FOOD MICROSTRUCTURE

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FOOD MICROSTRUCTURE

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Editorial

Announcing

Name change of the journal to

"Food Structure"

and

Frequency change to a Quarterly

During the 8 years of its existence, Food Microstructure has gained an international reputation for the quality of papers published, both as far as the scientific content and the form (including the superb reproduction of half-tones, Discussion with Reviewers, etc.) are concerned. The number of manuscripts submitted has increased to a point which makes it possible to publish the Journal as a quarterly (from volume 9, number 1, 1990) and, in this way, shorten the time between the submission of a paper and its publication.

Since Food Microstructure was first published, food structure studies have made considerable progress in both quantitative and qualitative aspects. It is increasingly recognized that food structure is affected by different factors and is closely related to many properties of the foods important to the manufacturer and the consumer. In recent times, structural studies in food science have developed into a sophisticated discipline in which food structure and micro-chemical composition may be studied by a variety of techniques. In addition new techniques continue to develop.

Food structure is studied at various levels of resolution. Over the 8 years, the contents of papers published in this journal have evolved from various aspects of microscopic structure to an emphasis on an integrated view leading to elucidation of correlation between physical and chemical structures of food. In recognition of these changes, this journal will be published under a new, broader name of Food Structure (new ISSN: 1046-705X; Coden: FSTUE2), and a new sub-title: "An International Journal on the Structure of Foods and Feeds with Special Emphasis on Relation Between Processing, Molecular Properties, Microstructure, and Macroscopic Behavior". The editors and publisher of Food Structure will take care that the quality of journal is maintained and further improved.

Suggestions and comments from our readers are invited, please contact any one of the editors or the publisher (see inside front cover of a recent issue of the journal).

Editors
Following is a list of papers on milk, cheese, yoghurt, and other dairy products published in Food Microstructure during the past five years (1985-1989). This index gives an abridged (or complete title), name of the first author, first page and the part number. Please see below for reprints availability.

**Food Microstructure, Volume 8, 1989**

- Electron Microscopic Localization of Cholesterol in Bovine Milk Fat Globules; R.W. Martin (31/1)
- Heat-Set Gels Based on Oil/Water Emulsions ... Whey Protein Functionality; R. Jost (23/1)
- Protein Concentration in ... Milk Retentates ... Microstructure of Gels; D.D. Gavric (53/1)
- ... Adherence of Lactobacillus Acidophilus to ... Intestinal Cells ...; S.K. Hood (91/1)
- Rheology and Microstructure of Strained Yoghurt (Labneh) ... Cow's Milk; A.Y. Tamine (125/1)
- Grittiness in a Pasteurized Cheese Spread: A Microscopic Study; H.W. Modler (201/2)
- The Size Distribution of Casein Micelles in Camel Milk; Z. Farah (211/2)
- Effect of Heating to 200 Degrees on Casein Micelles in Milk ...; V.R. Harwalkar (217/2)
- ... Spray-Dried Retentates Obtained by Ultrafiltration of Milk ...; M. Kalab (225/2)

**Food Microstructure, Volume 7, 1988**

**REVIEW:** Lacost Crystalization in ... Whey Powders and in Spray-Dried Lactose; Z. Saito (75/1)
- ...Microstructure in Raw, Fried, & Fried & Cooked Paneer ... from ... Milks; M. Kalab (83/1)
- Amino Acid Composition and Structure of Cheese Baked as a Pizza Ingredient ...; A. Paquet (93/1)
- Changes in Microstructure of Saint Paulin Cheese During Manufacture ... SEM; M. Rousseau (105/1)

**REVIEW:** Applications of Cold Stage SEM to Food Research; J.A. Sargent (123/2)
- Porosity, Specific Gravity and Fat Dispersion in Blue Cheeses; K.M.K. Kebany (153/2)
- Role of Beta-Lactoglobulin in ... Structure of Casein Particles in ... Gels; V.R. Harwalkar (173/2)

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- Encapsulation of Viscous Foods in Agar Gel Tubes for Electron Microscopy; M. Kalab (213/2)

**Food Microstructure, Volume 6, 1987**

- Product Morphology of Fatty Products; I. Heertje (1/1)
- ... Effect of Citrate on the Growth of Group N Streptococci; S. Ito (17/1)
- Crystallization of Calcium Phosphate at the Surface of Mould-Ripened Cheeses; B.E. Brooker (25/1)
- ... Curd Granules in Traditional Swiss Hard and Semi-Hard Cheeses; M.W. Ruegg (35/1)

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Influence of the Extrusion Process on Characteristics and Structure of Pasta

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Abstract

The effects of the kneading and forming processes of pasta quality have been investigated. Using the same blend of wheat flour and keeping mixing and drying conditions constant, three types of spaghetti were produced using the following three kneading and forming processes: A) kneading with a continuous press and forming by pressure-extrusion; B) kneading and forming by sheeting-rolls; C) kneading by hand and forming with sheeting-rolls. These three processes impart different mechanical work on the dough.

The three types of dried spaghetti exhibited differences in cooking requirements and in cooking quality. Spaghetti (A) absorbed water more slowly and showed poor cooking quality. Spaghetti (B) and (C) had a shorter cooking time, no stickiness and good firmness after cooking. Spaghetti compactness seems to control water absorption during cooking, i.e., the greater the compactness, the longer the cooking time. Nevertheless, compactness does not explain differences in cooking quality. On the contrary, this characteristic is clearly related to the different organization of proteins, as transmission and scanning electron microscopy images revealed. In fact both spaghetti (B) and (C) showed a compact and continuous protein network, probably as a consequence of the mild and ordered kneading obtained by the sheeting process. Spaghetti (A), produced by dough kneading implying strong mechanical stresses, exhibited a protein network breakage which may account for its poor cooking quality.

The results of the present work indicate that industrial kneading and extrusion, as performed today in the continuous press, are unsuitable for making the best use of poor quality raw materials. The pressure-extrusion process requires technological innovations which could ensure spaghetti with cooking quality comparable to that produced in the traditional sheeting process as well as high productivity.

Introduction

It is well known that pasta quality and its cooking characteristics, as far as consumer acceptability is concerned, are affected by the quality of the raw material as well as by the processing conditions. Numerous studies in this field have specified the properties of the raw material (2, 4, 6, 8, 14, 23, 36, 38) and the conditions of the drying cycle (11, 13, 25, 35) that ensure a good quality product.

On the contrary, there have been few investigations regarding the role that the first steps of the pasta-making process (kneading and extrusion) have on pasta quality. Papers published some years ago (34, 37) reported that hand-made and roll-sheeted spaghetti always exhibit higher quality compared with pasta produced from the same raw material by the usual pressure-extrusion process. This is well known by pasta-making technologists (19, 24).

Recently, some authors observed that noodles prepared by a sheeting process (10) show a higher gluten development in comparison to those obtained in a continuous industrial press (26). However, no explanation has been found for the differences in quality due to the kind of kneading and forming processes used.

Therefore, this study was designed to control the effect of various technological processes used for dough-forming on pasta quality. In particular, the continuous industrial press was compared to the more traditional working methods, such as roll-sheeting and hand-sheeting.

Materials and Methods

Spaghetti processing

A wheat flour blend (12% protein dry mass, particle size less than 100 micrometers), obtained by mixing a commercial soft wheat flour with a low protein content and a commercial hard wheat flour in the ratio of 70/30 w/w, was used.

The flour and water (at 36°C) were blended in order to produce a mixture with a final water content of 38%. Mixing was performed in a Braibanti laboratory mixer at 500 r.p.m. for 60 sec and then at 200 r.p.m. for 40 sec. After a resting period of 20 min in the mixer at room temperature, the flour/water mixture was kneaded and formed into spaghetti according to the following processes (Fig. 1): A) Extrusion under pressure with a continuous press (Braibanti laboratory press), using the following conditions: extrusion pressure, 60 atmospheres; dough...
temperature, 37°C; teflon lined die with 1.5 mm square holes. The time required for passing the dough through the cylinder and the head of the extruder was 2-3 minutes.

B) Roll-sheeting by means of pairs of stainless steel cylinders (12 cm diameter). The mixture was sheeted by passing it 10 times in the same direction at a roller setting of 12 mm. The sheet was folded in half before each passage. The roll setting of 12 mm assured good kneading without tearing the surface. The dough sheeting was then completed with 1 passage at 6 mm, 1 passage at 3 mm and 2 passes at 1.5 mm. These last 4 passages were performed, without folding the sheet, in order to reduce the sheet thickness gradually. The time required for the sheeting process was about 15-18 minutes.

C) Hand-made sheeting. The dough was carefully kneaded by hand for 10 min, wrapped in aluminium foil, rested for 30 min and rolled out in a sheet with a rolling pin. It was then passed twice through the rollers used in process B) set at 1.5 mm to obtain a uniform thickness. The time required for this process was about 1 hour.

The final dough sheets obtained in processes B and C were cut into strips (1.5 mm thickness) with a home noodle cutter. The three types of fresh spaghetti, with the same shape and size ("spaghetti alla chitarra", 1.5 mm thick) were dried at the same time at low temperature (45°C for 16 hours).

A modified extrusion process was also done in the continuous press to obtain samples for ultrastructural studies. A well-kneaded dough, produced under the same conditions as reported for process B, was prepared with 10 passages through the rollers (12 mm spacing). This roll-sheeted dough was then cut into small cubes, fed into the continuous press and processed as reported above for process A. The press was stopped after 5 min of working and the die removed. The dough in the interior of the extrusion-head could be easily and carefully removed (with a sharpened lancet) prior to its extrusion.

Porosity

Porosity was evaluated with a Carlo Erba mercury intrusion porosimeter (model 2000), connected to a computer for complete automatic operation, collection and processing of data.

Mercury behaves as a non-wetting liquid. Thus, it will not flow into small cavities of a porous solid unless it is forced by pressure. The higher the pressure, the smaller the pore (18). In our test,
some strands of dried spaghetti (0.5-1 g) were placed in the porosimeter vessel, evacuated, filled with mercury and then subjected to increasing pressure up to 2000 x 10^5 Pa. This pressure range allows pores with a radius from 4 to 7500 nm to be determined. The characteristics of compactness were expressed as cumulative volume (mm^3 of pores per g of solid) and as porosity % (mm^3 of pores per 100 mm^3 of solid).

Breaking strength

The breaking strength was measured with a bending test using the Instron testing machine (model 4301) equipped with a compression cell of 1000 g; the crosshead speed was fixed at 20 mm/min. A single strand of dried spaghetti (8 cm length) was placed on two cylindrical supports (1.2 cm diameter) 3.5 cm apart. The spaghetti strand was broken in the middle by a blade made from a piece of plexiglass (1.5 mm thick with a flat cutting profile) attached to the crosshead. The breaking strength was expressed as the force (g) required for breaking the strand of spaghetti.

Evaluation of Cooking Quality

Organoleptic test. The cooking quality was determined with a standard test for durum wheat spaghetti. 100 g of spaghetti were cooked in 1000 ml of boiling mineral water for the optimal cooking time (disappearance of the central ungelatinized core, ref. 35). It was then drained for 1 min in a strainer of 169 mesh. Stickiness (spaghetti adhesiveness to teeth during mastication) and firmness (force required to compress the spaghetti between the molar teeth) were evaluated by 5 expert tasters using appropriate adjectives (5, 7). The panel also gave an overall rating of quality: 3 for the poorest, 9 for the best.

Rheological test. The instrumental evaluation of spaghetti cooking quality was obtained with the compression test proposed by Dalbon et al. (8). The Instron testing machine (model 4301) was fitted with a 10,000 g compression cell, and with a circular plunger of 3.55 cm diameter. The crosshead and chart speeds were 5 mm/min and 100 mm/min, respectively. Spaghetti was cooked at its optimal time, drained for 1 min and then placed in a Petri box. Two min after draining, 6 strands of spaghetti were placed in the center of a steel plate and compressed to a maximum pressure of 2000 g. When this value was reached, the crosshead movement was reversed and the sensitivity of the Instron machine was expanded to maximum to measure stickiness with greater precision (5, 12). Stickiness was evaluated from the negative area of the force-deformation diagram and was expressed in N x mm. The same test was repeated on 6 other strands of cooked spaghetti after fixed times of draining in order to check the increase in stickiness with time.

Water absorption during cooking

The water absorbed by dried spaghetti during cooking was determined by measuring the increase in the spaghetti weight as a function of cooking time. Cooking was performed in a small vessel provided with an appropriate strainer, divided into 4 sections. 2.5 g of spaghetti (strands 2-3 cm length) were placed in each section and plunged into the boiling water. After 2.5 min of cooking, the sample in the first section was removed, drained on a filter paper for 13 min and weighed. The procedure was repeated on the spaghetti in the other sections after 5, 7.5 and 10 min of cooking. Water absorption was referred to 100 g of dried spaghetti. The material lost in the cooking water was not considered.

Transmission Electron Microscopy (TEM)

Samples were soaked in a 30% glycerol-water solution at room temperature. The imbibition times for fresh spaghetti (prior to drying) and cooked spaghetti were 20 min and 10 min, respectively. A small quantity of soaked samples was then placed on the gold specimen holders, frozen in super-cooled liquid nitrogen (LN_2) and transferred into a Balzers 301 unit. Defrosting was carried out for 20 min at -95°C. Fracturing was performed at ~105°C, immediately followed by shadowing with Pt/C (film thickness: ca. 2 nm, angle: 45°) and C (film thickness 20-25 nm). Replicas were cleaned in 70% and 30% sulfuric acid solutions for at least 48 h, then washed in distilled water, acetone, and double distilled water. Replica observation was performed with a Philips EM 4301 electron microscope at 80 or 80 kV.

Scanning Electron Microscopy (SEM)

Both fresh and cooked samples were immediately frozen in super-cooled LN_2 and then freeze-dried using an Edwards tissue drier (model EPD3). Freeze-drying was performed at ~60°C for 24 h. Fracturing was done in two ways: 1) cryo-fracturing the samples directly in LN_2, using a pair of tweezers, and then freeze-drying. The resulting fracture plane was smooth and even. 2) dry-fracturing by fracturing the sample after freeze-drying. In this case, the fracture plane was uneven and ragged. The freeze-dried samples were mounted on stubs, sputter-coated with gold and then observed in a Cambridge Stereoscan 150 microscope at 10 or 20 kV.

Results and Discussion

Conventional production of pasta involves the fixed steps of blending flour and water into a very stiff mixture, kneading, forming by extrusion and drying. For reasons of productivity, these steps take place on line and rapidly in the modern press. The process is continuous and completely automated with a time interval of only 2 minutes from kneading to the end of extrusion. In the more traditional systems, such as roll- or hand-sheeting, the steps of kneading and forming are performed separately. Therefore, it is possible, however roughly, to isolate the effect of each technological step on final pasta quality. In this regard, kneading and forming have been studied by subjecting the same mixture of wheat flour and water (without working and dough development) to different forming processes, as shown in Fig. 1:

- kneading with a continuous press and forming by extrusion (process A);
- kneading and forming with pairs of sheeting-rolls (process B);
- kneading by hand-working and forming with pairs of sheeting-rolls (process C).

As in our previous work (35), we used only wheat flour instead of durum wheat semolina. The low porosity of this raw material makes the pasta-making process critical, and allows the effects of the three different technological processes to be more easily assessed for the spaghettis produced.

An organoleptic test was first performed to evaluate the quality of the three types of spaghetti, all with the same shape and thickness and obtained...
Table 1 - Cooking Quality as Evaluated by Sensory Panel

<table>
<thead>
<tr>
<th>Process</th>
<th>Optimum Cooking Time</th>
<th>Stickiness</th>
<th>Firmness</th>
<th>Overall Rating*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Pressure-extruded spaghetti</td>
<td>9 min</td>
<td>High</td>
<td>Weak</td>
<td>6.0</td>
</tr>
<tr>
<td>B Roll-sheeted spaghetti</td>
<td>7.5 min</td>
<td>Absent</td>
<td>Good</td>
<td>8.0</td>
</tr>
<tr>
<td>C Hand-made spaghetti</td>
<td>7.5 min</td>
<td>Absent</td>
<td>Good</td>
<td>8.5</td>
</tr>
</tbody>
</table>

*Rating scale: 3 = poorest quality; 9 = best quality

Table 2 - Characteristics of Compactness of Dried Spaghetti

<table>
<thead>
<tr>
<th>Process</th>
<th>Cumulative Volume* (mm³/g)</th>
<th>Porosity* (%)</th>
<th>Breaking Strength (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Pressure-extruded spaghetti</td>
<td>26.95</td>
<td>3.75</td>
<td>88.0</td>
</tr>
<tr>
<td>B Roll-sheeted spaghetti</td>
<td>51.22</td>
<td>6.94</td>
<td>77.1</td>
</tr>
<tr>
<td>C Hand-made spaghetti</td>
<td>53.51</td>
<td>5.17</td>
<td>71.5</td>
</tr>
</tbody>
</table>

*See Porosity, Materials and Methods.

Figure 2 (at left). Stickiness of cooked spaghetti evaluated with the Instron rheological test. A) pressure-extruded spaghetti; B) roll-sheeted spaghetti; C) hand-made spaghetti.

Figure 3 (at right). Water absorption during cooking (g/100 g dried spaghetti). A) pressure-extruded spaghetti; B) roll-sheeted spaghetti; C) hand-made spaghetti.

from the same dough. Table 1 reports noticeable differences among the three samples. The cooking quality decreases (i.e., spaghetti becomes stickier and softer) as the working time of the dough decreases and the mechanical stress of the process increases. The worst quality pasta was obtained with the continuous pressure-extrusion process, whereas the best spaghetti was produced with the hand-working process. The high quality of this last product is surprising, considering that it was obtained from the same blend of wheat flour and at the same water content (36%). No other industrial process used today produces this result, including the roll-sheeting process which is based on the same mechanical principle.

The rheological evaluation of stickiness performed with an Instron testing machine (Fig. 2) also showed the same ranking of quality for the cooked spaghetti. Process A yielded the stickiest while process C gave the least sticky spaghetti.

Certain physical characteristics of spaghetti, such as compactness and water absorption during cooking, are clearly influenced by the kind of kneading and forming. The mechanical work performed under different conditions induces a different physical stress that obviously causes a different compactness in dried spaghetti. This last parameter, evaluated by means of breaking strength and porosity (Table 2), is highest for the extruded spaghetti and lowest for the roll-sheeting product.

Porosity affects the kinetics of water
Extrusion process and pasta quality

Figures 4 and 5. Freeze-fracture TEM. All the spaghetti images were taken in the center of the strand.

Figure 4. Roll-sheeted spaghetti. A) Fresh spaghetti: the continuous protein network (PN) surrounds the starch granules (SG). B) Cooked spaghetti: the starch material (SM), entrapped inside the protein network (PN), is organized in fibrils and small regular aggregates. Arrows show thin protein fibrils.

Figure 5. Pressure-extruded spaghetti. A) Fresh spaghetti: the protein (P) forms aggregates unevenly dispersed among the starch granules (SG), some of which appear broken (BSG). B) Cooked spaghetti: the starch material (SM) is highly dispersed inside a weak and discontinuous protein network (P).

absorption during cooking (Fig. 3) and contributes to the longer cooking time needed for pasta products submitted to high pressure which are thus less porous and more compact. Contrary to what has been reported by other authors (31, 32), the compactness of the dried pasta does not appear directly related to cooking quality.

These observations emphasize that the kind of process used for kneading and forming greatly influences the macro-structure of spaghetti and its cooking behaviour. These characteristics cannot be explained on the basis of our analytical data. However, clear suggestions seem to arise from careful observations, with TEM and SEM, of the fine structure of spaghetti at various processing steps.

Some ultrastructural features seem to be related to the kind of kneading and forming. The different structures may explain the cooking behaviours of spaghetti, as reported in the literature (33, 35).

The compact and continuous protein network observed in fresh roll-sheeted pasta (Fig. 4A) is comparable to that observed in durum wheat spaghetti (33) and likely traps the starch granules which swell during cooking (Fig. 4B). Under these conditions the starch material is retained inside the spaghetti strands during cooking, thus limiting its stickiness. Owing to the reduced swelling of the granules, the starch subunits are arranged into chain-like fibrils and ordered groups. This pattern, a probable consequence of the retrogradation phenomena (15), is always observed in pasta of superior cooking quality (5, 33, 35), and is never seen in
Extrusion process and pasta quality

Figure 8. Ultrastructure of dough just before its extrusion through the die. A) Freeze-fracture TEM image; B) SEM image after dry-fracturing. (SG) starch granules; (SM) starch material (probably from broken starch granules); (P) and arrows indicate the broken protein fibrils.

Figure 6 (on the facing page, left panel). SEM images of roll-sheeted spaghetti. A) cryo-fracturing and B) dry-fracturing of fresh samples; C) cryo-fracturing of cooked spaghetti. Symbol definitions are the same as in Figure 4. Arrows show location of protein network.

Figure 7 (on the facing page, right panel). SEM images of pressure-extruded spaghetti. A) cryo-fracturing and B) dry-fracturing of fresh spaghetti; C) cryo-fracturing of cooked spaghetti. Symbol definitions are the same as in Figure 5. Arrows indicate the broken protein network.

products obtained with soft wheat flour processed under industrial conditions (35). Hand-made spaghetti presents the same ultrastructural features as the roll-sheeted pasta.

On the contrary, in freshly extruded spaghetti (Fig. 5A) the protein matrix looks discontinuous with protein aggregates unevenly distributed among the starch granules. During cooking (Fig. 5D), the lack of a continuous protein network causes high hydration of starch material and its marked dispersion outside the spaghetti (33, 35), resulting in stickiness (16, 29).

SEM images confirm and complement TEM results, especially when the sample is prepared by cryo-fracturing, a sectioning technique comparable to the freeze-fracturing technique. Moreover, the larger field of view of the SEM images allows the continuity of the protein framework in fresh roll-sheeted spaghetti before (Fig. 6A, 6B) and after cooking (Fig. 6C) to be more easily observed.

The presence of broken protein fibrils in freshly extruded spaghetti is just as clearly seen (Fig. 7A, 7B). A similar organization in dough taken from the press has been reported by Matsuo et al. (26). After cooking, a dramatic swelling of the starch granules can be seen. They are often so coalesced and clustered together that their individual, original edges are no longer visible (Fig. 7C). This supports the idea that during cooking, extensive starch swelling prevails, with little protein interaction. This could account for the poor cooking quality of the pressure-extruded spaghetti (35).

Since the raw material and the other technological steps of pasta-making are held constant, the results show that the processes of dough kneading and extrusion significantly affect the starch and protein macromolecular organization inside the product. This fact clearly influences spaghetti cooking quality.

The lower quality characteristics of a pressure-extruded pasta, consisting of a discontinuous protein network, can already be seen in the dough taken from the extrusion-head just before extrusion through the die holes (Fig. 8A, 8B). Therefore, the step of mere extrusion does not seem to result in ultrastuctural differences between extruded and sheeted spaghetti.

By feeding the continuous press with a "worked" dough obtained by roll-sheeting, it was possible to demonstrate that dough transformation takes place before reaching the die. The "ideal" continuous protein network that this dough had when first put into the continuous press (Fig. 9A, 9B) is already lost by the time it reaches the die. The area of protein fiber breakdown is extensive in the entire field of view (Fig. 9C, 9D).

All the electron micrographs, together with the other experimental data from this work demonstrate that, in comparison with the more traditional processes, the technical step of kneading and forming carried out in the continuous press breaks down those dough structures which may affect spaghetti quality. In our case, the drop of quality is enhanced by using wheat flour whose protein, due to unsuitable gluten properties (17, 20, 21, 27) does not withstand the physical stress imposed upon it.

An explanation for protein fibril breakage could be insufficient dough development produced by auger. This phenomenon is probably related to short kneading time (28, 34) and low mechanical efficiency (10, 22) as well as the limited water content of the dough. It is well known that these factors prevent the possibility of good gluten development (9, 10, 26,
Figure 9. SEM ultrastructure of roll-sheeted dough (see text) before (A and B) and after (C and D) kneading in the continuous press by the auger. A) and C) cryo-fracturing; B) and D) dry-fracturing. The fibrils of the protein network (PN), that the dough showed after roll-sheeting, are weakened and broken (P) inside the continuous press. Arrows indicate the protein fibrils in all images.

34). In fact, the working time in a continuous press is reduced to only a few minutes in comparison with about an hour for the hand-sheeting process. The mechanical work done by the auger consists mainly in shifting the dough under pressure with coaxial rotations and laminar flow in the extrusion cylinder (26), followed, probably, by a less orderly transfer inside the extrusion-head. During sheeting, instead, the dough is formed by means of numerous passages through the rolls. As the sheet thickness is gradually reduced (by narrowing the gap between the cylinders), the dough is submitted to uni-directional and mild "stretchings" which promote an ordered and uniform gluten development. The same kind of dough structure results with the hand-working process.

The different mechanical stresses of the two kinds of dough forming could also be an explanation. Strong mechanical work takes place inside the press and induces stretching and remixing. This work is probably unsuitable for the plastic characteristics of the "developing" dough. The non-homogeneous pressure (1, 19) and the disordered movements with shearing and tearing of the dough in the last part of the machine may negatively affect the continuity of the protein matrix. It should be pointed out that, over the years, the industrial press has been improved for speed and extrusion capacity (hence for productivity - 3, 13, 34), but only somewhat improved for the regular and ordered flowing of the dough (28, 30, 34). The dough, in fact, is a non-Newtonian system whose viscoelastic characteristics and behaviour during mechanical transfer are mostly unknown. On the contrary, neither the hand-working nor the roll-sheeting processes impose negative physical stress to the dough which can thus freely expand and gently form. Finally, we can presume that insufficient kneading, together with mechanical stress, may produce the micro-breakdown of the gluten network found after the continuous pressure-extrusion.
Extrusion process and pasta quality

In view of the final quality of the product, kneading and forming under pressure undoubtedly represent a critical factor in the industrial process, whereas extrusion through the die, even if performed at high pressure, does not seem detrimental to pasta cooking quality. The final aim in the optimization of the kneading and forming process in the industrial press is to reach the level of spaghetti quality obtained with hand-working or, at least, with roll-sheeting, keeping all other conditions constant.

Conclusion

The results of the present investigation, obtained from cooking tests, physical-mechanical tests and ultrastructural observations, demonstrate that the kneading and forming of a pasta dough with a continuous press induces a decrease in spaghetti quality in comparison with roll-sheeting or hand-processing processes. The lower quality, related to the reduced working time and to the intensity of the mechanical stress, can be explained by ultrastructure breakage of the protein matrix observed in the dough before extrusion. This finding strongly indicates that industrial pressure-extrusion used today for pasta-making is not an ideal process for making the best use of poor quality raw materials and suggests that an improvement in final product quality can be expected by improving this production step. More research is needed in order to make kneading and forming a real "biotechnological" process. In this regard, a careful examination of the ultrastructure modifications of the dough during the various steps of the process proves to be indispensable for a correct and complete evaluation of the processing conditions.

References

trafile e della vite di compressione nelle prese continue per pasta alimentari. Tec. Molitoria 9(8), 59-61.

Discussion with Reviewers

R.R. Matsuo: Why did the authors choose a blend of common wheat (T. aestivum) for their study and not durum wheat (T. turgidum var. durum)? It is particularly surprising since Italy is one of the two countries in the world that specifies 100% durum wheat for pasta products. All Italian pasta manufacturers maintain that durum wheat, especially varieties produced in Puglia, produces the best pasta with excellent texture and little surface stickiness. Therefore the obvious question is how would pasta processed by the three methods described in this paper but made with high quality durum semolina be rated?

R. Moss: Could the authors confirm that it was flour (i.e., less than 130 micrometers), and not semolina from bread wheat, that was used? Are their comments equally applicable to extruded pasta vs. semolina? Please comment on the relative amounts of pasta made from flour versus that from the various types of semolina.

Authors: Our aim was to study the effects of different kneading and forming technologies on pasta cooking quality, the effects of which were expected to be limited. According to our experience (35), by using a raw material with poor pasta-making properties (in this case a blend of wheat flour, particle size less than 100 micrometers), it is possible to better identify the effect of a technological step on the quality of the final product.

It is, however, important to emphasize that, as the production of high quality semolina is limited, the world pasta manufacture mainly consists in products obtained from durum wheat semolina of medium quality and from T. aestivum flour (21).

Presently we cannot extend our experimental results to durum wheat semolina, even if, according to reputed Italian pasta technicians (24, 34), the semolina products obtained with a sheeting process always show higher cooking quality in comparison with pressure-extruded pasta.

R. Moss: The authors conclude by stating that more work needs to be done to make the kneading and forming a real biotechnological process. They indicate that the intensity of work input in the press has an adverse effect on the protein. Have they investigated the effect of different extrusion pressures or screw design on pasta structure and porosity and hence on quality? Also, though the effect of extrusion on starch is largely ignored in the text, Figure 5A shows damaged starch granules. Does more starch get damaged during extrusion and, if so, would this not adversely influence pasta quality?

Authors: In preliminary pasta-making trials, we produced spaghetti at two different extrusion pressures: 50 and 100 atm. SEM images of both products showed a marked breakage and discontinuity in the protein network. Both types of spaghetti were judged as having poor cooking quality, even if the pasta produced at 100 atm had a little lower stickiness. In the present research, extrusion was carried out at 60 atm of pressure. This value, lower than the industrial ones (ca. 100 atm), is justified by the higher humidity of the dough we used (36% compared with 30%); that is, however, a normal humidity for sheeting processes.

The screw used in the present work had a design comparable to that of an industrial press. We have not yet investigated the effect of other screw designs on pasta quality; this aspect is related to the optimization of the press and, hence, it will be the object of future studies.

The EM images of pressure-extruded spaghetti clearly exhibit an extensive break-down of the protein network. Therefore, even if some altered starch granules have been observed, it is not possible to relate spaghetti cooking quality to these observations. We agree that starch granule damage, which can take place during extrusion [Lintas C, D'Appolonia BL (1973). Effect of spaghetti processing on semolina carbohydrates. Cereal Chem. 50, 563-570], negatively affects pasta cooking quality as the broken granules swell more quickly during cooking [Collison R (1968). Swelling and gelation of starch. In: Starch and its derivatives. JA Radley (ed.), Chapman and Hall Ltd., London, 168-192].

R. Moss: What role does increased cooking time, per se, have on pasta quality and is the time influenced by porosity? How were the number of sheeting roll passes determined, what is the influence of this factor on pasta porosity, cooking time and quality?

Authors: The optimal cooking time is evaluated as the disappearance of the central ungelatinized core of spaghetti. This factor depends on several chemical and physical characteristics of the pasta, such as protein and water content, porosity, component organization and, obviously, size of the spaghetti. For this reason we suppose that the optimal cooking time has no direct relationship with cooking quality.

The sheet of dough was fed manually into the sheeting rolls. The number of passes was chosen by visually evaluating the surface of the sheet that should appear "velvety". This aspect assures good kneading. The influence of the number of the sheeting roll passes on pasta porosity, cooking time and quality was not studied.
VACUOLE FORMATION IN WHEAT STARCHY ENDOSPERM

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Abstract

The formation of vacuoles in wheat (Triticum aestivum cv. Highbury) starchy endosperm cells was studied using electron microscopy. Some vacuoles were always present, even in the coenocytic cytoplasm. The first formed endosperm cells were highly vacuolated, but became filled with cytoplasm as they grew older. Various-sized pieces of cytoplasm were found in vacuoles of developing endosperm cells, probably as a result of autophagic sequestration. The membranes of the autophagic vacuoles appeared to originate from the rough endoplasmic reticulum and from extensions of already-formed vacuoles. Autophagic activity was confirmed by localizing the hydrolytic enzyme acid phosphatase within the vacuoles. The rough endoplasmic reticulum (RER) also stained positive for this enzyme.

Introduction

Despite numerous studies, the precise mode of deposition of storage proteins in the developing starchy endosperm of wheat remains unclear. While this process has been studied by numerous investigators, a great deal of controversy exists concerning the process. One aspect is the route by which the storage proteins enter the vacuoles or whether they are deposited into vacuoles at all. An early hypothesis suggested that protein bodies were independent organelles capable of protein synthesis and deposition. This theory was supported by Morton et al. (1964) who reported that protein bodies contained DNA and ribosomes (with lower sedimentation values than those of the cytoplasm) within a distinctive membrane, and were capable of in vitro protein synthesis. Similarly the suggestion that vacuoles are the site of protein synthesis is based on the presence of ribosomes associated with protein bodies (Barlow et al., 1974). These results are not consistent with more recent studies, which have led to the proposal of two different mechanisms. The first is that the protein is synthesized on the rough endoplasmic reticulum (RER) and transported possibly via the Golgi apparatus into vacuoles (Greene, 1981; Donovan et al., 1982). Alternatively, the protein may be deposited directly within the RER lumen to form a body surrounded by a non-intact membrane of RER origin (Miflin et al., 1981; 1983).

One problem in determining the precise mechanism is obtaining reproducible and artifact-free specimens. For example, some workers have reported direct connections between RER and vacuoles (Campbell et al., 1981; Parker, 1982) while others have found none (Bechtel et al., 1982a; Bechtel and Barnett, 1986a, b). Furthermore, workers differ in their views as to what constitutes "good fixation" of specimens (Miflin et al., 1981; 1983).

In the present paper we report electron microscopic studies which show that storage proteins are present within vacuoles during the initial stages of protein deposition in developing wheat endosperm. We also show that the enlargement of these vacuoles during the early stages of development occurs by an autophagic process involving the RER and at least one hydrolytic enzyme (acid phosphatase) may be present within the vacuoles when protein deposition is occurring. The results differ from studies conducted on root tips.
are the earliest stages of differentiation. Our results may explain some of the problems experienced by early workers in interpreting the relationships between RER, vacuoles, and protein bodies.

Materials and Methods

Wheat (Triticum aestivum cv Highbury) used for this study was grown either in field plots or in glasshouses at Rothamsted Experimental Station, Harpenden, England during the summer of 1987. Individual heads were tagged at anthesis and harvested at various times during development, typically; day of flowering, 2, 4, 7, 9, 11, 14, 18, 21, 28, 35, 42, and 50 days after flowering (DAF). The middle florets were used for microscopy. Fixation for microscopy was conducted as previously described (Bechtel et al., 1982a) using 2% glutaraldehyde (v/v) and 2% paraformaldehyde (w/v). Acid phosphatase activity was localized at the ultrastructural level using a modified Gomori method (Gomori, 1952). Wheat endosperm tissue was fixed 1 h in 2% glutaraldehyde-2% paraformaldehyde in 0.05 M cacodylate buffer at pH 6.8 and 19 C. Tissue was washed 3 times for 10 min each with buffer and once overnight to remove fixative. Localization media consisted of 8.0 mM β-glycerol phosphate or 2.7 mM para-nitrophenyl phosphate in 0.05 M sodium acetate buffer (pH 5.5) and 2.4 mM lead nitrate. Incubation of wheat endosperm was at 37 C for 1 h followed by 4 washes in acetate buffer. Lead contamination was checked for by adding a drop of ammonium sulfide to the used wash buffer. Contamination was detected by the presence of a dark precipitate. No lead was detected following the second buffer rinse. Controls used for acid phosphatase localization were incubation medium minus the substrate and medium minus substrate and lead nitrate. Samples were then prepared for light and electron microscopy as previously described (Bechtel et al., 1982a).

Results

A light microscopic study confirmed previous results concerning the presence of vacuoles early in endosperm formation (Morrison and O'Brien, 1976; Nares et al., 1977). In order to study the formation of new vacuoles and enlargement of existing vacuoles, an examination of developing tissues was conducted by electron microscopy. The time frame for observation was from 14-17 DAF when cell division in the outer endosperm tissue had ceased and these cells possessed little storage protein. Examination was limited to the subaleurone region, the first two cell layers beneath the aleurone. A feature that was frequently observed was the presence of areas of cytoplasm surrounded by membranes (Figs. 1-3). The amount of cytoplasm within the membranes was extremely variable in size and the cytoplasm was composed only of groundplasm, ribosomes, and occasional membranes (Figs. 1-3). Organelles such as plastids, mitochondria, Golgi bodies, lipid droplets, and RER, that were typically present within endosperm cells, were never observed in these sequestered regions. Some regions were less than a micrometer in diameter while others were several micrometers across (Figs. 1 and 2). Present in the cell during the sequestration of cytoplasm within membranes was RER which had lost some or all of its ribosomes and had become distended (Figs. 3 and 4). Other membranes seemed more directly involved in sequestering the cytoplasm and were connected to pre-existing vacuoles (Fig. 5) or surrounded portions of cytoplasm (Fig. 6). Another phenomenon observed was vesicles and RER surrounding portions of cytoplasm which were devoid of most organelles (Fig. 7). These partially surrounded regions matched closely to those found within membranes (compare Figs. 1-3, 6 to 7). By 17 DAF possible remnants of cytoplasm were observed within vacuoles along with protein bodies (Fig. 8).

