FOOD MICROSTRUCTURE

An International Journal on the Microstructure and Microanalysis of Foods, Feeds and their Ingredients

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CONFOCAL SCANNING LASER MICROSCOPY IN FOOD RESEARCH:
SOME OBSERVATIONS

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Abstract

Confocal Scanning Laser Microscopy (CSLM) has advantages over conventional light microscopy and electron microscopy. In particular the possibility to perform optical sectioning, allowing the disturbance free observation of the three-dimensional internal structure, offers new possibilities in microstructural studies of food systems. The technique is further considered to be very valuable in the study of dynamic processes and transport phenomena. Preliminary results are discussed of an investigation into the microstructure of fat spreads, mayonnaise, cheese and rising dough using this CSLM technique.

Introduction

Light and electron microscopy play an important role in the observation of the microstructure of food systems. Electron microscopy (EM) has the advantage of a high resolution (~1 nm), but it is generally very laborious and requires elaborate sample preparation which may lead to artifacts. In addition, samples are observed under high vacuum and at high radiation doses. Classical light microscopy (LM) is easy to apply but its resolution is rather limited and undisturbed observation of samples is difficult, especially when deeper layers must be probed.

In this context, and in view of the desirability to perform measurements under dynamic conditions, alternative techniques such as acoustic microscopy, X-ray microscopy and confocal scanning laser microscopy (CSLM) may be considered.

The CSLM technique has already been discussed extensively (1, 2, 6, 8, 9). The basic idea of the confocal principle is that a point in the object is optimally illuminated and also optimally imaged in a detector pinhole, which leads to an increased resolution and a reduced depth of field because off-focus levels in the specimen will hardly contribute to the image. The latter property enables an excellent optical sectioning (i.e., the in-depth imaging of the structure) and a disturbance-free three-dimensional observation of a non-deformed sample, which cannot be achieved with classical LM. In addition, computer control and data acquisition create excellent possibilities for image analysis and processing.

Preferably, specimens are studied by fluorescent light because the different structure elements can best be distinguished by using specific fluorochromes. Also in this case, the point illumination and pinhole detection system is a great advantage of CSLM over conventional LM because it effectively suppresses contributions from off-focus levels of the object.

The aim of the present contribution is to present some of our first results on the observation of food systems by CSLM.

Experimental

General

Samples were observed by the CSLM developed by the Department of Electron Microscopy and Molecular Cytology, University of Amsterdam. Observations were made with oil-immersion optics (N.A. = 1.3) in the reflection / fluorescence mode (1, 2, 8), allowing visualization of structures to a depth of

Key words: Light microscopy, confocal microscopy, laser, three-dimensional imaging, food microstructure, margarine, butter, mayonnaise, cheese, dough, dynamic processes.
more than 100 μm under the surface of thick, non-deformed specimens.

Fat spreads

The water distribution in spreads containing 80% fat (commercial margarine and butter) was investigated. Samples of the product were taken by means of a sampling tube (Fig. 1) fitted with a sharp edge to prevent working of the sample as much as possible. When the tube was full, a stopper was inserted on the other end and the sample pushed out. The material protruding from the tube was cut into slices 2 mm thick by means of a thin platinum thread (0.1 mm).

Two or three of such slices (diameter = 3 mm) were placed on a cover glass, which was introduced in to a container containing a fluorescent dye. For a proper observation of the water globules in a fat spread, a fluoroscer has to be added either to the oil or the water phase. Nile Blue is a suitable dye for the localization of lipids by fluorescence light microscopy (3, 10). The samples of fat spreads were immersed into an aqueous solution of Nile Blue (0.001 % w/v) for at least 16 hours. This solution contains a number of lipophilic components, which diffuse into the liquid lipid phase, thus generating a deep yellow fluorescence. The lipid phase becomes fluorescent without altering the structure of the emulsion.

Mayonnaise

In order to maintain the structural integrity of the original mayonnaise as much as possible, nine parts of a mayonnaise sample were carefully mixed with one part of a Nile Blue solution (0.1 %). In contrast to fat spread samples, the components in the continuous water phase as well as in the interface become fluorescent (or at least more fluorescent than the discontinuous dispersed fat phase). In this case, the fluorescence of the hydrophilic components of the Nile Blue dominated that of the lipophilic components, which might be attributed to hindered transport. After staining, the mayonnaise sample was placed between two glass slides.

Cheese

A small piece of young Gouda cheese was placed on a glass slide and introduced in a 0.01 % Nile Blue solution to stain the fat phase or, alternatively, in 0.01 % solution of 1-anilino-8-naphthalene sulfonic acid (ANS) to stain the protein.

Rising Dough

To establish the capabilities of confocal instrumentation for analyzing dynamic processes in situ, the structural changes in rising dough were studied. To this end, 10 g of wheat flour was mixed with 5 ml of an 0.05 % fluorescein isothiocyanate solution in water and 0.5 g of yeast. After kneading for 10 min and a first rise for 30 min, a small piece of dough (thickness 2 mm) was placed between two glass slides and observed at room temperature (20°C) as a function of time.

Results and Discussion

Figs. 2 and 3 show the water distribution in margarine and butter samples, respectively. The water droplets appear as dark non-fluorescent structures embedded in a fluorescent fat phase. Because of the high resolution and high dynamic range, structures as small as 0.3 μm can be discerned without difficulty. This cannot be achieved by normal light microscopy, although special imaging modes may come very close (7). The main advantage of CSLM is that pictures can be taken at different depths without deformation of the samples. This possibility to make optical sections along the optical axis is demonstrated in Fig. 4 for a mayonnaise sample.

Mayonnaise is an oil-in-water emulsion containing a high percentage of oil (80 % or more). This high volume of oil causes the formation of a honeycomb structure of closely packed and often distorted oil droplets (the closest packing of equal spheres would lead to a volume fraction of 0.72). The interface layers between the droplets are imaged with a dimension of 0.2 μm, which is very close to the theoretical resolution limit of the instrument.

The depth resolution of the optical sectioning is about 0.7 μm (2), so that elements which are that distance apart, are imaged completely independently of each other and do not interfere with adjacent image planes. Consequently, large oil droplets (size ~ 10 μm) are detected on different image planes, whereas small droplets (~ 1 μm) appear or disappear from the image (Fig. 4).

Figs. 2 and 3. Water distribution in margarine (Fig. 2) and butter (Fig. 3). Fat phase is fluorescent.

Fig. 4. Mayonnaise obtained at (a) 2, (b) 4, (c) 6, and (d) 8 μm depth below the surface of the sample.
Depending on the optical density of the sample, optical sectioning can be applied up to depths of about 100 μm from the surface (2). A set of images thus formed at specific depths in the specimen can be used to obtain a stereopair of images. A three-dimensional observation of the microstructure requires the storage of data from such a set of images and the generation of stereoscopic images by a specific image processing algorithm (2, 9). Examples of such stereoscopic images are shown in Fig. 5 and 6. Microstructural studies of cheese by LM and EM have been the subject of numerous papers (11). The structural matrix in cheese is protein in which the fat is embedded as a dispersed phase (4, 5, 11). The three-dimensional structures observed by us both with protein staining (Fig. 5) and fat staining (Fig. 6), perfectly agrees with these earlier observations.

The spatial network of protein strands as well as the presence of dispersed fat globules and agglomerates are clearly observable and give an impressive image of the internal structure of the cheese. As already mentioned, this image is obtained without disruption of structure and laborious sample preparation. Moreover, the whole operation takes only a few minutes. It is these performance aspects which make the confocal equipment well-suited to perform in situ observations under dynamic conditions: structure formation in different types of gels, formation of emulsions, coalescence phenomena, and effects of deformation. An example is the study on the kinetics of rising dough, which are related to dough rheology and baking performance.

When mixing flour and water, starch and proteins are hydrated and form a visco-elastic matrix. This matrix can occlude air bubbles. In the rising stage, carbon dioxide produced by yeast, diffuses to the air cells occluded during mixing, thus causing expansion of the dough. The four micrographs (Fig. 7a–7d), obtained 1, 2, 4, and 6 minutes after the first dough rise, show the results of monitoring this process by CSLM about 20 μm under the surface of the sample. The protein mass and the starch granules generate strong and faint fluorescence respectively. Gas cells are discernable as dark structures in the protein matrix. Expansion and coalescence of gas cells responsible for dough rise, can also be clearly observed. It is possible to do these time-resolved observations on the internal dough structure without any perceptible photo-bleaching of fluorescer taking place.

Fig. 5. Gouda cheese. Protein is stained. Stereo-micrographs.

Fig. 6. Gouda cheese. Fat is stained. Stereo-micrographs.
Concluding Remarks

Confocal Scanning Laser Microscopy appears to be a very useful tool in the study of food microstructure. An improved resolution and a high dynamic range are no doubt advantages over conventional light microscopy. In particular, the possibility of optical sectioning, which allows a disturbance-free observation of the three-dimensional internal structure, offers new possibilities to do microstructural studies of food systems. In addition, CSLM enables the monitoring of dynamic processes as a function of variables such as temperature, pH, concentration, pressure, etc. In this context, the possibility to study transport phenomena by fluorochromes or gold-labeled compounds - should also be mentioned. These options may mark a new era in the study of food products.

References

Discussion with Reviewers

S.H. Cohen: What are the advantages and disadvantages of stage scanning as compared to laser scanning?
Authors: Advantages and disadvantages of on-axis stage scanning versus off-axis laser scanning have been discussed by Brakenhoff (2) and Petran et al., (6). An important aspect in favor of on-axis stage scanning is the somewhat higher attainable resolution. On the other hand, a scanning stage can be a nuisance when manipulation of specimen is required.

S.H. Cohen: What is the nature of the staining agent Nile Blue?
Authors: Nile Blue is a commercial preparation, containing protonated oxazine base, oxazine and free oxazine base.

D.P. Dylewski: I assume that all samples examined in this paper were studied at room temperature. Is it technically feasible to apply a hot or cold stage to the CSLM to enhance its versatility in studying dynamic processes?
Authors: Just as in normal light microscopy it is possible to use hot or cold stages. However, in case of stage scanning, dynamic experiments or micro-manipulation can be troublesome.

J.D. Fairing: In Fig. 2 many of the water droplets appear elliptical rather than circular. Is this an optical effect or is it due to the working of the sample?
Authors: We are not quite sure about the cause of this distortion. Most likely it is due to the working of the sample. On the other hand, it cannot be excluded that the scan speeds in both directions are not completely matched.

J.D. Fairing: What is the cause of the difference in appearance of the water-fat interface in Fig. 3 as compared with Fig. 2?
Authors: We cannot offer an explanation for this difference.

J.D. Fairing: In Figs. 5 and 6, what is the equivalent parallax angle of the stereopair?
Authors: The angle is ten degrees.

J.D. Fairing: In Fig. 7, why are the individual pixels so clearly visible when they are not seen in the other micrographs? What is the difference in the instrumental and recording conditions that produces this undesirable effect?
Authors: This effect is caused by the image format of 256-256 pixels, used in producing these pictures. These conditions were applied because rapid recording, of the dynamic behavior of dough rise, was required. Normally, a format of 512-512 pixels, and for high resolution images 1024-1024 pixels, is used.

J.D. Fairing: What is the wavelength of the exciting radiation in your experiments?
Authors: The wavelength of the exciting radiation is 512 nm.
AN ELECTRON SPIN RESONANCE STUDY OF STEARIC ACID INTERACTIONS
IN MODEL WHEAT STARCH AND GLUTEN SYSTEMS

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Abstract

Electron spin resonance (ESR) was used to examine interactions of 16-Doxyl stearic acid in wheat starch-water (starch:water = 1:1), vital wheat gluten-water and gluten-starch-water model systems. Immobilization of the 16-Doxyl stearic acid, shown by broad line ESR powder patterns, occurred in wheat starch model systems. In contrast to the starch systems, 16-Doxyl stearic acid in gluten-water systems did not display broad line powder patterns. Broadened 3-line ESR spectra were recorded for the gluten-water-16-Doxyl stearic acid. This result was probably due to spin probe which was bound, and spin probe which was in different water microenvironments of the gluten proteins. Stearic acid interactions in the starch-water or gluten-water systems were not changed after heating from 45-95°C.

Stearic acid interacted with both the starch and gluten components in gluten-starch-water-16-Doxyl stearic acid systems. Stearic acid binding with starch in the gluten-starch-water-16-Doxyl stearic acid was dependent upon the amount of gluten in the system. As increased levels of gluten were incorporated in the system, decreased binding of stearic acid occurred. Mobility of the 16-Doxyl stearic acid was increased after the gluten-starch-water-16-Doxyl stearic acid systems were heated from 45-95°C and cooled to room temperature. The stearic acid interactions in a gluten-starch-water-16-Doxyl stearic acid system were the same whether the ratio of gluten-starch:water-16-Doxyl stearic acid was 1:1 or 1:2.

Introduction

Electron spin resonance (ESR) spin probe techniques have been used to examine interactions of stearic acid with the starch component of wheat flour (Pearce et al., 1985, 1987; Nolan et al., 1986). In these studies a stable free radical containing stearic acid as a functional group was used as a spin probe. The spin probes were added to starch systems and used to monitor interactions between the stearic acid and starch. Strong binding of the stearic acid spin probes with starch in aqueous systems occurred at room temperature and after heating.

ESR techniques have also been used to examine the interactions of native flour lipids with gluten (Nishiyama et al., 1981; Nishiyama and Kunii, 1985). By incorporating stearic acid derivatives in gluten, defatted gluten, and flour lipids, Nishiyama et al. (1981) concluded that strong interactions were formed between native flour lipids and protein in hydrated gluten.

In the present study, 16-Doxyl stearic acid was used as a spin probe to examine stearic acid interactions in a hydrated vital wheat gluten system at room temperature and after heating from 45-95°C. Starch-water-16-Doxyl stearic acid studies were expanded from previous studies (Pearce et al., 1985, 1987) to include starch:water ratios equal to 1:1. Stearic acid interactions were then examined in gluten-starch-water-16-Doxyl-stearic acid systems at room temperature and after heating from 45-95°C.

Materials and Methods

Gluten, starch and solvent

Wheat starch (Aytex P, General Mills; 11.20% moisture, 0.25% protein, 0.01% fat, 0.01% ash) and vital wheat gluten provided by Continental Baking, Ralston Purina (7.30% moisture, 60.20% protein, 4.00% fat, 0.70% ash) were used. Glass distilled water was used as the solvent.

Spin probe

The structure of the spin probe used, 16-Doxyl stearic acid (2-(14-carboxytetradecyl)2-ethyl-4,4-dimethyl-3-oxazolidinylx) (Aldrich Chemical) is shown in Fig. 1. The probe was used as received and prepared for experimental studies by slurrying in water at room temperature for 24 h prior to use (water:probe weight ratio = 1:0.001). The slurried sample was a dispersion
of spin probe in water, as the probe solubility was much lower than 0.1 wt %.

Starch-water-16-Doxyl stearic acid sample preparation

Starch was added to the prepared water-spin probe mixture (water:spin probe weight ratio = 1000:1) and slurred with stirring for 24 h at room temperature (starch:water-spin probe weight ratio = 1:1). Samples were transferred immediately while slurring into 2 mm glass tubes. The 2 mm glass tubes were put in 5 mm NMR tubes for ESR analysis.

Gluten-water-16-Doxyl stearic acid sample preparation

Gluten powder was prepared for spin probe studies by mixing gluten and the slurred 16-Doxyl stearic acid-water mixture (gluten:water-spin probe weight ratio = 1:1). Samples were manually mixed with glass stirring rods until all of the gluten powder was wetted and formed a cohesive mass. Thin strands of the gluten sample were wound around the outside of an empty 2 mm glass tube. The 2 mm glass tube was placed in a 5 mm NMR tube, and the NMR tube was sealed.

Gluten-starch-water-16-Doxyl stearic acid sample preparation

All gluten-starch-water-16-Doxyl stearic acid samples were prepared by first mixing together starch and gluten powder. The prepared water-16-Doxyl stearic acid (weight ratio = 1000:1) was added to the gluten-starch mixture and slowly stirred for 24 h at room temperature. Samples were transferred into 2 mm glass tubes for ESR analysis.

Three gluten-starch combinations were examined resulting in weight ratios of gluten powder to starch equal to 0.075:1, 0.15:1, and 0.30:1. Water-16-Doxyl stearic acid was added so that each gluten-starch combination was examined at gluten-starch:water-16-Doxyl stearic acid weight ratios equal to 1:1 and 1:2.

ESR

All samples were scanned in the ESR spectrometer immediately after preparation at room temperature. The specified temperature for 4 min followed by quenching to room temperature. Temperatures from 45-95°C, at 5°C intervals, were utilized, using a fresh sample at each temperature. Spectra were collected on a Varian E-3 spectrometer at about 9.33 GHz. No attempt was made to exclude oxygen from the samples. The spectra were recorded in the vicinity of 3.2 KG with microwave power low enough to avoid any saturation. Correlation times \( \tau \) were calculated for three line spectra (Fig. 2) based on the Kivelson theory (Kivelson, 1960) using peak to peak line height \( h(0) \) ratios of first derivative spectra and the line width of the central line \( T_2(0) \) \(^{-1} \) (Stone et al., 1965).

\[
\tau = 4 \left[ \frac{h(0)}{h(1)} \right]^4 + b^4 \left[ \frac{h(0)}{h(-1)} \right]^4 - 2b^2 T_2(0) \]  

(1)

where \( b=M_h \) are the line heights corresponding to the nitrogen nuclear spin state \( M_h = +1, 0, -1 \) and \( b \) is a constant determined from the components of the electron nuclear hyperfine tensor, given by

\[
b = \left( \frac{4 \pi}{3} \right) \left[ A_{xx} - \frac{1}{2} (A_{xx} + A_{yy}) \right]
\]

All correlation times were calculated assuming isotropic motion of the nitroxide. Correlation times were calculated only when a simple 3-line spectrum was observed.

Results and Discussion

Starch-water-16-Doxyl stearic acid

An ESR spectrum for a saturated solution of water-16-Doxyl stearic acid, free of non-dissolved spin probe is shown in Fig. 3a. The free spin probe in water had a sharp 3-line spectrum with a rotational correlation time \( \tau \) equal to \( 1 \times 10^{-9} \) sec. Stearic acid-starch interactions were examined earlier for systems having a starch:water-16-Doxyl stearic acid weight ratio equal to 1:2 (Pearce et al., 1985, 1987). Because gluten systems were examined at gluten:water-16-Doxyl stearic acid weight ratios equal to 1:1, a starch system with a stearic acid-starch interaction was studied for baseline data. Stearic acid spin probes were highly immobilized in wheat starch-water-16-Doxyl stearic acid systems (starch:water-16-Doxyl-stearic acid = 1:1) at room temperature as shown by the broad line powder pattern recorded in Fig. 3b. The stearic acid spin probe remained highly immobilized after heating at 95°C and cooling (Fig. 3c) with only small changes in the fast motion component of the low field line. ESR spectra for samples that had undergone heating at 45-90°C were similar to Figs. 3b and 3c. The spectra for the 1:1 starch-water-16-Doxyl stearic acid systems were similar to those of earlier studies (Pearce et al., 1985, 1987) for starch-water-16-Doxyl stearic acid systems having a starch:water-16-Doxyl stearic acid weight ratio equal to 1:2. Thus, the general pattern of immobilization was not altered by a change in the water or spin probe concentrations used in these systems.

Gluten-water-16-Doxyl stearic acid

ESR spectra for gluten-water-16-Doxyl stearic acid at room temperature and for samples that had undergone heating at 95°C are shown in Fig. 4. Spectra for these samples were broadened 3-line spectra with apparent rotational correlation times of \( \tau = 2 \times 10^{-9} \) sec (Fig. 4a) and \( \tau = 1 \times 10^{-9} \) sec (Fig. 4b). The build-up of intensity to the left of the low field maximum is indicative of a distribution of motional times and may even indicate some fall off. The left of the low field maximum is indicative of a distribution of motional times and may even indicate a contribution from a small amount of completely immobilized probe. Composite spectra such as those in Fig. 4 possibly could result from a combination of 16-Doxyl stearic acid which is bound, and that which is in different water microenvironments of the gluten proteins. However, it is evident based on the absence of a low and high field extrema (see for example Fig. 3b) that a majority of the spin probe stearic acid molecules are not totally immobilized. The spectrum recorded for the gluten-water-16-Doxyl stearic acid system after heating at 95°C (Fig. 4)
ESR Study of Stearic Acid Interactions

Fig. 1. Structure of 16-DoxyI stearic acid.

Fig. 2. Typical 3-line ESR spectrum; h is peak to peak line height, h(0) is the central line, h(1) is the low field line and h(-1) is the high field line.

Fig. 3. ESR spectra for: (a) water-16-DoxyI stearic acid; (b) wheat starch-water-16-DoxyI stearic acid at room temperature; (c) wheat starch-water-16-DoxyI stearic acid after heating at 95°C and cooling to room temperature. The arrow suggests a minor fast motion component in the low field line.

Fig. 4. ESR spectra for gluten-water-16-DoxyI stearic acid: (a) at room temperature; (b) after heating at 95°C and cooling to room temperature.

4b) was not different from the room temperature spectrum as shown by their similar line shape and rotational correlation times. Although the ESR spectra did not change after heating at any temperature from 45-95°C, it is possible that small changes did occur in the gluten system that were not differentiated in the composite spectra.

Gluten-starch-water-16-DoxyI stearic acid ESR spectra for the gluten-starch-water-16-DoxyI stearic acid having a gluten:starch weight ratio equal to 0.15:1 and a gluten-starch:water-16-DoxyI stearic acid ratio equal to 1:1 are shown in Fig. 5. The spectra in Fig. 5 show the stearic acid interacted with both the starch and gluten in a combined system. The spectrum for the sample at room temperature (Fig. 5a) is a composite spectrum having components of the broad line powder pattern of starch-water-16-DoxyI stearic acid (Fig. 3) and the broadened 3-line spectrum of gluten-water-16-DoxyI stearic acid (Fig. 4). The low field line for the gluten-starch-water-16-DoxyI stearic acid (Fig. 5a) consists of an immobilized, slow motion component (A) and a small, faster motion component (B). The high field line is split into three components: an immobilized, slow motion component (F), and two faster motion components (D) and (E). Composite spectra similar to Fig. 5a having immobilized, partially immobilized and

Fig. 5. ESR spectra recorded at room temperature for samples with gluten:starch weight ratios equal to 0.15:1 and gluten-starch:water-16-DoxyI stearic acid weight ratios equal to 1:1 (a) prior to heating; (b) after heating at 60°C; (c) after heating at 95°C. A and F indicate the strongly immobilized component; B, D, and E the faster motion, unbound components; C the central line.
free, unbound components have been reported in biomedical studies (Piette and Hsia, 1979). After heating at 60 and 95°C (Figs. 5b and 5c, respectively) the B component of the spectrum becomes more pronounced indicating that there was decreased binding of 16-Doxyl stearic acid. Amplitudes of the faster motion components increased as the heating temperatures increased from 45-95°C.

The partitioning and interactions of 16-Doxyl stearic acid in gluten-starch-water-16-Doxyl stearic acid systems were affected by the amount of gluten in the system. ESR spectra for systems with a gluten-starch:water-16-Doxyl stearic acid weight ratio equal to 1:1 but containing either one half or twice the amount of gluten from that of the system having a gluten:starch weight ratio equal to 0.30:1 (Figs. 6a and 6b) were shown in Fig. 6. When the weight ratio of gluten:starch in the system was 0.075:1, the ESR spectrum (Fig. 6a) was similar to the broad line powder pattern of the starch-water-16-Doxyl stearic acid systems shown in Figs. 6b and 6c. ESR spectra for systems having a gluten:starch weight ratio equal to 0.30:1 (Fig. 6b) were composite spectra with lines from bound stearic acid (A and F) and free, unbound stearic acid (B and D). The proportion of unbound 16-Doxyl stearic acid in the 0.30:1 = gluten:starch sample (Fig. 6b) was, however, increased from that of the 0.15:1 = gluten:starch sample (Fig. 5a). The increase in the faster motion components of the 0.30:1 = gluten:starch sample can be shown by the increased ratio of the low field fast motion line height (B) to the center line height (C) from that of the 0.15:1 = gluten:starch sample. The ratio of the low field fast motion line height to the center line increased from 0.32 for the 0.15:1 = gluten:starch sample at room temperature (Fig. 5a) to 0.41 for the 0.30:1 = gluten:starch sample (Fig. 6b). The decrease binding of 16-Doxyl stearic acid in the 0.30:1 = gluten:starch sample was also reflected by the increased amplitude of the high field fast motion line (D) and diminution of the line from immobilized 16-Doxyl stearic acid (F). Thus, although the starch component of the system has the capability of binding almost all of the 16-Doxyl stearic acid in a starch-water-16-Doxyl stearic acid system (Figs. 3b and 3c), the spectra for the gluten-starch-water-16-Doxyl stearic acid systems show the extent of stearic acid-gluten interactions that will occur are dependent upon the amount of gluten in the combined system.

The 16-Doxyl stearic acid became more mobile after heating at 45-95°C in the systems that had a gluten:starch weight ratio equal to either 0.075:1 or 0.30:1. The changes after heating were similar to the trends displayed by the 0.15:1 = gluten:starch samples in Fig. 5. Amplitudes of the fast motion components increased as the sample was heated at higher temperatures. The decreased binding of 16-Doxyl stearic acid after heating could possibly be due to several factors. Conformational changes of the gluten proteins during heating could alter 16-Doxyl stearic acid interactions with the proteins or lipids associated with the proteins. Additionally as protein conformation changed with heating and more hydrophobic sites in the protein were exposed (Pearce et al., unpublished data), it is possible that 16-Doxyl stearic acid bound to starch prior to heating but which was shown to become unbound at elevated temperatures (Pearce et al., 1987) preferentially partitioned into the gluten component of the system.

An ESR spectrum for an unheated starch-gluten-water-16-Doxyl stearic acid system containing a weight ratio of gluten:starch equal to 0.15:1 and having a ratio of gluten-starch:water-16-Doxyl stearic acid equal to 1:2 is shown in Fig. 6c. The spectra are similar to that of the 0.15:1 = gluten:starch sample which had a ratio of gluten-starch:water-16-Doxyl stearic acid equal to 1:1 (Fig. 5a). The similarity of the spectra shows that the proportions of the 16-Doxyl stearic acid that partition and interact with either the gluten or starch component are not dependent upon the water and 16-Doxyl stearic acid concentrations in these two systems. Spectral line shape also was not altered in either the 0.075:1 or 0.30:1 = gluten:starch weight ratio samples when the ratio of gluten-starch:water-16-Doxyl stearic acid was increased from 1:1 to 1:2.

**Conclusions**

The interactions of 16-Doxyl stearic acid that occur with wheat starch or gluten depend upon whether the 16-Doxyl stearic acid is examined in starch-water, gluten-water or gluten-starch-water systems. In starch-water systems, 16-Doxyl stearic acid is bound at room temperature and after subsequent heating and cooling. Broadened 3-line ESR spectra were recorded at room temperature and after heating for gluten-water-16-Doxyl stearic acid and were a result of different types of stearic acid-gluten interactions occurring within the system. Specific stearic acid-gluten interactions could not be identified due to the composite nature of the spectra for gluten-water-16-Doxyl stearic acid.
ESR Study of Stearic Acid Interactions

Gluten-starch-water-16-Doxyl stearic acid systems showed that less stearic acid binding with starch occurs when gluten is incorporated in the system. As the proportion of gluten in the system increases, stearic acid binding with starch decreases. Stearic acid binding was further diminished after gluten-starch-water-16-Doxyl stearic acid systems were heated and cooled. Stearic acid interactions in the combined gluten-starch-water-16-Doxyl stearic acid systems were not different for gluten-starch:water-16-Doxyl stearic acid ratios of 1:1 or 1:2.

Acknowledgements

Published as paper no. 15450 of the Scientific Journal Series of the Minnesota Agricultural Experiment Station on research conducted under Minnesota Agricultural Experiment Station Project 18-027 and 18-063 supported in part by Minnesota General Agricultural Research Funds.

References


Discussion with Reviewers

J. Nishiyama: Why is there no numerical analysis for the broad line powder pattern ESR spectra? Authors: The gluten and starch systems we examined were not purified systems since we wanted to look at intact systems. Detailed numerical data could possibly be given for these spectra only after extensive studies of simple model systems. Additionally, calculation of correlation times for powder pattern spectra is difficult since it is hard to distinguish between anisotropic and isotropic motionally slowed spectra or between anisotropic motionally slowed spectra and composite spectra due to superposition of motionally slowed and motionally narrowed ESR spectra. Thus, it would be difficult to determine which theories and equations should be used to calculate true correlation times (Miller WG. 1979. Spin-labeled synthetic polymers, In: Spin Labeling II. Theory and Applications, LJ Berliner (ed.), Academic Press, New York, 173-221).

D.N. Holcomb: Is it known how the spin probe, 16-Doxyl stearic acid, binds to gluten and starch? Could binding of the probe alter the gluten and starch structures? Authors: The binding of 16-Doxyl stearic acid to gluten and starch can be through the hydrocarbon or hydroxyl group of the stearic acid. It is not likely to be binding through the nitroxide moiety. Conformation and mobility of polymers adsorbed on oxide surfaces by ESR spectroscopy, In: Polymer Characterization by ESR and NMR, AE Woodward and PA Bovey (eds.), ACS Symposium Series 142, American Chemical Society, Washington, D.C., 3-18. Based upon the comparison of ESR spectra for wheat starch-water-5-Doxyl stearic acid and wheat starch-water-methyl-5-Doxyl stearate, a probe which had the carboxyl group replaced by a methyl ester, it was concluded the probe must be binding through the hydrocarbon portion of the stearic acid (Pearce et al., 1987). Amylopectin and, to a greater extent, amylose components of the starch granule in studies using waxy and high amylose starches have been shown to bind 16-Doxyl stearic acid (Pearce et al., 1987). The results of this study cannot answer the question of whether probe binding alters the gluten and starch structures. The potential for structure alteration is present due to the hydrophilic and hydrophobic properties of the gluten-starch system. However, hydrophobic interactions can be weak or surface related, and in this case are not thought to have any significant effect on structure.

R.C. Hoseney: How were the gluten-starch-water-16-Doxyl stearic acid samples prepared? Authors: Gluten-starch-water-16-Doxyl stearic acid samples were prepared by weighing sample constituents into 20 ml beakers and magnetically stirring very slowly for 24 h. The sample size was large enough so that there was little headspace for sample moisture loss due to condensation on the sides of the beaker. The beaker was sealed with four layers of plastic wrap and two layers of paraffilm to prevent evaporation.

R.C. Hoseney: How do you envision that gluten, which apparently only interacts slightly with the probe, interferes with the binding of the probe with starch?
Authors: A possible explanation for the reduced probe binding with starch observed in the combined gluten-starch systems is shown in the above figure. The majority of the probe in the unheated system is bound to the starch. At elevated temperatures during heating the probe is released from the starch (Pearce et al., 1987). At temperatures where the probe is released from starch, ESR spectra from gluten-water-TEMPO systems have indicated that conformational changes are occurring in the protein and more hydrophobic regions of the protein are exposed. It is possible that upon cooling, increased amounts of the probe preferentially interact with the newly exposed hydrophobic regions of the gluten proteins.

R.C. Hoseney: What is the significance of your findings?
Authors: The implications of these findings are that competition may exist between product ingredients for interactions with stearic acid emulsifiers in a batter or dough. The data also show that the dynamics of stearic acid interactions with starch and gluten change as a product formulation is heated and then cooled.
Imbibition is a critical phase in germination and processing of legume seeds because cellular disruption during imbibition may influence seedling vigor and processing quality. Cellular disruption of cotyledony surfaces of beans (Phaseolus vulgaris L.) and peas (Pisum sativum L.) and the cellular contents released during imbibition were examined with scanning electron microscopy. Two types of cellular disruption were observed during imbibition: ruptures and fractures. Individual cells and small groups of cells on the surfaces of cotyledons ruptured after immersion in water. Ruptured cells had flaps of cell walls which remained attached to intact portions of cell walls. Fractured cells split in half, and remnant portions of cell walls were completely separated from each other. Disrupted cells on the interior surfaces of blister cavities were of the fractured type. Materials released from cotyledony tissues consisted of both dense aggregates of protein bodies and a dispersed phase of protoplasm. In some cases, protoplasm and protein bodies on cotyledony surfaces were found adjacent to single cell ruptures and, in others, the sites of losses were not found. The presence of protoplasm and protein bodies and absence of sites for their release indicate an additional mechanism other than fracture or rupture may contribute to losses of intracellular substances during imbibition.

Key words: beans, Phaseolus vulgaris (L), peas, Pisum sativum (L), cellular rupture, protoplasm extrusion, nutrient loss, solute leakage, soaking injury, imbibitional stress, imbibitional injury, protein bodies.

Introduction

Legume seeds and excised cotyledons which are soaked in water or sown in wet soil release intracellular substances into the surrounding water (Schroth and Cook, 1964; Duke and Kakefuda, 1981, Duke et al., 1983, Spaeth, 1987). Substantial quantities of nutrients can be lost from seeds during soaking, e.g., 60% of K content in 24 h (Simon, 1984). In crop production, losses of intracellular materials are associated with poor seedling vigor and stand (Larson, 1968, Powell and Matthews, 1978) because cells are ruptured and storage reserves are not available for growth of seedlings. Loss of intracellular substances is also associated with increased pathogen growth (Schroth and Cook, 1964) because nutrient sources become available to soil-borne pathogens.

