FOOD MICROSTRUCTURE

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FEATURES OF FOOD MICROSCOPY

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Abstract

The value of food microscopy is judged by its practical application to food handling. Hence microscopists must explain the relevance of their findings to food technologists. The food microscopist has to deal with materials that are particularly difficult to prepare for microscopy because they often contain high levels of fat, air, sugar, salt, starch or acid. Sometimes the methods used are unorthodox and could be regarded as questionable by microscopists in more traditional disciplines. This paper considers the relationship between food microscopists, food technologists and other microscopists.

An approach to interpretation of images is based on the following features.

1) All interpretations should consider preparation processes, however simple these may be.
2) Interpretations should be based, where possible, on differences between treated and control samples processed in the same way.
3) Microscopical observations should be linked to technological, chemical or physical observations.
4) Key observations should be checked by more than one microscopical technique.

This paper uses past and recent work at the Leatherhead Food R.A. to demonstrate how these criteria have been applied to fats, vegetables, meats, proteins, confectionery, surface fouling and foreign bodies.

The future of food microscopy is an exciting prospect, applying recent microscopical techniques to novel manufacturing problems.

Prologue

"Any fool can look down a microscope." The attitude to this statement has a profound influence on the operation of a microscopy department. Is the role of microscopists to maintain 'foolproof' techniques for others to apply, or should microscopists involve themselves with all aspects of research programmes?

Introduction

Food microscopy is the application of microscopy to technology. In this sense the microscopist has to act as the bridge between food science and technology, and the classical branches of microscopy. This role requires a mastery of the skills of microscopy and sufficient understanding of the relevant processes to be able to recognise and explain the significance of microscopical observations to food technologists. These sentiments may seem obvious but they establish the relationships between food microscopy, technology and other branches of microscopy. In this paper I should like to consider these relationships and demonstrate the contributions of microscopy to food science.

Dealing with Food Technologists

In some establishments the microscopy staff is regarded as 'just a service section', who provide pictures of relevant samples on request. Indeed, in some cases the sample is accompanied by a note of the observation required. In other places the microscopists appear to operate in isolation, with little attempt being made to integrate their observations into applied research. In my view, using a microscopy department in these ways is a quite inefficient use of a most valuable asset. The most important feature that a microscopist can add to any project is that of considering the problem from a different perspective. The food industry embraces a wide range of disciplines but most food scientists tend to view food from a background in chemistry, physics, microbiology or engineering. The result of this is that problems are tackled at two levels: either as a consideration of molecular interactions or in terms of bulk properties. At both levels the approach is normally numerical. Microscopists tend to view foods in concepts rather than numbers and at a level of organisation which is intermediate between molecular interaction and bulk behaviour. Hence the microscopist's viewpoint should add an extra dimension to the consideration of any problem. However, it is often difficult for the non-microscopist
to deal with pictures rather than numbers and to appreciate intermediate levels of structure. It is therefore essential for the microscopist to be able to relate his findings to bulk behaviour or molecular interactions in order to communicate effectively. This requires that the microscopist is fully involved with the planning and background of any project and provides opinions as well as photographs. This approach can lead to problems; in particular, 'demarcation disputes' can occur and the practical applications of microscopy often depend as much on the personalities involved as on technical considerations.

One of the problems that a microscopist faces is the question of artefacts. This fear of artefacts is often advanced as a reason for not incorporating microscopy into a research programme. In my experience a technical defence explaining preparation techniques and precautions has only limited effect. A positive approach with more impact is to compare microscopical procedures with other scientific approaches. In this way a microscopist's function is to interpret images which are produced by a controlled process in the same way as a biochemist may interpret a reading from an oxygen electrode placed in a slurry of tissue, or a chemist may interpret a burette reading as relating to a particular level of a component. In the case of the microscopist, the thought process is more obvious since a picture is, in general, not immediately interpreted numerically. With the advent of many 'black box' instruments, even in microscopy, all scientists need to be aware that their 'results' are in fact interpretations, even if the interpretations are carried out electronically. Microscopists should take a lead here.

In dealing with food technologists, therefore, microscopists need to show that they can contribute to the development of ideas and should make every effort to integrate their findings into the overall research programme.

Dealing with Other Microscopists

As I have previously mentioned, food microscopy is very much an applied branch of microscopy. Because of this, food microscopy is heavily dependent on methodology and technology developed by microscopists in other disciplines. However, food microscopy has special problems associated with specimen preparation compared with conventional biological methods. Almost invariably, processed foods are 'dead' and so the conventional view of fixation as preserving the living structure of cells does not apply. Many foods contain high levels of fat, sugar, air, salt, starch or acid, and conventional biological procedures may be inappropriate. The methods adopted to cope with particular food problems would probably horrify microscopists used to dealing with more conventional tissues. Examples of processes adopted to meet specific problems include fixation with hot uranyl acetate to follow the setting process in jam, long-term (several weeks) fixation in osmium tetroxide to preserve fat in chocolate and periodic acid/thiocarbohydrazide/osmium tetroxide fixation to preserve and stain starch gels. Where conventional fixatives are used it is sometimes necessary to use them in the presence of quite high levels of salt, sugar or acid to allow fixation before extraction. Sometimes fixation and dehydration need to be combined, e.g. using glutaraldehyde in ethanol, in order to restrict extraction of components.

In the case of an applied microscopist trying to help with a particular problem, I think that the approach 'at all costs prepare the sample for microscopy' is valid since microscopy is often the only way to obtain some structural information on the sample. This approach needs to be tempered by two concepts - respect for the microscope and respect for the sample. The first of these requires that the sample should be produced in a suitable form to allow reasonable interpretation and the second requires safeguards to be applied in considering the validity of the techniques used. The type of technique sometimes needed will, quite naturally, present a fear of artefact production in most biological microscopists and many food microscopists. In the same way as a positive approach to artefacts is useful in dealing with food technologists, so a positive approach to artefacts should be adopted in dealing with other microscopists: 'having prepared your sample for microscopy, remember what you have done to it'. In my view, if the sample has been prepared for microscopy, the image obtained will invariably reflect the preparation technique as well as the intrinsic structure of the specimen. This view is probably acceptable to most microscopists concerning electron microscopy but it also applies to light microscopy. At a simple level the refractive index and dispersivity of a mounting medium affect the image produced in the light microscope. Even as I write this paper I see that the Proceedings of the Royal Microscopical Society contains an article by Robinson (1986), making a similar point and using the appearance of soft marginal by different illumination techniques as an example. So every image should be interpreted with the preparation procedures in mind, even where those procedures are regarded as the best available.

A second safeguard in dealing with artefacts is to base interpretations wherever possible on differences between control and treated samples prepared for microscopy in identical ways. In this manner differences in structure can be related back to the treatment.

Thirdly, all microscopical observations should be linked to parallel technological, chemical and/or physical observations on the samples. This is the 'integrative approach' recommended by Davis and Gordon (1982).

Finally, key observations should be checked where possible by alternative preparation techniques. For example, where the method of choice is thin sectioning, some samples could be prepared by freeze-etching and vice-versa. Where possible, light and electron microscopy should be used to complement each other.

I hope that by now I have made my views clear on the role and philosophy of microscopy in food science. A particular problem arises where certain microscopy facilities are not directly available and the services of an essentially medical, biological or materials science department have to be used. In this case, persuading the microscopist operating the instruments of this general philosophy may be particularly difficult, but in my view it is also vital.

I should now like to illustrate the contribution of microscopy to food studies using examples from work at the Leatherhead Food R.A. before presenting my opinions on the future of food microscopy.

Some Fast Work at the Leatherhead Food R.A.

Creaming Power of Fats in Cakes

One of the early applications of electron microscopy at the Food R.A. was a study of the creaming power of fats (Meara et al., 1974). This is a good example of the integrative approach to microscopy as the work combined light and electron microscopy with X-ray diffraction, dilatometry, nuclear magnetic resonance and creaming power measurements. Several different fats were examined, thereby meeting the control
against treated criterion. The methods used were innovative, examining replicas of frozen samples and in some cases using a detergent wash prior to freezing in order to remove the liquid phase and reveal the crystal structures. An awareness of the effects of specimen preparation was needed since if the detergent was too concentrated the whole sample was dispersed, whilst if the detergent was not concentrated enough the liquid phase was not removed.

The first observation was based mainly on light microscopy but was confirmed by electron microscopy. Small individual crystals (~1 μm), mostly in the form of small uniformly sized clusters (3–4 μm), were found to be beneficial in producing a good creaming power fat. Polymorphic form of the crystals seemed less important although, on the whole, B crystals were smaller than B crystals and so produced fats with higher creaming power. The overall level of crystallinity was also important as the development of a good batter involved a liquid fat film around the air cells stabilised by a network of crystals. Smaller crystals could pack into the network better than large crystals; hence lard with very large crystals creamed very poorly whilst commercial shortening with smaller crystals creamed well. An anomaly was observed with rearranged lard which had large crystals but creamed quite adequately. This anomaly was resolved by electron microscopy, which showed that the crystals in rearranged lard had wavy edges (Fig. 1) and readily broke down during creaming to give small crystal fragments (Fig. 2). This work led to a much clearer understanding of the role of fats in creaming and to a better definition for the specification and monitoring of fats for use in cakes.

At about the same time as this work, studies on chocolate fats were underway. This has already been reported in some detail (Jewell, 1974; Berger et al., 1979; Lewis, 1981).

Fruit and Vegetable Products

Unlike the work on the creaming power of fats, which studied a precise and well-defined phenomenon, the work on vegetable products has been more diverse and has been spread over a longer period. Early work on pickles related changes in microscopical appearance to Instron measurements during various stages of pickle manufacture (Saxton and Jewell, 1969; Jewell, 1972). Again, an innovative approach to methodology was used, adapting the periodic acid/thiocarbohydrazide/silver proteinate technique to study cell wall structure (Jewell and Saxton, 1976). The development of methodology indicated that hemicelluloses were most readily stained by this technique.

The work demonstrated that initial changes in texture on brining could be attributed to plasmolysis, but after several days in brine the protoplasts were completely disrupted and further textural changes during freshening and acidification were due to changes in the plant cell wall constituents. Even when the protoplasm had not been degraded by brining, acidification produced breakdown of the membrane, thereby destroying any turgor pressure effect on texture. Pectin staining decreased with storage in brine and on acidification, whilst 1,2-glycol staining (presumed to be mainly hemicelluloses) decreased on storage in brine but not on acidification.

Rantsios and Jewell (1971) and Jewell et al. (1973) considered factors responsible for the breakdown of strawberries in jam making. From a study of the structure of strawberries they considered that strawberries with more vascular tissue would be more resistant to breakdown. The make-up of a strawberry is such that each achene (or seed) on the surface of the strawberry is linked to the centre of the strawberry with a vascular strand; hence the relative density of achenes on the surface of the strawberry should give a measure of its likely breakdown on processing. Therefore, small strawberries with many seeds should be more resistant than large ones with few seeds. Experimental results tended to support this hypothesis although other factors such as berry ripeness and general firmness of the tissue are also active in controlling tissue breakdown.

Work on processing plant tissues continued with a study of different blanching regimes and their effects on carrots (Mirza and Jewell, 1976). This work confirmed the role of cell walls in control of texture; blanching regimes, such as microwave blanching, produced much cell wall damage, producing soft carrots, whilst those causing less cell wall damage, such as direct steam blanching, produced firmer carrots. More recently, a study of the effect of various enzymes on cell wall structure and consequent texture change has been carried out (Holgate, personal communication). These observations showed that pectinase treatment had a major effect on cell walls, causing layering of cell walls and separation of cells in potato (Figs 3–6).
these changes were accompanied by excessive softening. Cellulase had a minor effect on both structure and texture and promoted fracture of cell walls rather than cell separation. Hemicellulases, pectinesterase and proteases had little effect on texture or cell wall structure. General observations on softening processes in plant tissue produced by ripening, spoilage, heating and other processes have shown changes in the cell wall similar to those seen in pectinase-treated tissue. This tends to confirm the view that pectin behaviour is the most important feature in controlling texture in processed plant foods. The use of calcium and calcium chelating agents to produce firmer or softer products or to shorten processing times has been shown to be feasible.