Cytochemical analysis for acid phosphatase was conducted to determine if this general hydrolase, which is typically associated with lysosomal systems, was localized within any of the structures associated with sequestered cytoplasm. A modified Gomori technique was used to localize acid phosphatase activity in developing endosperm cells. Para-nitrophenyl phosphate gave more consistent staining, than did β-glycerol phosphate with the large, extremely dense deposits that obscured cell ultrastructure. The buffer-only control lacked any staining of the endosperm (Fig. 9). Controls using incubation media minus the substrate all showed a small amount of background staining consisting of small, dense dispersed deposits in RER, mitochondria, cell walls, and nuclei (Figs. 10 and 11). In contrast, the samples with substrate present showed selective staining of the RER, small vesicles, vacuoles, portions of some protein bodies, and cytoplasm present within vacuoles. Vacuoles containing cytoplasm showed reaction product on, in, and around the sequestered cytoplasm (Figs. 12-14). The periphery of some protein bodies and portions of the protein granules
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also stained positively (Figs. 15-17). Staining of the RER was not uniform. RER associated with vacuoles stained positive while that further away did not stain (Fig. 18). In many cases positively-stained ER was found to be continuous with the tonoplast, with expanded regions of ribosome-less ER, or connecting vacuoles with expanded ER (Figs. 19-22). These ER-vacuole connections were apparently limited to the period of vacuole development because none were observed past this period. The thin filament-like extensions of vacuoles also stained positive (Fig. 23). Golgi bodies were not usually stained, but the few that were had reaction product associated with the RER was not uniform.

When the RER was not uniform.

The thin filament-like extensions of vacuoles also stained positive (Fig. 23). Golgi bodies were not usually stained, but the few that were had reaction product associated with the RER was not uniform.

Also, the process of autophagy has been documented for meristematic root tissue (see Marty et al., 1980 for a review). Our results suggest that a similar system may function in the initial production of some of the wheat endosperm vacuoles. The finding of regions of cytoplasm within intact "vacuole" membranes could possibly result from a particular plane of sectioning through the endosperm cell. We feel this explanation is highly unlikely because of several observations. First, the observed regions of cytoplasm never possessed any organelles other than free ribosomes. Since the membranes surrounding the cytoplasm always lacked ribosomes, and smooth ER in wheat endosperm was limited to tubular elements that interconnected with RER (Bechtle and Barnett, 1986a, b), it would not be possible to obtain planes of section that would resemble what we have observed. Furthermore many of our micrographs are very similar to those from root meristems for which corresponding high voltage electron microscopy has confirmed the three-dimensional structure of the autophagic system (Marty et al., 1980). Fixation artifacts could also account for various membranous configurations.

We have previously investigated our fixation scheme using freeze-fracture, freeze-etch techniques on fresh, glycerol treated and chemically treated wheat endosperm tissue and found that while, our routine fixation could result in breakage of the tonoplast (Bechtle and Barnett, 1986a), we did not observe this phenomenon with the present samples. Probably the most convincing evidence for the operation of autophagy in wheat endosperm is the acid phosphatase localization within the sequestered cytoplasm.

Acid phosphatase activity was localized within ER, vesicles, vacuoles, and protein bodies and activity was highest during meristematic activity and vacuole growth. The nonuniform localization of the enzyme may be caused by poor penetration of the reagents (Moore et al., 1987) or by uneven enzyme distribution. The presence of hydrolytic enzymes in vacuoles of plant tissues is a common phenomenon (Henry and Steer, 1985; Wittenbach et al., 1982) and associated with autophagy (Marty et al., 1980). The working hypothesis for autophagy and vacuole formation in root meristems in that tubular provacuoles (= the trans Golgi network of Griffiths and Simons, 1986; = GERL of Novikoff, 1976) wrap themselves around portions of cytoplasm to eventually form a double membrane bounded structure. The enclosed piece of cytoplasm then becomes digested (Marty, 1978; Marty et al., 1980). We have observed a very similar situation in wheat endosperm except that the "provacuole" membranes are derived from RER and pre-existing vacuoles rather than from the trans Golgi network. As the entire endomembrane system tends to be interrelated and interconnected, this difference may not be of fundamental significance.

In fact, the Golgi apparatus is preparing to package storage proteins at this time (Bechtle et al., 1982a; Bechtle and Barnett, 1986a, b) and may not be able to handle the extra burden of participating in autophagy. This is supported by the fact that very few Golgi bodies stain positive for acid phosphatase whereas the RER stains heavily. Other plant systems also show varied acid phosphatase localization (Henry and Steer, 1985; Gartner and Nagl, 1980; Pyliotis et al., 1979) indicating that a single mechanism of autophagy in plants probably does not exist.

The observations of vesiculization of RER and of vacuoles continuous with ER may be related to vacuole initiation. Previously we had been unable to identify these continuities using U.S. wheats grown under field conditions conducive to fast growth (35 days from flowering to combine harvest; Bechtle et al., 1982a; Bechtle and Barnett, 1986a, b). As these continuities are limited to the period of vacuolation prior to storage protein deposition, they probably contribute little to the direct flow of storage proteins into the vacuoles as previously reported (Simmonds, 1978; Campbell et al., 1981; Parker, 1982). Furthermore, the localization of acid phosphatase within the RER lumen lends credence to the view that ER functions in vacuole production.

Figs. 9-17. Acid phosphatase cytochemistry of 14 DAF wheat endosperm.

9. Buffer only control showing lack of staining of mitochondrion (M), RER, protein body (P), and cytoplasm (C) in vacuole (V).

10. Incubation medium without substrate shows minimal staining of cell wall (CW), mitochondrion (M), and RER (*). 11. Nitro substrate control exhibits a small amount of staining of mitochondrion (M) and nucleus (Nu). 12. Early stage of autophagy with cytoplasm in vacuole (V) surrounded by thin membrane (arrows) and with acid phosphatase localized in vacuole (*). 13. Autophagic vacuole in 12 DAF wheat endosperm showing acid phosphatase localization (*). 14. Two autophagic vacuoles (A and B) and a vesicle (*) staining positive for acid phosphatase in 12 DAF endosperm; A in a more advanced stage of digestion than B. 15. Acid phosphatase localization (*) around protein granule (P) and in vesicle (arrow). Note dense inclusion (I) and positive vesicle localization. 16. Positive localization of acid phosphatase (*) in protein body (P) and RER. 17. Protein body (P) with positively-stained regions (*).
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Localization of acid phosphatase within protein bodies presents an interesting dilemma. The function of protein bodies as autophagic organelles during seed germination had been hypothesized (Matile, 1968) and since proven (Van der Wilden et al., 1980); the presence of hydrolytic enzymes is necessary for the mobilization of storage reserves (Van der Wilden et al., 1980). Most enzymes present during germination, however, are synthesized de novo and are different from the ones present during seed maturation. It appears that autophagy is active only in endosperm cells for a short period of time and probably does not contribute much membrane to the overall tonoplast system as observed in more mature cells; the bulk of the vacuole enlargement results from fusion of various vesicles during grain filling (Bechtel et al., 1982b). The process is important because autophagy is a normal cellular process that assists in ridding cells of obsolete metabolic machinery and aids in recycling components. Although the vacuoles are produced by endosperm cells for the deposition of storage proteins, it is surprising to find that protein reserves are being deposited into newly formed vacuoles which also contain the hydrolytic enzyme acid phosphatase. Little is known about the substrate specificity of hydrolytic enzymes that are present in developing cereal endosperms, and other hydrolyases are probably also present within the vacuoles. This is supported by the similar activity profiles given by the bulk protease and acid phosphatase assays (data not shown). What effects these enzymes have on the protein storage reserves is unknown. Separation of the hydrolyases and protein granules in subcompartments of the vacuole could prevent mixing of the enzymes and storage proteins.

A possible function for the phosphatases is to break down phytic acid, a major component of the aleurome cell, the outermost endosperm tissue. We have frequently observed subaleurone cells that appear to be intermediate in position and character between aleurome and starchy endosperm cells in young developing endosperms. Since the mature wheat grain has a single layer of aleurome cells, the intermediate cells must have become typical starchy endosperm cells. Hydrolytic enzymes including acid phosphatase could have a role in regulating the differentiation of such cells by digesting aleurome-specific cytoplasmic components.

Acknowledgment
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References

Figs. 18-25. Acid phosphatase localization in 14 DAF wheat endosperm. 18. Section through wheat endosperm showing only the RER (arrows) next to vacuole (V) staining positive for acid phosphatase while RER further away does not stain. 19. Positively-stained RER continuous with tono-plast (arrow) of vacuole (V). 20. RER in process of vesicle formation exhibiting positive staining for the enzyme. 21. Acid phosphatase positive RER and vesicle (*) continuous with a vacuole (V). 22. Two vacuoles (V) connected by positively-stained RER (arrow). 23. Positive localization in long, thin extension (arrow) of vacuole (V). 24. Golgi bodies in wheat endosperm with acid phosphatase localized in the trans Golgi network and budding vesicles (arrows). 25. Vesicles (*) near Golgi body (G) and protein body (P) that have stained positive for enzyme.
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Morrison IN, O'Brien TP. (1976). Cytokinesis in the developing wheat grain; Division with and without a phragmoplast. Plants 130:57-67.


Discussion with Reviewers

Reviewer I: Couldn't the phenomenon you describe be compared to the extensive membrane complexes described by Mares et al., 1976, Plant Sci. Lett. 7:305-317?

Authors: The process you refer to was observed in modified aleurone cells very early in development and involves the proliferation of RER. Our system occurs much later in development, is for the starchy endosperm cells, and the RER may possibly function in autophagy. The two systems are not comparable.

M.G. Smart: Was any attempt made to reconstruct membranes "surrounding" portions of cytoplasm (or high voltage TEM of thick sections attempted)?

Authors: We have a limited number of serial section reconstructions, but they have not been of much help in visualizing the three dimensional aspects of this part of the cell. Our freeze-fracture work has been very useful, however, and shows close relationships between RER and vacuoles.

R.A. Stone: In assessing the acid phosphatase cytology can it be certain that the ER has, or does not have, associated ribosomes?

Authors: RER is RER when ribosomes are attached and proteins are secreted into its lumen. When ribosomes fall off and are not replaced the ER becomes smooth ER by definition. It no longer is capable of participating in protein synthesis.

Reviewer IV: What is the frequency of this proposed mechanism of vacuole formation?

Authors: We do not have any numbers for its occurrence, but feel it is prominent during the starchy endosperm cell growth stage when these cells undergo a large expansion in cell volume.
USE OF IMAGE ANALYSIS TO PREDICT MILLING EXTRACTION RATES OF WHEATS

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Abstract

Image analysis of grain morphological characteristics was examined as a possible means of predicting extraction rates of a wide variety of wheat types.

Two elevations of grain were examined and measured. For the top view whole grains were used, while for the lateral view grains were sagittally bisected in the plane of the crease. Extraction rate was assessed on laboratory mills and expressed as flour yield.

Milling extraction rate correlated with one shape factor with a coefficient of 0.76. Inclusion of a second factor in a stepwise regression increased the correlation coefficient to 0.926.

No satisfactory predictor of extraction rate exists at present although some grain traders believe that high grain specific weight indicates good milling quality. Specific weights of the wheats involved in the present study were measured and a significant negative correlation with flour yield, was found.

Introduction

Extraction rate - the weight of flour produced per 100 parts of wheat milled - is an important factor in milling economics. Hence, millers would like to be able to predict extraction rates of samples offered. Apart from knowledge of characteristics of individual cultivars, gained through experience or experimental evaluation, there is no reliable means of predicting milling yield. Specific weight measurement is performed routinely as a means of eliminating samples with much shrivelling - a factor which clearly reduces extraction rate - but if shrivelled samples are excluded correlations between specific weight and flour yield are generally acknowledged to be poor. A reliable means of predicting extraction rate would undoubtedly eliminate one of the major deficiencies in wheat trading requirements.

Prediction of milling extraction rate from a knowledge of grain morphological characteristics has been investigated in several studies. Characteristics such as grain length and breadth, crease depth and embryo size have been considered. In some studies endosperm content has been determined and related to milling extraction rate and grain size (Simmons and Meredith, 1979).

It is well known that the presence of shrivelled grains reduces milling yield. In addition to endorsing this, grain measurement and segregation showed that even well-filled small grains contained less endosperm than large, well-filled grains, which gave better milling yields (Simmons and Meredith, 1976).

Marshall et al (1984) used simple geometric models to examine the effects of grain shape and size on milling yields. Their conclusions emphasized the importance of grain size rather than shape as being the most important. In spite of this the same authors (1986) later found that the significant linear relationship between milling yield and grain size found in samples of one cultivar grown at one site, broke down when different cultivars were included, even when they too were grown at the same site. These findings confirmed others which were cited, showing that they represent general experience. The universality of the simple notional relationship between grain size and endosperm content and hence extraction potential is thus not confirmed by experimentation.

Image analysis provides a means of rapidly assessing a range of morphological parameters that can be correlated individually or together, with quality factors measured by other methods.
The process of image analysis occurs in a series of stages as follows:

1. **Image capture** - in which a specimen is presented to a TV camera which acquires an image that is capable of conversion into electronic signals suitable for digital processing and storage.

2. **Segmentation** - in which regions of interest within the image are separated from one another, and from the background. The amount of processing at this stage may be considerable. Its extent depends upon the complexity of the task - that is the number of different regions requiring separation and the quality of the image obtained from the camera.

3. **Measuring** - the image, having been digitized consists of an array of picture points or pixels. Segmentation permits object pixels to be distinguished from non-object pixels. By counting pixels of different types area relationships can be quantified and by noting relationships of pixels (especially those at boundaries) one to another, linear measurements and angular relationships can be defined.

4. **Analysis** - Measurements taken on individual objects can be related one to another and to the total image. They are presented in a form that can be logged and further analysed in data sets acquired from a group of similar images.

Different image analysis applications require emphasis to be placed at different stages in the process. Even when applied to the same problem different philosophies are adopted by users. Thus, in applying image analysis to cereal species and cultivar identification several approaches have been adopted. Symons and Fulcher (1988) examined grain sections so that only a small portion of a grain filled the field of view. Keefe and Draper (1988) relied upon information acquired form side elevations of single whole grains while Sapirstein et al. (1987) imaged top elevations of several grains at one time. Increasing the number of grains in a visual field speeds up the analysis and facilitates consideration of a statistically acceptable number of grains within each sample. However, it reduces the number of pixels allocated to each grain and this reduces the resolution. The decision on approach is a subjective and largely arbitrary for little guidance is available on what constitutes a representative sample for cultivar identification and what degree of resolution is required to provide the necessary discrimination among types.

In the clearly similar context of the present study the choice of imaging procedure is equally subjective. In view of the marked lack of success achieved in previous attempts to relate grain morphology based on manual measurements to extraction rate, the approach we adopted was to acquire as much digitized information on a grain as possible from the camera by examining a maximally magnified image and to retain that information by limiting image processing to a minimum. The latter requirement can only be fulfilled if an image capable of segmentation without processing is obtained, that is to say the areas of interest must be clearly defined in the original image.

The lack of success of manual methods also influenced the decision to maximize the number of parameters recorded by attempting to present internal as well as external structural details for analysis. Some manual measurement studies had, in fact, measured the crease depth. However, this defined the distance at one point only and, as grain shape varies, the relationship between this single measurement and the depth of endosperm above the crease also can vary.

To increase grain shape definition further both the lateral view of the sagittally bisected grain and the top view were examined. Since each grain could be identified individually measurement from two directions not only doubled the information to be considered, it also provided a means of assessing the reproducibility of the measurements, such as length, common to both elevations.

### Materials & Methods

**Image analysis**

The Seescan I3000 image analysis system was used. The digitized image is 256x256 rectangular pixels with an aspect ratio of 3:2; and 128 grey shades are provided. The system is mouse driven, the operator making selections of functions from menus. Manus can be changed through mouse operations and additional routines may be added through programming as all programmes are accessible. Routine operations are carried out through task-lists which can be tailored by the operator, via the mouse to suit his requirements. Once initiated, task-lists proceed to completion without further intervention although provision may be made in a task-list for labelling or interactive processing.

For the two elevations examined different task lists were compiled. In the top-view task-list, only the silhouette of the whole grain was required to be measured and a single threshold was adequate. In the side-view task-list, grain silhouette and endosperm area had to be measured separately. Interaction was provided for, only on silhouette outline in both cases. The spatial resolution was constant throughout all examinations.

**Preparation of specimens for examination**

Presentation of grains in a consistent orientation is vital for valid comparisons to be made. Because no suitable alternative could be devised, embedding of grain was the method of grain preparation adopted. Embedding is inevitably time consuming and could not be considered as a method in routine analysis. However, at the stage when worthwhile parameters are being identified, the embedding method has considerable merit. When cast in plastic the grains are held in robust blocks that are easy to handle. The regular shape of blocks makes them easy to register for precision machining and for location on a "stage" for presentation to the camera. Blocks can be retained for further examination if necessary, and a clear embedding medium permits the detection of an uninterrupted outline, facilitating segmentation of the image captured against back-lighting.

The details of the system adopted for aligning grains and casting blocks are as follows:

- **Casting chambers** as shown in Figure 1 were used. The grains were laid, with the embryo end consistently oriented, crease downward so that it registered with the rib on the base of the mold. The grains were retained in alignment and observation from the top revealed any misaligned grains. L.R. soft resin (London Resins Limited) was poured gently into the mold. It is of low viscosity and its hydrophilic properties permitted rapid flow and close adhesion to the grains with a minimum of disturbance. Grains which became disturbed could be easily realigned with forceps. The resin becomes solid within a few minutes but is best left for several hours to harden completely. For removing the cast, lowering the temperature of the mold by placing it in melting ice caused the mold to contract and separate form the cast.
Image analysis to predict milling extraction rates.

The mold was dismantled and the block removed. The use of ice gives considerable advantages over alternative methods, including use of grease as a barrier or clear adhesive tape on mating surfaces.

Some air bubbles trapped, particularly at the brush ends of grains corrupted the outline and required interactive definition of the silhouette during processing of the image.

Casts bear a groove on the underside corresponding to the rib on the base of the chamber. Correct alignment can be confirmed by reference to this.

Material lying to the narrower side of the groove was removed by use of an engineer's milling machine, which gives a clean finish and can be controlled with precision. Alternative methods, requiring less skill were tried.

Conventional band saws and cutting wheels (even diamond wheels) caused burning and smearing of the surface.

Presentation of grains for imaging

Batches of 100 grains were randomly chosen by means of a "Numigral" seed counter (Falling Number Co Ltd). Damaged grains were discarded and replaced with sound grains. All accepted grains were embedded in blocks of 10 as described above.

Top elevations were examined and measured before removal of the unwanted plastic and half-grains by engineers' milling machine. Endosperms were stained with iodine/potassium iodide before presentation for lateral elevation analysis.

A Phillips Video 4 (Newvicon) camera was used with a 16mm F1.4 lens. The necessarily short working distance was achieved by use of a 10mm extension ring.

Cameras of this type are best used in a horizontal position, hence the presentation set-up (Figure 2) was designed to permit this. The camera was mounted in a fixed position on a base. Also on the base was a mechanical microscope-stage permitting movement in the direction of the optical axis and from left to right at right angles to it. A perspex block fixed to the mechanical stage allowed blocks to be mounted at a suitable height for the camera. The entire cast was first placed on the perspex block with the top of the grains towards the camera, and the grains were identified and measured in sequence. After machining to reveal the central plane-face the blocks were re-examined with the machined face towards the camera. Before each measuring session a standard length was measured to ensure that calibration remained valid.

Figure 1. Exploded view of mold and cast.

Figure 2. System of presentation of embedded grains to T.V. camera.

Lighting

External measurements can be made from the image of a grain in silhouette. Hence, for this purpose, back-lighting is suitable. A 450 x 100mm light box fitted with fluorescent tube, was mounted vertically behind the sample stand, on the same base as the camera and stand. For visualization of the exposed endosperm it is necessary to provide incident lighting. In meeting this requirement several difficulties exist. Uniform lighting is difficult to provide under any circumstances but, in the present set-up, the working distance of the camera lens, adapted to achieve a large image of the grain, imposed additional problems. A solution to the problem has been found by use of a Lieberkunn mirror; a parabolic reflector mounted in the cowl surrounding the camera lens (Thomson & Bradbury 1987). Light transmitted through and around the specimen block and the perspex holder, is reflected on to the specimen face. Adjustment of the focal length of the camera permits coincidence of the focal planes of lens and mirror, and the specimen can
be examined in that position. Under these conditions the two areas required could be defined.

Because endosperm varies in its light transmittance/reflectance properties as a result of variation of mealiness/vitreousness, it has not been possible to achieve sufficiently consistent imaging conditions for providing contrast between endosperm and bran or embryo regions. To provide greater consistency in endosperm characterisation, iodine/potassium iodide solution was applied to the exposed face. All starch granules become stained dark purple, permitting starchy endosperm to be distinguished from surrounding tissues. Before applying the stain it has been found necessary to apply a thin coat of resin (methacrylate is water permeable) to the presence would reduce demarcation among tissues.

Different lighting conditions have been found to be optimal for the different views of grains. For top elevations a mask placed over the light box with a window 130 mm x 50 mm high centered on the optical axis, gave the best definition of the silhouette. For side elevations, in which two thresholds were needed, an annulus of external diameter 40 mm placed on the unmasked light box was favored. A Wratten 12 (deep yellow) optical filter was placed within the camera for both elevations.

The lighting conditions were adopted after much trial and error and the principles governing their effectiveness are not understood in all cases. They did however cope well with variations in bran color among wheat types.

Image analysing

Task-lists were constructed to provide a sequence of operations such as that below.

e.g. Sideview

<table>
<thead>
<tr>
<th>Automatic</th>
<th>Operator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Display identification facility</td>
<td>Start</td>
</tr>
<tr>
<td>Display grey image</td>
<td>Enter label</td>
</tr>
<tr>
<td>Capture image</td>
<td>&quot;Capture&quot;</td>
</tr>
<tr>
<td>Threshold at standard grey levels</td>
<td>Vary or accept</td>
</tr>
<tr>
<td>Measure silhouette &amp; geometric parameters</td>
<td>Identify endosperm</td>
</tr>
<tr>
<td>Measure endosperm</td>
<td></td>
</tr>
<tr>
<td>Send data to computer</td>
<td></td>
</tr>
<tr>
<td>Display, store and print</td>
<td></td>
</tr>
</tbody>
</table>

The automatic aspects of the entire sequence for each grain took approximately 15 s. Operator activities took perhaps another 30 s. It was rarely necessary to alter threshold levels and indeed much of the effort put into imaging was to avoid this. Occasionally grains could be seen to be incorrectly orientated and hence cut in the wrong place. An "abort" facility was written into the task lists to reject such grains.

Wheat samples

Details of the wheat types milled and examined are given in Table 1.

Table 1 Wheat types milled and examined by Image Analysis

<table>
<thead>
<tr>
<th>Where grown</th>
<th>Texture</th>
<th>Colour</th>
<th>Milling quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>cv Apollo</td>
<td>UK</td>
<td>Soft</td>
<td>Red</td>
</tr>
<tr>
<td>cv Avalon 1</td>
<td>UK</td>
<td>Hard</td>
<td>Red</td>
</tr>
<tr>
<td>cv Avalon 2</td>
<td>UK</td>
<td>Hard</td>
<td>Red</td>
</tr>
<tr>
<td>*CWRS No.1</td>
<td>Canada</td>
<td>V.hard</td>
<td>Red</td>
</tr>
<tr>
<td>cv Fortress</td>
<td>UK</td>
<td>Hard</td>
<td>Red</td>
</tr>
<tr>
<td>Ungraded</td>
<td>Germany</td>
<td>Soft</td>
<td>Red</td>
</tr>
<tr>
<td>cv Hornet 1</td>
<td>UK</td>
<td>V.soft</td>
<td>Red</td>
</tr>
<tr>
<td>cv Hornet 2</td>
<td>UK</td>
<td>V.soft</td>
<td>Red</td>
</tr>
<tr>
<td>cv Mercia</td>
<td>UK</td>
<td>Hard</td>
<td>Red</td>
</tr>
<tr>
<td>cv Yecora</td>
<td>Spain</td>
<td>V.Hard</td>
<td>White</td>
</tr>
</tbody>
</table>

* Canada Western Red Spring Grade No. 1

Milling

Millings were performed on a Buhler laboratory mill (MLU 202) fitted with flour covers (160 mm aperture) throughout. Wheat samples (2 kg) were conditioned, and milled after a lying time of 24 h. A feed rate of 100 g min⁻¹ was employed and flour yields were expressed as a percentage of feed stock. The fine offal fraction was re-treated by one passage through a Buhler laboratory Impact Finisher (MLU 302) so as to bring flour yields more closely in line with commercial practice. The millroom was maintained at a temperature of 18°C ± 1°C and 64 ± 2% relative humidity throughout.

Processing of Grain Data

In order to facilitate the analysis of the grain data, the I.3000 was connected to an Amstrad PPC640 portable computer, via the serial port. The Amstrad was used as a terminal to the I.3000, replacing its usual terminal, using a program written in C. This program also had the facility to save all incoming data to a disk file. The data thus saved could then be analysed on an IBM AT computer. Since no single software package was available which could perform all the necessary analysis, several packages were used, including Lotus 1-2-3, for basic analysis and data manipulation; GLIM, for more detailed analysis, and SAM (Statistical Analysis for Microcomputers), for stepwise regression. A certain amount of preprocessing of the data was necessary, using custom-written Basic programs.
Image analysis to predict milling extraction rates.

**Results**

**Milling Results**

Details of the millings of the ten wheat types are shown in Table 2.

<table>
<thead>
<tr>
<th>Wheat type</th>
<th>Break</th>
<th>Reduction</th>
<th>Finisher</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>cv Apollo</td>
<td>12.99</td>
<td>54.15</td>
<td>6.62</td>
<td>73.76</td>
</tr>
<tr>
<td>cv Avalon 1</td>
<td>16.98</td>
<td>57.65</td>
<td>3.38</td>
<td>78.01</td>
</tr>
<tr>
<td>cv Avalon 2</td>
<td>16.56</td>
<td>57.61</td>
<td>3.06</td>
<td>77.23</td>
</tr>
<tr>
<td>CWRS No.1</td>
<td>16.59</td>
<td>56.71</td>
<td>2.84</td>
<td>76.14</td>
</tr>
<tr>
<td>cv Fortress</td>
<td>14.39</td>
<td>57.82</td>
<td>4.10</td>
<td>76.31</td>
</tr>
<tr>
<td>German</td>
<td>13.98</td>
<td>57.89</td>
<td>5.18</td>
<td>77.05</td>
</tr>
<tr>
<td>cv Hornet 1</td>
<td>14.56</td>
<td>53.55</td>
<td>6.21</td>
<td>74.32</td>
</tr>
<tr>
<td>cv Hornet 2</td>
<td>15.29</td>
<td>45.03</td>
<td>9.64</td>
<td>69.96</td>
</tr>
<tr>
<td>cv Mercia</td>
<td>13.71</td>
<td>56.31</td>
<td>3.63</td>
<td>73.85</td>
</tr>
<tr>
<td>cv Yeora</td>
<td>13.48</td>
<td>54.76</td>
<td>4.26</td>
<td>72.50</td>
</tr>
</tbody>
</table>

Extraction rates varied between 69.96% and 78.01%. In all cases the first reduction provided the most flour and in all cases but one the first two reductions produced more than 60% of the total. The exception was Hornet 2. This wheat gave the lowest total flour yield and the first two reduction flours contributed less than 50% of the total. Examination of the milling details led to the conclusion that the sample had behaved exceptionally, probably as a result of sieves having been blocked, and that this had led to an artificially low recorded flour yield. Insufficient stock remained for a repeat milling. The results of Hornet 2 were excluded from data analysis.

Ideally, extraction rates should be expressed at a consistent grade colour figure (G.C.F) since when extraction rate is controlled by variation of milling conditions, changes in G.C.F. are almost inevitable. A basis for correction has been suggested (Dexter and Martin 1986) but in the present exercise the milling conditions were identical for all wheat types and extraction rates were accepted "as found"; no attempt being made to adjust for G.C.F, which is also influenced by other factors such as bran color.

**Image analysis results**

At least 100 grains of each wheat type were embedded in London resin (soft) as described above. All grains were identified and examined by image analysis in both top and side elevation. When necessary intervention by the operator took place to define part of the outline where the boundary of the silhouette was not clear.

The parameters that were measured for each grain are explained in Figure 3.

---

**Table 2** Flour yields as a proportion of grain weight

<table>
<thead>
<tr>
<th>Wheat type</th>
<th>Break</th>
<th>Reduction</th>
<th>Finisher</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>cv Apollo</td>
<td>12.99</td>
<td>54.15</td>
<td>6.62</td>
<td>73.76</td>
</tr>
<tr>
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<td>16.98</td>
<td>57.65</td>
<td>3.38</td>
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<tr>
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<td>15.29</td>
<td>45.03</td>
<td>9.64</td>
<td>69.96</td>
</tr>
<tr>
<td>cv Mercia</td>
<td>13.71</td>
<td>56.31</td>
<td>3.63</td>
<td>73.85</td>
</tr>
<tr>
<td>cv Yeora</td>
<td>13.48</td>
<td>54.76</td>
<td>4.26</td>
<td>72.50</td>
</tr>
</tbody>
</table>

---

A number of grains had to be eliminated owing to poor image resolution, and only those grains for which both views provided adequate images were considered for statistical analysis.

The final numbers accepted for all types are shown in Table 3. Measurements of CWRS appeared to be prone to considerable error and cv Apollo and cv Mercia also fell considerably below 100 grains each.
The three best relationships were length ratio (side), length ratio (top), Angle 1 (top) and embryo slope (side).

The length ratio defines the relative distance of the grain's widest point (strictly the mid-point of the pixels in the highest row) from the embryo tip. The high correlations with extraction rate achieved by this ratio measured both in top and side elevations suggests that it is an important characteristic of grain shape in the present context. Only the better relationship is shown in graphic form (Figure 4). Most points lie close to a notional line, with CWRS as an outlier. The two next-best relationships found were with slope length 1 measured in the top elevations and embryo slope, measured in the side elevation. In the former, (Figure 5) the correlation is very dependent upon the lowest point, while in the latter, most points lie close to a line, with one outlier (Figure 6).

<table>
<thead>
<tr>
<th>Wheat type</th>
<th>No. of accepted grains</th>
</tr>
</thead>
<tbody>
<tr>
<td>cv Apollo</td>
<td>45</td>
</tr>
<tr>
<td>cv Avalon 1</td>
<td>94</td>
</tr>
<tr>
<td>cv Avalon 2</td>
<td>167</td>
</tr>
<tr>
<td>CWRS No. 1</td>
<td>22</td>
</tr>
<tr>
<td>cv Fortress</td>
<td>87</td>
</tr>
<tr>
<td>German</td>
<td>102</td>
</tr>
<tr>
<td>cv Hornet 1</td>
<td>95</td>
</tr>
<tr>
<td>cv Hornet 2</td>
<td>113</td>
</tr>
<tr>
<td>cv Mercla</td>
<td>60</td>
</tr>
<tr>
<td>cv Yecora</td>
<td>156</td>
</tr>
</tbody>
</table>

Table 3 Nos. of grains accepted for analysis

Correlation coefficients for the regression of each parameter on extraction rate are shown in Table 4 and the details of selected relationships are shown in Figures 4-8.

Table 4 Correlations with milling extraction rate and measured or derived characteristics - 9 samples (Hornet 2 removed).

<table>
<thead>
<tr>
<th>Top elevation</th>
<th>Side elevation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length ratio</td>
<td>0.685*</td>
</tr>
<tr>
<td>Angle 1</td>
<td>0.551</td>
</tr>
<tr>
<td>Angle 2</td>
<td>0.122</td>
</tr>
<tr>
<td>Area</td>
<td>0.209</td>
</tr>
<tr>
<td>Length</td>
<td>-0.026</td>
</tr>
<tr>
<td>Width</td>
<td>0.387</td>
</tr>
<tr>
<td>Fullness</td>
<td>-0.018</td>
</tr>
<tr>
<td>Aspect ratio</td>
<td>0.439</td>
</tr>
<tr>
<td>Specific weight</td>
<td>-0.737*</td>
</tr>
<tr>
<td>1000 grain wt</td>
<td>-0.089</td>
</tr>
</tbody>
</table>

* = significant at 5% level

Figure 4. Relationship between flour yield (% of weight of feedstock, 14% moisture basis) and length ratio measured on the side elevation of grains. The points are identified with the name of the wheat type to which they relate. On other graphs identification is possible by reference to respective positions on the "yield" axis.

Figure 5. Relationship between flour yield and slope length 1 as measured on the top elevation.
Image analysis to predict milling extraction rates.

Table 5 Stepwise correlation between extraction rate and morphological descriptions. (Hornet 2 excluded).

<table>
<thead>
<tr>
<th>Step</th>
<th>Variable</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Length ratio (side)</td>
<td>0.779</td>
</tr>
<tr>
<td>2</td>
<td>Length</td>
<td>0.925</td>
</tr>
<tr>
<td>3</td>
<td>Upper slope (side)</td>
<td>0.951</td>
</tr>
<tr>
<td>4</td>
<td>Fullness (top)</td>
<td>0.994</td>
</tr>
</tbody>
</table>

For comparison with the relationships established through image analysis measurements, particularly that with length ratios, that between extraction rate and specific weight is also shown (Figure 9). While the correlation coefficient for the latter is relatively high, it is a negative one; the reverse of that normally relied upon in predicting milling extraction rate. The high coefficient appears to depend mainly on the influence of three points representing two wheat types (Avalon, characterised by a low specific weight, and Yecora with a high specific weight).

Figure 6. Relationship between flour yield and embryo slope as measured on the side elevation. \( r = -0.55 \)

Figure 7. Relationship between flour yield and grain length. \( r = -0.185 \)

Figure 8. Relationship between flour yield and upper slope measured on the side elevation. \( r = -0.026 \)

Because the three measurements depicted in Figures 5 and 6 are apparently closely related to the length ratios they do not contribute greatly to the establishment of a step-wise correlation. The greatest contribution here comes from the length itself, which, on its own, is correlated poorly with extraction rate (Figure 7). The best further introduction into the stepwise correlation is the side upper slope (Figure 8). The entire stepwise correlation is shown in Table 5.

Figure 9. Relationship between flour yield and specific weight of grain. \( r = -0.737 \)

Discussion

The unusual relationship between specific weight and flour yield demonstrates the inadequacy of this measurement as a predictive factor when several wheat types are under consideration. It also demonstrates the hazards involved in considering a small number of grain types in a comparison of this nature. Clearly a larger number would be more reliable for giving an indication of the consistency of the relationships involved. The removal of Hornet 2 due to its anomalous milling made a substantial difference to all the correlation coefficients, which demonstrates the distorting influence of a single result when so few results are available.

In spite of all the experimental deficiencies that came to light in the analysis the correlation between length ratios and flour yield are encouraging. A regression based on the 9 samples falls close to all the European wheats in the set, leaving only CWRS as an outlier. While a universal relationship would be highly
desirable, one describing European wheats only will be extremely useful and worthwhile. The present results give rise to far more optimism than have previous morphology-based systems of prediction (see introduction for references).

As well as being time consuming the embedding method of grain preparation and presentation proved unsatisfactory in that interactive definition of length was too frequently required because of air bags, particularly in the brush area. This led to an unexpectedly high difference between the lengths measured in the two grain orientations and the consequent rejection of many grains from the analysis. The number rejected was particularly high in the case of CWRS wheat, and this factor may have contributed to its outlying position in Figure 4. The smallness of the grains and their narrow crease gave rise to problems in orientation since the rib on the mold base did not fit the crease in some cases.

Consideration of more than one factor to give a stepwise regression was expected to be necessary to achieve a correlation of 0.9 or better. In the event only two descriptors were needed. Steps beyond two have little meaning when such small numbers of samples are involved but the possibilities for considering several steps in dealing with larger numbers in the future remain.

The small set of samples that we were able to analyse produced results which suggest that the method should be pursued. Greater numbers of samples should be examined taking the same measurements but using improved methods for preparation and presentation. Although it is difficult to justify continued examination of endosperm area/total area relationships on the basis of the correlation attained, it would be premature to exclude this as a possible factor at this stage, even though the preparation of the surface is complicated by this requirement.

The morphological factors which appear to be important in determining extraction rate were unexpected and no explanation can be offered for their manner of influence. Should they prove to be as well related to milling quality as this preliminary survey suggests, not only will they offer a basis of mill intake assessment and early selection screening for breeders but may ultimately lead to a better understanding of the fundamental influences on milling quality.

Acknowledgements

The work described was supported by the Home Grown Cereals Authority and this report is published with their permission. The authors wish to acknowledge the technical assistance they received from Miss Jackie Croft. The milling was performed under the direction of Dr. S.C.W. Hook, Mr. Alan Leer and his workshop staff greatly assisted through design and construction of presentation devices. Mr. Chris Moffett produced the diagrams.

References


Discussion with Reviewers

R. Angold: Although the authors see a benefit in gaining as much morphological data as possible, is there not a penalty in effort in embedding and milling the grains, which reduces the number of replicates that can be studied for each cultivar whilst also generating errors in the determination of L and A3?