The loss of intracellular substances has been attributed to large scale disruption of tissues manifested as transverse cracks (Morris et al., 1970), displacement of tissue strips (Duke and Kakefuda, 1981), and blistering of embryonic axes (Dunn et al., 1980) and cotyledony surfaces (Spaeth, 1987). Multicellular blisters on surfaces of bean cotyledons ranged from less than 0.4 to more than 0.8 mm in diameter (Spaeth, 1987). One mechanism for release of intracellular materials from blisters is pressure-driven extrusion (Spaeth, 1987). Partially hydrated protoplasm, protein bodies and, in some cases, starch grains (granules) exhibit characteristics of a viscous fluid which is extruded as streams of material through irregular orifices in blisters.

Solute leakage from cotyledony tissue is not always accompanied by visible tissue disruption. While cotyledons of one bean cultivar produced surface blisters, a second bean cultivar and two pea cultivars extruded small diameter streams of intracellular material but did not form visible blisters (Spaeth, 1987). Seeds with intact seed coats may leak substantial amounts of intracellular substances, yet not exhibit visible tissue disruption (Simon and Raja Harun, 1972, Duke and Kakefuda, 1981, Simon, 1984). The existence of cellular ruptures has been inferred from the appearance of intracellular substances, e.g. organelle specific enzymatic markers (Duke...
and Kakefuda, 1981), which are too large for diffusion through intact membranes. Evans Blue stain has been used to demonstrate plasma membrane rupture during imbibition (Duke and Kakefuda, 1981; Schoettle and Leopold, 1984). Detailed microscopic studies of cell rupture and viability would help to elucidate mechanisms of solute leakage and its role in reduced legume seed viability.

The first objective of this research was to examine cellular disruption on a scale smaller than transverse cracks, crescent cracks, and blisters. The second and third objectives were to identify various types of cellular disruption, and to identify some materials lost from cotyledons during imbibition. Scanning electron microscopy (SEM) was used to examine excised cotyledons and protoplasm lost during imbibition.

Materials and Methods

Seeds of two bean cultivars (Phaseolus vulgaris L. cv Apollo, a snap bean, and cv Royal Red Mexican, a dry bean) and two pea cultivars (Pisum sativum L. cv OSU 605, a wrinkled pea, and cv Garfield 81, a round pea) were obtained from local commercial or research sources. Seeds were equilibrated to constant water content over a saturated solution of potassium carbonate. Water content of seeds was determined by oven drying at 105°C for 48 h. Initial water contents of bean and pea cotyledons were 11 and 10% of seed dry weight, respectively.

Coats of dry seeds were carefully removed with a scalpel. Individual cotyledons were immersed in 6 ml of distilled, deionized water at 24°C. Cotyledons and extrusion streams from cotyledons were observed with a dissecting microscope during imbibition.

Cotyledons for SEM were prepared in several different ways. To prepare unfixed samples for SEM, some cotyledons were simply excised from dry seeds. Other cotyledons were excised, soaked in water for 30 min, blotted to remove excess water, and then allowed to dry at ambient temperature and humidity. In one set of Apollo bean cotyledons, a droplet of water was applied to abaxial surfaces. After the water was absorbed into the cotyledon or evaporated, the sample was reequilibrated at ambient relative humidity. Another set of bean cotyledons was fractured dry. Fracture surfaces were washed free of protoplasm and cellular inclusions as described by Wolf and Baker (1980). Dry tissues of all unfixed samples were sputter coated with gold (Hummer·Technics) 1.

To prepare fixed samples for SEM, cotyledons were immersed in 5 ml distilled, deionized water until blisters or extrusion streams appeared (20 min). To preserve the structure of extruded protoplasm during fixation, a glutaraldehyde:water solution (25%) was gently added to Petri plates containing the soaking seed, and mixed with the soak water to create a 5% glutaraldehyde solution for fixing tissue and extruded protoplasm. Thirty min after addition of the glutaraldehyde solution, cotyledons were rinsed and transferred to a 2% solution of osmium tetroxide in water for 30 min. Samples were dehydrated in a graded ethanol series (30 to 100%) (Hughes and Swanson, 1985). Fixed samples were dried in carbon dioxide with a critical point drier (Bomar SPC-1500) 1. sputter coated with gold (Hummer·Technics) 1. All samples were viewed and photographed at 20 KV with an ETEC U-1 Scanning Electron Microscope 1.

Results

In addition to the large scale cracking and multicellular blisters previously reported, epidermal cells of legume cotyledons were ruptured during imbibition. Areas of Apollo bean cotyledons which were not blistered exhibited ruptured cells and the contents from the ruptured cells on adjacent unblistered tissue (Fig. 1). Only a few of the total number of cells on the surface were ruptured (Fig. 1). Holes consisted of individual or small groups of ruptured cells. Flaps of cell walls, which were formed by the rupture process, were attached to remaining walls of ruptured cells. Cell contents released from cells did not contain recognizable constituents (Fig. 1). Cell contents from ruptures did not form extrusion streams as did cell contents from blisters (Spaeth, 1987).

The scale of individual cell rupture was too small to be seen without SEM, therefore, it was not possible to use light microscopy to directly confirm that individual cells or small groups of cells ruptured during soaking. To...
Cell Rupture In Cotyledons During Imbibition

1. 

2a. 

3. 

4a. 

b. 

b.
ensure that ruptures of individual cells were not caused by glutaraldehyde induced modification of cell walls, cotyledons were prepared for SEM by applying isolated drops of water to cotyledonary surfaces and allowing the drops to be absorbed or evaporate. This treatment also partially simulated the wetting and drying cycles employed by Powell and Matthews (1981).

Cells on the surfaces of Apollo cotyledons ruptured and lost intracellular materials when drops of water were applied to surfaces (Fig. 2a). Spots with irregular outlines were formed where water drops lay on cotyledon surfaces (Fig. 2a). The rough-textured areas around spots did not come into contact with water. Three zones were observed where water was applied, a rough center with ruptured cells and cellular contents, a bumpy surface without ruptures, and a perimeter which was smooth and cracked (Fig. 2a).

Cellular ruptures and intracellular materials were clearly visible at higher magnification (Fig. 2b). The intracellular materials on unfixed cotyledons were more consolidated than that on fixed cotyledons, but constituents were no more recognizable. Air-drying of soaked tissues without fixation resulted in formation of cracks and other predictable artifacts. Thin layers, or films of materials covered entire cotyledonary surfaces (Fig. 2a, 2b). Films were probably mixtures of soluble and insoluble components of cell contents which dried in a thin coating. Cracks in the film and cell walls probably formed during dehydration of the tissue. Dry films were similar to films found on particles of soy flour which were moistened with buffer and dried (Wolf and Baker, 1972).

When cotyledons of OSU 605, a wrinkled pea, were soaked in water, blotted dry, and allowed to dry in air, isolated cells on the surface also ruptured (Fig. 3). Remnant flaps of cell walls hung over the empty cells. Cotyledon surfaces immersed in large quantities of water and not subsequently fixed lacked protoplasm and inclusions (Fig. 3). Protoplasm and inclusions, which in other cases were fixed to cotyledonary surfaces or restricted to small volumes of water in drops, apparently were washed away in the larger quantity of soak water. Ruptured bean and pea cells contrasted with another form of damage to cotyledonal tissues, which also contributed to losses of intracellular materials. When cotyledonal tissues of beans fracture as a result of tensile stress, e.g., crack transversely, cells in the fracture plane split completely apart (Morris et al., 1970, Spaeth, 1987) (Fig. 4a) and parts of cell walls separated completely. When protoplasm, protein bodies and starch grains were washed out of fractured cells, incomplete cell wall fragments comprised most of the surface (Fig. 4b). When holes in cell walls were small, starch grains were retained in fractured cells while protein bodies and protoplasm were washed out (Fig. 4b). Cells at fracture surfaces did not exhibit remnant flaps characteristic of ruptured cells. Blister separation surfaces (Fig. 5), were similar to tensile fracture surfaces (Morris et al., 1970, Spaeth, 1987).

The shape of OSU 605 (wrinkled pea) cotyle-
dons differed from those of bean and round pea. Before imbibition, small, isolated particles of intracellular material were observed on the wrinkled surfaces of OSU 605 cotyledons (Fig. 6a). Epidermal cells gave the surface a rough texture. During imbibition and fixation, the wrinkles changed to creases, cotyledonary surfaces became smoother, and large quantities of flecks appeared on the central portion of the cotyledon, near the creases (Fig. 6b). Blisters were not found on the surfaces of pea cotyledons as in earlier results (Spaeth, 1987).

Creases in surfaces of OSU 605 cotyledons were filled with dense particles and surfaces adjacent to the creases were covered with dense particles and patches of less dense material (Fig. 6c). Large numbers of single cell ruptures or small tissue ruptures were not found.

Isolated epidermal cells on cotyledonary surfaces of OSU 605 (Fig. 6d) were ruptured. Flaps of cell walls remained attached to the main portion of the wall. Protoplasm released from ruptured cells was in two forms. Dense particles were roughly spherical or irregular in shape and ranged from 1 to 10 μm in effective diameter (Fig. 6d). The disperse phase of cellular contents often covered large areas of cotyledonary surfaces and consisted of small particles and a network of interconnecting material (Fig. 6d). Particle size distribution of the dispersed phase was similar to the granular cytoplasm found in unaged dry bean cells (Varriano-Marston and Jackson, 1981).

Dense particles from cotyledonary tissues were aggregates of spheroidal particles which ranged from 1 to 2 μm in effective diameters (Fig. 6d, 7). Spheroidal particles included protein bodies which were a major component of materials from ruptured cells. Although some protoplasm and protein bodies released from cotyledonary tissues clearly came from single...
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cell ruptures (Fig. 6d), substantial quantities of protoplasm and protein bodies appeared on cotyledonary surfaces where cell ruptures were not observed (Fig. 6c,d,7).

A summary indicates the absence or presence, and frequency of various features observed in each of the cultivars (Table 1). Note that Royal Red Mexican and Garfield 81 had relatively few ruptures and substantial quantities of protoplasm and protein bodies on the surfaces of cotyledons after imbibition.

**Table 1. Frequency of various features of injury and losses of intracellular substances from bean (Apollo and Royal Red Mexican) and pea (OSU 605 and Garfield 81) cultivars during imbibition.**

<table>
<thead>
<tr>
<th>Crop:</th>
<th>Beans</th>
<th>Peas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivar:</td>
<td>Apollo RRM, OSU 605, Garfield 81</td>
<td></td>
</tr>
<tr>
<td>Type:</td>
<td>Snap, Dry, Wrinkled, Round</td>
<td></td>
</tr>
<tr>
<td>Feature:</td>
<td>Transverse Cracks, Blisters, Cell Ruptures, Extrusion streams, Protoplasm, Protein bodies</td>
<td></td>
</tr>
<tr>
<td>Presence and Frequency:</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

A summary indicates the absence or presence, and frequency of various features observed in each of the cultivars (Table 1). Note that Royal Red Mexican and Garfield 81 had relatively few ruptures and substantial quantities of protoplasm and protein bodies on the surfaces of cotyledons after imbibition.

**Discussion**

Micrographs of cell ruptures (Figs. 1-3,6d) corroborated histochemical evidence for single cell ruptures (Duke and Kakefuda, 1981). Hypotheses about cellular rupture with complete loss of cell contents (Simon, 1984) were partially confirmed; however, cellular rupture demonstrated here was more deleterious than the membrane rupture that Simon (1984) and Powell and Matthews (1981) discuss. Cell walls as well as plasma membranes were ruptured during imbibition. Duke et al. (1983) suggested that cells might be able to repair mild cases of membrane rupture. Rupture of bean and pea cell walls during imbibition (Figs. 1-3,6d) was sufficient to preclude recovery or repair. However, it must be remembered that seed coats were not present to modify water uptake.

Cellular ruptures were observed in cotyledons which were imbibed but not fixed, and to a lesser extent in cotyledons which were fixed in aqueous solutions. Therefore, ruptures in fixed cotyledons were caused by imbibition of water from the fixation solution and were not caused by glutaraldehyde in the fixation processes. Fixation with glutaraldehyde was useful because protoplasm and protein bodies were retained on cotyledonary surfaces which otherwise would have been lost in the soak water.

Large holes were produced in walls of ruptured cells, but flaps of cell walls were attached to the undisturbed portions of cell walls (Fig. 1,3,6d). Ruptures appeared to be caused by plasmolytic, osmotically induced rupture of cells. The presence of cell wall flaps opening to the surface was characteristic of wall rupture and explosive release of the contents.

Rupture of single cells contrasted with another process by which intracellular materials were completely lost from cotyledonary cells, cell fracture in the plane of transverse cracks. Fractures of cells in transverse cracks are caused by tensile stresses in dry tissue (Spaeth, 1986, 1987) which split cells through the middle and leave empty cell walls after protoplasm and inclusions wash out (Morris et al., 1970; Wolf and Baker, 1980). Walls of cells in the fracture plane are split in half and no longer attached to one another. Fractured cells lacked flap remnants of cell wall (Fig. 4b). Separation surfaces in blister cavities were similar to fracture surfaces associated with transverse cracks (Fig. 5). The similarity indicates that tensile stresses were probably important in the formation of blisters. Holes in epidermal cells of soybean cotyledons and interior surfaces of seed coats observed by Yaklich et al. (1984) apparently were formed by a different (unidentified) process.

In contrast to the extrusion streams observed on fixed Apollo bean cotyledons and in water surrounding unfixed OSU 605 pea cotyledons (Spaeth, 1987), protoplasm and protein bodies from fixed OSU 605 cotyledons did not form extrusion streams. The disperse phase released from ruptures of OSU 605 cells was protoplasm fixed by the glutaraldehyde solution. Protoplasm quickly dispersed into soak water which did not contain glutaraldehyde. Rapid dispersion of protoplasm will make it difficult to determine to what extent fracture, rupture, extrusion, and diffusion each contribute to the aggregate quantities of material released from cotyledons.

The intracellular material observed on the surfaces of Royal Red Mexican, OSU 605, and Garfield 81 but not associated with cellular ruptures was also not in the form of extrusion streams. The dense particles from cotyledons were individual or aggregates of protein bodies (Fig. 6d,7). The disperse phases on the surfaces of Royal Red Mexican, OSU 605, and Garfield 81 differed slightly in form. Differences in protoplasm might reflect differences in composition of protoplasm from different genera and cultivars. Protoplasm and protein bodies may be one of the primary constituents of 'deposits' which have been observed on surfaces of excised cotyledons (Wolf and Baker, 1972).

The low frequency of cellular rupture in Royal Red Mexican, OSU 605, and Garfield 81 cotyledons did not appear to account for the quantities of protoplasm and protein bodies lost from cotyledons of these cultivars. Some ruptures may have been hidden by the protoplasm released from ruptured cells, or protoplasm and protein bodies may have been fixed to surfaces at some distance from ruptured cells. However, the low frequency of single cell ruptures on cotyledonary surfaces in which protoplasm was not fixed

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(Fig. 3) indicated that protoplasm and protein bodies may have been released from cotyledonary cells by some mechanism other than complete rupture of cell walls.

Powell and Matthews (1981) demonstrated that substantial quantities of intracellular substances are lost from cotyledonary tissue during imbibition and that cells are able to recover from such losses. A process by which cells could lose protoplasm and protein bodies and not rupture and completely discharge cell contents has yet to be identified. The distinctions between cellular rupture and fracture, and among the forms in which intracellular substances were released, will help efforts to develop breeding, cultural, and processing strategies which minimize the problems of tissue disruption during imbibition.

Conclusions

Disruption of cotyledonary cells during imbibition occurs on both large and small dimensions. Large disruptions include transverse cracking and blistering. Small disruptions were cell wall ruptures. Cellular fracture is caused by tensile stresses induced during imbibition by surrounding tissue. Fractured cells split open and most of their contents were released into soak water. In contrast, individual and small groups of cells discharged cellular contents under pressure during imbibition. Cellular contents discharged from ruptured cells included protoplasm, and protein bodies in dispersed and dense phases, respectively. Efforts to measure and control leakage from seeds should take into account variation among processes by which intracellular substances are released from cotyledons during imbibition.

Acknowledgements

A contribution from the Grain Legume Genetics and Physiology Research Unit, U. S. Department of Agriculture, Agricultural Research Service in cooperation with the Agricultural Research Center, Washington State University. The Electron Microscope Center, Washington State University provided facilities and technical assistance. Dr. M. J. Silbermagel and Crites-Moscow Co. supplied seeds. Jerry Coker provided technical assistance.

1Mention of a trademark, proprietary product, or vendor does not constitute guarantee or warranty of the product by the U. S. Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

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Discussion with Reviewers

W. J. Wolf: One would expect to be able to discern starch grains and protein bodies as constituents of cellular contents on the surfaces of cotyledons. Were the cell contents shown in Figs. 1 and 2b examined at higher magnification?

Authors: We did not find starch grains among the contents of ruptured cells in any of the seeds examined. Viewing the cell contents shown in Fig. 1 at high magnification revealed protein...
bodies similar to those of OSU 605 and Royal Red Mexican (Figs. 6d and 7). However, we could not discern protein bodies in contents from ruptured cells of air-dried samples, including Fig. 2b.

E. Varriano-Marston: Structures in Fig. 3 bear some similarity to stomates. How did you distinguish between cellular ruptures and stomates on the surfaces of cotyledons?
Authors: Stomates are generally bilaterally symmetric. Cellular ruptures were often asymmetric. The large flaps of cell wall which often remained attached to ruptured cells also served to distinguish them from stomates.

K. Saio: You have shown that some cells rupture while others do not. What differences exist between ruptured and unruptured cells?
Authors: Rupture of an individual cell is probably influenced by numerous factors including the movement of water, characteristics of the cell itself and characteristics of adjacent tissue. Epidermal tissues influence water movement into subepidermal cells and may diminish cellular rupture there. Variation in strength of individual cell walls might predispose some cells to rupture. Finally, the tendency of a cell to rupture may be influenced by surrounding cells. If compressive stresses in hydrating tissue contribute significantly to pressures which cause cellular rupture, then rupture of some cells may partially relieve the stresses and, thereby, diminish the number of neighboring cells which rupture.

K. Saio: Hard beans, which cannot absorb water, can be made to absorb water if the seed coat is scarified. The rupture of cells on the cotyledonary surface may act in a similar manner to promote rapid absorption of water while also resulting in the loss of nutrients from the seeds. Please comment.
Authors: The cellular ruptures which result from imbibitional stresses should be of interest to other areas of legume seed research including the serious problems associated with hard beans. Hardness in beans is caused by different factors including the inability of water to penetrate seed coats and failure of cotyledons to soften even when seeds imbibe water. Cotyledonary cell rupture would not appear to play a role in hardness resulting from seed coat impermeability where water does not enter cotyledons, but may be involved in forms of hardness where bean seeds remain hard even after cotyledons imbibe water.

K. Saio: Losses of solid and chemical components during immersion in water clearly increase depending on time and conditions during storage (Saio et al., 1980, Cereal Chem. 57, 77-82). Are such losses related to the frequency of cellular rupture?
E. Varriano-Marston: Cultivars differ in leakage and seedling vigor. Do differences in cellular rupture cause differences in leakage and vigor?
Authors: Given the variety of processes which can contribute to losses of intracellular substances, we would not necessarily expect to find strong correlations between any one form of imbibitional injury and total losses of intracellular materials. Differences in protein leakage among cultivars were consistent with the extent of cellular fracture in excised cotyledons of susceptible and resistant cultivars (Spaeth, 1987).

R. W. Yaklich: Are the results obtained in this research applicable to seeds with intact coats?
Authors: Seed coats certainly can influence water movement into cotyledonary cells. However, fractured cells associated with transverse cracks and multicellular blisters are found in some cultivars even when seed coats are intact (Morris et al. 1970, Spaeth, 1987). Rupture of individual cells seems to be a less severe case of imbibitional injury so we expect it will be found in seeds with intact coats of some cultivars.
THE MICROSTRUCTURE AND REHYDRATION PROPERTIES OF THE PHOENIX OYSTER MUSHROOM (PLEUROTUS SAJOR-CAJU) DRIED BY THREE ALTERNATIVE PROCESSES

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Abstract

The aim of this investigation was to observe the effects of three drying methods on the microstructure and rehydration properties of the Phoenix Oyster mushroom. Mushrooms were dried at 55°C by air-, freeze-, and vacuum-drying pilot plant processes. At the microstructural level, the hyphae of the air-dried samples were more flattened and collapsed than the vacuum- and freeze-dried samples. The basidia were distorted by all three treatments to a certain extent, but there seemed to be less damage on the gill surface of the vacuum-dried samples. Appearance, rehydration time and capacity were similar for freeze- and vacuum-dried mushrooms; air-dried mushrooms were shrunken and darker in colour, and their rehydration time and capacity were lower. Sensory evaluations indicated better flavour retention of the vacuum-dried product as compared to the air-dried material.

Introduction

Edible mushrooms can be processed by various methods including canning, drying and freezing. The effects of these processes on the mushroom macrostructure are well documented. However, few studies have been concerned with the microstructure of processed mushrooms. Jasinski et al. (1984) observed the ultrastructural changes of blanched and canned mushrooms of Agaricus bisporus, while Keresztes et al. (1985) studied the effect of ionizing radiation on the gill tissue of Agaricus bisporus and Pleurotus ostreatus.

Two methods of commercial importance for the drying of mushrooms are air drying and freeze drying (Cho et al., 1982). Air drying is more commonly used for preserving many wild varieties of edible mushrooms and some cultivated species such as Lentinus edodes and Volvariella volvacea. The main advantage of dehydrated mushrooms is that they are light and thus can be packaged, stored and transported cheaply. Air-dried products are often of poor quality due to shrinkage, textural changes, browning and incomplete rehydratability (Van Arsdale, 1973). The time taken by the dried mushrooms to rehydrate depends on the species concerned. Air-dried Chanterelles, Cantharellus cibarius, take up to 8 hours while air-dried Pleurotus ostreatus take about forty minutes (Gormley and O’Riordain, 1976). In contrast, freeze-dried mushrooms rehydrate within a few minutes because of their porous, non-shrunken structure. They also have a good colour and flavour (Fang et al., 1971). However, there are problems with freeze drying of mushrooms resulting from the damage incurred by the freezing stage of the process (King, 1975). Oddson and Jelen (1981) observed that rehydrated freeze-dried Pleurotus florida were limp, probably due more to the inability of the delicate structure of the mushrooms to withstand the freezing process than the drying itself.

During freezing, mechanical injury can result from the growth of ice crystals which occupy a larger volume than water. If the ice...
crystals are large enough, physical rupture can occur (Kuprianoff, 1962). Slow freezing favours the formation of large extracellular ice crystals while rapid freezing promotes the formation of small intracellular ice crystals which are less likely to cause structural damage (Pennema, 1975). Bello et al. (1984), however, showed that even with rapid freezing, damage to the nuclei and mitochondria of skeletal fish muscle could occur.

Smaller ice crystals may not be necessarily desirable for the freeze-drying process, since they result in pores of smaller diameter which offer a greater resistance to heat and mass transfer during drying (Cllobanu et al., 1976). Also, rapid freezing results in a greater loss of volatile components. Bartholomai et al. (1975) reported a significant reduction in the retention of a natural volatile component in liquid mushroom extract frozen rapidly in liquid nitrogen compared to the extract frozen slowly in still air at \(-40^\circ\text{C}\). Flink (1975) suggested that slow freezing allows a greater incorporation of volatiles into the solute phase and favours the formation of microregion structures in which the volatiles are entrapped. Thus rapid freezing may minimize structural damage but it does not maximize volatiles retention.

Since the freezing step appears to be the main cause of quality impairment of freeze-dried mushrooms, it was decided to investigate the effects of bypassing that stage and only subjecting fresh mushrooms to the drying conditions usually used for freeze drying, i.e., heating under high vacuum. The aim of this study was to compare three drying methods (i.e., air-drying, freeze-drying, and high vacuum-drying) for their effects on the microstructure and rehydration properties of the Phoenix Oyster mushroom, *Pleurotus sajor-caju*. The mushrooms were also evaluated for sensory characteristics such as texture, flavour and overall acceptability.

**Materials and Methods**

**Samples**

Fresh *Pleurotus sajor-caju* (Strain P60, Hauser Champignonkulturen AG, Switzerland) mushrooms were obtained from Mountain Mushrooms Research Farm (Airdrie, Alberta). Fruit bodies of 7-10 cm diameter were selected. Only the caps were used.

**Drying Treatments**

Gormley and O’Riordan (1976) recommended a temperature of 51-65°C for air-drying of *Pleurotus ostreatus*. Bartholomai (1974) reported obtaining dried mushrooms of satisfactory sensory quality from mushrooms dried by a combination of freeze-drying (contact freezing at \(-40^\circ\text{C}\); heating plate at 60°C) and air-drying at 80°C. A temperature of 55°C was used in our work for all three drying methods as a compromise and uniform treatment parameters.

Nine hundred grams of whole mushroom caps on a perforated tray were dried to a moisture content of 7-8% by the three methods investigated. Each treatment was replicated three times using three different batches of mushrooms. The methods used were: (i) Air drying in a forced convection oven (model LDB1-69, Despatch Industries Inc. Minneapolis, MN). The drying time was approximately 10 h. (ii) Freeze drying, for approximately 20 h. Mushrooms were previously frozen in a walk-in freezer (air-blast) at \(-30^\circ\text{C}\). They were next dried in an RePP freeze drier (Virtis Co. Inc., Gardiner, NY); the shelf temperature was maintained at 55°C and the condenser temperature at \(-60^\circ\text{C}\); the vacuum was less than 1 mm Hg. (iii) High-vacuum drying, using the equipment and drying conditions as in (ii), but omitting the freezing step.

**Temperature Changes in Mushrooms**

The temperature changes in a mushroom cap of 10 cm diameter were monitored by several thermocouples inserted at various locations during vacuum-drying.

**Scanning Electron Microscopy (SEM)**

Samples were prepared according to the method described by Nickerson et al. (1974) for fungal spores. Small pieces (5 mm³) of gill tissue and inner tissue of a cap were exposed to osmium tetroxide vapour overnight and air dried at room temperature. The dried samples were then sputter coated with gold (Nanotech, Semrep 2), and examined with the scanning electron microscope (Cambridge stereoscan model S250, Cambridge Industries, England) at 20 kV.

**Moisture Content of Mushrooms**

Approximately 3 g samples of dried mushrooms, crushed into small pieces, were dried at 105°C for 3 days. Moisture loss was then calculated. Determinations were carried out in duplicate.

**Rehydration Time**

About 2 g of dried mushrooms were soaked in 500 mL of water at 20°C. After a specific time interval, the mushrooms were removed and excess water gently blotted out by paper towel. The samples were weighed and then placed back into water. The time interval used for repeated determinations of air-dried mushrooms was 10 minutes while for the freeze-dried and high vacuum dried mushrooms, it was 30 seconds. Readings were taken until there was no further change in weight, at which time the mushrooms were considered to have reached full rehydration capacity. Determinations were made in triplicate.

**Rehydration Capacity**

Rehydration capacity was calculated as the maximum amount of water absorbed (g) per g of dry material as determined at the end of the rehydration time experiment. Determinations were made in triplicate.

**Sensory Evaluation**

Mushrooms were rehydrated and cooked in butter for two to three minutes. They were evaluated for texture, flavour and overall acceptability by a group of twenty-six panelists on a hedonic scale (Larmond, 1977) of 1 (dislike extremely) to 9 (like extremely). Results were analyzed by two way Analysis of Variance (ANOVA). Differences between means were tested by the Tukey’s test.
Microstructure of Dried Mushrooms

Results and Discussion

The freeze-dried and vacuum-dried mushrooms were similar in several aspects. They both exhibited good colour, little or no shrinkage (Fig. 1), and rehydrated within two minutes to a similar rehydration capacity (Table 1). In contrast, the air-dried mushrooms were darkened and shrunk; they took forty minutes to rehydrate and their rehydration capacity was lower.

At the microstructural level, the basidia of all three samples (Fig. 2) were distorted to a certain extent but there seemed to be less damage on the gill surface of the vacuum-dried samples. When rehydrated, the gill surface of the air-dried sample was more wrinkled than that of the freeze-dried product while the surface of the vacuum-dried sample was fairly smooth (Fig. 3). The hyphae of the air-dried samples were more flattened and collapsed than those of the freeze-dried and vacuum-dried (Fig. 4) samples. The rehydrated hyphae of the air-dried samples were more folded while those of the freeze-dried and vacuum-dried products were fairly smooth (Fig. 5).

According to Van Arsdel (1973), during drying the solid structural elements of the plant tissue are pulled closer together under the influence of surface tension as water evaporates from the wet surface. This effect eventually spreads to the deeper layers while water migrates to the surface where it is evaporated. Additional removal of water at the surface leads to further deformation as the structural elements are crumpled or folded to occupy a shrinking volume. Thus, the air-dried mushrooms were shrunk, the hyphae flattened and the basidia collapsed.

During freeze-drying, the original dimensions of the product are maintained first by freezing. The ice is then sublimed, usually under a high vacuum. Since there is no aqueous phase, there is no migration of water to the surface but instead a recession in face of frozen and dry layer. The effects of shrinkage and concentration of water soluble components due to the mobility of the aqueous phase are thereby prevented (Karel, 1975), and the resulting product is not shrunk but porous. At the microstructural level this was observed as hyphae which had retained to some degree their tubular structure (Fig. 4B). The basidia, however, were more damaged and some showed perforations in their wall (Fig. 2B). In this experiment, the mushrooms were frozen by air-blast freezing at -30°C. At this rate of freezing, there could have been the formation of ice crystals large enough to cause structural damage. To assess the extent of the damage, further observations at the ultrastructural level by electron transmission microscopy need to be carried out.

Table 1. Moisture Content and Rehydration Characteristics of Dried Mushrooms (at 20°C).

<table>
<thead>
<tr>
<th>Dried Sample</th>
<th>Moisture* (%)</th>
<th>Rehydration Time (minutes)</th>
<th>Rehydration Capacity** (g H₂O/g mushrooms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>7.4 (±0.1)</td>
<td>40</td>
<td>6.39 (±0.06)</td>
</tr>
<tr>
<td>Freeze</td>
<td>7.6 (±0.3)</td>
<td>1.5</td>
<td>7.21 (±0.05)</td>
</tr>
<tr>
<td>Vacuum</td>
<td>7.6 (±0.1)</td>
<td>2</td>
<td>7.16 (±0.07)</td>
</tr>
</tbody>
</table>

* Mean ± standard error (n=9)
** Mean ± standard error (n=6)

after fifteen minutes when the pressure within the dryer reached one Torr. As the pressure within the dryer was being reduced, water evaporated rapidly from the mushroom. Heat was lost from the tissue as latent heat of vaporisation but at the same time there was a heat gain from the heating plate and the surroundings. While the other parts of the mushroom cap remained above 0°C at the time intervals recorded, the temperature dropped to -8.2°C in the centre of the cap. Whether that area became supercooled or frozen has to be investigated further. The porosity of the vacuum-dried mushroom indicates the possibility of a similar mode of mass transfer in the tissue as that occurring during freeze-drying. Instead of a migration of water which would pull the tissues together, there could have been a receding interface of moist and dry layer which resulted in a porous structure, as shown for the vacuum-dried hyphae (Figs. 4B and 4C). The gill surface of the vacuum-dried sample, on the other hand was not as damaged as the freeze-dried sample (Figs. 2B and 2C). This may be due to the omission of the freezing process. The kinetics of heat and mass
Fig. 2. Gill surface of *P. sajor-caju* mushrooms. A - Air dried. B - Freeze dried. C - Vacuum dried.

Fig. 3. Gill surface of rehydrated *P. sajor-caju* mushrooms. A - Air dried. B - Freeze dried. C - Vacuum dried.
Fig. 4. Hyphae from *P. sajor-caju* mushroom caps. A - Air dried. B - Freeze dried. C - Vacuum dried.

Fig. 5. Hyphae from rehydrated *P. sajor-caju* mushroom caps. A - Air dried. B - Freeze dried. C - Vacuum dried.
transfer in the mushroom tissue would have to be investigated further before an explanation can be given for the similar porous structure of the freeze-dried and vacuum-dried samples.

Panelists used in the sensory test indicated no significant difference between freeze-dried and vacuum-dried mushrooms with respect to texture, flavour and overall acceptability (Table 2). However, flavour and acceptability of the vacuum-dried mushrooms were significantly better than that of the air-dried products, while the freeze-dried mushrooms were not significantly different from the air-dried mushrooms. This indicated that flavour might be better preserved in the vacuum-dried mushrooms than in the freeze-dried products but this was not significant enough to be detected by the panelists. The freeze-dried mushrooms could have lost more of the flavour components by leakage through the damaged cell walls when they were being rehydrated prior to cooking. In all tests, however, fresh mushrooms scored significantly higher than any of the three dried products.

### Conclusion

High vacuum drying is a method of potential importance for drying mushrooms. Vacuum-dried Phoenix Oyster mushrooms had similar features to those of the freeze-dried mushrooms such as good colour, non-shrunken structure and rapid rehydration rate to a high rehydration capacity. The absence of a freezing stage would avoid the undesirable characteristics associated with freezing and would also reduce the processing cost for obtaining quality dried mushrooms. This method could be applied to other mushrooms such as the Chanterelles, (Cantharellus cibarius) which give poor quality air-dried products while freeze-dried products are too expensive. A preliminary study on the Chanterelles (Li-Shing-Tat and Jelen, manuscript in preparation) indicated that the freeze-dried and vacuum-dried mushrooms were similar in appearance. The high vacuum drying method merits further investigation, especially for the edible mushrooms commanding premium prices.

### References


Effects of ionizing irradiation and storage on microstructure of mushrooms. J. Food Technol. 3, 333-349.