Fig. 3. TEM of thin-sectioned raw potato showing compact cell wall (CW).

Fig. 4. TEM of thin-sectioned potato which had been treated with pectinase enzyme showing diffuse cell wall splitting into layers.

Fig. 5. SEM of cut surface of raw potato showing cell walls fractured across (arrow). Prepared by solvent dehydration followed by vacuum drying from amyl acetate.

Fig. 6. SEM of cut surface of pectinase-treated potato showing cell walls separating along middle lamella. Preparation as Fig. 5.

Proteins

Light microscopy observations on pre-formed 'emulsions' prepared with a soya isolate were related to cooking losses by Parker and Lewis (1976). Salt had a large effect on the cooking losses of these systems and in particular the time of addition of salt during the chopping process was important. Dissolving salt in the water before adding the soya isolate and fat produced high cooking losses. When salt was added at the end of the chopping process the cooking losses were lower than when no salt was present. Microscopically, stabilisation of the fat was seen to be due to the formation of a protein network entrapping clumps of fat cells and free fat. When salt was present in the water before the soya isolate then less protein was dispersed from spray-dried particles and so a coherent network was not formed. When salt was added at the end of the mixing process the soya isolate had already dispersed and the salt appeared to improve the heat stability of the network.

These observations have been followed by studies on gelation of soya isolates, in this case using mainly electron microscopy and gel strength measurements (Groves, personal communication). Early observations indicated that the fixation step was not critical in handling cooked and cooled gels although
fixation with hot glutaraldehyde was useful in following changes during cooling of the gels. Working with soya isolate at a level of 16% m/V in water, it was found that a substantial amount of the protein remained in spray-dried particles even after prolonged mixing and standing overnight. During cooking the particles swelled and more protein was extracted to form a network. The soya isolate gel was a composite structure of swollen spray-dried particles held together by an extracted protein cement (Figs 7 and 8). In this sense soya isolate gels resembled starch gels reported earlier (Lewis, 1981). The formation of a network in the extracted protein was partly a non-reversible aggregation during heating and partly a reversible aggregation on cooling. Heating to retort temperatures (110°C and 120°C) produced much greater aggregation during heating to both the extracted protein and that remaining in the spray-dried particles (Figs 9 and 10).

Salt had a marked effect on the gelation of soya isolate, particularly if the salt was dissolved in the water before the soya isolate was added. In this case the gel strength was lower than with no salt at temperatures up to 80°C but was increased at temperatures above 80°C. The gel at higher temperatures was found microscopically to be made up of swollen spray-dried particles which had very little cementing network but which interlocked together – rather like a three-dimensional jigsaw (Figs 11 and 12).

Different soya isolates were examined and some differences were observed in the extent of dispersion from spray-dried particles and the effects of salt. However, in all cases the behaviour of the spray-dried particles was a significant feature in the gels.
Fig. 11. TEM of thin-sectioned soya isolate gel with salt, heated to 98°C. Note very little extracted protein (arrow) between spray-dried particles.

Fig. 12. SEM of soya isolate gel with salt, heated to 98°C. Note swollen spray-dried particles (S).

Meat Products

Some observations on the effects of salt and polyphosphate on pork meat have already been reported (Lewis, 1981) and further investigations are being reported elsewhere. (Lewis et al., 1986).

Various meat cuts have been surveyed to assess their fat-binding ability. In the methodology used, cryostat sections were stained with acid fuchsin to show protein and osmium tetroxide to show fat. Osmium staining was found to produce less ‘smearing’ than the more usual stains such as Sudan Black B. This work showed that meats behaved in one of four ways.

Type I meats broke down into small pieces during chopping but did not form a network. These meats did not retain fat well. Type II meats formed a coarse network after a short period of comminution but this broke down on longer chopping to give isolated meat fragments surrounded by fat; again these meats did not retain fat well. Type III meats formed a coarse network after a short period of comminution and this developed into a finer, more extensive network on prolonged chopping; these meats generally retained fat quite well. Type IV meats formed a fine network after a short period of comminution and this network was retained on further comminution; these meats were best at retaining fat.

All samples characterised as types I and II had a pH of 6.0 or less, whilst the pH of type III and IV meats ranged from 5.75 to 6.35.

A meat which showed type II behaviour was comminuted with polyphosphate present in addition to salt and water, and in this case the behaviour became that of type III and fat retention was considerably improved.

This work indicated that the performance of some meats could be improved by the addition of polyphosphates and suggested that meats with lower pH values might be more amenable to polyphosphate action. This idea is developed further by Lewis et al. (1986).

Toffee and Sugar Confectionery

Campbell et al. (1951) studied the keeping qualities of boiled goods. They placed spots of boilings between cover slips and observed the change at the edge of these spots when the coverslips were stored in a moist atmosphere and the edge was examined daily. They showed that crystallisation started as the viscosity was reduced near to the surface by absorption of water and that the zone of reduced viscosity spread through the mass of the boiling as further crystallisation released more moisture. The effectiveness of various dusting agents in preventing moisture uptake by boiled sweets was also studied, as was the effect of porosity of the structure of sugar on moisture uptake. Finer dusting powders and less porous structures were found to reduce the moisture uptake.

This work established guidelines for composition and storage conditions of boiled goods that form the basis of present-day commercial practice. In these studies microscopy was used mainly to illustrate the changes occurring in boiled goods so that Grover (1941) and Lees (1965) use micrographs to illustrate features of crystallisation.

The use of microscopy as a truly explanatory tool was not revived until the advent of electron microscopy at the R.A. in the late 1960s. Stansell and Jewell (1975) studied crystallising systems with microscopy and proposed the following hypothesis of sugar crystal growth.

“Nucleation in sucrose solutions is related to the availability of free hydrogen bonding sites to permit sucrose/sucrose interaction and this occurs above 75% sucrose (s = 1.20 supersaturation). Nucleation is followed by the formation of aggregates of particles of dimensions of 25-30 nm which undergo further association to produce crystals and thence crystals. If the mother liquor concentration falls below a supersaturation of 1.20 then growth proceeds as layer growth.”

An example of aggregates packing into larger structures is shown in Fig. 13.

This hypothesis helped to explain the rapid growth of crystals during the early stages of sugar crystallisation and led to the development of a procedure for growing small crystals to make an icing sugar rather than grinding large crystals (Stansell, 1977). The technique involved shearing sugar syrups at controlled concentrations and temperatures to induce regions of crystallisation within the mass of syrup. Icing sugar produced by this technique, known as microcrystalline sugar, consisted of clean-faced crystals, as revealed by microscopical examination (Fig. 14). The properties of icing sugar were related to the microscopical characteristics of the sugar, and a large number of very fine particles (less than 0.2 μm) were found in milled icing sugars (Fig. 15). It was considered that these very fine particles could lead to the formation of a compacted cake and thus account for some of the poor handling properties of icing sugar. Microcrystalline sugar which had very few fine particles had
rather better handling and dispersibility properties than conventional icing sugar. In this case particle sizing was carried out by image analysis techniques and by Coulter Counter. It is interesting to note that the 'fines' in icing sugar represented an insignificant proportion by weight of the sugar although they represented a large proportion of the number of particles present in the samples. This indicates the importance of specifying whether a size distribution is based on weight (or volume) or on numbers of particles.

Toffee has also been studied microscopically. Grover (1937) used microscopy to study emulsification of oil in toffee making. Mineral oil incorporating a red dye was used to prepare toffees and the size of droplets was assessed at the pre-mix stage and in the finished toffee. Droplet size in finished toffee was assessed by dispersing the toffee in warm water, mixing with a little gelatin and allowing a drop of the dispersion to set under a coverslip on a microscope slide. This procedure is similar to the one currently in use at the R.A. except that agar is used instead of gelatin as this tends to produce less coalescence with some of the modified milk proteins now available. In Grover's study, three different skimmed condensed milk samples of differing consistency were used to produce toffee and the thinnest consistency milk was not quite so good as the others in producing an emulsion at the pre-mix stage. However, the emulsion that was formed by thin-consistency condensed milk was more stable on cooking to produce toffee. Later work on toffee involved electron microscopy and again the effects of different condensed milks on the structure and flow properties of the toffee were examined. This work indicated that toffee made from condensed milk with small casein micelles tended to have a small fat droplet size in the finished toffee; this led to a high-yield stress and produced toffee that was difficult to deposit. Hence it was considered that some breakdown of the emulsion during toffee manufacture was desirable. Recently, a wide range of modified milk proteins has been studied as ingredients in toffee making. In this work the light microscopy technique was adapted for electron microscopy and this has allowed the interactions between proteins occurring at the fat-droplet membrane to be studied in more detail. The toffee dispersion in agar was fixed, dehydrated and embedded for thin sectioning. Care must be exercised in interpreting results from dispersed toffees. In this case, freeze-fracture studies were carried out in parallel to the thin sectioning and light microscopy was also used to assess the toffees. This approach indicated that whey proteins tend to form a flexible membrane at the surface of the fat globule membrane and in toffees made only with whey this results in a fairly stable emulsion during toffee making. When casein is present it associates with whey at the interface and this makes the membrane more brittle, allowing some breakdown of the emulsion during cooking (Figs 16 and 17). The size of casein micelle is also important: in calcium-reduced milks the casein micelles are very much smaller than those in standard skimmed milk powders (Fig. 18). The smaller micelles do not appear to produce brittleness in the membrane and the emulsion remains stable during cooking. These findings were linked to properties of the toffees, in particular, viscosity, colour, storage behaviour and texture. The state of the emulsion and fat-droplet membranes related to the viscosity, those toffees with small droplets and flexible membranes producing toffees with lower viscosities.

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![Fig. 13. TEM of freeze-etched preparation of 80% sugar syrup. Note aggregates of microcrystals (A) and glass regions (G). Shadowing direction approximately top to bottom, printed through intermediate.](image)

![Fig. 14. SEM of microcrystalline sugar. Note clean crystal surfaces.](image)

![Fig. 15. SEM of icing sugar. Note many fine particles (arrows) on surface of crystals.](image)
Fig. 16. TEM of thin-sectioned toffee (caramel) made with whey protein. Prepared by gently dispersing toffee in agar solution and embedding blocks of agar containing toffee dispersion. Note membrane (arrows) around fat droplets (M).

Fig. 17(a). TEM of thin-sectioned toffee made with skimmed milk powder. Prepared as Fig. 16. Note casein aggregates (C) on membrane. (b). TEM of freeze-etched toffee made with skimmed milk powder. Shadowing direction approximately top to bottom, printed through intermediate.

Fig. 18. TEM of thin-sectioned toffee made with calcium-reduced skimmed milk powder. Prepared as Fig. 16. Note small size of casein micelles (arrows).

Some Recent Work at the Leatherhead Food R.A.

Fouling of Heat Exchangers by Foods

The build-up of deposit on heat exchangers is a matter of considerable economic consequence to the food industry. The deposit results in poor heat transfer, which in turn means that higher temperatures and more energy are required for a given heat process. The deposit also increases the amount of cleaning time required and may break off to contaminate the product.

Most of the previously reported work on this topic has been carried out on dairy products and in these studies very little attention has been given to a study of the microstructure of the deposits.

The work at the Food R.A. combined microscopy with food engineering. A pilot-scale fouling rig was designed and built and consisted of a hot water or steam-heated pipe placed inside an insulated tube through which the product was circulated. The steam pressure or water temperature in the heating pipe could be controlled and monitored and the product inlet and outlet temperatures were recorded throughout each experiment to determine the change in heat transfer efficiency as a deposit builds up on the pipe. Blood plasma was used as the protein for most of the experimental work but critical observations were checked with soya isolate, whey protein isolate, egg albumen and gelatin solutions.