Authors: Yes. There is a dilemma which has to be resolved by personal judgement. As we collect more data a sounder basis for such decisions will emerge.

R. Angold: Does the milling accurately define the centre of the crease? Very small errors of registration might be expected to generate large variations in the value of A1.

Authors: We have not attempted to quantify the errors involved in variations of this kind. Our subjective judgement is that those grains whose data were accepted for inclusion in the analysis, were comparable in this regard. The high level of rejection reflects our attention to this factor.

R. Angold: Could the inclusion of the soft cultivars Hornet and Apollo have given rise to differing extraction rates for reasons other than grain shape factors? Would there have been greater benefit in more replicates of a single hard variety?

Authors: Our experiment included two cultivars which were duplicated in an effort to include intra-varietal comparisons. Unfortunately Hornet 2 was eliminated leaving only Avalon with two replicates. There are many comparisons still to be made and the experiment we reported must be regarded as preliminary.
Image analysis to predict milling extraction rates.

W. Bushuk: What is the basis for selecting the best three relationships?
Authors: The method of selection used was forward stepwise regression: at each stage, the measurement explaining the most variation not explained by measurements already chosen was included. Thus although one measurement may have a higher simple correlation with extraction rate than others if that measurement is also highly correlated with a further measurement, it might not provide any further reduction in variation when this third measurement is taken into account.

W. Bushuk: I do not think that one should expect a strong relationship between extraction rate (using a constant milling procedure) and specific weight for a set of samples that differ so widely in hardness.
Authors: We agree, although we were surprised that it was negative. The reason for including this was to show how difficult it is to predict extraction rate. Specific weight is the only existing parameter that we know of which might even be considered as a possibility.

R. Angold: Why are Newvicon cameras best used in a horizontal position?
Authors: All tube-technology cameras are best used in an orientation in which debris from the electron tube cannot fall on to the face-plate and damage the phosphor.

S.J. Symons: Did the authors consider vacuum infiltration of the resin to remove air bubbles trapped by the brush?
R. Angold: Had they tried to overcome the problem of air bubbles trapped in the beard hairs by the use of vacuum during embedding?
Authors: Our attempts to eliminate bubbles by this means were unsuccessful. Air was dislodged from the brush in some cases but it rarely migrated far enough from the grains before the viscosity of the resin increased enough to immobilise it. Handling the system inside a chamber was very inconvenient.

S.J. Symons: Are the authors aware of statistical packages such as GENSTAT or SAS, which would provide a more comprehensive statistical analysis procedure than that obtained from the multitude of packages listed?
Authors: Yes we now have GENSTAT and wish we could afford SAS.

S.J. Symons: The authors find length ratio as the best prediction of flour yield. This suggests kernel shape is of significance here. CWRS are reported as small kernels with a narrow crease. Does this suggest to the authors that CWRS kernels are fundamentally different in shape to kernels of UK or European origin?
Authors: We would not reach this conclusion on the basis of the limited data available but the point is an interesting one.
GRITTINESS IN A PASTEURIZED CHEESE SPREAD: 
A MICROSCOPIC STUDY

H.W. Modler, S.H. Yiu, U.K. Bollinger*, and M. Kalf

Food Research Centre, Research Branch, Agriculture Canada
Ottawa, Ontario, Canada K1A OCS, and
*Rideau Valley Foods, Inc., North Gower, Ontario, Canada K0A 2T0

Abstract

Coagulation of pasteurized (63°C for 30 min) milk and blending of the resulting curd with high-fat cream followed by heat treatment (44° to 60°C for 10 min) of the blend in preparation of a hot-pack cheese spread led to the formation of a gritty product. Microscopic examination of hard particles causing the grittiness indicated that they consisted of compacted protein. Staining for calcium failed to detect any elevated concentration of this element in the particles. They were amorphous and contained no crystalline structures. Encapsulation of the gritty curd in agar gel tubes made it possible to freeze-fracture the small hard particles and to examine their internal structure by scanning electron microscopy. The hard or gritty particles consisted of compacted protein. This was confirmed by transmission electron microscopy of thin sections of the product embedded in a resin.

The unpasteurized (unheated) cold-pack cheese spread prepared from the same ingredients was smooth with no grittiness defect. Based on the findings, grittiness was avoided in the hot-pack product by using curd obtained by coagulating milk which had previously been heated to 90°C for 10 min.

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KEY WORDS: Cheese spread, Curd, Electron microscopy, Fluorescence microscopy, Grittiness, Heating of milk, Milk coagulation.
4 h and 20 min after starter addition, the coagulated milk was cut using 1.25 cm knives. Immediately after cutting, the vat was cooled to 10°C and left overnight under quiescent conditions. Whey was drained from the vat on the following morning. The curd was placed in 20 kg hoops and allowed to drain for an additional period of 24 h before use.

Acid curd was prepared by the same procedure except rennet was omitted and the curd was not cut until the pH had declined to 4.02 (11 h after starter addition). Flow diagrams for both types of curd are shown in Fig. 1.

Preparation of cheese spread

The cheese spread product was formulated to contain approximately 85% curd (approximately 35% solids), 8 to 10% cream (50 to 60% milkfat), 5% sucrose, and 0.2% sodium alginate. Cream was added to a dry blend of sugar and stabilizer contained in a Green processing kettle (Model TDC/TA-20SP). The mixture was pasteurized at 80°C for 10 min and then cooled to 30° to 35°C. Cheese curd was then blended with the cream, sugar, and stabilizer mixture by means of a Polytron blender, Model 4 PK. The mixture, with the exception of the control treatment, was pasteurized by heating to 63°C. After holding for 30 min at 63°C, the product was homogenized in a two-stage Gaulin homogenizer, Model 769441, at total pressure of 17.2 MPa. The product was packaged directly into 100-ML containers and refrigerated at 4°C. In the subsequent discussion, the pasteurized product will be referred to as the "hot-pack product" whereas the control or unpasteurized cheese will be referred to as the "cold-pack product". Fig. 2 shows the flow diagram for both the cold-pack and the hot-pack products.

Analytical procedures

Fat and nitrogen were determined according to the AOAC procedures [2] and total solids were determined using a CEM Model AVC-80 microwave moisture oven. Nitrogen was converted to protein using a factor of 6.25.

Evaluation of grittiness

Grittiness of various degrees which developed in several experimental variations was evaluated subjectively by mouthfeel and by visual examination under a low-magnification dissecting microscope.

Fluorescence microscopy

The cheese samples were fixed with 2.5% glutaraldehyde for 24 h, embedded in Histo Prep (Fisher Scientific Co., Fair Lawn, NJ, USA) support medium for cryo-sectioning, mounted on cold object disks, and frozen immediately at −25°C. Frozen sections, 4-6 μm thick, were obtained using a cryo-microtome (Reichert-Jung Scientific Instruments, Belleville, Ontario, Canada) and affixed to glass slides for subsequent staining and microscopic examination.

To reveal the distribution of gritty particles in the cheese preparations, the frozen sections were stained with 0.1% (w/v) aqueous Fast Green FCF according to a method similar to that described by Chayen et al. [5]. Alternatively, the sections were stained with 0.1% (w/v) Acidine Orange [14].

To detect the presence of calcium-containing structures in the cheese spread, the sections were treated with 5% (w/v) silver nitrate followed with 5% (w/v) sodium thiosulphate according to the Van Kossa’s procedure described by Thompson [13] and Yiu [14]. All stained sections were rinsed with distilled water, air-dried, and mounted with non-fluorescent immersion oil under cover slips. Stained sections were then examined using a Zeiss Universal Research Photomicroscope (Carl Zeiss Ltd., Montreal, Quebec, Canada) equipped with polarizing, bright-field, and fluorescence optics.
A fluorescence filter combination with a dichroic beam splitter and an exciter/barrier filter set for maximum transmission at 450-490 nm/520 nm was used for the fluorescence analysis. Micrographs were recorded on 35 mm Ektachrome 400 daylight diapositive film from which colour prints were obtained.

Scanning electron microscopy (SEM)
The samples were encapsulated in agar gel tubes (inner diameter of 1.0 mm) using a method described earlier [1,7], fixed in a 2.5% glutaralddehyde solution for 24 h, dehydrated in a graded ethanol series, frozen in melting Freon 12 at -150°C, and freeze-fractured under liquid nitrogen. The fragments were thawed in absolute ethanol at 20°C-25°C and were critical-point dried from carbon dioxide. Dry fragments were mounted on aluminum stubs, sputter-coated with gold, and examined in an ISI D5-130 microscope which had been equipped with an external oscilloscope [3], and operated at 20 kV. Micrographs were taken on 35-mm 125-ASA film.

Transmission electron microscopy (TEM)
Also for TEM, the samples were first encapsulated in agar gel [7] in order to prevent them from disintegrating during preparatory steps. The encapsulated samples were fixed in a 2.5% glutaraldehyde solution for 24 h, postfixed in a buffered (0.05 M veronal-acetate buffer, pH 6.75) 2% osmium tetroxide solution for 2 h, dehydrated in a graded ethanol series, and embedded in a Spurr's low-viscosity resin (J. B. EM Service, Inc., Pointe Claire-Dorval, Quebec, Canada). Sections stained with uranyl acetate and lead citrate solutions were examined in a Philips EM-300 electron microscope operated at 60 kV.

Results and Discussion
The curd used in the preparation of the cheese spread contained approximately 35% solids, 17-18% fat, and 14% protein and had a pH of 4.5 ± 0.1. The addition of cream and sugar increased the total solids and fat contents to approximately 40% and 20%, respectively, in the finished cheese spread. There was about 12% protein and 10% fat in the final product and its pH was 4.60 ± 0.05.

The texture of the cheese spreads produced by this process varied from smooth for the cold-pack product to gritty for the hot-pack product. Reasons for the development of the gritty texture during the initial part of this experimentation remain unclear. A reduction in the temperature of heat treatment, after the curd was blended with the pasteurized cream, sugar, and stabilizer mixture, failed to resolve the problem: grittiness was observed even when the product was held at 44°C for 30 min. Attempts to reduce the grittiness through double or even triple homogenizing were only partially successful. Other measures taken, including the use of buffering salts such as trisodium phosphate (Na$_3$PO$_4$) to raise the pH to 5.8 prior to heating, using acid-coagulated curd rather than renneted curd, and fermenting from the Rosell 5270 mixed culture to the C$_3$ culture failed to resolve the grittiness problem.

From the fact that the cold-pack cheese spread was smooth, it is apparent that the heat treatment at 44° to 63°C for 30 min may have led to the formation of small hard particles causing the hot-pack product to be gritty. However, pasteurization of the cheese spread is necessary to extend the shelf life of the product.

The presence of hard particles which caused grittiness in the hot-pack cheese spread was easily recognized by sensory evaluation, in particular by mouthfeel, and also visually by viewing a smear under a dissecting microscope.

Rapid screening of the structure, size, and distribution of the gritty particles present in the cheese samples was conducted by fluorescence microscopy using Fast Green FCF and Acridine Orange as staining reagents. The structure of the smooth cold-pack cream cheese spread is shown in Fig. 3. Fast Green FCF provided the best visual contrast between the particles causing grittiness and the surrounding matrix when the samples were excited at the 450-490 nm wavelength. The gritty particles imparted a yellow fluorescence against the red fluorescing background of the matrix. Positive interactions between Fast Green FCF and the gritty particles as well as their surrounding matrix suggested that both the gritty particles and the matrix contained protein. At a low magnification, the particles appeared as irregular clusters, varying in size and shape, embedded in a homogeneous matrix (Fig. 4). The particle sizes ranged from 10 to 100 µm in diameter and their shapes varied from being elongated, near-spherical, to irregular. They were distributed at random throughout the cheese spread samples. At a higher magnification, they appeared as densely aggregated masses which lacked visible microstructural details (Fig. 5). The different colour fluorescence detected after staining with Fast Green FCF indicated that the gritty particles differed from the surrounding protein matrix in terms of their affinity for the dye. Changes in the dye-binding properties may imply chemical modification of the protein curd resulting from the heating process or they may reflect a difference in chemical composition such as the presence of components other than protein.

No birefringence was noticed when the particles causing grittiness were examined under polarized light, indicating that the particles were not crystalline. They also failed to give a positive reaction with a silver nitrate solution in the Von Kossa method which is recommended for the detection of calcium phosphate crystals in cheese [4,34]. Thus, the grittiness of the hot-pack cheese spread was not caused by the presence of crystalline mineral salts or salts containing calcium.

A higher resolution such as that provided by a scanning electron microscope was used to reveal more detail of the gritty particle structure. Encapsulation of the viscous samples in agar gel tubes made it possible to handle them as solid samples, i.e., to fix them, dehydrate, and freeze-fracture them. Freeze-fracturing of the samples imbedded in a paraffin embedding medium produced smooth fracture planes (Fig. 6) running through the casein particles, thus opening their interior for examination. The smooth gels (cold-pack) had relatively uniform protein matrices (Fig. 7) which...
Grittiness in Cheese Spread

Fig. 6. Encapsulation in an agar gel tube (arrow) makes it possible to examine a freeze-fractured cold-pack cheese spread sample at a low magnification using conventional SEM. Freeze-fracturing produces a smooth fracture plane (P).

Fig. 7. SEM of a smooth cold-pack cheese spread made from pasteurized (63°C, 30 min) milk shows a protein matrix to be composed of relatively uniform small particles.

Fig. 8. Detail of the fluffy particles constituting a smooth cold-pack cheese spread made from pasteurized (63°C, 30 min) milk.

Fig. 9. stain with Acridine Orange shows a uniform protein matrix (green fluorescence, large arrows) with evenly distributed lactic acid bacteria (yellow fluorescence, small arrows).

Fig. 10. Detail of a particle causing grittiness in hot-pack cheese spread made from pasteurized (63°C, 30 min) milk. Particles causing grittiness (arrows) appear bright yellow on a reddish background of the uniform protein matrix. Staining with Fast Green FCF.

Fig. 11. Fluorescence microscopy of a hot-pack cheese spread made from pasteurized (63°C, 30 min) milk containing a stabilizer (large arrow) shows a heterogeneous structure. Casein particle aggregates (small arrows) vary in dimensions. Staining with Fast Green FCF.

Fig. 12. Fluorescence microscopic detail of stabilizer particles (arrows) present in a hot-pack cheese spread sample. Staining with Fast Green FCF.

Fig. 13. Homogenization of the curd which contained casein particle aggregates led to a uniform structure of the product (green fluorescence, large arrows). In this sample stained with Acridine Orange, yellow dots (small arrows) indicate lactic acid bacteria.

Consisted of fluffy particles as was demonstrated at a higher magnification (Fig. 8). In contrast, the gritty samples (hot-pack) had structures, the coarseness of which was visible even at a low magnification (Fig. 9). Examination of the larger particles in greater detail showed them to be compact (Fig. 10) with a higher incidence of lactic acid bacteria (Fig. 11) than the surrounding medium consisting of smaller fluffy particles. This increase in the incidence of the bacteria may have been caused by the contraction of the porous protein matrix.

TEM confirmed the findings made by SEM concerning the presence of compact protein particles in the gritty hot-pack cheese spread. Fig. 12 shows both kinds of structure in the cheese spread. The body of the product consisted of casein particles aggregated in the form of chains and clusters with relatively evenly distributed pores filled with the liquid phase (whey). Only the porous structure was present in the smooth cold-pack cheese spread. The hard particles causing the grittiness problem in the hot-pack cheese spread, however, were compact, as had already been shown by SEM.

Since grittiness was found in the hot-pack cheese spreads made using either acid-coagulated...
or renneted curd, it appeared that the reasons for grittiness to develop in the product should be sought in the treatment of milk prior to its coagulation. It is known that curd made from unheated milk is composed of large clustered casein particles. This structure favours exclusion of whey and rapid compaction of the casein particle clusters which is important in cheese manufacture. In contrast, the structure of curd made from milk heated to a minimum of 85°C consists of smaller casein particles which are coagulated in the form of chains. This structure is more resistant to syneresis than the former structure and is thus the basis of yoghurt where it is important to retain the liquid phase [8].

In another series of experiments, therefore, the milk was heated to 90°C in an APV-UHT Pilot Plant Apparatus (APV-Gaulin, Inc., Everett, MA, USA) and held for 10 min at that temperature (Fig. 13). This heat treatment of milk [12] successfully prevented the development of grittiness in the final product, i.e., in the hot-pack. The curd, which was made using one half of the amount of rennet used in the first series, consisted of grains (Fig. 14) which had a uniform structure formed by casein particle chains apparent at higher magnifications (Figs. 15 and 16). This structure differed from the structure of curd made from pasteurized milk in that the latter curd consisted of casein particle clusters (Figs. 7 and 8). Blending of the curd made from milk heated at 90°C with high-fat cream in the Polytron blender resulted in the disintegration of the curd grains (Fig. 17). Homogenization in the two-stage Gaulin homogenizer produced a smooth cheese spread, the microstructure of which was uniform (Fig. 18). At a higher magnification, however, a corpuscular microstructure was apparent (Figs. 19 and 20).
Grittiness in Cheese Spread

Flow diagram showing the preparation of curd made from milk heated at 90°C for 10 min.

Fig. 13. Flow diagram showing the preparation of curd made from milk heated at 90°C for 10 min.

Fig. 14. Curd made from heated (90°C, 10 min) milk consisted of grains (C) having a uniform structure. Fig. 15. Uniform structure of curd grains at a higher magnification. Fig. 16. Detail of the casein particle matrix in curd made from milk which had been heated at 90°C for 10 min. Fig. 17. Blending of curd made from heated (90°C, 10 min) milk with cream resulted in the disintegration of the curd grains into smaller particles (arrows).

This structure is the result of the disintegration of the considerably larger grains by blending and homogenization. It is important to note that all casein particle clusters were porous and that no compact particles were found either by sensory evaluation or by SEM of the hot-pack product.
An examination of the effects of stabilizers on the microstructure showed that some stabilizers, although not causing grittiness, may have contributed to the development of a coarse texture of the product. One such example was a stabilizer based on a red seaweed (Gigartinaeae) extract which contained galactomannans. Its use in the preparation of the cheese spread resulted in a heterogeneous microstructure which, although not noticeable by the mouthfeel, was clearly detected by fluorescence microscopy. This heterogeneity was characterized by the presence of a low concentration of casein particle aggregates (Fig. 21) and also fibrous particles of the stabilizer (Fig. 22). The aggregates included fat globules and were neither as compact (Figs. 23 and 24) nor as large as the particles which caused grittiness (Figs. 9-11). TEM confirmed the less compact internal structures of the aggregates (Fig. 25). The casein particle aggregates developed in the presence of the stabilizer were susceptible to partial disintegration by homogenization (Figs. 26 and 27). The mouthfeel of the resulting hot-pack cheese spread was smooth.

In conclusion, the development of grittiness in the products described was found to be related to the heat treatment of milk. Fluorescence microscopy was used to rapidly detect hard particles causing grittiness. SEM contributed to the solution of the grittiness problem because it helped to establish that the gritty particles were composed of compacted protein. The examination of their internal structure was made possible following the encapsulation of the viscous product in agar gel tubes and freeze-fracturing. TEM of thin sections confirmed the SEM findings.

Acknowledgments

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References


Fig. 18. Homogenization of the curd and cream blend produced a cream cheese spread which had a uniform structure.

Fig. 19. At a higher magnification, the disintegration of the uniform grain matrices into small casein particle clusters is noticeable leading to the formation of a spreadable product.

Fig. 20. Detail of casein particle clusters in a homogenized hot-pack cheese spread made from heated (90°C, 10 min) milk.
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Discussion with Reviewers

R. T. Marshall: Since the curd, which produced a smooth cheese spread, was made with one half as much rennet as the curd giving a gritty spread, this introduces two variables, i.e., heat treatment and rennet concentration. How was it determined that heat alone was the causative factor?

Authors: Several measures to reduce the grittiness were taken and have been mentioned, e.g., lower temperature of pasteurization, triple homogenization, use of another bacterial starter culture, and use of acid-coagulated curd instead of renneted curd. All of these measures failed to prevent grittiness from developing. Since heating of the milk to 90°C eliminated the defect, insufficient heating of the milk prior to coagulation was identified as the factor causing grittiness in the product under study.

R. T. Marshall: That homogenization reduced the sizes of larger grains is not apparent in comparison of Figs. 16 and 20. These are of the same magnification and appear to have the same sizes of casein micelles. Fig. 20 appears to show a higher degree of aggregation of the micelles and other particulates. This point needs clarification.

Authors: Fig. 16 shows detail of a large grain, featured in Fig. 14 and marked with letter C. These grains have a uniform microstructure consisting of a continuous matrix of casein micelle chains and clusters. As a result of homogenization, the grains were broken into considerably smaller particles. These particles are shown in Fig. 20. Homogenization also led to some compaction of the matrix but the casein micelle dimensions remained unaffected.

M. L. Green: You state that the porous structure of the spread contains whey-filled pores. Where is the relatively large amount of fat in this product located? As you have extracted the fat during the preparation of most samples, can you be sure that fat is not involved in the formation of the gritty particles?

Authors: Preliminary examination of the gritty product by fluorescence microscopy pointed to protein rather than to fat as the cause of grittiness. Fat was in the form of small globules, the distribution and dimensions of which are evident from Fig. 12. This micrograph was obtained by TEM of a thin section.

D. N. Holcomb: You state that grittiness was evaluated subjectively by mouthfeel. Was such evaluation done by a trained panel or by expert cheesemakers? Was it done by sensing particles between the tongue and palate or between the teeth? Can you provide enough details so a reader could repeat this evaluation? In my experience, “experts” may disagree as to whether or not a product is gritty.

Authors: Grittiness was detected by examining the cheese spread between the tongue and the palate. It was so pronounced in the initial product that it was not necessary to use the services of a trained panel.

D. N. Holcomb: The authors note that bacteria were associated with the compact particles in hot-pack cheese with a higher incidence than in the surrounding medium. Can they postulate an explanation as to why the bacteria should be preferentially associated with the particles? Could the bacteria contribute to formation of the compact particles?

Authors: Gritty particles originated by the shrinkage and compaction of larger areas of curd. During this act of shrinking, the bacteria were concentrated into the smaller volume of the compact particles and gave the appearance of being in higher numbers per unit volume.

D. N. Holcomb: Is there any correlation between particle size and grittiness? Would the same correlation hold for compact protein particles, crystals, and other particles?

Authors: Probably there is a correlation, but it was not the subject of this study.
THE SIZE DISTRIBUTION OF CASEIN MICELLES IN CAMEL MILK

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Abstract

The size distribution of casein micelles in camel milk has been determined by electron microscopy. Individual and pooled samples were cryo-fixed by rapid freezing and freeze-fractured. Electron micrographs of the freeze-fracture replica revealed a relatively broad size distribution, with an average micelle diameter around 280 nm in the volume distribution curve. The distribution was significantly broader than that of the particles of cow's or human milk and showed a greater number of large particles. The submicelles were also somewhat larger than those observed in cow's and human milk (approx. 15, 10 and 7 nm, respectively). The average values for the gross composition of camel milk were similar to those of cow's milk. Partition of mineral salts between the serum and micellar phase of camel milk was studied by means of ultrafiltration. The proportion of soluble forms of the minerals expressed as percentage of their total concentrations were 33% for calcium, 69% for magnesium, 52% for phosphorus and 60% for citrate.

Introduction

According to FAO statistics, there are 17 million camels in the world, of which 12.2 million are in Africa and 4.8 million in Asia (22). The camel is a potentially important source of milk. Indeed, in some countries hosting large camel populations, camel milk is one of the main components of the human diet. Milk production varying between 1,800 and 12,700 kg during a lactation period between 9 and 18 months has been reported (13). Information on the characteristics of camel milk is limited. Data available show, however, significant differences between cow and camel milk proteins in properties such as electrophoretic mobility, molecular size (8) and rennet coagulation (7).

While a considerable amount of data is available on micellar casein of bovine milk, very little is known about casein micelles of camel milk. Ali and Robinson (2) have analyzed the size distribution of casein micelles in six samples of camel milk. They determined a number average diameter of 160 nm on electron micrographs of ultra-thin sections. This value, however, overestimates the true mean, because particles with diameters smaller than 14 nm could not be measured. It was therefore considered useful to determine the complete size distribution of casein micelles in camel milk by using freeze-fracture replica of cryo-fixed samples and to compare it to that observed in milk of other species. The freeze-fracture technique allows counting and sizing of the smallest casein micelles including submicelles. Other basic data on the chemical composition of camel milk are also given.

Materials and Methods

Milk samples

Camel milk samples were taken at Ngare Ndare Camel Farm which is situated just north of the equator in Kenya's Laikipia District, at an altitude of 1,730 to 1,890 m above sea level. The animals of indigenous breed (Camelus dromedarius) were all fed exclusively by grazing. The milk samples A and B were collected from 10 individual camels, on two different occasions. On each occasion, the 10 milk samples were pooled, kept refrigerated, and transported to our laboratory within 36 hours. Upon arrival, the milk samples were skimmed, freeze-dried and stored in sealed plastic bags until analysis. Two individual fresh milk samples (numbers 52 and 56) were also used for the analysis. For these samples the time...
Table 1. Average chemical composition of camel and cow’s milk

<table>
<thead>
<tr>
<th>Component</th>
<th>unit</th>
<th>camel milk</th>
<th>cow milk</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>mean (x)</td>
<td>std (s)</td>
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<tr>
<td>Dry matter</td>
<td>g/100 g</td>
<td>12.2</td>
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<tr>
<td>Protein</td>
<td>g/100 g</td>
<td>3.11</td>
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<td>mg/100 g</td>
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<td>Casein N</td>
<td>% of TN</td>
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<tr>
<td>Non-casein N</td>
<td>% of TN</td>
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<tr>
<td>Non-protein N</td>
<td>% of TN</td>
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<tr>
<td>Lactose</td>
<td>g/100 g</td>
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<tr>
<td>Fat</td>
<td>g/100 g</td>
<td>3.15</td>
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<tr>
<td>Ash</td>
<td>g/100 g</td>
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</tr>
<tr>
<td>Calcium total</td>
<td>mg/100 ml</td>
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<td>9</td>
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<tr>
<td>Calcium dissolved</td>
<td>% of total</td>
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<td>Magnesium total</td>
<td>mg/100 ml</td>
<td>8.3</td>
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<tr>
<td>Magnesium dissolved</td>
<td>% of total</td>
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<tr>
<td>Phosphorus total</td>
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<td>4</td>
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<tr>
<td>Phosphorus dissolved</td>
<td>% of total</td>
<td>52</td>
<td></td>
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<tr>
<td>Citrate total</td>
<td>mg/100 ml</td>
<td>177</td>
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<tr>
<td>Citrate dissolved</td>
<td>% of total</td>
<td>60</td>
<td></td>
</tr>
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</table>

a Walstra and Jenness (20); b Jenness and Patton (12); c N x 6.38; d as citric acid.

Chemical analysis

Total solids, fat, protein, lactose and ash were determined according to AOAC standard methods (4).

The nitrogen distribution in the milk was determined by the procedure of Aschaffenburg and Drewry (5). The following N-fractions were determined: total protein nitrogen (TN), non-casein nitrogen (NCN) and non-protein nitrogen (NPN), soluble in 12% trichloracetic acid. The amount of casein nitrogen (CN) was calculated by difference.

In order to study the distribution of salts between the dissolved and colloidal phases in milk, it was filtered through a diaflo ultrafiltration membrane (Amicon PM10). The ultrafiltration was carried out under nitrogen at a pressure of 0.35 MPa. In both the original milk and the collected ultrafiltrate the following minerals were determined: calcium and magnesium by atomic absorption spectrophotometry (19), phosphorus by the phosphomolybdate method described in the International Dairy Federation Standard (11) and citrate enzymatically by using a commercially available test kit (Boehringer, Mannheim, West Germany, catalog number 139076).

For amino acid analysis, casein was precipitated from skimmed milk with 0.01 mol/l acetic acid at pH 4.5 - 4.6. The precipitate was washed three times with water and freeze-dried. 20-30 mg of this acid casein were hydrolyzed with 6 mol/l HCl for 24 hours at 110°C under vacuum. The hydrolysate was analyzed on a model Liquimat III amino acid analyzer (Kontron Instruments AG, Zürich) according to the procedure of Amado et al. (3).

Electron microscopy

The reconstituted and fresh skimmed milk samples were cryo-fixed using the propane jet-freezing technique. This technique basically involves the rapid freezing (approximately 10,000 K.s⁻¹) of a very low mass specimen in a jet of liquid propane at 88 K (14, 15). Freeze-fracture replicas were then obtained as described earlier (16). Fourteen to sixteen electron micrographs of each sample were taken at a magnification of approximately 20,000x and the negatives were enlarged 2.6 times for counting and classifying the particles. The total surface area of milk observed for the four samples was 742 micrometers². 6,618 particles were counted on this surface. A diameter class width of 20 nm was chosen for the classification of the particles on the prints. A transparent sheet with bars corresponding to the different size classes was placed over the prints. The size class of each particle was found by fitting it into the appropriate diameter range. Particles smaller than about 5 nm in diameter were not considered.

Statistical analysis

Conversion of the observed size distribution of plane sections into real distribution of spherical particles was made using a method proposed by Goldsmith (10). The original FORTRAN program was modified and translated into GW-BASIC for use on MS-DOS microcomputers. Copies of the program are available on request from one of the authors (M.R.). A slice thickness of 5 nm was assumed. Preliminary calculations revealed rather broad size distributions with relatively low frequencies in the larger size classes. The class width was therefore increased from 20 to 40 nm.
Casein micelles in camel milk

Table 2. Amino acid composition of whole casein from camel and cow’s milk

<table>
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<th>Constituent</th>
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<th>cow</th>
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<tr>
<td>Aspartic acid</td>
<td>7.28</td>
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<td>Threonine</td>
<td>4.87</td>
<td>4.42</td>
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<td>Serine</td>
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<td>Glutamic acid</td>
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<td>Proline</td>
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<td>Glycine</td>
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<td>Alanine</td>
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<td>Valine</td>
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<td>6.48</td>
</tr>
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<td>Cysteine</td>
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<td>0.65</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.70</td>
<td>2.51</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.23</td>
<td>5.54</td>
</tr>
<tr>
<td>Leucine</td>
<td>10.89</td>
<td>8.41</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.64</td>
<td>5.59</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.01</td>
<td>4.73</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.53</td>
<td>7.33</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.44</td>
<td>2.70</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.63</td>
<td>3.62</td>
</tr>
</tbody>
</table>

\[\text{amino acid}^a\]

1 Alais and Blanc (1); recalculated on a % basis. Tryptophane was not determined.

The equations used to calculate the various mean diameters (\(d_m, d_V, d_{VS}, d_{VM}\)), the distribution width \((c_8)\) and volume fraction \((V)\) are explained in detail elsewhere (16). It should be remembered, that the number average and volume average diameters \(d_n\) and \(d_V\) are sensitive to shape, errors at both ends of the distribution function, and total number of particles. These values are meaningful only for corresponding symmetrical distribution curves. The weighted mean diameters \(d_{VM}\) and \(d_{VS}\) are more useful averages for the characterization of the casein micelle distributions. The distribution width \(c_8\) corresponds to the coefficient of variation of the surface-weighted distribution (21). Distribution curves from different samples were compared using a standard chi-square test for multiway frequency tables.

Results and Discussion

Chemical composition of camel milk

Table 1 shows data on the chemical composition of the camel milk used for this study. Values for cow’s milk from the literature (12, 20) are presented for comparison. In general, the gross composition of camel and cow’s milk is similar. The values of CN, NCN and NPN expressed as percentage of the total N appear also to lie in the same ranges.

Concentrations of calcium, magnesium, phosphorus and citrate, along with their partition between the dissolved and colloidal phases are also given in Table 1. As generally reported in the literature (9, 12), about one third of the calcium and phosphate, 75% of magnesium, and 90% of the citrate of fresh cow’s milk are present in the serum phase. In camel milk, the distribution of calcium, magnesium and phosphorus is similar. However, the amount of citrate in the serum phase was found to be lower in camel milk.

Fig. 1. Freeze-fractured casein micelles in camel milk (cm: casein micelles; sm: submicelles).

Fig. 2. Number of particles observed in freeze-fractured camel, cow’s and human milk. The ordinate is logarithmic and gives the number of particles per mm² fractured area and per nm class width.

The amino acid compositions of pooled camel and cow’s milk casein are presented in Table 2. A similar pattern can be observed for both species. The most pronounced differences were found for glycine and cysteine, both being significantly lower in camel milk casein.

Size distribution of casein micelles

Fig. 1 shows a typical electron micrograph of casein particles in freeze-fracture replica of camel milk. The mean diameter of the submicelles was on the average 15 nm. This is a rough estimate, because of uncertainties in the technique (plastic deformation of proteins etc.).

The average number of particles observed on such freeze-fractured surfaces is shown graphically in Fig. 2. The ordinate gives the normalized frequency of particles per unit area, i.e., the average number of particles per mm² fractured area and per nm class width. The distribution is significantly broader than that of cow’s or human milk and shows a greater number of large particles.
Table 3. Size distribution of casein micelles in camel milk compared to cow’s milk

<table>
<thead>
<tr>
<th>Parameter</th>
<th>unit</th>
<th>herd milk</th>
<th>individual</th>
<th>pooled</th>
<th>ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A*</td>
<td>B*</td>
<td>52*</td>
<td>56*</td>
</tr>
<tr>
<td>Average micelle diameter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$d_m$, number average</td>
<td>nm</td>
<td>28</td>
<td>28</td>
<td>27</td>
<td>26</td>
</tr>
<tr>
<td>$d_v$, volume average</td>
<td>nm</td>
<td>63</td>
<td>57</td>
<td>51</td>
<td>50</td>
</tr>
<tr>
<td>$d_{vs}$, volume/surface av.</td>
<td>nm</td>
<td>165</td>
<td>131</td>
<td>113</td>
<td>114</td>
</tr>
<tr>
<td>$d_{wm}$, weight average</td>
<td>nm</td>
<td>288</td>
<td>222</td>
<td>212</td>
<td>237</td>
</tr>
<tr>
<td>Distribution width</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$c_B$ % of $d_{vs}$</td>
<td>%</td>
<td>0.5</td>
<td>0.6</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Volume fraction, $v$</td>
<td>%</td>
<td>3.2</td>
<td>2.6</td>
<td>2.4</td>
<td>2.9</td>
</tr>
<tr>
<td>Submicelles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$d_n$, number average</td>
<td>nm</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>15</td>
</tr>
</tbody>
</table>

* A and B: pooled samples, freeze-dried and reconstituted; 52 and 56: fresh samples.

a From Røegg et al. (16) and Schmidt et al. (17,18); b 14-16 nm; c calculated from size distribution

**Fig. 3 (at left).** Size distribution of casein particles in camel milk compared to cow’s and human milk (volume frequency histogram).

**Fig. 4 (to the right).** Cumulative particle volume distribution of casein micelles in camel milk (pooled data from two individual and two herd milks).

The differences between the distribution curves of the two individual camel milks and the herd milk samples were most pronounced in the diameter range of about 200 to 500 nm. However, the differences were statistically not significant.

The particles in the lowest size class with diameters smaller than 40 nm comprise about 80% of the observed total number of particles but represent only 4-8% of the mass or volume of the casein in camel milk. It is therefore meaningful to consider the weight or volume frequency distribution. Fig. 3 shows the volume frequency of the pooled data of the four milk samples, compared again with the distributions found in cow’s and mature human milk (16). The volume distribution curve of casein micelles in camel milk is broad and shows a maximum around 280 nm.

As can be deduced from the cumulative distribution curve in Fig. 4, micelles with diameters between 125 and 310 nm comprise about 50% of the volume or mass of the casein.

Some statistical data derived from the distribution curves, such as mean diameters, width of the distribution, and volume fraction are summarized in Table 3. For comparison, the ranges of the corresponding values for cow’s milk are also included.

In earlier investigations, camel milk, after rennet addition, was found to coagulate 2-3 times slower than cow’s milk. The coagulum obtained was a precipitate in the form of flocks and no homogeneous clot formed (7). The present investigation revealed a relatively broad size distribution of casein micelles in camel milk with a greater number of large micelles.
Casein micelles in camel milk

than in cow’s milk. The poor rennetability could be related to these differences in the size of casein particles. Coagulation time varies with the micelle size and reaches an optimum in the medium and small size micelles. This appears to be related to the availability of k-casein. The content of k-casein decreases with increasing micelle size (6, 20).

From the results obtained it can be concluded that camel milk casein differs from cow’s milk casein in terms of micellar size distribution. However, it would be premature to discuss the impact of this difference in relation to the preparation of products from camel milk. Various biochemical aspects must also be considered and additional studies are necessary to correlate any special feature of product structure with the findings in this investigation.

Acknowledgements

The authors express special thanks to Dr. E. Wehrli, ETH Zürich, for the preparation of the freeze-fracture replicas and to Mrs. M. Farah and U. Moor for their help in the analysis of the micrographs.