Discussion with Reviewers
D.L. Rinker Substrate, culture and environment influence the quality of the fresh product. Would the result of the experiment be any different under different substrate, culture and environmental parameters? Also, there are many species of Pleurotus cultivated commercially. How would your technique or results vary with the different species?

Authors: Mushrooms subjected to the three drying treatments all came from the same batch. The relative difference between the dried products would probably be similar for mushrooms cultivated under a different set of conditions and for mushrooms of different Pleurotus species.

D.L. Rinker: Why were caps only used? Pleurotus sajor-caju is a cultivar of choice by some for the edibility of the stems. There is considerable weight loss (dollar loss to the producer) if the stipes are trimmed short.

Authors: Air-dried stems are tougher in texture than the caps. Also, because of the differences in structure between the caps and the stems, they would dry at different rates. In this experiment, it was decided to concentrate on the caps. Oddson and Jelen (1981) indicated that freeze-drying was damaging particularly to the structure of the stems.

E. Kovacs: Were the ultrastructural changes observed correlating with texture measured by an objective method (Instron) or only by sensory evaluation?

Authors: The textural characteristics of the rehydrated uncooked mushrooms were measured by the Ottawa Texture Measuring System of Vosey (1971, J. Inst. Can. Technol. Aliment. 14 (3), 91-103). There was no significant difference between the vacuum-dried and freeze-dried products (Table 3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Force (kg)/25g</th>
</tr>
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<tbody>
<tr>
<td>Air-dried mushrooms</td>
<td>73.54 (±1.43)*</td>
</tr>
<tr>
<td>Vacuum-dried mushrooms</td>
<td>51.55 (±1.27)</td>
</tr>
<tr>
<td>Freeze-dried mushrooms</td>
<td>50.11 (±5.64)</td>
</tr>
</tbody>
</table>

Mean values ±S.E. (n=9)
*Significantly different at 5% level

E. Kovacs: Do you plan to investigate other mushrooms, for example wild mushrooms, which are very rich in flavour components? Pleurotus species are generally poor in
flavour components; why did you choose *Pleurotus sajor-caju*?

**Authors:** *Pleurotus sajor-caju* was chosen in this study because of its rising importance in the exotic mushroom industry in Canada, and the current industrial interest in finding suitable processing technologies for the excess production. However, the final objective was to find a method of drying that could be applied to high value mushrooms such as the Chanterelle. Preliminary tests on *Cantherellus cibarius* indicate that the vacuum-drying method gave a similar product to that obtained by freeze-drying.

**E. Kovacs:** Were the dried samples investigated right after drying or after storage? If the dried product was stored, please clarify the storage conditions.

**Authors:** After drying, the products were stored in moisture-proof bags at room temperature. Samples for investigation were prepared within a week after drying treatments.

**E. Kovacs:** It would be interesting to know whether there are any differences in brittleness of the dried, freeze-dried and vacuum-dried products. It is important from a consumer's point of view.

**Authors:** The differences in brittleness between the three dried products were not tested. The vacuum-dried and freeze-dried products were both fragile and would require protective packaging.

**P.T. Atkey:** Have the authors considered any other methods of preparing specimens for SEM which might be less damaging to the tissue?

**Authors:** Specimens were dried samples which had already been damaged by the drying treatments. It is possible that the preparation technique employed here will cause further damage to the tissue but it is expected that all samples will suffer the same degree of damage and that the relative difference between the samples can still be assessed. Another method that could be used for the specimen preparation is Cryo-SEM.

**P.T. Atkey:** Have the authors looked at fresh tissue as control test for the techniques they used for specimen preparation for the SEM?

**Authors:** Fresh tissue was not included as control since the aim was to compare dried samples. A technique that preserves the structures of the fresh tissue may not be necessarily suitable for the dried samples. In retrospect, fresh tissue as control might have been useful to assess the structure of the rehydrated samples.

**P.T. Atkey:** An increasingly important method of short-term mushroom preservation is vacuum cooling. Have the authors examined the effects this may have on *Pleurotus* tissue?

**Authors:** No, this was not investigated.
FLUORESCENCE AND LIGHT MICROSCOPIC ANALYSIS OF DIGESTED OAT BRAN

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Abstract

The structural and chemical compositions of digested oat bran were analyzed by fluorescence and other types of light microscopy. The digestion of oat bran was carried out under two conditions - in vitro, by incubation with human saliva, and in vivo, by feeding rats with an oat-bran supplemented diet. Comparison of the digestible breakdown among different structural components of oat bran was conducted by microscopically examining samples obtained from the salivary digestion and from the ileum and contents of the large intestine (LICh) of the rats. Results of the examinations revealed that the 1-3(1-4)-β-D-glucan-rich cell wall of the subaleurone layer, along with its cellular content, were relatively more digestible than the comparable structures of the aleurone layer. Most of the aleurone structural components, including the cell wall and the phytin globoids, as well as the outer fibrous layers of the bran, remained detectable in the ileum samples, indicating that they were poorly digested by the endogenous gastrointestinal enzymes of the rats. Partial to complete degradations of the aleurone cell wall and the phytin globoids were detected in the digested oat bran tissues found in the LIC of the rats. Microscopic examination of the LIC samples also revealed the presence of the rat intestinal microflora, undigested outer tissues of the bran with its detectable phenolic content, and a large amount of crystalline structures that differed morphologically and chemically from oat phytin globoids. Energy dispersive X-ray microanalysis of the LIC samples revealed their elemental composition to be high in both phosphorus and calcium along with traces of magnesium, potassium and iron.

Introduction

Oat bran is a product separated by mechanical processing of dehulled oat grains (groats). Oat bran is rich in cell wall fibers, particularly (1-3)(1-4)-β-D-glucan (about 8% - Wood, 1986) which appears to play a major role in lowering serum cholesterol levels in humans and rats (Kirby et al., 1981; Chen et al., 1981; Anderson et al., 1983). Oat bran is also rich in minerals (Peterson et al., 1974), most of which occur as subcellular phytin globoids within the aleurone layer (Pomeranz, 1973). However, interactions between phytate and other dietary components, such as cell wall fibers and various minerals may significantly lower the bioavailability of minerals in animals and humans (Taylor, 1965; Morris, 1986). Hence, the role of oat phytin globoids as a source of dietary minerals and the effect of phytate on mineral availability in humans who ingest oat bran for physiological benefit have to be considered.

Past studies on the nutritional aspects of oat bran mainly regarded the bran as a single entity rather than an organized array of chemically and structurally distinct tissues. Like most plant fibers, oat bran fiber is generally believed to be poorly digested by the human digestive enzymes owing to the lack of specific enzymes such as cellulases in their digestive systems. Hence, degradation of the fiber relies mainly on the microbial fermentation that occurs in the large intestine of the human or the rat (including the cecum) (Nyman and Asp, 1986). However, a recent review on oat morphology by Pulcher (1986) describes oat bran components as chemically distinct structures, characterizable by their different cell wall fibers. Studies of plant fiber digestion indicated that some cell walls are more readily digested than the others, depending on their chemical and structural compositions (Akin et al., 1984). Direct comparison between the different oat cell wall components and their digestibilities under comparable conditions is lacking. A clear understanding of the relationship between the oat bran structures and their digestibilities would improve the knowledge of the role of oat bran as a source of dietary fibers, leading to better utilization of the oat fibers for maximum physiological benefits.
insufficient understanding of the nutritional quality of oat bran is partly due to the difficulty of extracting individually pure cell wall fractions for enzymatic digestion and to the inadequacy of effective methods capable of analyzing both chemical and structural compositions of the product at the same time. Two different types of microscopic techniques appear suitable for investigating the digestibility of different oat bran components under comparable conditions. Fluorescence microscopy proved to be effective for simultaneously analyzing food structures and their chemical compositions in various food products (Fulcher, 1982, Yiu et al., 1983; Yiu, 1985, 1986). The technique of energy dispersive x-ray microanalysis coupled with scanning electron microscopy enables concomitant analyses of structures and their elemental compositions (Pomeranz, 1973). Using these two techniques as analytical tools, the present study aimed at following structural and chemical changes of oat bran components subjected to in vitro (human, salivary) and in vivo (rat intestinal) enzymatic digestion.

Materials and Methods

Oat Bran

Mothers Oat Bran, Creamy High-Fiber Hot Cereal, was obtained from the Quaker Oats Company, Barrington, IL.

In Vitro Digestion of Oat Bran

In vitro digestion of oat bran by enzymes present in the human saliva was conducted according to the method described by Yiu et al. (1987). Oat bran (6 g wet weight), either cooked (6 g bran in 60 ml water) for three minutes or soaked in distilled water for the same period of time, was incubated at 37°C with 2 ml human saliva and 5 ml distilled water inside a dialysis tubing (Spectrapor Membr., 32 mm, molecular weight cutoff: 6,000-8,000) and simultaneously dialyzed against 200 ml distilled water with constant stirring. After 3 h, the digested contents were removed and immediately prepared for microscopic examination.

In Vivo Digestion of Oat Bran

Five Sprague-Dawley weanling female rats were fed a diet containing 22.4% casein, 0.3% D-L-methionine, 0.2% choline, 3.5% mineral mixture (AIN mineral mixture 76, ICN Nutr. Biochem. Ltd., Cleveland, OH.), 1.1% vitamin mixture (AIN vitamin mixture 76, ICN Nutr. Biochem. Ltd., Cleveland, OH.), 11.1% corn oil, 38.3% corn starch and 22.7% oat bran (containing an equivalent content of 4% dietary fiber). The animals were housed in wire-bottom cages. Distilled water was provided ad libitum. After 27 weeks on the diet (to observe the long-term effect of ingesting oat bran in the rat), the animals were anesthetized and later sacrificed with 2% halothane (Ayerst, Montreal, PQ) in oxygen. The whole gastrointestinal tract (GI) was removed and placed in ice-cold Ringer solution. The cecum was separated from the small intestine. The digesta from the distal portion of the ileum (0-6 cm from the cecum) was collected in a plastic scintillation vial, freeze-dried and stored at -70°C until microscopic examination. Pellets, or LIC, from the distal portion of the large intestine were collected and stored as for the ileum digesta.

Light Microscopy

All samples were first encapsulated in solvent agar in a petri dish according to a previously described method by Yiu et al. (1983), and were cut into 1-2 mm blocks after the agar was set firm. They were then fixed in 3% glutaraldehyde (in 0.01M phosphate buffer, pH 7.0) for 24 h according to the method described by Yiu (1986). Fixed samples were dehydrated through methyl cellosolve, ethanol, n-propanol, and t-butanol followed by infiltration with glycol methacrylate monomer for 3-5 days at room temperature prior to polymerisation at 55°C in gelatine capsules. Sections were cut 2 μm thick using an ultramicrotome (Sorvall Inc., Newtown, CT) equipped with a glass knife. All sections were affixed to glass slides for subsequent staining and microscopic examination.

Depending on the histochemical content of the sample, each section was stained with one of the following reagents according to the methods previously described (Yiu, 1986; Yiu et al., 1987): (1) 0.1% (w/v) aqueous Acriflavin Orange (BDH Chem. Ltd., Poole, England) for 1-2 min; (2) 0.1% (w/v) aqueous Acriflavin HCl (BDH Chem. Ltd., Poole, England) for 1-2 min; (3) 0.1% (w/v) Cellulfluor (Polysciences Inc., Warrington, PA) in 50% ethanol for 1 min; (4) 0.01% (w/v) aqueous Congo Red (Fisher Scientific Co., Fair Lawn NJ) for 1-2 min; (5) 0.5% iodine (w/v) in 5% aqueous potassium iodide solution (IKI) for 2 min; (6) 0.05% (w/v) Toluidine Blue for 1-2 min. All stained sections were rinsed in distilled water, air-dried, mounted in immersion oil, and examined by fluorescence, bright-field, or polarizing optics using a Zeiss Universal Research Photomicroscope (Carl Zeiss Ltd., Montreal, PQ.). The microscope was equipped with both a conventional bright-field illuminating system and a II1 RS excitation illuminating system and condenser combination. A 100 W mercury-arc burner for fluorescence analysis. The II1 RS condenser contained three fluorescence filter systems with a dichromatic beam splitter and an exciter/barrier filter set for maximum transmission at 365 nm/418 nm (FL I), 450-490 nm/520 nm (FC II) and 546 nm/590 nm (FC III). Micrographs were recorded on 35 mm Ilfordchrome 400 Daylight film.

Energy Dispersive X-Ray Microanalysis

Unfixed samples of oat bran, the feed, and contents of the large intestine of two rats were mounted on carbon holders with silver paste. Three specimens were prepared for the examination of each of the above samples. The specimens were coated with carbon using a Speedivac Coating Unit (Model 1286/1258, Edwards High Vacuum, Oakville, Ont.) and examined on an ISI-DS130 scanning electron microscope (Rayonics Inc., Downsview, Ont.). The microscope was equipped with an energy dispersive X-ray detector and analyzer (TN-5500, Tracor Northern Canada, Rexdale, Ont.), with the detector set at 45° angle to the specimen stage and a working distance of 35 mm. The microscope was operated at an accelerating voltage of 20 keV, no tilt, 5 x 10^-9 A probe current and a probe size of 180 nm in diameter.
Structures of Digested Oat Bran

spectra were collected for 100 s at each of five selected sites per specimen, and their mineral element contents were qualitatively analyzed.

Quantitative Elemental Analysis

The LIC samples from the two rats were analyzed by Atomic Absorption Spectrophotometry (Model 975, Varian Canada Inc., Ottawa, Ont.). The analysis was conducted at the Land Resources Research Centre, Agriculture Canada, Ottawa, Ont.

Results and Discussion

Structural and Chemical Compositions of Oat Bran

Most of the oat bran structural components and some of their chemical contents can be revealed simultaneously by simple fluorescence microscopic techniques. For example, using Congo Red as a staining reagent for revealing cell wall structures and the FC I filter system for short-wavelength excitation and subsequent fluorescence analysis, it was possible to detect autofluorescence emitted by the outer layers, which include the outermost pericarp, testa and the nucellus, (bluish white fluorescence), the aleurone and part of the sub-aleurone cell walls (blue fluorescence), indicating the presence of phenolic compounds (Fig. 1). Although the identity of these compounds is not fully established, ferulic acid is believed to be one of the major phenolic compounds present on the oat cell walls (Collins, 1986; Fulcher, 1986). The above examination also revealed the protonated location of (1-3)(1-4)-β-D-glucan (β-glucan) (red fluorescence), on the sub-aleurone cell wall (Fig. 1). In addition, the aleurone protein bodies embedded with visible globoids and the sub-aleurone reserves of protein and starch were also revealed in the same section.

Digestion of Oat Bran by Human Salivary Enzymes

Both uncooked and cooked oat bran samples were incubated with human saliva and the effect of cooking on the amylolytic digestion of some of the oat bran components was determined by the following microscopic analysis. Sections from the digested and undigested samples were stained with Cellfluor to detect changes in the cell wall structures. Similar to what was shown previously in rolled oats (Yiu, 1986), cooking induced more cell wall breakdown in the sub-aleurone layer, resulting in a decrease of fluorescence intensity in the cellfluor-bound cell walls. The decrease was probably due to the release of β-glucan from the cell wall into the cooking medium (Yiu et al., 1987). However, the diminished fluorescence intensity was not attenuated after the incubation with saliva (results not shown). The result was not unexpected, since enzymes capable of digesting the β-glucan are absent in human saliva. However, more cell wall breakdown in the sub-aleurone layer would lead to more release of its cellular content, thereby increasing the surface area for other enzymatic digestion, such as starch hydrolysis by amylases which are abundant in human saliva. Hence, the most noticeable difference of the oat bran components after the salivary incubation was the structural changes of the starch granules. After the incubation, the cooked oat bran had virtually no detectable starch granule structures, whereas the uncooked samples contained many partially digested starch granules (Fig. 2). This finding, which is in agreement with previous reports (Snow and O'Dea, 1981; Yiu et al., 1987), confirms that uncooked oat bran can be digested by human salivary amylases to a certain extent and that cooking greatly enhances its digestibility. Cooking also induced some release of the aleurone cell contents, but much less than that of the sub-aleurone layer, owing to the relatively sturdy aleurone cell wall. This was demonstrated by a decrease in the amount of phytin globoids, the structure of which can be detected by staining with Acriflavine HCl (Fig. 3) and by viewing under polarized light. However, whether the decrease was due to a loss of the cell contents going into the incubation medium or was caused by enzymatic breakdown remained uncertain. In view of the fact that phytase has never been detected in human saliva, the degradation of oat phytin globoids is expected to be low.

Digestion of Oat Bran by Rat Intestinal Enzymes

Rats fed with an oat-bran-supplemented diet were used as experimental models to investigate the digestive breakdown of various oat bran cell wall fibers and the phytin globoids in the gastrointestinal tract of rat. Digestion of oat bran in the rat ileum

Microscopic examination of the rat ileal contents (the digesta) revealed that most of the sub-aleurone cell wall and its cellular components were partially or completely degraded (Fig. 4), whereas the aleurone cell wall was relatively intact and many phytin globoids remained detectable (Fig. 5). This finding indicates that the former tissue is more susceptible to the environment of the rat digestive system, and it further confirms that the two cell walls are structurally and chemically different from each other. Aside from the host's digestive enzymes, microbial enzymes may also play a role in degrading the oat bran components, even though the number of microflora present in the ileum is relatively small (Borriello, 1986). The manifestation of degradation by the microflora is demonstrated in Fig. 6 which shows penetration of microorganisms through an opening on the sub-aleurone cell wall into the interior of the cell. The opening on the wall was probably caused by mechanical rupture induced during processing or chewing.

Digestion of oat bran in the rat large intestine

Examinations of the contents of the large intestine revealed a different pattern of oat bran digestion. Unlike those present in the salivary-digested samples and in the ileum digesta, most aleurone cell walls were degraded while there was no trace of any detectable sub-aleurone cell wall structure (Fig. 7). Residual bran materials were mostly components of the outer fibrous tissues, including the trichomes (the oat hairs) (Figs. 8a and 8b). The low digestibility of these tissues probably resulted from their chemical composition which includes lignin, cutin and phenolic acids (Fulcher, 1986).
The microbial population present in the large intestine of the rat was undoubtedly one of the major factors for degrading the aleurone and sub-aleurone cell wall fibers. In fact, close contact between the microflora and the cell wall materials was observed in the LIC samples (Figs. 8a and 8b). Although not identified individually, the shapes of these microorganisms, mostly spherical or elongated rods of various sizes and lengths, were revealed by the present microscopic examination (Fig. 9a). Previous studies on the microflora of the large GI tract suggest that they are mostly streptococci, bacteroides, and lactobacilli, all of which can produce enzymes capable of digesting cereals including fibers and phytin (Hill, 1986; Nayini and Markakis, 1986). Furthermore, it appears that the bran materials tended to attract certain types of microorganisms (Fig. 9b) to their vicinity, but more studies would be required in order to confirm this observation.

Many of the phytin globoids remained detectable after degradation, by staining with Acriflavine HCl (Fig. 10a), or by viewing the same sample under polarized light. The polarizing-microscopic examination also revealed the presence of other crystalline compounds that were structurally different from the phytin globoids and reacted differently to the staining reagent, Toluidine Blue (Fig. 10b). Similar crystalline structures (in much smaller numbers) were detected in the feed sample, and they were presumably the added mineral salts that constituted part of the rat diet. The above speculation was supported by results obtained from energy dispersive X-ray microanalysis of the feed, oat bran and the LIC samples. The major elements that were detected in the feed included sulfur, phosphorus, magnesium and calcium (Fig. 11a) while those detected in oat bran were phosphorus, potassium, sulfur, chlorine and silicon (Fig. 11b). In comparison with the above samples, the LIC contained predominantly phosphorus and calcium, in addition to magnesium, sulfur, potassium, chlorine and a trace of iron (Fig. 11c). These findings were confirmed by data obtained from the atomic absorption analysis which quantitatively estimated the concentrations of some of the above elements in the feed and in the LIC samples of both rats. All four major minerals (Ca, Mg, P, and Fe) were at least ten times more concentrated in the metabolic waste products of the rats than those in the feed (Table 1). Similar findings for figures on the opposite page.

Fig. 1. A Congo Red stained section of oat bran showing the composition of its structural components which include the seed coat layers (between arrows, upper left), the aleurone layer (A) with its subcellular globoids (small arrows), and the sub-aleurone layer (S) with its cellular contents of protein bodies (large arrows) and starch granules (*). Photographed using FC I.

Fig. 2. A section of uncooked oat bran stained with IKI to reveal the effect of salivary digestion on the structure of starch (arrows). Photographed using bright-field optics.

Fig. 3. A section of cooked oat bran stained with Acriflavine HCl to reveal the presence of phytin globoids (arrows) inside the aleurone cells. Photographed using FC III.

Fig. 4. A section of rat ileum contents stained with Cellflour, showing the partially digested cell wall (large arrows) of the sub-aleurone layer and the relatively intact aleurone cell wall (small arrows) of oat bran. Photographed using FC I.

Fig. 5. The same section (as in Fig. 4) stained with Acridine Orange and viewed under polarized light to reveal presence of phytin globoids (arrows) inside the aleurone layer of oat bran.

Fig. 6. A section of the rat ileum content stained with Acridine Orange, revealing the penetration of intestinal microorganisms (arrows) through an opening of the cell wall into the interior of the aleurone cell. Photographed using FC II.

Fig. 7. A section containing contents of the large intestine of the rat stained with Congo Red to show the partially digested aleurone layer (A) of oat bran. Photographed using FC II.

Fig. 8. Sections containing contents of the large intestine of the rat stained with Acridine Orange, revealing the presence of the undigested (a) outer bran layers and (b) the trichomes (*) among the intestinal microorganisms (arrows). Photographed using FC I.

Fig. 9. Different sections of the same contents shown in Fig. 8 stained the same way to reveal the various forms of microorganisms; (a) those near the digested remnants of the bran structural components (*) are predominantly spherical or oval rods (small arrows) and (b) those away from the bran materials are mainly long, slender rods (large arrows). Photographed using FC II.

Fig. 10. Sections containing contents of the large intestine of the rat stained with (a) Acriflavine HCl to reveal the presence of undigested phytin globoids (arrows), and (b) Toluidine Blue to reveal the presence of crystalline structures (arrows). Photographed using FC I for (a) and polarizing optics for (b).

(Scale bars on the micrographs represent 1 μm.)

### TABLE 1

Concentration of Selected Elements in Rat Feed and Contents of the Large Intestine

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Ca</th>
<th>% Mg</th>
<th>% P</th>
<th>% K</th>
<th>μg/g Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6.39</td>
<td>0.53</td>
<td>6.39</td>
<td>0.66</td>
<td>906</td>
</tr>
<tr>
<td>B</td>
<td>6.31</td>
<td>0.58</td>
<td>6.88</td>
<td>0.59</td>
<td>752</td>
</tr>
<tr>
<td>C</td>
<td>0.51</td>
<td>0.06</td>
<td>0.80</td>
<td>0.52</td>
<td>60</td>
</tr>
</tbody>
</table>

A = LIC from rat 1
B = LIC from rat 2
C = feed for both rats
were reported in an unrelated study which showed that the levels of several mineral elements, including Ca and Fe, were higher in the fecal matter than in the diet enriched with corn bran (Dintzis et al., 1985). The present result indicates that mineral elements present in the diet were not completely absorbed by the rats. Although it is known that cereal phytate can chelate dietary calcium to form insoluble calcium phosphate complexes (Taylor, 1965), there is no direct evidence in the present study to suggest that the detected crystalline structures may contain the above complexes. However, more investigations are currently being conducted in order to determine the chemical composition of these structures and the effect of low and high levels of dietary cereal fiber on mineral absorption in rats.

Fig. 11. Energy dispersive X-ray analysis spectra from (a) the feed, (b) oat bran, and (c) contents of the large intestine of one rat.

Conclusion

The present study demonstrates that the digestive breakdown of various oat bran components differs, depending on the structural and chemical compositions of the component and its location in the gastrointestinal system. The subaleurone layer, which had high polysaccharide concentrations mainly in the forms of starch and β-glucan, was most susceptible to the host’s digestive enzymes in the saliva (for the starch hydrolysis) and in the proximal intestinal tract (for the β-glucan-rich cell wall degradation). Degradations of most of the aleurone cell wall and some of the phytin globoids occurred chiefly in the lower intestine where the microflora played a significant role in degrading the above materials. In contrast with the aleurone and sub-aleurone layers, the outer bran tissues of oats (including the trichomes and the pericarp) were mostly undigested by the animals. In addition, the present study also revealed that contents of the large intestine of the rats contained numerous insoluble crystalline structures and had high mineral concentrations, particularly phosphorus and calcium. The findings suggest that the mineral supplement that was incorporated in the diet was not completely absorbed by the rats.

Acknowledgements

The authors thank Dr. W. Ihnat and Mr. Richard Cloutier of the Land Resources Research Centre, Agriculture Canada, for performing the atomic absorption spectrophotometric analysis and
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the staffs, particularly Mr. Ernie Bond, of the Electron Microscopy Unit, Plant Research Centre, Agriculture Canada, for assisting the EDX-microanalysis. This publication is contribution 731 from the Food Research Centre, Agriculture Canada, and contribution 255 from the Nutrition Research Division, Health and Welfare Canada.

References


Discussion with Reviewers

F.R. Dintzis: It would be helpful for the authors to comment about the 22.7% oat bran content in the diet, especially since I, and probably most readers, do not know if this is a high dose of bran. Was the transit time for the rats about normal once they adjusted to the diet? I ask this because the fermentation of oat bran in the gut should be dependent on transit time and the reader should know if physiological status of the rats was about normal.

Authors: Oat bran contains about 17% dietary fiber (Mongeau R, Brassard R. 1986. J. Food Sci. 51:1333-1336.) and its presence in the diet (22.7%) amounts to a total concentration of approximately 4% fiber. This is not a high dose of bran. The intestinal transit time for the animals averaged about 13-15 h, not significantly different from that of rats fed with a diet containing rat chow.

F.R. Dintzis: Could the authors give the reader some estimate of the weight percent of 'oat bran' recovered from LIC?
Authors: About 20.9% ± 1.7% of the ingested oat fiber was recovered from LIC of the five rats.

P.R. Dintzis: The peaks in Figs. 11a and 11b are relatively convincing. The detectability of Mg and Ca in Fig. 11c seems questionable. I wonder if it would be appropriate to include a blank for a control, i.e., just the EDX spectra taken from a silver paste coated carbon stub.

Authors: Both Mg and Ca peaks consistently appeared in the X-ray spectra of the LIC samples, but were absent from the spectra taken from a carbon coated specimen stub. The X-ray spectra of the background are not shown because there was no evidence of any spectral interferences.

D.J. Gallant: The peak/background ratio (Fig. 11c) seems very high, comparing to Figs. 11a and 11b, and is more comparable to a bran (or aleurone layer) spectrum than a spectrum from organic-rich material. Generally, the spectrum of bran is about ten times richer in K and P (and in Mg) than the other elements, but it does not appear so in Fig. 11b. Could you explain that? What was the surface topography of your sample during the analysis?

Authors: X-ray spectra taken from protein bodies of the aleurone layer of freeze-fractured oat grains contained the 3 major peaks (at a ratio of P:K:Mg/4:3:1, results are not presented in this paper), and all of them had high peak/background ratios, similar to what you suggested. However, similar spectral profiles were not recorded on samples taken from the feed or processed oat bran due to the presence of starch granules which greatly masked details of the bran structures. No attempt was made to extract the bran from the samples for the sake of preserving as much chemical and structural contents as possible. The background counts shown in Figs. 11a, 11b and 11c are similar but are presented at different scales. The low peak/background ratios shown in Figs. 11a and 11b were probably due to the presence of elements at low concentrations.

P.R. Dintzis: I find it most interesting that the phytin globoids are still detectable after passage through the small intestine where most mineral absorption is thought to occur. I do hope the authors are obtaining some measure of recoverable globoid content in digesta retrieved from the ileum and perhaps from the colon.

Authors: We did not try extracting undigested globoids from the digesta but we measured the phytate content in LIC using the AOAC method. About 60% of the ingested phytate was recovered in the LIC of the rats.

L.U. Thompson: What happened to the phytic acid which no longer is detectable in the bran?

Authors: Phytic acid may form soluble or insoluble complexes with minerals. Alternatively, it may be degraded by enzymes present in the intestine.

L.U. Thompson: The presence of crystalline salts in the LIC suggests that the minerals which were added to the diet were not completely solubilized in the gastrointestinal tract. To confirm this, did you try testing in vitro the solubility of the mineral mix under the pH conditions of the GI tract?

Authors: No, we did not test the solubility of the mineral mix under the pH conditions (around pH 6.8-7) of the GI tract. Since its presence was detected in samples which had been fixed in glutaraldehyde at pH 6.8-7, we assumed that its solubility was low.

L.U. Thompson: What was the [phytic acid] [calcium] / [zinc] molar ratio in the diet? Would you expect the results to be different if the calcium content was more or less?

Authors: The molar ratio was [5.97] [150] / [0.84] = 186. The high ratio may affect the zinc absorption. The apparent absorption of Zn in the five rats was ~5.1%. Different results could be expected if the calcium content was changed; the higher the value of the ratio, the lower the absorption of Zn.

L.U. Thompson: Zinc, which is known to tightly bind to phytic acid, was not detected in the LIC or ileus contents. Does it mean that zinc was not preferentially bound by phytic acid in oat bran, or was the zinc concentration just below the sensitivity of the methods used for measurement?

Authors: Atomic absorption spectroscopy revealed that the zinc content present in the fecal matters of the five rats was 879 ± 56 μg/g fecal dry weight. It is possible to detect very low levels (<0.1%) of zinc by x-ray microanalysis (Chandler et al. 1977. Histochem. J. 9:103) provided that ideal operating conditions, such as higher accelerating voltage (>20 keV), thin specimen, and longer time of analysis (>100 s.), are met. The present study focussed on detecting the presence of lighter elements, such as P, K, and Ca. No attempt was made to detect Zn either microscopically or via X-ray microanalysis.

L.U. Thompson: Can you conclude from your data that phytic acid does not affect the fiber breakdown in the GI tract?

Authors: About 60% of the ingested phytate and <20% of the original oat fiber were recovered from the LIC samples (measured by the AOAC methods). Neither the above result nor data presented in this study can conclude the relationship between phytic acid and fiber degradation.
PHYSICAL AND MOLECULAR PROPERTIES OF LIPID POLYMORPHS
— A REVIEW —

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Abstract

The physical and molecular properties of the polymorphism of stearic acid, oleic acid and SOS (1,3-dioleoyl-2-oleoyl glycerol) are comparatively discussed. Temperature dependence of Gibbs energy (G-T relation) of three polymorphs of stearic acid; A, B and C, revealed close relationships to each other. The molecular structures subtly differed in these polymorphs. In contrast, three polymorphs of oleic acid, α, β and γ, exhibited remarkably different characteristics. G-T relation showed more diversified features: in particular, the melting points of α and β differ by 3°C. An order-disorder transformation occurred between α and γ, as a result of conformational disordering in the portion of the oleic acid molecule from the double bond to the terminal methyl group in α. Finally, five polymorphs of SOS were newly presented, α, γ, pseudo-β', β2 and β1. X-ray spectra and thermal behaviors proved that the above five forms are the independent polymorphs. The author discussed the multiple polymorphism of SOS, taking into account the lamellar sorting of stearic/oleic acids chains accompanied with the change in the chain length structure. In relation to the polymorphism of SOS and other 1,3-disaturated-2-oleoyl triglycerides, the author emphasizes the possibility that the conversion from Form V to VI in cocoa butter might be caused principally through the polymorphic transformation from β2 to β1 of the higher-melting fat fractions of cocoa butter.

Introduction

The physical and molecular properties of lipid polymorphs have drawn attention of many investigators in the fields of biological sciences and oil chemical technology. This is due to the fact that the polymorphism of lipids is highly relevant in biological systems, and also decisive to the physical properties of foods, cosmetics, etc., which comprise lipids as the main compounds of solid fats.

The polymorphism may be discussed in terms of thermodynamic stability, crystal packing and molecular conformation as far as the physical aspects are concerned. The fundamentals of the macroscopic features of polymorphism, such as morphology, solidification kinetics and so on, may be explained in terms of these physical aspects. Each of the above factors is highly dependent on the molecular species which constitute the lipid, under a given set of external conditions. This is easily seen if one compares the melting points of stearic acid (69.6°C) with oleic acid (16.2°C of the high-melting polymorph). Obviously, this difference is the result of a drastic reduction in the chain-packing energy by the introduction of one cis-double bond at the central position of the polyethylene chain.

Very recently, many investigators have tried to elucidate the physical and molecular properties of some principal fatty acids and triglycerides. Particular effort has been devoted to the lipids containing unsaturated fatty acids as the main and functionally active constituents. Accordingly, this paper gives a brief review of the polymorphism of stearic acid and oleic acid, being representative of the saturated and unsaturated fatty acids, respectively. Furthermore, new findings are presented on the polymorphism of SOS, 1,3-dioleoyl-2-oleoyl triglyceride, as representative of the symmetric S₀S₀ (S₀: saturated acid, O: oleic acid) triglycerides. First, we briefly discuss some conceptual and methodological backgrounds.

Polymorphism: Thermodynamic Stability and Crystallography

In order to discuss the physical properties of different polymorphic forms of certain fatty acids and triglycerides, a precise knowledge of thermodynamic stability is prerequisite. For this purpose, measurements on the solubilities, melting points and transformation pathways of all the polymorphs are most characteristic. The less stable polymorphs melt at lower temperatures,
are more soluble in solvent, and transform to more stable ones either via solid-state (Verma and Krishna, 1966) or via solution-mediated (Cardew and Davey, 1985) or via melt-mediated transitions (Sato and Kuroda, 1987). The latter two transformations may actually occur if the solid-state transformation is kinetically hindered. Many long-chain compounds reveal these transformations. Knowing the thermal data, one may draw the relationship between the thermodynamic stability using a crystal Gibbs energy (G) and temperature (T) diagram.

