pieces and sets as a gel on heating, thereby binding meat pieces together (see Jolley and Purslow, 1988). Thirdly, polyphosphates greatly reduce the loss of water on cooking.

Since myofibrils occupy about 80% of the volume of living muscle fibres, most of the water in the muscle cell is present in the myofibrils in the spaces between thick and thin filaments (Figure 5). Only a small fraction of this water (perhaps about a tenth) is actually at any instant bound to protein (see Offer and Knight, 1988a). The simplest hypothesis to explain changes in the water held by meat is to suppose that they have their origin in changes in the volume of the myofibrils. In the raw state, where the filament lattice is regular, we suppose that uptake of water occurs by the entry of water into the myofibrils as they swell laterally by an expansion of the filament lattice. Conversely, we suppose that loss of water occurs by the expulsion of water from the myofibrils as they shrink laterally when the filaments get closer together (Figure 4).

Drip Loss

X-ray diffraction has the dual advantages of being non-invasive and being capable of determining the centre-to-centre spacing between neighbouring thick filaments with a high precision. Figure 5 shows the filament lattice spacing in beef rectus abdominis muscle as a function of time post mortem. The spacing does not change much with time initially, but at rigor onset there is a rapid decline to a new lower spacing, about 4.4% smaller than the original spacing.

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Figure 5. Changes in the filament lattice spacing in beef rectus abdominus muscle post mortem determined by X-ray diffraction. (Reproduced from Offer et al. (1989) by permission of Marcel Dekker).

Figure 6. Lateral shrinkage of a myofibril post mortem. (a) Living muscle. The myosin heads are detached and the filaments are widely spaced. (b) Normal rigor muscle. The myosin heads have attached to the thin filaments drawing the filaments closer together. (c) PSE rigor muscle. Shortening of the myosin heads prior to rigor has caused the filaments to be drawn still closer together at rigor onset. At the bottom of the figure two myosin molecules are shown on the same scale, one normal and the other denatured under PSE conditions. The heads of the latter are shorter. (Knight, unpublished results). This corresponds to a 9% decrease in the cross-sectional area, and therefore volume, of the myofibrils. The decrease is partly due to a fall in pH and partly due to the attachment of myosin heads at rigor onset (see Offer and Knight, 1988b) (Figure 6a).

Myofibrillar shrinkage is faster in pig muscles than in beef corresponding to the shorter times to rigor onset in pig (Knight, unpublished results). Preliminary results show that in PSE meat the myofibrils shrink about twice as much as in normal meat accounting for the higher drip in this state (Knight, unpublished results). What is responsible for this greater shrinkage? Penny (1967a) and Stabursvik et al. (1984) showed that myosin was denatured in PSE muscle. We have recently found by negative staining that if myosin is exposed to conditions (pH 6.0, 35°C for 5 or 10 minutes) similar to those experienced in PSE meat and which cause substantial loss of myosin ATPase (Penny, 1967b), the head length decreases from 19 nm to 17 nm (Sharp, Walker and Offer, unpublished results). This is sufficient to draw the thick and thin filaments even closer together at rigor onset and give rise to increased expulsion of water and drip. Calculations show that this small change in head length is sufficient to account for the increased myofibrillar shrinkage and therefore the increased exudation in the PSE state.

We can now ask where the water expelled from the myofibrils accumulates. Ideally, to observe this, we need a structural technique with a moderately high resolution (say 5 nm), which could non-invasively image water compartments in a whole muscle. Perhaps in the future nuclear magnetic resonance imaging may prove helpful, but in the shorter term we have studied water compartments in sections of muscle fixed at various times post mortem and embedded in paraffin wax (Offer and Cousins, unpublished results). We find that in beef sternomandibularis muscle at 2 h post mortem there are no large channels in the meat; the fibres fill the endomysial network and the fibre bundles fill the perimysial network (Figure 7a). At 6 h post mortem, however, gaps of variable width appear between fibre bundles (Figure 7b). At this stage there are few gaps between fibres. Finally, after 24 h post mortem, gaps also appear between fibres (Figure 7c), as was first shown by Heffron and Hegarty (1974).

Evidently the fibres shrink as their constituent myofibrils shrink and the water that is left behind accumulates first around the perimysial network and later around the endomysial network, giving rise to two extracellular water compartments.

Finally, we can ask which water compartment is the source of drip, along what channels does it flow to the surface, and what force drives it out? A slice of meat held with the fibres vertical produces much more drip on the lower than the upper surface (Offer, 1984a). Similarly, if the slice is placed with the fibres horizontal, the lower part of the cut surface can be seen to be much wetter than the upper. If a piece of meat is placed in an organic solvent of the same density as meat so that hydrostatic pressures due to gravity inside and outside the meat are equalised at every level, drip is
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producing a small hydrostatic pressure on that region. If the column is small, say 1 cm diameter, the solution passes through the meat parallel to the fibre direction washing out myoglobin and other soluble proteins from a cylinder of the meat, and a circle on the opposite face of the meat appears nearly colourless (Starr, Almond, Knight and Ofer, unpublished results). If the solution contains fluorescently-tagged bovine serum albumin and the opposite face is observed with a binocular microscope equipped with fluorescence optics, the first appearance of fluorescence is quite local and confined to the primary perimysial network (Figure 8b) together with regions of the secondary perimysial network opening from it. With time, fluorescence emerges over a larger area but still is confined to the perimysial network (Figures 8c-f). This confirms the existence of continuous longitudinal channels through the meat between the fibre bundles. We suppose that drip arises predominantly by the action of gravity draining the fluid in these channels to the cut surfaces.

Meat Processing

We have tested the hypothesis that water uptake in meat processing is due to lateral expansion of myofibrils. This was done by observing the effect on individual myofibrils of successive irrigations of salt (Offer and Trinick, 1983; Knight and Parsons, unpublished results) by the technique of Hanson and Huxley (1955). Phase-contrast light microscopy allows the myofibrils to be viewed in their hydrated state without staining. In our earlier work the myofibrils were attached to cover-slips but in more recent work they have been suspended between the bars of an electron microscope grid. When rabbit or beef myofibrils are treated with a series of NaCl solutions of increasing concentration and buffered to pH 5.5 to simulate rigor conditions, lateral swelling of the myofibrils starts in 0.6 M NaCl and is accompanied by extraction of myosin from the centre of the A-band, approximately the H-zone (Figure 9). Further swelling occurs in 0.8 and 1 M NaCl. The response of different myofibrils is somewhat variable, but the average swelling in 1 M NaCl is 90% ± 5% for rabbit myofibrils and 44% ± 60% for beef myofibrils (Knight and Parsons, unpublished results). This is more than sufficient to explain the uptake of water during meat processing.