References


Discussion with Reviewers

W. Buchheim: Apparently reconstituted (freeze-dried) skim milk was used for electron microscopy work. Is there any danger that freeze-drying might affect size, shape, and distribution of micelles?

P. Resmini: It is written that both fresh and freeze-dried milk samples have been analyzed, but no data are reported concerning these two different products. Freeze-fracturing techniques suggest that the usual freeze-drying of liquid milk may modify the structure of casein micelles, due to the low freezing rate that promotes ice crystal formation inside the micelles, therefore freeze-drying of milk does not seem to be a suitable technique for ultrastructure studies of casein. Please comment.

Authors: The freeze-dried samples were reconstituted to 12.2 % dry matter at 30 - 35°C. There is a certain risk that freezing and thawing or reconstitution of the freeze-drying affects the structure of casein particles. To our knowledge, no statistically significant differences between size distribution in fresh and reconstituted preparations has been reported in the literature and no significant difference was observed in the present investigation.

W. Buchheim: In my opinion, the number and sizes of micelles and non-micellar casein, visible in Fig. 1 contradict the frequency values given in Fig. 2 because the micrograph shows approximately equal number of small particles and cross-sections of large micelles, instead of 100- or 1000-fold. Please comment.

Authors: Fig. 1 is not a "random picture". A sector has been chosen which shows both large and small micelles. Therefore, the size distribution on this Fig. cannot be used to estimate the real distribution. The area of Fig. 1 represents about 7.9 micrometers².
This is only about 1/100th of the total area that has been measured.

W. Buchheim: In case that the amount of non-micellar casein ("submicelles") has been overestimated, some average values (e.g., $d_m$, $d_v$, and even $d_{vm}$) would be too small. According to reviewer's own experience (see e.g., Food Microstructure 5(1), 181-192, (1986)) direct determination of $d_{vm}$ from micrographs (via circumferences and areas of particles) is the best way for testing such possible discrepancies.

Authors: The unweighted mean diameter $d_m$ and to some extent the other measures of the mean which are based on the lower moments of the distribution function are sensitive to both ends of the distribution as well as to the total number of the particles counted. The higher the power of the moments, the less is the sensitivity to the uncertainty in the estimation of the smallest particles. $d_{vm}$ is therefore the most robust estimate of the mean diameter. Considering the very broad size distribution of the casein particles in camel milk, the meaning of an "average diameter" should not be overestimated.

W. Buchheim: I have some doubts as to how meaningful size values for so-called submicelles are. Protein molecules are plastically deformed when freeze-fractured, so that we identify primarily only their existence in the plane of cleavage. Slightly modified fracturing and shadowing conditions may influence their apparent size so that measurements of "diameters" and comparisons in different experiments are questionable.

P. Walstra: Conclusions about the size of submicelles are, in my opinion, rather questionable because of the uncertainties in the technique.

Authors: We agree with the reviewers' comment. The diameter of the submicelles is a rough estimate. It has mainly been added for comparison and because of the pronounced difference to that of cow's milk.
EFFECT OF HEATING TO 200°C ON CASEIN MICELLES IN MILK:
A METAL SHADOWING AND NEGATIVE STAINING
ELECTRON MICROSCOPE STUDY

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Ottawa, Ontario, Canada K1A 0C6

Abstract
Milk was heated to 200°C for 3 min in sealed inverted-Y-shaped glass vials and reacted with a glutaraldehyde solution at that temperature. Electron microscopy of the metal-shadowed and negatively stained samples revealed that casein micelles in the milk did not disintegrate extensively at the high-temperature used but, rather, became enlarged. Some of them were found to be either clustered or distorted.

Introduction
Ultra-high temperature (UHT) treatment of milk has been developed to significantly increase its shelf life. The treatment, which destroys bacteria and their spores, consists of heating milk to a high temperature for a short period of time (e.g., 141°C for 15 s [29]). The effects of UHT and high-temperature short-time pasteurization (HTST, 92°C for 20 s) on casein micelles in milk were studied by several authors. A slight increase in the mean dimensions of casein micelles was found in unheated milk [1, 7, 10, 11, 18, 24, 29] but in UHT-sterilized concentrated skim milk the mean dimensions of the casein micelles increased twice to three-fold [5, 12]. This increase has been reported [2, 6, 24, 29, 35] to be accompanied by an increase in the number of particles smaller than casein micelles. Several authors [5, 7, 24, 29] suggested that these small particles are composed of heat-denatured whey proteins which were not adsorbed on the casein micelles. An increased concentration of soluble casein was found following heating of a system consisting of whey protein-free casein micelles [2]. Heating of milk was also reported to lead to the release of macropeptides [15] and to shifts in the mineral constituents from the colloidal to the soluble state [2, 10, 11]. Hostettler and Imhof [18] hypothesized that casein micelles disintegrate into submicellar particles at high temperature and, upon cooling, the latter particles reaggregate into particles larger than the original casein micelles.

The composition, structure, and dimensions of casein micelles in UHT-sterilized milk may play a role in its gelation during prolonged storage [3, 12, 13]. It is now generally accepted on the basis of current knowledge that age-gelation of UHT-sterilized milk is preceded by changes taking place at the surface of casein micelles. Questions concerning whether such changes are caused by enzymatic action of heat-stable proteases or are the result of purely physico-chemical alterations have not yet been unequivocally answered [12]. However, clarification of even some facets of the problems may contribute to the understanding of the age-gelation of UHT milk.

Changes in the casein micelle dimensions in milk and their distribution have been studied by a variety of techniques and the results were
reviewed [3, 16]. The techniques used include light scattering [17], size exclusion chromatography [24], chromatography on controlled pore glass [1, 21], light microscopy [34], and electron microscopy [4, 6, 7, 11, 13, 22, 28-33]. In the electron microscopic studies, the casein micelles were examined either unfixed or fixed after the heated milk was cooled. No attempts to study the micelles at the high temperature were reported. For example, Rüegg and Blanc [29] examined UHT-treated milk samples by electron microscopy following their cooling to room temperature. Glycerol added to the milk was used as a cryoprotective agent. The samples were freeze-fractured and then replicated with platinum and carbon [32]. In the milk that had been heated, the number of submicellar particles was increased and there was also a slight increase in the number of large casein particles at the expense of medium-sized micelles. The authors hypothesized that the large particles originated as a result of the reaggregation of submicellar casein and that the submicelles preferentially associated with large micelles although they recognized that non-specific precipitation of heat-denatured serum proteins on casein micelle surfaces may also have contributed to the micelle enlargement.

The objective of this study was to subject milk to extreme conditions, i.e., heating to 200°C for up to 3 min, and to examine the integrity of the casein micelles by electron microscopy. Such high temperatures may not be used in dairy processing but may be attained in other areas such as the manufacture of confectioneries. In order to visualize the casein micelles in the state as they existed at the high temperature, a simple aid was developed which made it possible to interact the casein micelles with glutaraldehyde at the high temperature.

Materials and Methods

Heating of milk

The skim milk used was either of commercial origin or was prepared from fresh pooled whole milk obtained from the Central Experimental Farm dairy cattle herd. Fat was separated from the fresh milk by low-speed centrifugation (415 g for 30 min). Inverted-Y-shaped tubes (Fig. 1) made from Pyrex glass were used to heat both the milk and a 2.8% aqueous glutaraldehyde solution separately at the same time. The milk (0.2 mL) was placed in one arm of the tube and the glutaraldehyde solution (0.2 mL) was placed in the other arm. The tube was flame-sealed and the contents were heated in a sand bath for varying periods of time. The temperature of the sand bath was maintained at 150±2°C or 200±3°C. The glass tubes were completely immersed in the sand bath, otherwise water vapour would have condensed on the cooler wall and the condensate would have flowed down in either compartment. When the heating of the milk was completed, the tubes were removed from the sand bath and immediately inverted, which allowed the milk to react with the glutaraldehyde solution at the high temperature. Then the mixtures were cooled to 25°C. In parallel experiments, the milk and the glutaraldehyde solution were allowed to cool to 25°C before they were combined. The tubes were opened and the reacted milk was prepared for electron microscopic examination. Unheated milk fixed with glutaraldehyde at 25°C was used as the control.

Electron microscopy

Two electron microscopic methods were used to examine the integrity of the casein micelles: (a) shadowing with platinum and carbon (replica technique) and (b) negative staining.

In the replica technique, a droplet of the treated milk was spread on a freshly cleaved mica sheet (5 x 8 mm) pretreated with an aqueous 0.1% poly-L-lysine hydrobromide solution (60,000-120,000 daltons; Polysciences, Inc., Warrington, PA, USA) and after 1 min, the excess milk was washed off with distilled water. Corpuscular milk proteins (casein micelles and submicellar particles) adhering to the mica sheet [23, 25] were then dehydrated in a graded ethanol series and critical point-dried from carbon dioxide. Following shadowing at a fixed angle of 27° or rotary shadowing by evaporating platinum and carbon in vacuo [20], the replicas thus prepared were separated from the mica support by floating on a 0.17:1 mixture of sodium hypochlorite solution. Milk proteins adhering to the replicas were digested within 30 min. The replicas were then washed with water, picked up on 400-mesh grids, and examined in a Philips EM-300 transmission electron microscope operated at 60 kV. Micrographs were taken on 35-mm film (Eastman Fine Grain Release Positive Film 5302) and intermediate negatives were made on Kodak Plus-X 35 mm film [20].

For negative staining, the fixed milk was diluted with a 0.01 M CaCl₂ solution in the ratio of 1:50 [28, 33]. A droplet of this mixture was applied to a 200-mesh (hexagonal) grid which had been coated with Formvar and carbon films and then treated by the glow-discharge method in order to make the carbon surface hydrophilic [9]. Then, a
Results and Discussion

Visualization of casein particles, as they exist in milk while it is heated to temperatures above the boiling point, is difficult to achieve. Such heating can be accomplished only under elevated pressure, and fixation should be done at the high temperature in order to preserve all changes in the integrity of the micelles which may have been caused by the high-temperature treatment.

This objective could be approached from two directions. One approach was to physically fix the hot milk by freezing it rapidly in Freon 12, at -150°C, freeze-fracture it, and to examine platinum- and-carbon replicas by transmission electron microscopy (TEM). Such experiments were carried out, but it was impossible to freeze the hot milk rapidly enough to prevent the development of ice crystals and the distortion of the casein micelles.

The other approach involved chemical fixation [14] of casein present in the milk, in either the micellar or submicellar forms, at the high temperature using a glutaraldehyde solution and then cooling the mixture as described in the Methods section. Although it is not common to fix biological materials with glutaraldehyde at 200°C [27], it is believed that this reaction followed by cooling of the mixture would not result in any severe changes in the state of the protein particles present.

Metal-shadowing

Replication of casein micelles in fresh unheated milk using a metal-shadowing technique showed that they were mostly spherical (Fig. 2). At a high magnification, they appeared to be composed of submicelles (Fig. 3). A low concentration of submicellar particles in the milk was also noticeable. Intermediate negatives were made from the original negatives in order to show areas coated with platinum as light in colour.

In milk that had been heated, some casein micelles were aggregated and the incidence of submicellar casein was somewhat increased. Also, the shapes of some of the casein micelles were found to have been altered. The severity of the changes depended on the intensity of the heat treatment. In milk heated at 100°C for 3 min, the casein micelles acquired a ragged surface topography (Fig. 4). Aggregation of protein and the formation of so-called "spikes" or "hair" were reported earlier also by other authors [8, 11, 13, 19, 35]. Changes were also found in the casein micelle ultrastructure: the submicelles were more clearly visible (Fig. 4) than in casein micelles in unheated milk (Fig. 3). This was probably the result of some loosening of the bonds between the submicelles that took place due to the effect of the heat treatment and this loosening may also be responsible for the enlargement of the micelles. In milk heated at 200°C for 3 min, casein micelles with a tight packing density (Fig. 5) as well as the looser packing density (Fig. 6) were found. Aggregated casein micelles with spiked surfaces (Fig. 7) were also present but the incidence of the particle clusters was low as evident from Fig. 8. Severely distorted casein micelles (Fig. 9) were occasionally noticeable even in the vicinity of undistorted micelles in milk heated at 200°C. Also occasionally, clusters of two or three casein micelles connected by fibre-like material (Figs. 10 and 11) were seen in the heated milk.

The images of casein micelles present in milk which had been heated to 200°C and then cooled to 25°C prior to fixation with glutaraldehyde resembled the images of casein micelles interacted with the glutaraldehyde solution at 200°C.

Casein micelles shadowed with platinum and carbon using either the stationary (unidirectional) or rotary techniques reveal their three-dimensional structure in great detail. Tilting the replicas in the electron microscope and taking pairs of micrographs each at a different angle makes it possible to view the three-dimensional structure as was shown earlier [20]. However, the technique used poses the risk that the attachment of the casein micelles to the mica sheet treated with polylysine may have been selective. It is possible that only unchanged casein micelles would be attached to the support whereas altered casein micelles would not be attached. This would mean that even if most of the casein micelles had disintegrated during heating of the milk, only the remaining intact casein micelles would become attached to the polylysine-treated support, and thus give an impression that there were no changes in the integrity of the micelles. In the replication technique, milk is applied to the polylysine-treated mica sheet and a few minutes later the milk is washed off with water. Thus, only a small proportion of the protein particles (casein micelles and submicelles) present in the milk remain attached to the mica support. Subsequently, the mica sheet with the protein particles attached is treated with increasing concentrations of ethanol in order to dehydrate the protein. Finally, the material adhering to the mica support is exposed to liquid carbon dioxide during critical-point drying. Because of the preparation steps involving the use of liquids such as ethanol and carbon dioxide, it is possible that some protein particles are washed off. For these reasons, it was necessary to use a method which prevents the loss of protein particles during the preparative steps and allows the observation of the protein particles in their initial distribution ratio.

Negative staining

To overcome problems associated with shadowing, negative staining was carried out. This method was used by several authors [21, 33] to study casein micelles in milk. It was necessary, however, to establish the optimal conditions for this study. Of the several stains tested, a 3% phosphotungstic acid solution at pH 7.2 gave the best results. Uranyl acetate applied at various concentrations and pH values reacted with the micelles and allowed them to be stained. Wherever ammonium molybdate caused an increase in background staining.

The milk was diluted in order to obtain a suitable distribution of the casein micelles on
Rotary shadowing of casein micelles in unheated milk with platinum and carbon reveals their spherical shapes and size distribution.

Detail of the structure of a casein micelle in unheated milk. Rotary shadowing shows the submicelles (small arrows). The perimeter of the casein micelle is excessively light (large arrow) and lacks detail because the electron beam was absorbed by passing through the platinum coating on the vertical wall; this phenomenon is characteristic of the rotary coating technique.

Detail of the structure of a casein micelle in milk heated at 100°C for 3 min. Casein submicelles (large arrows) and aggregated protein particles (small arrows) may be seen.

Figs. 5 and 6. In milk heated at 200°C for 3 min, some casein micelles show a tight packing density (Fig. 5) and other micelles show a looser packing density (Fig. 6). Aggregated casein micelles were present in milk heated at 200°C for 3 min. Stationary shadowing reveals spiked micellar surfaces.

Fig. 8. The incidence of aggregated casein micelles (large arrows) is relatively low in milk heated at 200°C. Small arrow points to casein submicelles.
Casein Micelles in Heated Milk

Fig. 9. Severely distorted casein micelles (small arrows; rotary shadowing) in milk heated at 200°C for 3 min. Another casein micelle (large arrow) has a regular appearance and shows the submicellar ultrastructure.

Figs. 10 and 11. Occasionally, protein strands connecting two or more micelles were noticeable in milk heated to 200°C for 3 min.

Fig. 12. Negative staining of casein micelles in unheated milk.

Fig. 13. Casein micelles in milk heated at 200°C for 3 min and mixed with a glutaraldehyde solution at the high temperature.

Fig. 14. Aggregated casein micelles in milk heated at 200°C for 3 min and mixed with a glutaraldehyde solution at the high temperature.

the grids for electron microscopic examination so that it could be observed whether or not the casein micelles were connected to each other. The best results were obtained by diluting the milk with a 0.01 M CaCl₂ solution in the ratio of 1:50 as initially suggested by Nitschmann [26]. Casein micelles disintegrated in milk that had been diluted with distilled water.

With the optimal conditions determined, casein micelles were observed both in unheated milk (Fig. 12) and in milk heated to 200°C for 3 min, irrespective of whether the milk had been mixed with the glutaraldehyde solution at the high temperature (Fig. 13) or after both the milk and the glutaraldehyde solution had been cooled to 25°C. Similar to metal shadowing, casein micelle clusters (Fig. 14) were found in heated milk by negative staining. In all milk samples, submicellar casein was also noticeable.

Electron microscopy of milk which had been heated at 200°C for 3 min and reacted with glutaraldehyde at that temperature revealed that casein micelles in that milk were not disintegrated, at least not to any noticeable extent. Metal shadowing using the stationary or rotary technique [20] showed the casein micelles as three-dimensional entities, the shapes of which were slightly distorted and the submicellar structure somewhat loosened as the result of heating. The question of whether samples prepared by the shadowing technique would give an accurate representation of the effect of heat on the integrity of the micelles has been answered by the results of the negative staining method, which produced images of casein micelles similar to those found in the control (unheated) milk. Thus, the results presented do
not support the hypothesis [18] that upon severe heat treatment casein micelles disintegrate into submicelles which reassociate upon cooling.

Acknowledgments

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References

Casein Micelles in Heated Milk


Discussion with Reviewers

V. Kakuda: Does all the liquid (water from the skim milk and glutaraldehyde and glutaraldehyde itself) vaporize under these conditions? If most of the water vaporizes, then dry caseins would be left on the bottom of the tube. Is it possible for unhydrated casein micelles to be less prone to disintegration, hence the results you see?

Authors: The water in the sample and the glutaraldehyde solution vaporizes only if the vial is not completely immersed in the sand bath; the water then condenses on the inside wall at the cooler top of the vial. No water condensation was observed provided that the vial was fully immersed. In earlier experiments, where this defect occurred, the evaporated milk was not examined but was discarded.

Reviewer 1: Why was size distribution not established?

Authors: Extensive experiments would be required to correctly establish the size distribution, but the data would not be important in this particular study, the objective of which was to only examine whether casein micelles would disintegrate when exposed to 200°C for 3 min.

N. A. Carrell: Can you quantify the aggregation and incidence of submicellar casein? This is not evident on the fields shown.

Authors: Quantitation of aggregated casein micelles could be based on electron micrographs provided that a sufficiently high number of casein micelles is examined. The incidence of submicellar casein could be established by examining electron micrographs of negatively stained samples.

N. A. Carrell: Is it possible that the ragged surface of heated casein micelles is due not to attached protein particles but to voids created due to released protein particles?

Authors: Although this may be possible, protein aggregation rather than release has been reported by other authors to be the cause of the ragged casein micelle surface.

N. A. Carrell: The structures shown in Figs. 10 and 11 are intriguing. Did you find many of these structures? What do you think they are?

Authors: Their incidence was a fraction of a percentage point of all casein micelles present; we, too, find them intriguing.

N. A. Carrell: Can you estimate what proportion of the casein micelles in the heated milk were aggregated or were altered in shape? Did you find a similar proportion in the milk which was fixed after it was cooled?

Authors: There were approximately 5% of casein micelles clusters in heated milk irrespective of whether it was mixed with glutaraldehyde while hot or cooled. Less than 2% of the micelles were distorted.

N. A. Carrell: Could the smaller structures which you refer to as submicelles, or the roughness on the micelle surface, actually be due to aggregates of whey proteins? If you were to heat washed micelles, not milk, would you expect any differences in structure?

Authors: The surface of washed casein micelles showed no protein aggregates (roughness) due to heating to 100°C.

V. Kakuda: Could the glutaraldehyde vapour fix the unhydrated casein micelles during the heating process and prevent disintegration as well?

N. A. Carrell: The authors state that the milk was reacted with glutaraldehyde at 200°C. However, the milk was mixed with glutaraldehyde at that temperature and there are no data to prove that the reaction did not occur at a lower temperature due to vaporization of glutaraldehyde. Nor is there any evidence that glutaraldehyde, that had been subjected to high heat and pressure, functions in the same manner as glutaraldehyde that had not been treated in this manner. Since the conclusion is based entirely on the premise that the micelles were fixed at high temperature, and since the effect of high heat treatment on milk is such an important issue, I feel that the premise must be substantiated. Two simple experiments would provide this proof:

To test the effect of heat and pressure on glutaraldehyde functionally, milk (at room temperature) could be fixed with glutaraldehyde which has not been heated and, separately, with glutaraldehyde which has been heated in the sealed tube.
to 200°C and then cooled. Then, the resulting structures could be compared.

To test whether the fixation did indeed occur after mixing, a simple biochemical experiment could be carried out. Milk could be (1) not mixed with glutaraldehyde, (2) mixed with glutaraldehyde at 200°C as described in the manuscript, and (3) heated to 200°C in the special vial containing glutaraldehyde in the other arm but not mixed with the fixative after cooling. The samples could then be analyzed by gel electrophoresis or by HPLC. Either technique would indicate whether the caseins in the milk from Treatment 3 were crosslinked by glutaraldehyde as those in Treatment 2 would be, or were not crosslinked, as those in Treatment 1 would be.

Secondly, all the experiments were done with milk. The whey proteins in the milk can, and do, attach to casein micelles as a result of heating. This could contribute to an increase in micellar dimensions. Additionally, both heat and glutaraldehyde fixation cause aggregation of whey proteins. Is it possible that these aggregates, and not submicelles, are what we see on the surface of the micelles in Figs. 4 and 6? If washed micelles were heated and fixed as the milk was and the same structures were observed, then the effect of whey proteins could be ruled out.

Authors: Whey protein aggregates as well as casein submicelles may be seen in Figs. 4 and 6.

We agree that the suggestions for additional experiments outlined above are excellent and should be followed. However, we cannot extend the study at this time and will return to this subject at a later date.
COMPOSITION AND SOME PROPERTIES OF SPRAY-DRIED RETENTATES OBTAINED BY THE ULTRAFILTRATION OF MILK

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Abstract

Retentates containing 20, 27, and 34% total solids, obtained on commercial scale by the ultrafiltration of milk, were spray-dried on laboratory scale using centrifugal atomization and single-stage drying with the inlet air temperature of 220°C and the outlet air temperature of 90°C.

The protein content in the powders was 31% to 35% compared to 24.8% protein in the control whole-milk powder. Lactose contents were markedly lower in the retentate powders (~10.6%) than in the milk powder (40.4%). Storage of the powders at 37°C resulted in a marked increase in the 5-hydroxymethylfural contents with doubling of this content in the retentate powders and tripling in the spray-dried retentate powder particles.

When viewed by scanning electron microscopy, the spray-dried retentate powder particles had smooth surfaces free from wrinkles usually seen in spray-dried milk powders. When the same products were exposed to atmospheres having 75%, 85%, or 100% relative humidity, the retentate powders exhibited less lactose recrystallization than the milk powder.

The melting temperature (T_m) (as determined by differential scanning calorimetry) of lactose present in the retentate powders was not affected by the reduced lactose content in the powders but the fusion enthalpy (ΔH_fusion) of lactose was reduced in the retentate powders compared to the control milk powder.

Introduction

To facilitate the spray-drying of milk, a great part of water present in the milk is removed by evaporation whereby the total solids content of the milk is increased to 35-50% [5]. All the components of the milk, i.e., casein, whey proteins, lactose, minerals, as well as fat in the case of whole milk, are retained in the evaporated milk. Consequently, less water has to be removed in the spray-drier from the milk thus concentrated than from milk that has not been preconcentrated. Retentates obtained by the ultrafiltration of milk represent another product which may easily be spray-dried. As the result of the ultrafiltration of milk, high-molecular substances such as proteins are retained in the retentate on one side of the ultrafiltration membrane used, and low-molecular substances such as lactose and mineral constituents pass into the filtrate, which is called permeate. In the retentates, the concentrations of the low-molecular substances are reduced and the relative concentration of the proteins is increased. The altered chemical composition may affect a number of functional properties of the spray-dried retentates compared to milk powders. For example, in regular milk powders, the presence of anhydrous lactose in a glassy form leads to their hygroscopicity [7, 22] which is particularly noticeable in spray-dried whey powders having a high lactose content [24]. Anhydrous lactose in spray-dried milk powders rapidly absorbs water when the powders are exposed to humid atmosphere and may crystallize in the a-monohydrate form. The development of lactose crystals on the surface of milk powder particles during storage indicates that the moisture content had exceeded the safe level. Associated with exposure to excessively high humidity is the development of lumpiness and caking which reduce the quality of the milk powders [26] during storage under such conditions. To better understand these phenomena, crystallization of lactose in milk powders has been studied by many authors [3, 13, 16, 18, 20, 22-26]. As the use of ultrafiltration in modern dairy technology is extended, it is important to find proper conditions for storage and transportation of the ultrafiltration retentate which would be economical and yet would ensure good quality of the product.

The two objectives of this study were (a) to characterize the composition and structure of
spray-dried ultrafiltration milk retentates as a consequence of different degrees of concentration prior to drying, and (b) to evaluate the effect of differences in composition of the retentates on their functional behaviour under elevated relative humidity storage conditions, as compared to ordinary milk powder spray-dried under the same conditions as the retentates. Scanning electron microscopy (SEM) was used to calibrate the analyser for temperature and enthalpy calculations.

Scanning electron microscopy (SEM)
Each spray-dried powder was spread in a thin layer on the sticky surface of a dry-moist film disc attached to an SEM aluminum stub using a silver-based cement (Ladd Industries, Burlington, Vermont, USA). Additional spreading was done with a fine sable brush. The powders were sputter-coated in a Hummer II Technics sputter coater to form a gold layer approximately 20 nm thick and were examined in an ISI OS-130 scanning electron microscope operated at 20 kV. Micrographs were taken on 125 ASA 35-mm film [2, 11].

The effect of humid atmosphere on the crystallization of lactose in the powders was studied by exposing the powders (mounted on the sticky film discs or spread on microscope glass slides in layers less than 0.5 mm thick) to 75%, 85%, and 100% relative humidity in glass dishes (55 mm in diameter and 35 mm high) having ground lids. The experiments were carried out at 25°C and 40°C for periods ranging from 2 to 72 h [24]. Atmosphere with 75% relative humidity developed in the presence of a saturated solution [17, 24] of NaCl, atmosphere with 85% humidity was provided by a saturated KCl solution, and 100% relative humidity was provided by saturation of the atmosphere over distilled water.

Results and Discussion

Chemical composition

Chemical composition of the retentates prior to spray-drying is listed in Table 1. The concentrations of the individual constituents increased in proportion to the intended differences in the total solids contents except total proteins and lipids.

Table 1. COMPOSITION OF RETENTATES OBTAINED BY THE ULTRAFILTRATION OF MILK

<table>
<thead>
<tr>
<th>Component</th>
<th>Approximate solids contents (%) in the retentates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34</td>
</tr>
<tr>
<td>Total solids</td>
<td>34.2</td>
</tr>
<tr>
<td>Mineral matter</td>
<td>1.26</td>
</tr>
<tr>
<td>Lipids</td>
<td>15.00</td>
</tr>
<tr>
<td>Lactose</td>
<td>3.65</td>
</tr>
<tr>
<td>Total proteins</td>
<td>21.21</td>
</tr>
<tr>
<td>Non-casein nitrogen</td>
<td>0.159</td>
</tr>
<tr>
<td>Non-protein nitrogen</td>
<td>0.014</td>
</tr>
<tr>
<td>Proteose-peptone</td>
<td>0.070</td>
</tr>
<tr>
<td>Casein</td>
<td>10.3</td>
</tr>
<tr>
<td>Whey proteins</td>
<td>0.96</td>
</tr>
<tr>
<td>True proteins</td>
<td>11.1</td>
</tr>
<tr>
<td>5-HMF* (μmol/L)</td>
<td>11.5</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
</tr>
</tbody>
</table>

* 5-Hydroxymethylfural
5-HMF in the 34% total solids retentate. Data on the composition, pH, and solubility of the spray-dried retentates compared to those of spray-dried milk are presented in Table 2. There were no major differences in most micro and macro constituents among the spray-dried retentates. However, the initial total solid contents of the retentates before spray-drying and the resulting differences in the thermal treatment during spray-drying led to some variations in the physico-chemical characteristics of the powders. For example, there was a trend toward a higher protein content in the powders made from the retentates which were concentrated to higher total solids contents. The powders, made from the 20%, 27%, and 34% total solids retentates and subsequently stored at 4°C, contained 32.3%, 34.7%, and 36.5% total proteins per 100% total solids, respectively.

Table 2 also shows the composition of the control milk powder. As a result of ultrafiltration, ash (mineral matter) was lower in the dried retentates (3.42% in 100% total solids) than in the milk powder (5.55% in 100% total solids). The effect of ultrafiltration was also clearly evident from indices related to the lipid content (45.7% fat in the retentates compared to 27.5% fat in milk per 100% total solids basis) as well as the protein content (34.3% protein in the retentates compared with 25.5% protein in milk). However, whey proteins were approximately the same in the retentates (1.34%) as in the dried milk (1.35%) based on the same 100% total solids level.

Lactose concentrations were sufficiently high in all retentates to initiate the Maillard reaction with proteins. The concentration of 5-HMF was used to monitor the rate of the early stages of this reaction before the brown pigments started to develop. The lowest 5-HMF concentrations (10.2-16.7 μmol/L) were found in fresh retentates (Table 1). There was little difference in spray-dried milk stored at 4°C (14.5 μmol/L) but an almost threefold increase (40.7 μmol/L) was found in milk powder stored at 37°C for 8 months. In spray-dried retentates, the 5-HMF concentrations were considerably higher than in spray-dried milk (Table 2). They varied between 59.6 and 70.3 μmol/L in spray-dried retentates stored at 4°C and were increased approximately twice to 117.9-125.6 μmol/L in retentate powders stored at 37°C.

Solubilities of the retentate powders stored at 4°C fluctuated between 90.9% and 94.2% but were decreased to 78.6-80.1% in powders stored at 37°C indicating an adverse effect of an elevated storage temperature.

Structure of the retentate powders

Macroscopically, all retentate powders had a moist-looking appearance. The small particle aggregates, however, separated easily on contact. Under a scanning electron microscope, the particles of fresh spray-dried retentates used in this study had relatively smooth surfaces irrespective of the extent of concentration by ultrafiltration prior to spray-drying. There were only a few major topographic features of note. Shallow "dimples" (Figs. 1, 11, and 12) were seen on most retentate particles. Simple "venation" (Fig. 1) was also frequently present. Smaller globules, either individual or in clusters, were occasionally seen to be fused to larger particles. Only a small part of the globular retentate particles had deeply wrinkled surfaces. In this respect, the retentate particles differed from skim milk and whole milk powders (Fig. 2) [4, 6, 22, 25, 26] which were characterized by both deep and shallow wrinkles on most particles. The retentate particles also differed from spray-dried buttermilk of commercial origin, the particles of which featured characteristic crater-like rims surrounding small globules attached to the larger particles (Fig. 3) [10], and from spray-dried whey powders which had deep narrow wrinkles [24]. The differences in topography of the various particle surfaces and their
Fig. 1. Fresh spray-dried milk retentate powder particles have smooth surfaces with shallow dimples (small arrows) and occasional simple "venation" (large arrow).

Fig. 2. Fresh spray-dried whole-milk powder particles have wrinkled (arrows) surfaces.

Fig. 3. Commercial spray-dried buttermilk particles have crater-like (large arrow) surface topography with smaller globules attached and surrounded with rims (small arrows).

Figs. 4 to 6. Whole-milk powder exposed for 24 h to an atmosphere having 75% (Fig. 4), 85% (Fig. 5), and 100% (Fig. 6) relative humidity at 25°C. Arrows in Fig. 4 point to needle-like crystals.
Spray-dried Ultrafiltration Milk Retentates

Figs. 7 to 9. Whole-milk powder exposed for 3 d to an atmosphere having 75% (Fig. 7), 85% (Fig. 8), and 100% (Fig. 9) relative humidity at 40°C.

Spray-dried buttermilk exposed for 2 h to association with the technology of spray drying were discussed earlier by Caric and Kalab [6]. Particle surfaces free of crystals were seen under a scanning electron microscope in all freshly spray-dried milk and retentate powders. Lactose present in the powder particles was apparently in the amorphous (glassy) form which resulted from the rapid dehydration of milk and retentate droplets during spray drying [26].

Exposure of milk powders to a humid atmosphere led to the absorption of water by the amorphous α- and β-lactose forms and their conversion into crystalline α-monohydrate. The crystallization rate increased with the increases in lactose concentration in the powder and the relative humidity of the atmosphere. Saltmarch and Labuza [24] studied lactose crystallization in hygroscopic spray-dried sweet whey which contained 66% lactose. Lactose crystallization was already noticeable after a week at 53% relative humidity at 25°C. Milk powders which were used as a control in our studies, started developing α-monohydrate lactose crystals after exposure to 75% humidity at 25°C for 24 h (Fig. 4). The development was slightly more advanced at 85% humidity (Fig. 5). At 100% relative humidity, however, the milk powder particles were almost completely covered with lactose crystals (Fig. 6). By extending the exposure of the milk powder to 75%, 85%, and 100% humidity to 3 days, the incidence of the lactose α-monohydrate crystals on the particle surface was increased and was even higher in powders exposed to the humid atmospheres at 40°C for 3 days (Figs. 7 to 9). Commercial spray-dried buttermilk made from sweet cream [10] was more susceptible to lactose crystallization than the milk powders and the buttermilk particles were almost completely covered with α-monohydrate crystals following an exposure to 100% humidity at 25°C for only 2 h (Fig. 10).

In contrast to the milk and buttermilk powders, the retentate powders exhibited less lactose crystallization when exposed to humid atmosphere. In powders obtained from retentates which
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contained 34% total solids prior to spray-drying, no lactose crystals developed following exposure of the powders to 75% and 85% humidities at 25°C or even at 40°C for 3 days (Figs. 11 and 12). In retentate powders exposed to 100% humidity for 3 days, plate crystals developed on particle surfaces (Fig. 13). At 40°C, the double adhesive tape, to which the powder particles were attached during their exposure to humidity, softened considerably and the smaller powder particles sank into the sticky layer.

Compared to powder particles which remained separated from each other during their exposure to humidity because they were mounted on an adhesive tape, powders in the form of compact layers spread on microscope glass slides which had been exposed to the humid atmosphere, fused into aggregates in a process similar to the manufacture of instant milk powders or the process which takes place during lumping and caking of milk powders stored in an atmosphere having excessively high humidity. Differential scanning calorimetry

In spray-dried milk powders, lactose is present in both microcrystalline (α- and β-forms) and amorphous states. The amorphous state develops when lactose dissolved in milk is dried rapidly [15]. Amorphous (glassy) lactose is slowly transformed into crystalline lactose on exposure to moisture. Characterization and determination of crystalline lactose in milk powders by differential scanning calorimetry (DSC) was suggested by several authors [9, 16, 19, 20].

When subjected to DSC, all milk and retentate powders under study produced thermograms, each showing one major endothermic peak. The melting temperatures (Tm) ranged from 194°C to 208°C. In addition, two minor endothermic peaks occurred at approximately 150°C and 175°C (Figs. 14-16). The Tm values observed markedly differed from the melting temperatures of anhydrous lactose published in the literature [15, 19]. The melting temperature Tm of the anhydrous α-form was reported to be -215°C and the melting temperature Tm of the β-form was reported to be -235°C [15, 19]. Since crystallization water in the lactose hydrate evaporates at temperatures below 150°C [15, 19], the melting points observed are those of the anhydrous forms [19]. The Tm values obtained in this study are lower than those listed above and correspond more closely to the Tm values of the complexes of α- and β-lactose reported in the literature [15]. Although α- and β-lactose differ in their Tm values and the difference has been used to determine the levels of these lactose forms in whey powders [20], two separate melting points, one for the α- and the other for the β-form were not observed with the retentate powders. It is not possible, therefore, to estimate the α/β ratio from the Tm data.

The fusion enthalpy (ΔHfusion) of lactose present in the milk and retentate powders was
Spray-dried Ultrafiltration Milk Retentates

**Table 3.**

LACTOSE FUSION CHARACTERISTICS ($\Delta H_{\text{fusion}}$) IN MILK AND ULTRAFILTRATION RETENTATE POWDERS

<table>
<thead>
<tr>
<th>Powder</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H_{\text{fusion}}$ (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>206.6 ± 0.5</td>
<td>125.0 ± 10.8</td>
</tr>
<tr>
<td>Retentate (20% TS*)</td>
<td>208.1 ± 5.6</td>
<td>73.5 ± 14.2</td>
</tr>
<tr>
<td>Retentate (27% TS*)</td>
<td>204.7 ± 2.0</td>
<td>61.2 ± 8.9</td>
</tr>
<tr>
<td>Retentate (34% TS*)</td>
<td>194.4 ± 2.9</td>
<td>49.5 ± 11.3</td>
</tr>
</tbody>
</table>

* Total solid levels before spray-drying.

related to the total solids content of the retentates prior to spray-drying (Table 3). However, as these total solids contents affected the concentration of lactose in the retentates, it was concluded that the $\Delta H_{\text{fusion}}$ values reflected the lactose concentration.