The crystallographic aspects of the polymorphism of fatty acids and triglycerides are reflected in the lateral packing and lamella stacking of the hydrocarbon chains, which are most easily measured by X-ray diffractometry or by Infrared (IR) and Raman spectroscopy. Indicative of the lateral packing, characterized by the subcell structure, are the X-ray short spacings. These have so far exhibited three specific subcells; orthorhombic perpendicular (O1), triclinic parallel (T0) and pseudooctahedric parallel (O0′) as shown in Fig. 1 (Abrahamsson et al., 1984). In addition, a hexagonal subcell is reported to occur in highly metastable states (Abrahamsson et al., 1978). The saturated aliphatic chains are principally packed in O1 and T0 according to the mode of crystallization and thermodynamic stability, whereas O0′ is reported for the low-temperature polymorph of oleic acid (Abrahamsson et al., 1962). Hence this may be one of the subcells characteristic to the cis-ununsaturated acyl chain.

The lamellar stacking is indicated by the X-ray long spacing spectrum which equals the inter-lamellar distance between the terminal CH₃ groups of the lipid lamellae. The long spacing also can be a measure of the chain length structure, in particular, of triglycerides (Small, 1986). Normal triglycerides, such as monosaturated acids, reveal a double chain length structure where the long spacing equals the length of two fatty acids and one glycerol group. A change from the double to triple chain length structures occurs when the fatty acid moieties become mixed: i.e., large differences in the numbers of carbon atoms, (Kodali et al., 1984), saturated/unsaturated mixed acids, (Lutton, 1972) etc. The triple chain length structure is caused by a sorting of one chain acid from the other two chain acids (Fig. 2). Thus, the long spacing equals the sum of the lengths of three fatty acids and two glycerol groups. Even a six chain length structure is proposed (Fuhey et al., 1985), consisting of two triple chain length lamellae in a polytype relation (Verma and Krishna, 1966).

Last, molecular information about the hydrocarbon portions of the molecules can be elucidated with IR, Raman, high-resolution NMR, etc. The data on chain packing within the lamellar plane, inter-lamellar end packing, configuration and conformation of the carbon chains and hydrogen bonding, etc., obtained with these spectroscopic methods are not only complimentary to those from X-ray diffraction but also diagnostic of subtle structural properties of the polymorphism on a molecular level.

**Stearic Acid**

Three typical polymorphs of stearic acid, A, B and C, have been known for 30 years (von Sydow, 1956). The fourth, D, was found later by the spectroscopic methods (Holland and Nielsen, 1962). Form A is triclinic, and B, C and D are monoclinic. The subcell structures of B and C are reported to be O1. As for the thermodynamic stability, the solubilities of A, B and C were measured independently (Table 1) (Beckmann et al., 1984). B has the lowest solubility below 32°C (Sato et al., 1985), whereas C is the least soluble above that temperature. Form A has a higher solubility than the lowest value at all temperatures. Accordingly, the thermodynamic stability of the A, B and C polymorphs of stearic acid may be depicted by the G-T diagram shown in Fig. 3. The G values of B and C are the same around 32°C. This is apparently contradictory to the features of the solid-state transformation. Stenhagen and von Sydow (1953) reported that the transformation temperatures on heating from A to C, and from B to C are 54°C and 46°C, respectively. Another report (Garti et al., 1980) says that B transforms to C at 54°C. All these values are higher than the actual crossing points of the Gibbs energies of A, B and C. This is attributed to a kinetic hindrance of the transformation in the solid-state. So, the actual polymorphic transformation in the crystal may depend on the heating rate, which differs in the above two papers.

Structural analyses using single crystals of B and C showed that the hydrocarbon chains of C are in the all-trans conformation (Malta et al., 1971), whereas the C1-C3 carbons closest to the carboxyl group of B are in gauche conformation (Goto and Asada, 1978).

**Oleic Acid**

Three polymorphs of oleic acid, α, β and γ, were recently confirmed by means of DSC, X-ray diffraction (Suzuki et al., 1985), IR and Raman spectroscopy (Kobayashi et al., 1986). The transformation circuit of the three polymorphs is depicted in Fig. 4 (Sato and Suzuki, 1986). α is crystalized by chilling the melt, although it is thermodynamically metastable. The preferred crystallization and metastability for α resemble those of α of glycerides. α and γ undergo a reversible transformation in the solid-state at ~2.2°C on heating. γ is the form on which the structural determination was done (Abrahamsson et al., 1962). β, the most stable polymorph, crystalizes with very slow rates, both from solution and the melt. There is no solid-state transformation from α (or γ) to β in the melt-grown crystal due to a steric hindrance. Instead, the solution mediates the conversion. Lutton's high-melting form (Lutton, 1946) is equivalent to β in both the melting point and X-ray diffraction spectra. α, however, is contradictory to his X-ray data on the low-melting form, although the melting point is the same.

Table 2 summarizes the melting points and the enthalpy and entropy of fusion, dissolution and transformation. Solubility measurement (Sato and Suzuki, 1986) made it possible to depict the G-T relationship as shown in Fig. 5. The G values of γ and β are parallel to each other, whereas those of α and γ come close together with increasing temperature. Far below their crossing point, they melt. This multiple melting is characteristic of oleic acid and is not observed in saturated fatty acids.

The morphology and X-ray diffraction patterns of the three forms are shown in Figs. 6 and 7. All the crystals were of a tabular shape with a well-developed basal surface in a slightly supersaturated solution. α reveals a slender hexagonal shape, while β shows a truncated lozenge shape. The truncation occurs normal to the bisectrix of 55°. γ reveals a rectangular shape which is consistent with the subcell structure of O1/2 (Abrahamsson et al., 1962). The morphology of the three forms changes
Physical and Molecular Properties of Lipid Polymorphs

Fig. 1. Subcell structures of O\|, T\| and O'\| of aliphatic chains.

Fig. 2. Double, (a), and triple, (b), chain length structures in triglycerides.

Fig. 3. Relationships between Gibbs energy (G) and temperature (T) of A, B and C polymorphs of stearic acid, from solubility data (Beckmann et al., 1984).

Fig. 4. A transition circuit among the polymorphs of \(\alpha\), \(\beta\) and \(\gamma\), and melt of oleic acid (Sato and Suzuki, 1986).

Fig. 5. Relationships between Gibbs energy (G) and temperature (T) of \(\alpha\), \(\beta\) and \(\gamma\) polymorphs of oleic acid, from solubility data (Sato and Suzuki, 1986).

Fig. 6. Crystal shapes of \(\alpha\), \(\beta\) and \(\gamma\) polymorphs of oleic acid (Sato and Suzuki, 1986).

Fig. 7. X-ray diffraction spectra of \(\alpha\), \(\beta\) and \(\gamma\) polymorphs of oleic acid (Suzuki et al., 1985).

Table 1. Enthalpy (\(\Delta H\)) and entropy (\(\Delta S\)) of dissolution of A, B and C polymorphs of stearic acid in decane (Beckmann et al., 1984).

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\Delta H) (kJ/mol)</td>
<td>65.7</td>
<td>69.0</td>
<td>64.4</td>
<td>193.4</td>
<td>205.0</td>
<td>189.9</td>
</tr>
</tbody>
</table>
drastically to needle shape when the supersaturation of solution or supercooling is increased. The short spacing spectra of \( \gamma \) corresponds to the subcell of \( O' \). The other two forms reveal remarkably different patterns, implying different subcell structures. The long spacings are 4.34 nm (\( \alpha \)), 4.12 nm (\( \beta \)) and 4.19 nm (\( \gamma \)).

Vibrational spectroscopic studies on \( \alpha \), \( \beta \) and \( \gamma \) forms of oleic acid have resulted in the following (Kobayashi et al., 1986): (a) the \( \gamma \)-\( \alpha \) transformation is of an order (\( \gamma \))-disorder (\( \alpha \)) type which is accompanied by a conformational disordering in the portion of aliphatic chain between the double bond and the terminal methyl group (methyl-sided chain). The most conspicuous spectral change is seen in the low-frequency Raman spectrum (Fig. 8a). All the sharp bands of \( \gamma \) collapse to a broad band in \( \alpha \) due to a loss of translational symmetry. This originates from a disorder-ordered structure. Additionally, a peculiarity, indicating the same conformational disordering, appeared in two strong bands due to a C-C stretching mode. This mode is reflected in a single band for saturated acids. After the \( \gamma \)-\( \alpha \) transition, the 1125 cm\(^{-1}\) band of methyl-sided chain drastically decreased in intensity, whereas the 1095 cm\(^{-1}\) band due to the carboxyl-sided chain remained unchanged. (Fig. 8b). Thus, introduction of one cis-double bond at the central position of the alkyl chain induces an increase in the chain mobility, resulting in a new type of transformation of an interfacial melting. Conformational disordering of this kind was not detectable in \( \beta \). (b) The conformation of the polyethylene chains of \( \gamma \) and \( \beta \) is all-trans. It is likely that gauche conformations occur in the disordered methyl-sided chain of \( \alpha \). (c) \( \beta \) and \( \gamma \) differ most in the characteristic bands of the olefin groups; skew-cis skew' for \( \gamma \), whereas \( \beta \) may take skew-cis skew type conformation. (d) As for the subcell structure, \( \gamma \) shows typical spectral bands characteristic of \( T \). This supports the \( O' \) subcell, because \( O' \) may be included in the category of \( T \). The \( \beta \) form assumes a specific subcell structure differing from \( O' \) and \( T \) according to C-C progressive bands reflected in IR spectra. The inferred subcell of \( \beta \) suggests that the C-C zigzag planes of neighboring chains are not parallel to each other but, instead, somewhat inclined.

It is worth noting that the transformation of interfacial melting of \( \gamma \)-\( \alpha \) of oleic acid was also observed in \( \gamma \)-\( \alpha \) of palmitoleic acid and in \( \gamma \)-\( \alpha \) of erucic acid (Suzuki et al., to be submitted). This indicates a characteristic kind of chain disordering in unsaturated fatty acids having one cis-double bond.

Comparing the polymorphism of stearic and oleic acids, differences are seen both in the crystal structures and the thermal behaviors as described above. In addition, the kinetic behaviors of crystallization are different. The polymorphs of stearic acid crystallize in a different manner, depending on solvent, supersaturation and temperature. The quantitative differences in the nucleation rate (Sato and Boistelle, 1984) and the crystal growth (Beckmann and Boistelle, 1985) are up to approximately 50% under normal conditions of crystallization. In the case of oleic acid, however, the rate of crystallization of \( \alpha \) and \( \beta \) are very different. For example, \( \alpha \) exclusively solidifies from the melt with moderate cooling rates, e.g., 2°C/min as examined by DSC. On the contrary, only \( \beta \) occurs when the cooling rate is lower than 0.05°C/min (Suzuki et al., 1985). These differences demonstrate more diversified characteristics of both the thermodynamics and kinetics of the polymorphism of oleic acid. From this, one may conclude that the complexity can exist in the polymorphism of mixed saturated/unsaturated triglycerides. Independent or cooperative changes in the chain packing ad molecular motions of the saturated and unsaturated alkyl groups are likely to occur while sharing the common glycerol backbone. This is discussed in the next section.

**SOS**

The polymorphism of triglycerides has been studied extensively because of their importance in lipid chemistry (Small, 1986). The polymorphism of monosaturated acid triglycerides
has been well established, as far as the thermodynamic properties are concerned. In the literature, however, results are rather contradictory for mixed saturated/unsaturated acid triglycerides, despite the importance of these compounds in confectionery fats. For the same compound, inconsistent results are reported for the number and nomenclature of polymorphs, structures of the subcell and chain length, melting points and thermal behaviors, etc. This is shown in Table 3 which summarizes the nomenclature and the long spacing data of the polymorphs of SOS. Although not presented here, there is a wide variation in the melting point. This confusion might be attributed to the purity of sample employed, or experimental methods and instruments.

The authors have recently studied the polymorphism of a series of S_{\text{t}}O_{\text{s}} triglycerides (\text{S}\text{t}: \text{C}_{18}, \text{POP}; \text{C}_{18}, \text{SOS}; \text{C}_{20}, \text{AOA}, \text{and C}_{32}, \text{BOB}). Particular concern was given to purity of the samples and techniques for identification of individual polymorphs. Two samples, lower-purity (91%) and higher-purity (99%), were examined for each compound using the same thermal treatments to reduce the effect of purity. Polymorphs were identified by their characteristic X-ray diffraction patterns after obtaining a single melting or solidification peak. In taking these data, two thermal treatments were used: transformation from the polymorphs which were directly solidified from the melt at various temperatures and the melt-mediated transformation. The latter transformation is a re-solidification from the melt which was formed by rapidly raising the temperature to just above the melting point of a less stable form (Sato and Kuroda, 1987). The X-ray and DSC measurements were carried out simultaneously for each sample during the above thermal treatments. Polymorphs were named by taking into account the characteristic X-ray diffraction patterns, DSC data, and related previous work. This paper presents a summary of new results of SOS and POP (Sato et al., submitted to J. Am. Oil Chem. Soc.) and other S_{\text{t}}O_{\text{s}} compounds (Wang et al., 1987).

Five polymorphs of SOS were obtained at ambient temperatures (above 15°C): \(\alpha\), \(\gamma\), pseudo-\(\beta'\), \(\beta_2\), and \(\beta_1\), all of which occurred both in higher- and lower-purity samples. Additionally, the lower-purity samples contain another intermediate polymorph having X-ray short spacing spectra, e.g., three peaks of 0.435 nm, 0.419 nm, and 0.393 nm, similar to \(\beta_1'\) of tristearin (Simpson and Hagmann, 1982). This form, however, did not appear in the higher-purity samples and should be disregarded as a polymorph of pure SOS.

Each polymorph has the following characteristics: (a) DSC (2°C/min) reveals a single melting peak except for \(\alpha\) whose melting peak (around 23°C) was followed by solidification of \(\gamma\). (b) Thermal treatments exhibited successive irreversible transformations in the solid-state. (c) The X-ray short spacings reveal unique patterns for all polymorphs as shown in Fig. 9. Higher- and lower-purity samples gave identical short spacing spectra and there was no doubt in discriminating between specific spectra of \(\alpha\), \(\gamma\), pseudo-\(\beta'\). This is also true for long spacings, melting points and enthalpies of fusion, \(\Delta H_f\) (Table 3). The distinction between \(\beta_2\) and \(\beta_1\) is subtle, yet it has an important relevance. The short spacings, melting points and \(\Delta H_f\) are distinctly different but the long spacings have the same value. For short spacings, a strong peak for 0.458 nm is common, but the intensity ratios of other peaks show a clear contrast. Furthermore, the two peaks in \(\beta_2\) denoted by arrows (0.400 nm and 0.390 nm) are split into two in \(\beta_1\) and a peak in \(\beta_2\) denoted by

### Table 3. Long spacing (LS, nm), melting point (\(T_m\), °C) and enthalpy of fusion (\(\Delta H_f\); kJ/mol) of five polymorphs of SOS obtained in the present study and corresponding polymorphs in the literature.

<table>
<thead>
<tr>
<th>Form</th>
<th>Purity</th>
<th>LS</th>
<th>(T_m)</th>
<th>(\Delta H_f) (a)</th>
<th>(\Delta H_f) (b)</th>
<th>(\Delta H_f) (c)</th>
<th>(\Delta H_f) (d)</th>
<th>(\Delta H_f) (e)</th>
<th>(\Delta H_f) (f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)</td>
<td>91%</td>
<td>5.05</td>
<td>28*</td>
<td>-</td>
<td>IV</td>
<td>(\alpha)</td>
<td>(\alpha)</td>
<td>(\alpha)</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>99%</td>
<td>5.05</td>
<td>35.4</td>
<td>98.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(\gamma)</td>
<td>91%</td>
<td>7.37</td>
<td>37*</td>
<td>-</td>
<td>II</td>
<td>(\beta')</td>
<td>(\beta')</td>
<td>(\beta')</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>99%</td>
<td>7.35</td>
<td>35.4</td>
<td>98.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudo-(\beta')</td>
<td>91%</td>
<td>7.06</td>
<td>38*</td>
<td>-</td>
<td>II</td>
<td>(\beta')</td>
<td>(\beta')</td>
<td>(\beta')</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>99%</td>
<td>7.00</td>
<td>36.5</td>
<td>104.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\beta_2)</td>
<td>91%</td>
<td>6.50</td>
<td>42*</td>
<td>-</td>
<td>I</td>
<td>(\beta)</td>
<td>(\beta)</td>
<td>(\beta)</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>99%</td>
<td>6.50</td>
<td>41.0</td>
<td>143.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\beta_1)</td>
<td>91%</td>
<td>6.50</td>
<td>43*</td>
<td>-</td>
<td>I</td>
<td>(\beta)</td>
<td>(\beta)</td>
<td>(\beta)</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>99%</td>
<td>6.50</td>
<td>43.0</td>
<td>151.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) Daubert and Clarke, 1944), (b) Filer et al., 1946), (c) Lutton and Jackson (1950), (d) Malkin and Wilson, 1949), (e) Lavery (1958), (f) Landmann et al., 1960). (*) Examined by viewing transparency temperature of sample with eyes.
a filled triangle (0.375 nm) disappeared in \( \beta_1 \). The difference in \( \beta_2 \) and \( \beta_1 \) is also manifest in the melting curves examined by DSC. Figure 10 depicts DSC curves of the five polymorphs of SOS recorded while heating (2°C/min). When a sample revealing the superimposed X-ray short spacing spectra of \( \beta_2 \) and \( \beta_1 \) was heated, double melting peaks were detected. Subsequent results confirmed that the higher-melting peak increased in size as the duration of tempering increased.

Typical thermal treatments for obtaining the five polymorphs of SOS are as follows. \( \alpha \) is formed by chilling the melt below 23°C. \( \gamma \) is formed either by transformation from \( \alpha \) (e.g., over 12 h at 17°C) or by the solidification from the melt in a temperature range of 24–28°C. The melt-mediated transformation from \( \alpha \) also yielded \( \gamma \) in the same temperature range. \( \gamma \) transformed to pseudo-\( \beta' \) by tempering over 1 h at 30°C. The melt also solidified into pseudo-\( \beta' \) around 29–36°C, although very slowly. The transformations of pseudo-\( \beta' \) to \( \beta_2 \) and \( \beta_2 \) to \( \beta_1 \) occurred over 10 h at 35°C and 11 days at 40°C, respectively. These data confirm the five individual polymorphs of SOS.

The chain length structure of \( \alpha \) is double, Fig. 2, but in contrast, triple chain length structures are revealed in the other four forms. This means that a conversion in the chain length structure occurs during the transformation in the solid-state.

Analysis of other \( \text{SOS}_1 \) compounds show that AOA and BOB possess the same five polymorphs as SOS with regard to X-ray short and long spacings (Wang et al., 1987). POP, however, displayed unusual behavior. \( \alpha, \gamma, \beta_2 \) and \( \beta_1 \) are found as in all the \( \text{SOS}_1 \) compounds, but three more intermediate forms were also obtained. They were named \( \delta, \text{pseudo-} \beta'_2 \) and pseudo-\( \beta_1 \). The latter two forms show the X-ray short spacing spectra similar to pseudo-\( \beta' \) of SOS, but subtle differences were also observed. Furthermore, their chain length structure was double chain-length. Pseudo-\( \beta'_2 \) and pseudo-\( \beta'_1 \) were formed either via the transformation from \( \gamma \) or via the melt solidification and differed in melting point by about 2.5°C. Pure \( \delta \) occurred during the melt solidification in a narrow range of the solidification temperature. Consequently, a total of seven polymorphs were obtained in POP.

The description of the polymorphism of the \( \text{SOS}_1 \) compounds is concluded with justifications for the nomenclature of the different polymorphs. \( \alpha \) corresponds to what is commonly seen in glycerides, being characterized by a single short spacing around 0.42 nm. \( \gamma \) has a strong short spacing line at 0.472 nm, which is not seen in any polymorphs of saturated fatty acids and glycerides. Therefore names of \( \beta' \) (Filer et al., 1946), \( \beta'' \) (Malkin and Wilson, 1949), and sub-\( \beta \) (Lutton and Jackson, 1950, Lavery, 1958) may be inadequate. This peak was observed only in \( \gamma \) of oleic acid (Fig. 7). The name of pseudo-\( \beta' \) is used because the short spacing spectra are similar to patterns of \( \beta' \) for \( \text{SOS}_1 \), and because Gibon et al. (1986) gave the same name to the polymorph of POP having features equivalent in the present study. \( \beta_1 \) and \( \beta_2 \) have the X-ray short spacings similar to \( \beta \) of \( \text{SOS}_1 \) with increasing subscripts denoting decreasing melting point. Use of Roman numerals (I, II, etc.) (Daubert and Clarke, 1944; Landmann et al., 1960), or of A, B, etc., for POP (Lovegren et al., 1971) is disregarded since they are far from the nomenclature traditionally employed in glycerides.

At present, the structures of the subcell and unit cell, and the molecular conformations of the \( \text{SOS}_1 \) polymorphs are unknown. Due to subtle differences in X-ray diffraction patterns, slight variations in molecular structure are possible. In this regard, a possible variation may be a change within the saturated or unsaturated lamellae which are separated into the different layers in the triple chain length structure. Since the polymorphic behaviors of the unsaturated and saturated acids are quite different, it is highly feasible that multiple polymorphic forms of \( \text{SOS}_1 \) may occur due to independent or cooperative molecular changes in the two different lamellae. Vibrational spectroscopic measurements and X-ray structure analyses using single crystals should give decisive data. Work is in progress.

**Cocoa Butter Polymorphs**

The peaks of medium strength in the X-ray patterns of \( \beta_2 \) and \( \beta_1 \) are very similar to those of Forms V and VI, respectively, of cocoa butter (Fig. 11) (Wille and Lutton: 1966, Garti et al., 1986). Furthermore, the difference in the melting point is of the same order of magnitude; 2.5°C between Forms V and VI of cocoa butter (Wille and Lutton, 1966) and 2.2°C between \( \beta_2 \) and \( \beta_1 \) (Table 3).
Physical and Molecular Properties of Lipid Polymorphs

To verify these similarities, the polymorphism of a mixture of POP/POS/SOS (wt% ratio, 18.2/47.8/34.0; purity: POP, 99.2; SOS, 99.0; POS, 98.3) was examined using the thermal treatments previously described (Sato et al., unpublished). The same DSC and X-ray short spacing patterns were obtained as those of \( \beta_2 \) and \( \beta_1 \) of SOS and POP. The melting points of \( \beta_2 \) and \( \beta_1 \) of the mixture were 33.2°C and 35.3°C, respectively. In addition, mixtures of POS/SOS with a lower content of POS, or without POS, also revealed essentially the same results. From this it is concluded that the \( \beta_2 \) and \( \beta_1 \) polymorphs are characteristic forms in pure \( \text{SOS} \) triglycerides and their mixtures, and that the transformation of Forms V \( \rightarrow \) VI in cocoa butter is the result of a polymorphic transformation of the \( \text{SOS} \) fractions from \( \beta_2 \) to \( \beta_1 \). Wille and Lutton (1966) compared the X-ray data of a mixture of SOS (25%), POS (50%) and SOO (25%), and reported the similarity of its pattern to the \( \beta \) polymorph of SOS and POP. No distinction was made, however, between “two \( \beta \)” forms of the two triglycerides.

As for the mechanism of the V \( \rightarrow \) VI transition in cocoa butter, the present consideration agrees better with ideas based on the solid-state transformation (Garti et al., 1986), yet contradicts those which assume that the V \( \rightarrow \) VI change is caused by the separation of a portion rich in high-melting fat from a lower-melting portion of cocoa butter (Manning and Dimick, 1985). Obviously, the actual process of the V \( \rightarrow \) VI transformation in cocoa butter is rather complicated. At elevated temperatures (around 30°C), the higher-melting fractions of cocoa butter, mainly POP/POS/SOS, coexist with liquid oil which consists of the lower-melting fractions. Therefore, transformations mediated in liquid oil may also take place: dissolution of \( \beta_2 \) (Form V) in oil, and re-crystallization of \( \beta_1 \) (Form VI). The transformations mediated via oil (solution)-mediated and via solid-state may concurrently take place. In both processes, however, the basic physical features may be regulated by two different polymorphs of the \( \text{SOS} \) triglycerides.

Conclusion

The polymorphism of stearic acid, oleic acid and SOS has been discussed. It was shown that, physical and molecular characteristics in the polymorphism is quite different between stearic and oleic acids. This difference is caused by introduction of one cis-double bond at the center of the aliphatic chain in oleic acid. Therefore, one may expect that the molecular natures of the polymorphs of various unsaturated fatty acids may be more complicated, depending on the number, position and configuration of the double bond. Extensive work should be devoted to the study of these unsaturated fatty acids, since data are quite lacking.

Knowledge about the polymorphism in mixed saturated/unsaturated acid triglycerides is rather scarce or contradictory. This should be overcome, since many naturally-important triglycerides consist of saturated and unsaturated acyl chains, e.g., cocoa butter. Examples presented in this review, POP and SOS, indicate that the numbers of individual polymorphs are increased and their molecular natures become more complicated in comparison to monoacid triglycerides. More detailed analysis for the crystal and molecular structures of all polymorphs of \( \text{SOS} \) or other mixed triglycerides must be necessary.

In these researches, one may have to employ pure sample to get essential features of the polymorphism of each compound to reduce the effects of impurities. Then, the complicated polymorphism of lipids in real systems which consist of varying fats and fatty acids can be analyzed using the data of pure substances. Furthermore, multiple techniques for identification of the individual polymorphs must be applied to the polymorphism of complicated triglycerides, since the differences in thermal and structural behaviors between the polymorphs may be rather subtle. In this regard, to get the molecular properties, spectroscopic methods are very convincing.

References


**Discussion with Reviewers**

**J.W. Hagemann:** Saturated monoacid triglycerides of even chainlength less than 16 carbons exhibit a third intermediate melting polymorphic form. Using this analogy, do you perceive that the three new intermediate forms of POP reflect an effect due to the shorter chainlength acids? Since the new forms are double chainlength structures, is it possible that chain reordering is occurring between the carboxyl group and double bond? **Author:** Complex intermediate polymorphs were found only in POP. Therefore, we think that this complexity is caused by certain interaction between shorter length of saturated acyl chain with oleic acid. Among three intermediate forms of POP, pseudo- and pseudo- are double chainlength but is of triple chainlength, although all of their X-ray short spacing spectra are similar to that of pseudo- of SOS. There are two transition circuits in the crystal after melt crystallization: . Both undergo conversions in the chainlength structure; (double →) triple → double → triple. Presumably these conversions may be accompanied with reordering of oleic and palmitic acyl chains. Thus, it is possible that, in forms of POP, specific lateral packings consisting of oleic and palmitic acyl chains may exist in the same lamella.

**K. Larsson:** The name α-form in paraffins, simple esters and glycerides corresponds to crystals with a hexagonal (or pseudo-hexagonal) subcell. The α-form of oleic acid seems to have the triclinic chain packing. Thus the type of disorder is different from that of triglycerides, and might motivate a different name not to cause confusion. What is your view? **Author:** We intend to give a proper nomenclature for the polymorphism of unsaturated fatty acids using Greek characters, knowing that the similar nomenclature for glycerides (α, β and β) has been established in lipid chemistry. Primary concern was paid to discriminate between saturated (even-numbered, A, B etc.; odd-numbered, A’, B’, etc.) and unsaturated fatty acids. In doing so, Greek character was chosen because of its convenience more than Roman numerals, etc. First, we actually feared.
that some confusion might arise, e.g., between α of glycerides and α of oleic acid as you pointed out. No similarity is seen both in molecular conformation and subcell structure between these two α-forms, although solidification behaviors look alike. I hope, however, that this confusion will be solved in accordance with progress in research for varying unsaturated fatty acids. I would note that the α-form was observed in a few unsaturated fatty acids, being characterized by an interfacial melting as briefly mentioned in the text. In addition, it was found that the γ-form also exists in erucic and palmitoleic acids, exhibiting the same molecular conformation and subcell structure as γ of oleic acid.

**K. Larsson:** Your proposal of independent molecular change in the two different lamellae of SOS is interesting. Do you think it would be possible that the unsaturated chain layer even could "melt" below the saturated chain layer, corresponding to a liquid-crystal formation?

**Author:** In each polymorph of SOS, no significant change was detected in X-ray short spacing spectra taken at 5°C and just below its melting point. This means that the lateral packings of stearic and oleic lamellae are uniquely fixed in each polymorph. However, we have no convincing information on oleic acyl chains in any forms, e.g., whether in crystalline state or in liquid-crystalline state, since discrimination in X-ray short spacing patterns between the two lamellae is very difficult. Yet, preliminary studies with Raman technique indicate that the oleic chains are rather disordered in γ and pseudo-β′, but more ordered in β2 and β1. Further systematic study is needed to solve this important problem.
Abstract

Differences in clotting between human and cow's milk in the stomachs of infants are discussed. Gastric pH, after ingesting milk, of an infant up to 6 months of age stays at a pH range of 4-5, near the isoelectric point of casein, and never reaches the value of 2, which is found in adults. Pepsin (or gastricsin) can hydrolyze proteins at this pH range. Gastric emptying time is shorter with human milk than with cow's milk which appears to be correlated to the smaller size of human milk clots. Elimination of a readily coagulable fraction of casein from cow's milk by restricted rennet action produced a-casein-rich milk with similar coagulating properties to that of human milk. Although pepsin digestibility at pH 2 was greater for bovine whole casein than bovine β-casein-rich fraction or human casein, this difference was minimized or even reversed at pH 4. This was ascribed to the difference in clotting behavior of αs1-casein and β-casein, namely a harder clot of the former. Therefore, the difference in clotting and proteolytic properties between human milk and cow's milk in an infant's stomach can be explained from the difference in chemical properties of their major caseins, i.e., β-caseins and αs1-caseins in human milk and cow's milk, respectively.

Introduction

Digestion of protein in the stomach by pepsin is generally considered as a preliminary step to the digestion in the small intestine by more powerful proteases, i.e., trypsin, chymotrypsin and several peptidases. Since pepsin hydrolyzes the sites in peptide linkages which are different from the sites of hydrolysis by proteases in duodenal juices, the role of digestion in the stomach cannot be ignored, though it may be supplemental in the complete digestion of proteins in the digestive tract. In the case of infants, it is generally agreed that up to 3 months of age pepsic activity is low and that minimal protein digestion occurs in the stomach (Berfenstam et al., 1955). Buchs (1973), however, suggested that the main physiological role of pepsin was to split off a few amino acids or peptides which stimulated the release of gastrointestinal hormones after they had reached the duodenal lumen.

Because of the important roles played by stomach digestion at the early, but essential, stage of complete digestion of proteins, it may be useful to know the meaning of clotting in the stomach in the digestion of milk (Ruegg and Blanc, 1982). The main concerns are the significance of clotting of cow's milk in comparison to that of human milk within the stomachs of human infants.

Gastric Functions in Infants

Gastric pH

Hydrochloric acid production is observed in the stomachs of infants soon after birth. Although newborn infants have a neutral to slightly alkaline gastric pH, within 24 hours after birth, gastric acid secretion reaches a peak comparable to that in a 3-year-old child. However, 2 days after birth, gastric acid secretion decreases rapidly, and a low level is maintained for at least 3 weeks (Harris and Fraser, 1968). The mean pH in the stomachs of 1-2 day old infants has been reported to be 3.0-3.1 but after the intake of milk the pH quickly rises due to a strong buffering capacity of milk. Hydrogen ion concentration in the stomach of full-term infants is estimated to be less than 30% of that in the adult stomachs (Lebenthal et al., 1983). Because of this difference, while pH in the stomachs of adults decreases to below 2 within 2 hours after ingestion of milk, in the case of infants up to 5 months of age, pH frequently stays between 4-5, even 2-3 hours after the intake of milk (Nakai, 1962).
Fig. 1. pH-Activity profiles of pepsins in stomach juices from different mammals.  
(a) Hemoglobin profile: a mixture of diluted stomach juice and 2% hemoglobin after pH adjustment was incubated at 35°C for 30 min and A280 of the 12% trichloracetic acid filtrate was measured. A280 value for the original juices before dilution was plotted.
(b) Edestin profile: a mixture of diluted stomach juice and 0.5% edestin after pH adjustment was incubated at 35°C for 30 min. The turbidity after addition of sulfosalicylic acid was measured as A£60 and digestion rate (%) was computed from a standard curve (linear for A660 vs edestin).

It is interesting to note that immunoglobulins (Ig), which are useful for preventing pathogenic infection in an infant's intestine, can pass through the stomach without destruction as Ig are not stable at pH below 4, but are stable above pH 4 (Kaneko et al., 1985).

Pepsin secretion
Agunod et al. (1969) and Deren (1971), reported that during the first day of life, the pepsic activity was one-fifteenth that in the adult. During the next 4-month period, the pepsin output increased sevenfold. Over this period, the pepsin secretion changed in accordance with the hydrogen ion secretion. In infants who were 2 years old, pepsin output per kg body weight became roughly comparable to that observed in adults.

When the pH-activity profile was measured, human stomach juice had 2 peaks compared to a single peak for stomach juices from most other mammals (Fig. 1). This double-peak property is dependent on the substrate. For example, infantile stomach juices had pH optima at 1.5 and 3.2 for edestin and 1.9 and 2.8 for hemoglobin, while only one peak of pH 2.2 was observed with casein (Nakai, 1962). However, this double peak property was not absolutely distinct, as purified hog pepsin showed a double-peak property at pH 1.8 and 2.6 using edestin with a smaller pH difference between the 2 peaks than human pepsin, while a single peak was observed for hemoglobin and casein.