If pyrophosphate is included in the irrigation medium, extraction of myosin occurs at lower NaCl concentrations and the extraction is more complete. In the presence of 10 mM pyrophosphate and 1 mM MgCl₂, very little happens when the myofibrils are irrigated with 0.3 M NaCl, but in 0.4 M NaCl the A-band is completely or nearly completely extracted commencing from both edges eventually leaving strings of I-segments (Offer and Trinick, 1983). In the presence of pyrophosphate, swelling is markedly less than that produced in NaCl alone (42% in rabbit, 14% in beef) (Knight and Parsons, unpublished results).
When meat is processed, it is often treated with small volumes of very concentrated brines so that initially myofibrils may be exposed to high concentrations of NaCl. During curing, for example, near the sites of brine injection there will initially be a very high salt concentration which progressively diminishes, whereas further from these sites the concentration of NaCl increases progressively from zero. It is therefore important to know how myofibrils respond to very high NaCl concentrations. While naively one might think that higher NaCl concentrations would have a greater effect than lower salt concentrations, this is not so. As the concentration of neutral salts like NaCl increases, initially there is an increasing tendency for protein assemblies to dissociate (the 'salting-in' range) but at higher concentrations this tendency diminishes (the 'salting-out' range). Callow (1932) found that the water uptake by meat in brines was maximal at 1 M NaCl and no water was taken up at concentrations above about 4 M NaCl. Correspondingly, myofibrils do not swell and show little change in structure when treated with 5 M NaCl buffered to pH 5.5, although at lower

Figure 8. Demonstration of longitudinal channels through meat. A 12 cm head of a solution of fluorescently-tagged serum albumin has been applied to one surface of a 1.5 cm transverse slice through beef semitendinosus in the rigor state and the emergence of this solution on the opposite face followed with time. (a) Opposite face observed by visible light showing perimysial network. (b-f) observed with fluorescence optics (b) 8 minutes (c) 16 minutes (d) 20 minutes (e) 24 minutes (f) 32 minutes after application of hydrostatic head.
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Figure 9. Swelling of rabbit myofibrils in salt solutions. The same myofibril is seen throughout by phase-contrast microscopy. (a) After preparation in pH 7 preparation medium. (b) After irrigating with 0.1 M NaCl, 1 mM MgCl₂, 10 mM sodium acetate, pH 5.5. (c) – (i) after further 3 minute irrigations with NaCl at the molar concentrations shown on the left hand side together with 1 mM MgCl₂, 10 mM sodium acetate, pH 5.5. Sarcomere length 2.3 μm. (Reproduced from Offer and Trinick (1983) by permission of Elsevier Applied Science).

Concentrations (e.g., 1 M) they swell and the A-band is partly extracted (Knight and Parsons, 1988).

We may conclude that during the curing process the sites where swelling and protein solubilisation first occur are not immediately adjacent to the injection sites but at some distance from them. The difference in the course of changes in NaCl concentrations in different parts of the meat is likely to be the source of the approximately periodic stripes often present in bacon slices (Voyle et al., 1986).

We commented above on the variability of the response of myofibrils to salt. In a field of myofibrils prepared from rabbit plantaris muscle irrigated with 0.45 M NaCl plus pyrophosphate at pH 5.5, some myofibrils are extracted and swell, others are only slightly extracted and do not swell (Figure 10) (Knight and Parsons, unpublished results). This is at least in part due to the difference between fibre types. Myofibrils prepared from muscles with a high proportion of white fibres respond to a lower salt concentration than those from muscles like the soleus with a high proportion of red fibres. By antibody labelling of myofibrils from a muscle such as rabbit plantaris, which comprises a mixture of fibre types, we have shown that myofibrils from fibres containing slow myosin require a higher concentration of salt for extraction than myofibrils from fibres containing fast myosin (Knight and Parsons, unpublished results). The suitability of meat for processing may therefore be influenced by variation in the content of fibre types from muscle to muscle and animal to animal.

Mechanism of Swelling. In a previous publication we supposed that chloride ions binding to the filaments increased their negative

Figure 10. Variability in response of myofibrils to salt treatment. A field of rabbit plantaris myofibrils is shown (a) before and (b) after treatment with 0.45 M NaCl, 1 mM MgCl₂, 10 mM pyrophosphate, pH 5.5. In most of the myofibrils shown, the A-bands are extracted but the arrowed myofibril is resistant to this salt concentration. Bar indicates 10 μm.

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charge, causing increased long-range electrostatic repulsive forces and therefore swelling (Offer and Trinick, 1983). However, at high salt concentrations, the excess of counterions balancing the negative charge on the filaments is localised so closely to the filament surface that the charge on the filaments would be effectively screened. This screening effect would more than outweigh the effect of increased charge due to Cl binding (Ledward, 1983; Offer, 1984b; Offer et al., 1989).

In collaboration with Dr B Millman and Dr B Nickel of Guelph University, we have proposed a new kind of hypothesis in which salt-induced swelling is entropically rather than electrostatically driven (Offer et al., 1989). This is illustrated in Figure 11. It is well known that moderately high salt concentrations (0.5 to 1 M) depolymerise thick filaments into myosin molecules. In the region where thick and thin filaments overlap, these myosin molecules would tend to remain attached to actin filaments (Figure 11b), although in the H-zone they would be free to diffuse away. The myosin tails, being flexibly attached to the heads, would tend to explore space but this motion would, we suppose, in the unswellen lattice be severely restricted by the presence of neighbouring thin filaments and the myosin molecules attached to them (Figure 11b). If the lattice swells (Figure 11c) the tails would have greater freedom of motion and therefore higher entropy. Since systems tend to move to a state where they have highest entropy, there would be a marked tendency for the lattice to expand, which, compared with the electrostatic swelling pressure, would diminish only slowly with expansion.

On this hypothesis, depolymerisation of the thick filaments liberates the myosin tails necessary for the driving force to be developed. By contrast, dissociation of the myosin from actin would reduce the swelling pressure by reducing the amount of bound myosin. This effect would be particularly great if there were an excess of brine such as in the irrigated myofibrils, since much of the myosin would then escape.

The effectiveness of polyphosphates in promoting water uptake and protein solubilisation in meat processing is in part due to the small increase in pH alkaline polyphosphates produce (Lewis et al., 1986; Trout and Schmidt, 1986) but there is also a specific effect (Bendall, 1954). It is very well established that pyrophosphate in the presence of magnesium ions weakens the association of actin and myosin heads (see Offer and Knight, 1988a). It is also known that pyrophosphate assists chloride in causing the depolymerisation of thick filaments (Harrington and Himelfarb, 1972). This gives us a basis for understanding the effects of pyrophosphate. Because depolymerisation is promoted by pyrophosphate, extraction of myosin and swelling occur at a lower concentration of NaCl than in its absence. But because pyrophosphate promotes dissociation of myosin from actin, maximum swelling in the presence of pyrophosphate would be expected to be lower than in its absence, especially in systems, like irrigated myofibrils, where myosin would be lost. In systems where the brine:meat ratio is much smaller, such as occurs in meat processing, the swelling with pyrophosphate would be greater since more myosin would remain bound to actin.