Exposure of the milk and retentate powders for 3 days at 40°C to atmosphere having 100% relative humidity did not affect the fusion thermograms (Fig. 17), except that the $T_m$ values were slightly lower than with the freshly spray-dried powders.

**Conclusions**

Higher concentration rates used during the ultrafiltration of milk resulted in increased protein, fat, and ash contents in the retentates and lower lactose contents. These shifts in chemical composition of the retentates were reflected by the composition of the resulting spray-dried powders. The lower lactose content in the retentate powders caused the powders to exhibit less crystallization and recrystallization of lactose on exposure to humid atmosphere. The low lactose content was also apparent when the retentate powders were subjected to differential scanning calorimetry: the fusion enthalpy values ($\Delta H_{\text{fusion}}$) expressed in J/g were smaller with retentate powders than with whole-milk powders. Storage of the powders at an elevated temperature of 37°C intensified the Maillard reaction (noticeable from the 5-HMF values) in the retentate powders and had a deleterious effect on their solubility as compared to storage at 4°C.
Acknowledgments

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References


Discussion with Reviewers

N. Singer: The evidence of an apparent increase in Maillard reaction somehow induced by the ultrafiltration process needs to be emphasized.

M. Saltmarsh: Could the 5-hydroxymethylfural (5-HMF) levels noted for the 34% retentate correspond to the level on non-protein nitrogen (NPN) in the retentate, i.e., lower NPN, lower rate of 5-HMF development?

Authors: We doubt that the 5-HMF levels in the retentates would be affected by the NPN concentrations. Casein participates most actively in Maillard reactions. β-Lactoglobulin also reacts to some extent [14]. Primary reactive groups in proteins are non-ionized amino groups of branched diamino acids or terminal α-amino groups of proteins. About 30% of all free amino groups in milk proteins are e-amino groups of lysine.

No significant differences were found in the 5-HMF levels in various retentates. However, the 5-HMF contents in retentate powders stored at 4°C and 37°C were approximately 4 and 3 times, respectively, as high in milk powders stored at the two corresponding temperatures.

M. Saltmarsh: What could have contributed to the lesser degree of lactose recrystallization in the retentates than in the whole milk powder?
Spray-dried Ultrafiltration Milk Retentates

Authors: A markedly lower lactose content in the retentate powders is the most probable reason.

P. Jelen: What does the term "moist-looking appearance" of the retentate powders mean? The powders were presumably dry.
Authors: Yes, they were dry, yet they looked and behaved as if they were moist, i.e., the particles stuck together when taken out of the pouch and handled.

P. Jelen: The comment on the double adhesive tape seems odd – why bother mentioning it?
Authors: A double adhesive tape is convenient in order to mount powders for SEM examination. However, the powders are seldom exposed to a high temperature (40°C) for several days under high relative humidity (100%) conditions prior to SEM. Our observation may be important to someone who intends to study particles smaller than spray-dried retentates at high temperature and high relative humidity. For that purpose, it would be advisable to replace the tape with another material.

N. Singer: Fig. 3 raises the following questions: How do rims form around minute globular particles which are seen attached to larger particles of spray-dried buttermilk? Could some light be shed on this structure by performing cross sections? Would we see a "root" or a shallow cup-like interface?
Authors: In the spray-drier, minute buttermilk droplets dry and solidify more rapidly than larger particles. It is probable that the minute solid droplets collide with the still-liquid but already highly viscous larger particles. As a result of the impact, the minute solidified droplets form craters, in which they become embedded. The mass pushed aside by these colliding solid droplets surrounds them in the form of the rims seen. If this is true, we would see shallow cup-like interfaces such as the one marked with an arrow in Fig. 3, that was left on the surface of the larger particle when a smaller droplet broke off, rather than "roots". It is interesting to note, however, that the rims were observed only with spray-dried buttermilk but not with other milk powders.

Shallow dimples were seen in spray-dried milk retentates. It is probable that in this product, small solidified droplets collided in the spray-drier with the larger particles at a stage when the larger particles were no longer liquid but were still soft. The small solid droplets left their imprints ("dimples") on the surface but, unlike in spray-dried buttermilk, did not become attached to it.

P. Jelen: If there was any recrystallization at the 3-day storage, why were the Tm values the same?
Authors: The Tm values have been tabulated by Morrissey for pure lactose and may not exactly apply to spray-dried milk and retentate powders, where proteins and other constituents are present at high concentrations.
FLUORESCENCE MICROSCOPY STUDIES ON (1,3)-\(\beta\)-D-GLUCAN IN BARLEY ENDOSPERM

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Abstract

Development of (1,3;1,4)-\(\beta\)-D-glucan and (1,3)-\(\beta\)-D-glucan in kernels of Himalaya and Bonanza barley has been followed by fluorescence microscopy using calcofluor and aniline blue fluorochromes. Specific enzymes were used to confirm the identity of these two polysaccharides in sections of endosperm tissue. All barley lines tested contained both types of \(\beta\)-glucan but (1,3;1,4)-\(\beta\)-glucan was synthesized at an earlier stage of development than was (1,3)-\(\beta\)-glucan. Small bead-like deposits of (1,3)-\(\beta\)-glucan were detected in all cultivars examined. These deposits were present throughout the endosperm and appeared to be associated with the inner walls of endosperm cells. After treatment of sections with (1,3)-\(\beta\)-glucanase, these deposits could not be detected with aniline blue. Himalaya barley contained, in addition to these bead-like deposits, larger deposits of (1,3)-\(\beta\)-glucan that appeared to be associated with the inner surface of the cell walls of the outermost cells of the starchy endosperm. The deposits were concentrated at the aleurone-endosperm junction and those present in immature kernels were susceptible to hydrolysis by (1,3)-\(\beta\)-glucanase. Enzymic analysis indicated that, in mature Himalaya kernels, the large deposits also contained (1,3;1,4)-\(\beta\)-glucan and other material of, as yet, unknown identity.

Introduction

The major component of barley endosperm cell walls is a (1,3;1,4)-\(\beta\)-D-glucan (Fincher, 1975; Ballance and Manners, 1978) containing approximately 70% (1,4)-\(\beta\)-glucosyl linkages and 30% (1,3)-\(\beta\)-glucosyl linkages. This polysaccharide is composed mainly of cellotriosyl and cellotetraosyl units linked by single (1,3)-\(\beta\)-glucosyl linkages (Parrish et al., 1983). A small number of blocks containing up to ten glucose residues linked predominantly by (1,4)-\(\beta\)-glucosyl bonds may be present also in the \(\beta\)-glucan (Woodward et al., 1983). The presence of contiguous (1,3)-\(\beta\)-glucosyl linkages has been reported in some \(\beta\)-glucan preparations (Bathgate et al., 1974; Fleming and Kawakami, 1977) but other workers have not found such linkages in \(\beta\)-glucan extracted from barley at 40°C (Woodward et al., 1983). It is possible that \(\beta\)-glucan remaining in the cell wall after a 40°C aqueous extraction and requiring even more severe conditions for extraction may contain some contiguous (1,3)-\(\beta\)-glucosyl linkages. The existence of such linkages in mixed linkage \(\beta\)-glucan from barley is still, therefore, a matter for debate.

The mixed linkage \(\beta\)-glucan has been implicated in a number of problems that can arise during barley processing and utilization such as poor endosperm modification during malting (Aastrup, 1983), filtration problems during mashing (Luchsinger, 1987), unsatisfactory beer stability during storage (Gjertsen, 1966) and digestive problems in chickens on barley diets (Burnett, 1966). Because of the commercial impact of these problems, a large body of information now exists in the scientific literature (Fincher and Stone, 1986) on the occurrence and properties of the mixed linkage \(\beta\)-glucan present in barley endosperm cell walls.

The presence of another type of \(\beta\)-glucan containing predominantly (1,3)-\(\beta\)-glucosyl linkages (Fulcher et al., 1977) has been reported in cereal grains. This \(\beta\)-glucan, commonly called (1,3)-\(\beta\)-D-glucan, has been found associated with the aleurone cell walls of wheat (Bacic and Stone, 1981a,b) and barley (Taiz and Jones, 1970; Bacic and Stone, 1981a,b) and the endosperm cell walls of wheat (Bacic and Stone,
1980) and barley (Fulcher et al., 1977). Unlike the mixed linkage β-glucan, (1,3)-β-glucan does not appear to be distributed uniformly throughout the barley endosperm. It has been detected as discrete deposits at the aleurome-endosperm junction in Himalaya (Fulcher et al., 1977), Clipper (Bacic and Stone, 1981a) and Sonja (Boissonnet and Scriban, 1982) barley cultivars, as an almost continuous layer at the aleurome-endosperm junction of Vanier barley (Fulcher et al., 1977) and as irregular, very small deposits on the interior surface of endosperm cell walls of Betzes (Fulcher and Wong, 1980), Clipper (Bacic and Stone, 1981a) and Himalaya (Fulcher et al., 1977) barley cultivars. Results from these studies suggest that the amount of this β-glucan present in barley endosperm and the type of deposit it forms may vary from one cultivar to another.

The (1,3)-β-glucan appears to represent only a small proportion of the total material in barley endosperm (Tiunova et al., 1988). It is not surprising, therefore, that the physical and possible functional properties of this polysaccharide have not been investigated. Because the largest deposits of (1,3)-β-glucan appear to be concentrated around the exterior of the barley endosperm, the polysaccharide, despite being present in relatively small amounts, could play an important role during germination by limiting the movement of hydrolytic enzymes from the aleurome to the endosperm. Also, there is on-going interest from the malting, brewing and poultry-feeding industries on the negative effects of β-glucan on the end-use quality of barley intended for these industries. Recent research on the effect of dietary fibre on blood cholesterol levels has pointed out the possible beneficial effects of cereal fibre, including β-glucan, in the human diet (Newman et al., 1977; Klopfenstein, 1988). It seemed worthwhile, therefore, to re-investigate the occurrence and location of (1,3)-β-glucan in a number of different barley cultivars and to follow formation of this β-glucan during kernel growth and maturation.

Materials and Methods

Barley Samples

The following samples of mature barley kernels were used in this study: Himalaya (6-rowed) grown at Pullman, Washington (1979, 1985, 1986), and the Glenlea Research Station, Manitoba (1985); Bonanza, a 6-rowed barley (Glenlea Research Station, 1985, 1987); Argyle (5-rowed) and Ellice, a 2-rowed barley (Glenlea Research Station, 1987); Harrington, a 2-rowed barley (Lethbridge, Alberta, 1982); Klages, a 2-rowed barley (Vulcan Research Station, Alberta, 1982); Sonja, a 2-rowed barley (grown in France, 1982); Compana (2-rowed), Titan (5-rowed), Betzes (2-rowed) and their isogenic hull-less lines (Sidney, Montana, 1979); hull-less cultivars Tupper (5-rowed), Scout (2-rowed) and Yukon (2-rowed). All samples were from the interior surface of the endosperm. Kernels were cut into sections, as well as the remaining untreated sections, were treated with 100 μL of phosphate buffer (0.2 M, pH 6.5). One section was washed briefly in distilled water. Two of these sections, as well as the remaining untreated section, were treated with 100 μL of (1,3;1,4)-β-glucanase from Bacillus subtilis in phosphate buffer (0.2 M, pH 6.5) for 4 h at 20°C. Sections were drained and washed with distilled water. Two of these sections, as well as the remaining untreated section, were treated with 100 μL of (0.2 units of Endo-α-(1,3;1,4)-β-glucanase was purified from Bacillus subtilis as described previously (Ballance, 1985). One unit of enzyme activity is defined as the amount of enzyme that releases one micromole of reducing sugar (as glucose equivalents) per min from 0.6% lichenin in 0.2 M maleate buffer at pH 6.5 and 37°C.

Enzyme Treatment

Slides containing five sections were placed on wet filter paper in a petri dish. One section was used as a control and was treated with 100 μL of phosphate buffer (0.2 M, pH 6.5). Three other sections were treated with 100 μL (one unit of activity) of (1,3;1,4)-β-glucanase from Bacillus subtilis in phosphate buffer (0.2 M, pH 6.5) for 4 h at 20°C. Sections were drained and washed with distilled water. Two of these sections, as well as the remaining untreated section, were treated with 100 μL of (1,3;1,4)-β-glucanase from Bacillus subtilis in phosphate buffer (0.2 M, pH 6.5) for 4 h at 20°C. Sections were drained and washed with distilled water. Two of these sections, as well as the remaining untreated section, were treated with 100 μL of (0.2 units of (1,3;1,4)-β-glucanase from Bacillus subtilis in phosphate buffer (0.2 M, pH 6.5) for 4 h at 20°C. Sections were drained and washed with distilled water. Two of these sections, as well as the remaining untreated section, were treated with 100 μL of (1,3;1,4)-β-glucanase from Bacillus subtilis in phosphate buffer (0.2 M, pH 6.5) for 4 h at 20°C. Sections were drained and washed with distilled water.
activity) of (1,3)-β-D-glucanase in acetate buffer (0.2 M, pH 5.0) for 6 h at 20°C. Stock xylanase solution was diluted 500x with 0.2 M sodium acetate buffer at pH 5.5, was prepared. Sections were treated with 100 µL of diluted enzyme for 5 h.

A pectinase solution containing 8 mg of pectinase in 10 mL of 0.2 M acetate buffer, pH 5.5, was prepared. Sections were treated with 100 µL portions of this solution for periods of up to 16 h. After enzyme incubation, the slides were drained and washed with distilled water. They were then stained with aniline blue and a duplicate set was stained with calcofluor. Several replicates were prepared for each barley sample examined.

Microscopy

Stained sections were examined with a Wild Leitz Orthoplan fluorescence microscope using Filter Block A (exciting filter BP 340-380 nm, barrier filter LP 430 nm) for both stains. Illumination was provided by a Super Pressure Mercury Lamp HBO 50W. Micrographs were taken on Kodakcolour VR 400 film.

Results and Discussion

Criteria for Identifying (1,3)-β-D-Glucan Deposits

A sample of (1,3)-β-glucan has not yet been isolated from barley endosperm and characterized. Evidence for the presence of this polysaccharide in the endosperm has been based on fluorescence microscopy studies using aniline blue as a fluorochrome (Fulcher and Wong, 1980; Fulcher and Wood, 1983). There has been some controversy over the specificity of this stain but recent work (Evans et al., 1984; Wood and Fulcher, 1983, 1984) has shown that aniline blue does contain a minor component that has a high specificity for polysaccharides containing contiguous (1,3)-β-glucosyl linkages. This minor component of the fluorochrome has been isolated, characterized and shown to react with (1,3)-β-glucans (Evans and Hoyne, 1982; Evans et al., 1984). Complementary studies have shown that the ability of deposits in sections of barley and wheat endosperms to react with aniline blue can be removed by treating the sections with exo-(1,3)-β-glucanase (Fulcher et al., 1977; Bacic and Stone, 1980). Therefore, there is reasonable evidence that aniline blue, used under appropriate conditions, is a specific fluorochrome for this polysaccharide and so it was used in the current study. An endo-enzyme with a specificity for (1,3)-β-glucans is produced naturally in barley kernels (Ballance and Meredith, 1974), presumably to hydrolyze the (1,3)-β-glucan. This endo-(1,3)-β-glucanase has been purified (Ballance and Svendsen, 1988) and was utilized in this study to confirm that deposits that stained with aniline blue were susceptible to degradation by (1,3)-β-glucanase. The fluorochrome Sirofluor, purified from aniline blue (Evans and Hoyne, 1982), was used in some experiments in this study. Because it gave similar results to those obtained with aniline blue only micrographs obtained with aniline blue have been used.

β-Glucan Deposition During Seed Development

No information is available on the formation of (1,3)-β-glucan during the development of barley kernels. A growth study was initiated, therefore, to follow the synthesis of this material in two barley cultivars, Himalaya and Bonanza, during kernel development and maturation. Because of certain differences observed, these cultivars will be discussed separately. For comparative purposes, formation of (1,3;1,4)-β-glucan in developing kernels was studied also using the fluorochrome, calcofluor.

No cell walls, as depicted by calcofluor staining, were detected in endosperms of Himalaya at 4 days after anthesis but a continuous network of thin cell walls was apparent throughout the endosperm at 7 days after anthesis (Fig. 1). This network extended to the testa because, at this stage, aleurone cells could not be distinguished from those of the starchy endosperm. The apparent empty space between the endosperm and testa was produced during preparation of the section. No aniline blue staining material was detected in the endosperm at this stage. Over the next few days, the peripheral cells of the starchy endosperm differentiated to form the aleurone tissue, which usually contains 2-4 layers of cells in barley (Fig. 2).

Small amounts of (1,3)-β-glucan deposits were detected first at 12 days after anthesis and were readily apparent at 14 days (Fig. 3). These deposits were associated with endosperm cells adjacent to the aleurone. Small bead-like deposits of aniline blue staining material were also detected in the endosperm at this stage. Treatment of 14-day sections with (1,3)-β-glucanase resulted in complete removal of all aniline blue staining material but the calcofluor stained material was not affected. The aleurone cell walls at this stage had not thickened appreciably and were only weakly autofluorescent. Calcofluor-stained sections (not shown), however, indicated thickening of the endosperm cell walls. This endosperm cell wall thickening only reached a maximum near seed maturity, confirming the results of Coles (1979).

At 20 days after anthesis, the (1,3)-β-glucan deposits associated with the periphery of the endosperm had increased in size and number (Fig. 4). After treatment with (1,3)-β-glucanase, these deposits no longer stained with aniline blue (Fig. 5). The aleurone cell walls had thickened and appeared to be losing their ability to stain with calcofluor. The reason for this is not clear but it may be due to the mixed linkage β-glucan complexing with and being diluted by other cell wall components, such as arabinoxylans, that must be laid down rapidly at this stage because they become the major components of aleurone cell walls of mature kernels (Bacic and Stone, 1981b).

No significant increase was detected in the apparent amount of (1,3)-β-glucan present at 24 days after anthesis and deposits of this material...
Figs. 1-7. Sections of Himalaya barley endosperm. Fig. 1: 7 days after anthesis, calcofluor stain. Fig. 2: 10 days after anthesis, calcofluor stain. Fig. 3: 14 days after anthesis, aniline blue (AB) stain. Fig. 4: 20 days after anthesis, AB stain. Fig. 5: 20 days after anthesis, treated with (1,3)-β-glucanase, AB stain. Figs. 6 and 7: Mature kernel, AB stain. T = testa, Al = aleurone, ◄ = large (1,3)-β-glucan deposits, ➔ = bead-like (1,3)-β-glucan deposits. Bar = 25 μm.
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Figs. 8-13. Sections of mature Himalaya barley endosperm. Figs. 8 and 9: Treated with (1,3)-β-glucanase, aniline blue (AB) stain. Fig. 10: Calcofluor stain. Fig. 11: Treated with (1,3,1,4)-β-glucanase, calcofluor stain. Fig. 12: Treated with (1,3;1,4)-β-glucanase, AB stain. Fig. 13: Treated with (1,3;1,4)-β-glucanase, followed by (1,3)-β-glucanase, AB stain. Al = aleurone, ▲ = large (1,3)-β-glucan deposits, ▲ = bead-like (1,3)-β-glucan deposits. Bar = 25 μm.
Figs. 14-19. Sections of Bonanza barley endosperm. Fig. 14: 11 days after anthesis, aniline blue (AB) stain. Figs. 15 and 16: Mature kernel, AB stain. Fig. 17: Mature kernel treated with (1,3)-β-glucanase, AB stain. Fig. 18: Section of mature Harrington barley, AB stain. Fig. 19: Section of mature Tupper barley, AB stain. Al = aleurone, \( \Rightarrow \) = bead-like (1,3)-β-glucan deposits. Bar = 25 \( \mu \text{m} \).
throughout the endosperm were still susceptible to (1,3)-β-glucanase.

The physical appearance and frequency of (1,3)-β-glucan deposits in mature Himalaya kernels were similar to those described in a number of publications (Fulcher and Wong, 1980; Fulcher and Wood, 1983). Unlike (1,3;1,4)-β-glucan, which is an integral part of barley endosperm cell walls and so is distributed more or less uniformly throughout the endosperm, (1,3)-β-glucan appears to exist as discrete deposits of varying size and frequency in the endosperm. The largest deposits were found at the aleurone-endosperm junction and appeared to be associated with the inner surface of the interior cell walls of the endosperm (Fig. 6). These deposits formed a discontinuous line of material that almost encircled the endosperm of some kernels examined. In addition, much smaller deposits, similar to strings of beads, were present throughout the endosperm (Fig. 7). These, too, were associated with the inner walls of endosperm cells and were similar to those described by Fulcher et al. (1984).

Although these bead-like deposits varied in frequency, they were more frequent in the subaleurone region of the cheek area of the endosperm. Similar results were found with a number of Himalaya samples that had been grown under different environmental conditions (see Materials and Methods). All samples were characterized by wide variation in the distribution of the (1,3)-β-glucan deposits. Treatment of sections of mature kernels with (1,3)-β-glucanase removed the ability of the bead-like (1,3)-β-glucan deposits to stain with aniline blue (Fig. 8) as has been described previously for barley (Fulcher et al., 1977) and other plants (Fulcher et al., 1976). However, the larger deposits at the endosperm periphery were not noticeably affected by the enzyme (Fig. 9) even after a 24 h treatment. Calcofluor staining clearly shows that these deposits are enclosed within the wall of the endosperm cell (Fig. 10) and that there is also visible intense blue stained sections (Fig. 9) and appears as a continuum of non-fluorescent wall surrounding the fluorescent deposits. When sections were treated with (1,3;1,4)-β-glucanase, all calcofluor staining material was removed (Fig. 11), in agreement with published work (Wood et al., 1983) but deposits of (1,3)-β-glucan were not affected (Fig. 12). Subsequent treatment of these sections with (1,3)-β-glucanase removed the ability of the deposits to stain with aniline blue (Fig. 13). It should be stressed that complete removal of these deposits was not achieved by the enzyme treatments; some material remained but it did not stain with aniline blue or with calcofluor. Treatment of sections with xylanase had no apparent effect on the deposits or on the endosperm cell walls. Aleurone cell walls, which are composed mainly of arabinoxylans (McNell et al., 1975), were hydrolyzed, however, and lost their ability to autofluoresce. Calcofluor treatment did not affect the (1,3)-β-glucan deposits either but (1,3;1,4)-β-glucan in the endosperm cell walls of treated sections was hydrolyzed and aleurone cell walls were partially hydrolyzed. This suggests that the pectinase sample contained both xylanase and cellulase or endo-(1,3;1,4)-β-glucanase activities.

Results from the enzymic studies indicate that the large aniline blue staining deposits found at the endosperm periphery close to the aleurone layer of Himalaya barley are a complex containing (1,3)-β-glucan, (1,3;1,4)-β-glucan and other material of, as yet, unknown identity.

Kernels of Bonanza barley, harvested at different stages of development, were analyzed also for the two types of β-glucan. Endosperm cell walls containing mixed linkage β-glucan were detected first at 4 days while (1,3)-β-glucan was not found in significant amounts until 11 days after anthesis (Fig. 14). Changes in the endosperm occurred faster in Bonanza than in Himalaya as described previously (MacGregor and Dushnicky, 1989). The characteristic, large deposits of (1,3)-β-glucan found at the periphery of Himalaya barley endosperms were not detected in Bonanza even in mature kernels (Fig. 15). Small, bead-like deposits similar to those detected in the interior of Himalaya endosperms were found also in Bonanza (Figs. 15 and 16), predominantly in the cheek region. These deposits increased in amount as the kernels developed and matured. Bonanza appeared to contain higher levels of these deposits than did Himalaya but a wide variation in levels was observed for both cultivars. Treatment with (1,3)-β-glucanase of sections of Bonanza from all developmental stages, including mature (Fig. 17), resulted in removal of all aniline blue stainable material. In both Himalaya and Bonanza barleys, therefore, (1,3)-β-glucanase was able to hydrolyze the small bead-like deposits of (1,3)-β-glucan found in the interior of endosperms. Himalaya thus differs from Bonanza in having large peripherally located deposits which, in the mature seed, are resistant to (1,3)-β-glucanase degradation.

The absence of large (1,3)-β-glucan deposits at the periphery of Bonanza endosperms was unexpected in the light of previous publications and suggested that the (1,3)-β-glucan content of barley was even more variable than had been suspected. This prompted a wider study on the presence of this material in barley cultivars.

Comparison of (1,3)-β-D-Glucan Deposits Among Barley Cultivars

A number of malting cultivars currently grown in Canada, including Argyle (6-rowed), Harrington (2-rowed), Ellice (2-rowed) and Klages (2-rowed), were examined. No large discrete (1,3)-β-glucan deposits were detected at the endosperm periphery of any of the cultivars but all cultivars contained the much smaller bead-like deposits described already in this paper (Fig. 18).

Himalaya is a hulless barley. To determine if the characteristic large deposits of (1,3)-β-glucan found at the periphery of Himalaya barley endosperms were associated with the hulless characteristic, three barley cultivars, Compana,
Titan and Betzes and their hulless isogenic counterparts as well as the hulless cultivars Tupper and Scout were examined. The large \( \beta \)-glucan deposits, so characteristic of Himalaya, were not detected in any of these cultivars. Small bead-like deposits, similar to those described for other barley cultivars studied, were visible throughout the endosperms of all cultivars (Fig. 19). No differences were detected between the pairs of isogenic cultivars in either the frequency or size of the \((1,3)\)-\( \beta \)-glucan deposits. Previous reports have described the presence of \((1,3)\)-\( \beta \)-glucan deposits at the endosperm periphery of hulled cultivars such as Vanier (Fulcher et al., 1977), Clipper (Bacic and Stone, 1981a) and Sonja (Boissonnet and Scriban, 1982). Currently available evidence suggests, therefore, that these deposits are not associated directly with the hulless characteristic of some barley cultivars. In the current study, only a few of the large deposits of \( (1,3)\)-\( \beta \)-glucan were detected at the endosperm periphery of a sample of Sonja barley.

A recent report (Galway and McCully, 1987) suggests that formation of \( (1,3)\)-\( \beta \)-glucan deposits can be induced in root tissues during preparation of sections for microscopy. It is possible that some of the small, bead-like deposits of \( (1,3)\)-\( \beta \)-glucan could be formed in this way and further work is required to investigate this possibility. However, it is unlikely that the large deposits, located at the aleurone-endosperm interface of Himalaya barley, are formed during section preparation.

Barley-based diets are utilized poorly and can cause digestive problems when fed to young chickens. These problems have been traced to the relatively high viscosity induced by barley \((1,3;1,4)\)-\( \beta \)-glucan as the feed passes through the digestive tract of the chicken (White et al., 1983). An important element in current barley breeding programs is to minimize such viscosity effects by reducing \( \beta \)-glucan levels in barley. In one such program it was noted that some barley lines with apparently similar total \( \beta \)-glucan content produced extracts with widely varying viscosity levels (Bhaty and Rossnagel, private communication). Variation in the proportions of \((1,3;1,4)\)- and \((1,3)\)-\( \beta \)-glucans would offer one explanation for these results. Again, however, no obvious differences were observed in the \((1,3)\)-\( \beta \)-glucan content of the breeders’ lines studied. No large, discrete deposits of aniline blue staining material were found at the endosperm periphery. Therefore, it is unlikely that \((1,3)\)-\( \beta \)-glucan had a significant effect on barley viscosity levels.

On the evidence of fluorescence microscopy, it would appear that \((1,3)\)-\( \beta \)-glucan represents only a small proportion of the total \( \beta \)-glucan in barley endosperm. However, the relative fluorescence intensities of \((1,3;1,4)\)-\( \beta \)-glucan/calcofluor and \((1,3)\)-\( \beta \)-glucan/aniline blue complexes have not yet been determined. It is possible that there is a significant difference between them so that fluorescence studies could give a misleading idea of the relative amounts of the two \( \beta \)-glucans present. Results of a recent quantitative study (Tunova et al., 1988) indicate that barley grain contains about 1% of \((1,3)\)-\( \beta \)-glucan. This compares with levels of 4-6% for the mixed linkage \( \beta \)-glucan (Anderson et al., 1978).

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Discussion with Reviewers:

P. J. Wood: Some (1,3)-β-D-glucans are inaccessible to certain (1,3)-β-D-glucanases as a result, possibly, of a triple-helical conformation which may be disrupted by prior alkali treatment [Catley and Fraser, Carbohydr. Res. 183 (1988) 83-88]. Did the authors determine if their (1,3)-β-D-glucanase was active against (1,3)-β-D-glucans other than laminaran, such as scleroglucan, pachyman and (in tissue sections) callose?

Authors: Sections of Himalaya barley were treated with 0.5 M NaOH for 10 min prior to digestion with (1,3)-β-D-glucanase for 24 h. The large β-glucan-containing deposits were not removed by this treatment. The (1,3)-β-D-glucanase hydrolyzed hydrated pachyman but susceptibility of the other substrates to the enzyme was not determined.

Reviewer III: What would the solubility potential of (1,3)-β-D-glucan likely be in cold, warm, hot water or diluted alkali compared with the mixed linked β-D-glucan of the endosperm cell wall?
Authors: We have no information on the relative solubilities of the two types of β-glucan in these solvents.

Reviewer III: Since the endosperm cell walls also contain protein, could the pectinase preparation used contain proteolytic enzymes?
Authors: It is quite possible that the pectinase preparation used in this study contained proteolytic enzymes. If so, these enzymes could hydrolyze the protein present in the endosperm cell walls.

Reviewer III: Do the authors have any information on the relative location of the materials in the bead-like bodies since this would influence enzymic degradation?
Authors: We have limited information on the bead-like bodies in barley endosperm. They were present in all barley cultivars examined, appeared to be associated with the inner walls of endosperm cells and were hydrolyzed with relative ease by (1,3)-β-glucanase.

M. E. McCully: In the light of the known induction of "wound" callose by conventional fixation procedures (Galway and McCully, Protoplasma 139, 77-91, 1987), how do you know for certain that all your observed (1,3)-β-glucan deposits are endogenous?
Authors: We cannot be certain that all of the small, bead-like deposits are endogenous because of Dr. McCully's findings. All kernel sections were treated in the same way and we found the regular occurrence of large (1,3)-β-glucan deposits only in Himalaya barley. If these deposits were caused by wound induced callose formation, then we would expect to find them in other barley cultivars.
Abstract

The utilization of valuable by-products of seed processing residues as coextrusion materials was investigated. By mixing sunflower, pumpkin, corn or rice germ presscake with cereals (wheat, corn, rice), the good protein quality of the former group might improve the biological value of the resulting coextrudates. The microstructure of such coextruded products was analysed with reference to their chemical compositions, nutritional characteristics and functional properties. As seed processing residue was increased, the microstructure of the products became more compact and uniform and the air/solids ratio decreased considerably. The results showed that the highest acceptable concentration of the additive was not more than 20-40 % for the applied high temperature short-time procedure.

Introduction

Extrusion technologies are being used increasingly in the processing of cereal-based foods, snacks and related products. Usage has grown rapidly because of the development of technologies capable of producing a wide range of products economically. High-temperature, short-time (HTST) procedures are versatile and continuous. The resulting products are sterile and very digestible (Seib 1976).

Because mechanical and heat treatment can or has the potential to disorganize the original structure of raw materials (Björck et al 1984), the physicochemical character, ultrastructure and texture of the main components of the starting materials (starch, proteins and lipids) can change significantly during extrusion (Seib 1976, Williams 1977).

The modifications of starch due to extrusion were detailed by several workers (Mercier and Feillet 1975, Faubion and Hoseney 1982a, Owusu-Ansah et al. 1983, Goodman and Rao 1984, Villareal and Juliano 1987). Some of changes expected to occur in proteins during extrusion have been reviewed as well (Faubion and Hoseney 1982b). Heat-sensitive components (vitamins, fatty acids, etc.) are effectively preserved by short term thermal processing while the relative amount of dietary fiber slightly increases under the extreme conditions of temperature and pressure (Varo et al. 1983, Björck et al. 1984).


In spite of these successes, the use of HTST extrusion cooking for the texturization of certain oilseed proteins or mixtures of plant materials has not been investigated satisfactorily. The objective of the present work was to study the effect of extruding seed processing residues (sunflower seed, pumpkin, maize germ, rice germ) with cereals (wheat, maize, rice). The changes in chemical composition, microstructure, functional properties (expansion, water- and oil-absorption capacity, emulsion activity index) and nutritional

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KEY WORDS: Microstructure, coextrusion, cereals, oilseed residues, functionality, nutritive value, food additive.

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quality of the extruded products were investigated as a function of concentration of the seed processing residues. This study aimed at the utilization of these valuable industrial by-products (seed processing residues) as food ingredients in human nutrition.

Materials and Methods

Materials

Oilseed press cakes, sunflower seed, pumpkin seed, maize germ, and rice germ were obtained from the Herbaria Co., Budapest. The fresh cold pressed (350 atm) by-products were ground by Cyclotec 1093 (Tecator, Sweden) sample mill to an average particle size < 0.32 mm and were used immediately for extrusion. The commercially available cereal meals (hard wheat, maize, rice) were obtained from Hungarian Grain Trust (Budapest).

Extrusion

The seed processing residues and cereal components were mixed, pairwise in a cylindrical powder mixer of an industrial extruder (CMVO1, CMV Székesfehérvár, Hungary) with a 35 mm diameter single screw (1/4 ratio=6/1, pitch 46 mm) was used to extrude the mix. The extrusion parameters were as follows: 155 °C barrel temperature (heated by an electrical induction system), 100 rpm screw speed and six 4 mm restriction dies. The total residence time of the product in the extruder was about 8 sec. The products were cooled by air and ground into powder for analysis.

Proximate analysis

Raw materials and extrudates were analysed by AACC (1983) and AOAC (1980) methods: Crude protein (Kjeldahl; AACC method 46-10), moisture (AACC method 44-19), ash (AOAC method 08-01), crude fiber (AOAC method 14020) and lipid (AOAC method 14018).

Functional properties

Bulk density was estimated by measuring the weight of 10 cm³ unextruded (ground) and 1000 cm³ extruded material. Graduated cylinders containing the extrudates were tapped 30 times to allow uniform compaction of the material. The mean of nine replicates was reported. The water absorption capacity (WAC) was determined by a modified capillary absorption method at ambient temperature according to Enslin (1933). Samples of 50 mg were placed on the surface of a glass filter and the amount of absorbed water was determined from the changes of the water level in the capillary. The WAC value was expressed as g water absorbed/g sample. The results represent the mean of three replicate measurements.

Oil absorption capacity (OAC) was measured by modified method described by Lin et al. (1974). A 0.5 g sample was mixed (1 min) with 3 cm³ sunflower oil in a plastic centrifuge tube and allowed to stand 20 min at ambient temperature. The slurry was centrifuged at 4000 rpm (1800 g) for 25 minutes. The tubes were inverted on filter paper for 10 minutes and then reweighed. The measured data were expressed as g absorbed oil/g sample. Samples were measured in triplicate.

Results and Discussion

Chemical composition

The raw materials used (Table 1) allowed us to vary the chemical composition of the coextrudates over a wide range. By altering the amount of macromolecules present (protein, starch, lipid), parameters of nutritional importance could be changed accordingly. The nutritional properties of the products were determined by their amino acid composition and by the amount and ratio of essential amino acids (Table 2).

<table>
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<th>Meal</th>
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<th>Lipid</th>
<th>Moisture</th>
<th>Ash</th>
<th>Crude fiber</th>
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<td>0.33</td>
<td>6.0</td>
<td>0.44</td>
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<td>6.2</td>
<td>0.80</td>
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<td>0.11</td>
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<td>8.78</td>
<td>6.3</td>
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<td>7.1</td>
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<td>5.6</td>
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<td>13.73</td>
<td>7.3</td>
<td>8.23</td>
<td>13.90</td>
</tr>
</tbody>
</table>

- triplicate determinations of each sample
The lipid and crude protein determination of the products confirmed that during the extrusion process no loss of protein or lipid occurred.

Based on a given raw material composition, any required product can be realized. However, sensitive amino acids did change during extrusion which resulted in a decreased nutritive value of the products (see below).

Functional properties

While the bulk density (BD) of the products decreased due to extrusion in all cases (Table 3), there were large differences in BD depending on the cereal components used. This could be due to different behaviors by the various starches (Mercier and Feillet 1975) and proteins (Faubion and Hoseney 1982b) tested. In all cases, the BD values were considerably influenced by the addition of the seed processing residues. The changes of BD as a function of additive level were less pronounced in mixtures containing wheat. Using maize and rice as cereal components, considerable changes were observed. In the mixtures of maize + rice germ 20%, rice + sunflower seed 20%, and rice + rice germ 20%, the BD values of the products were lowest and most comparable to that of extruded pure wheat.