It was postulated that there could be another protease present in the stomach with a higher optimum pH than pepsin, which would explain digestibility in the infantile stomach even at pH higher than the optimum pH < 2 of pepsin. This other enzyme has been isolated and identified as gastricin by Tang et al. (1959). Since gastricin is produced from the same zymogen as pepsin depending on gastric pH (Tang, 1970), and also it is as strongly proteolytic as pepsin (pepsin A, EC 3.4.23.1), it was categorized as pepsin C (EC 3.4.23.3) by the Nomenclature Committee (1984) for the International Union of Biochemistry.

Despite having an optimum pH (pH 2.8) similar to that of chymosin (pH 3.8 on hemoglobin), gastricin is proteolytic, and not as milk clotting as chymosin (EC 3.4.23.4). There was no evidence for the presence of chymosin in the stomach of infants, even in those fed with cow's milk-based formula (Komura et al., 1957; Malpress, 1967).

Gastric emptying
Effects of age on the gastric emptying time are evident, although comparison of published data from different studies must be made with caution in order to match data using identical liquid meals in volumes appropriate to the size of the stomach. Gastric emptying time is usually measured from the percentage of a meal remaining in the stomach (residual volume, V%) plotted against time after consumption of the meal. The stomach contents are withdrawn at certain time intervals after intake of the test meals containing an indicator, e.g., phenol red. From the
concentration of the indicator in the samples withdrawn, V is calculated. The half time is frequently used as the time required for V to fall to 50%.

Hunt and Spurrell (1951), Blumenthal et al. (1979), and Pildes et al. (1980) reported an average half-emptying time of 21.8 min and 44.6 min for adults and infants, respectively, when carbohydrate solutions were ingested. For cow's milk which clots in the stomach, the half time was extended to 45 min (Heading et al., 1976) and 87 min (Signer and Fridrich, 1975), respectively.

It is generally accepted that gastric emptying is delayed in premature infants compared to full-term infants. Gupta and Brans (1978) showed that during the first 12 hours of life, preterm infants emptied a smaller portion of dextrose solution than full-term infants.

In infants it appears that the type of meal affects emptying time. Most infants receiving breast milk had a rapid early phase with logarithmic decline followed by a linear phase of emptying (Cavell, 1979). In contrast, most of the infants fed a cow's milk formula had either a delayed early emptying phase followed by a linear emptying pattern or a linear emptying pattern from the beginning. In general, the overall gastric emptying time was slower with cow's milk than with human milk. An example with premature infants showed the half-emptying time of 25.1 min vs. 51.9 min for human milk and cow's milk, respectively (Cavell, 1979).

**Milk Clotting and Proteolytic Activity**

**Pepsin vs. chymosin**

Although almost all proteinases clot cow's milk, the enzymes which have optimum pH in the acidic side (pH < 5) have a similar molecular structure and contain aspartic acid residues essential for proteolysis. Therefore, the enzymes in this category are called "aspartyl proteinases".

These enzymes have similar sequences: a high level of similarity (57%) was observed between pepsin and chymosin, while 25% similarity was observed between chymosin and a bacterial rennet (Mucor miehei proteinase). However, penicillopepsin (the acid proteinase produced from the mold Penicillium janthinellum) had similarity values of only 23% and 22% with chymosin and pepsin, respectively (Yada, 1984). The milk clotting activity of proteinases, in units, has been defined as the amount of proteinase which clots 10 ml of reconstituted skim milk in 100 sec at 30°C; the specific activity is then expressed as milk clotting activity per mg proteinase. Proteolytic activity may be determined as the ability to hydrolyze sodium caseinate and expressed as amount of tyrosine released per mg proteinase (Yada and Nakai, 1986).

Milk clotting abilities expressed as the ratio of milk clotting to proteolytic activity were 95.8 and 47.0 for chymosin and pepsin, respectively, compared to 60-70 for microbial rennets and less than 0.3 for the proteinase from Asp, saltol and for penicillopepsin. It appears, therefore, that the protein sequence itself may be relatively unimportant for milk clotting activity.

The three-dimensional structures of aspartyl proteinases are bean shaped with a long substrate binding cleft between two domains (Breath, 1981). The major catalytic functions of chymosin are derived from the carboxyl groups of Asp 32 and 215.

![Fig. 2. Hypothetical illustration of the action of chymosin on \( \kappa \)-casein.](image)

The ionized carboxyl group of Asp 32 activates a water molecule. The proton donor is Tyr 75 which is rather mobile; upon binding with the substrate, its phenolic OH-group moves near the NH-group of the susceptible peptide bond of the substrate (Fig. 2). Discriminant analysis of electrical and hydrophobic parameters, zeta potential, surface hydrophobicity, and circular dichroism data showed the importance of \( \beta \)-sheet, \( \beta \)-turn, and random structure for milk clotting activity of proteinases (Aishima et al., 1987). For the coagulation of casein micelles, the \( \kappa \)-casein component, particularly its hydrophobic moiety, i.e., para-\( \kappa \)-casein portion, should be located near the two active Asp residues and Tyr residue in the vicinity of the cleft area of the enzymes (Fig. 2). Hydrophobic property inside the cleft may assist in the access to and proper orientation of \( \kappa \)-casein. More detailed information on the three-dimensional structures of the substrate and enzymes is required for explaining the milk clotting activity, especially the difference in the mechanisms between pepsin and chymosin which are similar in sequence.

**Clotting of Cow's Milk and Human Milk**

Clotting of milk in the abomasum by chymosin is important for the digestion and absorption of protein in calves fed with milk. It has been well established that chymosin splits the peptide bond between Phe 105 and Met 106 of the \( \kappa \)-casein molecule yielding para-\( \kappa \)-casein and a macropeptide. This destroys the protective functions of \( \kappa \)-casein, resulting in precipitation of \( \kappa \)-caseins and \( \kappa \)-casein in the presence of \( \text{Ca}^{++} \). Stomach clots delay gastric emptying and thus
improve protein and fat digestion (Huber, 1969). When calves were fed with whole milk, clot prevention treatments resulted in a decrease in weight gains, feed efficiency and digestibility of dry matter, and marked increase in post-feeding levels of plasma amino acids and urea nitrogen (Jenkins and Emmons, 1982). Therefore, clotting of milk in the stomach is a physiological prerequisite for the completion of the protein digestion mechanism in calves.

In the case of human infants, the situation is reversed. The gastric emptying time is faster with human milk than with cow's milk (Cavell, 1979). Slight or no clotting of human milk is observed in the infantile stomach.

In vitro experiments using adult rats to study the effect of clotting on gastric emptying and digestion of bovine caseins have been reported (Miranda and Pellissier, 1981). The diet containing bovine skim milk clotted in the rat stomach, and resulted in a significantly higher amount of sediment remaining in the stomach 30 minutes after ingestion, indicating a reduction in the rate of gastric emptying compared to an unclotted diet based on 3% whole bovine casein solution in water. Electrophoresis of the remaining stomach contents indicated little proteolytic degradation in the clotted diet, whereas breakdown products from αs1-, β- and κ-casein could be identified from the unclotted diet.

Ultrastructural studies of the milk curd in the gastric lumens of suckling rats (Berendse, 1982) indicated an appearance similar to that reported for bovine milk curd and cottage cheese (Kalab, 1981). There was no ultrastructural evidence of intragastric proteolysis in suckling rats up to 15 days of age.

To reproduce milk clotting in the human stomach, the American Dairy Science Association (1941) proposed a standard method for measuring curd tension using a pepsin-HCl solution simulating gastric juice. "Soft curd milk" was defined as milk with a curd tension value below 20 g compared to values of 50-60 g for regular cow's milk. Curd tensions of both human milk and evaporated cow's milk were 0 g, meaning very fine coagulum formation or no clotting at all.

However, the pH of the clot according to this method was about 6.2 which was excessively high, thus corresponding only to a very early stage of stomach digestion even for infants. When an in vitro digestion test was carried out using a pepsin-HCl solution to simulate the physiological conditions, it was found that "soft-curd milk" no longer produced soft curd, and even evaporated cow's milk formed discernible curds while human milk showed either no clot or almost undetectable very fine curds (Fig. 3). None of the methods suggested for making soft-curd milk, i.e., dilution, heating, calcium reduction, and homogenization, were effective in simulating the clot of human milk under the physiological conditions in the infantile stomach (Nakai, 1963a). To withstand the stomach pH of a young infant, prevention of acid clot of casein, in which calcium is not involved in the coagulation mechanism, is essential.

**Humanization of Cow's Milk**

Cow's milk is reported to contain approximately 3.5 g protein/100 ml, whereas human milk usually averages 1.2 g/100 ml (George and Lebenthal, 1981). In addition, there are significant differences in the protein composition of the two milks, human milk having a much lower ratio of casein to whey proteins. Furthermore, it is generally agreed that the major casein in human milk is a β-casein-like fraction. Traditionally, casein has been classified into α-, β- and γ-caseins based on their electrophoretic mobilities. Nagasawa et al. (1967) found that casein was the major casein in human milk, which did not contain calcium-sensitive α-casein. Toyoda and Yamauchi (1973) concluded from the sedimentation rate, optical rotary dispersion and circular dichroism data that the major fraction of human casein was similar to bovine β-casein, based on the temperature-dependent polymerization and molecular structure. Human β-casein, like bovine β-casein, produced a γ-casein-like degradation product as a result of pepsin hydrolysis (Azuma et al., 1985). In contrast, cow's milk contains about 45% αs1-casein (Packard, 1982; Schmidt, 1982), a fraction which is either absent or present in minute quantities in human milk.

Upon acid precipitation, human milk produces a much finer protein flocc than cow's milk (Fig. 4). The fine clot of human milk in the stomach apparently shortens the gastric emptying time as compared to the coarser cow's milk clot. Although the casein micelles of human milk are much smaller and presumably more digestible than those of cow's milk, they remain unchanged for 3 hours after nursing (Hadorn, 1981). In vitro studies demonstrated that the initial rate and extent of hydrolysis by pepsin were much greater at pH 2 for bovine milk than for human milk (Li-Chan and Nakai, manuscript submitted). At pH 4, the initial rate of hydrolysis was higher for human milk, but the extent of hydrolysis after 60 minutes was greater for bovine milk.

Preliminary experiments indicated that upon acidification to pH 4 in the presence of 11 mM CaCl₂, a fine soft floc was formed in the case of bovine β-casein, in contrast to a sticky hard clot for bovine αs1-casein. In the absence of calcium ions, clear solutions were formed at pH 2 with both caseins, while at pH 4, a hard clot was observed for αs1-casein, whereas β-casein solution was turbid with no visible evidence of clotting. Figure 5 shows the time course of hydrolysis of these casein fractions by pepsin, measured as absorbance at 280 nm (A₂₈₀) of the 2.5% trichloroacetic acid-soluble fraction during proteolysis. At pH 2, the increase in A₂₈₀ was more rapid and extensive for αs1-casein than β-casein (Fig. 5a); on the other hand, at pH 4, most rapid and
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Fig. 4. Micrographs of (a) 1.25% human milk, and (b) 1.25% bovine milk samples acid clotted at pH 4.

Fig. 5. Pepsin hydrolysis curves at (a) pH 2 and (b) pH 4 of bovine α51-casein and β-casein with and without 11 mM CaCl2. (Hydrolysis conditions: 0.49% casein, 0.005% pepsin, 37°C).

extensive hydrolysis was observed with β-casein, especially in the absence of CaCl2 (Fig. 5b). Tan and Whitaker (1972) also reported that the initial rates and extents of hydrolysis of caseins by both chymosin and pepsin generally decreased with increasing pH from 3.0 to 6.0; the initial rates of hydrolysis decreased in the order of α-, κ- and β-casein, at pH 3.0, 5.5 and 6.0. However, for β-casein, the extent of hydrolysis by 4 different enzymes (chymosin, pepsin, M. pusillus protease and E. parasitica protease) was greater at pH 3.5 than at 3.0, and hydrolysis at pH 3.5 was more extensive for β-casein than the other caseins. This is probably related to the formation of harder clots with α51-casein than with β-casein as the clotting pH approaches the isoelectric points of these proteins.

By careful control of the reaction conditions for chymosin activity to give only "partial" clotting, it was possible to prepare a modified milk with coagulability similar to that of human milk (Fig. 3). A limited amount of rennet was used which would result in coagulation of part of the protein after heating (Nakai, 1963b).

Recently, we have re-investigated the soluble casein fraction, recovered after mild rennet modification at neutral pH for partial coagulation of bovine casein, especially with respect to studying its clotting behavior and hydrolysis by pepsin at acidic pH (Li-Chan and Nakai, manuscript submitted). The process for rennet modification of a 2% bovine casein

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4 Rennet is a common term referring to the commercial crude preparation which contains chymosin. In the work described here, the source of enzyme was "rennin" (Product R7751 from Sigma Chemical Co., St. Louis, MO) and the terms "rennet" and "rennin" have been used interchangeably.
solution is shown in Figure 6. Analysis of electrophoretic patterns demonstrated that the ratio of \( \alpha_1 \)- to \( \beta \)-casein was increased from 0.7 in the unmodified bovine casein, to higher than 3.0 in the soluble fraction recovered after rennet treatment (Fig. 7). Protein recovery in this fraction, rich in soluble \( \beta \)-casein, was 20-25%, yielding a 0.5% protein solution. This falls within the average range for casein concentration normally found in human milk. Thus, the preferential coagulation of \( \alpha_1 \)-casein by this process can result in a \( \beta \)-casein-rich cow’s milk that more closely resembles human milk with respect to casein concentration and composition.

Upon acidification of these casein solutions, it was observed that hardness of the clot formed at pH 2 or 4 decreased in the order of bovine, rennin-modified and human casein (Fig. 8). Large hard clots were formed by the acidification of bovine casein, especially at pH 4, whereas smaller clots were observed with rennin-modified casein. Turbid suspensions containing very fine protein flocs were observed upon acidification of human casein solutions.

Scanning electron microscopy of the fine flocs of human casein in solution acidified to pH 4 showed very tightly clustered protein particles (Fig. 9a). In contrast, the larger bovine casein clots consisted of a fairly dense lattice-like network of protein particles (Fig. 9b), while rennin-modified bovine casein formed a looser network (Fig. 9c). Rennin modified bovine milk also formed a loose structure but the globular particles were larger than those observed for casein (Fig. 9d).

At pH 2, the extent of hydrolysis by pepsin after 60 min incubation followed the order bovine > rennin-modified > human casein (Fig. 10a). On the other hand, at pH 4, the extent of pepsin proteolysis was greatest for rennin-modified casein (Fig. 10b). Both experiments were carried out at a protein concentration of 0.5%. The trends for pepsin hydrolysis of bovine control and rennin-modified caseins thus resemble those for \( \alpha_1 \) vs. \( \beta \)-caseins, respectively (Figs. 5a, 5b). Both bovine whole casein and \( \alpha_1 \)-casein showed much greater susceptibility to pepsin hydrolysis at pH 2 than pH 4, which may be related to the harder clot formed as well as the lower activity of pepsin at the higher pH. On the other hand, \( \beta \)-casein and rennin-modified casein (which is predominantly \( \beta \)-casein) were still hydrolyzed at a moderate rate, even at pH 4, which may be related to the open loose structure of these caseins. Similar trends were observed when the protein concentration of casein solutions as substrate for pepsin hydrolysis was increased to 2% or when milk samples (1.25% protein) were used as substrates. However, when the protein substrate concentration was only 0.1%, none of the casein samples yielded large clots at either pH 2 or 4, and the differences in their rates and extent of hydrolysis were also minimal. These results suggest that both the clot formation as well as the composition of the casein fraction (e.g., \( \alpha_1 \)- vs. \( \beta \)-casein) affect proteolysis, especially at pH 4 which is similar to the gastric conditions of infants.

A possible reason why bovine \( \alpha_1 \)-casein was more digestible than \( \beta \)-casein at pH 2 may be due to the accessibility of peptide linkages susceptible to pepsin hydrolysis. Since A280 was used to monitor release of 2.5% TCA soluble peptides, the higher aromatic amino acid content of \( \alpha_1 \)-casein than \( \beta \)-casein may also partly explain the differences in A280 as a function of hydrolysis time. However, reversal of
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Fig. 8. Micrographs of (a) 0.5% human casein, (b) 2% bovine casein, and (c) 2% rennin-modified casein, clotted at pH 4.

Fig. 9. Scanning electron micrographs of (a) human casein, (b) bovine casein, (c) rennin-modified casein and (d) rennin-modified milk clotted at pH 4. Black bar = 2 μm; 20kV; arrows in (a) point to dense clusters of casein particles. Samples acidified to pH 4 were fixed with 2.5% glutaraldehyde. After rinsing with 0.1M sodium cacodylate buffer (pH 7.4), the particles were treated with 1% osmium in cacodylate buffer, rinsed, then further fixed with 2% uranyl acetate, prior to dehydration (30-100% alcohol) and critical point drying. Dried samples, mounted on slabs with conductive silver cement, were coated under argon with 30nm thickness of gold and observed in a Cambridge 250T scanning electron microscope.
this situation at pH 4 may have been due to the clotting difference at pH close to the isoelectric points of the caseins. Because the αs1-casein clots were firmer, pepsin could not penetrate through and the degree of hydrolysis was considerably decreased.

The reasons for the difference in clotting at the isoelectric points between the two caseins are unknown. In general, protein solubility is explained based on the interrelation between charge and hydrophobicity (Hayakawa and Nakai, 1985). If this concept is accepted, the solubility of proteins at the isoelectric point should be controlled solely by hydrophobicity, as the net charge is minimal at the isoelectric point. However, the content of hydrophobic amino acid residues is higher in β-casein than αs1-casein (Eigel et al., 1984). This means that β-casein should form harder clots than αs1-casein, which is opposite to what is observed. A possible explanation is that most of the hydrophobic side chains in β-casein are not fully exposed due to some steric hindrance. The degree of phosphorylation is in the order of αs1-casein, β-casein and human β-casein (8, 5 and 0-5 moles of phosphate per molecule, respectively). Although the formation of strong salt bridges at the isoelectric point in the presence of calcium ions is unlikely, it is possible that some salt bridge formation due to a strong dissociating ability of the phosphate radicals at pH 4.8 contributes to the formation of firm clots.

Although human and bovine β-caseins have been reported to be homologous with respect to the amino acid sequences (Greenberg et al., 1984), the temperature of polymerization of human β-casein is higher than that of bovine β-casein, i.e., 20°C vs. 8.5°C (Toyoda and Yamuchi, 1972) and antigenic reactivities are different between the two β-caseins (Otani et al., 1984). Furthermore, human κ-casein differs from its bovine counterpart in having a much higher carbohydrate content, i.e., 40% vs. 5%, and shows a greater stabilization of αs1-casein than bovine κ-casein in the presence of calcium ions (Yamauchi et al., 1981). Unlike bovine κ-casein, human κ-casein is present in a monomeric form at pH 7 (Azuma et al., 1984). These differences in β- and κ-caseins may be a reason for differences in casein micelle formation and acid clotting between human and cow's milks. The tight protein clot structure observed by SEM for acidified human casein compared to the looser structure of the bovine casein clot (Fig. 9) may explain the lower extent of proteolysis by pepsin of human milk than cow's milk.

Preferential coagulation of bovine αs1-casein fraction by rennet treatment yields a β-casein rich milk which forms softer clots and is more susceptible to pepsin digestion at pH 4 than bovine milk. β-casein enrichment in cow's milk has also been reported by Rose (1968) by ultra-centrifuging the milk at 4°C to sediment αs1-casein rich micelles. Thus simulation of human milk clots in the infant stomach is now feasible to a certain extent. However, the significance of the more detailed differences between human and bovine species in their α- and β-caseins as well as other protein and non-protein constituents requires further studies, before the true meaning of this simulation for human feeding can be assessed.

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Discussion with Reviewers

Reviewer IV: In Figs. 5 and 10, have the authors considered (or corrected for) effects of differing aromatic amino acids in \( \alpha_{\text{s}1} \) and \( \beta \)-caseins? This might affect the relative positions of their curves.

Authors: It is true that the differing aromatic amino acid contents of \( \alpha_{\text{s}1} \) and \( \beta \)-casein would affect their absorbance at 280 nm (Tyrr). The contents are approximately 14 and 24 residues per monomer for \( \beta \)-casein and \( \alpha_{\text{s}1} \)-casein, respectively (Thompson, 1971), while A280 values at 1% are 4.6 and 10.1, respectively, (Sober, 1972). However, it is not possible to directly correct the absorbance values shown in Figs. 5 and 10 for these differences since there may not be any direct correlation between the total content of aromatic amino acid residues in a protein and the extent of their release by pepsin in the form of 2.5% TCA-soluble peptides. The differing aromatic amino acid contents may explain in part the observation that A280 values of the soluble fraction after pepsin hydrolysis at pH 2 for 20-60 min have been almost double for \( \alpha_{\text{s}1} \)-casein compared to \( \beta \)-casein (Fig. 5a).
However, the reversal of this trend at pH 4 (Fig. 5b), which shows higher A280 for the soluble fractions from \( \beta \)-casein than \( \alpha_{\text{s}1} \)-casein, strongly suggests that the extent of hydrolysis was greater for \( \beta \)-casein at pH 4. To confirm this, a different method to quantitate hydrolysis in the TCA-soluble fraction would be required, e.g., Kjeldahl nitrogen or biuret reaction. In this regard, the results of Tam and Whitaker (1972, text reference) using 2,4,6-trinitrobenzenesulfonic acid to follow peptide bond hydrolysis indicated higher extents of hydrolysis of \( \alpha \)-casein at pH 3.0 and 5.0, but higher extents of hydrolysis of \( \beta \)-casein at pH 3.5.

Reviewer IV: What is the yield of rennin-modified casein (in the process shown in Fig. 6)? What do the authors propose to do with the residual \( \alpha_{\text{s}1} \)-k-casein complex?

Authors: The yield of rennin-modified casein is approximately 20-25% of the starting casein. The residual casein complex could be useful as a food ingredient, or perhaps as a process cheese ingredient.

P.B. Berendsen: Although the magnifications in Figures 9a, b, c, and d are the same, the casein micelles in Figure 9d appear to be larger. Has this simply resulted from choice of areas or did the treatment of the sample shown in Figure 9d enlarge the casein micelles?

Authors: Firstly, the particles in these samples should not be referred to as micelles. The caseinate samples (Figs. 9a and b) were prepared by acidifying milk to pH 4.6 at 40°C. The rennet modification process to prepare samples in Figs. 9c and d would also destroy the micellar structure. The differences in particle sizes were definitely not due to a choice of area; the micrographs depict typical sizes of particles in each of the samples after clotting at pH 4. The difference between samples in Figs. 9a, b, c, d, versus that in Fig. 9d, is that the latter represents rennin-modified milk at pH 4 whereas the other three were casein samples at pH 4. The basis for these differences is not clear to us.

P.B. Berendsen: Have you done, or are you aware of any studies which compare the caseins of colostral milk or milk at the initiation of suckling with those later during milk secretion? If so in what way do they differ?

Authors: Ruegg and Blanc (1982, text reference) have published a review on the structure and properties of particulate constituents of human milk, including changes at different stages of lactation. These changes include the absence of the characteristic band of \( \beta \)-casein in electrophoretic patterns of colostral or early milk (1 to 4 days post partum), and a decrease in citrate concentration and increase in average diameter of casein particles with advancing lactation.

J.J. Strandholm: What, if anything, is known regarding the effects of longer gastric emptying time of cow's milk compared to human milk on the nutrition or health of infants?

Authors: We are not aware of any published studies that deal with this question.

Additional references

EFFECTS OF DRYING TECHNIQUES ON MILK POWDERS QUALITY AND MICROSTRUCTURE:
A REVIEW

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Abstract

The quality of milk powders is markedly affected by the composition and properties of the milk, the manufacturing procedures, thermal processing during manufacture and, in particular, the drying technique itself. A variety of physico-chemical analytical methods, including scanning electron microscopy, has been used to obtain information on the effects of the various factors on the microstructure of the milk powders. Roller-drying, which has recently lost its commercial importance, produces a sheet of dried milk that is powdered in a hammer mill. The resulting powder consists of compact particles with sharp edges. Powders obtained by spray-drying are in the form of more or less regular globules which may have their surface convoluted to a varying extent. Inside, the particles are porous. Lactose present in the particles is in an amorphous glass form. In instant milk powders, the powder particles are agglomerated and lactose is partly converted into microcrystalline form.

Introduction

The technique of drying of milk was developed many centuries ago. Initially, the milk was dried primitive ly in the sun. One of the earliest reports was made by Marco Polo. In his 13th century journeys, he described the production of dried milk products and their subsequent rehydration and consumption [in reference 14]. However, industrial production of dry milk was initiated only in 1810. At that time, Nicholas Appert in France developed and described a procedure of evaporating milk to one third of its initial volume in an open vessel [14]. This product was made in a pill form and was air-dried to remove residual moisture [33]. The first commercial dry milk production was based on a British patent granted to Gimson in 1855. Sodium or potassium carbonates and sucrose were used as ingredients in the dried milk [14]. The manufacture of a milk powder free from any alien ingredient was first reported in 1898 for which a number of patents was granted following extensive experimentation. Soon afterwards, in 1902, the first drum-drying equipment was designed and put into service. The development of spray-drying equipment and procedure can be traced to a patent granted to Percy in 1872 in the U.S.A. [in reference 30]. Improvements have been made since then.

The introduction of an instantizing procedure characterized by two-stage drying [18, 34-36] has markedly improved the drying technique and improved the quality of the milk powder. More recently, a three-stage spray-drying procedure is used to produce a superior product at a low energy consumption [2, 3, 8, 18, 24, 26, 30].

Under certain conditions, dried milk products have several major advantages over fresh milk products. These advantages include lower mass and volume than the fresh products which means that they may be stored in a smaller storage space with no special storage requirements, and may be transported more easily and at a lower cost.

Drying of milk balances the supply of milk with the demand for it. Dried surplus milk can be stored for long periods of time. Thus, it forms a stable food reserve for future use. The demand for dried milk is rapidly increasing in developing countries. There, the milk is reconstituted in recombining dairy plants and is used to combat malnutrition [11, 25]. Because dried milk products retain their high quality for a long time, they are irreplaceable in human nutrition in hot climates. They are also of importance in averting starvation in catastrophic situations such as earthquakes.
epidemics, or wars. It is essential, of course, that the local population can digest milk. For those who cannot, it is possible to produce low-lactose milk powders. Thus, milk powders are used on a global scale to correct the imbalance between the need for high-quality nutrients and their temporary scarcity.

The drying technique markedly affects the physical, chemical, and microbiological properties of the finished product. The objective of this review is to compile information on the effects of the drying techniques most commonly used on the structure and related properties of powdered milk.

Principles and Techniques of the Drying of Milk

An objective of modern dairy technology is to produce high-quality milk powders. Following reconstitution, the products should closely resemble fresh milk particularly as far as nutritional and sensory properties are concerned. However, milk powders manufactured for various uses in the food industry may differ in quality parameters. The following powders are produced most commonly: skim-milk powder, partly skimmed milk powder, and whole-milk powder. Of the three types listed, skim-milk powder is the dairy ingredient most frequently used in the food industry. Thus, it represents the largest part of the milk powder production. There are several reasons for its popularity, all of which are associated with the absence of milk fat in the product. One reason is the resistance of the skim milk powder to the development of rancid off-flavour during long-time storage. Another reason is the easy recombination of the powder with vegetable fats which makes it possible to adjust the fat content in the reconstituted milk product to the desired level.

Skim milk powder is graded according to the heat treatment of the skim milk during production. The amount of undesired whey protein present in the powder, expressed in mg N/g of powder as the 'whey protein nitrogen index' (WPNI), is closely related to the heat treatment. In low-heat powders, WPNI exceeds the value of 6.0, in medium-heat powders, WPNI ranges from 1.51 to 5.99, and in high-heat powders, WPNI is below 1.5. Varying heat treatments impart various properties to the skim-milk powders produced. Low-heat powder is used, e.g., in recombined pasteurized milk and cream production because it ensures that the resulting products are free of a cooked flavour and thus resemble fresh milk products [44]. High-heat powder is commonly used as an ingredient in bakery products. Bread produced with this powder has an increased water-binding capacity and improved flavour and texture characteristics [13], with the loaf shrinkage considerably reduced. To produce reconstituted evaporated milk products from milk powders, the desired heat stability is achieved by high-temperature heat treatment of the skim milk prior to powder production [8, 44]. Thus, because of their functional and nutritional properties, various milk powders are used as ingredients in many foods. The reasons leading to the use of the powders in the foods have been discussed in greater detail elsewhere [13, 17, 24, 27-29, 31].

Functional properties of milk powders include emulsification, fat absorption, system stabilization, water binding, viscosity, gelation, texturization, consistency formation, plastic properties formation, fibrous structure formation, adhesion and cohesion, aeration, foam stabilization, flavour enhancement, and increased yield of the product. Because of these functional properties, it is beneficial to use milk powders as ingredients in many foods to improve their quality and appeal. A wide range of applications of milk powders was recently reviewed by several authors [8, 9, 13, 7, 27-29, 31]. Milk powders are used directly or indirectly in foods made at home, in bakery products, confectionery products, meat products and meat substitutes, convenience foods, beverages, and dietetic products. They are also used in pet foods and animal feed.

Currently, there are several technological systems used in the dairy industry to dry milk:

- Roller drying
- Spray drying:
  - Centrifugal atomization
  - Pressure atomization
  - Foam spray drying
  - Steam swept wheel atomization
  - Venturi spraying
  - Two-stage spray-drying system producing non-agglomerated powders (1970)
  - Three-stage spray-drying system producing either agglomerated or non-agglomerated powders (1990)
- Freeze drying

Only systems which are commonly used in the dairy industry will be discussed in greater detail in this paper.

Spray-drying systems have been the subject of intensive research and development in the past few years although consideration has also been given to some other drying systems. However, these attempts failed. The current trend is to continuously modify and improve the spray-drying systems.

A decade ago, a two-stage drying system with an external vibrating fluid bed was developed for the production of non-agglomerated powders [39]. Using this system, processing has been improved economically and the products have attained higher quality because lower drying temperatures were used during the final phase of drying. This method is also used to produce milk powder which is less hygroscopic than regular milk powder.

The introduction of a stationary fluid bed integrated in the drying chamber is the most recent innovation of the spray-drying technique. The stationary fluid bed was developed and introduced in industrial use in the 1980's. This development makes it possible to maintain a higher moisture content in the milk during the initial stage of drying and is characterized by a lower outlet air temperature in comparison with the other spray-drying systems. This means that the economic as well as the quality parameters are improved and a free-flowing agglomerated powder is produced. The flow chart of this three-stage drying process is shown in Fig. 1.

The newest spray-drying procedure has been described in detail by several authors [1, 39, 40, 43] and the corresponding processing performances are presented in the technical documentation by the producers of the equipment [2, 3].

Effects of Drying Techniques on the Microstructure and Related Properties of Dried Milk

Initial electron microscopic observations of milk and milk products were made in Switzerland by Hostettler and Imhof [16] in 1952. Buma and Henstra [7] were the first to publish micrographs obtained by scanning
Milk Powder Quality and Microstructure

Quality, i.e., a complex of physico-chemical and sensory attributes of the dried milk products, is influenced by the following factors acting on milk during the manufacturing process, or later, during storage:

(a) chemical composition of the raw materials (high fat or protein content in the dairy product, presence of non-dairy ingredients),
(b) manufacturing techniques and parameters (separation of fat, preheat treatment, homogenization, evaporation),
(c) drying techniques and conditions (roller drying, various methods of spray drying, instantization, multi-stage drying),
(d) storage conditions (packaging materials and methods, temperature and duration of storage, water activity in the product).

Of all the factors mentioned above, the drying techniques and conditions affect the microstructure (dimensions, shapes, and density of the particles) to the greatest extent. Main definitions and a possible classification of food powders according to their physical characteristics have been published in detail by Peleg [37]. Bulk properties of the powders are determined by their physical and chemical characteristics as well as by the processing system (Fig. 2) [30].

Roller Drying

Roller drying is accomplished by a direct heat transfer from a hot drum into a thin layer of evaporated milk. After the water present in the milk evaporates, the solids are scraped off and pulverized in a hammer mill. Consequently, the dry particles have a characteristic structure (Figs. 3 and 4). The irregular shapes and sharp edges of the particles reveal that the powder was produced as a result of crushing. The particles are compact and contain no occluded air. Even if some aeration takes place in the pipelines while evaporated milk is being conveyed to the drums, the air escapes from the milk during roller drying. The milk is


electron microscopy (SEM) of spray-dried milk powders in 1981. SEM has been used more frequently [6, 7, 39-22, 38] than transmission electron microscopy (TEM) [5, 32] to study the structure of milk powders. This is because each electron microscopic technique provides a different type of information. SEM is appropriate to study the surface morphology as well as internal structure of the milk powder particles whereas TEM techniques such as thin-sectioning [32] or freeze-fracturing [5] make it possible to identify the individual powder constituents such as casein micelles and fat particles.

Fig. 2. Effects of various factors on the bulk density of spray-dried milk powders. Source: [30].
deactivated irrespective of whether the atmospheric pressure or the vacuum roller-drying procedures are used. This is evident from the equally compact structures of the resulting powders. Although the thickness of the evaporated milk layer on the hot drum may affect the particle size, the final dimensions of the particles are determined by the hammer mill. Despite the compactness of the roller-dried milk particles, their irregular structure contributes to a powder. Low powder transportation costs per kg of powder. Bulk densities of non-fat milk powders produced by various processes are presented in Table 1 [14].