Microscopical methods. Samples were examined by light microscopy and transmission electron microscopy. Several different methods were used depending on the nature of the deposit.

1) Direct observation of deposit on heated pipe by SEM. Three-inch lengths were unscrewed from the length of the pipe and the deposit was allowed to air dry. The pipe section was carbon coated and examined in a Cambridge S250 scanning electron microscope.

2) Removal of dried deposit. When the pipe was removed from the rig whilst still hot the deposit dried on the pipe within a few minutes. Generally, the deposit could be removed from the pipe with a scalpel. The removed deposit could be examined directly in the SEM after sputter-coating with platinum.

3) Removal of wet deposit. In most cases the pipe was cooled before removal from the rig and the deposit took over an hour to dry on the pipe. In order to allow a rapid 'turn round' time of experimental runs the deposit was removed whilst still wet. The removed deposit was floated in a petri-dish containing distilled water, collected on cellulose acetate sheet and allowed to air dry for SEM examination. Samples for TEM and light microscopy were transferred to a fixative; either 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, or Minsky’s fixative (a commercial fixative available from National Diagnostics Ltd) was used. It was found that samples could be stored in the fixatives for long periods at room temperature without noticeable deterioration. After fixation, the samples were dehydrated through a graded alcohol series, embedded in LR White resin and sectioned at 2 μm thick for light microscopy and less than 100 nm thick for electron microscopy. Some samples were transferred from absolute alcohol to amyl acetate and vacuum dried, or to Freon 113 and air dried for SEM. The structures seen were essentially similar for both fixatives used and for direct air drying and solvent vacuum or air drying. Sections for light microscopy were stained with acid fuchsin or mounted and stained in 0.2% toluidine blue, 30% glycerol/0.66% phenol mountant. For electron microscopy, sections were stained in 4% aqueous uranyl acetate followed by 0.2% lead citrate.
Features of Food Microscopy

or 7% methanolic uranyl acetate without counterstain or 2% alcoholic phosphotungstic acid.

4) Replica technique. In some cases the deposit was very difficult to remove from the pipe in pieces large enough to examine by SEM. Sometimes a segmented pipe was used (method 1) but a replica technique was also developed. This technique was essentially as described by Scott (1982). A primary replica of the dried surface was prepared with Xantopren Blue (Bayer Ltd) – silicon rubber dental impression material. A secondary replica was prepared from the primary replica using Stycast 1225 (Emerson and Cumming Ltd). The directions given by the manufacturers of these products were followed. The secondary replica was sputter-coated and examined by SEM.

The replica technique was compared with some direct observations and found to give good correlations for 'low-profile' deposits, which were generally the most difficult to remove from the pipe. Thicker deposits with larger undulations gave some problems with air bubbles in the replica; however, these could be easily recognised by SEM.

Electron microscopy was carried out on a JEOL 1200 EX electron microscope operating at 80 kV in the TEM mode and 40 kV in the SEM mode. Larger samples and samples for X-ray microanalysis were examined at ERA Technology, Leatherhead, using a Cambridge S350 SEM equipped with a Link 860 analysis system; this microscope was operated at 20 kV.

Observations. The initial conditions for operating the fouling rig used steam at low pressure as the heating medium and a heating time of 45 minutes using 1% blood plasma in tap water constantly recycled through the plant to build up a deposit. The essential structure of the deposit was shown in Figs 19–21. Figure 19 shows a stereo pair of the surface of the deposit as seen by SEM and reveals a deposit with many cavities. The nature of these voids is revealed by examining cross-sections; Fig. 20 shows the light microscopy view of the deposit and the montage presented in Fig. 21 shows the TEM view. X-Ray microanalysis spectra from the top and bottom surfaces of the deposit are shown in Figs 22 and 23. Our interpretation of these results is as follows. The deposit consists of at least two layers. A primary layer is formed close to the heated surface as a compact layer of protein, which is also associated with calcium either in the form of protein complexes or as 'scale' deposited independently of the protein. A secondary layer (or layers) is formed on top of the primary and this layer is much less compact, containing protein flocculates. The overall structure of the deposit is highly aerated, suggesting that air or steam bubbles forming at the pipe surface are acting as nuclei for the denaturation of the protein film.

This interpretation, once accepted by all those involved with the project, led to two main avenues of investigation: (i) the influence of calcium ions in the water on the deposit; and (ii) the influence of air/steam bubbles on the deposit.

The influence of calcium ions was studied using double-deionised water with calcium chloride added at various levels. In general terms the deposit was thicker at higher calcium levels and at low calcium concentrations the deposit was confined to a primary layer structure (Fig. 24). Increasing calcium levels increased the amount of secondary layer produced. Runs with tap water and no protein produced a scale deposit on the pipe (Fig. 25). The effects of steam/air bubbles was pursued in two ways. By using lower heating temperatures (e.g. water at 80°C and 90°C) it was possible to obtain similar deposit structures to those obtained with low-pressure steam, indicating that air bubbles rather than steam were the more likely cause of the deposit build-up. The sequence of events seems to be that air comes out of solution at the heated pipe surface and protein denatures at the hot air surface, trapping the air in place and building up the deposit layer. Air is a good insulator and so heat transfer efficiency falls off quite rapidly as the deposit builds up. The second approach was to apply a back pressure to the product side of the fouling rig. As the applied back pressure was increased the amount of deposit decreased until at about 10 KPa over pressure the deposit was hardly present (Fig. 26).

Conclusion. The work on fouling is a good illustration of the practical application of microscopy. The methods used, especially allowing samples to air dry, may be considered crude but they are effective in dealing with a type of material which is probably not widely encountered outside the food industry. Apart from the technical problems of preparing samples, the presentation of the results in a convincing way was also important: in this respect the stereo pairs, which give excellent 3-D effect, were a major asset. Once the interpretation had been accepted, the follow-up experimentation to confirm the findings could be planned. Throughout the work microscopy was undertaken alongside heat transfer and thickness measurements with generally good agreement. The work presented here is an extract from a more comprehensive study of the factors affecting protein fouling of heat exchangers.

Fig. 19. SEM of deposit of blood plasma on heated surface. Stereo pair. Note large air inclusions (arrows).
Fig. 20. (above) LM of section through blood plasma deposit from tap water. Note air inclusions (arrows) in layer.

Fig. 21. (left) TEM montage of thin-section through blood plasma deposit from tap water. Note dense primary layer (P), flocculant secondary deposit (F), outer membrane (O) and air inclusions (A).
Features of Food Microscopy

**Fig. 22.** Top surface of pipe deposit.

**Fig. 23.** Bottom surface of pipe deposit.

**Fig. 24.** TEM of thin-section through blood plasma deposit from deionised water. Note thin compact primary layer (arrow), same magnification as Fig. 21.

**Fig. 25.** SEM of scale from tap water. Prepared by replica technique.

**Fig. 26.** SEM of deposit of blood plasma from water at 22 psi back pressure and 10 psi steam pressure. Note fewer air inclusions (arrows) in deposit.
Foreign Bodies in Foods

Foreign bodies is a constant cause of concern to food manufacturers, and people who use microscopes in the food industry are often expected to assist in the investigation of foreign bodies. Consequently, the first contact between a food technologist and a microscopist often relates to a foreign body investigation, and so the technologist's first impression of food microscopy will often depend on how well a foreign body is identified. It is well worth while for the food microscopist to develop skills in handling foreign body problems since these will establish his or her reputation in the eyes of many food technologists. One of the main problems in foreign body identification is the difficulty in specifying which test or tests should be carried out since each occurrence requires an individual approach.

The literature of general approaches to foreign bodies in food is fairly limited although Grahame (1981) covers a wide range of contaminants; Walls (1965) and "The Particle Atlas" (McCrone and Dely, 1973) are also extremely useful and cover a range of materials likely to be found as foreign matter in foods. Specific types of foreign matter, such as insects, hairs and wood, have a more extensive literature, and a recent bibliography (White and Shenton, 1984) gives a good introduction to this literature.

Two recent areas of study at the R.A. have related to foreign bodies in foods. The first of these was prepared by Smith (1983) and is presented as a scheme for examining foreign material contaminants in foods. The scheme gives a good approach to foreign bodies and suggests the following stages in identification. A preliminary examination should be carried out to obtain as much information of the sample as possible with as little disturbance as possible. This generally involves the use of a low power stereomicroscope and checking any observations against the recorded history of the sample. During the preliminary examination an attempt should be made to classify the sample, and the following categories are suggested, along with some comments to aid classification: recognisable objects; metal; metal/non-metal composite; animal/vegetable (biological); crystalline; fibrous; laminar; amorphous (hard); amorphous (soft); composite.

Once the sample has been placed in a category, a more specific identification can be attempted. This can be usefully based on checklists of possible foreign bodies for each category and confirmatory tests can be applied for each suspected item. If identification is not possible, an attempt should be made to reclassify the sample in the light of the tests carried out. A simple example may help to clarify this scheme. Some black cigar-shaped objects about 5 mm long and 2 mm wide were submitted. At first sight these were considered to be mouse droppings. However, when they were cut open they were found to be quite firm and to have white centres. These were sectioned and found to consist of masses of filaments. The bodies were now classified as fungal in origin and this was checked by staining in cotton blue in lactophenol. Finally, our mycologist confirmed that they were indeed mould sclerotia.

Checklists and suggestions for confirmatory tests are included in Smith (1983) and both the initial classification and confirmatory tests make extensive use of microscopical techniques. Many of the microscopical techniques are given in a Manual of Microscopical Methods (Lewis, 1978).

Glass fragments. Glass fragments are among the more common foreign bodies found in foodstuffs. In most years about 10% of prosecutions for foreign bodies in food relate to glass (Martin, 1984). There is a wide range of possible sources for glass contamination and it is in the food manufacturer's interest to identify the origin so that action can be taken to prevent further occurrences.

The first stage in the analysis of glass is to examine the sample in a stereoscopic light microscope at magnifications of 10 x to 40 x, asking the following questions: Are any original surfaces present? Are the surfaces moulded, polished or drawn? Are there chips or scratches which are likely to have been caused before breakage or during a food-processing operation? Is any debris present on the surfaces; is it present on all surfaces? What can be deduced about the shape of the original items; in particular can a diameter of curvature or thickness be estimated?

Typical features seen by low-power microscopy are shown in Figs 27–35. The initial examination of the glass will often give a strong indication of the glass origin and confirmatory tests can be applied. Debris from the surfaces can be removed and identified using a compound microscope; this will often reveal the nature of the foodstuffs with which the glass has been in contact. The diameter of curvature may be estimated; larger pieces can be estimated by fitting to standard curves whilst examining at low magnification, whilst smaller pieces can be derived by interference fringes. Locke (1984) describes equipment for obtaining such fringes; a less elaborate method using the interference fit between the sample surface and a glass slide is described in Lewis (1978). Some interference patterns are shown in Figs 36 and 37.