The water and oil absorption capacities of the pure cereals generally increase upon extrusion (Lawton et al. 1985). The values obtained for extruded maize and rice (Tables 4 and 5) did not differ significantly in either parameter while wheat showed lower WAC and OAC values compared to extruded maize and rice. On comparing the WAC of pure extruded cereals to that of coextrudates, WAC values were found to decrease with increasing the amount of the oilseed component (Table 4). Low concentrations of seed processing residues (20%) resulted in 10-30% decreases in the WAC of extrudates. WAC was least affected when maize germ residue was used as the seed processing residue component. Higher substitutions resulted in more drastic lowering of the WAC, e.g., 65% decrease in wheat; pumpkin seed (40:60).

![Image of Table 2: Amino Acid composition of raw materials (mg amino acid/g sample)]

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Wheat</th>
<th>Maize</th>
<th>Rice</th>
</tr>
</thead>
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<tr>
<td>ASP</td>
<td>5.41</td>
<td>7.52</td>
<td>9.32</td>
</tr>
<tr>
<td>THR</td>
<td>4.08</td>
<td>5.12</td>
<td>5.46</td>
</tr>
<tr>
<td>SER</td>
<td>4.56</td>
<td>4.38</td>
<td>3.48</td>
</tr>
<tr>
<td>GLU</td>
<td>34.97</td>
<td>20.98</td>
<td>13.10</td>
</tr>
<tr>
<td>PRO</td>
<td>12.55</td>
<td>4.33</td>
<td>2.40</td>
</tr>
<tr>
<td>GLY</td>
<td>4.37</td>
<td>3.31</td>
<td>3.55</td>
</tr>
<tr>
<td>ALA</td>
<td>3.48</td>
<td>7.46</td>
<td>4.17</td>
</tr>
<tr>
<td>CYS</td>
<td>0.87</td>
<td>0.81</td>
<td>0.98</td>
</tr>
<tr>
<td>VAL</td>
<td>3.02</td>
<td>3.80</td>
<td>2.81</td>
</tr>
<tr>
<td>MET</td>
<td>1.08</td>
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<tr>
<td>ILE</td>
<td>3.08</td>
<td>2.43</td>
<td>2.10</td>
</tr>
<tr>
<td>LEU</td>
<td>5.85</td>
<td>12.32</td>
<td>5.81</td>
</tr>
<tr>
<td>TVR</td>
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<td>5.92</td>
<td>4.98</td>
</tr>
<tr>
<td>PHE</td>
<td>5.32</td>
<td>6.74</td>
<td>4.34</td>
</tr>
<tr>
<td>LYS</td>
<td>2.91</td>
<td>3.30</td>
<td>4.32</td>
</tr>
<tr>
<td>HIS</td>
<td>2.83</td>
<td>4.04</td>
<td>2.25</td>
</tr>
<tr>
<td>TRP</td>
<td>0.06</td>
<td>0.05</td>
<td>0.13</td>
</tr>
<tr>
<td>ARG</td>
<td>4.43</td>
<td>4.88</td>
<td>7.07</td>
</tr>
</tbody>
</table>

![Image of Table 3: Bulk density of raw materials and their extrudates prepared from pure cereals or mixtures of cereals and seed processing residues]

<table>
<thead>
<tr>
<th>Additive</th>
<th>Wheat</th>
<th>Maize</th>
<th>Rice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>raw extr.</td>
<td>raw extr.</td>
<td>raw extr.</td>
</tr>
<tr>
<td></td>
<td>kg/dm³</td>
<td>kg/dm³</td>
<td>kg/dm³</td>
</tr>
<tr>
<td>None</td>
<td>0.715</td>
<td>0.710</td>
<td>0.646</td>
</tr>
<tr>
<td>Sunflower seed</td>
<td>20%</td>
<td>0.704</td>
<td>0.661</td>
</tr>
<tr>
<td></td>
<td>0.642</td>
<td>0.674</td>
<td>0.674</td>
</tr>
<tr>
<td></td>
<td>0.640</td>
<td>0.680</td>
<td>0.674</td>
</tr>
<tr>
<td>Pumpkin seed</td>
<td>20%</td>
<td>0.670</td>
<td>0.661</td>
</tr>
<tr>
<td></td>
<td>0.649</td>
<td>0.674</td>
<td>0.661</td>
</tr>
<tr>
<td></td>
<td>0.615</td>
<td>0.674</td>
<td>0.661</td>
</tr>
<tr>
<td>Maize germ</td>
<td>20%</td>
<td>0.638</td>
<td>0.661</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>0.674</td>
<td>0.661</td>
</tr>
<tr>
<td></td>
<td>0.586</td>
<td>0.674</td>
<td>0.661</td>
</tr>
<tr>
<td>Rice germ</td>
<td>20%</td>
<td>0.677</td>
<td>0.661</td>
</tr>
<tr>
<td></td>
<td>0.652</td>
<td>0.674</td>
<td>0.661</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>0.674</td>
<td>0.661</td>
</tr>
</tbody>
</table>

![Image of Table 4: Microstructure of extrudates](Image of Table 4: Microstructure of extrudates)

<table>
<thead>
<tr>
<th>Additive</th>
<th>Wheat</th>
<th>Maize</th>
<th>Rice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>raw extr.</td>
<td>raw extr.</td>
<td>raw extr.</td>
</tr>
<tr>
<td></td>
<td>kg/dm³</td>
<td>kg/dm³</td>
<td>kg/dm³</td>
</tr>
<tr>
<td>None</td>
<td>0.715</td>
<td>0.710</td>
<td>0.646</td>
</tr>
<tr>
<td>Sunflower seed</td>
<td>20%</td>
<td>0.704</td>
<td>0.661</td>
</tr>
<tr>
<td></td>
<td>0.642</td>
<td>0.674</td>
<td>0.674</td>
</tr>
<tr>
<td></td>
<td>0.640</td>
<td>0.680</td>
<td>0.674</td>
</tr>
<tr>
<td>Pumpkin seed</td>
<td>20%</td>
<td>0.670</td>
<td>0.661</td>
</tr>
<tr>
<td></td>
<td>0.649</td>
<td>0.674</td>
<td>0.661</td>
</tr>
<tr>
<td></td>
<td>0.615</td>
<td>0.674</td>
<td>0.661</td>
</tr>
<tr>
<td>Maize germ</td>
<td>20%</td>
<td>0.638</td>
<td>0.661</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>0.674</td>
<td>0.661</td>
</tr>
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<td></td>
<td>0.586</td>
<td>0.674</td>
<td>0.661</td>
</tr>
<tr>
<td>Rice germ</td>
<td>20%</td>
<td>0.677</td>
<td>0.661</td>
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<tr>
<td></td>
<td>0.652</td>
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</tr>
<tr>
<td></td>
<td>0.625</td>
<td>0.674</td>
<td>0.661</td>
</tr>
</tbody>
</table>

![Image of Table 5: Microstructure of extrudates](Image of Table 5: Microstructure of extrudates)
cereal. Fig. 1a shows the flaky sheet type matrix being characteristic of extruded rice whereas extruded maize (Fig. 1b) had a loose structure with uniform cell sizes. The location of large and small cells was random. The largest compartments were about 1 mm in diameter. The loose microstructure of these two cereal products explains their low bulk density. Extruded wheat (Fig. 1c) had fibrous surfaces and contained irregularly shaped cells. The different coextrudates showed a significantly different microstructure at low and high magnification. The structure of each particular coextrudate varied within the sample. Only typical changes due to the addition of seed processing residue are presented in Figures 2 to 4.

The effect of increasing processing residue content on extrudate microstructure is shown in Figs. 2a–c. The addition of 20% rice germ disrupted the flaky structure of extruded rice with the appearance of small granules (Fig. 2b). With increasing amounts of the additive, a compact, granular structure (Fig. 2c) was observed at higher magnifications. Upon the addition of seed processing residues, the ratio of dispersed air was reduced (bulk density increased) and the structure became more compact and the cell walls showed less variability within the sample.

Effects similar to the above mentioned were observed for coextrudates of processing residues and maize. Adding rice germ, pumpkin, maize germ and sunflower seed processing residues at 60% concentration caused the original microstructure...
Microstructure of coextrudates

Table 4. Water absorption capacity of extrudates from cereals and from mixtures of cereals and seed processing residues

<table>
<thead>
<tr>
<th>Additive</th>
<th>Wheat</th>
<th>Maize</th>
<th>Rice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g water/g material</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sunflower</td>
<td>20%</td>
<td>4.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Seed</td>
<td>40%</td>
<td>2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maize</td>
<td>60%</td>
<td>1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pumpkin</td>
<td>20%</td>
<td>2.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sunflower</td>
<td>40%</td>
<td>2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Seed</td>
<td>60%</td>
<td>1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maize</td>
<td>20%</td>
<td>3.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Germ</td>
<td>40%</td>
<td>3.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rice</td>
<td>60%</td>
<td>2.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maize</td>
<td>20%</td>
<td>3.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Germ</td>
<td>40%</td>
<td>2.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rice</td>
<td>60%</td>
<td>2.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

- a - significantly different from the 20% mixtures of the same additive, p<0.05
- b - significantly different from the 20% and 40% mixtures of the same additive, p<0.05
- c - values of pure extruded product significantly (p<0.05) different from values of coextrudates with the same cereal

to disappear. The coextrudates had compact structure containing intact starch granules 5-15 μm and in some areas gelatinized starch was observed (Fig. 3a-e).

On adding seed processing residues to wheat, the original lamellar structure (observed at higher magnification) completely disappeared (Fig. 4a-e). With 20% seed processing residue addition some fibrous-lamellar structure at the surface of compartments could still be observed (Fig. 4b-c). Increasing the amount of the additive to 60%, a rippled structure with randomly distributed granules of different size was observed in some areas of the sample. These granules, appearing only with the addition of 60% residues seem to be intact starch particles with a maximum diameter of 20 μm.

Nutritional evaluation

Protein nutritional quality indices (Norup and Olesen 1976) of extrudates prepared from cereals and from mixtures of cereals and seed processing residues were calculated from the samples' amino acid compositions (Table 6). The relatively low biological values of cereals were increased by the addition of seed processing residues. The biological value of extrudates made with rice germ and sunflower seed increased considerably. The identity of the cereal component played a role as well. All three cereals are well complemented with rice germ at concentrations of 20-40%. Sunflower seed improved the biological value of extrudates at all seed processing residue concentrations. Wheat and maize improved only when supplemented with large amounts of pumpkin seed. The nutritional quality of these coextrudates was not significantly improved by the addition of maize germ.

There were significant (p<0.05) differences (Table 6.) between measured and calculated biological values in extruded products. These results suggest that the destruction of limiting amino acids (tryptophan, cysteine and methionine) during extrusion has to be taken into consideration.

Conclusions

Functional properties and microstructural evaluations suggested that WST extrusion is an applicable technology to process various cereals containing seed processing residues as coextrudates.

The bulk density of the coextrudates increases with an increase in the amount of seed processing residues in the mixture. A pronounced decrease was observed in the WAC and OAC of the coextrudates. Because of the above unfavorable tendencies, the amount of seed processing residues in the products is limited (max. 20-40%). In contrast, an advantageous increase in nutritional value was observed by addition of seed processing residues.

The extrudates of pure cereals showed cellular microstructures of different character but became more compact when the processing residues were added. Increasing the seed processing residue content reduced the proportion of air in the products which paralleled a shift in the ratios of gelatinized/intact starch.

The recommended cereals and seed processing residues to form coextrudates of acceptable quality are shown by our results to be composed of
Figure 3. Scanning electron micrographs of a) extruded maize and coextrusions of maize with seed processing residues of b) 60% rice germ; c) 60% pumpkin; d) 60% maize germ; e) 60% sunflower. (s) starch, (gs) gelatinized starch.

Figure 4. Scanning electron micrographs of a) extruded wheat and coextrusions of wheat with different amounts of seed processing residues; b) 20% rice germ; c) 20% pumpkin; d) 60% rice germ; e) 60% pumpkin. (s) starch, (gs) gelatinized starch.
Table 6. In vitro biological value of proteins in extrudates calculated by Morup and Olesen (1976) index

<table>
<thead>
<tr>
<th>Additive</th>
<th>Wheat</th>
<th>Maize</th>
<th>Rice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>calcu-</td>
<td>calcu-</td>
<td>calcu-</td>
</tr>
<tr>
<td></td>
<td>rated</td>
<td>rated</td>
<td>rated</td>
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<tr>
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<td>35.8</td>
<td>33.6</td>
<td>29.8</td>
</tr>
<tr>
<td></td>
<td>su.</td>
<td>su.</td>
<td>su.</td>
</tr>
<tr>
<td>Rice 20%</td>
<td>97.6</td>
<td>86.2</td>
<td>90.4</td>
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<tr>
<td>germ 40%</td>
<td>97.3</td>
<td>106.4</td>
<td>86.6</td>
</tr>
<tr>
<td></td>
<td>78.5</td>
<td>89.9</td>
<td>76.6</td>
</tr>
<tr>
<td>Pumph-</td>
<td>53.3</td>
<td>47.2</td>
<td>68.4</td>
</tr>
<tr>
<td>kin 40%</td>
<td>59.3</td>
<td>55.3</td>
<td>66.1</td>
</tr>
<tr>
<td></td>
<td>56.5</td>
<td>59.4</td>
<td>63.3</td>
</tr>
<tr>
<td>Maize 20%</td>
<td>47.4</td>
<td>42.2</td>
<td>62.2</td>
</tr>
<tr>
<td>germ 40%</td>
<td>50.6</td>
<td>49.9</td>
<td>57.5</td>
</tr>
<tr>
<td></td>
<td>46.3</td>
<td>50.5</td>
<td>54.1</td>
</tr>
<tr>
<td>Sun- 20%</td>
<td>61.7</td>
<td>56.1</td>
<td>77.5</td>
</tr>
<tr>
<td>flower 40%</td>
<td>71.0</td>
<td>67.3</td>
<td>79.5</td>
</tr>
<tr>
<td></td>
<td>69.6</td>
<td>73.9</td>
<td>80.3</td>
</tr>
<tr>
<td>seed 60%</td>
<td>76.0</td>
<td>68.7</td>
<td>72.7</td>
</tr>
</tbody>
</table>

a - calculated values represent the theoretical predictable biological values
b - measured values were calculated by amino acid composition of extrudates

References


Glossary

Wheat: Key term explained.
Maize: Key term explained.
Rice: Key term explained.

Discussion with Reviewers

D.J. Gallant: Cytochemistry would helpfully resolve the question "how the oil seed interacts with the other components of cereals and oil seed residues". Did you try to study it either under the light or transmission electron microscope?

Authors: Specific staining is planned for cytochemical evaluation of coextrudes to reveal the interaction of macromolecules during extrusion. Light microscopy results will be presented in a separate paper.

W.J. Wolf: Sunflower seeds contain chlorogenic acid and other phenolic acids. Does the presence of these compounds cause problems of undesirable colors in the coextrudes?

Authors: The color of the products was basically determined by the color of the cereal component obtained after hydrothermal treatment. The white or yellow color of the cereal components turned into clear brown or green due to addition of sunflower seed and pumpkin, respectively. The colors were stable during storage. For these reasons the chlorogenic acid and phenolic components of the seed processing residues were not measured.

W.J. Wolf: The oilseed press cakes contain appreciable amounts of lipids (Table 1). Do these residual lipids cause stability problems in the coextruded materials?

Authors: The organoleptic properties of the coextrudes are certainly influenced by the characteristics of the lipids. For this reason coextrusion was done from freshly pressed oilseed residues. The oxidation of the sensitive components will have to be prevented by adequate packaging and by adding antioxidants. Storage experiments are planned to investigate the stability of the products.

W.J. Wolf: For a food product to be successful, it must have organoleptic properties that are acceptable to consumers. Have you evaluated any of the extruded materials for their organoleptic characteristics, i.e., flavor and texture?

Authors: Preliminary experiments were carried out to analyze the organoleptic properties of the coextrudes. The flavors were present.

J.M. Faubion: What are the small granules (about 1 μm) shown in Fig. 3b-e?

Authors: The 1-2 μm size granules are believed to be protein bodies according to previous works of Pagani et al. (1986).

J.M. Fabubion: Why does the addition of the seed processing residues inhibit or prevent starch gelatinization?

Authors: The elevated seed processing residue content (higher amount of lipids) presumably prevented the water migration that paralleled the reduced heat conduction. These unfavourable temperature conditions and the decreased amount of available water resulted in reduced gelatinization.

P.D. Richards: The main body of the work relates the microstructure to the functional and nutritional aspects. The authors should explain why the nutritional and related data are essential for this work?

Authors: The purpose of the coextrusion is to obtain products with increased nutritive value. Measuring the change of these parameters to evaluate the products is essential.

P.D. Richards: Have the authors made any investigations of biodigestibility in vivo to determine the nutritional value of the products?

Authors: Parallel in vivo rat trials and in vitro measurements for determination of the biological value and the true digestibility of proteins for extruded products were performed. The correlations between in vivo and in vitro data were determined for both parameters and will be reported elsewhere.

Additional Reference

A METHOD FOR LIGHT AND SCANNING ELECTRON MICROSCOPY OF DROUGHT-INDUCED DAMAGE OF RESTING PEANUT SEED TISSUE

Clyde T. Young* and William E. Schadel

Department of Food Science
North Carolina State University
Raleigh, North Carolina, 27695-7624

Abstract

Tissue damage creates numerous problems for the microscopic examination of resting peanut (Arachis hypogaea L. cv. Florunner) seed physical structure. This paper presents a method to deal with specific problems encountered in light microscopy (LM) and scanning electron microscopy (SEM) of drought-induced damage of peanut tissue. Major findings include: (1) improved SEM imaging through reduction of charging by increasing coating thickness from 25 nm to 30 nm; (2) improved stain affinity for LM tissue preparations by decreasing osmium tetroxide fixation time from 2 hours to 1 hour; and (3) improved tissue fixation for LM and SEM by application of a modified Karnovsky fixative (glutaraldehyde/formaldehyde combination) which proved more successful than glutaraldehyde alone for tissue preservation.

Introduction

Light microscopy (LM) and scanning electron microscopy (SEM) of native resting peanut involve a fixation-dehydration-drying methodology that seeks to preserve the tissue so that it will retain its morphology for microscopic examination. Tissue which is damaged before fixation presents unique problems which hinder this effort. Such damaged tissue may result in poor SEM imaging, poor LM stain affinity, and poor tissue fixation for both LM and SEM.

Our laboratory was first involved with microscopic examination of drought-induced damage of resting peanut seed tissue in connection with evaluation of peanuts for a severe off-flavor problem (Young and Schadel, 1984). During that study, numerous problems with SEM imaging and LM staining preparations were encountered while adapting undamaged resting peanut seed tissue fixation-dehydration methods for use on resting peanut seed tissue with drought-induced damage. The present investigation was undertaken to discover the cause of those problems and to devise a method to improve the microscopy for evaluation of drought-induced damage of resting peanut seed tissue.

Materials and Methods

Cotyledons of resting peanut (Arachis hypogaea L. cv. Florunner) seed were examined with a dissecting microscope for physical structural characteristics. They were divided into two groups: (1) cotyledons with normal physical characteristics; and (2) cotyledons with drought-induced tissue damage. Tissue blocks (1 mm³) of both groups were most suitably fixed in a modified Karnovsky's fixative (Karnovsky, 1965). This modified fixative was prepared by mixing 25 mL of 8% formaldehyde, 3.6 mL of 70% glutaraldehyde, and 28.6 mL of 0.2 M sodium phosphate buffer (hereafter referred to as buffer). The pH of the mixture was adjusted to 7.0. Tissue blocks were fixed under vacuum for 30 minutes at room temperature to promote initial infiltration and then fixed (not under vacuum) for 48 hours at 4°C. Following a 24 hours wash in 6 changes of 0.1 M buffer (4°C, pH 7.0), the material was post-fixed for 1 hour in 1% osmium tetroxide in 0.1 M buffer at 4°C. After post-fixation, material for LM was washed for 30 minutes in 0.1 M buffer (4°C, pH 7.0), dehydrated in a graded series of aqueous ethanol (10, 25, 50, 75, 95) to 100% ethanol, and embedded in...
Spurr's low viscosity medium (Spurr 1969). Sections were cut on a Reichert ultramicrotome and stained differentially with 1% acid fuchsin and toluidine blue (Feder and O'Brien, 1968). Identically fixed tissues for SEM were dehydrated in an identical series of aqueous ethanol followed by a graded series of ethanol—acetyl acetate (10, 25, 50, 75, 95, and 100% acetyl acetate). Carbon dioxide was used as the transitional fluid in a Ladd Critical Point Dryer (Ladd Research Industries, Burlington, VT). After critical point drying, the specimens were mounted on aluminum stubs with aluminum tape. Two groups of tissue blocks for SEM were then coated with a gold-palladium alloy of 25 nm and 30 nm respectively in a Technics Hummer V sputter coater fitted with a Technics digital thickness monitor (Technics, Alexandria, VA). Samples were observed and photographed at 20 keV with an ETEC Autoscan Microscope (ETEC Corp., Hayward, CA).

The specific changes in our present method from our previous preparations and the reasons for those changes were:

1. Use of a modified Karnovsky's fixative rather than 4% glutaraldehyde to allow the formaldehyde component to penetrate faster than the glutaraldehyde component and temporarily stabilize structures which were subsequently more permanently stabilized by the glutaraldehyde;

2. Initial fixation under vacuum rather than at atmospheric pressure to achieve a more thorough and uniform fixation;

3. Post-fixation in 1% osmium tetroxide for 1 hour rather than 2 hours to allow less blackening of the tissue and enhance stain affinity for LM preparations;

4. Use of a Technics Hummer V Sputter Coater fitted with a Technics Digital Thickness Monitor to measure the thickness of coating that best reduced the charging of the specimen when examined with the SEM.

**Results and Discussion**

**Scanning Electron Microscopy**

Our SEM work of resting peanut seed tissue with drought-induced damage was initially hindered by problems with excessive charging of the specimen. By using the digital thickness monitor to measure the thickness of the gold-palladium coating applied to the specimen by the sputter coater, we were able to determine that the charging problem of the tissue with drought-induced damage was a result of the gold-palladium coating being too thin.

SEM of undamaged resting peanut seed epidermal tissue could be accomplished without charging artifacts when the gold-palladium coating was 25 nm thick (Fig. 1). Tissue with drought-induced damage, however, would charge when coated with 25 nm thickness (Fig. 2). By increasing the thickness of the gold-palladium coating by 20% (from 25 nm to 30 nm), we were able to minimize charging (Fig. 3). The charging problem was most likely the result of increased roughness in the surface of the samples (compare Figures 1 and 2).

**Light Microscopy**

LM tissue preparation using 1% osmium tetroxide for 2 hours were initially hindered by excessive blackening not only of damaged cells, but of the undamaged cells beneath the damaged cells (Fig. 4). Excessive blackening of tissue prevented us from evaluating the depth of damaged tissue because our LM staining procedures for undamaged tissue were ineffective. By reducing the length for post-fixation in 1% osmium tetroxide from 2 hours to 1 hour, we were able to use the LM stains of 1% acid fuchsin and toluidine blue more effectively since the blackening of undamaged tissue was lessened. By using the phase contrast optics on the light microscope, we observed that the fixation of the cytoplasmic network had been effectively accomplished during the decreased post-fixation period. The cytoplasmic network has a net-like appearance as a result of the oil being removed from the lipid bodies by alcohol dehydration and leaving spaces in the cytoplasm (Fig. 5).

**Fixation**

While initial use of a 4% glutaraldehyde fixative in 0.05 M sodium cacodylate (pH 7.0) for peanut tissue with drought-induced damage was adequate, we felt that better integrity of the cytoplasmic network in undamaged cells could be attained. This would more readily enable us to distinguish damaged cells from undamaged cells, as preservation of the cytoplasmic network is lost in severely damaged cells. Horisberger and Vonlanthen (1980) achieved excellent fixation of soybean seed using a mixture of glutaraldehyde and formaldehyde in 0.1 M phosphate buffer (a modified Karnovsky's fixative) and a post-fixation in 1% osmium tetroxide. We have made our own modifications of a mixture of glutaraldehyde and formaldehyde followed by a post-fixation in 1% osmium tetroxide that most effectively achieves fixation of resting peanut seed tissue. Our fixative modifications proved to be excellent for observing the cytoplasmic network both with phase contrast LM (Fig. 5) and SEM (Fig. 6).

**Figures 1 and 2.** Scanning electron micrographs of undamaged (Fig. 1) and drought-induced damage of (Fig. 2) resting peanut seed epidermal tissue coated with 25 nm thick gold-palladium coating. Note the absence (in Fig. 1), and the presence (in Fig. 2, arrows) of charging defects (which obscure surface details). Bar = 20 micrometers.

**Figure 3.** Scanning electron micrograph of drought-induced damage of resting peanut seed epidermal tissue coated with 30 nm thick gold-palladium coating. Bar = 10 micrometers.

**Figure 4.** Phase contrast light micrograph of peanut tissue with drought-induced damage. The cellular organization of the damaged tissue is disrupted (arrows). Bar = 20 micrometers.

**Figure 5.** Phase contrast light micrograph of undamaged peanut tissue. Note the fixation of cytoplasmic network (c), protein bodies (p), and starch grains (st). The cytoplasmic network has a net-like appearance as a result of the oil being removed from the lipid bodies by alcohol dehydration and leaving spaces in the cytoplasm. Bar = 20 micrometers.

**Figure 6.** Scanning electron micrograph of undamaged peanut cell. Note the integrity of cytoplasmic network (arrows) at high magnification. Bar = 1 micrometer.
Light and scanning electron microscopy method for peanuts
Acknowledgments

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References


Discussion with Reviewers

R. Taber: Would the authors distinguish between a resting peanut seed, a dormant seed, and a quiescent seed?
Authors: Resting seed, dormant seed, and quiescent seed are all synonymous terms.

R. Taber: Do the authors prefer LM examination of thick sections of peanut seed that have been embedded for transmission electron microscopy over the traditional paraffin sections? If so, what additional information is gained.
Authors: We prefer plastic-embedded sections over paraffin embedded sections because plastic embedded sections can be stained and examined without the removal of the plastic embedding medium. Paraffin-embedded sections require the removal of paraffin before staining. Our experience has been that cellular components may be lost during staining procedures which require prolonged rinsing of deparaffinized sectioned tissue.

R. Taber: Would it be difficult to use a freeze-fracture technique and cold stage to view damaged kernels? Would you expect this technique to provide the information desired?
Authors: The technique you mention should provide information about damage to lipid bodies because it would enable the lipid bodies to be examined without extracting the oil within the lipid bodies.

R. Taber: What is the advantage of using the digital thickness monitor over simply performing sequential coatings for routine examination?
Authors: The digital thickness monitor enables an investigator to reproduce an optimum coating thickness with reliability. Our experience has been that sequential coatings are time-consuming and may result in a lack of uniformity of coating thickness among different samples.
OBSERVATION OF SEEDING EFFECTS ON FAT BLOOM OF DARK CHOCOLATE

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Abstract

Surface microstructures and polymorphism of seeded dark chocolate were observed with cryo-SEM, to clarify the effects of seeding on fat bloom stability of dark chocolate. Two thermal tests, cycling between 32 and 20 C (32/20) and 38 and 20 C (38/20), were applied to examine the fat bloom stability of the chocolate. We used three crystalline powders: Form VI of cocoa butter; the most stable β₁ form of SOS (1,3-distearoyl-2-oleoylglycerol); and the second stable β₂ form of BOB (1,3-dibehenoyl-2-oleoylglycerol) as seed materials. Seeding with cocoa butter (Form VI) and SOS (β₁) at concentrations of 0.5 % % showed good fat bloom stability in the 32/20 test. In the case of the 38/20 test, however, fat bloom was not prevented. Seeding with BOB (β₂) gave the best fat bloom stability in both thermo-cycles; in particular, 5 wt.% BOB (β₂) completely prevented fat bloom after the 38/20 test.

KEY WORDS: Cryo-scanning electron microscopy, seeding effect, fat bloom, dark chocolate, polymorphism, cocoa butter, surface microstructure, 1,3-dibehenoyl-2-oleoylglycerol.

Introduction

In chocolate production, pre-crystallization influences viscosity, demolding, and qualities of the final product such as gloss, snap, heat resistance, fat bloom resistance and so on (5,6,14,16). The tempering process, necessary to stabilize the cocoa butter crystals, involves well-controlled cooling/heating/cooling (5,18). In comparison with tempering, seeding is simpler and more convenient since cocoa butter crystallization is caused by adding seed crystal powders while simply cooling the molten chocolate. The seeding method, however, has not been used for two major reasons. First, crystals having small dimensions were difficult to produce on the factory scale. Second, details of the seeding conditions have not been examined, although some attempts have been made to clarify the solidification kinetics of tempering (1,3,7-9,13).

We have recently studied seeding effects on the solidification of cocoa butter and dark chocolate, aiming to use the seeding technique in factory-scale chocolate production. In previous reports, we developed a method to mass produce seed crystals of small dimensions using a cryo-mill at -50 °C using liquid nitrogen (10). The crystallization kinetics of cocoa butter were measured in relation to the concentration and polymorphism of various seed crystals (12). We related the physical properties of seeded dark chocolate to demolding and anti-bloom phenomena, showing that seed materials of thermodynamically stable polymorphs of St-O-St type triacylglycerols (S=unsaturated acyl chain) have the most favorable physical properties.

In this paper, we carefully observed the effects of seeding on the fat bloom stability of dark chocolate. We used three seed materials, cocoa butter (Form VI), SOS(β₁) and BOB (β₂) in which S, O and B stand for stearoyl, oleoyl and behenoyl acyl chains, respectively, since these three accelerated the crystallization of cocoa butter most effectively.

Materials and Methods

The three types of crystal were pulverized with a cryo-mill at -50 °C using liquid nitrogen. The dimensions ranged from 20 to 70 μm as measured with cryo-scanning electron microscopy (cryo-SEM) at -100 °C. The desired polymorphic forms of the above fat
Seeding effects on fat bloom of dark chocolate

materials were prepared by the method of Wang et al. (23). The polymorphism of the fats was determined by DSC at a scanning rate of 10°C/min, and X-ray diffraction (XRD). The purity of the fat materials were analyzed with high-performance liquid chromatography (HPLC). Major triacylglycerols involved in cocoa butter employed were P90 (55.8%), POS (32.7%), in which P stands for palmitoyl acyl chain, and SOS (22.5%). The purity of the seed crystals was 78.7% (SOS) and 72.5% (BOB). SOS and BOB were of industrial purity. The melting points of the seed crystal powders, defined as temperatures at the peak of DSC melting, were cocoa butter (Form VI) 34.0°C, SOS (β1) 42.0°C and BOB (β2) 51.4°C. Dark chocolate was manufactured by the following formulation: chocolate liquor 33.00 wt.% sugar 41.65 wt.%, cocoa butter 24.85 wt.% and soya lecithin 0.50 wt.%. We examined the fat bloom stability of seed-solidified dark chocolate by the following methods. The molten dark chocolate, 60°C, 250 g weight, was cooled to 30°C within 10 minutes with agitation (194 rpm) in a rotational viscometer (11). Seed crystals were added at different concentrations with respect to the cocoa butter content of dark chocolate (43 wt.%), at the time when the temperature of dark chocolate reached 30°C. Seed concentration ranged from 0.01 to 5 wt.%. After seeding, the dark chocolate was agitated for 5 minutes at 30°C in order to homogeneously disperse the seed crystals in the molten chocolate. Thereafter, 4 g of the seeded dark chocolate was put in a mold (10 mm x 20 mm x 3.5 mm) made of ethylene chloride resin (thickness of 0.5 mm). The dark chocolate in the mold was immediately cooled to 15°C and solidified for 15 minutes in a cooling box. After cooling, the molded samples were aged by storing at 20°C for one week.

After aging, thermo-cycle tests were carried out using a thermostated chamber. The cycle involves heating at 32 or 38°C for 12 hours and cooling at 20°C for 12 hours, referred to us 32/20 or 38/20, respectively. During the thermo-cycle test, we observed changes in the appearance of the samples, both on the surface and interior, using an optical microscope and cryo-SEM (-100°C to 130°C). Polymeric modification of the seed-solidified chocolate was measured with XRD and DSC at a scanning rate of 5°C/min, during the thermo-cycle test. Prior to the XRD study, sugar in the sample was dissolved by the method of Giddley and Clerc (9): the sample was sliced with a knife into powder below 8 mesh size, and put in 120 ml of water (7% x 10°C) to dissolve the sugar.

Results and Discussion

32/20 Thermo-cycle test

Figures 1, 2, 3 show the surface appearance of the seeded dark chocolate after the 32/20 test, in which the fat bloom is revealed in white-color areas. Cocoa butter (Form VI), Fig. 1, and SOS (β1), Fig. 2, showed similar trends with increasing number of cycles and seed concentration; fat bloom was prevented in the range of the seed concentration from 0.05 to 1 wt.% and below 4 cycles. At 0.01 wt.%, fat bloom stability decreased slightly. Similarly, the high-concentration seeding of 2.5 and 5 wt.% lowered stability against fat bloom. In the case of the 5 wt.% seeding, fat bloom occurred after one cycle. In contrast, seeding with BOB (β2), Fig. 3, improved fat bloom stability with increasing seed concentration. No fat bloom was observed after 6 cycles, when the seed concentration exceeded 1 wt.%. Figure 4 shows cryo-SEM photographs of the surface microstructure of a non-bloomed sample with 0.5 wt.% of SOS (β1) seed taken prior to the thermo-cycle test (normal sample), bloomed sample seeded with 5 wt.% of cocoa butter (Form VI) and SOS (β1) after 6 cycles, and a non-bloomed sample seeded with 5 wt.% BOB (β2) after 6 cycles. The normal sample showed a very smooth surface even at high magnification (Fig. 4a). The bloomed samples, however, showed rough surfaces (Fig. 4b and c). In the case of the non-bloomed sample seeded with 5 wt.% BOB (β2), Fig. 4d, a smooth surface was observed, although it was slightly rougher than the normal untreated sample (Fig. 4a).

The polymorphic conversion from Form V to Form VI was observed during this 32/20 cycle test. The polymorphs of the sample seeded with cocoa butter (Form VI) and SOS (β1) were both Form VI, whereas Forms V and VI at similar concentrations were confirmed in the chocolate seeded with 5 wt.% BOB (β2).

Legends for figures on the next page.

Fig.1 Photographs showing dark chocolate, seeded with powder crystals of cocoa butter (Form VI) at seven concentrations, during the 32/20 thermo-cycle tests up to 6 times (see text).

Fig.2 Photographs showing dark chocolate, seeded with powder crystals of SOS (β1) at seven concentrations, during the 32/20 thermo-cycle tests up to 6 times.

Fig.3 Photographs showing dark chocolate, seeded with powder crystals of BOB (β2) at seven concentrations, during the 32/20 thermo-cycle tests up to 6 times.

Fig.4 Cryo-SEM photo-micrographs showing the surface microstructures of dark chocolate after 6 cycles of the 32/20 test: (a) non-treated sample; (b) bloomed sample seeded with 5 wt.% cocoa butter (Form VI); (c) bloomed sample seeded with 5 wt.% SOS (β1); and (d) bloomed sample seeded with 5 wt.% BOB (β2) (Scale bar=20 μm).

Fig.5 Photographs showing dark chocolate, seeded with cocoa butter (Form VI), SOS (β1) and BOB (β2), after one cycle of the 38/20 test.

Fig.6 Cryo-SEM photo-micrographs showing the surface microstructures of dark chocolate after one cycle of 38/20 test: (a) bloomed sample seeded with 5 wt.% cocoa butter (Form VI); (b) bloomed sample seeded with 5 wt.% SOS (β1); and (c) bloomed sample seeded with 5 wt.% BOB (β2) (Scale bar=20 μm).
38/20 Thermo-Cycle Test

Figure 5 shows photographs of seeded dark chocolate after the 38/20 test. Significant fat bloom occurred in all samples seeded with cocoa butter (Form VI) and SOS (β1) after one cycle. In the seeding of BOB (β2), however, high seed concentration improved fat bloom stability. In particular, a seed concentration of 5 wt.% BOB (β2) completely prevented fat bloom after one cycle.

As to the 38/20 test, a white network was formed on the bloom surface. The interior of the bloomed dark chocolate changed into a very rough structure look like closely packed small spheres, whose sizes ranged from 0.3 to 1.5 mm. Figure 6 shows cryo-SEM photographs of the surface microstructures of bloomed samples seeded with 5 wt.% of cocoa butter (Form VI) and SOS (β1), and a non-bloomed sample seeded with 5 wt.% BOB (β2), after one cycle. Numerous needle-like crystals were formed on the surface of the bloomed samples (Fig.6a and b). In contrast, the non-bloomed sample in Fig.6c, seeded with BOB (β2), reveals a smooth surface which is almost the same as that of the non-bloomed surface in Fig.4d. As to polymorphism, all bloomed and non-bloomed samples were Form V.

Mechanism of Fat Bloom

In Table 1, we summarize the features of fat bloom formation through the above two thermal treatments. It appears that the mechanisms of fat bloom formation differ between 32/20 and 38/20 tests. The difference may be related to whether or not the thermal cycle goes above the melting point of cocoa butter 34 (C). How­

ever, the growth of large crystals of cocoa butter which scatter light, resulting in a white appearance on the chocolate surface, may occur in both cases as the basic mechanism (16,17).