The importance of myosin to swelling has been tested by irrigating myofibrils, from which myosin has been previously extracted, with a solution of myosin. The thin filaments bind the

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Figure 11. Mechanism of swelling of myofibrils in salt. (a) Longitudinal section through the overlap region of a myofibril showing two thin filaments and one thick filament with its myosin heads attached to the thin filament. (b) As (a) but showing the depolymerisation of the thick filament as a result of treatment with NaCl before swelling has occurred. The motion of the myosin tails is restricted. (c) After swelling of the filament lattice, the myosin tails are able to move through a larger angle. (Reproduced from Offer et al. (1989) by permission of Marcel Dekker).
myoglobin and the myofibrils swell substantially. Though the hypothesis appears plausible and we will be testing it critically.

Effect of the Endomysium on Swelling. Having discussed the swelling of myofibrils in salt solutions, we can now consider the swelling of whole fibres, and consider the influence of the connective tissue. The degree of swelling of dissected muscle fibres in salt solutions is in our hands very variable from fibre to fibre. Wilding et al. (1986) had shown that, if the endomysial sheath surrounding a fibre is damaged at one point, much more swelling takes place there and they concluded that the endomysium acts as a mechanical restraint to swelling. It seemed possible that the variability we observed was due to some fibres being dissected with an endomysial sheath and some without. Whether or not a fibre has a sheath can be determined by dissolving out muscle proteins from the muscle fibre with SDS. After irrigation of a sheathed fibre, the endomysial sheath is left, but with an unsheathed fibre the endomysium dissolves. We found that the sheathed fibres swelled only a little in salt, and the unsheathed fibres swelled much more (Knight et al., 1989; Offer et al., 1989). This reinforced the conclusion of Wilding et al. that the endomysium could act as a mechanical constraint and also showed that fibres can be dissected without a sheath.

We further found that the remarkable increase in swelling over a narrow period of time post mortem observed by Wilding et al. could be entirely explained by the change with time of the ease of stripping of the endomysium; there was no other effect of time (Knight et al., 1989). Stanley (1983) showed that emptying of the contents of muscle fibres when exposed to calcium ions requires an extended period of conditioning. All this suggests that the optimum time post mortem for processing of meat may depend on proteolytic weakening of the connection between muscle fibres and connective tissue allowing stripping of the endomysial sheath during comminution, and therefore greater myosin extraction and water uptake.

Appearance of Meat

The colour of meat is determined not only by the quantity and oxidation state of the pigment myoglobin, but by the light scattering properties of the meat (MacDougall, 1982). In samples with a high light-scattering ability, such as PSE meat, light does not penetrate far into the meat before being scattered; hence there is relatively little absorption by myoglobin and so the meat appears pale. In contrast, dark, firm and dry (DFD) meat, formed when the final pH is high, say >6.0, scatters light only to a small extent. Incident light is therefore able to penetrate the meat for a substantial depth and is strongly absorbed by myoglobin. Such meat therefore appears dark. We shall now discuss the structural features responsible for this light scattering.

It has long been appreciated that there is a broad relationship between the appearance of the meat and its water-holding characteristics (Hamm, 1960), although drip loss and light scattering do not accurately parallel one another (Warriss and Brown, 1987). We have supposed that the light scattering property of muscle resides in the myofibrils and depends on their degree of expansion (Offer and Trinick, 1983). Recent experiments have enlarged and modified this view. Jeacocke (1984) had shown that the light-scattering ability of muscle increases substantially at rigor onset, presumably due to the shrinkage of myofibrils occurring at this time. He also showed that the light-scattering ability is little affected by treatment of muscle with detergent, suggesting that membranes and membrane-lined organelles contribute little to the light-scattering by muscle.

Dark field microscopy provides a rather direct way of investigating light scatter since the image is constructed from light scattered by the object. Dark-field images of isolated myofibrils in suspension indicate that the A-band, and to a lesser extent the Z-disc, are bright sources of scattered light (Jeacocke, unpublished results). The intensity within the A-band is not uniform; the intensity distribution is consistent with an origin in light scattered principally at the surfaces between the A-band and the suspending medium and at the A-I junction (Figure 12c). In general, light scattering occurs at interfaces between two phases that have different refractive indices. The observations on myofibrils are therefore explained by the steps in refractive index occurring at their surfaces and at the A-I interfaces. Light scattered from a suspension of myofibrils is maximal at a pH of about 5 and decreases at lower or higher pH's (Jeacocke, unpublished results). This is consistent with light scattering depending on myofibrillar volume which would be expected to be minimal at pH 5, the isoelectric point of myosin actin.

In muscle the light scattering properties of myofibrils are modified because they are packed alongside one another in register with a small but variable gap between neighbours (Figures 12b, c,d). If neighbouring myofibrils touched (Figure 12b), there would be no step in refractive index at their junction and therefore no light scattering at this interface. A small gap, say greater than a tenth the wavelength of light (Figure 12c), would be sufficient to create substantial scattering and the degree of scatter would increase with the width of these gaps. The step in refractive index would be greatest at the intermyofibrillar gap between A-bands.

The confocal scanning light microscope produces reflectance images of skeletal muscle which differ substantially from those produced by conventional transmission light microscopy (Jeacocke, unpublished results). The particular advantage of this technique is that artefacts due to fixation, dehydration and embedding are avoided and that the optical section providing the image can be as thin as 0.8 µm, less than the diameter of a myofibril. This allows the light
scattering features in a thin layer within a sample of meat to be viewed without superposition effects from structures above and below the section. Figure 13 shows the appearance of a thin optical section within a fibre bundle from rabbit psoas muscle. The most obvious feature of this image is the highly speckled appearance. Small, bright sources of light substantially smaller than 1 μm in width are distributed throughout the section but are particularly numerous along transverse bands about 1 μm wide. These bands often extend right across the fibre without dislocations and are arranged with the same periodicity as the sarcomere. Comparison between reflectance and fluorescence confocal images of skinned muscle in which the myosin has been labelled fluorescently suggest that each speckled band coincides axially with the central region of an A-band. The speckled appearance varies along the length of a fibre and also between fibres. In PSE meat the speckles are larger and there appear to be more of them. The speckled appearance is not substantially altered by treatment with detergents, suggesting that membrane-bound organelles, such as mitochondria and the sarcoplasmic reticulum, are not directly responsible for the scattering.