Refractive index and density are used widely in forensic science, particularly to compare samples. Hot-stage methods and dispersion-staining techniques are often used for determining refractive index, and extremely good discrimination between samples is possible (McCrone, 1974; Locke, 1985). However, in tracing the origin of an unknown glass sample, refractive index and density are of less value because of the overlap of values for different glass types. Borosilicate glass (e.g. Pyrex) can generally be identified by its low refractive index and density, but window glass and container glass tend to overlap each other. A list of refractive index and density ranges for glass types is given in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Glass Type</th>
<th>RI</th>
<th>Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borosilicate</td>
<td>1.47–1.48</td>
<td>2.2–2.3</td>
</tr>
<tr>
<td>Container glass</td>
<td>1.51–1.52</td>
<td>2.5–2.7</td>
</tr>
<tr>
<td>Window glass</td>
<td>1.51–1.54</td>
<td>2.4–2.6</td>
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Energy-dispersive X-ray microanalysis was investigated as a possible means of examining glass with a view to determining its origin. A total of 280 samples was examined, including food-container glass, domestic table and kitchenware glass, lighting glass, 'Pyrex' glass and some specialised glasses. Small fragments broken from the glass were mounted, fracture side upwards, in Tempfix on a piece of microscope slide. Care is taken to align the fracture surface parallel to the slide; this is done by manipulating the sample under a dissecting microscope whilst the Tempfix is still warm. The fragments were carbon coated by evaporation from four carbon fibre sources at a pressure of ~ 1.10^-4 Torr. The glass samples were examined in a Cambridge S250 scanning electron microscope operated at 20 kV and equipped with a Link
Fig. 27. Irregularities (arrows) in moulded surface.

Fig. 28. Container glass sample showing Rockware logo.

Fig. 29. Groove (arrow) produced by wire in reinforced window glass.

Fig. 30. Scratches (arrows) on rim of 'Pyrex' basin.

Fig. 31. Backscattered electron image of white metallic flecks (arrow) on 'Pyrex' glass. X-Ray microanalysis showed the flecks to be iron and chromium.

Fig. 32. Chips caused by machinery.

Fig. 33. 'Stretch marks' (arrows) which run around the side of drinking glasses.

Fig. 34. Fine capillary (arrow) in drawn tubing such as fluorescent light tubes.

Fig. 35. Optically polished lens (L) and ground edge (G).
All glasses were examined under the standard conditions with an ultra-thin carbon window, and additionally some glasses were examined at 40 kV accelerating voltage and/or with a beryllium window. This made it easier to discern low levels of barium, chromium and iron. With some glasses (e.g. light bulbs), inside and outside surfaces were examined in addition to fracture faces.

Currently, glass samples are analysed routinely using a beryllium X-ray window and efficiency factors used to relate the counts to those obtained with a carbon window. Two standard glasses are included with each batch of analyses to check the efficiency factors.

Estimates of variation were made by repeatedly examining the same area, analysing different pieces of the same item, analysing pieces from different items in the same manufacturing batch and analysing pieces from different manufacturing batches. These suggested that within-item and within-batch variations were likely to be as great as between-batch variations and indicated that, where possible, several different pieces of glass from each sample should be analysed.

Examination of the analyses of glasses in the different categories, e.g. Pyrex, container, domestic, etc., suggested that the calcium and sodium peaks formed a basis for some discrimination. Lead-containing glasses could also be separated as specialised glass. With regard to non-lead-containing glasses, Pyrex and heat-resistant glasses had low sodium contents (3–6% of total peak area) and very low calcium levels (< 0.2%). Non-Pyrex glasses had higher sodium levels (> 7%). U.K. container glass had fairly high calcium levels (mostly 8.5–12%), whilst domestic glass was slightly lower (mostly 7–9%) and lighting glass was lower still (mostly 4–6.5%). Window glass had a variable calcium distribution, although a number of samples (including Pilkington and most car windscreen) had calcium levels around 7–8% (of total peak area) and a smaller group of window samples had levels around 11–14%. Modern window glass, produced by the float process, could often be recognised by a small tin peak on one surface. Some further differentiation seemed possible based on potassium levels and magnesium levels, and a scheme was drawn up to give a guide to the likely origins of glass samples. (Figures 39–43 show typical spectra for selected glass types.) The scheme allowed glasses to be categorised into twelve categories. Some of the categories were quite distinctive. For example, forty-seven of the fifty-one glasses in one category were container glass; other categories contained mixtures of glass types. On the whole, the scheme seems to give a reasonable separation for the samples examined. However, the classification should not be applied too rigidly since some variation has been found between different pieces of the same glass. Also, only fairly limited numbers of samples in some categories have been examined, and it is not always possible to deduce the likely distribution of glasses as foreign bodies. However, the scheme seems to provide a guide to the likely origins of glass samples. In some cases, carrying out several analyses may help to decide between container or domestic glass but, in other cases, the overlap has to be accepted. Where possible, it is preferable to include possible sources of the glass for comparative analysis with the unknown sample, and in this way it may be possible to obtain evidence linking a particular container or other glass object with a foreign body. This can be particularly useful where a piece of glass is found within a glass container.

X-ray analysis is a useful technique for helping to identify the origin of glass but it should always be combined with as many other observations as possible.

<table>
<thead>
<tr>
<th>Window label</th>
<th>Window centre</th>
<th>First channel</th>
<th>Last channel</th>
<th>Net integral</th>
<th>Percentage total</th>
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<tr>
<td>Na</td>
<td>1000</td>
<td>880</td>
<td>1140</td>
<td>1224</td>
<td>8.96</td>
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<tr>
<td>Mg</td>
<td>1240</td>
<td>1180</td>
<td>1320</td>
<td>1368</td>
<td>1.00</td>
</tr>
<tr>
<td>Al</td>
<td>1460</td>
<td>1400</td>
<td>1540</td>
<td>626</td>
<td>0.46</td>
</tr>
<tr>
<td>Si</td>
<td>1760</td>
<td>1580</td>
<td>1960</td>
<td>106789</td>
<td>78.26</td>
</tr>
<tr>
<td>S</td>
<td>2200</td>
<td>2240</td>
<td>2380</td>
<td>40</td>
<td>0.03</td>
</tr>
<tr>
<td>Cl</td>
<td>2600</td>
<td>2540</td>
<td>2680</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>K</td>
<td>3300</td>
<td>3180</td>
<td>3440</td>
<td>879</td>
<td>0.64</td>
</tr>
<tr>
<td>Ca</td>
<td>3960</td>
<td>3500</td>
<td>3840</td>
<td>14392</td>
<td>10.55</td>
</tr>
<tr>
<td>Ba</td>
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<td>4340</td>
<td>4620</td>
<td>17</td>
<td>0.11</td>
</tr>
<tr>
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</tr>
<tr>
<td>Mn</td>
<td>5880</td>
<td>5800</td>
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<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Fe</td>
<td>6400</td>
<td>6200</td>
<td>6600</td>
<td>110</td>
<td>0.09</td>
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</tbody>
</table>
Fig. 39. Spectrum from laboratory 'Pyrex' glass.

Fig. 40. Spectrum from light-bulb envelope.

Fig. 41. Spectrum from window glass.

Fig. 42. Spectrum from glass mug.
The scheme has been used in practice for two years and the majority of glasses received have been recognised based on analysis and examination of surface features. In cases where known samples have been supplied (for comparison with unknowns), the scheme has accurately categorised the glasses.

Conclusion. Foreign bodies cover a very diverse range of materials and consequently no hard and fast schedule can be drawn up. Some general guidelines can be applied: firstly, gather as much information as possible whilst disturbing the sample as little as possible. From this information try to categorise the item and make a selection of confirmatory tests. If the confirmatory tests prove negative, re-assess all the available information, recategorise the sample and select some more confirmatory tests; repeat this process until the sample is identified - or no more material remains for analysis. In the identification of foreign bodies, microscopy and microscopic analytical techniques are invaluable as they allow information to be gained from a limited amount of material.

Future Prospects for Food Microscopy

There are many areas of 'conventional' food technology which are incompletely understood and where microscopy will undoubtedly be involved in providing explanations; the sources of variation in meats, protein functionality, behaviour of polysaccharide gelling agents and gums, textural consequences of low-fat and low-salt products and crystal properties of sugars, fats and water are examples of such areas. So, for a while yet, the microscopical approach which I have illustrated earlier will continue to be useful.

Taking a longer-term view, however, I see the food microscopist being asked rather more difficult questions. I think that these questions will arise from two sources: firstly, novel methods and ingredients will require understanding to allow optimisation of food processes and, secondly, microscopic techniques will allow more precise localisation of components. Some examples of the new technologies are irradiation, extrusion cooking, microwave heating, intensive processes and ingredients made by biotechnology and genetic engineering. The main difference in approach for the microscopist compared with dealing with more conventional operations will be that both the technology and the microscopy will be exploring new ground. In applying microscopy to present-day food science much of the technological behaviour of foods is well known, and hence microscopical observations can be related to well-known facts. In exploring new technologies it may well be that the microscopist will be able to guide the progress of technology rather than just aid process optimisation. As an example, Cassens et al. (1984) present the possibilities of producing custom-made meats with, say, a predominance of one type of fibre. The role of the food microscopist in this case could be to demonstrate the performance of each fibre type on processing and so advise on how to produce meat custom-made for particular products. This should provide an exciting role for those food microscopists bold enough to accept the challenge.

I see an equally exciting and challenging prospect when I look at the advances being made in microscopical techniques. X-Ray microprobe and other microanalysis methods, various cryotechniques, gold and fluorescent labelling, autoradiography and chemical location reactions are obvious candidates for applying to food systems and should allow the microscopist to approach more difficult problems involving the precise role of individual components in complex food mixtures. Other techniques such as scanning ion microscopy, scanning tunnelling microscopy, acoustic microscopy, scanning light microscopy, NMR microscopy, and X-ray microscopy, as yet in the early stages of development, may open up entirely new prospects for the food microscopist.

However, the food microscopist will need to adapt most of these methods in order to apply them to food systems with their high levels of salt, air, fat and sugar. Adaptation will not always be easy and will involve first mastering the techniques on conventional samples before attempting to apply them to foods. An example of one of our current problems may help to illustrate this point. An area of concern is the distribution of crystalline sodium pyroantimonate in bacon. At a fairly crude level the distribution can be determined by energy-dispersive X-ray microanalysis of 10 µm thick cryostat sections. This indicates a crucial role of meat connective tissue in controlling distribution, especially in injected meat samples. In order to obtain a more precise distribution of salt, particularly differences in concentration within and between different cell types, a chemical localisation technique was attempted. Pyroantimonate is widely reported as a precipitant for many cations including sodium (Simpson and Spicer, 1975). Small pieces of bacon were processed according to some of the recommended methods for sodium, in the anticipation that the likely problem would be that the precipitation would be too great throughout the tissue to allow differences in composition to be seen. In fact, the problem was that no precipitation at all was observed within the samples since all the pyroantimonate was precipitated outside the tissue by salt diffusing from the meat. As yet the problem remains unsolved although possibly cryoultramicrotomy could help if it were a more routine technique.

The explosive development of computer techniques in recent years has made it possible for image analysis to be used
Features of Food Microscopy

routinely. This has advantages and disadvantages. The advantages are that digital imaging allows many microscopical features to be presented in a more convincing way – for example using computer-generated colours or producing electronically ‘enhanced’ images – and also allows data from micrographs to be presented numerically. There is a danger, however, that these computer interpretations will be accepted as absolute fact. As with all aspects of food microscopy, the microscopist must use new techniques but must use them thoughtfully.

Conclusions

Microscopy has played a vital role in the explanation of food behaviour and will continue to do so. To be more effective, microscopy must be fully integrated into all stages of research projects. The future for food microscopy is exciting for those who are prepared to accept the challenge of guiding food technologists and are prepared to learn and apply advances in other branches of microscopy. Every food microscopist should have a clear philosophy to guide his approach. The current state of food microscopy is largely exploring the effects of major structural features on food behaviour, and this suggests to me that a bias towards the use of results, as opposed to seeking ultimate structural detail, is justified. The maturation of food microscopy as an innovative science may lead to a need for more subtle variations to be understood and the emphasis may need to change. However, the need for a clear philosophy will remain.

Epilogue

The trouble with allowing fools to look down microscopes is that they are likely to come to foolish conclusions. The food microscopist has a duty to ensure that conclusions produced from images are sensible in terms of the microscopical techniques used and relevant in terms of food processing.