In the case of the 32/20 test, the bloom-causing grain growth may be initiated by Form VI crystals of cocoa butter which are formed either through solid-state transformation or by the seed crystals themselves. The large grains of Form VI, which was converted from Form V through thermal treatment, grew at the expense of small grains of Form VI via Ostwald ripening (15). This process may provide the solute molecules of cocoa butter which are incorporated in the growing Form VI crystals.

The increasing concentration of the two seed crystals, cocoa butter (Form VI) and SOS (β1), may not interrupt the stable grain growth of Form VI. Hence, the excess seed crystals accelerated the growth of the large grains of Form VI. This may be justified by the fact that Form VI of cocoa butter and β1 of SOS are same with respect to chain length structure and subcell packing (19,21,23).

In the case of the 38/20 test, fat bloom may form by the same mechanism as that in non-tempered chocolate (16). There is the possibility that thermal unstable seed crystals completely melt or dissolve in the molten chocolate, due to high temperature (38 C) and long treatment (12 hours); this might occur in the seed crystals of SOS (β1) and cocoa butter (Form VI). When the completely molten dark chocolate was subsequently cooled to 20 C, it solidified in the same manner as non-seeded crystallization, which readily causes bloom-related growth of Form V by transformation from the first crystallized, metastable forms of cocoa butter, presumably Form III and IV (2,4,22,24). There is a trend that, with increasing rate of crystallization, less stable forms of triacylglycerols crystallized more readily than more stable forms (20). This is the reason why only Form V was detected in the bloomed samples after the 38/20 test. As to BOB (β2), the seed crystals do not melt at 38 C for 12 hours, since the melting point is reasonably high. Hence, the remaining BOB (β2) crystals induce stable crystallization of Form V without causing the formation of other metastable forms.

Concluding Remarks

BOB (β2) seed crystal exhibited the most favorable solidification behavior, with no fat bloom even after the 38/20 thermal treatment. We assume that BOB (β2) is the most suitable seed crystal for chocolate production, since it also accelerates the crystallization rate of dark chocolate. Studies to determine optimal conditions will be needed to apply the seeding method to industrial chocolate production.

Acknowledgment

Fuji Oil Co. kindly provided the fat samples.

References


4) Chapman GM, Akehurst EE, Wright WB. (1971) Cocoa
Seeding effects on fat bloom of dark chocolate


Discussion with Reviewers

G.Ziegler: Cocoa butter (CB) Form VI crystals are more heat-resistant than cocoa butter Form V, arising during the industrial pre-crystallization process. Therefore, chocolate seeded with CB Form VI is rather stable at a 32/20 cycle test. Is the chocolate seeded with CB Form VI more bloom-resistant in comparison to industrial chocolate at milder temperature below 30 °C?

Authors: It is highly possible, as you indicate, to observe that seeding of CB Form VI may reveal better fat bloom stability than CB Form V after thermal test below 30 °C. However, we did not examine this phenomenon in the present study.

G.Ziegler: There may be different origins of fat bloom: the polymorphic transition (as you describe) or some fractionation effects in mixtures of CB with non-compatible fats. Could BOB retard fat bloom of the second type, too?

Authors: In a separate experiment, we examined fat bloom stability of a blended dark chocolate involving CB and non-lauryl cocoa butter replacer (CBR) having concentration ratios of CB/CBR=0.5 < 5/75 < 20 both by usual tempering and seeding. The results showed remarkable fat bloom stability by using BOB (β₂).

J.Shlichter: The BOB gave excellent fat bloom stability which is not surprising if crystal structure compatibility of the impurity with the fat host is considered as some possible mechanism for this action. The authors could examine their findings in view of the proposed mechanism of the "button syndrome". What do you think about this?

Authors: We agree with your idea of "button syndrome" in the present case of seeding of BOB (β₂), in a sense that the affinity in the molecular structure between host molecules (CB) and guest molecules (BOB) plays decisive roles.

J.de Mun: Apparently, the seeding of chocolate with BOB was effective because of the high melting point of BOB (53 °C). This would probably impart sandiness to the chocolate when added at a level of 5%.

Authors: The BOB β₂ crystal powders did not impart sandy mouth feel at all when their dimensions did not exceed ca. 70 µm as we have examined in the present study, since the dimensions of cocoa mass particles dispersed in chocolate are of the same order of magnitude.

J.de Mun: The BOB seeding does not appear to be of any practical value, because this compound would no doubt be a permitted additive.

Authors: In Japan, an approval for the use of lipase-catalyzed interesterified fats in food products like BOB has been given. So, chocolate seeded with BOB crystals has already been placed on the market in Japan.

Editor: How may be a reader obtain ref. 6, 14 and 17?

Authors: These Proceedings of the Pennsylvania Manufacturing Confectioners Association Production Conferences are available from: P.M.C.A., P.O Box 68, Route 29, Perkiomenville, PA 18074, USA.
Microstructure of Nuñas: Andean Popping Beans

(Phaseolus vulgaris L.)

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Abstract

Nuñas, popping beans (Phaseolus vulgaris L.), burst and expand when heated rapidly. Differences in seed microstructure between popping and conventional (non-popping) bean genotypes conceivably contribute to popping in nuñas. However, the microstructural characteristics which contribute to the popping attribute and sites of expansion have not been identified. Seeds and excised cotyledons of unpopped and popped nuñas were examined using scanning electron microscopy (SEM). Protoplasts of unpopped nuñas were similar to protoplasts of conventional beans. Intercellular spaces of unpopped nuñas were occluded by schizogenous cell walls. The occluded form of intercellular spaces differed distinctively from the open form in popped nuñas and untreated conventional beans. The expansion of cotyledon mesophyll in popped nuñas came primarily from expansion of cell walls and secondarily by expansion of the intercellular spaces. Cell wall thickness and dimensions of protoplasts were not changed during popping. Expansion of cell walls away from protoplasts created intracellular voids. SEM images indicated that starch granules (grains) in popped nuñas were generally not altered by popping. Starch granules did not gelatinize or melt during popping as indicated by retention of birefringence. In contrast to popcorn (Zea mays L.), starch granules did not contribute to expansion of popped nuña cotyledons.

Introduction

Nuñas are a type of common bean (Phaseolus vulgaris L.) which burst and expand during rapid heating (Hyland, 1968; Zimmerer, 1985; National Research Council, 1989). Popping beans have been and still are grown and consumed almost exclusively at high elevations in the Andes of Peru and Bolivia. In traditional Andean cooking, nuñas are roasted in sand or oil (Zimmerer, 1985, 1987). Nuñas can also be popped in hot air or microwave ovens (National Research Council, 1989; van Beem and Spaeth, 1989). The advantage of nuñas over conventional (non-popping) bean genotypes is a shorter cooking time. This difference is especially important at fuel-scarce high elevations. Popping of nuñas requires less than 5 min. While cooking of beans in boiling water may require more than an hour for cotyledons to become soft. Nuñas do not require additional cooking after popping. While nuñas are an ancient crop (Zimmerer, 1987; National Research Council, 1989), they are relatively unknown outside of the Andean Region and have received little scientific study. The mechanism of popping and sites which contribute to volumetric expansion during popping of nuñas are unknown. In contrast, the mechanism and sites which contribute to expansion of popcorn have been studied (Hoseney et al., 1983). The expansion of popped tissue relative to unpopped tissue is substantially greater for popcorn (Hoseney et al., 1983) than the doubling of volume observed for nuñas (van Beem and Spaeth, 1989). In popcorn, the starch granules (grains) expand and contribute substantially to bulk expansion of the popped tissue (Hoseney et al., 1983). Large numbers of small fractures in popped corn endosperm also contribute to volumetric expansion (Hoseney et al., 1983). While the microstructure of conventional common bean cultivars has been studied (Varriano-Marston and Jackson, 1981; Swanson et al., 1985; Hughes and Swanson, 1985; Spaeth, 1987a, 1989), no information on the microstructure of nuñas was found in the literature.

Characteristics which allow nuñas to pop are also unknown. Zimmerer (1985) suggested that differences in density between nuñas and conventional beans might be partially responsible for popping. A National Research Council report (1989) derived from an informal survey of researchers familiar with nuñas indicated that the quality or quantity of starch in nuñas may differ from conventional non-popping beans and be responsible for expansion of nuñas during popping.

The mechanism of popping is of interest for analysis of popping and because characteristics which permit popping may also influence physiological processes in seeds during maturation, storage, imbibition and germination. Scanning electron microscopy (SEM) is an excellent tool for examining...
structural characteristics which may contribute to popping of *nuïa* and sites of expansion.

The objective of this research was to observe tissue and cellular structure of *nuïa* seeds before and after popping. *Nuïa* microstructure was compared with published descriptions of popcorn microstructure (Hoseney et al., 1983) to identify features which may allow *nuïa* to burst and expand. SEM was used to examine starch granules, protein bodies, protoplasm, cell walls and intercellular spaces of un popped and popped cotyledons of *Nuïa* Pava and unpopped cotyledons of five additional *nuïa* cultivars and three conventional non-popping bean cultivars.

**Materials and Methods**

Sources of the beans (*Phaseolus vulgaris* L.) examined in this study are given in Table 1. *Nuïas* were field-grown in the highlands of Peru where cultural practices and environmental conditions are conducive to production of *nuïas* which pop well. *Nuïas* were held at ambient temperature and relative humidity before popping and preparation for SEM. *Nuïas* and conventional beans had water contents of 0.05 to 0.06 g H₂O/g seed (dry wt. basis) before popping. Seeds were dried in an oven for 24 h at 100°C for determination of water content.

Individual beans were popped by heating for 2.5 to 4 min at full power in a 1600 watt, 2450 MHz Kenmore microwave oven (Sears Roebuck and Co.). Beans were observed visually during heating and removed from the oven after complete expansion. Microwave preparation of beans for SEM avoided contamination with oil (Hoseney et al., 1983). Microwave preparation also minimized artifacts caused by agitation or redistribution of material by air currents during hot-air popping.

Beans studied with SEM were mature air-dry or popped oven-dry tissues, therefore, fixation, freeze fracture, and critical-point drying were not required (Spaeth, 1987a). Cotyledons were fractured by hand using single-edge razor blades. Beans were sputter coated with gold and observed and photographed at 20 kV on an Hitachi S-570 Scanning Electron Microscope.

Birefringence of starch granules was examined using a Zeiss Photomicroscope II.

Cotyledons from six *Nuïa* Pava seeds and two seeds each of the five remaining *nuïas* and three conventional cultivars were examined.

**Results**

The cotyledons of *nuïas* heated in a microwave oven not only burst the testa and expanded, but also cracked so that the shape of beans was altered as a result of the heating treatment (Fig. 1). The expansion of microwave popped mesophyll excluding the large cracks was similar to that observed for *nuïas* heated in oil or hot air (van Beem and Spaeth, 1989).

The cracks in microwave popped cotyledons ranged from large, easily visible cracks to small checks. The large cracks were generally transverse with respect to the long axis of the bean (Fig. 1). The epidermis near the large cracks exhibited many small checks roughly parallel to the larger cracks (Fig. 2a). Outside this region, the epidermis was not checked. The mesophyll tissue fractured through cell walls rather than separating between cells at the middle lamella (Fig. 2b). Protoplasts adhered to the fracture cell walls or were pulled away and left empty cell walls. Fractures also pulled vascular bundles apart.

Cotyledon tissue of unpepped *nuïas* fractured through protoplasts of mesophyll cells (Fig. 3a). Cell walls surrounded protoplasts that contained starch granules, protein bodies and starch bodies. Changes were noted in the nature of the cellulae and the shape of the cells. The visual evidence of the presence of these changes was consistent with the appearance of the protoplasts. The protoplasts were not stained by the dye used. The birefringence of starch granules was examined using a Zeiss Photomicroscope II. The results showed that the birefringence of starch granules was not affected by the heating process.

**Table 1. Sources of beans.**

<table>
<thead>
<tr>
<th>Cultivar Name</th>
<th>Country, Province</th>
<th>Biological Status</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nuïa</em> Pava</td>
<td>Peru, Cajamarca</td>
<td>Landrace</td>
</tr>
<tr>
<td><em>Nuïa</em> Parcoyana</td>
<td>Peru, Cajamarca</td>
<td>Landrace</td>
</tr>
<tr>
<td><em>Nuïa</em> Iuevo de Huanchaco</td>
<td>Peru, Cajamarca</td>
<td>Landrace</td>
</tr>
<tr>
<td><em>Nuïa</em> Mani Roja</td>
<td>Peru, Cajamarca</td>
<td>Landrace</td>
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<tr>
<td><em>Nuïa</em> Cuzco Café</td>
<td>Peru, Cuzco</td>
<td>Landrace</td>
</tr>
<tr>
<td><em>Nuïa</em> Cuzco Blanco</td>
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</tr>
<tr>
<td>Calima</td>
<td>Colombia, Valle</td>
<td>Bred line</td>
</tr>
<tr>
<td>Liboronino Redondo</td>
<td>Colombia, Nariño</td>
<td>Landrace</td>
</tr>
<tr>
<td>Bolon Blanco</td>
<td>Colombia, Nariño</td>
<td>Landrace</td>
</tr>
</tbody>
</table>

**Fig. 1.** Popped *nuïa* with cracked cotyledons (C), testa (T) and hilum (H). Bar = 10 mm.

**Fig. 2.** *Nuïa* Pava cotyledon which fractured during heating in the microwave oven. Low magnification (2a) with larger cracks (C) extending into cotyledon mesophyll, the epidermal surface (E), and surface checks (SC) in the epidermis. Bar = 1 mm. Detail of large crack (2b) with protoplast (P), empty cell walls (W) and a fractured vascular bundle (VB). Bar = 100 μm.

**Fig. 3.** Fracture surfaces of *Nuïa* Pava cotyledons (fractured for SEM, not a result of popping). Unpopped (3a) with cell walls (W), interior of contact cell wall (ICW), starch granules (SG), large concavities (LC), protein bodies (arrow), and intercellular spaces (IS). Popped (3b) with exterior of protoplast (EP), contact cell wall (CW), schizogenous cell wall (SW), voids (V) between the protoplast and cell walls, intercellular spaces (IS), and projections (Pj) from the surfaces of protoplasts. Bar = 50 μm.

**Fig. 4.** Transverse fractures through intercellular spaces (IS) of *nuïa* cotyledons. Unpopped *Nuïa* Parcoyana (4a) with exterior of protoplast (EP), schizogenous cell wall (SW), contact cell wall (CW) and vertices of the intercellular spaces (arrows). Popped *Nuïa* Pava (4b) with interior surfaces of empty cell walls (ICW) and circular depressions (arrows) in contact cell walls. Bar = 10 μm.
Nuiñas: Popping Beans

and protoplast. Cotyledons of unpopped nuiñas exhibited only a small volume of intracellular voids. Large concave depressions in the fracture surfaces of protoplasts (Fig. 3a) were left by starch granules in the complementary fracture surface. Intercellular spaces were small.

Cotyledon tissue of popped nuiñas was fractured through cell walls but was not fractured through protoplasts (Fig. 3b). During popping, cell walls expanded, separated from protoplasts, and created intracellular voids. The thicknesses of cell walls were not changed. The separation of walls from protoplasts exposed the exterior surfaces of protoplasts and interior surfaces of cell walls. Popped tissue contained many more voids than unpopped tissue. The largest voids opened between the surfaces of protoplasts and the interiors of cell walls. The distances between the exterior surfaces of protoplasts and interior surfaces of cell walls differed within and among cells but the maximum separation ranged from 10 to 20 μm.

The second contribution to expansion in popped tissue came from the opening of intercellular spaces (Fig. 3b). Schizogenous cell walls occluded intercellular spaces in unpopped nuiñas (Fig. 4a). Schizogenous walls are the parts of cell walls which separate during seed growth and development, and surround intercellular spaces in mature seeds (Kollöffel and Linsen, 1984, Spaeth, 1989). In contrast, schizogenous cell walls did not occlude intercellular spaces in popped nuiñas (Fig. 4b). Longitudinal sections through intercellular spaces of unpopped nuiñas show that the spaces were occluded along much of the length of the spaces (Fig. 5a). Bent or folded segments of schizogenous wall filled the lumens of intercellular spaces. Longitudinal sections through intercellular spaces of popped nuiñas show that the spaces were open from end to end (Fig. 5b).

The open, continuous network of intercellular spaces in popped nuiñas (Fig. 5b) was also observed in untreated conventional cotedyledons (Fig. 5c). Intercellular spaces in unpopped cotedyledons of the additional five nuiña cultivars exhibited the occluded form of intercellular spaces while the three conventional (non-popping) cultivars exhibited the open form as observed in popped nuiñas. The epidermal layer of unpopped nuiñas had fewer voids than the mesophyll tissue of unpopped nuiñas (Fig. 6).

While occasional intercellular spaces in mesophyll of nuiñas remained closed or partially closed after popping (Fig. 7a), most intercellular spaces in popped tissue formed an interconnecting network of open spaces. Cracks intermediate in size between macroscopic cracks (Figs. 1, 2) and intracellular voids (Figs. 3b, 5b, 7a) were not observed in popped Nuiña Pava other than the checks in the epidermis (Fig. 2a).

Protein bodies were not distinguishable from protoplasm in popped nuiñas (Fig. 7a). Numerous projections were observed on the surfaces of protoplasts in popped Nuiña Pava. Depressions in the interior surfaces of contact cell walls (Figs. 4b, 7a) corresponded to the sites where the projections were observed on the surfaces of protoplasts.

Starch granules were not directly visible in popped nuiñas, because cells of popped beans did not fracture through protoplasts. Starch granules in popped nuiñas were sometimes evident as bumps beneath the surfaces of protoplasts (Fig. 7a). Size and shape of most starch granules beneath the surface of protoplasts fell within the range of sizes and shapes of starch granules in unpopped nuiñas (Fig. 7a,b). We observed only one starch grain in the beans studied with SEM that changed markedly during popping (Fig. 7b). The outside diameter of the altered starch granule was larger than the diameters of other starch granules in popped or unpopped nuiñas. A hole in the surface of the altered starch grain revealed an internal cavity.

The failure of the popping process to alter the external appearance of starch granules prompted an examination of birefringence of starch granules in unpopped and popped nuiñas. Starch granules in popped nuiñas exhibited birefringence patterns similar to birefringence patterns of starch granules from unpopped nuiñas and conventional beans.

Discussion

The volumes of intracellular and extracellular voids in cotedyledon mesophyll and epidermis of unpopped nuiñas were small. The major difference between unpopped nuiñas (Figs. 3a, 4a, 5a, 6) and conventional beans (Fig. 5c; Spaeth, 1989) was the smallest volume of intercellular spaces in nuiñas due to occlusion of the spaces by schizogenous cell walls. The relative absence of voids in nuiña cotyledons was similar to the absence of voids in vitreous endosperm of unpooped corn (Hoseney et al., 1983).

Popping in corn requires steam pressure which builds in the kernel during heating (Hoseney et al., 1983). The occlusion of intercellular spaces in unpopped nuiñas (Figs. 3a, 4a, 5a, 6) may contribute to popping of nuiñas by increasing the resistance to flow of steam out of cotyledons, or by absence of space into which steam may expand. Intercellular spaces in popped nuiñas (Figs. 3b, 4b, 5b) were more similar to the open, continuous network of intercellular spaces observed in conventional cultivars (Fig. 5c; Hughes and Swanson, 1985; Spaeth, 1987b, 1989) than they were to the intercellular spaces of unpopped nuiñas. The open network of intercellular spaces of popped nuiña cotyledons may be pathways for escape of steam under pressure. The observation that some intercellular spaces were still occluded after popping (Fig. 7a) indicated that occluded intercellular spaces were present before popping. Cell walls may also constitute barriers to flow of steam during rapid expansion. Resistance to steam flow by occluded intercellular spaces in nuiñas is a plausible mechanism of popping. Tests of the resistance hypothesis will require nuiñas and conventional beans produced in one location at one time.

Protoplasts of unpopped nuiñas (Figs. 3a, 6) were similar to protoplasts of conventional non-popping beans (Fig. 5c; Varriano-Marston and Jackson, 1981; Hughes and Swanson, 1985; Spaeth, 1989). The appearance of nuiña protoplasts was altered by popping as the appearance of conventional bean protoplasts is during imbibition (Varriano-Marston and Jackson, 1981; Hughes and Swanson, 1985; Spaeth, 1989). Projections from the surfaces of protoplasts and depressions in cell walls of popped nuiñas were similar to

![Fig. 5. Longitudinal fractures through intercellular spaces (IS) in bean cotyledons. Unpopped Nuiña Pava (5a) with occluded sections of intercellular spaces (between arrows). Bar = 10 μm. Popped Nuiña Pava (5b) with contact cell wall (CW), interior surface of contact wall (ICW), schizogenous cell wall (SW), exterior surface of schizogenous wall (ESW). Bar = 50 μm. Calima (5c), a conventional non-popping cultivar of Andean bean with open section of intercellular space (between arrows). Bar = 50 μm.](image)
those observed in beans after imbibition. The sizes of protoplasts did not change during popping (Figs. 3a,b,5a,b).

The expansion of nurias during popping came primarily from changes in the space between cell walls. Cell walls expanded and created intracellular voids around protoplasts (Figs. 3b,5b,7a,b). Schizogenous cell walls of the occluded form in un popped nurias (Figs. 4a,5a) separated to the open form in popped nurias (Figs. 4b,5b). Intracellular voids contributed more to cotyledon expansion of popped nurias than the open form of intracellular spaces.

The smaller expansion of nurias (van Beem and Spaeth, 1989) relative to the expansion of popcorn (Hoseney et al., 1983) was primarily due to differences in starch granules and the frequency of small cracks. Most starch granules in nurias were not visibly changed during popping of nurias (Figs. 5,6,7) while most of the starch granules in the vitreous endosperm of popcorn expand during popping (Hoseney et al., 1983). The structure of the altered starch granule in Nutia Pava (Fig. 7b) was more similar to starch granules of popped corn (Hoseney et al., 1983) than to other starch granules of either unpopped (Fig. 3a) or popped (Fig. 7) nurias.

The reasons starch granules did not gelatinize during popping of nurias is not clear. The high temperature inside of nurias during time of popping may be lower than the temperature inside of popcorn, or the starch granules in nurias may have a higher temperature for gelatinization than starch granules in the vitreous endosperm of popcorn. Since gelatinization of starch granules depends on temperature and moisture content of the starch, analyses will require measurements of both.

The crack processes in popped nurias were generally transverse with respect to the long axis of the bean (Fig. 2a) similar to transverse cracks formed during imbibition (Spaeth, 1986). Cotyledon mesophyll tissue of popped nurias generally lacked the small cracks (Figs. 2,3,7) which make a substantial contribution to the expansion of popped corn endosperm (Hoseney et al., 1983). The epidermal surface of popped nuria cotyledons exhibited small checks (Fig. 2a) which contributed little to expansion.

Nurias which pop immediately after harvest sometimes fall to pop after a period of aging (National Research Council, 1989). Another aging process of common bean cultivars is the development of the hard-to-cook phenomenon (Varriano-Marston and Jackson, 1981; Swanson, Hughes and Rasmussen, 1985; Hincks and Stanley, 1986). Several mechanisms are reported to contribute to development of hard-to-cook beans including changes in cell wall properties (Hincks and Stanley, 1986) after storage at high temperatures and high humidities. Changes in cell wall properties may affect the popping process and could explain why popping is sometimes incomplete and variable, even within a nuria line.

The occluded intercellular spaces in nuria cotyledons constitute an interesting modification of cotyledon tissue from the perspective of seed physiology. The diffusion of gases through dry nuria tissue may differ from diffusion of gases through cotyledons of conventional beans because of the differences in pathways for diffusion. The condition of intercellular spaces in nurias before and during dehydration, and during and after rehydration is not known. The occluded intercellular spaces may also alter the hydraulic conductivity and consequently transport of liquid water into cotyledons.

Conclusions

SEM analysis of nurias indicated that the primary sites of expansion were the intracellular spaces between the protoplast and cell wall, and intercellular spaces which changed from an occluded form in un popped nurias to an open form in popped nurias. The expansion differed from popcorn in that neither starch granules nor small multicellular cracks contributed significantly to the expansion of nurias. The occluded form of intercellular spaces in un popped nurias differed distinctively from the open form of intracellular spaces in popped nurias and unpopped conventional beans.

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, College of Agriculture and Home Economics, Washington State University, and CIAT. Drs. P. M. Cortes, and J. S. Hughes provided valuable discussions, and Dr. G. G. Galvez supplied the seeds of the nuria cultivars from Peru. The Electron Microscopy Center, Washington State University provided facilities and technical assistance.

References


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Discussion with Reviewers

W. J. Wolf: If the cell wall expands during popping, wouldn't you expect a narrowing of the cell wall? Have you looked for evidence of changes in the structure of the cell wall as a result of the expansion?

Authors: For a homogeneous, isotropic material, one would expect a decrease in thickness after expansion. Since cell walls are neither homogeneous, nor isotropic, we did not know what to anticipate. We have not observed marked changes in thickness of cell walls. We have not studied changes in cell wall structure as a result of popping in greater detail.

R. W. Yaklich: Do you think the differences you observed in microstructure of popped nuñas would be the same at a higher elevation?

Authors: The cooking time required for popping of nuñas is reported to increase at high elevations (K. S. Zimmerer, personal communication). Since elevation can influence popping time, elevation may also influence microstructure of popped nuñas.

R. C. Hoseney: What is the significance of the fact that popping only takes 4 min to produce a soft bean while cooking in water takes a much longer time?

Authors: Beans become soft during boiling because cells in cotyledons separate from each other along the middle lamella (Hincks and Stanley, 1986). We observed no evidence of cell separation along the middle lamella in popped nuñas. Therefore, the process by which nuñas become soft during popping differs from the process during boiling. Two different processes of softening apparently develop at substantially different rates.

B. G. Swanson: Is the moisture content of nuñas prior to popping similar to unpopped corn?

Authors: The water content of nuñas before popping in the current research was below the range of water contents conducive to optimal popping of popcorn (Hoseney et al., 1983). We have not determined the range of initial water contents conducive to popping of nuñas.

B. G. Swanson: Is moisture expansion, as steam, responsible for changes in intercellular spaces?

Authors: We hypothesize that steam pressure is responsible for the observed expansion of cells and intercellular spaces, however, tests of this hypothesis need to be conducted.

B. G. Swanson: Is moisture or structure primarily responsible for popping of nuñas?

Authors: Moisture and structure both seem to be essential for popping. We suspect that constricted intercellular spaces limit the escape of moisture and cause the nuñas to pop after pressure increases in the cotyledons.

B. G. Swanson: Do nuñas have a shorter cooking time than conventional beans in water?

Authors: When nuñas and conventional beans are both boiled, nuñas do not cook more quickly than conventional beans (CIAT, 1989). Softening of nuñas during boiling probably takes place by the cell separation mechanism, so the popping mechanism confers no advantage during boiling.

Additional References


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THE EFFECT OF CHLORIDE SALTS ON THE TEXTURE, MICROSTRUCTURE
AND STABILITY OF MEAT BATTERS

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Abstract

The stability, texture and microstructure of six mechanically deboned chicken meat batters prepared with NaCl (1.25 and 2.5%) and replacement of the 2.5% NaCl with MgCl₂, CaCl₂, KCl and LiCl based on isoionic strength were examined. The uncooked MgCl₂ batter showed the poorest fat binding. The monovalent chloride salts produced stable cooked batters, whereas both divalent salts did not. CaCl₂ produced a more unstable batter than MgCl₂. High correlation was found between water and fat loss and total cookout losses from cooked batters. Texture was significantly affected by the type of chloride salt used. The divalent chloride salt batters had low brittleness and were similar in texture. They had a different texture profile from monovalent chloride salt batters. Hardness and springiness were found to be related to batter stability.

Microstructural differences between treatments reflected differences in batter stability and appeared to explain some of the textural differences. The protein matrices of the monovalent chloride salt batters were all similar. However, LiCl produced a more tightly interwoven matrix than the others. Extensive coalescence was evident in the batters made with MgCl₂ and CaCl₂ which resulted in the formation of fat channels. In addition, their protein matrices were highly aggregated. Batter stability and texture appear to depend on the structure and integrity of the matrix as well as the formation of a stable protein film around fat globules.

Introduction

The production of finely comminuted sausages such as frankfurters requires the availability of adequate amounts of extracted myofibrillar proteins in the meat batter. Myofibrillar proteins are principally responsible for binding added moisture and fat and thus for the desirable texture associated with these products. The solubilization of myosin and actomyosin is of great importance in the formation of stable meat batters (Gillett et al., 1977). Sodium chloride (NaCl) is the salt most widely used to enhance the extraction of myosin and actomyosin during comminution (Aghar et al., 1985; Barbut and Findlay, 1989).

Two main models have been proposed to explain fat stabilization in such batters. According to the emulsion theory, the proteins form a film or membrane around the fat globule which stabilizes them (Jones, 1984), whereas the no-emulsion theory proposes that fat globules are physically entrapped within the protein matrix (Lee, 1985). During cooking, protein coagulation helps to immobilize the fat, water and other constituents.

Unstable batters are infrequent, but bothersome problems in routine meat processing operations. Research has therefore been directed at the mechanisms by which batters are stabilized. Early research demonstrated the existence of an interfacial protein film (IPF) around fat globules and suggested that it was responsible for the stability of meat batters during cooking (Hansen, 1960; Swift et al., 1961, Borchert et al., 1967). Several workers have since confirmed the presence of a protein film around fat globules (Theno and Schmidt, 1978; Lee et al., 1981; Carroll and Lee, 1981; Svasdee et al., 1982). In addition, Jones and Mandigo (1982) have investigated the ‘pores’ found in the protein film in the cooked batters and suggested that they serve as a pressure release mechanism during cooking. The matrix as well as the chemical and physical nature of the fat have also been shown to play an important role in emulsion stability (Meyer et al., 1964; Townsend et al., 1968).

Growing concern has been expressed over the link between hypertension and dietary sodium intake (Kent, 1991). This has fueled a drive to reduce the levels of NaCl in meat products (Anon., 1980). However, reducing NaCl levels in
meat products will reduce protein extraction which will result in an unacceptable product (Thiel et al., 1986; Whiting, 1987; Hand et al., 1987; Barbut and Findlay, 1989). In order to successfully reduce the sodium levels, an effective NaCl substitute must be found. It has been suggested that the chloride ion is responsible for the functional efficacy of NaCl in meat products (Hamm, 1970; Offer and Trininck, 1983; Asghar et al., 1985). Consequently, the potential use of other chloride salts (KCl, MgCl₂, LiCl) has been studied (Seman et al., 1980; Hand et al., 1982a,b; Barbut et al., 1988). This approach has met with some success, however, its application has been limited because of some off-flavour problems and insufficient knowledge of the mechanisms by which NaCl stabilizes meat batters.

There is currently an increasing body of information on the functional aspects of NaCl and other chloride salts in meat products. However, a fundamental understanding of the mechanisms involved can greatly speed up the process of developing NaCl alternatives. Very little work on the effect of chloride salts on the microstructure and/or texture of meat emulsions can be found in the literature (Knipe et al., 1985; Whiting, 1987; Barbut and Mittal, 1988; Barbut, 1989). Thus, the objectives of this work were to use a model system to obtain basic information on the effect of different chloride salts on emulsion stability and investigate their relations to product microstructure and texture.

**Materials and Methods**

**Treatments**

Five different chloride salts were used to produce six different poultry meat batters in three separate trials. NaCl was used at levels of 1.5% and 2.5% of the total weight of the batter. Four other chloride salts (MgCl₂, CaCl₂, KCl, LiCl) were used at levels which gave an ionic strength equivalent to that of 2.5% NaCl (Table 1.). The 2.5% NaCl represented the most widely used level for comminuted products today. The 1.5% NaCl represented a 40% reduction that would still provide a borderline stability (Whiting, 1984).

**Ingredients and Product Manufacture**

Batters (0.5kg batches) were made from mechanically deboned chicken meat (MDCM) obtained from a commercial processing plant. The meat was kept frozen (-18°C) for up to one month prior to use. Proximate analysis of the raw meat as determined in duplicate (AOAC, 1980) was: 66.7% moisture, 16.1% fat, 14.3% protein and 1.1% ash. The composition of the cooked batters was not determined. All treatments were formulated with 6.0% added water (based on a finished product weight). What was not added was the sole source of salt. The level of chloride salts (Fisher Co., Ontario) varied among treatments.

Batters were chopped for 4 min in a non-vacuum bowl cutter (Hobart, model 84142, Troy, OH) using the high speed setting (33 rpm). Final chopping temperatures did not exceed 8°C in any of the trials. To remove air bubbles, batters were later vacuum tumbled in a pre-cooled 40 litre table top vacuum tumbler (Lycot, Columbus, WI) for 30 sec at a pressure of 0.15 atm. For cooking, 34g of batter was accurately weighed into 50ml plastic test tubes (to prevent evaporation) which were centrifuged (Fisher Centrifuge, Fisher, Ont.) at a low speed (600 g) for 5 min in order to evacuate any air trapped during stuffing (Whiting, 1984). Six tubes per treatment were cooked to an internal temperature of 69°C in a water bath gradually heated from 50°C to 80°C within 1.5 hr. Three tubes were used to determine emulsion stability (ES) and the rest were stored in a cooler (2°C) overnight.

**Sampling and Testing**

The pH of raw batters was determined in triplicate (Hemacat J-398, Cole Palmer, Chicago IL). Emulsion stability was assessed in triplicate on both the raw and cooked batters. For the raw batters, 34g of each batter was weighed into 50ml plastic centrifuge tubes and centrifuged at 18,000 g. The amount of oil separated was determined and used as a measure of the stability of the raw batter. Stability of the cooked batter was measured by the amount of liquid and fat released from a 34g sample during cooking (Townsend et al., 1968).

Texture profiles were determined on seven cooked samples per treatment (Bourne, 1978). A central core (10mm high x 10mm in diameter) was removed from each specimen and compressed twice to 25% of its original height (Keeton et al., 1984; Zeigler et al., 1987) using the Instron Universal Testing Machine (Model 1122, Instron Corp., Canton, MA). Cross-head speed was 20mm/min and chart speed was 200mm/min. Hardness (force to maximum deformation, N), brittleness (force to initial fracture, N), cohesiveness (ratio of the area of the second curve to the area of the first curve, mm²/mm²), springiness or elasticity (distance from gauge length to slice surface of second curve, mm), gumminess (hardness x cohesiveness, N) and chewiness (gumminess x springiness, Nmm) were determined.

**Electron Microscopy**

Samples for scanning electron microscopy (SEM) were prepared by a modification of the procedure of Jones and Mandigo (1982). Two cooked batters per treatment were randomly selected from each treatment. A block (2 x 1 x 1cm) was cut from the centre of each and sliced into 3 mm cubes. They were broken using forceps such that the broken side would be used for SEM. The specimens from each treatment were fixed in 2% glutaraldehyde + 1% paraformaldehyde in HEPES buffer (pH 6.0) for 2 hrs, rinsed five times for 10 min with the buffer, and post-fixed with 1% OsO₄ for 4 hrs. This was followed by five more rinsings with buffer after which specimens were dehydrated through a graded series of ethanol of 50, 70, 80, 90, 95% and three changes of 100% for 10 min each. Specimens were then critical point dried using CO₂, mounted on stubs and sputter-coated with palladium/gold (Hummer VII, Anatech Ltd., Va.). The samples were examined by SEM (Hitachi S-570, Tokyo) at 10 kV.

For transmission electron microscopy (TEM), a standard procedure was used. The fixed and dehydrated samples were then dehydrated through a graded series of ethanol at ratios of 3:1, 1:1 and 1:3 for 30, 30 and 45 min, respectively. Samples were then infiltrated with 100% Spurr's overnight in a...
Effect of Chloride Salts on Meat Batters

Table 1: Emulsion stability and pH of cooked and uncooked meat batters prepared with different chloride salts.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NaCl (1.5%)</th>
<th>NaCl (2.5%)</th>
<th>MgCl₂ (1.35%)</th>
<th>CaCl₂ (1.58%)</th>
<th>KCl (3.19%)</th>
<th>LiCl (1.18%)</th>
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<tr>
<td>Ionic Strength</td>
<td>0.26</td>
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<td>0.43</td>
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<tr>
<td>pH (raw) (S.D.)</td>
<td>6.74bc</td>
<td>6.76abc</td>
<td>6.66c</td>
<td>6.08e</td>
<td>6.83c</td>
<td>6.69cd</td>
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<td>Uncooked Fat Loss (S.D.)</td>
<td>0.59</td>
<td>0.53</td>
<td>0.61</td>
<td>0.18a</td>
<td>0.62b</td>
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<td>Cooked Gel water Loss (S.D.)</td>
<td>4.62c</td>
<td>3.09d</td>
<td>21.06b</td>
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<td>4.09c</td>
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<td>Fat Loss (S.D.)</td>
<td>0.15bc</td>
<td>0.08bc</td>
<td>0.49a</td>
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<td>0.21b</td>
<td>0.06c</td>
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<td>Total Liquid Loss (S.D.)</td>
<td>5.06c</td>
<td>3.32d</td>
<td>22.47b</td>
<td>25.56a</td>
<td>4.71c</td>
<td>2.11d</td>
</tr>
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</table>

Means followed by different superscripts in the same row are significantly different (P<0.05).