Assuming that the speckled character is not an artefact of coherent light illumination, we may conclude that light scattering from muscle is predominantly not from molecular features (for example, cross-bridges) but rather from much larger structures. It seems possible that the speckled appearance arises from variations in the closeness of packing of adjacent myofibrils. On this basis the light scattering sources would correspond to small regions where the gap between adjacent myofibrils was wider than elsewhere. Adjacent myofibrils are joined together at Z-disc (and M-line) level by proteins such as desmin (Granger and Lazarides, 1978) (Figure 12d). The speckles may therefore arise from light scattering at the boundaries of intermyofibrillar compartments bounded by these structures. It is at present unclear whether the T-tubules and the terminal cisternae of the sarcoplasmic reticulum, which are present as a collar around each myofibril at the ends of the A-bands, play any role.
role in producing light scattering, for example by acting as spacers between myofibrils. Further work is required to test these notions and to determine the precise nature of the scatterers.

**Toughness**

The most important attribute of the eating quality of meat is its tenderness. When we chew meat, it is pulled apart, and the difficulty with which this is achieved is perceived as the sensation of toughness. A variety of mechanical tests has been applied to meat, but tensile tests, in a direction either parallel or perpendicular to the fibre axis, are the simplest to interpret (Bouton et al., 1975). The longitudinal breaking strength of raw meat correlates reasonably well with the sensory perception of toughness of cooked meat (Stanley et al., 1972), although no such comparison seems to have been done on the longitudinal breaking strength of cooked meat.

A particularly useful approach is to examine the structural events occurring when samples of meat are extended to breaking point (Carroll et al., 1978; Purslow, 1985, 1987). In a tensile test, the progressive breakdown of the structure leading to its final fracture will depend not only on the stiffness, breaking strength and breaking strain of its components, but on their spatial arrangement and the connections between them.

When a transverse slice of cooked meat is pulled apart in a direction perpendicular to the muscle fibre axis, the first event seen is the opening up of cavities throughout the slice in regions between fibre bundles (Purslow, 1985, 1987) (Figures 14a,b). Histological investigation shows that the site of fracture lies between the perimysial network and the endomysia of fibres on the surface of a bundle (Purslow, 1987). A striking demonstration that the cleavage pathway is between perimysium and endomysium is obtained by viewing the cleaved surfaces by scanning electron microscopy (Purslow, 1987). Evidently the weakest component of the cooked meat, and therefore the first to break, is the junction between the endomysium and perimysium. As the load is further increased, some of the cavities join up in a fracture path which runs along the boundaries between fibre bundles, with strands of perimysium, presumably originating from nodes of the perimysial network, bridging the gap at intervals and carrying the load (Figure 14b,c). When a still higher load is applied, further extension occurs and these perimysial strands rupture (Figure 14c,d). Thus, in this transverse direction, the breaking strength (maximum stress) of the meat is simply determined by the amount and strength of the perimysium, the last structure to break.

We shall now consider what happens when strips of raw and cooked meat are subject to tensile testing parallel to the muscle fibre direction. In raw meat, after an initial stiff phase for small extensions, the material becomes more compliant, and only when the meat is stretched to twice its original length, does the stiffness rise steeply due to stretching of the perimysium (Davey and Dickson, 1970). Cooking dramatically changes the shape of the load-extension curve. After cooking at 80°C, the initial stiffness is much higher, but after loads of approximately 1 to 1.5 kg/cm² and extensions of 5 to 20% (the yield point), the stiffness falls markedly (Bouton et al., 1975; Locker et al., 1983). The breaking strength of meat cooked at 70 or 80°C is appreciably greater (1.5x) than that of raw meat (Bouton et al., 1975; Locker et al., 1983), whereas breaking extensions have been reported to decrease slightly on cooking (Locker et al., 1983) or to increase (Bouton et al., 1975). Cold-shortened samples have a far greater breaking extension than an unshortened control, and stretched muscles have a smaller breaking extension (Bouton et al., 1975). For meat cooked at 80°C the peak force is largest for cold-shortened samples, followed by stretched samples and is least for unshortened controls.

If cooked muscle is stretched to about 65% extension, then the tension is removed, the strip returns almost to its original length (Locker et al., 1983). However, on reloading the strip, the load-extension curve is markedly different: the load required for small extensions is much smaller, although as would be expected, the load required to return to a 65% extension is the same as for the first run. The form of the curves is consistent with elastic structures progressively failing at extensions right up to 65% and not only at or near the yield point.

Ageing for 7 d at 2°C prior to cooking almost halves the initial stiffness without affecting the stiffness at higher extensions (Locker et al., 1983). The breaking strength is slightly reduced. After cooking for 3 h at 100°C, which causes the solubilisation of a substantial fraction of the collagen as gelatin, the yield point survives but the longitudinal breaking strength is greatly reduced (Locker et al., 1983). This suggests that connective tissue contributes substantially to the breaking strength but is not implicated in the structural changes responsible for the yield point.

The precise sequence of structural events that occurs as the cooked meat is stretched is not yet clear, although we do know many of the elements of the overall picture. At the macroscopic level, the first clearly observable event upon extending unaged cooked meat is the separation (debonding) of fibre bundles from each other (Purslow, 1985, 1987) (Figure 15b), showing that the endomysial-perimysial junction is a weak component in this testing direction, as well as in the transverse direction.

The result is that each fibre bundle and the perimysial network become isolated from one another and independently bear the tension. The fibre bundles and perimysium can then be regarded as elements acting in parallel. In this case, the element with the least extensibility (least breaking strain) is the first to break and the load is then thrown onto the remaining elements. Structures progressively break down and the most extensible component finally remaining determines
Fracture behaviour of a transverse slice of cooked meat when it is pulled apart in a direction perpendicular to the fibre axis. The stippled dark grey areas represent the fibre bundles and the dark lines show the perimysial network. (a) Prior to the application of tension the fibre bundles are joined to the perimysium by tenuous connective tissue threads. (b) After extension, cavities develop due to the partial separation of fibre bundles and perimysium. (c) On further extension, a fracture pathway connects some cavities but is bridged by perimysial strands. (d) Complete fracture.

Figure 14.

the load and extension at failure. Let us consider the extensibility of these two elements. Muscle fibres isolated from cooked muscle can be stretched by greater amounts before they break than can raw fibres, in some cases to more than 100%, although considerable variability is observed (Wang et al., 1956; Hostetler and Cover, 1961; Jeacocke, unpublished experiments). Ageing the meat decreases the extensibility of the cooked fibres. Perimysium isolated from cooked muscle is also very extensible. Breaking extensions are very variable, but in some cases exceed 150% (Lewis and Purslow, unpublished results).

Thus when a cooked muscle strip continues to be stretched longitudinally, after the point where debonding of fibre bundles from the perimysium occurs, both the perimysial network and the fibre bundles will contribute to the load-bearing at substantial extensions. It seems most likely that fibre bundles progressively fail as independent units (Purslow, 1985) (Figure 15c), finally leaving perimysial strands as the last structures to break (Carroll et al., 1978 (Figure 15d). However, it is not known whether peak loads have been passed at the stage when only perimysial strands remain. In other words it is not yet clear what contribution fibre bundles make to the longitudinal breaking strength (maximum stress).