Acknowledgements

All figures are reproduced by courtesy of the Leatherhead Food R.A. and I am grateful to the Director, Dr A.W. Holmes, for permission to present this paper. I am also grateful to the past and present members of the Microscopy Section whose efforts have helped to make this paper possible. Much of the work included was funded by the Ministry of Agriculture, Fisheries and Food.

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D.F. Lewis


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Discussion with Reviewers

R.J. Carroll: Could you comment on the future role of energy-dispersive X-ray (EDX) analysis and electron energy loss spectroscopy (EELS) in food microscopy research?

Author: I think there are two aspects to this question. Firstly, EDX is a technique which impresses chemists and food technologists and is useful in helping to overcome reservations about the use of microscopy. The image processing packages with modern systems also enhance the presentation of microscopy and encourage application of results. Although this is not strictly scientific, I think food microscopists would be well advised to take advantage of this aspect of modern instrumentation.

The second aspect is the more scientific application of these techniques. Currently, I think EDX has more applications because of its versatility, being useful in the SEM, STEM and TEM modes. I can see further developments in windowless detectors and combined use of EDX and backscattered electron detectors as leading to great improvements in the usefulness of EDX in the future.

EELS has slightly more restricted applications as it can only be used with thin specimens. However, the recently announced Zeiss microscope with integrated EELS and offering quite high resolution elemental mapping may indicate the way TEMs will develop.

Both techniques have the potential to locate elements in foodstuffs and this could lead to technological advances in food handling. For example, a knowledge of the distribution of salts in meat could lead to better microbiological control; monitoring the distribution of calcium or potassium ions in gels and plant tissues could lead to improved texture control of foods, and localising the elements of emulsifiers could lead to optimisation of recipes and processes.

The use of both EDX and EELS systems for elemental mapping in foods will, of course, require the solution of many specimen preparation problems.

D.P. Dylewski: Are only whey proteins incorporated into the membrane surrounding emulsified lipid in toffee? Or might other proteins be involved?

Author: Toffees made with whey protein as the only protein source show a membrane round fat droplets. I believe that casein, when present, associates with the whey protein in the membrane; where the casein micelles are large this results in the membrane becoming more brittle. With small casein micelles, such as in calcium-reduced milks, I suspect that the casein may be more directly involved at the fat/syrup interface. Of course, all the above is my interpretation of what I see and how the toffee performs; other explanations may be possible.

D.N. Holcomb: Are there any 'Food Microscopists' organisations? Would such a group be of value if they do not already exist?

Author: The Food Microstructure Journal and meetings are the only regularly organised events for food microscopists that I know about. I believe that the American Association of Feed Microscopists and Sektionen Futtermittelmikroskopie und Futtermittelmiikrobiologe der internationalen Arbeitsgemeinschaft fur Futtermitteluntersuchung exist to promote feed microscopy. I think that an organisation for food microscopists would be most useful and the renewed interest in food microscopy in recent years suggests that it should be possible to start such an organisation. Possibly the Royal Microscopical Society could be persuaded to foster such a group.
FIXING CONDITIONS IN THE FREEZE SUBSTITUTION TECHNIQUE FOR LIGHT MICROSCOPY OBSERVATION OF FROZEN BEEF TISSUE

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Abstract

The freeze substitution histological technique allows the indirect light microscopic observation and the quantitative evaluation of ice crystal size in frozen tissues. The use of chemical fixatives in substituting fluids improves morphological and histochemical preservation of the tissue. Fixation conditions become important since this step can introduce modifications in crystal sizes. Effects of temperature on: a) diffusion rate of fixing solution in tissue, b) recrystallization rate of ice in frozen beef and c) variations of the frozen water fraction were analyzed, establishing that isothermal freeze fixation constitutes an appropriate method for histological observation. Fixing at temperatures lower than that of the sample, in an attempt to reduce recrystallization effects, involves an increase of the frozen water fraction in the tissue leading to modifications in size of ice crystals and to changes in the histological structure.

Introduction

Examination of frozen tissue by light microscopy shows differences in the cell structure with regard to the location of ice crystals (intra- or extra-cellular) and fiber disruption. This examination can be performed in two ways: a) direct observation of ice crystals formed in the tissue (microscope equipped with cryo-stage) and b) indirect observation which considers voids to represent the spaces occupied by ice crystals in the specimen (freeze - substitution and freeze - drying techniques). Indirect observation is the more common method of choice.

Freeze substitution is based on the dissolution of ice within the tissue at low temperature in a fluid solvent containing chemical fixing agents which contribute to the morphological and histochemical preservation of the tissue. Fixing agents act mainly by introducing new cross-linkages between and within the molecules of the tissue constituents, thus immobilizing its structure (Feder and Sidman, 1958).

Traditionally, freeze substitution for both light and electron microscopy required that frozen tissues be cooled to very low temperatures, down to -20°C (Fernández-Morán, 1960; Van Harreveld and Crowell, 1964; Van Harreveld et al., 1965; Rebhun, 1972; Asquith and Reid, 1980). Richardson and Scherubel (1908) first reported observations on the use of ethanol as a substituting agent to examine ice crystal formation in frozen tissues. Van Hulle et al. (1965) compared direct observation with the fixing - staining procedure showing that tissue voids represent the size and location of ice crystals and that the freeze substitution technique did not result in any gross misrepresentations of the frozen structure. If fixation temperature differs from that of the sample, melting of ice or additional freezing of water can occur during fixation step, thus changing the ice crystal measurements (Kaess and Weidemann, 1961; MacKenzie, 1972; 1975; Mackenzie et al., 1975; Lampila et al., 1985).

Bevilacqua et al. (1979) and Bevilacqua and Zaritzky (1980) used the isothermal freeze substitution technique to analyze the effect of freezing on the ice morphology of beef muscle.

Freezing is one of the more common methods of food preservation. Storage temperatures lower
than - 18°C are indicated to retard frozen food deterioration during prolonged periods but involve considerable energy costs (Jul, 1984). When only short storage times are required the technique of partial freezing (super-chilling or light freezing) with storage temperatures close to - 5°C can be used to reduce energy consumption (Alemán et al., 1982).

Histological analysis of partially frozen tissues can be performed using isothermal freeze fixation technique. Since the diffusion rate of a fixing solution is not instantaneous, one concurrent phenomenon such as recrystallization could modify crystal sizes and shapes during fixation, thus altering subsequent measurements (Mackenzie et al., 1975).

Recrystallization (grain growth) is determined by the average grain size enlargement without increasing the total crystalline mass. Driving forces are essentially differences in surface energy of the grain boundaries, which allow the growth of the larger crystals at the expense of the smaller ones (Luyet, 1966; Love, 1966, 1968; Fennema, 1973). Recrystallization rate in muscle tissue increases with temperature and becomes important at thermal levels above - 7°C (S-y and Fennema, 1973; Voyle, 1974; Gerrits and Jansonius, 1975; Bevilacqua and Zaritzky, 1982).

The objective of the present study was to establish adequate fixing conditions (time and temperature) for application of the freeze substitution technique in the measurement of ice crystal sizes in frozen beef tissues especially at high subzero temperatures. Analysis was based on the evaluation of the following phenomena occurring in the samples: variation of the frozen water fraction with temperature, diffusion of the fixing solution and ice recrystallization rate in tissue.

**Materials and Methods**

**Freezing Procedure**

Beef samples (Semimembranosus muscle, 60 hours post-mortem), were frozen in a meat exchanger where methanol from a Lauda UK 50 D-W Cryostat was circulated with a temperature control of + 0.1°C. The freezing rate was regulated by modifying the temperature of the refrigerant (-20 to -30°C) and interposing acrylic slabs between the meat and the heat exchanger. Heat flux from both sides was unidirectional and parallel to the muscle fibers. Lateral insulation was provided by the use of expanded polystyrene 5 cm thick. The corresponding thermal histories were monitored by copper-constantan thermocouples which were inserted in the border and center of the meat pieces. Freezing rates were represented by the time necessary for the central point of the sample to change its temperature from -1 to -5°C (characteristic freezing time, t°).

**Determination of the Fixative Diffusion Coefficient**

The freeze substitution method was applied using Carnoy fluid as the fixative (absolute ethyl alcohol 60%, chloroform 30% and glacial acetic acid 10%, v/v). Ethanol solutions of picric acid (1%) and mercuric chloride (1%) were also assayed. According to the results of Bello et al. (1981) picric acid denatures the proteins of the tissues and coagulates them, but causes extreme shrinkage; mercuric chloride distorts the tissue less than picric acid does. The general appearance of the tissue was improved using Carnoy fluid as in previous works (Bevilacqua et al., 1979; Bevilacqua and Zaritzky, 1980, 1982).

The diffusion coefficient of the Carnoy fluid in frozen beef tissue was measured at different temperatures in meat samples simulating unidirectional mass transfer conditions. Beef samples of 3.5 x 3.5 x 1 cm were frozen as described above reaching final uniform temperatures of -4, -10, -20 and -26°C. Carnoy fluid with Methylene blue (0.1% W/V), used as an indicator to facilitate visualization of diffusion through the tissue, was allowed to cool in storage chambers to the same final temperatures. Specimens were transferred to a volume of fixative that was 100 times the tissue volume and freeze fixation was allowed to proceed isothermally. Depth of penetration (δ) of the fixative was measured photographically under an S.R. Zeiss stereomicroscope equipped with a Zeiss MC 63 Camera at 6 hour intervals. Experiments at the assayed temperatures were performed in triplicate.

**Histological Method. Effect of Fixating Temperature**

Beef samples 5 cm in diameter and 1 cm thick were frozen as described above reaching a uniform final temperature of -5°C (typical of a partially or lightly frozen tissue). For histological analysis (freeze substitution method) small pieces of 0.5 cm in diameter and 1 cm thick were cut from the initial specimens in a cold chamber at -5°C to avoid thermal changes. These specimens were fixed at two different temperatures (-5 and -15°C) to analyze the effects of this step on ice crystal morphology. Experiments were performed in quadruplicate.

Fixation times at each temperature (t°) were obtained from the results of the diffusion coefficient experiments previously described. Once this step was accomplished, samples were brought to room temperature, dehydrated using a series of gradually increasing concentrations of ethyl alcohol, cleared in benzene and embedded in paraffin (56-58°C). The sectioning was done with a rotary microtome (American Optical, model 820) using steel knife; section thickness ranged from 8-10 μm. Sections were mounted on glass slides and stained with haematoxylin-eosin.

**Ice Crystal Measurements**

Micrographs of the frozen specimen sections were obtained with a Leitz Ortholux II microscope equipped with a Leitz Vario-Dphthalm Camera. A Zeiss Morphomat 30 semi-automatic image analyzer working on the principle of coordinate measurement by quantifying the propagation time of magnetically induced waves (magnetostriction), was used to obtain, using an adequate internal program, the equivalent diameter of the holes left by the crystals in the tissue and the total area occupied by ice. The equivalent diameter (De) was defined as the diameter of the circle which has the same surface area as the measured figure.

Mean crystal diameters and standard deviations were obtained from a frequency distribution of more than one hundred crystals per field. Confidence limits for the mean crystal diameter were calculated using the Student "t" test (P > 0.05).
Fixation in Freeze Substitution Technique

Results and Discussion

Effects of Fixing Temperature on Histological Structure of Frozen Beef Tissue

Histological transverse sections of bovine semitendinosus muscle (60 hours post-mortem), frozen at -5°C with a characteristic freezing time \( t_f = 20 \text{ min} \), and fixed at -5 and -15°C, are illustrated in Fig. 1a) and b) respectively. The analysis of the micrographs in the image analyzer equipment showed that samples fixed at -15°C have a significantly (\( P < 0.05 \)) higher proportion of ice and larger mean crystal diameter than those at -5°C. The experimental ratio of areas occupied by ice at -15°C with respect to -5°C was 1.20 and the corresponding mean equivalent diameters were \( D_{15} = 20.46 \pm 1.71 \mu \text{m} \) at -15°C and \( D_{5} = 12.41 \pm 2.84 \mu \text{m} \) at -5°C. These results are attributed to the effect of temperature on the frozen water fraction in the tissue. According to Fig. 2, 85% of total water in beef muscle is frozen at -15°C and only 74% at -5°C (Riedel, 1957), leading to a theoretical ratio of frozen water fraction at these temperatures of 1.18 close to the experimental value of 1.20 obtained from the micrographs.