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</thead>
<tbody>
<tr>
<td>Spray-drying</td>
<td>0.50 - 0.60</td>
</tr>
<tr>
<td>Roller-drying</td>
<td>0.30 - 0.50</td>
</tr>
<tr>
<td>Foam spray-drying (USDA*)</td>
<td>0.32</td>
</tr>
<tr>
<td>Spray-drying (commercial)</td>
<td>0.26</td>
</tr>
<tr>
<td>Instant spray-drying (commercial)</td>
<td>0.59</td>
</tr>
</tbody>
</table>

*United States Department of Agriculture.
Source: Hall and Hedrick [14].

Spray Drying

Atomization of fluid milk into small droplets usually causes the spray-dried milk powder particles to be spherical with diameters in the range of 10 to 250 μm. The particles contain occluded air in the form of vacuoles varying in dimensions. Some powders have a large central vacuole (Fig. 5) whereas in other powders, e.g., those produced by centrifugal atomization, small vacuoles are distributed relatively evenly across the particles (Fig. 6).

The surface of spray-dried particles is usually smooth but may also be wrinkled. The vacuoles in both the smooth and wrinkled particles occasionally contain minute dried milk particles. The external appearance as well as the internal structure of milk powders were studied by Buuma and Henstra [7]. These authors used a nozzle atomization pressure of 7.85-9.80 MPa (80-100 kg/cm²). They found that even particles of the same powder, e.g., skim-milk powder, may have different superficial structures (Figs. 7 and 8). The presence of smooth particles as well as particles having severe surface folds in the same sample is attributed to the different drying conditions to which the individual particles were exposed. Buuma and Henstra [7] have suggested that the formation of the deep surface folds in the skim-milk powder particles is caused by the presence of casein in the skim milk. This suggestion is based on a comparison of micrographs of a spray-dried skim-milk powder (Figs. 7 and 8) with those of a spray-dried whey powder (Fig. 9). As far as the drying conditions are concerned, there is a greater tendency to form wrinkles as the temperature of the inlet air is increased. Also, large temperature differences between the hot air and the milk powder particles [15] contribute to the formation of the wrinkles. Generally speaking, centrifugal and nozzle atomization of milk destined for spray-drying result in the production of powder particles that have similar morphology. As far as the bulk density and the free-flowing characteristics of the powders are concerned, the nozzle atomization system appears to be preferred to the centrifugal disk atomization. On the other hand, higher feed concentrations may be used with the latter system. This means that the energy consumption is lower with the centrifugal disk atomization system [43] than with the nozzle atomization system.

De Vilder et al. [45] studied the effect of the spray-drying conditions on the physical characteristics of whole-milk powder using the centrifugal atomization system. When the inlet air temperature was raised to 225 °C from 155 °C, the bulk density and the mean powder particle density were decreased whereas the vacule volume was increased. This was evidently caused by rapid drying and moisture expansion in the powder particles. A similar finding was made earlier by the same authors [45] with powders produced by nozzle atomization. These authors also studied the effects of outlet temperatures varied from 70 °C to 105 °C while the inlet temperature remained constant at 195 °C. At a low (70 °C to 95 °C) outlet air temperature, uniform drying was achieved throughout entire powder particles. Because there was little expansion of air at that temperature, the combined volume of the vacules was small. Higher outlet temperatures (95 °C to 105 °C) led to overheating of the milk, expansion of the trapped air bubbles, formation of cracks in the particle surface, a higher combined vacule volume, and a low mean density of the powder particles. Consequently, the bulk density of such milk powder was low. High outlet air temperature may lead to high porosity of the powder particles if the concentration of total solids in the milk to be dried is low [11]. The fact that the outlet temperature has the greatest effect on the overall milk powder quality has been well known [8, 12, 26, 30, 38]. De Vilder et al. [45] also found that the bulk and mean powder particle densities were decreased as the number of the atomizer revolutions was increased.

Bloore and Boag [4] studied the effect of nozzle atomization on the quality of the resulting skim milk powder using an empirical approach. They reported regression equations for the solubility index, bulk density, powder particle density, and powder particle dimensions as related to operation variables of the spray-drying equipment used. The regression equations obtained were in the form of second-order polynomials having the following 5 variables: (1) the total solids content of the concentrate to be spray-dried, (2) the feed rate, (3) the atomization pressure and (4) temperature, and (5) the inlet air temperature. The authors concluded that a high atomization pressure was preferable.

Müller [32] studied the distribution of casein micelles in spray-dried milk powders and found that they retained their globular nature. This finding was confirmed for skim-milk powder by Kalab and Emmons [21] who examined spray-, roller-, and freeze-dried skim-milk powders by TEM and found 3 kinds of microstructure present. They found that powder microstructure failed to reflect the differences in the preheat treatment of the milk destined for spray-drying. Low-heat, medium-heat, and high-heat powder particles appeared to be similar.
Fig. 3. SEM of a roller-dried skim milk powder shows irregular particles with sharp edges.

Fig. 4. Detail of a roller-dried skim milk powder particle showing minute lactose crystals (arrow) on its surface.

Figs. 5 and 6. Large central vacuoles (Fig. 5, asterisk) or evenly distributed small vacuoles (Fig. 6, asterisks) are present in spray-dried skim milk powder produced by centrifugal atomization of the skim milk.

Fig. 7. SEM of spray-dried skim milk powder produced by nozzle atomization of the skim milk shows various extents of surface wrinkling. (Courtesy of T. J. Buma and S. Henstra [7]).

Fig. 8. Fractured particle of spray-dried skim milk. The interior of the particle is compact with a few small vacuoles (arrows). The surface of the particle is wrinkled. (Courtesy of T. J. Buma and S. Henstra [7]).
Various dairy products, e.g., buttermilk, subjected to the same spray-drying technique were characterized by diverse morphological features (Fig. 10). Such differences may be used to detect adulteration of spray-dried milk products with less costly products [20, 21].

Ultrafiltration may be used to increase the protein content in milk destined for spray-drying. During ultrafiltration, milk undergoes membrane fractionation of the constituents and the chemical composition of the ultrafiltered milk (retentate) differs from that of the original milk. In our experiments, milk retentates containing 20%, 27%, and 34% of total solids were produced on a UF Module Type 35-GR-6-P (DDS, Pasilac, Denmark). The retentates were dried [47] in a spray-drier Type LAB 1 (APV Anhydro, Denmark) at an inlet air temperature of 220°C and an outlet air temperature of 90°C. Centrifugal atomization was used in conjunction with one-stage drying. SEM of the spray-dried retentates showed that they possessed all external (Fig. 11) and internal (Fig. 12) structural features characteristic of regular spray-dried milk powder particles and were indistinguishable from them.

Fuchheim [5] freeze-fractured milk powders, replicated the fractures using platinum and carbon, and examined the replicas by TEM. Micrographs of low-heat (Fig. 13) and high-heat skim-milk powders (Fig. 14) and micrographs of whole-milk powder (Fig. 15) show casein micelles and fat globules as distinct entities dispersed in a lactose/salt matrix. The observations are in agreement with King's [23] finding that amorphous lactose partly forms the continuous phase in spray-dried milk powder particles. Although spray-drying is rapid, a small number of lactose crystals may be formed inside the powder particles. Warburton and Pixton [46]
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reported the presence of prism-shaped lactose crystals in freshly produced milk powder particles mounted in the Heinz fluid (Fig. 16). Saito [42] found no lactose crystals in freshly produced milk powders examined by SEM and X-ray diffraction analysis. However, he observed numerous prismatic and diamond-shaped lactose crystals after the milk powder particles were mounted in the Heinz fluid and concluded that the crystals developed during mounting. The presence of lactose crystals in milk powder is the sign of improper storage associated with exposure of the powder to high humidity.

Fig. 16. Light microscopy of prism-shaped lactose crystals in freshly produced spray-dried milk mounted in the Heinz fluid. (Courtesy of S. Warburton-Henderson [46]; copyright Slough Laboratory; magnification not shown).

Roetman [41] studied lactose crystal formation in spray-dried milk powders and the effect of lactose crystallization on the milk powder particle structure based on experiments with spray-drying various model solutions. His electron micrographs showed that crystalline lactose present in the milk powders was either 'precrystallized' (i.e., the crystals originated in the milk concentrate prior to drying) or were 'postcrystallized' (i.e., the crystals developed after spray-drying). The origin of the crystals was reflected by their shapes. Precrystallization resulted in a tomahawk form of the lactose α-hydrate. Needle-like crystals of the lactose β-anhydride were formed in postcrystallized products.

In spray-dried whole-milk powder, fat is present both at the surface of the particles in the form of pools and inside the particles in capillary pores and cracks. The portion of the which may be reached by fat solvents was called extractable fat by Buma [6] who studied it in greater detail. Another portion of the fat, which is present in larger amounts in homogenized than in nonhomogenized milk, is encapsulated in the powder particles where it is complexed with milk proteins; it cannot be extracted easily and is resistant to oxidation [6]. Because homogenization stabilizes the fat in this way, it is an important step in the production of spray-dried whole-milk powder; it is not, ordinarily, a required step in the processing schedule.

Dimensions of the spray-dried particles are influenced by the temperature of drying, concentration, and viscosity of the milk to be dried, and the atomizing system used. Foam spray drying produces the largest

Figs. 13 and 14. Freeze-fracturing reveals the internal structure and composition of skim milk powders: low-heat powder (Fig. 13) and high-heat powder (Fig. 14). C: casein micelles, CA: cavity in a fat globule, FG: fat globule, LS: lactose/salt matrix. (Courtesy of W. Buchheim [51]).

Fig. 15. Freeze-fracturing shows a high concentration of fat globules (FG) in whole-milk powder. C: casein micelles, CA: cavities in fat globules. (Courtesy of W. Buchheim [51]).

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Characterization of Spray-Dried Skim Milk Powders

<table>
<thead>
<tr>
<th>Atomization method</th>
<th>High-capacity single nozzle</th>
<th>Medium-capacity multiple nozzles</th>
<th>Low-capacity nozzles</th>
<th>Steam-swept wheel</th>
<th>Rotary wheel A</th>
<th>Rotary wheel B</th>
<th>Rotary wheel C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed conc. [% total solids]</td>
<td>42</td>
<td>44</td>
<td>42</td>
<td>45</td>
<td>46</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Inlet air temperature (°C)</td>
<td>195</td>
<td>170</td>
<td>170</td>
<td>180</td>
<td>200</td>
<td>200</td>
<td>250</td>
</tr>
<tr>
<td>Moisture content [%]</td>
<td>3.2</td>
<td>3.6</td>
<td>3.4</td>
<td>3.7</td>
<td>3.8</td>
<td>3.6</td>
<td>3.9</td>
</tr>
<tr>
<td>Bulk density of powder tapped 100x</td>
<td>0.62</td>
<td>0.72</td>
<td>0.72</td>
<td>0.79</td>
<td>0.62</td>
<td>0.76</td>
<td>0.71</td>
</tr>
<tr>
<td>Bulk density of powder tapped 1250x</td>
<td>0.65</td>
<td>0.76</td>
<td>0.77</td>
<td>0.83</td>
<td>0.65</td>
<td>0.80</td>
<td>0.74</td>
</tr>
<tr>
<td>Solubility index (mL)</td>
<td>0.3</td>
<td>0.8</td>
<td>0.2</td>
<td>0.3</td>
<td>0.1</td>
<td>0.05</td>
<td>0.1</td>
</tr>
<tr>
<td>Vol. of vacuoles [mL/100 g]</td>
<td>26.0</td>
<td>13.5</td>
<td>4.0</td>
<td>5.5</td>
<td>21.5</td>
<td>11.8</td>
<td>14.3</td>
</tr>
</tbody>
</table>

* A limited number of samples was available from commercial production. The listed operating conditions and other results may not represent the optimum.

Source: Plíšeký [38].

Powder particles. They are followed, in size, by particles obtained by centrifugal atomization. The nozzle atomization system produces the smallest powder particles.

A high quantity of occluded air in spray-dried milk particles is undesirable because it results in low bulk density of the powder. The quantity of occluded air is about the same in milk powders obtained by centrifugal and high-capacity pressure atomization. However, the bulk density of powders obtained by nozzle atomization in low-capacity plants is higher and this is evident from Table 2 [38]. Vacuoles in spray-dried milk particles originate as the result of air incorporated in concentrated milk prior to spray-drying and during the drying process. Evaporation acts like a deaerator, while atomization during spray-drying introduces air into the milk. Plíšeký et al. [38] designed a special atomizer called a 'steam-swept wheel'. Here, steam is introduced into the atomizer and, consequently, the air-liquid interface is replaced with a steam-liquid interface. The effect of atomization on the shrinkage of the milk particles is evident from Figs. 17 to 19 and from Table 2. A two-stage spray-drying system has a similar effect on particle shrinkage.

So-called instantization of milk powders is an additional development in the drying of milk. The objective is to agglomerate the primary milk powder particles in order to make their dissolution in water easier. Agglomeration changes the microstructure of the powders (Figs. 20 and 21). Partial conversion of the amorphous glass form of lactose into a microcrystalline form also takes place as the result of instantization. This is accomplished by maintaining a higher moisture content in the powder particles during the two-stage drying process. This moisture is removed following the agglomeration of the powder particles.

Milk powders produced in the three-stage spray-drying system are similar in appearance [15, 39, 40, 43] to instant powders obtained in the two-stage system (Figs. 20 and 21). The primary powder particles are agglomerated to a varying extent depending on the equipment and the drying process used.

**Figs. 17-19.** SEM micrographs showing the effect of atomization on the appearance of skim milk powders. Fig. 17: Steam-swept wheel atomization. Fig. 18: High-capacity nozzle atomization. Fig. 19: Medium-capacity nozzle atomization. (Courtesy of J. Plíšeký [38]).
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Conclusions

Modern, sophisticated dairy technology makes it possible to dry milk in powder form. Nutritional and sensory properties of the reconstituted products resemble fresh milk very closely. However, the quality of the final product is affected by a number of factors such as the chemical composition of the raw milk and its treatment, drying techniques, and storage conditions. The most recent developments in the spray-drying technology are aimed at producing a wide variety of milk powders at a low energy consumption and capital costs as low as possible. The powders produced should have excellent properties (free-flowing property and solubility) and retain high quality during storage.

Although many effects are interrelated, the drying conditions affect the microstructure and related properties most severely. SEM has been used more frequently than TEM to investigate the microstructure of the milk powders. Particle dimensions, shapes, and densities as well as the presence of lactose crystals can be easily evaluated by SEM. TEM is more suitable to identify the particular powder constituents. TEM studies have shown that casein micelles and fat globules are dispersed in an amorphous matrix composed of lactose, whey proteins, and salts. In whole-milk powders, fat is present both at the surface and in the capillary pores as well as in cracks inside the powder particles.

Spray-dried particles are mostly spherical and contain vacuoles of occluded air inside. The surface of spray-dried particles is usually smooth with a high occurrence of wrinkles and deep folds. The tendency to form wrinkles is greatest at high inlet air temperature and also when large temperature differences occur between the hot air and the milk particles. Centrifugal atomization as well as nozzle atomization, followed by spray-drying, produces powder particles of similar morphology. Steam-swept wheel atomization leads to particle shrinkage and a high bulk density of the powder. Physical characteristics of the powder particles such as vacuole volume, bulk density, free-flowing properties etc. are closely correlated with the particle microstructure. In contrast to spray-dried powders, roller-dried milk powder particles are compact and have characteristic irregular shapes with sharp edges.

Powder particle dimensions are influenced by the concentration and viscosity of the milk, the atomizing system, and the temperature of drying. Foam spray-dried particles are largest, particles obtained by centrifugal atomization and smaller and particles produced by nozzle atomization are smallest.

With instantization and integrated fluid bed technologies well established, it is assumed that the future trends in milk powder production will be in the modification of processes and equipment used. Electron microscopy will be a tool to evaluate the effects of the innovations. It will continue to provide information about the microstructure of the powders as related to their other physical properties.

Acknowledgments

The authors thank Dr. W. Buchheim for providing the micrographs in Figs. 13-15, Dr. T. Buma and Dr. S. Henstra for the micrographs in Figs. 8-10, Dr. J. Pisecky for the micrographs in Figs. 17-19, and Dr. S. Warburton-Henderson for Fig. 16. The authors also thank APV Anhydro A/S and A/S Niro Atomizer for supplying technical documentation. Appreciation is expressed to Prof. Dr. Greta Dominić for help with the manuscript and Dipl. Ing. Ljiljana Kulić for technical assistance. Electron Microscope Unit, Research Branch, Agriculture Canada in Ottawa provided facilities. Contribution 751 from the Food Research Centre.

References

TEXTURAL PROPERTIES AND MICROSTRUCTURE OF PROCESS CHEESE FOOD REWORK

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Abstract

Process cheese food was made using sodium citrate (2.7%) or trisodium phosphate (TSP, 2.7%) as emulsifying agents. No precooked cheese (rework) was used in some samples whereas in others the rework (20%) consisted of a cheese blend emulsified with sodium citrate (2.7%) and (a) briefly heated to 82°C, (b) heated to 82°C for 1 h, (c) heated to 82°C for 5 h, and (d) heated to 82°C for 5 h, frozen at -10°C for 24 h, and thawed at 4°C. Heating for extended periods of time produced so-called hot melt. When used as rework, hot melt considerably decreased the meltability of the product made. All samples under study were examined by light microscopy (LM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Gradual solubilization of the emulsifying agents in the cheese blend and emulsification of fat were visualized by LM and SEM. TEM revealed considerable changes in the protein matrices of the hot melt and the process cheese food made with TSP. Small electron-dense areas having a high affinity for osmium developed in both products, but their shapes and degrees of affinity for osmium were different. It was possible to detect the presence of hot melt used as rework in the process cheese food samples under study.

Introduction

Process cheese food is an emulsion prepared by cooking one or more varieties of natural cheese with other ingredients such as water, milk solids including butter, emulsifier, salt, and colouring agents to produce a homogeneous product. According to Canadian regulations [12], process cheese food should contain no less than 51% cheese and the final product should have a minimum 46% moisture and a minimum 23% of fat. Process cheese food can be packaged in cans or as blocks but is marketed most commonly in a slice form for convenience of use.

Processing conditions vary depending on the equipment used and the type of the cheese product to be made. A minimum temperature of 65°-70°C is desirable for processing [7]. In general, a high cooking temperature such as 80°-90°C with sufficient stirring and an appropriate holding time renders a uniform product and generally ensures its microbiological safety [9]. However, the heat treatment applied during cooking and holding varies and can become excessive at times. Excessive heating can occur, e.g., if there is a delay or stoppage in packaging lines. Consequently, the process cheese food emulsion thickens considerably and becomes difficult to extrude to form slices. The stiffened mass, which is called 'hot melt', is removed from the pipes using compressed air and is frozen until re-used. To be re-used ('reworked' or 'reprocessed' [7]), hot melt is thawed, shredded, and added as an ingredient into a fresh cheese blend. Thus, hot melt is a type of process cheese food rework, which can be defined as any process cheese food that is not packaged for sale although it meets the product specifications but is mixed with a fresh blend and processed again. Unlike regular rework (i.e., precooked cheese), however, hot melt sometimes increases the viscosity of a freshly cooked emulsion and changes the quality of the final product in an unpredictable way. It is not understood how hot melt affects the texture of the product.

The objective of this study is to examine the effect of hot melt (used as rework) on the microstructure and rheological properties of process cheese food.

Materials and Methods

Two series of experiments were carried out. In the first series, hot melt taken out from the pipes of a commercial plant was used as rework. Three types of rework were examined: (1) Regular process cheese food slice, (2) quickly frozen cooked process cheese food emulsion, and (3) hot melt.

Key Words: Electron microscopy, Hot melt, Light microscopy, Microstructure, Osmophilic protein areas, Pre-cooked cheese, Process cheese food, Rework.
The regular process cheese food slice used in this present study was a product of the usual kind from a process cheese plant. Hot melt was produced in the plant by slowly cooling (from 82°C to 4°C in 5 h) and freezing cooked process cheese food emulsion that was too thick to extrude. The quickly frozen process cheese food emulsion was prepared by rapidly cooling (from 82°C to 4°C in 10 min) and freezing a process cheese food emulsion cooked in the laboratory using the same cheese blend.

Preparation of process cheese food on laboratory scale

Mixtures of natural cheeses were shredded in a Hobart cutter/mixer (Model N-50, The Hobart Manufacturing Company, Troy, OH) and blended with other ingredients. Water, butter, and salt were added to adjust the composition of the product to 45% water and 24% fat, and sodium citrate (2.7%) was used as an emulsifier. The blend was cooked in a stainless steel container thermostatically controlled by a boiling water bath. With the agitation provided by a Crafco stirrer (Type ZR21, Canlab, Waterloo, Ontario, Canada), the internal temperature of 82°C was achieved in 3-5 min. Cooked emulsions were stored in 500 g plastic tubes at 4°C.

Hot melt was prepared on laboratory scale by holding the process cheese food emulsion made with sodium citrate at 82°C for 1 h and for 5 h to study the effect of two different heat treatments. The hot melt was then frozen at -10°C for 24 h, thawed at -4°C, shredded, and used as rework at 20% (w/w) in a fresh cheese blend. Sodium citrate or trisodium phosphate (TSP, 2.7%) was used as the emulsifying agent for the mixture.

Measurement of apparent viscosity

Immediately after the emulsion had been cooked, its apparent viscosity was measured using a Brookfield viscometer (Brookfield Engineering Laboratories, Stoughton, MA). The T-b type spindle was used at 4 rpm with the Helipath feature in operation. At the time of measurement, the temperature of the emulsion was 71 ± 2°C. Results are presented in poises.

Determination of firmness

The firmness of the process cheese food was determined after 24 h of storage at 4°C using a precision penetrometer (Precision Scientific Co., Chicago, IL). The cone penetrating into the cheese under a load of 150 g was 2.0 cm in diameter and was 3.2 cm high. Results are presented in depth of the cone penetration in millimeters.

Estimation of meltability

After the penetration test, samples were cut into disks 6 mm high and 40 mm in diameter and equilibrated for 2 h at 20°C in petri dishes. The equilibrated disks were then placed in an oven heated at 140°C and kept there for 6 min to melt. After cooling to 20°C for 30 min, the diameters of the melted disks were measured using a ruler. Results are expressed in millimeters.

Chemical analysis

Moisture of the process cheese food was determined by a vacuum oven method [1] and the fat content was determined using a modified Babcock test [6].

Microscopy

All process cheese food samples submitted for microscopic examination were first cooled in a refrigerator at 4°C for 48 h before vacuum packaging and shipping using a cooled container.

For light microscopy (LM), 1-2 mm cubes of unfixed process cheese food samples were frozen at -20°C and sectioned into sections 6-8 μm thick using a Cryo-Cut E microtome (Reichert-Jung Scientific Instruments, Belleview, Ontario, Canada). The sections were affixed to glass slides, air-dried, stained, and examined under a Zeiss Universal Research Photomicroscope (Carl Zeiss Ltd., Montreal, Quebec, Canada). The microscope was equipped with both a conventional brightfield illumination system and a III epil RS epil-illuminating condenser combined with an HBO 100 W mercury-arc illuminator for fluorescence analysis. An exciter/barrier filter set for maximum transmission at 450-490 nm/520 nm was used for fluorescence examination of sections stained with Acrimed Orange (BDH Chem. Ltd., Poole, England), or Nile Blue A (Eastman Kodak Co., Rochester, NY) according to the methods described by Yiu [13]. Unstained or stained sections were also viewed under crossed polarizers to examine the birefringence of crystalline structures. Photomicrographs were taken with Ektachrome 400 Daylight film and were processed as black-and-white prints.

For scanning electron microscopy (SEM), the process cheese food and the hot melt samples were cut into prisms, 1 x 1 x 10 mm. The prisms were fixed in a 3.5% aqueous glutaraldehyde solution at 20°C for 2 h, washed in water, dehydrated in a graded ethanol series, and dehydrated in carbon dioxide. The samples were then returned into absolute ethanol, frozen in Freon 12 cooled to -150°C with liquid nitrogen, and fractured under liquid nitrogen. The fragments were melted in absolute ethanol at 20°C and were critical point-dried in a modified Sandri PVT-3 critical-point drier (Tousimis Research Corp., Rockville, MD) using carbon dioxide. Dry fragments were mounted on aluminum stubs, sputter-coated with gold (a layer of gold approximately 20 nm thick) using a Technics Hummer II sputter coater (Soquelec Ltd., Montreal, Quebec, Canada) and examined in an AMR-1000A scanning electron microscope (AMRay Inc., Bedford, MA) operated at 10 kV. Micrographs were taken on 35-mm Kodak Plus-X film.

For transmission electron microscopy (TEM), the samples initially fixed in the 3.5% glutaraldehyde solution for SEM examination were cut into particles approximately 1 mm in diameter and these were postfixed at 20°C for 2 h in a 2% osmium tetroxide solution in a 0.05 M veronal-acetate buffer, pH 6.75, washed with the buffer, and dehydrated in a graded ethanol series. The dehydrated samples were embedded in a Spurr's low-viscosity medium (J. B. EM Service, Inc., Pointe Claire-Dorval, Quebec, Canada). Sections, approximately 90 nm thick, were taken using a diamond knife mounted in an OM U2 microtome (Reichert Optische Werke AG, Vienna, Austria) stained with uranyl acetate and lead citrate solutions [4, 5] and examined in a Philips EM-300 electron microscope (N. V. Philips, Eindhoven, the Netherlands) operated at 60 kV. Micrographs were taken on 35-mm Eastman Kodak Fine Grain Release Positive Film 5302.

Fat globule dimensions were evaluated from micrographs using an MOP-3 Digital Image Analyzer (Carl Zeiss, Inc., Don Mills, Ontario, Canada). The fat globules were grouped into size classes at 1.0 μm intervals. Particles in each range were assigned the mean diameter of that range which was used to calculate the total area of the sections. Corrections for true diameters were not used.

Results and Discussion

The commercially made regular process cheese food slice and the quickly frozen process cheese food emulsion were homogeneous soft products with a smooth texture. The hot melt sample was dry and crumbly, especially in the core region. Physical and chemical changes may take place in the hot melt before it is used as rework. Firstly, the core receives the most heat treatment before cooling due to the difference in the cooling rate depending on the
position in the container into which hot melt is placed. Secondly, solutes are known to migrate towards the centre of hydrated materials upon freezing [3]. The changes thus induced may contribute to the characteristics of the hot melt. When the hot melt is added to a fresh batch, it may lead to the formation of a thick and undesirable texture of the finished product.

Textural properties

Using the three types of rework of commercial origin mentioned above, five kinds of process cheese food were prepared in the laboratory (first column in Table 1). All the process cheese food contained 43 ± 1% moisture and 24 ± 1% fat, and its pH ranged from 5.5 to 5.7. The results of the apparent viscosity, firmness, and meltability tests are summarized in Table 1.

Table 1. Textural properties of process cheese food with rework.

<table>
<thead>
<tr>
<th>Composition of process cheese food samples</th>
<th>Apparent viscosity (penetrometer test)</th>
<th>Firmness (in mm)</th>
<th>Meltability (in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular PCF* (no rework)</td>
<td>200</td>
<td>12.9</td>
<td>62.8</td>
</tr>
<tr>
<td>Regular PCF* containing:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20% quickly frozen PCF*</td>
<td>400</td>
<td>11.4</td>
<td>48.5</td>
</tr>
<tr>
<td>20% PCF* slice</td>
<td>490</td>
<td>12.0</td>
<td>46.5</td>
</tr>
<tr>
<td>10% hot melt</td>
<td>750</td>
<td>11.3</td>
<td>45.9</td>
</tr>
<tr>
<td>20% hot melt</td>
<td>840</td>
<td>10.2</td>
<td>40.0</td>
</tr>
</tbody>
</table>

* Process cheese food

The apparent viscosity of cooked process cheese food emulsion was increased in general when any rework was added; the increase depended on its type and amount. The process cheese food slice and the quickly frozen process cheese food emulsion used as rework increased the apparent viscosity of fresh batches. The increase was considerably greater with the hot melt used as rework and was directly related to the amount of the hot melt added. At 71°C, the cooked process cheese food emulsion was easily pourable whereas the emulsion made with hot melt was too thick to pour.

Results of the penetrometer test show that rework in general decreased the depth of penetration, indicating that the firmness of the product was increased. The sample with the greatest amount of hot melt was the firmest, whereas the regular process cheese food with no rework, used as a control, was the softest. There is a positive correlation between apparent viscosity of the cooked emulsion and firmness of the product.

Products that are easy to melt, such as the regular (control) process cheese food containing no rework, have large melted diameters (Table 1). The finished product containing 20% hot melt almost did not melt at all under experimental conditions and, hence, had the smallest melted diameter. In the samples under study, the melted diameter showed a negative correlation with apparent viscosity and firmness of the process cheese food.

Microstructure

The process cheese food samples examined by microscopy are listed in Table 2.

Light microscopy. Clearly distinguishable mixtures of cheeses were revealed by LM in samples #11 and #20. Differences were found in the structure and chemical composition of both samples. A relatively dense protein matrix of hot melt (sample #4) and a less dense protein matrix with thin protein strands of the regular process cheese food blend (sample #1) were demonstrated using Acridine Orange (Figs. 1 - 4). Staining with Nile Blue A showed that hot melt contained an apparently lower concentration of fat globules than the regular process cheese food blend (Figs. 5 and 6). An absence of crystalline structures in the hot melt component and their abundance in the regular process cheese food base was noticeable when both samples were examined under polarized light (Figs. 7 and 8). It is evident that sodium citrate crystals used to make the hot melt component had been dissolved in the aqueous phase of the cheese during the preceding processing treatment. Crystals of the emulsifying salts added to the fresh cheese blend, i.e., sodium citrate in sample #11 and TSP in sample #17, were still noticeable at the beginning of processing when the temperature of the cheese blend reached 71°C.

Table 2. Process cheese food samples examined by microscopy.

<table>
<thead>
<tr>
<th>Number: Composition:</th>
<th>Temperature:</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1 PCF* + NaCit**</td>
<td>cooked to 82°C</td>
</tr>
<tr>
<td>#2 PCF + NaCit</td>
<td>held at 82°C for 1 h</td>
</tr>
<tr>
<td>#3 PCF + NaCit</td>
<td>held at 82°C for 5 h</td>
</tr>
<tr>
<td>#4 PCF + NaCit</td>
<td>frozen at -10°C for 24 h, thawed at 4°C</td>
</tr>
<tr>
<td>#5 PCF + NaCit</td>
<td>71°C</td>
</tr>
<tr>
<td>#6 PCF</td>
<td>76°C</td>
</tr>
<tr>
<td>#7 PCF</td>
<td>82°C</td>
</tr>
<tr>
<td>#8 PCF + NaCit + 20% of #1</td>
<td>71°C</td>
</tr>
<tr>
<td>#9 PCF</td>
<td>76°C</td>
</tr>
<tr>
<td>#10 PCF</td>
<td>82°C</td>
</tr>
<tr>
<td>#11 PCF + NaCit + 20% of #4</td>
<td>71°C</td>
</tr>
<tr>
<td>#12 PCF</td>
<td>76°C</td>
</tr>
<tr>
<td>#13 PCF</td>
<td>82°C</td>
</tr>
<tr>
<td>#14 PCF + TSP***</td>
<td>71°C</td>
</tr>
<tr>
<td>#15 PCF</td>
<td>76°C</td>
</tr>
<tr>
<td>#16 PCF</td>
<td>82°C</td>
</tr>
<tr>
<td>#17 PCF + TSP + 20% of #1</td>
<td>71°C</td>
</tr>
<tr>
<td>#18 PCF</td>
<td>76°C</td>
</tr>
<tr>
<td>#19 PCF</td>
<td>82°C</td>
</tr>
<tr>
<td>#20 PCF + TSP + 20% of #4</td>
<td>71°C</td>
</tr>
<tr>
<td>#21 PCF</td>
<td>76°C</td>
</tr>
<tr>
<td>#22 PCF</td>
<td>82°C</td>
</tr>
</tbody>
</table>

* Process cheese food
** Sodium citrate
*** Trisodium phosphate

Both the fluorescence and polarizing microscopy studies revealed a distinct boundary between the hot melt component and the regular process cheese food base in samples that had been heated to 71°C. However, no distinct boundary was observed in samples heated at higher temperatures. Apparently, hot melt became uniformly dispersed in the blend soon after the temperature of 71°C had been exceeded.

Scanning electron microscopy. Process cheese food samples examined by SEM were considerably smaller than those examined by LM. This difference in size is
stipulated by the technique used. Dehydration in ethanol, freezing, and critical point-drying of samples destined for SEM require that the samples be small. In small samples, however, it was difficult to find borders between the two kinds of material, i.e., the regular cheese food blend and hot melt.

The starting material, which was obtained by cooking a fresh cheese blend to 92°C (sample #1, Table 2) contained a great number of crystals of sodium citrate [8] used as the emulsifying agent (Fig. 9). By holding the blend at this temperature for 1 h (sample #2) and also for 5 h to give it an excessive heat treatment and to produce hot melt (sample #3), the occurrence of the sodium citrate crystals was markedly reduced (Figs. 10 and 11). Following freezing to −10°C for 24 h and thawing, there was almost a complete absence of sodium citrate crystals in sample #4 (Fig. 12). No structural difference between the base and the rework was detected in samples #8, #9, and #10, all of which consisted of a regular cheese blend containing 20% of sample #1, but differed slightly in terms of the degree of processing. A similar finding was made in samples #17, #18, and #19, all of which contained the same ingredients as those present in the samples mentioned above except the emulsifying agent which was TSP.