This kind of model can explain in general terms the differences between the longitudinal and lateral breaking strengths of meat and the effect of ageing. In unaged meat the longitudinal breaking strength of cooked meat is more than ten times higher than the lateral breaking strength (Bouton and Harris, 1972; Purslow, 1985). This can be understood if in unaged meat the fibre bundles make a substantial contribution to the longitudinal breaking strength and none to the transverse breaking strength. As meat is aged, the lateral breaking strength is unaltered (Bouton and Harris, 1972; Purslow, unpublished experiments). This
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Figure 15. Fracture behaviour in a strip of cooked, unaged meat when pulled apart in a direction parallel to the fibre axis. The grey areas represent fibre bundles. The heavy lines represent the perimysium and the fine diagonal lines represent fine connective tissue strands between perimysium and endomysium. (a) Prior to the application of tension. (b) After extension, separation of fibre bundles and perimysium occurs. (c) On further extension, fibre bundles break. (d) Complete fracture.

indicates that although the perimysium may be subtly altered during conditioning (Stanton and Light, 1987), its strength after cooking is unchanged. However, the longitudinal breaking strength declines considerably during ageing (Bouton and Harris, 1972; Purslow, unpublished experiments). This can be understood if the relative contribution of the fibre bundles to the longitudinal breaking strength declines with age. This may come about because when the meat is aged, fibre bundles tend to fail at smaller extensions and therefore contribute less to the maximum stress.

It is of considerable interest to know what happens in the myofibrils when a cooked fibre is stretched. In muscle cooked at temperatures above 60°C, the thick filaments fuse to form an apparently amorphous A-band (Schmidt and Parrish, 1971) probably consisting of a myosin gel (Ishitoroshi et al., 1982; Hermansson and Langton, 1988) in which gap filaments are embedded. The thin filaments in the I-band lose their identity on cooking above 60°C (Schmidt and Parrish, 1971) but it is not yet clear whether the actin and regulatory proteins form a gel. When unaged cooked meat is stretched to breakage, Locker and Wild (1982) considered that there was no visible damage to the integrity of the myofibril. At extensions up to 30%, the I-band increases in length, but at extensions up to 60% the A-band also stretches. Unfortunately no information is available for larger extensions. It appears that up to 60% extension at least, the length changes in the muscle strips are accommodated by a uniform, proportional increase in the length of all sarcomeres. However, the point does not seem to have been rigorously tested. Sarcomeres from muscle that had been stretched to breaking point and allowed to recover appear similar to those from rest length muscle (Locker and Wild, 1982) but the available resolution may be inadequate to detect breaks in the structure. On stretching up to 30%, filaments of unknown origin, possibly gap filaments, appear in the I-band. At 50% extension, the stretched A-band develops a speckled appearance due to fragments superimposed on an array of fine filaments, which have been assumed to be gap filaments, although this...
remains to be proved.

When cooked beef muscle that had been aged 7 d at 2°C is stretched to the breaking point, the extension occurs by the stretching of the I-bands alone; the A-bands are unaltered (Locker and Wild, 1982).

At present, it is therefore far from clear what structural events are responsible for the yield point, what structures in the A and I bands are load-bearing and where in the sarcomere failure occurs. It is also unclear when the fibre contents fall, whether structural continuity is provided by the endomysium; Street and Ramsey (1965) show that, at least in red muscle fibres, following rupture of the fibre, the endomysium is capable of maintaining the integrity of the fibre at nominal loads of 3.7 kg/cm², a value close to the breaking strength of cooked meat.

Clearly more information is needed on the relative contributions of fibre bundles and perimysium to the stress-strain curves of muscle aged for different times, stretched or shortened to different extents and cooked under different conditions. We especially need to establish at what point different structural elements fail in a longitudinal tensile test.

The structural approach therefore is valuable in focussing attention on the components that determine the strength of the meat and enables the wealth of biochemical information on the nature of the collagen making up the connective tissue, and in particular its cross-linking, to be integrated into the mechanical picture. It is also useful in providing a framework within which to investigate the effects of other processes which affect toughness, such as cooking, ageing and cold-shortening.

References


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Water-holding, appearance and toughness of meat


Discussion with Reviewers

R. Hamm: The authors explain the high drip loss of PSE pork by denaturation of myofibrillar proteins, particularly by changes in the head porosity of the myosin molecule. On the other hand, Honikel and Kim (1986) concluded from their results that the fast release of drip post mortem from PSE muscle must be mainly due to changes in the muscle cell membranes, and not more than 25% might be due to denaturation of myofibrillar protein. Do you agree that increased permeability of cell membranes contributes considerably to the wateriness of PSE pork?

Authors: No, we are not convinced by their arguments. Honikel and Kim (1986) found that in pig psoas muscles considered to be PSE in that they had a pH < 5.8 at 45 min post mortem, only 25% of the myosin was denatured as judged by loss of ATPase or by differential scanning calorimetry. But their argument, that wateriness cannot be due to myosin denaturation because it is so limited, is logically flawed. PSE is not an all-or-none phenomenon. Drip loss increases progressively with decrease of pH. In studies on pig longissimus dorsi muscles, maximum drip loss occurred at a pH of 6.0 or below (Warriss and Brown, 1987), although the extent of denaturation, and its dependence on pH, would be expected to depend on the chilling rate and may well depend on the muscle, for example due to fibre type differences. Honikel and Kim excised their muscles soon after death and then kept them at 35°C. The conditions experienced by these muscles were therefore less severe than would have been encountered had the muscles remained on the carcass, where in the PSE state the temperature can exceed 40°C at 1 h post mortem. It is not therefore surprising that the degree of myosin denaturation they observed was low. By contrast, Stabursvik et al. (1984) showed that in muscles kept in a PSE carcass, a larger fraction (about 50%) of the myosin was denatured.

Honikel and Kim did not measure the drip from their muscles and were therefore not in a position to prove that their muscles gave the maximum amount of drip that pig psoas muscles are capable of. In other words they did not establish that their muscles were in an extreme state of
PSE, and therefore could not expect the degree of myosin denaturation to be maximal. Even if it were established that, in PSE muscles giving maximum drip, the degree of myosin denaturation was only about 25%, this would not demonstrate that myosin denaturation was unimportant. We do not know the dependence of myofibrillar shrinkage on the degree of myosin denaturation. There is no reason to think that maximum shrinkage requires all the myosin to denature. It could be that when only a fraction of the heads has denatured, their tendency to shorten over-rides the undenatured heads and causes the lattice to shrink maximally. What is urgently needed is to determine the degree of myosin denaturation and myofibrillar shrinkage for a wide range of pH values.

With regard to membrane damage, it is known that if a muscle is cut very soon after death and centrifuged, very little water is lost (Ling and Walton, 1976); in other words its water-holding capacity is high. Thus despite the cell membrane having been severed, water is well retained by the cell contents (by the myofibrils in our view). This shows that breakage of the cell membrane by itself is not the cause of drip. The fact that drip is a solution of sarcoplasmic proteins (Lawrie et al., 1980; Brisset, personal communication), suggests that even in normal rigor the cell membrane becomes leaky. Our own experiments have shown that in PSE muscle the myofibrils have shrunk by an amount that can explain the increased drip and there does not seem to be a need to invoke any other explanation.