It can be observed that fixing at temperatures lower than that of the sample in an attempt to reduce recrystallization effect at high subzero temperatures, involved an increase of the frozen water fraction of the tissue which led to a different histological structure.

Determination of the Diffusion Coefficient of Fixative in Beef Tissue

To compare the use of low fixing temperatures with isothermal freeze fixation at high subzero temperatures, fixative diffusion coefficient and influence of recrystallization during fixation have to be evaluated.

A typical photograph indicating Carnoy fluid penetration in frozen beef tissue is shown in Fig. 3. The system can be mathematically considered as a semi-infinite media since during the

![Fig. 2. Equilibrium freezing point curve for beef tissue. Initial water content of the tissue on wet basis: 74%. From Riedel, (1957) and Mascheroni and Calvelo, (1978).](image)

![Fig. 3. Diffusion of the fixative in frozen tissue. The light zone shows penetration depth (6) of Carnoy fluid with methylene blue (6 = 0.34 after one day of fixation at -10°C). Arrow in the border of the tissue indicates direction of the diffusion flux.](image)
experiment fixing penetration depths were small in comparison with sample thickness in the direction of mass transfer. Values of fixing solution penetration (δ) as a function of time were used to determine diffusion coefficients (Crank, 1957) according to:

$$\delta = 4 \sqrt{Dt}$$  \hspace{1cm} (1)

where: δ : depth of fixative penetration (m); D : diffusion coefficient of fixative (m²/s), t : time (s). Values of D were calculated at different temperatures (Table 1). Activation energy (Ea) for the diffusion process of fixing solution was determined according to an Arrhenius type dependence on temperature:

$$D = D_0 e^{-Ea/RT}$$  \hspace{1cm} (2)

where $D_0$ is the pre-exponential factor. The activation energy was estimated from the linear regression of In D versus 1/T (Fig. 4) obtaining $Ea = 57,560 \pm 2390$ J/mole. Eqs. (1) and (2) allowed the calculation of total fixation times ($t_f$) expressed in hours at different temperatures:

$$t_f = \frac{\delta^2}{D_0} 1.736 \times 10^{-5} t$$  \hspace{1cm} (3)

Values of $t_f$ (Table 2) were obtained considering $\delta = 0.25 \times 10^{-2}$ m, which corresponded to the radius of the samples used for the histological treatment.

The determination of diffusion coefficients for the fixing solution in frozen beef tissue allowed estimation of total fixation times at different temperatures, accelerating the operation of the freeze substitution technique.

Recrystallization Effects during Fixation

Recrystallization could modify the crystalline structure during the diffusion of the fixative particularly at temperatures near -1.1°C (initial freezing point of beef tissue). To estimate this effect, increments in mean ice crystal diameters during fixation time were calculated according to the ice recrystallization kinetics in frozen beef proposed by Bevilacqua and Zaritzky (1982):

$$\frac{dD}{dt} = K/D$$  \hspace{1cm} (4)

that gives the following:

$$D^2 - D_0^2 = 2 K t$$  \hspace{1cm} (5)

where: $D$ : mean ice crystal diameter at fixation time; $D_0$ : initial mean ice crystal diameter; $K$ : recrystallization kinetic constant, with:

$$K = K' e^{-Ea/RRT}$$  \hspace{1cm} (6)

where:

$K'$ (preexponential factor) = 1.67 $\times 10^{-5}$ m²/s; $Ea$ (activation energy of recrystallization phenomenon) = 4.35 $\times 10^4$ J/mole.

The difference ($\Delta$) between the average ice crystal diameter at the total fixation time and the initial diameter ($\Delta = D - D_0$) should indicate the enlargement of ice crystals due to recrystallization during fixation.

Effects of initial crystal diameters and fixing temperature on $\Delta$ were estimated using Eq. (5) with $t = t_f$ (total fixation time). Two
typical average values of initial extracellular ice crystal with their confidence limits were considered (Table 2). As can be observed large initial crystal diameters led to smaller changes in Δ because the driving force for recrystallization increased with the average curvature of the surface (reciprocal of the mean crystal diameter, Eq. 4). Changes in the size of ice crystals during fixation at different temperatures were not statistically significant (p > 0.05) because increments in size due to recrystallization laid in the 95% confidence interval for the corresponding D0 value (Table 2).

Results confirmed that isothermal freeze fixation was an appropriate method for analyzing the histological structure and ice crystal pattern in frozen tissues. It was demonstrated that changes in crystal size caused by recrystallization were not statistically significant (p > 0.05) even when sample temperature was as high as -5°C since the simultaneous increase of the fixative diffusion rate and reduced total fixation times. When the temperature of the sample is lower than -15°C isothermal freeze fixation is also recommended. In this case, however, effects of recrystallization and percentage conversion of water to ice during the fixing step were notably reduced and an eventual fluctuation of the fixing temperature would not introduce any significant change in ice crystal sizes.

Conclusions

Determination of temperature effect on fixative diffusion coefficients allowed estimation of total fixation times thus accelerating the operation of the freeze substitution technique. Isothermal freeze fixation was an appropriate method for analyzing ice crystal patterns in frozen beef tissue even at high subzero temperatures (lightly frozen tissues). Fixing at temperatures lower than that of the sample in an attempt to reduce recrystallization effects (grain growth) involved an additional freezing of water in the tissue that changed ice crystal measurements. Experiments showed that recrystallization effects during isothermal freeze fixation produced non-significant (p > 0.05) changes in ice crystal diameters even when sample temperature was close to -5°C because fixation times were notably reduced.

Acknowledgements

The authors acknowledge Mrs. Marta Macias for typing the manuscript.

References


M.N. Martino and N.E. Zaritzky


Discussion with Reviewers

M. Brown: Why do you consider the evaluation of recrystallization effects important?
Authors: Reported data showed that recrystallization rates in frozen beef tissues are relevant at high subfreezing temperatures. Since fixative diffusion in freeze-fixation proceeds with a finite velocity, recrystallization phenomenon during fixation time can increase ice crystals modifying the initial pattern. Therefore, one of the objectives of this study was to evaluate this change. However, finally we demonstrated that recrystallization effect was negligible because diffusion rate of the fixative increases at high subfreezing temperatures reducing fixing time and the possibility of recrystallization during this stage of the histological analysis.

S. Cohen: How do the authors define lightly frozen tissue?
Authors: Lightly frozen or partially frozen beef tissue is that submitted to temperatures between -5 to -1.1°C (high subfreezing temperatures), having therefore a low proportion of water converted to ice. This kind of tissue becomes important since partial freezing improves the quality of the products with respect to the refrigeration process.
Changes in the Microstructure of Skipjack Tuna During Frozen Storage and Heat Treatment

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Abstract

Samples of fresh, frozen and heat-treated skipjack tuna muscle were observed by scanning electron microscopy. The photomicrographs were used to assess changes in the microstructure of fish muscle during frozen storage and thermal processing. Differences noted in frozen tissue could be related to the formation of gaps between fibers and the deformation of muscle fibers. No freeze mediated damage to the cell wall was observed at the lower limits, 20,000x, of high resolution scanning electron microscopy. The degree of muscle fiber shrinkage and erosion and the behavior of certain protein fractions was found to be affected by thermal processing. Granular material presumed to be primarily sarcoplasmic proteins aggregated at the surface of the muscle fiber and in the interstices between the sarcolemma and the muscle fiber. The sarcomere was disrupted upon precooking. The I-band and the A-band have congealed to form a single band which constitutes the majority of the sarcomere.

Introduction

Post-mortem fish muscle is susceptible to many physicochemical changes. Freezing and frozen storage of such tissue can have more profound effects. These changes can be broadly classified in the areas of texture, protein functionality or organoleptic quality. A slow rate of freezing and higher storage temperatures (-5 to -10°C) can distort cells and crush myofibrils (Bello et al., 1982). Freezing can cause deformation of the sarcoplasmic reticulum (Jarenback and Liljemark, 1975a,b,c; Liljemark, 1969). Further, to a limited extent, large ice crystals can rupture cell membranes thus initiating the outward movement of cellular constituents (Giddings and Hill, 1979; Hamm, 1960; Deatherage and Hamm, 1960). The investigation of structural damage has been accomplished more often with transmission than with scanning electron microscopy.

Scanomicrographs of rainbow trout muscle held under either chilled storage (Schaller and Powrie, 1971) or given thermal treatments (Schaller and Powrie, 1972) have been studied. Under refrigeration temperatures (3°C), for 6 days, the trout muscle showed a shortening of the transverse elements and a perforation of the sarcolemma (Schaller and Powrie, 1971). The majority of published work relating scanning electron microscopy (SEM) to muscle has generally involved heated beef muscle. Hoestetler and Landmann (1968) studied photomicrographs of beef longissimus dorsi during heating of muscle fiber fragments from 29 to 80°C. The results indicated that there was a decrease in muscle fiber width (23 to 27%) at temperatures from 53 to 77°C and that this change occurred within five minutes. Further, it was found that during the denaturation of myofilaments, soluble sarcoplasmic proteins were visible as amorphous particles around the muscle fiber. With this, the fiber became narrower, or shrank. The process was thought to be complete when the temperature reached 53°C. Schaller and Powrie (1972) observed a shrinkage of the muscle fiber and an accumulation of granular material between the muscle fiber and the sarcolemma in beef and chicken muscle heated to 97°C. This occurrence was not detected in trout dorsi heated to the
same end point. Granulation of the sarcolemma of bovine semitendinosus was found to occur at 60°C and edomysial collagen became nonfibrous at 60°C (Jones et al., 1977). Perimysial collagen was found to degrade at 70°C (Cheng and Parrish, 1976). Fish collagen deteriorates at lower heating temperatures.

In trout muscle heated to 97°C, a gap appeared at the H-zone and breaks across the fibers occurred at the Z-disc (Schaller and Powrie, 1972). Heating beef muscle to greater than 60°C also showed breaks to occur at the Z-disc and a wider or separated I-band region resulting (Jones et al., 1977; Cheng and Parrish, 1976).

The objective of this work was to assess changes in the microstructure of skipjack tuna during frozen storage and thermal processing. Attention was given more to the phenomena rather than to the biochemical mechanisms involved. The post-mortem biochemical changes and canning yields as affected by freezing and frozen storage are the subject of a separate paper (Lampila et al., manuscript submitted).

Materials and Methods

Brine-frozen raw, precooked and canned chunk style skipjack tuna (Euthynnus pelamis) was obtained from a commercial tuna processor. Tuna was received over a period of eighteen months from approximately fifteen different ships. Two to three skipjack tuna were evaluated from each ship. It was reported that the tuna were frozen according to commercially acceptable practices. At the time of microscopic examination, tuna samples had been frozen for ca. three months. Commercially frozen tuna (frozen at sea) was compared with fresh skipjack tuna obtained from Southern California and Hawaii.

A 2.5 cm thick steak was removed from behind the dorsal fin with the aid of a band saw. From this section a 2 cm² specimen was removed from the upper left quadrant of white loin with a clean, sharp scalpel. The cube of meat was placed into a clean petri dish and was sliced into 3 x 5 mm sections with an oil-free, sharp razor blade. Specimen sections were immediately immersed into fixative. The tissue, raw and precooked, in a frozen state was easier to slice into the appropriate size sections for fixation. Similar sampling procedures were followed for precooked and canned tuna.