There was a marked difference, however, in the processing conditions to which the two components were exposed in samples #11 to #13 and in samples #20 to #22. Although the small samples (6 mm) made it difficult to find sites which would show both components side by side, minute compact areas were occasionally found in some of the samples under study, e.g., in sample #12 (Fig. 13). The process cheese food blend emulsified with TSP (sample #14, Fig. 14) resembled in general the cheese blend emulsified with sodium citrate (sample #1). Fig. 9) except that the structure was coarser, e.g., the fat globules and the crystals of the emulsifying agent were larger. In addition, calcium phosphate crystals [2, 4, 8] were abundant (Fig. 14) apparently as a result of using a phosphate-based emulsifying agent. Compact areas were present in this product (Fig. 15) although no rework was used. It is doubtful, therefore, that compact areas noticeable by SEM also in other samples of this experimental series could be related to the presence of hot melt. Mixtures of the base cheese blend and hot melt emulsified with TSP, i.e., samples #20 to #22, contained a higher concentration of the compact areas, some of which were quite large (Fig. 16), than samples #11 to #13, where such areas were very rare. Gradual dissolution of TSP in these process cheese food samples was also evident.

It was assumed that using different emulsifying agents would make it easier to distinguish hot melt from the process cheese food base. However, LM showed (Figs. 7 and 8) that there was a structural difference between a briefly processed cheese blend and hot melt even if both products were made using the same emulsifying agent. The occurrence of sodium citrate crystals was high in the briefly processed cheese and low in the hot melt because most of the crystals had dissolved during the extended period of heating. This finding was confirmed by SEM.

LM studies showed that hot melt dispersed readily in the cheese blend emulsified with either sodium citrate or TSP. Hence, LM appears to be more suitable for detecting the dispersion of hot melt in the process cheese food blend than SEM. However, neither technique helped explain why physical properties such as firmness and meltability were changed in the finished product if it contained hot melt.

Transmission electron microscopy. The higher resolution provided by TEM, as compared with LM and SEM, made it possible to examine the structures of the process cheese food and hot melt samples in greater detail. At a low magnification, TEM was used to study the nature and the size distribution of emulsified fat particles whereas structural details of the protein matrix were examined at higher magnifications.

Fat particles were counted and measured in composite micrographs of total areas of approximately 6250 μm² using a Zeiss MOP-3 digital image analyzer. The fat particles, enlarged 4000x, were classified (class intervals of 1 μm) for diameters. Because the areas slightly differed from each other, the data were adjusted to the standard area of 6250 μm², related to the dimensions of the sections and the 60-mesh hexagonal grids used to support them.

In the hot melt (sample #4), emulsification appeared to be more advanced than in the regular process cheese food (sample #1) and the number of the fat particles in the hot melt was higher by almost 25% (Fig. 17). The mean diameters were converted into mean radii (shown in Figs. 17 and 18) and these were used to calculate the circular areas occupied by the fat particles. The latter distribution is presented in Fig. 18. Assuming that there was no change in the total volume of the fat present in the process cheese food samples, the total area occupied by the fat particles in the sections in the micrographs should have remained the same: the empirical difference of approximately 11% must, therefore, be considered as an acceptable experimental error. There was a higher number of...
coalescing fat particles in the hot melt (samples #3 and #4) than in sample #1. The large composite micrographs used to determine the fat particle size distribution showed this phenomenon better than the relatively small areas of individual micrographs presented in Figs. 19 and 22. It is probable that the fat particles coalesced in the process cheese food during the prolonged heating without stirring.

There were also structural differences in the appearance of the fat particles. In the initial process cheese food (sample #1), most fat globules appeared to be lined with a material that was less electron-dense than the surrounding protein matrix (Figs. 19 and 20). The occurrence of this material was lower in sample #2 (Fig. 21) heated for 1 h at 82°C and was almost completely absent in hot melt (samples #3 and #4, Figs. 22 and 25) heated for 5 h. The nature of this material is not known. It may be hypothesized that its incidence was associated with the addition of water to the initial cheese blend.

Prolonged heating of the process cheese food made with sodium citrate led to the development of characteristic electron-dense areas (Figs. 22 and 23). Repetition of the experiments confirmed that these areas developed in the process cheese food in the presence of sodium citrate as a result of prolonged heating and were not artefacts. These electron-dense areas had sharp outlines even at a higher magnification (Fig. 23) and were distinctly different from fat globule membrane fragments which were present in small quantities in the process cheese food samples. It is possible that the protein in the electron-dense areas was changed in such a way that it formed compact clusters or that its affinity for osmium, uranyl, and/or lead was increased. The increased local concentration of the heavy metals resulting from their more intense binding may have simulated a more compact structure. To establish whether there were differences in the intensity with which the clusters would bind the individual heavy metals, sections postfixed with osmium tetroxide but not stained, and sections stained with uranyl acetate or lead citrate, and with both reagents were examined. The dark areas are clearly visible in an unstained section (Fig. 24) indicating that their affinity for osmium was high. Other strongly osmophilic structures were found to form a part of the lining at the fat particles. Sections stained with only uranyl acetate or lead citrate did not differ in contrast from unstained sections and are therefore not shown.

Fig. 9. Process cheese food blend briefly heated to 82°C (sample #1). Round void spaces in the cheese matrix indicate the distribution and dimensions of fat globules.

Fig. 10. Process cheese food blend heated at 82°C for 1 h (sample #2). Sodium citrate crystals (C) are smaller and less numerous than in sample #1.

Fig. 11. Process cheese food blend heated at 82°C for 5 h (hot melt, sample #3). Most sodium citrate crystals are dissolved, arrows point to a few crystal residues.

Fig. 12. Process cheese food blend heated at 82°C for 5 h, frozen at -10°C for 24 h, and thawed at 4°C (hot melt, sample #4). In comparison to the preceding samples, the structure is characterized by the absence of crystals of the sodium citrate emulsifying agent.

Fig. 13. Sample #12 consisting of process cheese food blend sample #1) to which 20% of rework (hot melt, sample #4) was added and the mixture was briefly heated to 76°C. Right: compact area, arrows: residues of the emulsifying agent (TSP) crystals.

Fig. 14. Process cheese food blend emulsified with 2.7% trisodium phosphate (TSP). No rework was used (sample #14). C: crystals of the emulsifying agent, TSP. Arrows: calcium phosphate crystals are abundant.

Fig. 15. Sample #14 contains compact areas (R) although no rework was used. C: gradually dissolving crystals of the emulsifying agent, TSP.

Fig. 16. Sample #20 consisting of process cheese food emulsified with 2.7% TSP and 20% of rework (sample #4). The mixture was heated at 76°C. Right: compact area, arrows point to residues of the emulsifying agent (TSP) crystals.

Fig. 17. Histograms of the size distribution (radii in um) of fat particle sections in areas of 6250 um² in process cheese food samples #1 (basic process cheese food) and #4 (hot melt).

Fig. 18. Histograms showing the distribution of combined areas (in um²) occupied by fat particles classified by their radii (in um) in areas of 6250 um² in process cheese food samples #1 (basic process cheese food) and #4 (hot melt), calculated from the mean radii shown in Fig. 17.
**Fig. 19.** Process cheese food blend heated to 82°C (sample #1). m: lactic acid bacteria. Light arrows: fat globule membrane fragments. Dark arrows: fat particle (F) lining.

**Fig. 20.** Detail of fat particle (F) osmiophilic lining (arrow) in sample #1. C: calcium salt crystal.

**Fig. 21.** Process cheese food heated at 82°C for 1 h (sample #2). m: microorganisms. Light arrows: fat globule membrane fragments. Dark arrows: fat particle (F) lining.

**Fig. 22.** Process cheese food heated at 82°C for 5 h (hot melt, sample #3) contains many electron-dense areas (dark arrows). Light arrow: fat globule membrane fragments, C: emulsifying agent (sodium citrate) crystals, F: fat particles, m: microorganisms.

**Fig. 23.** Detail of electron-dense areas developed in hot melt (process cheese food heated at 82°C for 5 h, sample #3).

**Fig. 24.** Unstained section of hot melt (sample #3). Electron-dense areas (o) developed in the protein matrix of process cheese food heated at 82°C for 5 h and fat particle (F) lining (dark arrows) have strong affinity for osmium. C: emulsifying agent (sodium citrate crystal), light arrow: fat globule membrane fragment.
TEXTURE AND STRUCTURE OF PROCESS CHEESE REWORK

Process cheese food which contained rework in the form of the cheese blend briefly heated to 82°C (sample #10) had the structure (Fig. 26) similar to regular process cheese food blend emulsified with sodium citrate which was free of rework (samples #1 and #2, Figs. 19 and 21). The protein matrices in all three samples were uniformly stained and contained only a small number of fat globule membrane fragments.

It was possible to detect the presence of 20% of hot melt as rework in the process cheese food (emulsified at 82°C with stirring, sample #13) based on the presence of the characteristic dark areas (Fig. 27).

Interestingly, the process cheese food made with 2.7% TSP also had a characteristic, though different, appearance under the TEM. Although the blend had been heated only briefly to 82°C (sample #16), there was a high concentration of dark areas in the protein matrix (Fig. 28). These areas (Figs. 26-35) were larger but not as dark as the areas that developed in hot melt made with sodium citrate (Figs. 23 and 25). The use of 20% rework in the form of regular process cheese food, sample #1, led to a product which contained one component emulsified with sodium citrate and the other component emulsified with TSP (sample #19). The microstructure of this process cheese food (Fig. 29) resembled that of process cheese food emulsified with TSP containing no rework, i.e., sample #16 (Fig. 28). However, the presence of rework used in the form of hot melt (sample #22) resulted in a product which contained both kinds of dark areas in the protein matrix (Figs. 30-32, 35). Examination of unstained thin sections confirmed that the dark areas that had developed in the hot melt were either more compact or had a higher affinity for osmium than the less dark areas that had developed in the process cheese food in the presence of TSP (Fig. 32). This was confirmed by an examination of thin sections stained with uranyl.
detectable structural changes as revealed by TEM. The distinction between the dark areas and fat globule membrane fragments at high magnification (Fig. 34) was as clear as was the distinction between the two kinds of the dark areas (Fig. 35).

The causes for the development of the dark areas in the hot melt, i.e., in a cheese blend heated at 82°C for 5 h in the presence of 2.7% sodium citrate, and in a similar blend briefly heated at the same temperature in the presence of 2.7% TSP, are not known. It is possible that the protein underwent some changes [10] which either increased its affinity for osmium or made it interact in a way that would result in areas more compact than the rest of the protein matrix. It is probable that the increased affinity for osmium may also be related to detectable structural changes as revealed by LM (Figs. 1-4). It may be suggested that the development of the dark areas in hot melt as observed by TEM is related to a decreased meltability of the process cheese food which contains hot melt as rework.

Additional experiments are needed to explain the phenomena described in this paper. TEM of freeze-fractured process cheese samples, which shows the microstructure of unstained protein matrices [11], should be used in addition to the examination of stained thin sections. Ion etching followed by SEM may be useful for characterizing the nature of the electron-dense areas. Gel filtration and electrophoresis is suitable to characterize changes in the physical nature of the proteins constituting the matrices of the hot melt as well as other process cheese food samples. Amino acid analysis will elucidate changes in the chemical composition of the process cheese food proteins resulting from prolonged heating.
TEXTURE AND STRUCTURE OF PROCESS CHEESE REWORK

Fig. 33. Detail of dark areas (t) developing in process cheese food in the presence of TSP. F: fat particle. Fig. 34. Greater detail of the dark areas (t) developing in process cheese food in the presence of TSP. Dark arrows: fat globule membrane fragments. Fig. 35. Detail of two dark areas present in process cheese food made with 2.7% TSP using 20% rework consisting of hot melt (sample #4) made with sodium citrate. o: area developed in hot melt. t: area developed in the presence of TSP.

Acknowledgements

Skillful technical assistance with electron microscopy provided by Mrs. Paula Allan-Wojtas and Mr. J.A.G. Larose is acknowledged. Electron Microscope Unit, Research Branch, Agriculture Canada in Ottawa provided facilities. Effects of the rework on apparent viscosity, firmness, and meltability of the process cheese food were studied at the Canada Packers Research Centre in Toronto. The authors thank Mr. C. Farnum and Dr. H.W. Modler for useful suggestions. Contribution 735 from the Food Research Centre.

References


Discussion with Reviewers

D. N. Holcomb: I find the authors' distinction between 'hot melt' and 'rework' a little confusing. Can this be explained?
Authors: The product that had been once processed but not packaged for sale and is to be reprocessed, is called 'rework'. 'Hot melt' is defined in this study as the process cheese which solidified in the pipes due to excessive heat treatment. If the 'hot melt' is reusable, it becomes 'rework'.

D.N. Holcomb: The authors use a more-or-less 'standard' test for cheese meltability. Would additional useful
information be obtained by monitoring the actual internal temperature of the melting cheese?

Authors: The internal temperature of the melting cheese was not monitored. Separate experiments would be required to study the heat conductivity of the cheeses.

M. Carli: What were the types of cheese used to make the process cheese blend?

Authors: Part skim milk cheese and stirred curd were used in this study.

D.H. Holcomb: Could the authors explain why it is necessary to use osmium tetroxide when fixing samples for TEM but not for SEM? Presumably, fat is extracted for SEM, but is not extracted for TEM. Can the authors estimate how complete are the extraction and retention?

Authors: In order to obtain the most information from milk product samples, fat should either be completely removed or completely retained when preparing these samples for electron microscopy. Partial removal or retention constitutes an artefact [14]. Because TEM is used to demonstrate relationships between components in samples, it is important that the fat component be completely retained. Postfixation with OsO₄ is employed for this purpose. The reaction of unsaturated fatty acids with OsO₄ is accelerated in the presence of imidazole [15]. For SEM, the microstructure of the protein matrix can be studied using samples in which fat has been completely removed (extracted with chloroform after ethanol dehydration). The distribution of fat in the matrix is revealed by freeze-fracturing and is apparent from the distribution of void spaces which had initially been occupied by the fat. Postfixation with imidazole-buffered OsO₄ can be used to retain fat in samples destined for SEM. However, the micrographs of freeze-fractured samples show topographically flat fracture planes, occasional void spaces in the protein matrix indicate the presence of whey or air pockets.

R.E. Cartwright: The heat treatment of the product featured in Table 1 has not been revealed. Please clarify.

Authors: Process cheese food featured in Table 1 was made in the laboratory. After the internal temperature of the cheese reached 82°C (in 5 to 7 min of heating), the cooked cheese was cooled and stored. The hot melt used as rework at 10% and 20% levels was taken out from the pipes of a commercial plant. The amount of heat received by this batch of hot melt varied but is not known. Table 1 is used to demonstrate the considerable effects of hot melt on textural properties of the process cheese food. For the subsequent experiments, another batch of hot melt was made in the laboratory using a known heat treatment.

R.E. Cartwright: The rate of freezing for the laboratory samples must be very fast compared to industrial applications where the bulk quantity to be frozen is much greater. How do you feel a reduced rate of freezing would affect the microstructure of processed cheese and how would its reuse at a 10% or 20% level affect the finished product?

Authors: The rate of freezing was not determined, but it may be assumed that the rate for a small laboratory sample would be higher than the rate for bulk quantity. Slow freezing of cheese may result in the development of ice crystals. Should any ice be formed in slowly frozen 'hot melt', the melted water would be absorbed during processing and the void spaces would vanish as the 'hot melt' is dispersed in the cheese blend rapidly. This hypothesis should be confirmed by experiments. However, the rate of freezing probably does not have as much effect as the rate of cooling. The slowly cooled 'hot melt' rework receives more heat (more heat denaturation), particularly in the high-temperature region, than a rapidly cooled rework and would make the fresh batch of process cheese firmer and less malleable.

R.E. Cartwright: Light microscopy indicated that the cheese was more uniform when cooked to higher temperatures. Would you expect there to be less difference in the products listed in Table 1 if all were cooked to the higher levels?

Authors: Cooking to higher temperature means extended emulsification and, thus, a uniform structure. The cheeses listed in Table 1 were sampled when cooked at 71°, 76°, and 82°C in order to establish the extent at which the 'rework' becomes dispersed in the cheese blend.

R.E. Cartwright: How do you determine that the phenomenon seen in TEM micrographs of the hot melt samples is fat coalescing? Earlier you reported that the emulsification in the hot melt appeared to be more advanced with 25% more fat droplets and a smaller mean radius. The data seem to suggest just the opposite.

Authors: The fat particles in the hot melt were smaller and, thus, more numerous than in the control process cheese sample. This would suggest that additional dispersion of the fat took place during the excessive heat treatment. However, as the hot melt had been heated quiescently, i.e. without stirring as a form of mechanical energy required to break the fat particles [16], it is difficult to perceive that the emulsification would continue. The term 'coalescing' has been used based on the appearance of fat particle clusters. This comment is valuable as it may stimulate interest in studying the effects of quiescent heating and/or cooling on the dispersion of fat in process cheese.

M. Carli: Amino acid analysis of cheese proteins is carried out after acid hydrolysis of the peptide bonds, so all amino acids present in the system are simply quantitatively determined giving no information about their mutual interactions or interactions with other components. Chemical analysis of the protein fractions (protein nitrogen, soluble nitrogen) and Mallard's reaction products such as 5-hydroxymethylfurfural would provide more information about the amino acid interactions than the amino acid analysis.

Authors: We agree that a variety of analytical methods should be used to study the changes in the process cheese proteins. As excessive heat treatment may induce changes in the amino acid composition of the proteins (loss of individual amino acids and/or their racemization), amino acid analysis including the detection of D-amino acids should be one of the analytical procedures used.

Additional references

THE EFFECTS OF POLYSORBATE 80 ON THE FAT EMULSION IN ICE CREAM MIX: EVIDENCE FROM TRANSMISSION ELECTRON MICROSCOPY STUDIES

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Abstract

Emulsifiers are used in ice cream to produce a dry, smooth textured product with desirable melting properties. They function by promoting a partial destabilization of the fat emulsion. Polyoxyethylene sorbitan monooleate is used very commonly in the ice cream industry for this purpose. The objective of this research was to examine by transmission electron microscopy the differences in the fat globules in typical ice cream mix emulsions prepared with and without 0.08% polyoxyethylene sorbitan monooleate.

Ice cream mix was combined 3:1 with a 2% solution of ultralow gelling temperature agarose at 20°C, fixed with 4% glutaraldehyde, postfixed with 1% OsO₄ in imidazole/phosphate buffer, embedded in Spurr resin and thin-sectioned for viewing. The emulsifier reduced the number of casein micelles adsorbed to the fat globules as determined by both TEM and quantification of membrane protein with Kjeldahl analyses. The fat/surface interfacial tension was also significantly reduced by the presence of emulsifier in the mix. The data suggest that emulsifiers promote fat destabilization through reduction of membrane protein, based on their ability to reduce the fat/surface interfacial tension. In the presence of crystallized fat, the emulsion then becomes less stable to shear forces during the whipping and freezing of ice cream.

Key Words: agarose, casein micelles, emulsifiers, emulsion, fat destabilization, ice cream, milk fat globules, Polysorbate 80, transmission electron microscope, Tween 80

Introduction

Emulsifiers, such as mono- and di-glycerides or polyoxyethylene sorbitan esters, are used in ice cream to improve the whipping quality of the mix, to produce a drier ice cream with smoother body and texture, and to achieve good drawing qualities at the freezer (Arbuckle, 1976). These effects result from a destabilization of the fat emulsion (Keeney, 1958; Berger, 1976) as occurs during the whipping of heavy cream (Schmidt and van Hooydonk, 1980; Brooker et al., 1986) in which chains and clusters of partially ruptured fat globules form a network of fat that envelops and stabilizes the air cells.

During ice cream manufacture, the whipping and concomitant freezing process imposes large shear forces on the mix. The combination of ice crystallization and shear is responsible for destabilization of the milkfat globules (Lin and Leeder, 1974; Goff and Jordan, 1986). The magnitude of destabilization facilitated by an emulsifier is related to its hydrophilic/lipophilic balance (HLB) (Govin and Leeder, 1971), and/or to the interfacial tension between the serum and lipid phases in the presence of the emulsifier (Walstra, 1983). In a protein stabilized emulsion such as a dairy emulsion, the addition of surfactant prior to homogenization reduces the amount of protein adsorbed per surface area of fat (Oortwijn et al., 1977; Oortwijn and Walstra, 1979,1982; Barford and Krog, 1987).

Alsafar and Wood (1966) used transmission electron microscopy to examine the fat dispersion in ice cream. They reported that the surface layer of fat globules appeared darker and thicker without an emulsifier than with emulsifier. Berger and White (1971), using freeze-etching techniques, reported the presence of a granular coating, considered to be casein subunits, on the outside of the fat globules. They stated that the strength of the fat globule membrane is the overriding factor in predicting fat destabilization. It was suggested that the membrane is only weakly bound and is easily stripped off to expose a crystalline lipid shell composed largely of the high-melting glycerides (HMG). Liquid fat escaping from these ruptured HMG shells thus becomes the cementing agent in fat destabilization during freezing (Berger and White, 1976). However, this liquid core/solid shell model has been challenged (Walstra and van Beresteyn, 1975; Walstra, 1983).

Polyoxyethylene sorbitan monooleate (Polysorbate 80 or Tween 80) is the agent most commonly used by the ice cream industry for destabilization of the fat emulsion (Thomas, 1981; Keeney, 1982). This research project was initiated to elucidate the mechanism of emulsifier action in promoting fat destabilization during the manufacture of ice cream. The objective of this study was to examine the microstructural differences in ice cream mix emulsions prepared both in the presence and absence of polyoxyethylene sorbitan monooleate.
Materials and Methods

Ice cream mix preparation
Four replicates of ice cream mixes with the following compositions were prepared: 10% milkfat, 11% milk solids-not-fat (msnf), 10% sucrose, and 5% corn syrup solids; and 10% milkfat, 11% msnf, 10% sucrose, 5% corn syrup solids, and 0.08% polyoxyethylene sorbitan monooleate (Tween 80, ICI Americas Inc., Wilmington, DE). Fresh cream, skim milk, and nonfat dry milk were used as the sources of milk solids. The 8 kg mixes were blended, pasteurized at 74°C for 75E Homogenizer), cooled to 5°C, and aged 24 h, as in conventional ice cream manufacturing procedures.

Fat destabilization analysis
One two-litre aliquot of each recipe was frozen in a Taylor batch freezer to -5°C and held in the freezer for a total of 15 min of agitation. Samples (40mL) were removed from the barrel every 2.5 min and analyzed for the degree of fat destabilization by turbidimetry. Samples were diluted 1:500 with distilled water and absorbance was measured at 540nm. Percent fat destabilized was calculated as ((Amix-Aice)/Amix) x 100% (Keeney, 1958).

Interfacial tension
The interfacial tension was measured with a duNuoy ring apparatus (Fisher Surface Tensiometer) by placing the ring in water or in aqueous solutions of 11% milk solids-not-fat in a jacketed beaker at 70°C, with and without 0.08% polyoxyethylene sorbitan monooleate, layering the solution with anhydrous milkfat at 70°C, aging 10 min while maintaining constant temperature, and then drawing the ring from the one phase into the other.

Transmission electron microscopy
The two ice cream mix emulsions, after aging, were warmed to 15°C and combined with a 2% solution of ultralow gelling temperature agarose (SeaPrep agarose, FMC Marine Colloids Div.) at a rate of 3 parts sample to one part agarose. The agarose solution had been previously heated and cooled to 2°C. The ice cream mix and agarose combination was allowed to solidify at 4°C overnight. Pieces of the mix were then cut and fixed in 4% glutaraldehyde in 0.1M phosphate buffer (pH 7.0) overnight. After several rinses in phosphate buffer, the samples were postfixed in 1% osmium tetroxide in 0.1M phosphate/imidazole buffer (1:1 v/v; pH 7.0) overnight (Aagnermuller and Fahimi, 1982; Kalab, 1985). Following several buffer rinses, the samples were dehydrated in successive ethanol concentrations, embedded in Spurr resin molds, and held at 70°C overnight. Samples were then thin sectioned (Sorvall MT-2 Ultra-microtome), placed on carbon-coated Formvar copper grids, and viewed using a Philips EM-300 Transmission Electron Microscope.

Fat globule measurement
Cross section diameters of all of the fat globules in at least 40 different random fields from each treatment were measured from the micrographs. A total of 911 globule sections were measured and grouped in 0.5 μm categories. The number of discrete protein particles adhering to each of the globules was also counted. The average diameter of the globule cross sections was determined as was the average number of adsorbed protein particles per globule as a function of globule diameter.

Quantification of membrane protein
Total protein in the fresh ice cream mix was determined by Kjeldahl nitrogen. Mix samples were then centrifuged at 26,000g, 20°C, 30 min, the centrifuge tubes were then frozen, the fat layer was removed, and the serum was thawed, mixed, and reanalyzed for Kjeldahl nitrogen. Adsorbed protein was calculated as the difference between total protein and serum protein (Oortwijn and Walstra, 1979).

Results and Discussion
The differences observed in the degree of fat destabilization when the two ice cream mixes were concomitantly whipped and frozen into the ice cream state are illustrated in Figure 1. The addition of Tween 80 to the mix prior to homogenization clearly produced an emulsion which was less stable to the applied shear forces than an emulsion stabilized solely by the milk proteins. Fat destabilization also manifested itself in dryness, smoothness, and meltdown of the two ice creams when the ice cream mixes were frozen.

Both mixes, however, were equally stable following homogenization when not subjected to the freezing process (Table 1).

The interfacial tension between the aqueous phase and anhydrous butteroil was taken as representative of the interfacial tension at the surface of the globule in an emulsion. While the absolute numbers may be different due to the greatly different surface to volume ratio, the relative trends can be observed. The interfacial tension at the aqueous phase/oil surface was reduced by the milk proteins and was also reduced by the emulsifier (Table 2). This decrease in interfacial tension correlated with the increase in fat destabilization in the presence of the emulsifier. A good correlation has been shown between the destabilizing power of several emulsifiers and the serum/lipid interfacial tension in the presence of the emulsifier and has been reported elsewhere (Goff et al., 1987).

In examining electron micrographs of the two emulsions, differences in the number of casein micelles adhering to the fat globules were observed. In the absence of the emulsifier, more casein micelles adsorbed to the fat globules (Figure 2, Figure 4)
Effects of polysorbate 80 in ice cream mix

Table 2. Interfacial tension between anhydrous butteroil and an aqueous phase with or without 10% skim milk powder and with or without Tween 80 as determined by a dNuoY ring at 70°C.

![Image](image_url)

Table 3. Summary of the number of micelles adhering to fat globules in the presence and absence of Tween 80 as a function of the globule section diameter. Mean section diameters of the two emulsions are also shown.

Table 4. Differences in the amount of protein adsorbed to the fat globules in each of the mixes as determined by centrifugal separation and Kjeldahl analyses.

than when the emulsifier was included in the mix (Figure 3, Figure 4). This difference in micellar adsorption was quantified by counting number of micelles adsorbed per globule in random micrographs from each emulsion. The statistical analyses performed demonstrated a significant difference (p<0.01) between emulsions; nearly twice as many micelles were adsorbed to the fat globules in the mix without emulsifier than in the mix containing Tween 80 (Table 3).

Adsorbed protein was also determined by Kjeldahl analysis as described above. A small portion of the fat was found to remain in the serum after centrifugation as was found by Corwin and Walstra (1979). The results indicated that 15.9% of the total mix protein was adsorbed to the fat globule in the absence of emulsifier whereas only 7.8% was adsorbed to the fat globule in the mix containing Tween 80 (Table 4).
presence of Tween 80 (Table 4). This difference was statistically significant and the 95% confidence intervals are shown in Table 4.

The information gained from measurement of the fat globule diameters on the micrographs is relevant only to the cross-sectional diameters and not to the true diameters of the globule (Table 3). The mean section diameters and size distribution of the sections between the two emulsions, however, were not significantly different. Turbidity or absorbance of the two emulsions was also determined at both 400 nm and 540 nm using two different procedures (Table 5). There was no difference in light scattering between the two emulsions. Thus it appears that the addition of the emulsifier had no significant effect on fat globule size distribution of the two populations. The high homogenizing pressures involved were the overriding factor in size distribution.

Emulsifier action, therefore, may be summarized as follows. The added emulsifiers reduce the interfacial tension between the serum and lipid (fat globule) phases of the mix. It is thus more favourable for the emulsifiers rather than caseins to adsorb to the fat surface at the time of homogenization, as this leads to a lowering of the net free energy of the system. This reduction in the amount of casein, however, produces an emulsion which is less stable to the shear forces applied during ice cream freezing. As a result, the fat emulsion is destabilized to a greater extent in the presence of the emulsifiers. Destabilization leads to the production of a smoother ice cream with good melt resistance due to the enhanced structure of the foam caused by the fat network.

Examination of the electron micrographs revealed crystalline fat within the cross section of the fat globule. The needle-like crystals were distributed evenly and randomly throughout the globule (Figures 2-6) and no evidence of a solid shell around the fat globule was observed; however, a crystalline periphery was slightly evident in a few globules (Figure 5). This structure conforms to that proposed by Walstra (1981); however, concentric crystalline layers of fat at the globule boundary have been shown by freeze etch techniques (Berger, 1976; Buchheim and Precht, 1979; Precht and Buchheim, 1979).

Many of the globules also showed evidence of fat crystals which influenced the shape of the globule (Figure 6). van Boekel and Walstra (1981) have suggested a model for crystallization within a fat globule whereby tangentially oriented crystals protrude from the surface of the globule and are able to pierce the film between two existing droplets upon close approach, thus promoting rupture of the globule membrane and subsequent coalescence. This may partly explain the necessity of a partially crystalline fat to enhance fat destabilization or partial churning.

Table 5. Differences in fat globule size distribution of the two ice cream mixes as determined by light scattering techniques (Walstra, 1968).

<table>
<thead>
<tr>
<th>ICE CREAM MIX</th>
<th>ABSORBANCE (400nm)</th>
<th>ABSORBANCE (540nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>w/ Soln. A (2%)</td>
<td>w/o Soln. A (2%)</td>
</tr>
<tr>
<td>NO EMULSIFIER</td>
<td>0.83</td>
<td>0.92</td>
</tr>
<tr>
<td>0.08% TWEEN 80</td>
<td>0.83</td>
<td>0.92</td>
</tr>
</tbody>
</table>

n = 4

Soln. A = 0.375% Disodium EDTA
0.125% Tween 20
adj. to pH 10 with NaOH

Figure 5. Milkfat globule from an ice cream mix showing the intricate structure of needle-like crystals present in the cross section of the globule. F=fat globule, arrow points to fat crystal. Bar=1μm.

Figure 6. Milkfat globule from an ice cream mix. Arrow points to surface distortion of the globule caused by the presence of the internal crystalline structure. F=fat globule. Bar=1μm.

Figure 7. Transmission electron micrograph of an ice cream mix illustrating two fat globules joined together by the adsorption of a casein micelle onto each globule. F=fat globule, arrow points to casein micelle attachment. Bar=1μm.
under the influence of applied shear forces. The perpendicu-
lar and tangential crystal orientations observed in many of the fat globules
from the two ice cream mixes support this theory. The presence of
adsorbed casein micelles, however, would tend to physically block
the influence of these crystal protrusions on fat coalescence and
destabilization through steric repulsions.

Another observation made from the micrographs was the
presence of homogenization clusters formed by the sharing of an
adsorbed casein micelle between two fat globules, thus holding
them together (Figure 7). Odgen et al. (1976) have proposed this
type of structure as one of the possible mechanisms involved in
clustering of fat globules post homogenization. The two stage
homogenization process involved in the preparation of the mixes
for this experiment is intended to reduce the amount of fat globule
clustering. Thus, only a few such clusters were seen in all of the
micrographs examined.

Conclusions

In conclusion, a significant difference was observed in the
number of casein micelles adhering to the fat globule when the
ice cream mix was homogenized in the presence of polysorbate
80, more protein being adsorbed in the absence of the
emulsifier than in its presence. The total surface area of fat in the
emulsion was not affected by the presence or absence of the
emulsifier. Crystalline fat within the fat globule exhibited a random
distribution throughout the cross section of the globule. Several fat
globules exhibited crystals which appeared to influence the
peripheral shape of the globule.

Acknowledgments

The authors wish to thank the Wisconsin Milk Marketing
Board for their support of this research. The authors also wish to
thank Dr. M. Kalab for helpful suggestions.

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P. Walstra: Have you compared the results of Table 3, no emulsifier, with those of Walstra and Oortwijn, Neth. Milk Dairy J. 36(1982)103-113, Fig. 1? You find a similar decrease in number of micelles attached per μm circumference of the fat globule with increasing globular diameter.

Authors: Our findings do agree with the prediction by Walstra and Oortwijn that smaller globules acquire more large particles per unit surface area, although the globule diameters in Table 3 refer only to the cross sections and, since this was not systematically studied by us, there are insufficient observations in the larger size ranges to independently conclude this relation.

I. Heerlig: Ice cream is made by cooling to about -5°C while beating in air, and under these conditions coalescence of fat takes place. On the other hand, ice cream emulsions were analyzed for their microstructure by a preparation scheme involving a temperature regime with a lowest temperature of -4°C. This preparation scheme and these conditions are apparently very well suited to show the interaction between casein and the fat globules. Can these conditions be considered to be representative for the situation at -5°C, in particular for aspects such as the amount and orientation of fat crystals and the deformation of fat globules? If not, would it be advisable and possible to perform these types of microstructural observations at this temperature?