G.R. Schmidt: The authors mention that the amount of drip in PSE meat is due to a rapid breakdown of glycogen post mortem, or due to slow chilling of the carcass. Could there be other processes in the muscle which are genetically controlled which could affect the water holding capacity and appearance of the muscle? Do these genetically-controlled factors need to be specifically associated with glycogen breakdown and rate of chilling?

Authors: There are four circumstances in which PSE meat has been reported to form:
1) In animals that are stress-susceptible due to a genetic defect, there is a high rate of post-mortem glycolysis.
2) Even in stress-resistant animals, excessive stress at slaughter can also trigger a high rate of post-mortem glycolysis.
3) Certain pig carcasses have been shown to exhibit a near-normal rate of glycolysis but a larger-than-normal extent of glycolysis, so that the final pH is low (~5.1) (Lawrie et al., 1958; Monin and Sellier, 1985).
4) If the chilling rate is excessively slow, the temperature in the deep muscles of a carcass can remain high for a long time post-mortem (MacDougall, 1982). The formation of PSE meat when the post-mortem temperature is kept high can be demonstrated by holding meat post-mortem at 37°C (Wismeier-Pedersen and Briskey, 1961).

The common feature of these is that the muscles experienced a low pH while the carcass is still warm. As far as we are aware, all cases of PSE can be explained on the simple hypothesis that it is due to the time-dependent denaturation of protein occurring under these conditions.

Of the causes given above, (2) and (4) are due to bad handling of animals or carcass, rather than a genetic defect. The high rate of glycolysis in stress-susceptible pigs is well established as a genetic defect. The cause of the low ultimate pH in (3) is not known and has been rather little studied, but might be the result of another genetic defect, for example causing an increase in the amount of muscle glycogen at slaughter.

While paleness in meat might come about simply by a reduced myoglobin level (which might be genetically controlled), our current understanding is that the combination of pale, soft and exudative characteristics is caused only by thermal denaturation of proteins. It is difficult to envisage any other set of circumstances, genetically controlled or otherwise, that would bring about this thermal denaturation, and which would be the cause of PSE.

However, the severity of the PSE state resulting from a particular temperature and pH might be influenced genetically. For example, the myosin isoforms present in different muscle fibre types might have slightly different susceptibilities to denaturation. Apart from this, we think it unlikely that there are genetic differences in myosin between breeds which affect stability, but this cannot be ruled out.

R. Hamm: The authors propose a new hypothesis in which salt-induced swelling is entropically, rather than electrostatically, driven, and the depolymerisation of the thick filaments is the decisive factor. It was relatively easy to explain the effect of different anions and cations on the swelling of muscle and the water-holding capacity of meat by changes in electrostatic interactions between protein molecules. How can the new hypothesis explain these differences: by different effects of the ions on the depolymerisation of the thick filaments? Is it possible that the association of the tail moieties of myosin is caused by electrostatic forces and that short-distance electrostatic effects of ions result in more or less depolymerisation of the filament depending on the type of ion, rendering possible entropic swelling? In this case, both electrostatic and entropic effects would participate in the swelling.

Authors: Neutral salts have their effect on protein systems by affecting the stability not only of electrostatic bonds but also of hydrogen bonds and hydrophobic bonds (see von Hippel and Schleich, 1969). When the concentration of the salt is raised, hydrophobic groups make increasingly unfavourable interactions with the solution, whereas charged groups or polar groups make increasingly favourable interactions. The net result of increasing the concentration of the salt is first to weaken interactions between
protein molecules (or subunits). This is the salting-in effect and one of its manifestations is to increase the solubility of the protein. For example, raising the NaCl concentration to 0.6 M at pH 5.5 will cause depolymerisation of a myosin filament, and thereby bring myosin into solution. However, above a certain limit, raising the concentration of neutral salt has the opposite effect and interactions between protein molecules (or subunits) are enhanced. This is the salting-out range, where the solubility of the protein decreases to such an extent that it may precipitate. Thus at very high NaCl concentrations (~4 M), myosin filaments are not depolymerised and muscle and myofibrils do not swell (Callow, 1932; Knight and Parsons, 1988) and it is possible to precipitate myosin at such high salt concentrations (Edsall, 1930).

Although this behaviour applies to all cations and anions, they differ markedly in these abilities. Some ions, for example CNS⁻ or I⁻, are very effective at dissociating protein assemblies at low concentrations and will even cause protein subunits to unfold. They increase the solubility of proteins very greatly but maximum solubility requires relatively high ion concentrations and the salting-out effect may not be observed at practically realisable concentrations. Other ions, such as sulphate, are much less effective at salting-in, but achieve their maximum effect at relatively low concentrations so that salting-out can be readily demonstrated. The salting-out effect and one of its manifestations, as salting-in effect and one of its manifestations, as salting-out range, where the solubility of the protein decreases to such an extent that it may precipitate. Thus at very high NaCl concentrations (~4 M), myosin filaments are not depolymerised and muscle and myofibrils do not swell (Callow, 1932; Knight and Parsons, 1988) and it is possible to precipitate myosin at such high salt concentrations (Edsall, 1930).

It is not necessary to suppose that the interactions between the myosin tails in the filament are exclusively electrostatic in character; hydrogen bonds and hydrophobic bonds may also be involved. It is possible that salt depolymerises myosin filaments by increasing the interactions which polar, rather than charged, groups make with the medium. In the sense that electrostatic effects may be responsible for thick filament depolymerisation, we would agree that electrostatic effects may play a role in swelling, but it seems to us likely that the swelling pressure generated by the addition of salts is not electrostatic.

G.R. Schmidt: The authors mention the curing process where swelling and protein solubilisation occur extensively at the sites of needle injection. This phenomenon causes the appearance of periodic stripes in bacon slices and we also observe this in roast beef and hams from time to time. Do the authors have recommendations for processors to prevent the formation of these periodic stripes of variable protein solubilisation in meat products?

Authors: We have investigated the nature of these periodic stripes in bacon slices and shown that near the sites of brine injection there is actually rather little change in structure: the myofibrils are still clearly seen and thick and thin filaments are still apparent (Voyle et al., 1986). It is further from these injection sites where myofibrillar structure is grossly disrupted. We suggested that although the final salt concentration is likely to be uniform throughout the meat, the difference in behaviour might be due to the different time-courses of salt concentration at the two locations. Near the sites of brine injection, the salt concentration will rise very fast to a high concentration and then slowly fall to the final concentration. Further from the injection site, the salt concentration will be zero for some time after injection and then slowly rise to the final concentration as salt diffuses in from the injection sites. Experiments with myofibrils show that prior exposure to high salt concentrations reduces both swelling and extraction at moderate concentrations (Knight and Parsons, 1988), suggesting that salt-induced denaturation may have a role in the formation of the stripes.