Determination of Collagen

Samples of raw tuna were immersed into either a phosphate buffer alone or phosphate buffer plus collagenase (Sigma, Type VII). Specimens were incubated at 37°C for 4 h, using procedures as described by Eino and Stanley (1973).

Sarcoplasmic Proteins

Sarcoplasmic proteins were isolated as described by Hashimoto et al. (1979). These proteins were resolubilized and subsequently heat denatured by immersing specimens into a water bath at 100°C for 5 min.

Fiber Diameter

The diameters of muscle bundles were measured from photomicrographs. The mean of between sixty and ninety fiber bundles was calculated. Measurements were determined from the center one-third of photomicrographs taken at low magnification, with minimal tilt (less than 4°). Data were analyzed using the Analysis of Variance system of Madigan and Lawrence (1982) and Duncan’s Multiple Range test described by O’Mahony (1986). Formulas for unequal sample sizes were used.

Specimen Preparation

Specimens were fixed by a modification of the methods described by Sabatini et al. (1963). Fixation was conducted in a 0.1 M cacodylate buffer plus 2% glutaraldehyde (pH 5.8) for 16 h at 22°C. After washing in 0.1 M cacodylate buffer plus 5% sucrose, the samples were cryofractured. This step was followed by serial dehydration in ethanol and substitution with amyl acetate. Specimens were dried to critical point, mounted onto aluminum stubs and pulse-sputter-coated with gold. Specimens were viewed with either the Philips PSEM Model 501 equipped with a tungsten filament or the ISI OS 130 High Resolution Research Microscope equipped with a LaB₆ (lanthanum hexaboride) cathode.

Results and Discussion

Frozen Storage

As a rule, tuna destined for commercial canneries are frozen on board ship in the round in a concentrated (23%) NaCl brine to -10 to -12°C and may be held as such for as long as three months before further processing. In order to conserve energy and maximize brine freezing efficiency, the catch may be held in refrigerated sea water until ready for freezing. The actual on board freezing rate depends on the following factors: temperature of the fish and of the brine; the quantity of fish packed into a well; the circulation of the brine; and on the refrigeration systems and their efficiency. Both freezing time and the storage temperature affect the rate of growth and the final size of ice crystals. According to Stansby (1956) and Dyer (1971) the critical freezing range in which most of the water is frozen out is between -1 to -5°C. For an allied study in this laboratory, fresh skipjack tuna (1.5 to 2.5 kg) were frozen in a concentrated (23%) NaCl brine to simulate on board handling. The freezing rate was monitored and is presented in Figure 1 (Lampila et al., manuscript submitted). The length of time for the backbone temperature to decrease to -5°C and then to -12°C was 36 and 84 hrs, respectively (Figure 1). This is a relatively slow rate of freezing. Evidence of and conclusions regarding the effects of freezing rate and ice crystal damage on muscle structure are controversial.

Fresh, never frozen, tuna muscle fibers appear to be uniform in shape and fully contracted (Figure 2). Individual myofibrils and their transverse striations which delineate sarcomeres are well detailed. The diameters are ca. 2.2 µm in length. In contrast, after freezing to ca. -12°C, some deformation of tuna muscle fibers can be noted, a consequence presumably of ice crystal formation during freezing (Figure 3). Large spaces were noted between these muscle...
fibers, probably the site of ice crystal deposition. These findings are supported by published transmission electron micrographs of Bello et al. (1982); Jarenback and Liljemark (1975a, b, c) and Liljemark (1969). More recent scanning electron micrographs of rockfish isothermally freeze-fixed at either -5°C or -20°C present additional confirmatory evidence of the distortion of the fascicular grouping and a separation of the basal lamina from the muscle cell during freezing and frozen storage (Lampila et al., 1985). The deformation of the skipjack tuna tissue was however, not consistent. It is presumed that migration of sarcoplasmic fluid back into the cell upon thawing resulted in some cells returning to their native conformation. The consistency with which a uniform cellular shape is restored may be attributed to the original handling of the tuna; as practices of engineers on different ships may vary more than is reported.

Figure 1. This represents the freezing rate of tuna in a concentrated (23%) NaCl brine.

Figure 2. Longitudinal view of fresh, never frozen tuna.

Figure 3. The structural fibers of raw, previously frozen tuna muscle show deformation, a consequence presumed to be caused by ice crystal damage. Connective tissue (C) is indicated.

Figure 4. The connective tissue (C) or sarcotendina is shown surrounding a single muscle fiber from tuna frozen aboard ship.
The connective tissue surrounding a single tuna muscle fiber is shown in Figure 4. Presence of collagen was verified by treating tuna muscle samples with collagenase and establishing the disappearance of the tissues in question (Figure 5). At low magnification, the transverse striations characteristic of muscle tissue were evident. No ice crystal damage was evident to the structure below the surface of the cell membrane. The tissue was however fixed after being thawed, and, as previously suggested, the cells may have returned either to or very close to their native conformation.

Use of the SEM is limited to surface examination; therefore the condition of the material at the time of fixation is important. Due to the migration of fluids in and out of the cell during thawing, changes meditated by freezing and frozen storage may not be accurately preserved when the tissue is chemically fixed. As a consequence, some structural changes, such as a weakened sarcolemma, may not be detected. Further, the pliable nature of collagen may provide the extensibility needed for ice crystal growth, without showing direct evidence of cellular rupture.

Thermal processing

In the United States, tuna destined for human consumption is most commonly canned. Before canning, tuna is normally precooked to facilitate removal of the skin, bones and red meat. The precooking of tuna involves heating to a backbone temperature of about 60°C. As the standard retail size (No. 307x113) can, tuna is retorted at temperatures exceeding 111°C for periods of two to two and one-half hours.

Thermally processed (precooked and canned) tuna muscle underwent some expected changes in the structure. A granulation of the sarcolemma (Figure 6) proposed to be, on bee muscle proteins, a coagulation of collagenous and sarcoplasmic proteins (Cheng and Parrish, 1976; Schaller and Powrie, 1972), appears to have resulted from thermal processing as it was not detected on chemically fixed fresh or frozen tissue. Rupture of cellular membranes, or a weakening of the cell wall may have facilitated the outward movement of intracellular constituents and enhanced, to some extent, the degree of granulation noted. In cross-section, sarcoplasmic proteins, probable collagenous materials and other constituents were present in the interstitises between the shrunken muscle fiber and the endomysium (Figure 7). This material also appears to be granular in nature. Schaller and Powrie (1972) isolated this granular material by centrifuging isolated sarcoplasmic proteins and observing the same under the SEM. In this laboratory, sarcoplasmic proteins were isolated from raw tuna muscle by methods described by Hashimoto et al. (1979). The sarcoplasmic isolate was heat coagulated, fixed and observed under the SEM. The results concurred with those of Schaller and Powrie (1972) that the granular material formed between fibers during cooking could be, in part, precipitated sarcoplasmic proteins from fluids that collect at the interface of the sarcolemma during fiber shrinkage. Machlík and Drudt (1963) noted that beef collagen shrinkage is essentially complete between 57 to 59°C. Transformation of beef collagen into gelatin begins around 63°C (Hamm, 1966). The conversion of beef collagen into gelatin occurs at a temperature higher than for fish. It is therefore likely that the granular material observed may be a combination of heat coagulated sarcoplasmic proteins and gelatin.

After tuna was precooked and canned, there was noticeable erosion of the muscle fiber (Figure 8). Erosion can be defined as the disruption of the cell wall and a discharge of the heat coagulated sarcoplasmic constituents, resulting in more granular material in the interstitises between muscle bundles. Doty and Pierce (1961) found that erosion of the muscle fibers increased with the duration of heat treatment. Further, Hearne et al. (1978) studied the effects of the rate of heating of bovine semitendinosus to 40, 50, 60 and 70°C. The results indicated that rapid heating caused an acceleration and increase of myofibrillar fragmentation. The precooked and canned tuna specimens studied here however evidenced minimal apparent fragmentation when rapidly heated to ca. 60°C (precooking) and ca. 111°C (canning), respectively; the fibers appeared to be congealed and homogenous.

Muscle Structure

The size of the sarcomere (1.5 μm) indicates that the sarcomere is fully contracted. SEM of cryofractured myofibrils revealed the presence of Z-lines, A-bands and H-zones (Figure 9). There was disruption of the sarcomere after precooking and canning. The I-band and A-band have coagulated into a solid block of actomyosin which constitutes the majority of the sarcomere (Figure 10). These findings were in agreement with those described in beef muscle (Locke and Wild, 1982; Leander et al., 1980; Carroll and Jones, 1979; Schaller and Powrie, 1972). There has been
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some further distortion of the sarcomere during thermal processing. The damage is evident in this figure due to greater magnification and high resolution. The sarcomere has been extremely shortened and large spaces have developed between each structural unit. This may thus indicate the combined effects of freezing and thermal processing on the muscle structure.

**Fiber Diameter**

The mean fiber bundle diameters are presented in Table 1. The difference between the diameter of raw and either precooked or canned fibers was statistically significant (P < 0.05). There was, however, no difference between the diameters of precooked and canned muscle fibers. This does indicate that shrinkage of the fibers did occur upon the first thermal treatment, and can be attributed to altered hydration or cook-cool loss. Hatae et al. (1984), measured the diameters of heat-set muscle fibers from five different fish species. Their results indicated that the diameters varied between species and that the diameters correlated with drip loss and firmness. These researchers did indicate that distinguishing textural differences of different species was possible via discriminant analysis but not through individual parameters.

**Table 1. Mean Fiber Diameter of Raw, Precooked and Canned Skipjack Tuna Muscle.**

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<td>52.0±18.0ª</td>
<td>45.6±14.8b</td>
<td>46.3±15.6b</td>
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ª, bMeans within each row represented by different letters indicate significant differences (P<0.05). Data are presented as mean ± standard deviation of 62, 71 and 76 fibers.

**Conclusions**

In tuna, freezing caused more of either a shifting or a distortiog of muscle fibers than damage at the cell wall level observable at the lower limits, 20,000x, of high resolution scanning electron microscopy. During thermal processing of tuna, changes occurred manifested as a granulation of the sarcolemma. Also structural bands were altered and the migration of cellular constituents into the interstices of the muscle fiber and cell wall were detected. Further work is planned to investigate the effects of freezing and frozen storage on the muscle structure with the aid of isothermal freeze-fixation.

**Acknowledgements**

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**References**


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Alimment. 5:184-190.


Discussion with Reviewers

R.J. Carroll: The authors state the muscle fibers in Fig. 2 are fully contracted and the sarcomeres measured 2.2 μm. The authors also state a fully contracted sarcomere is 1.5 μm. Can you explain this discrepancy?

Authors: The muscle fiber shown in Figure 2 is of fresh fish that was never frozen. The sarcomere did measure 2.2 μm in length. The fully contracted sarcomere measuring 1.5 μm in length refers to Figure 9. The muscle shown in Figure 9 had been frozen. Further, the 2.2 μm measure relates to the exterior of the sarcomere. The 1.5 μm measurement refers indeed to the cryo-fractured specimen and a measure of the interior of the sarcomere.

R.J. Carroll: Is the change in appearance of the sarcomeres in Fig. 9 compared to those in Fig. 2 due to the freezing of the muscle?

Authors: Freezing may in part have caused some shrinkage due to drip loss upon thawing. Also, we reiterate that the two different figures represent surface versus interior views of the muscle sample.

G.M. Pigott: This paper could be improved if the damage or non-damage to cell walls, as assessed by the photomicrographs, could be correlated with water loss during processing and storage.