Statistical Analysis

The experiment was repeated three times. For the microscopical evaluation, samples from two of the trials were examined. The experiment was based on a complete randomized block design. Data were analyzed by analysis of variance using the General Linear Models (GLM) procedure (SAS Institute Inc., Cary, N.C.). Tukey’s test was used to detect significant differences between treatment means. Correlation coefficients were determined on textural and emulsion stability parameters.

Results and Discussion

Emulsion Stability and pH

Fat was the only component released from the uncooked batters during centrifugation; no water was separated. Only the MgCl₂ treatment showed significantly higher fat release (p<0.05) than other treatments (Table 1). Whiting (1984) and Patana-Anake and Foegeding (1985) also reported no significant water loss prior to cooking in reduced NaCl batters and batters to which different binders were added. The difference in fat binding in the uncooked state suggests that the mechanisms by which MgCl₂ and CaCl₂ act to destabilize batters may be different, possibly because of differences in the type and quantity of extracted protein and protein-cation interaction. Divalent cations are thought to reduce batter stability by increasing protein aggregation through the formation of salt bridges (Hamm, 1970; Asghar et al., 1985).

In the cooked batters, both MgCl₂ and CaCl₂ resulted in high fat and water losses; however, the latter produced a less stable product (Table 1). There was no significant difference in fat loss between the two divalent salt treatments but CaCl₂ caused significantly more water loss than did MgCl₂ (p<0.05). This suggests that unstable batters are more often accompanied by water loss than fat loss. This conclusion was supported by high correlations (p<0.01) between water loss and total cook-out losses (Table 2). Other researchers have suggested that water loss is the main effect of batter instability (Patana-Anake and Foegeding, 1985; Schmidt, 1984).

Magnesium ions have been shown to destabilize meat batters prepared from pork and beef as well as poultry (Seman et al., 1980; Knipe et al., 1985; Barbut et al., 1988). MgCl₂ and CaCl₂ have different effects on pH and water holding capacity (WHC) in ground beef (Wierbicki et al., 1957). Whiting (1987) found no significant differences in fat exudation between beef/pork frankfurters made with MgCl₂ and CaCl₂. However, CaCl₂ caused more water loss from cooked batters. These findings agree with the results in Table 1. On the contrary, he found that MgCl₂ formed a stable...
A. Gordon and S. Barbut

Table 2  Correlation coefficients for emulsion stability, pH and textural characteristicsa.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>pH</th>
<th>Gel Water Releasedb</th>
<th>Fat Releasedb</th>
<th>Total Liquid (cooked)</th>
<th>Fat Released (uncooked)</th>
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<td>Hardness</td>
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<td>0.302*</td>
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<td>N.S.</td>
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</tr>
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<td>Springiness</td>
<td>0.350*</td>
<td>-0.518**</td>
<td>-0.497**</td>
<td>-0.520**</td>
<td>N.S.</td>
</tr>
<tr>
<td>Cohesiveness</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>-0.384*</td>
</tr>
<tr>
<td>Gummness</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Chewiness</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>pH</td>
<td>-</td>
<td>-0.759**</td>
<td>-0.644**</td>
<td>-0.756**</td>
<td>N.S.</td>
</tr>
<tr>
<td>Gel Water Released (cooked)</td>
<td>-</td>
<td>0.913**</td>
<td>0.999**</td>
<td>0.285*</td>
<td></td>
</tr>
<tr>
<td>Fat Released (cooked)</td>
<td>-</td>
<td>0.924**</td>
<td>N.S.</td>
<td>0.284*</td>
<td></td>
</tr>
<tr>
<td>Total Liquid (cooked)</td>
<td>-</td>
<td>-</td>
<td></td>
<td>0.284*</td>
<td></td>
</tr>
<tr>
<td>Fat Released (uncooked)</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

a - total no. of observations = 54.
b - gel water, fat released from cooked frankfurters (ml/100g).
c - not significant.
* - significant (P<0.05); ** - significant (P<0.01).

batter, a conclusion which supports the findings of Hand et al. (1982a) for both pork/beef and mechanically deboned turkey meat frankfurters. A tenuous relationship (P<0.05) was found between fat lost from the uncooked product and the ES of the cooked product (Table 2). This suggests that fat binding prior to cooking may influence the stability of the cooked product.

The other salts resulted in the formation of much more stable batters (Table 1). LiCl formed the most stable batter when compared to the KC1 and 1.5% NaCl treatments but was not more stable than the 2.5% NaCl batter. In contrast, Hand et al. (1982a) and Whiting (1987) found no difference in stability between batters made with these salts. KC1 has been found to be as effective as NaCl in promoting ES (Hand et al., 1987; Barbut et al., 1988). Whiting (1984) found significantly greater water loss from batters made with 1.5% than 2.5% NaCl but significant fat loss only occurred at less than 1.0% NaCl. LiCl has been shown to have a positive effect on water retention (Hand et al., 1982b) but is not approved for use in food.

The lower pH of the MgCl2 and especially the CaCl2 treatments could possibly have caused lower protein extraction and greater protein-protein interaction resulting in the formation of a less open matrix. This could reduce water and fat binding, resulting in lower stability of the cooked batters (Table 1). Moderate correlation was found between pH, fat and water lost from the cooked products (Table 2). Clarke et al. (1987) have observed that a pH in the region of 6.0 resulted in poorer batters than a pH of about 6.3. However, Knipe et al. (1985) found that while MgCl2 reduced the meat batter pH and ES, it was still able to extract enough protein to facilitate the formation of a batter.

If the pH effect is significant, then the higher pH resulting from KC1 addition in this study should have facilitated greater protein extraction and better ES when compared to the standard 2.5% NaCl treatment. However, this was not the case. Further, LiCl produces a pH similar to that of MgCl2 and the 1.5% NaCl treatments but formed a much more stable batter (Table 1). One must therefore be cautious about the pH effect when different ions are compared.

Texture

The texture of the meat batter was significantly affected by the type of chloride salt (Table 3). The texture profile of the 2.5% NaCl treatment was different from that of all
other treatments but the texture of the 1.5% NaCl and KCl treatments was very similar. The elasticity of both NaCl treatments was higher than the others. The 2.5% NaCl and CaCl₂ treatments were harder (p<0.01) than all the other treatments; lithium chloride produced the least hard frankfurter (Table 3). The 1.5% NaCl, 2.5% NaCl and KCl treatments were the most brittle (p<0.01) while the divalent salts produced the lowest brittleness. MgCl₂ and 2.5% NaCl produced the most cohesive frankfurters. In general, the divalent chloride salts were similar in texture except for differences in hardness and cohesiveness.

The hardness of frankfurters is influenced by the protein concentration in the aqueous phase prior to cooking (Hamann, 1988). Therefore, the relative differences reported here may be due to different types and amounts of extracted protein as well as cation-protein interaction during cooking. The differences in hardness and cohesiveness between the Mg²⁺ and Ca²⁺ batters support the idea that they act by different mechanisms in destabilizing the batter. The MgCl₂ batter was more cohesive, a textural characteristic believed to be formed prior to cooking. However, the CaCl₂ batter was harder and this characteristic is thought to be formed during cooking (Montejano et al., 1984; Patana-Anake and Foegeding, 1985). The differences in elasticity between the NaCl treatments and the others probably resulted from microstructural differences in the matrix structure and fat dispersion, as discussed below. Correlations between texture and ES are reported in Table 2. Hardness and springiness were the only textural characteristics related to batter stability. Patana-Anake and Foegeding (1985) have also found that ES characteristics correlated with springiness and hardness but not with cohesiveness and brittleness. Hardness, springiness and brittleness appear to be the main characteristics which determine the final composite texture of the cooked batters. Szczesniak (1963) suggested that hardness and springiness were related to the attraction forces acting between particles and opposing disintegration. Patana-Anake and Foegeding (1985) suggested that springiness and brittleness have a similar microstructural basis and it is possible that they may be affected by the type and amount of protein extracted.

A small, inverse correlation was found between fat loss before cooking and cohesiveness (Table 2). Such a relationship would suggest that this textural parameter develops prior to cooking. Other workers have reported results which suggest that cohesiveness is among the intrinsic properties of the batter developed prior to cooking (Montejano et al., 1984; Patana-Anake and Foegeding, 1985). In addition, Hamann (1988) noted that cohesiveness depends on the functionality of the proteins extracted during manufacturing. Hence, it appears that protein-fat interaction in an uncooked batter may influence protein-protein binding, thereby affecting the cohesiveness of the final product.

**Table 3** Textural parameters of frankfurters prepared with different chloride salts.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NaCl (1.5%)</th>
<th>NaCl (2.5%)</th>
<th>MgCl₂ (1.3%)</th>
<th>CaCl₂ (1.5%)</th>
<th>KCl (3.1%)</th>
<th>LiCl (1.1%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardness 1</td>
<td>10.35cd</td>
<td>13.83a</td>
<td>11.80b</td>
<td>13.93a</td>
<td>10.82bc</td>
<td>9.27d</td>
</tr>
<tr>
<td>(S.D.)</td>
<td>1.80</td>
<td>2.82</td>
<td>1.63</td>
<td>2.97</td>
<td>2.07</td>
<td>1.86</td>
</tr>
<tr>
<td>Brittleness</td>
<td>11.11ab</td>
<td>11.86a</td>
<td>6.77c</td>
<td>7.60c</td>
<td>11.95a</td>
<td>10.43b</td>
</tr>
<tr>
<td>(S.D.)</td>
<td>1.72</td>
<td>2.59</td>
<td>0.95</td>
<td>1.52</td>
<td>2.43</td>
<td>1.80</td>
</tr>
<tr>
<td>Springiness</td>
<td>5.88a</td>
<td>5.91a</td>
<td>5.11b</td>
<td>5.06b</td>
<td>5.08b</td>
<td>5.23b</td>
</tr>
<tr>
<td>(S.D.)</td>
<td>0.34</td>
<td>0.44</td>
<td>0.23</td>
<td>0.21</td>
<td>0.21b</td>
<td>0.22ab</td>
</tr>
<tr>
<td>Cohesiveness</td>
<td>0.22b</td>
<td>0.24a</td>
<td>0.24a</td>
<td>0.22b</td>
<td>0.21b</td>
<td>0.22ab</td>
</tr>
<tr>
<td>(S.D.)</td>
<td>0.02</td>
<td>0.05</td>
<td>0.02</td>
<td>0.04</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Gumminess</td>
<td>2.22c</td>
<td>3.43a</td>
<td>2.88b</td>
<td>3.14ab</td>
<td>2.37c</td>
<td>2.05c</td>
</tr>
<tr>
<td>(S.D.)</td>
<td>0.36</td>
<td>1.25</td>
<td>0.5</td>
<td>0.98</td>
<td>0.88</td>
<td>0.71</td>
</tr>
<tr>
<td>Chewiness</td>
<td>12.97cd</td>
<td>20.68a</td>
<td>14.87bc</td>
<td>16.12b</td>
<td>12.02cd</td>
<td>10.91d</td>
</tr>
<tr>
<td>(S.D.)</td>
<td>1.95</td>
<td>8.25</td>
<td>2.70</td>
<td>5.42</td>
<td>4.51</td>
<td>3.76</td>
</tr>
</tbody>
</table>

a-d - means followed by different superscripts in the same row are significantly different (p<0.05).
e - IS of all treatments (except 1.5% NaCl) equivalent to 0.43.
f - standard deviation.

The differences in hardness and springiness between the NaCl and KCl treatments were very similar. The elasticity of both NaCl treatments was higher than the others. The 2.5% NaCl and CaCl₂ treatments were harder (p<0.01) than all the other treatments; lithium chloride produced the least hard frankfurter (Table 3). The 1.5% NaCl, 2.5% NaCl and KCl treatments were the most brittle (p<0.01) while the divalent salts produced the lowest brittleness. MgCl₂ and 2.5% NaCl produced the most cohesive frankfurters. In general, the divalent chloride salts were similar in texture except for differences in hardness and cohesiveness.

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A small, inverse correlation was found between fat loss before cooking and cohesiveness (Table 2). Such a relationship would suggest that this textural parameter develops prior to cooking. Other workers have reported results which suggest that cohesiveness is among the intrinsic properties of the batter developed prior to cooking (Montejano et al., 1984; Patana-Anake and Foegeding, 1985). In addition, Hamann (1988) noted that cohesiveness depends on the functionality of the proteins extracted during manufacturing. Hence, it appears that protein-fat interaction in an uncooked batter may influence protein-protein binding, thereby affecting the cohesiveness of the final product.

**Microstructure**

The 2.5% NaCl treatment showed an even distribution of fat globules throughout the matrix (Fig. 1A). Many of the fat globules seen under the TEM (Fig. 2A) were surrounded by a defined dark line which is believed to be the interfacial protein film encapsulating fat globules (Hansen, 1960). Borchert et al. (1967),
Lin and Zayas (1987) and Comer and Allan-Wojtas (1988) have also shown this protein film in TEM micrographs. The low NaCl treatment appeared to have a less even fat particle distribution with larger fat globules (Figs. 1C and 2C). The morphology of the low NaCl batter is probably a result of diminished protein extraction during comminution. This would make less protein available for interfacial film and matrix formation, which can lead to lower fat retention during cooking. Clarke et al. (1987) reported that an aggregated type matrix was formed as a result of high cook loss in a 1.3% NaCl low fat comminuted beef batter.

The batter made with MgCl₂ had a fairly even distribution of fat throughout the matrix with the fat globules generally assuming irregular shapes (Figs. 1B and 2B). Extensive coalescence was evident in both SEM (noted by arrows) and TEM micrographs. However, a few very small, round globules could also be seen. CaCl₂ produced a matrix in which the fat appeared to be relatively evenly distributed (Fig. 1E). There appeared to be a great variation in the size of the fat globules in this treatment. The gross coalescence of fat is strikingly evident in the TEM micrograph (Fig. 2E) and was responsible for the virtual continuity of fat throughout the protein matrix. Residues of protein film were visible within the large, irregular shaped fat mass which seemed to be in the intermediate stages of coalescence. Some small, round globules were also seen.

The formation of fat channels in both divalent chloride salt treatments appear to be closely linked to the instability of the batters formed (Figs. 2C, 2E; Table 1). Fat channel formation in meat batters as well as changes in the protein matrix have been found to accompany decreased ES (Townsend et al., 1968; Carroll and Lee, 1981; Lee et al., 1981). The formation of fat channels is thought to create discontinuity in the protein matrix which causes a general destabilization of the batter and results in extensive fat and water loss (Lee, 1985).
Effect of Chloride Salts on Meat Batters

Figure 2. Transmission electron micrographs of representative fields from the six chloride salt treatments. A = 2.5% NaCl (m-matrix, p-protein film); B = MgCl₂ (f-fat, c-coalescence, a-aggregated area, g-round globule); C = 1.5% NaCl (a-aggregated area); D = 1.39% KCl; E = 1.58% CaCl₂ (r-protein film residue, t-tunnels forming fat channels); F = 1.81% LiCl (i-interwoven matrix, f-fat globule). (Bar = 10µm).
particle size and distribution have also been found to affect ES; the smaller the fat particles and the more even their distribution, the more stable the resultant batter was (Lee et al., 1981).

The batters made with KCl and LiCl show fairly even fat particle distribution (Figs. 1D and 1F, respectively). The size distribution of particles within both treatments seemed to be fairly even. TEM micrographs showing their cross-sections (Figs. 2D and 2F) indicated that some of the globules were irregular in shape probably resulting from the structural constraint imposed by the gelled protein matrix. The LiCl batter seemed to have a less uniform fat particle distribution than the 2.5% NaCl treatment but was otherwise similar (Fig. 2A vs 2F). The microstructures of the 1.5% NaCl and KCl treatments were very similar (Figs. 1C and 1D) and relate well to their ES.

The Matrix Structure

The aggregation and denaturation patterns of the proteins in a frankfurter batter are of vital importance to the formation of the protein matrix as well as the protein envelope surrounding fat globules (Schmidt et al., 1981). While fat dispersion and fat particle size play a role in stabilizing the batter, some have suggested that the structure of the protein matrix may be the single most important factor determining batter stability (Jones, 1984). SEM micrographs of the matrices of the different treatments showed differences which might have influenced ES. The matrices of the stable batters had basically a similar appearance; a high magnification example is shown (Fig. 3). The protein matrix of the 2.5% NaCl treatment had a fine, thread-like structure which was highly interwoven and could have been the reason for its good water binding (Fig. 2A). The KCl and LiCl treatments had a similar appearance, but the LiCl appeared to produce a more highly interwoven, dense matrix (Fig. 2F). These microstructural differences seemed to be related to the observed differences in ES.

In contrast to the matrices formed by the more stable chloride salt treatments, those formed by MgCl₂ and CaCl₂ showed varying degrees of aggregation (Figs. 4, 2B and 2E, respectively). The MgCl₂ and CaCl₂ frankfurters had protein matrices which were highly aggregated with large tunnels evident throughout (Figs. 4 and 5). The matrix of the MgCl₂ batter appeared to have greater continuity and smaller tunnels than the CaCl₂ batter (Figs. 2B vs 2E and 4 vs 5). The matrix produced by 1.5% NaCl showed some aggregated areas interspersed with more open areas (Fig. 2C), suggesting lower protein extraction than the 2.5% NaCl. A constant feature observed in all treatments was the existence of small openings in the matrix (Figs. 3 and 4). These were noticeably fewer in the unstable treatments; the CaCl₂ batter had the least. These tiny openings were formed as a result of a finely interwoven, lacy matrix and are believed to hold water by capillary forces (Schmidt et al., 1981).

Jones and Mandigo (1982) and Schmidt et al. (1984) observed an apparent cooperativeness between fat and water loss from products during cooking. It may be that water is lost at a faster rate and

Figure 3. Scanning electron micrograph of the matrix of the 1.39% KCl treatment (representative of the matrices in stable emulsions); p-pore. (Bar = 2µm).

Figure 4. Scanning electron micrograph of the matrix of the 1.35% MgCl₂ treatment; t-tunnel, f-bound fat, p-pore. (Bar = 2µm).

Figure 5. Scanning electron micrograph of the matrix of the 1.58% CaCl₂ treatment; t-tunnel. (Bar = 2µm).
in greater amounts than fat because it is continuous throughout the matrix and more mobile. It is likely that interprotein interaction in unstable batters causes the matrix to contract thereby creating tunnels through which water loss occurs more readily. Water loss increases the availability of protein ligands formerly involved in water binding, thus increasing protein-protein interaction. This can cause further matrix contraction, more water loss and can in turn create even wider tunnels through which the slower moving fat is able to flow. These tunnels may become filled with molten fat, forming fat channels (Figs. 2B and 2E). This could be further facilitated by the weak, thin and often incomplete protein film surrounding the fat globules in unstable batters (Fig. 2E).

Deng et al. (1981) found a large amount of broken protein film and fat separation from large fat globules in which the interfacial film was forced to assume irregular shapes. This inability of the protein film to stabilize large globules can contribute to the cooperativity between fat and water loss from unstable batters. The fact that no water was separated from the raw batters suggests good water binding in this state. Therefore, it appears that the main reasons for fat and water loss in the unstable batters were the formation of tunnels in the matrix of the cooked product and the inability of the insufficient amount of protein to form a coherent film to stabilize the fat during cooking.

**Microstructure Related to Texture**

The microstructure of the chloride salt treatments in this study appeared to be related to their texture. Both NaCl treatments had similar springiness and brittleness which could be due to similarities in the structure of their matrices. Textural differences were possibly caused by what appears to be differences in fat particle size and dispersion as well as water loss during cooking. Angel et al., (1974) and Cassens and Schmidt (1979) have related firmness to fat globule size and distribution. The 2.5% NaCl and KCl treatments seemed to have similar fat particle distribution which may explain their similar brittleness values. However, their different texture overall may be the result of different degrees of interlinking within their matrices (Fig. 2).

The textural difference between LiCl and 2.5% NaCl treatments could possibly have resulted from the highly interwoven matrix of the LiCl treatment combined with its relatively uneven fat particle dispersion (Fig. 2A vs 2F). This would enhance its water holding properties and affect those textural characteristics (such as gumminess) which are affected by water content and brittleness which may be dependent on fat dispersion (Szczesniak, 1963; Zeigler et al., 1987). The low brittleness of the divalent chloride salt treatments may have been due to the continuity of the fat throughout their matrices. The percentage fat content of these treatments would have increased as a result of high water losses. Greater contribution from the plastic nature of the fat to batters could therefore influence brittleness (Zeigler et al., 1987). The low springiness was likely a result of the low degree of integrity of their matrices. Montejano et al. (1984) found that meat muscle gels with a sponge-like texture and large pores had low elasticity. They also indicated that aggregated-type surimi gels had lower shear stress at failure but higher shear strain than non-aggregated gels. Shear stress and shear strain have been correlated respectively with hardness and cohesiveness (Hamann, 1988). The gels formed by the divalent chloride salts in this study appeared to be very similar in texture to both the beef and surimi aggregated gels described above.

The results of this study supported the findings of some previous studies on beef or pork systems. However, there were clear differences with other studies especially with respect to the effect of different chloride salts on batter stability and texture. The texture of cooked batters was found to vary depending on the chloride salt and was significantly affected by batter stability. The monovalent chloride salts formed more stable batters than MgCl2 and CaCl2. KCl (3.19%) produced a batter similar in texture to 1.5% NaCl. The 3.19% KCl was more similar to 2.5% NaCl than was 1.18% LiCl. Although commercial conditions were not used, it appears that proper formulation may allow the successful use of KCl in combination with other salts in the production of high quality reduced-sodium frankfurters. Product texture was also related to microstructure. The results suggest that batter stability and texture depend on the structure and integrity of the protein matrix as well as the formation of a coherent interfacial protein film around the fat globule and fat particle dispersion.

**Acknowledgements**

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Food Technol. 22, 319-338.

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Discussion with Reviewers

Reviewer #4: Test tube experiments have their limitations. Have you prepared frankfurters in commercial type formulations, eg. 11% total protein, 25% fat, to verify conclusions reached from this study?
R.G. Cassens: On what grounds can the results of your model system be related to a commercial frankfurter despite the differences in formulation and preparation?
Authors: It is true that test-tube experiments have limitations. However, while the fat content of our batters (ca 16%) was low compared to many commercial formulations, the protein content (ca 14%) compares favourably to levels found in some commercial products (Ockerman, 1989). The fat in our formulation was derived solely from the MDCM and was not supplemented in order to simplify the interpretation of results. Although this experiment was based on a model system, products with similar formulations have been prepared under commercial conditions and have shown comparable results (Barbut, et al., 1988). Further, the results obtained for the 2.5% and 1.5% NaCl treatments are consistent with those of frankfurters prepared with standard formulations under commercial conditions (Whiting, 1984; Hand et al., 1987). In addition, Hand et al. (1982b) prepared poultry (mechanically deboned turkey meat) and pork/beef frankfurters with four of the salts used in this study and found great similarities in the behaviour of these salts in both meat systems. Their results showed pH differences between treatments similar to ours and their conclusions with respect to the effect of KCl and MgCl₂ on product texture agree with ours.

R.G. Cassens: Was nitrite used? What data for all three trials combined for statistical analysis? How many samples were actually viewed for microscopy in order to obtain a conclusion?
Authors: Nitrite was not used since we did not want to introduce additional ions into the system which could complicate the interactions that might affect product texture and microstructure. This is especially true in the case of the Mg²⁺, Ca²⁺ and Li⁺ ions where their nitrite salts are not commercially available. The data from all three trials were pooled for statistical analysis after being checked for significant block effects. Any block effect which existed was adjusted for in the model used for the analyses.

For microscopy, five (5) fields from each of three (3) specimens per treatment from the first two trials were viewed. This gave a total of 30 fields per treatment.

C.M. Lee: Would you explain why you used such a short chopping time (4 min) and low temperature (6°C) even at a high speed? How can you assume that this preparation regimen facilitated adequate solubilization and fat dispersion? Have you considered looking at the structure under a light microscope?
Authors: The deboning process involves separation of the meat from the bone particles and connective tissue by passing the ground mix through small diameter holes in a metal screen. This results in a comminuted product with a paste-like texture in which the original myofibrillar structure has been severely disrupted. Therefore, four (4) min of chopping at high speed can provide an acceptable batter in terms of adequate protein solubilization and fat dispersion. The chopping time used was found to be sufficient to give adequate fat dispersion when mechanically deboned poultry meat is used to produce meat batters (Barbut, 1988). The final low temperature of 6-8°C was used in order to avoid the melting of the poultry fat which is more unsaturated than pork or beef fat and therefore melts at lower temperatures (Townsend et al., 1968).

In this study, light microscopy (LM) was not used. However, we are planning to use LM together with an image analysis system to further investigate the fat dispersion patterns.

Reviewer #4: Have you carried out any sensory tests to support the statements on textural differences derived from the instrument values?
Authors: No sensory tests were carried out in this experiment for several reasons. The formulations used included KCl and MgCl₂, both of which have been consistently shown to be objectionable in terms of taste when used by themselves in frankfurter formulation as was done here (Hand et al., 1982b; Terrell, 1983; Barbut et al., 1988). In addition, LiCl is not Generally Recognized as Safe (GRAS). Studies relating sensory texture to instrumental measurements show high correlation between TPA fracturability and sensory springiness and firmness (Keeton et al., 1984) and between cyclic compressive forces at 70% deformation and sensory elasticity, firmness and chewiness (Lee et al., 1987). TPA cohesiveness relates well to sensory cohesiveness and TPA hardness has been related to sensory hardness (Montejano et al., 1985).

G.R. Schmidt: In Table 1, it is shown that fat loss only varied from 0.06 to 0.55% whereas water loss varied from 2 to 24%. Isn't this convincing evidence for the theory of water binding by the protein matrix being the factor of predominant importance in batter stability?
Authors: The results in Table 1 do provide overwhelming evidence that water binding by the
protein matrix is the predominant factor in determining batter stability. Nevertheless, at least two other factors must be considered. First, the proteins which are responsible for matrix formation and therefore water binding, are the same proteins which form the interfacial film (Galluzzo and Regenstein, 1978 a,b). Consequently, poor protein extraction or protein aggregation can both result in extensive water loss and cause a weak, unstable interfacial film to be formed. In addition, even if large amounts of broken interfacial film exist, fat will be lost at a much slower rate than the less viscous water. Another factor worthy of consideration is the effect of fat spread throughout the matrix on texture. The fat may not be lost from unstable batters because of reasons already discussed, but it will be redistributed throughout the matrix (Figs. 2B and 2E). This will have undesirable effects of the product texture and acceptability as has been shown by Barbut et al. (1988).

G.R. Schmidt: The pH was probably of little importance since all values were above the critical level of 6.0.

Authors: We agree with your conclusion on the effect of pH.

Reviewer #4: The fat losses and the SEM micrographs do not support the conclusion that MgCl₂ and CaCl₂ caused extensive fat coalescence. One disadvantage of electron microscopy is that the fields are small. Have you used light microscopy to compare the extent of fat coalescence?

Authors: Fat coalescence does not necessarily result in excessive fat losses in all cases. Moreover, it is difficult to discern coalescence in SEM because fat generally appears ovoid in the plane of fracture and many of the interconnecting channels are buried within the matrix. Hence, transverse sections as in TEM or light microscopy (LM) will more easily reveal the nature and extent of coalescence. We would like to point out that the arrows in Fig. 1B are pointing to fat channels connecting two ovoid "globules." Studies are currently under way in our laboratory to examine the fat dispersion of these chloride salt treatments using LM. It may also be of interest to note that during sample preparation for TEM, semi-thin sections are viewed by LM prior to the final trimming of the embedded sample for sectioning. The fields viewed during this process showed similar fat coalescence patterns to those shown by TEM in Figure 2.

G.R. Schmidt: There is a general question of cause and effect for channelling and coalescence of fat and water. The large deposits of fat and water shown in Figures 2B and 2E could be the result of free water and melted fat flowing in large pores of an aggregated protein matrix. Are these deposits of fat and water seen in the raw batter?

Authors: No water was expelled after centrifugation from any of the raw batters examined in this study. This alludes to the fact that there was no 'free' water in the raw batters of any of the treatments; all of the water was bound by the protein matrix. However, it seems that cooking caused matrix shrinkage especially in the Mg₂⁺ and Ca²⁺ batters which resulted in the water losses observed (Table 1). Conversely, fat was released from the MgCl₂ treatment prior to cooking and this may indicate that unstable fat does exist in the raw batter of this treatment. The microstructure of raw batters was not examined in this study. However, work is currently under way in our lab to examine the relationship between raw and cooked batter microstructure.

J.C. Acton: Brown and Toledo (J. Food Sci. 40:1061, 1975) demonstrated that fat stabilization and water stabilization were closely linked in meat batters cooked after chopping to different temperatures. If water and fat flow create the "tunnels" that you described in the matrix, what textural parameter(s) of the final product would likely be related to the degree of tunneling or channelling found? Would the described "small openings in the matrix" of the batters lead to more extensive appearance of tunnels in the case of treatments involving the chloride salts of Mg⁺ and Ca⁺?

Authors: The results obtained in this study indicate that the fracturability (brittleness) and the elasticity (springiness) were the textural parameters most likely affected by the degree of tunneling in unstable batters. Fracturability has been related to sensory springiness (Keeton et al., 1984). Further, the high moisture losses in the unstable batters should lead to a relative increase in the percentage of fat in these batters and, because of its plastic nature, the fat should cause a reduction in springiness (Lee et al., 1967; Zeigler et al., 1987). The "openings in the matrix" result from the interlaced nature of the protein network and are probably sites of water binding by capillary forces. We think that in Ca⁺ and Mg⁺ destabilized batters, cooking leads to extensive protein-protein aggregation and matrix shrinkage. This could result in an enlargement of some of these "openings" which may therefore be the source of the fat-filled tunnels observed.

Reviewer #4: The problem of small field size makes the interpretation of differences in the "protein matrices" difficult. Could you visibly detect, with the naked eye, major differences in the continuous phase of cooked homogenates prepared with divalent cations versus those prepared with monovalent cations? How would you describe these visual differences?

Authors: Visual differences between cooked batters made with the divalent cations and those made with monovalent cations were easily detectable. The monovalent chloride salt batters had continuous phases which were smooth and uniform in appearance. On the contrary, the divalent cations produced batters with a very coarse, uneven appearance.

G.R. Schmidt: The higher magnification shown in Figures 3 - 5 are more convincing than the lower magnifications shown in Figure 1. The fineness of the protein matrices shown in Figure 2 are the most convincing. At higher magnification, is the fineness of the protein aggregates shown in these
Effect of Chloride Salts on Meat Batters

treatments clearly associated with cook yield? Figures 2b and E appear to have very large aggregates and the others very fine aggregates, especially 2F.

Authors: It appears that there is a relationship between the fineness of the protein matrices (Fig. 2) and water and fat binding (Table 1) and therefore cook yield. The LiCl treatment had the most highly interwoven (fine) matrix and also had the least amount of liquid lost during cooking. On the contrary, NaCl and CaCl₂ produced highly aggregated matrices (Figs. 2b and 2E) and showed extensive fat and water losses.

J.C. Acton: What do you think was the cause or source of the interfacial film "pores" found at the surface of the dispersed fat globules by Jones and Mandigo (1982)?

Authors: Jones and Mandigo (1982) suggested that holes ("pores") were formed at weak points in the interfacial film as a result of the internal pressure built up by the expanding fat during cooking. They further suggested that increasing end-point chopping temperatures created thicker, less flexible protein films which were ruptured by thermally expanding fat. The results of our study indicate that the protein film around fat globules ruptured when it was not strong or elastic enough to restrain the expanding fat during cooking. Furthermore, a companion study on the mechanism of pore formation conducted in our lab (unpublished work) supports the theory of Jones and Mandigo (1982) and indicates that the occurrence of pores may be dependent on the nature of the protein film itself. Huber and Regenstein (1988) observed that not enough protein is extracted in meat batters to completely surround the fat globules. This may suggest that some pores are in existence prior to cooking and that fat exudation occurs through these pre-formed holes; however, additional information is needed to clarify this situation.

J.C. Acton: Do you think that the protein forming the interfacial film is interlinked with protein remaining within the aqueous phase, a) in the raw batter below 10°C of chopping, and b) in the final cooked product? The SEM micrograph of Theno and Schmidt (1978 - Figure 8) indicated a linkage between the film and matrix in a cooked frankfurter, but this is not readily evident in your SEM micrograph for cooked poultry meat batters.

Authors: It is our belief that the proteins of the interfacial film and those of the protein matrix are interlinked in both raw and cooked batters. Hermansson (1986) hinted that all of the proteins within a meat batter may be part of a continuous system. We have recently seen this binding using cryo SEM in both raw and cooked meat batters (Gordon and Barbut, submitted). It therefore appears that fat globules are bound to the protein matrix in the raw batter as well as in cooked batters. We have also seen this physical binding in conventional SEM preparations of cooked batters (unpublished data) but the micrographs in Figure 1 do not show it.

Additional References


Zeigler, GR, Rizvi, SSH, Acton JC. (1987). Relationship of water content to textural characteristics, water activity and thermal conductivity of some commercial sausages. J. Food Sci. 52, 901-905, 915.
The first edition of this well-known reference book appeared in 1931 and the revised 5th edition has now been edited by B. Hohmann and F. Deutschmann of the Department of Applied Botany, University of Hamburg (F.R.G.). Scientists working in the areas of foods and feedstuffs will find comprehensive information on the structural organization of every major plant species and their most important natural or processed products. Following an introduction on light microscopy, various techniques of preparation and especially a detailed summary of chemical reagents for selective treatments and specific staining of constituents are given. The following 15 chapters deal with all major groups of vegetable foods. The chapter on cereals also includes information on typical contaminants such as foreign seeds or fungi parasites. One chapter is devoted to the major types of starch, others to the most important starch-containing legumes and their mill-products. All relevant oil-containing seeds and fruits, along with the most common vegetables and fruits, including topical and sub-topical species are described in detail. Also included are products like coffee, cocoa and tea, and a great variety of spices made from seeds and fruits as well as from flowers and from vegetative parts of plants. Structure information on edible mushrooms, thickening materials (polysaccharide gums) and honey is presented along with some information on feed-stuff ingredients of animal origin. Most figures are detailed drawings of characteristic cell and tissue structures, generally given at a magnification of 2000:1. In addition, there are drawings and photographs of particulate matter like seeds and starch granules. Although information based on electron microscopy studies of plant materials has not been considered, Gassner's book represents a unique survey of the broad field of electron microscopy and has done so in 5 chapters and 4 appendices. The first two chapters include a review of light microscopy and introductions to TEM, SEM and STEM (transmission, scanning, and scanning transmission electron microscopy, respectively). Chapters 3 and 4 deal mostly with specimen preparation and micrograph interpretation, including a brief introduction to image analysis. The fifth chapter presents some applications of electron microscopy (no food applications) and X-ray analysis is discussed in an appendix. Many food scientists use electron microscopy only as a tool and have little interest in learning details of the field. For them, the "easily understood survey" given in this book should be useful. Some newer techniques which are becoming increasingly important in the food area are only briefly mentioned, if discussed at all. These techniques include cryomicroscopy, environmental scanning electron microscopy, and others which may help to avoid specimen preparation artefacts. (Artefacts of electron microscopy are discussed briefly, but, again, with no food applications). An appendix includes a clear introduction to energy dispersive X-ray micro-analysis. Food scientists often are concerned with the distribution of lower atomic number elements, and would therefore be especially interested in windowless and thin window detectors. However, those detectors - now commercially available - receive very little treatment in this book.

Full-time food microscopists may not find this book highly valuable for themselves, but may find it to be a valuable reference for their "clients" who need a general introduction to the area.

David N. Holcomb
DISCUSSION WITH REVIEWERS

Each paper in this journal contains a Discussion with Reviewers (DWR). This section follows the text (generally after references) and should be read as a part of the paper. Each paper submitted to Food Microstructure is reviewed by at least three, and often more, reviewers. The reviewers are asked to separate their comments and questions. The comments are used in determining the acceptability of papers. The comments require no written responses from authors, however, in several cases, the authors prefer to include responses to comments, or to questions phrased from, or based on, these comments (either as a result of editorial suggestion or on the author's own initiative) in DWR.

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The Editors
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Cover Photo: Casein in milk, known to exist as colloidal suspensions in a micellar state, are aggregates of submicelles. The stability of micelles during processing and storage plays a major role in the quality and shelf life of many milk products. The effect of high temperature treatment on the micellar integrity is examined in a paper by V.R. Harwalkar, P. Allan-Wojtas, and M. Kalab (pages 217-224). This electron micrograph by rotary shadowing shows the submicellar assembly in casein micelles from unheated milk.

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