Since the periodicity of the stripes is the same as the interval between needles, it would seem reasonable to attempt to combat the problem by using a smaller separation between needles.

R. Hamm: Is it possible to explain the effect of pH, in the absence of salt, on the swelling and water-holding capacity of meat only by the electrostatic hypothesis, or does pH exert an influence on the depolymerisation of the thick filament?

Authors: Although pH undoubtedly has a strong influence on the myosin molecule-myosin filament equilibrium (Josephs and Harrington, 1968), we doubt whether, in the absence of added salt, there is significant depolymerisation of myosin filaments in the pH region of greatest interest, pH 5.5 to 7.0. Rather, we suppose that raising the pH increases the negative charge on the filaments, thereby increasing the electrostatic force between them and expanding the lattice. This is well shown by Matsuda and Podolsky (1986). In their work, raising the pH from 5.5 to 6 expands the lattice considerably, but raising the pH further to 7 had little further effect. This latter result can be explained on the electrostatic mechanism by charge saturation (Offer and Knight, 1988), but would be hard to explain by depolymerisation.

We consider that the filament lattice spacing of myofibrils, and therefore the water-holding capacity of meat, depends on a number of factors: pH and ionic strength affect
the electrostatic repulsive force between filaments; the structural integrity of the myofibrillar components affects the mechanical constraints on swelling; the presence of osmotic agents such as sarcoplasmic proteins, partially or completely excluded from the lattice, lowers the chemical potential of the water outside the myofibrils relative to that inside and thereby causes shrinkage. In addition, entropic mechanisms, such as we have outlined, may come into play under conditions where the degree of molecular freedom would increase if there were an expansion of the filament lattice. At present, we can envisage such a mechanism operating only when the myosin filaments depolymerise and we think this would occur only in the presence of salt and polyphosphate, or with acid marinades (Offer and Knight, 1988a).

G.R. Trout: In the section on water-holding, no mention is made of the effect of heating and the interaction between heating, pH, salt type and concentration on the microstructural changes and their relationship to water-holding. Would you comment on the importance of these factors in light of the fact that the major objective of studying the effects of salts, such as sodium chloride and sodium tripolyphosphate, on water-holding is to determine how they affect the water-holding ability of the cooked meat product? Authors: We quite agree that it is very important to understand how NaCl and polyphosphates affect the water-holding ability of cooked meat products. But meat is treated with these salts in the raw state and we do need to know what is happening in this system first before tackling the experimentally harder problem of the structural changes occurring when salt-treated meat is cooked.

We think it likely that polyphosphates in the presence of NaCl decrease cooking loss by their action in solubilising myosin (Offer and Knight, 1988a). When myosin molecules are heated, they form a gel with good water retention (Tsai et al., 1972). The solubilised myosin is presumably capable of permeating the entire muscle, that is to invade the I-bands of the muscle fibres from which it was previously absent and also to invade the extracellular space. If this is correct, when the salt-treated meat is cooked, a myosin gel will be formed throughout the meat and is likely to play a major role in water retention (Bendall, 1954; Sherman, 1961; Hellendoorn, 1962; Kotter and Fischer, 1975; Offer and Knight, 1988a). We can explain the effect of polyphosphate in the presence of NaCl on cooking loss, since polyphosphates cause dissociation of actomyosin and more myosin will be solubilised. Greater solubilisation will also occur at higher pH.

G.R. Schmidt: The authors discuss extensively a previous paper by Offer and Trinick (1983) in which myofibrils are irrigated with an extraction medium. Generally, this research differs from applied meat processing in two ways. There is no mechanical action applied to facilitate the disruption of the myofibrils and the pH utilized is 5.5, whereas in meat processing the pH is normally 6.0. Would altering the mechanical action and the pH affect the interaction between the proteins and the irrigation medium? Authors: In those experiments we were not trying to duplicate precisely the conditions occurring in meat processing, but rather by simplifying the system to gain insight into the mechanisms involved in water uptake and myosin extraction. We chose a pH of 5.5 to mimic the conditions in fresh meat. We wanted to explore the specific effects of NaCl and polyphosphates without complicating matters by altering the pH as well. However, Dr Schmidt is right to emphasise the small rise in pH that occurs when alkaline polyphosphates are used and it would be desirable to determine more precisely what effect this might have.

A rise in pH would be expected to lower the NaCl concentration required to depolymerise the thick filament (Josephs and Harrington, 1968), and hence assist both swelling and myosin extraction.

With regard to mechanical action, it is at present far from clear precisely how much disruption of muscle structure occurs in different products. At one end of the spectrum, in a traditional ham, gross disruption of the muscle fibre probably occurs only at the surface of the meat and, in a British-style sausage, muscle fibres probably also survive intact. In a frankfurter, it seems that even the myofilament is grossly disrupted, although to what level is unknown. However, regardless of the size of the meat piece or fragment of myofibril, the underlying molecular interactions between the ions of the medium and the myofibrillar proteins are presumably the same as with isolated proteins.

G.R. Schmidt: The authors discuss the ageing of the endomysium and indicate that aged muscles empty the contents of their muscle fibres when exposed to the proper ionic environment more readily than fresh muscle. If this is so, how does this phenomenon interact with the property of myosin being more extractable pre-rigor when obviously, the endomysium is still in a very strong state? Authors: The work we have discussed was performed on rigor muscle. The pre-rigor state has quite different properties. We have found (Elsey and Knight, unpublished experiment) that it is quite easy to draw muscle fibres, free of endomysium, from a transversely cut surface of pre-rigor muscle (Schoenberg and Eisenberg, 1985), but this is difficult from rigor muscle. Perhaps, therefore, a high incidence of stripping accompanies comminution pre-rigor, but this needs to be tested. We suppose that removal of the endomysium from around a muscle fibre would promote extraction of solubilised myosin, in both pre-rigor and rigor muscle, by removing an impediment to outward diffusion.

The ATP present only in pre-rigor meat produces a high degree of dissociation of myosin from actin, permitting a quicker diffusion of myosin molecules once added. NaCl has
depolymerised the thick filaments. This factor will influence the outward movement of myosin even in fibres still enclosed in endomysium.

G.R. Schmidt: The authors discuss the changes that take place in muscle when myofibrils in a cooked fibre are stretched. The authors do mention gap filaments. What do the authors speculate is the contribution of titin to the longitudinal strength of cooked meat?

Authors: We discussed this important question recently (Offer et al., 1988). Titin is a very large protein (molecular weight 3 million) and titin molecules are very thin filaments about 4 nm wide and up to 1 μm long (see reviews by Squire et al. (1987) and Offer (1987)). Recent location studies using monoclonal antibodies to titin have shown that a single titin molecule spans all the way from the Z-disc to the M-line (Whiting et al., 1989). There is evidence that titin is the protein component of the gap filaments, the structures spanning the gap between A and I-bands in highly stretched muscle.