Authors: During ice cream manufacture, a mix is prepared by processes similar to the one employed by us. This mix is then aged at -4°C prior to being concomitantly whipped and frozen into the ice cream state. We were interested in determining what microstructure existed prior to freezing such that fat coalescence during freezing was enhanced in the presence of the surfactant. We did not examine samples of frozen ice cream using this technique, although more information regarding the time and temperature dependent fat crystallization process may be gained from such a study.

I. Heerlig: Differences in fat destabilization were found only when the mixes were subjected to the freezing process. Does this not imply that, apart from the fat crystals, at least ice crystals also may induce coalescence of fat globules, which also, in that case, may be prevented by steric repulsion of the adsorbed casein micelles?

Authors: Indeed that is the case. It was reported by us (Goff and Jordan, 1986) that the combination of ice crystallization, air incorporation, and shear forces were all necessary for fat destabilization to occur in ice cream. When the fat globules lack the physical protection of the adsorbed protein layer and are subjected to the conditions of the freezer, coalescence is enhanced. Fat crystallization is also necessary for this destabilization to occur as the fluidity or mobility of the globule is greatly reduced and the potential for interglobule contact through protruding fat crystals as hypothesized by van Boeckel and Walstra is enhanced.

I. Heerlig: Large differences are found in the amount of fat crystals in the different emulsion droplets (compare fig. 5 with 6 and 7). This is in accordance with earlier observations of Walstra (Neth. Milk Dairy J. 1967, 21, 166) by light microscopy and Buchheim and Precht (Milchwissenschaft 1979, 34, 657,720) by freeze fracture electron microscopy. There is some debate (Walstra, 1983) whether fat globules with birefringent outer layers as viewed by polarized light microscopy consist of small tangentially oriented crystals or of large concentric layers of fat crystals, forming a more or less solid shell. In fig. 5, a concentric arrangement of large crystalline layers is observed. Should this morphology, obtained by a chemical fixation procedure, be considered as independent proof for the existence of this type of arrangement?

Would the authors dare to speculate which type of globules would be more likely to be involved in coalescence: droplets with a strong crystallization with concentric layering as observed in fig. 5, or droplets with a crystal protrusion as indicated in fig. 6?
STRUCTURAL BINDING PROPERTIES OF SILVERCARP (HYPOPTHALMICHTHYS MOLTRIX) MUSCLE
AFFECTED BY NaCl AND CaCl₂ TREATMENTS

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Abstract

The texture of restructured muscle products is well known to be strongly affected by various salts. In the present work the effects of NaCl and CaCl₂ on the ultrastructure of both fresh and cooking salted silvercarp (Hyloptalanumichthys molitrix) muscle were examined, in order to elucidate the heat-initiated binding phenomenon. Sodium chloride at 0.3 and 1.5% caused swelling and fusion of the myofibrils and loss of arrayed structure. At 12% NaCl there was a compaction of myofibrillar structure. Calcium chloride at all the tested concentrations resulted in shrinkage of myofibrils. The present study demonstrated two main effects of salts on the ultrastructure of fish muscles: (i) swelling of myofibrils which, at relatively high NaCl concentrations (1.5%) resulted in conversion of the arrayed structure of the myofibrils into a homogeneous and amorphous mass, and (ii) shrinkage of myofibrils, which may result in compact and denser appearance of filaments within myofibrils and their shortening. The textural changes which resulted from the different salt treatments are explained by these findings.

Introduction

Sodium chloride and phosphates are commonly used in muscle food systems in order to reduce drip and to improve functional properties such as binding and emulsification.

Hamm (1960) stated that the effect of salts on muscle tissue may be understood by considering both the cations and the anions. Salts such as NaCl, the ions of which contribute electrical charges to the peptide chains, result in repulsion or attraction between the protein molecules and this brings about increased or decreased hydration. Divalent ions such as Ca²⁺ and Mg²⁺ are believed to cross-link protein chains and this narrows the intermolecular spaces available for water and results in decreased hydration. At high concentrations there is a decrease in water holding capacity for most salts, which is explained by cleavage of hydrogen bonds and excessive folding of the peptide chains ("salting out"). Various ions have been arranged in the so-called "Hofemeister" series which ranks them according to their effectiveness in salting out proteins. With this regard, increased conformational stability was associated with loss of solubility (Haschemeyer and Haschemeyer, 1973). Weinberg et al. (1984a) found a high correlation between the expressible moisture in cool muscle as affected by various salts and the texture of loaves that were prepared from ground cool. In that study 0.25M NaCl resulted in a cohesive and elastic texture in the cooked loaves; 2M NaCl (12%) resulted in compact and grainy texture; CaCl₂ at ionic strength range between 0.085 and 0.5M resulted in soft and crumbling loaves which expressed a great volume of moisture upon cooking. Similar results were also obtained with silvercarp (Weinberg and Angel, 1984; 1985).

The formation of a cohesive and elastic muscle matrix from small pieces is achieved by mixing small muscle cubes with NaCl and phosphates. A tacky and swollen batter is then obtained, and when heat is applied the proteins coagulate and binding between the separate muscle pieces occurs. This phenomenon is referred to as 'heat initiated binding' (Vadehra and Baker, 1970). The mechanism through which the heat initiated binding is achieved is believed to be
due to the solubilization and extraction of the myofibrillar proteins by the salts. These proteins then serve as binders between the muscle particles (Vadehra et al., 1970; Macfarlane et al., 1977; Siegel et al., 1978).

Offer and Trinick (1983) used light microscopy as well as transmission electron microscopy (TEM) to study the swelling behavior of isolated myofibrils from psoas rabbit muscle as affected by various NaCl and pyrophosphate solutions. They related the swelling of the myofibrils to the water retention properties of the muscle, and proposed a mechanism by which NaCl and phosphate resulted in swelling by adding electrical charges (Hemm, 1960), and by detachment of transverse cross bridges from the actin filaments. When Voyles et al. (1984) studied the effect of salt and phosphate bringing on the ultrastructure of pig muscle by TEM, they found swelling, extraction of A-band proteins and Z-line break-up; these results depended upon muscle age post mortem, pH, and duration that muscle samples were soaked in salt and phosphate solutions. In addition, loss of structure was attributed to either the extraction or detachment of thin or thick filaments from the myofibrils. In Leader et al. (1980) a study of structural changes in heated bovine muscle by SEM and TEM showed that heating muscle samples to temperatures between 63°C and 70°C caused sarcomere contraction and loss of the I-band. Some Z-line breakage was also noticed.

In the present work an attempt was made to shed light on the understanding of the heat initiated binding phenomenon by studying the ultrastructure of fresh and heated silver carp muscle tissue treated with NaCl and CaCl₂.

Materials and Methods

Preparation of the fish muscle

Fresh silver carp (Bypophthalmichthys molitrix) was purchased from a local dealer, filleted and ground through a 5mm end plate. Seventy five g of chilled (2°C) ground fish was mixed thoroughly by hand for 3 min, with or without various salts. The treatments were: control (no salt added), 0.3% NaCl (W/W), 1.5% NaCl, 12% NaCl, 0.55% CaCl₂, 1.25% CaCl₂, and 2.5% CaCl₂. Care was taken to avoid the formation of air pockets. After a homogeneous mass was achieved, a mixed sample from each treatment was taken for ultrastucture examination. The rest of the fish was stuffed into standard 100 ml Pyrex beakers covered with aluminum foil punctured with several small holes, and placed in an oven at 170°C for 20 min, to reach an internal temperature of 75°C. After these samples were cooled to room temperature, portions from the internal zone were also taken for ultrastructural examination.

Electron microscopy

Muscle samples were fixed for 2 h in 3.5% glutaraldehyde in 0.1M sodium cacodylate, pH 7.0, cooled within a water-ice bath, and postfixed for 2 h in 2% OsO₄ in 0.1M phosphate buffer. The fixed samples were dehydrated with increased concentrations (20, 40, 60 and 100%) of ethanol, each step lasting ca. 30 min, and this was followed by several rinsings with absolute acetone, for 1 h each. Then, the samples were embedded within Agar 100 resin (Agar Aid) at room temperature, and left overnight at 70°C for polymerization. Ultrathin sections were prepared with an LKB ultramicrotome with a diamond knife of Diatom, Switzerland, stained with uranyl acetate and lead citrate, and examined with a TEM (JEOL 100CX II-LEO) at 80 kV.

Results and Discussion

Sodium and calcium chloride at the various concentrations were chosen for the present ultrastructural study because of two main reasons: 1. these salts have been found to exert very different textural and water holding effects on fish muscle tissue (Weinberg et al., 1984; Weinberg and Angel, 1984, 1985). 2. differential scanning calorimetry studies on the effects of various salts on cod muscle revealed very different thermal transition curves of NaCl and CaCl₂, treatments (Weinberg et al., 1984b) which probably indicated different effects on the conformation of the proteins.

Both textural and thermodynamical analyses might indicate possible ultrastructural differences which may explain the physical properties of the treated muscle.

The myofibrillar arrangement of the silver carp is typical for muscle and characterized by distinct sarcomeres (Figs. 1a, 1b). In Fig. 1c the array structure is evident, as are the details at higher magnification (Fig. 1d). Heat treatment resulted in shrinkage of the sarcomere length accompanied by digestion of both the I-band (including the Z-line) and the H-zone, as indicated by areas of very low densities (Fig. 2a). Higher magnification reveals residues of filamentous elements in the I and H zones which might have served as binding structures between adjacent sarcomeric elements.

Based on the present study it seems that the digestion of the myofibrils into separate sarcomeric elements might explain the softening of the fish muscle obtained by cooking. It also appears that the heat treatment resulted in disappearance and fusion of the thick and thin filaments of the myofibrils (Figs. 1b, 1d) and converted them into a homogeneous matrix of amorphous pattern (Fig. 2b).

Johnson et al. (1981) discussed the role of textural elements in a rheological model of fish flesh. They envisioned fracture of textural elements upon heating, and the above mentioned

Figures 1a, 1b. Longitudinal ultrasection of fresh silver carp muscle. The typical sarcomeric zones (A, H, I, M, Z) are indicated. 1a - bar=800 nm; 1b - bar=50 nm.

Figures 1c, 1d. Cross ultrasections of the muscle described in Fig. 1a. 1c - bar=230 nm; 1d - bar=50 nm.

Figures 2a, 2b. Longitudinal ultrasection of heat treated muscle. Remnants of the typical sarcomeric zones (H, I, Z) are indicated. 2a - bar=700 nm; 2b - bar=67 nm.
Structural Binding of Silvercarp Muscle
Structural Binding of Silverscarp Muscle

disappearance of filaments from the myofibrils could support such ideas.

Myofibrillar proteins can be solubilized and extracted from muscle tissue at relatively high NaCl concentrations (0.25 M and above). However, in the present study NaCl concentrations as low as 0.3% resulted in swelling of the I-band (Fig. 3a). This significant change is illustrated by comparing Figures 1b and 3b. It is interesting to reveal that with 0.3% NaCl the A-band is composed of several bands of various densities (Fig. 3a). Figure 3c demonstrates a high magnification of the area around the H-zone. Fig. 3d shows the muscle array in cross section in which several densities may be observed. Higher magnification of the myofibrillar cross-section shows diverse densities within the myofibrils (Fig. 3e). Figures 3a, 3b show a typical pattern of the 0.3% NaCl treatment upon heating. The sarcomeric arrangement is still retained, however there is some fusion of adjacent sarcomeric elements which is apparent (Fig. 4b).

At 1.5% NaCl treatment both the myofibrillar and sarcomeric arrangement disappear in the fresh muscle (Fig. 5a), and a filamentous pattern was not observed (Fig. 5b). However, remnants of intermyofibrillar regions can be identified in the homogeneous mass of the both fresh and heat treated samples (Figs. 5a, 5b). It appears that this ultrastructure is a result of marked swelling of the myofibrils upon salting. Swelling of the myofibrils resulted in the loss of the array structure. Fusion of the myofibrillar proteins into an amorphous mass, and might explain the resulting tacky muscle batter which leads to a cohesive and elastic texture upon heating (Valdehra and Baker, 1970; Weinberg and Angel, 1984).

Upon heating, the 1.5% NaCl-treated samples exhibited two main patterns: 1. homogeneous amorphous pattern, and 2. residues of sarcomeric elements (Fig. 6). It is possible that where the salt has completely reacted with the muscle proteins a complete homogeneous mass was obtained. When the mixing with salt was incomplete some residual sarcomeric elements can still be identified with the disintegrated I-bands (Fig. 6). Voyle et al. (1984) suggested an accumulation of protein globules on the muscle fibers membranes of both control and salted samples. However, in the present study no such material was identified in the homogeneous amorphous mass. Fig. 7a shows the pattern of the 12% NaCl treated samples in which the borders between adjacent myofibrils may be identified. However, higher magnification of these samples (Fig. 7b) reveals a filamentous pattern which was undetectable at 1.5% NaCl, as seen in Fig. 5b) in the A-zones. The pattern of the filamentous material is very dense and compact. Indeed the texture of the fish loaves prepared with 12% NaCl was very compact, grainy and inelastic (Weinberg and Angel, 1984).

Calcium treatment of 0.55% resulted in some initial shrinkage of the I band and H-zones (Fig. 8). The I-bands were more susceptible to shrinkage and this formed a structural pattern of the myofibrils. The heat treated samples of 0.55% CaCl2 showed a narrow pattern of the myofibrils, with distinct z-lines and "empty" I-bands. In addition, the H-zones shrunk considerably (Fig. 9). This might explain the large amount of drip that resulted from the calcium addition. At higher calcium concentrations the sarcomeric pattern of the shrunken myofibrils is almost lost (Fig. 10a). At higher magnification the various sarcomeric zones can still be identified (Fig. 10b). A significant shrinkage is observed with the heat treated samples in which the sarcomeric pattern is lost (Fig. 11). At 2.5% CaCl2 the myofibrillar structure is distorted (Fig. 12).

Summary and Conclusions

The present study demonstrated two main structural effects of salts on the ultrastructure of fresh muscle: 1. swelling of the myofibrils at 0.3% NaCl treatment which increased at 1.5% NaCl treatment resulting in fusion and conversion of the myofibrillar arrayed structure into an amorphous pattern. 2. shrinkage of the myofibrils which occurred with the CaCl2 treatment (at all the treated concentrations) which resulted in a compact and denser appearance of the filaments within the myofibrils and their shortening. Particularly dense structure have been observed at 12% NaCl.

Based on the present study, it is suggested that the first effect is a result of conformational instability of proteins which leads to fusion of the myofibrils and to a loss of their arrayed structure. Such fusion might be related to the binding and cohesive properties of these proteins. On the other hand, the effect of shrinkage is a result of cross linking of the relatively open and solubilized protein structure which leads to denaturation of the myofibrils which are protected from denaturation. It is interesting to note that CaCl2 affects only shrinkage, while NaCl brings about both swelling and fusion at relatively low concentrations and compactness and shrinkage at high concentrations.

Acknowledgment


Figures 3a-3c. Longitudinal ultrasection of 0.3% NaCl treated muscle. Zones A, H, I, M, Z are seen. 3a - bar=800 nm; 3b, 3c - bar=60 nm.

Figures 3d, 3e. Cross ultrasection of the muscle described in Fig. 3a. 3d - bar=250 mm; 3e - bar=60 nm.

Figures 4a, 4b. Longitudinal and cross ultrasections of the heat treated muscle described in Fig. 3a. Remnants of the typical sarcomeric zones A, H, I, and Z are identifiable. 4a - bar=800 nm; 4b - bar=320 nm.
Structural Binding of Silvercarp Muscle

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Siegel DG, Thono DW, Schmidt GR (1978) Meat massaging: the effects of salt, phosphate and massaging on the presence of specific skeletal muscle proteins in the exudate of a sectioned and formed ham. J. Food Sci. 43, 327-333.


Discussion with Reviewers
G.R. Schmidt: In figures 1c and 1d, what causes the unusual banding pattern in these cross-sections?
Authors: These patterns are dependent on the sectioning plane in relation to the longitudinal direction of the myofibrils. An explanation for this is found in a diagrammatic illustration in "The Science of Meat and Meat Products" (1970), pp. 35 (by Price J.F. and Schweigert, B.S., PFP, Westport, CT.)

G.R. Schmidt: Can the authors explain how the striated banding pattern shown in figure 7b "returned"? Could this be the result of a non-homogeneous distribution of salt in the mixed fish muscle? Was there any attempt to check for homogenous salt distribution? Would it have been better to hold the raw mixture for 24 h equilibration period prior to sampling and cooking?
Authors: The various treatments with dry salts exerted immediate and obvious effects on the texture of the fish muscle after mixing. The purpose of the present study was to follow the structural properties of these changes. The word "return" used in the original manuscript to describe the effect of 12% NaCl is not quite adequate. It was actually related to compare the 12% NaCl and the 1.5% NaCl treatments. Anyway the striped pattern was obtained upon treatment with 12% NaCl. We did not follow here changes that might occur upon gradual increase of the salt concentration in the same sample.

G.R. Schmidt: Why is no data presented on drip since reference to this is made in the text?
Authors: Visual impression of the drip volume was very obvious; we did not record the amount of drip.

Reviewer II: Overall, the manuscript does not elaborate "binding" property, instead, it describes some simple salting-in and salting-cut phenomena in the presence of various levels of salt.
Authors: The description of heat initiated binding is given in the Introduction, and it explains how the present study is related to this phenomena. Indeed it is related to salting-in, salting-cut and water retention properties of the muscle proteins, as emphasized in the Introduction.

Reviewer II: Why did the authors use dry salt instead of brine? For uniform distribution of as low as 0.3% salt, a brine would seem to be more reasonable.
Structural Binding of Silvercarp Muscle

Authors: We used dry salts and not brine for the following reasons: 1) Dry salts exerted immediate and obvious effects on the fish muscle. 2) Dry salts are used in the industry to prepare ham and loaf-type products. 3) Additional water of the brine might have changed the structure as a result of dilution effect, or washing out of various components.

Reviewer II: A 12% salt is not practical for a restructured muscle food; the phenomenon described is nothing but a dehydration (cosmetic drying) rather than a protein solubilization.

Authors: Previous studies examined the effect of a 12% NaCl and found it to strongly affect the texture (Weinberg et al., 1984a; Weinberg and Angel, 1984). Therefore we examined in the present study the ultrastructure of the muscle tissue also under such extreme conditions. The purpose of this study was to follow the ultrastructure of fish muscle with various textural properties, rather than for practical implications. As mentioned in the introduction, textural properties of restructured muscle products is highly correlated with water holding properties (Weinberg et al., 1984a). This phenomenon involves also protein solubilization (Vadera and Baker, 1970; Macfarlane et al., 1977; Siegel et al., 1978). However, it could still be that a 12% NaCl treatment involves also dehydration besides conformational changes in the proteins.

D.F. Lewis: Swelling without dispersion is unlikely to cause fusion - indeed, swelling would be expected to move structures further apart.

Authors: According to the present study, the amorphous ultrastructure is a result of fusion.

D.F. Lewis: Why did the authors use minced fish which adds the difficulties of the interpretation?

Authors: Mincing through 5 mm grinder dose not affect the sarcomeric ultrastructure as obvious from the ultrastructure of the unsalted

Figure 8. Longitudinal ultra-section of 0.55% CaCl₂ treated muscle. Shrunken regions (A, I) are marked. Bar=800 nm.

Figure 9. Longitudinal ultra-section of the heat treated muscle described in Fig. 8. Remnants of sarcomeric regions (H, I) are clearly identifiable. Bar=800 nm.

Figures 10a, 10b. Longitudinal ultra-section of 1.25% CaCl₂, treated muscle. Shrunken sarcomeric zones H and I (A, I) can still be identified. 10a - bar=800 nm; 10b - bar=230 nm.

Figure 11. Ultrathin section of heat-treated muscle described in Fig 10a. Remnants of the various sarcomeric zones can be identified (were not marked). Bar=800 nm.

Figure 12. Ultrathin section of 2.5% CaCl₂ treated muscle. Sarcomeric patterns are distorted, but the filamentous structure is observed. Zone A and the inter myofibrillar region (IMR) can be identified. Bar=60 nm.

muscle. However, comparison with a non-minced and non-salted muscle as well as with the control treatment have been done to verify this point. We used ground muscle in order to ensure even distribution of the salts in all parts of the examined tissue.

S. Cohen: Muscle components are held together by structural entities. When treated with salts or other chemicals such as polyphosphates there can be a leeching out of substances such as sarcoplasmic proteins which then bind or "glue" the muscle fibers together. When heated, the various binding components of muscle such as the intermediate filaments vimentin, titin and nebulin are broken. The authors should address these differences.

Authors: 1) The present study, and previous data (Weinberg, Z.G. 1982. 'Heat Initiated Binding in Fish', Ph.D. thesis, Cornell University, Ithaca, N.Y.), does not suggest a leaching out (or extraction) of muscle proteins. In an experiment, fish muscle treated with NaCl released less proteins into solutions compared to control muscle. This is different from the suggestion of Siegel et al. (1978) which explained that there is extraction of proteins onto the surface of the muscle particles. 2) The literature suggests that the sarcoplasmic proteins contribute very little to the binding properties of muscle (e.g. MacFarlane et al., 1977). 3) Concerning the cytoskeletal proteins mentioned, some filamentous remnants can be observed in some heat treated samples along the I zones (Figs. 2a, 2b). However, no histochemical attempt was made to identify specific proteins.

C.A. Voyles: The concentration of NaCl which gives rise to the structural changes described in fish muscle is much less than that required to bring about similar changes in mammalian muscle (Offer and Trinick, 1983; Voyles et al., 1984). Do the authors consider this to be a significant species difference, or are other factors involved?

Authors: The same salt treatment (1.5%) resulted in different textures in a cooked product prepared from different fish and avian muscles. The fish product was significantly more tender than the avian products. Biochemical tests also revealed differences between the fish and the avian salt-soluble proteins (Weinberg and Angel, submitted). The comparison of different muscles treated with salts (including mammalian muscles) should be made now under the microscope as well.

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INTERNAL POROSITY OF CORN EXTRUDATE AIR CELL WALL

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Abstract

Transmission electron microscopy was used to observe structures within the air cell wall of a corn-based extrudate. Modified fixation and embedding techniques were employed to obtain optimum thin sections. Photomicrographs from these sections showed minute air cells measuring approximately 5 μm or less. The size of air cells might impose a limiting factor on the infusion of particulate materials throughout the extrudate matrix.

Introduction

The only conclusive method to differentiate physical change taking place within an extrudate as a result of the extrusion process is microscopy (Stanley, 1986a).

Gomez and Aguilera (1983) used polarized light microscopy to show that the morphology of extruded corn starch samples can be seen as a composite of gelatinized and dextrinized material. Using scanning electron microscopy (SEM) Gomez and Aguilera (1984) observed wall thinning in gelatinized corn starch extrudate and breakdown of the walls into flake-like structures as the extrudate became more gelatinized. Owusu-Ansah et al. (1983) used SEM to ascertain complete gelatinization in a corn starch extrudate. Harper (1986) compared the SEM microstructural differences between defatted soy protein and corn grit extrudates and found rougher cellular surfaces in the corn product compared with the soy extrudate. The general morphological characteristics of corn-based extrudates were described by Stanley (1986b) who, using SEM, found these extrudates to have a porous structure consisting of air pockets which are surrounded by laminar sheets of gelatinized starch. To further elucidate extrudate morphology, in this paper we describe the procedure used to obtain microstructural evidence of air spaces or voids within air cell walls of a corn extrudate.

Materials and Methods

Samples used in this study were from a single batch of corn-based extrudate which had been freeze-dried.

Light Microscopy (LM). 4 mm² fragments of air cell walls were placed on a glass microscope slide and examined using a Zeiss Ultraphot Microscope equipped with Luminar optics and a 16 mm objective lens. Photographs were taken with reflected and transmitted light using Polaroid 55 P/N film.

Scanning Electron Microscopy. 4 mm² fragments of air cell walls were affixed to an SEM stub with silver paste, sputter coated with gold palladium in a Hummer X sputter coater, and examined in the SEM mode of a Hitachi 600-2 scanning transmission electron microscope (STEM) at 50 kV. Photographs were taken using Polaroid 55 P/N film.

Transmission Electron Microscopy (TEM). 1 mm² fragments similar to those used above were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) overnight at 22°C (Table 1). The
fixation, dehydration and embedding schedule is given in Table 1. The composition of the embedding medium is given in Table 2. The fragments were removed from the Epon, placed into embedding capsules containing fresh Epon and polymerized overnight at 60°C in a vacuum oven. After polymerization had taken place the blocks were trimmed and 100 nm sections were cut with a Sorvall MT2B ultramicrotome using a glass knife. The sections were mounted on 3 mm copper grids, stained with uranyl acetate and lead citrate, and examined using the TEM mode of a Hitachi 600-2 STEM (operated at 50 kV). The photographs were taken using Kodak 2415 35 mm Technical Pan film. The STEM objective moveable aperture was set at 50 μm to obtain an image with greater contrast.

Table 1: Fixation, Dehydration and Embedding Schedule

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2</td>
<td>overnight</td>
</tr>
<tr>
<td>2</td>
<td>Buffer wash</td>
<td>1 hour</td>
</tr>
<tr>
<td>3</td>
<td>Buffer wash</td>
<td>1 hour</td>
</tr>
<tr>
<td>4</td>
<td>Buffer wash</td>
<td>1 hour</td>
</tr>
<tr>
<td>5</td>
<td>Osmium tetroxide</td>
<td>1 hour</td>
</tr>
<tr>
<td>6</td>
<td>Buffer wash as in steps 2, 3, 4</td>
<td>1 hour each</td>
</tr>
<tr>
<td>7</td>
<td>50% ethyl alcohol</td>
<td>30 minutes</td>
</tr>
<tr>
<td>8</td>
<td>70% ethyl alcohol</td>
<td>30 minutes</td>
</tr>
<tr>
<td>9</td>
<td>80% ethyl alcohol</td>
<td>30 minutes</td>
</tr>
<tr>
<td>10</td>
<td>90% ethyl alcohol</td>
<td>15 minutes</td>
</tr>
<tr>
<td>11</td>
<td>95% ethyl alcohol</td>
<td>15 minutes</td>
</tr>
<tr>
<td>12</td>
<td>Absolute alcohol</td>
<td>15 minutes</td>
</tr>
<tr>
<td>13</td>
<td>Absolute alcohol</td>
<td>15 minutes</td>
</tr>
<tr>
<td>14</td>
<td>Absolute alcohol (over silica gel)</td>
<td>15 minutes</td>
</tr>
<tr>
<td>15</td>
<td>Absolute alcohol / Epon mix (equal parts) without DMP-30</td>
<td>2 hours</td>
</tr>
<tr>
<td>16</td>
<td>Epon mix with DMP-30</td>
<td>2 hours</td>
</tr>
<tr>
<td>17</td>
<td>Polymerize at 60°C under vacuum</td>
<td>24 hours</td>
</tr>
</tbody>
</table>

Table 2: Composition of Embedding Medium (adapted from Dawes, 1979)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Epon 812</th>
<th>DDSA</th>
<th>Epon 812</th>
<th>NMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.1 ml</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
<td>4.9 ml</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Mixture:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solution A</td>
<td>7.0 ml</td>
<td>3.0 ml</td>
<td>0.15 ml</td>
<td></td>
</tr>
</tbody>
</table>

Results and Discussion

Examination with reflected light microscopy of the air cell wall portion of a highly porous corn-based extrudate showed an unremarkable rough surface (Fig. 1A); however, when the same sample was observed using transmitted light, air spaces (A) or voids could be seen throughout the sample (Fig. 1B). It appeared that the air spaces were separated by septa; however, none of the structures could be clearly distinguished at this relatively low magnification. At a higher magnification, using SEM, few pores were seen at the surface (Fig. 2). Although the possibility exists that the pores were covered by AuPd during sputtering, it is more likely that, in this sample, there was virtually no communication between the internal air spaces and the surface.

When sections of the air cell wall were examined by TEM the structures which were barely visible with LM could be readily seen (Fig. 3). Minute air spaces (A) or voids of varying sizes separated by septa (arrows) of varying thicknesses within the matrix of the cell wall are probably analogous to the observations of minute vacuoles in the walls of the larger vacuoles in SEM micrographs of wheat and rye bread crumbs made by Pomeranz and Meyer (1984). Expansive forces occurring during extrusion cause wall thinning (Gomez and Aguilera, 1984) and pore size as well as wall thickness were related to extrudate moisture content (Harper, 1986). These findings were based partially on SEM data. However, the same conditions affecting the morphology of large extrudate structures, whose dimensions are in the 50 to 100 μm range, probably contribute to structures of 5 μm or less within an air cell wall.

Finally, elucidation by TEM of the internal microstructure of the corn extrudate air cell wall permits a more informed evaluation of the effects of changing extrusion parameters on infusion (air cell size may limit diffusion of infusate particulates), extrudate morphology and texture.

References


Figure 1. Portion of an air cell wall. Reflected light micrograph (Figure 1A) showing unremarkable rough surface, and transmission light micrograph (Figure 1B) showing air spaces (A) or voids throughout the sample. Bar = 0.1 mm.

Figure 2. Scanning electron micrograph of portion of sample seen in Fig. 1. Some pores (P) can be seen within the rough surface. Bar = 50 μm.

Figure 3. Transmission electron micrograph of a section of air cell wall. Minute air spaces (A) are separated by septa (arrows) of varying thicknesses. Bar = 1 μm.

Proceedings of the Fifth Pfefferkorn Conference on

PHYSICAL ASPECTS OF MICROSCOPIC CHARACTERIZATION OF MATERIALS

27 papers; 254 + x pages; hardbound; all papers reviewed (and contain Discussion with Reviewers)

From the Foreword:

This first supplement to Scanning Microscopy contains most of the invited and contributed papers presented at the Fifth Pfefferkorn Conference held in Brueggen, West-Germany during October 2-7, 1986, on "The Physical Aspects of Microscopic Characterization of Materials." Correspondingly, the contributions deal with the physics of generation and detection of signals used for the characterization of materials on a microscopic level. It was attempted to provide an overview of the properties and potential of a large number of probes, be it electrons, ions, photons or phonons which have been used in the past or appear promising for use in the future. We succeeded in assembling a large number of experts in rather diverse fields. Each of them shows his latest achievements, of course, but efforts are made to make it understandable to the specialist in other fields, and to the reader not familiar with a particular field.

The number of topics and techniques is rather large. It ranges from established techniques which have reached an admirable state of perfection, to very recent techniques which are just emerging and the potentials of which have yet to be explored. Among the former are high resolution secondary ion and atom mass spectroscopy, high resolution electron energy loss spectroscopy and electron holography, to name only a few. On the other side there are such novel techniques as Scanning Tunneling Microscopy, Near-Field Optical-, X-Ray-, and Photoelectron Microscopy, or LEED-Microscopy and the Scanning Electron Microscope with spin polarization analysis for the study of magnetic microstructures. The hope of the organizers was to open new avenues by bringing people, topics and techniques together which are not normally familiar to each other. We would be happy if a reader of this book, perhaps a secondary ion microscopist would decide to incorporate a tunneling microscope to study ion-induced damage on an atomic level, or if an analytical electron microscopist decided to look more deeply into the potential of X-ray microscopy for his purposes.

Organizers:
Juergen Kirschner
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John A. Venables
Om Johari

Kernforschungsanlage, Juelich, West Germany
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Conference Director, Scanning Microscopy International

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Book Reviews


This book presents the proceedings of the Tenth Basic Symposium sponsored by the Institute of Food Technologists and International Union of Food Science and Technology. In fifteen chapters, nineteen authors describe basic aspects of water structure and binding and their effects on the chemical reactivity, texture, flavor, color, and microbiological stability of foods. In addition, methods for determining water activity are presented.

Chapters by M. Le Maguer, "Mechanics and Influence of Water Bindings on Water Activity" and S.J. Richardson and M.P. Steinberg, "Applications of Nuclear Magnetic Resonance" contain structural information which may be of interest to food microbiologists. In a chapter on "Effects of Water Activity on Textural Properties of Food", M.C. Bourne reviews data which show that the textures of various foods depend upon water activity. If food microstructure is a determinant of texture, then more studies of microstructure-water activity relationships would be of interest.

This book is intended to be read by food scientists and technologists and others involved with food products. For the beginner, it is a well-organized survey; for the experienced investigator, it is a valuable reference.

D. N. Holcomb


This slim text is a condensed survey of the chemistry of food. The subject is divided by chapter into: carbohydrates, lipids, proteins, colors, flavors, and preservatives. The concise treatment given each provides an overview particularly useful in organizing concepts and data in this extremely complex area of study, this aspect makes the text useful as a review to those experienced in the field and to students for whom this would serve as an excellent supplement to a more detailed treatment of the subject.

As much as possible each chapter is covered by developing a matrix of relationships including: the fundamental structure of the category; the effect of this structure on the chemical and physical properties of food; the cellular organization and function of the component; nutritional aspects; effects of modern processing on the components physical, chemical, and nutritional properties (and vice versa); and the complex interrelationships between the subject and those of the other chapters. This is not light reading, however, Dr. Coulitate draws on the reader's experience with food to keep the text from being dry or confusing.

This is an excellent book recommended to all for its broad overview of the subject.

Steven Huntoon
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, sea foods, meat, 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 acceptable for publication are allowed oral presentation. The latter related microscopy/microanalytical methods.

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 either 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 reviews). In addition to all the text, the manuscript may have to contain the author's publishable responses to questions raised by the paper’s reviewers (see the Discussion with Reviewers in papers published in FM).

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.

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 more than 1000 words.

LETTER TO THE EDITOR: Commenting on a paper already published in FM.

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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 discussed,” “are presented” etc. should be avoided.

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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.

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