When muscle is cooked, the thick filaments fuse together and the thin filaments disintegrate, although the A and I bands persist (Schmidt and Parrish, 1971). Recent studies by Coumac et al. (1987) have shown that, unlike the thick and thin filaments, gap filaments survive cooking and have argued that gap filaments alone provide the tension-bearing elements in the cooked myofibril.

It has been suggested that since titin is rapidly degraded by proteolysis on ageing, it could not support load (King and Kurth, 1980), but there is no reason why a break in the primary structure should necessarily cause the titin molecules to lose their secondary or tertiary structural integrity. Myosin filaments form a strong gel on heating, so that, in the A-band region, load is probably borne both by the myosin gel and by titin filaments. However, the structure formed from the thin filaments on cooking is not known, and it is possible that, at the high concentrations present in muscle, the thin filament components also form a gel. Thus we do not know if or not titin is the only component in the I-band that supports tension and thus whether titin alone determines the breaking strength of the cooked myofibril.

G.R. Schmidt: The authors comment that there is a variability of response of myofibrils to salt. They attribute this difference to muscle fibre types. Is it possible that the protein titin could be involved in this phenomenon?

Authors: Paterson et al. (1988) have found that titin is extracted from myofibrils under conditions of salt concentration that extract myosin and cause swelling. They have proposed that loss of titin may be a cause of swelling, so it is conceivable that variable loss of titin could underlie variable swelling. Variable loss of titin might arise either from variable conditioning or to differences in fibre type. It remains to be shown whether titin and/or nebulin do indeed constitute constraints on swelling of the myofibril, and whether extraction of titin correlates with the amount of swelling.

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FOOD MICROSTRUCTURE
INSTRUCTIONS TO AUTHORS

Papers for publication in the international journal Food Microstructure are invited. Papers can cover all types of foods, including vegetables, grains, seafoods, meats, dairy products and others. Topics of interest are: fundamental aspects of food microstructure such as the molecular and colloidal forces which determine it, and the practical relationship between food microstructure and processing, ingredient changes, shelf life, consumer acceptability, and other food-related areas. Techniques used may include transmission and scanning electron microscopy, light microscopy, x-ray microanalysis, or other related microscopy/microanalytical methods.

Papers for Food Microstructure (FM) may be offered at any time. Papers can be for publication only, or intended for oral presentation at the Annual Food Microstructure meeting in early spring. The latter papers are due two months prior to the start of the meeting; only papers acceptable for publication are allowed oral presentation. Oral presentation of a paper at some other meeting or publication as reviewed abstract (e.g., in proceedings, etc.) does not preclude consideration of a paper by FM.

The letter accompanying the paper should contain names and complete addresses of at least four persons competent to review the paper. Suggested reviewers: a. must neither be from author's current or recent affiliations, nor coworkers; b. should preferably be active researchers in the field (e.g., whose work is being extensively referred to); and c. need not be personally known or contacted by the authors. The editors will select the most suitable reviewers consistent with these criteria. Each paper will be intensely reviewed by at least three reviewers.

The initial paper (hereafter referred to as "paper") should conform to these Instructions. However, to be published after reviewing, the final manuscript (hereafter referred to as "manuscript") should be a: submitted on the model sheets conforming to the Manuscript Preparation Guidelines (mailed along with the reviewers' comments), or b. sent to SEM Inc. for preparation at a nominal cost (per details mailed with the paper).

The following types of contributions can be offered. A length limit is not imposed on papers. Short, but complete, papers are welcome.

RESEARCH PAPER: Presents new unpublished findings.

MANUSCRIPT PREPARATION:

REVIEW PAPER: Includes an extended literature review and complete bibliography, emphasizes author's new unpublished findings and in an extended discussion puts the topic in proper perspective.

TUTORIAL PAPER: Contains an organized comprehensive review of all relevant published material as for a teaching lecture.

TECHNICAL TIP: Paper should have no more than 1000 words.

LETTER TO THE EDITOR: Commenting on paper already published in FM.

The author should indicate the type of paper and carefully adhere to the applicable definition, since the reviewers and editors judge the paper accordingly.

INSTRUCTIONS FOR SUBMISSION OF PAPERS

Type paper in double-spaced format on standard size paper.

The paper should include title page, abstract, all headings and text. On the title page include: a. a short title which accurately represents the contents of the paper; b. an informative running head consisting of no more than 50 characters; c. names and affiliations of all authors, name and complete work and home addresses and phone numbers of the person to contact; d. 10 key words/phrases suitable for subject index; and e. for review papers, indicate page numbers containing new material (e.g., "new material will be found on pages ").

An Abstract (of 100-250 words) is required for all papers. The Abstract should be concise and include the purpose of the paper, major results obtained and conclusions. Phrases such as "will be described," "is presented," "are presented," etc. should be avoided.

The Introduction of the paper must contain a clear, concise statement of the purpose of the paper and the relationship of this paper to what is already in the literature. As applicable, a Materials and Methods section with complete specimen preparation information must be included (even if already published elsewhere), so that the work can be duplicated by others.

Equations should be numbered consecutively, using arabic numerals. Each symbol and abbreviation should be defined when first used. SI units must be used; other metric units or U.S. customary units (English), if used, must be given in parentheses.

REFERENCES

Include all references relevant to paper which are either readily available published works or papers in press. Work in progress, manuscripts submitted or in preparation, unpublished findings, personal communications etc. must be excluded from the reference list but may be acknowledged in the text (in parentheses).

The reference list at the end of the paper must be organized in alphabetical order by the first authors' names. Names of all authors (last names and initials only, with a comma between names and no other punctuation), full titles of papers, appropriate bibliographic information (with standard abbreviations for journals, and editors and publishers for books and proceedings), and inclusive pagination must be included. Availability information must be included for all non-journal references.


In the text, cite references in one of the following two styles: a. Cowley (1967) or (Cowley, 1967) or Cowley and Wall (1970). If there are three or more authors, use the form Venables et al. (1978). If more than one paper is published in the same year by the same author (or group of authors) use the year of publication first, followed by the initials of the authors. As long as there is consistency, either superscript or full-size numerals in brackets [I] can be used. In this case, the numbering must be in sequence in the reference list, but the references will generally not appear in sequence in the text.

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Cover Photo: Current health concerns add to the importance of localizing cholesterol in foods. This figure, from the paper "Electron Microscopic Localization of Cholesterol in Bovine Milk Fat Globules" by R.W. Martin, Jr. (pages 3-9), is a freeze-fracture transmission electron micrograph of a milk fat globule treated with cholesterol specific binding agent filipin showing the cholesterol distribution in the outer membrane. The 25 nm indentations are the regions where cholesterol is localized.

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