Authors: To some extent, "drip" data are available, as indicated by Table 1 and the text section entitled Fiber Diameter. Further, more detailed information is from allied studies which involved the handling, freezing and frozen storage of fresh skipjack tuna. The screening of tuna from commercial fishing vessels (described herein) and later, simulating on board conditions in the laboratory (a separate manuscript which is in review for publication) have provided some insight into the handling of tuna and subsequently the impact on the biochemistry and yield parameters. This screening work was preliminary in nature and provided the basis for the later work involving the handling of fresh fish.
FREEZE TEXTURIZATION OF PROTEINS: EFFECT OF THE ALKALI, ACID
AND FREEZING TREATMENTS ON TEXTURE FORMATION

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Abstract

The effects of alkali extraction, isoelectric precipitation, and rate of ice crystal formation on protein interaction in the freeze texturization process were studied. The protein isolate was obtained from mechanically deboned poultry meat residues by extraction with NaOH at pH 10.5 and precipitation by HCl at pH 5.0. The presence of large molecular weight subunits in the protein isolate was revealed by SDS-polyacrylamide gel electrophoresis. Scanning electron microscopy demonstrated the nature of the fiber formation and protein cross-linking as affected by freezing at -25°C or -196°C. The transformation of the level of protein organization from the lumpy or granular nature of the unfrozen sample to the highly spongy and randomly oriented protein mass (-196°C) or to the highly organized, parallel sheets of interconnected, unidirectionally frozen protein (-25°C) was illustrated.

Introduction

The primary aim of food protein texturization is to simulate the texture of meat or other structured foods. The three main processes employed in protein texturization are spinning, extrusion and freeze texturization. The recent work of Lawrence (1981) contains an extensive review of these protein texturization techniques.

A variety of protein materials from conventional (e.g. milk, fish, meat and egg) and non-conventional (e.g. beans, blood, offal and meat deboning residues) sources can be used as raw materials for the production of texturized food products. However, according to Hartman (1978) there are some common molecular structure requirements for a protein to be suitable for fiber formation such as high molecular weight (greater than 10,000), long linear chain length, high degree of linear symmetry, absence of high ratio of bulky side chains and high degree of polarity. These structural requirements are necessary for development of orientation and crystallization among the molecular chains.

The basic principle involved in any protein texturization process is to convert the native, non-fibrous protein into a fibrous form; this may be accomplished by a series of treatments that change intramolecular stereochemistry and develop intermolecular structural arrangement of polypeptides in the protein chains. Unfolding of the native globular protein followed by reorganizing into a more aligned and cross-linked state imparts a higher degree of physical strength in the resulting fiber (Huang and Rha, 1974). This transformation of the native globular protein into edible fibrous protein can be achieved by a strong alkali treatment which dissolves and denatures the protein resulting in a solution of random coils (Shen and Morr, 1979; Kelley and Pressey, 1966; Kinsella, 1978) to be processed by a suitable texturization technique. Further, protein polymerization via disulfide bond formation is also favored under the alkaline condition (Fukushima, 1980; Kelley and Pressey, 1966). It appears that the main differences between extrusion, spinning and freeze texturization lie in the extent to which the conformational changes

KEY WORDS: Freeze texturization, unidirectional freezing, protein structures, fiber formation, protein cross-linking, texture formation, Scanning Electron Microscopy.
in the protein molecule are achieved. The phenomenon of protein fiberization during texturization was demonstrated by microscopic techniques such as light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Kazemzadeh et al., 1982; Tananto et al., 1978a, 1978b; Legla et al., 1978; Tananto and Rhee, 1978; Aguiler et al., 1976; Maurice et al., 1976; Cumming et al., 1972).

In the production of freeze-texturized soy protein known as "Kori-tofu", unfolding of the globular proteins (2,7,11,155 components; molecular weights ranging from 32,000 to 320,000 daltons) is accomplished by heat treatment (Hashizume et al., 1971; Wolf, 1970). This process exposes the masked sulfhydryl groups needed for the disulfide bond formation (Fukushima, 1980). This stable covalent bond plays an important role in the texture of the product (Salo et al., 1971; Salo et al., 1989; Salo and Watanabe, 1978). Hydrophobic bonds (Hashizume et al., 1981; Fukushima, 1980). Hydrogen bond formation should be enhanced at this low temperature in view of the negative enthalpy that characterizes this process (Taborsky, 1979). These chemical reactions take place in the concentrated unfrozen liquid part of the material (Hashizume et al., 1971).

In practice, the soy protein curd or solution is frozen in such a way that heat removal takes place in all directions, resulting in a spongy product that lacks the fibrous orientation characteristic of meat structure. In contrast, a parallel oriented protein mass which is retained after freeze drying can be obtained following the freezing process introduced by Lugay and Kim (1981). The technique is to freeze the protein solution in a manner and at a rate such that elongated ice crystals are generated unidirectionally. Hence, the proteins and other solutes are concentrated in the spaces between the ice crystals forming distinctly aligned parallel zones. Lawrence and Jelen (1982) investigated the effects of pH, total solids and freezing rate on the texturization of proteins extracted from mechanically deboned poultry meat residues. In a review of the various freeze texturization processes, Lillford (1986) highlighted the following critical steps: separation of phases on freezing (integral or concentrated solute or suspension), orientation of ice crystals and hence passive orientation of the concentrated phases of fibrous final structures, and fixation of the structure by formation of new chemical bonds.

Freeze texturization of proteins is still poorly understood and little information on the exact nature of the events occurring in the process is available. The study reported herein was conducted with the following objectives:

a) to determine the molecular weight distribution in the alkali extracted, acid-precipitated chicken protein before subjecting it to the freeze texturization process; b) to determine the effect of freezing rate on the structural orientation of the freeze-texturized protein mass, and c) to illustrate, by scanning electron microscopy, the events leading to texture formation in freeze texturization of alkali solubilized poultry proteins.

Materials and Methods

Sample Preparation

Mechanically deboned poultry meat residues were obtained from a local poultry processing plant. The bone residues were collected during the deboning operation and immediately extracted following the procedure described earlier (Lawrence, 1981; Lawrence and Jelen, 1982). Briefly, the procedure consisted of mixing the ground bones with sufficient 20% NaOH to obtain pH 10.5, centrifuging (27,300 x g), acidifying the supernatant to pH 5.0, and centrifuging again (2,520 x g) in the same centrifuge (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Instrument, U.S.A.). About 150 grams of the fresh protein isolate (containing 9% protein by microKjeldahl) was frozen in an aluminum dish (7.0 cm x 3.3 cm) fitted at the center of a styrofoam block (17 cm x 17 cm x 10 cm) to insulate all sides except the top portion of the dish (Figure 1). This freezing method was adopted from Lugay and Kim (1981) and was used to achieve unidirectional freezing of the protein mass. The samples were frozen at -25°C and held at this temperature for 48 hours. After freezing
some samples were freeze dried while others were
heat set by autoclaving at 121°C, 1.034 bar for
15 minutes and then cooled at room temperature. Some samples were frozen by direct immersion of
the uninsulated dish in liquid nitrogen to illustrate the effect of the freezing rate on the
textural pattern formed by non-directional freezing. The frozen sample was thawed in 95%
ethyl alcohol for stabilization and dehydration. Some freeze texturized samples were freeze dried in order to fix the structure to see whether any possible alteration might have been caused by stabilizing the protein using ethyl alcohol.

Different sample preparations were used in order to demonstrate fiber formation and cross-linking in the acid-precipitated protein isolate. The protein isolate was washed with 500 ppm of EDTA, followed by washing with deionized water, to remove the extraneous, water-soluble materials that could have masked the protein fiber. To find out whether fiberization and cross-linking was present in the acid-precipitated protein, a fresh sample was analyzed by electron microscopy after immersion in 95% ethyl alcohol to stabilize and dehydrate the protein.

Microscopy
Texturized protein samples, fixed by freeze drying or heat setting, were fractured into about
0.5 cm³ and then dried by the critical point
drying technique with liquid CO₂ for microscopic analysis. The samples were mounted on aluminum stubs with silver conducting paint and sputter coated with gold to a thickness of 150 Å. Scanning electron microscopic examination was performed with a Cambridge Stereoscan 250 at 25 kV.

Determination of Molecular Weight
The molecular weight distribution of the protein in the isolate was determined using
sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein isolate was purified following the method described by Parsons and Lawrie (1972) wherein the extract was homogenized in three volumes of 0.1 N HCl in 75% ethyl alcohol and then centrifuged at 30,000 x g for 30 minutes. The supernatant was discarded and the pellet was resuspended in five volumes of acetone. The suspension was left for 3 hours and centrifuged again. The acetone extraction was repeated and the pellets were then dried at 50°C and finely ground. The protein content of the dried protein powder was 95%, as determined by the microKjeldahl method. The protein powder was dissolved in 1 ml
of sample buffer containing 0.05M Tris-HCl (pH 6.8), 1% SDS, 0.01% bromophenol blue, 30% glycerol and 20 μl of 2-mercaptoethanol. The buffer was heated in a boiling water bath for 3 minutes prior to use.

The proteins were resolved in acrylamide gels
using a vertical slab electrophoresis (Electrophoresis Cell Model 220) following the procedure
outlined by Bio-Rad Laboratories (1977), based on the design described by Akroyd (1967) and
Laemmli (1970). A more detailed description of the method had been reported by Consolacion
(1986).

The chemicals used in the SDS-PAGE were
obtained from Sigma Chemical Company (U.S.A.). The standard proteins (Pharmacia Fine Chemicals, Uppsala, Sweden) used in this study as standards were carbonic anhydrase (29,000), egg albumin (45,000), bovine albumin (66,000), phosphorylase
B (97,400), α-galactosidase (116,000) and myosin (205,000).

Electrophoresis was carried out at 30 mA for
3-4 hours or until the tracking dye reached the bottom of the gel. After the electrophoretic run, the gel was stained in a solution of 45%
methanol, 10% acetic acid and 0.35% Coomassie Blue R-250 for 12 hours and then destained in a solution of 10% acetic acid and 30% methanol. The mobilities of each protein subunit were calculated and the molecular weights estimated from the standard curve, obtained by plotting the log molecular weight versus mobilities of the standard proteins (Neber and Osborn, 1969).

Results and Discussion
The alkali treatment of the mechanically
deboned poultry meat residues solubilizes both
the myofibrillar and sarcoplasmic components of
the muscle proteins. Upon acidification of the
alkali extract from pH 10.5 to 5.0, the myofi-
brillar proteins are mainly precipitated while
the sarcoplasmic proteins remain in the super-
natant solution (Lawrence, 1981; Young, 1976; Young, 1975). A negligible amount of collagen
is extracted under these conditions (Lawrence,
1961).

The electrophoretograms of the resolved, acid-precipitated protein and the corresponding
molecular weights of the major bands are shown in
Figure 2. The numerous bands indicate a wide
range of protein subunits present in the isolate. Quite evident is the presence of the dark bands
which indicate high protein concentrations, two
of which should correspond to myosin and actin
with molecular weights of 220,000 and 46,000-
47,000 daltons, respectively (Hofmann and Hamm, 1978). In a similar study using poultry deboning
residues, Kijowski and Niewiarowicz (1985)
suggested that the presence of numerous protein
bands in the electrophoretogram was due to the
occurrence of proteins not only from meat
residues but also from the skin and bone marrow.

The majority of the salt soluble proteins recovered in the bands were of the molecular
weight 17-21 x 10^3 and 38-47 x 10^3 daltons, from
which they suggested the presence of the following proteins: actin, tropomyosin, the
light chains of myosin and troponin and the
degraded products of the heavy chains of myosin, possibly due to enzymic breakdown resulting from liberation of lysozomal enzymes by the deboning
process.

The occurrence of quite large molecular

35
weight components in our protein isolate may be indicative of protein-protein interactions during the alkali extraction. Protein cross-linking is an important requirement for fiber formation and texturization (Hartman, 1978; Kelley and Pressey, 1966). The tensile strength of the fibers increases with increasing subunit chain length until a limiting plateau is reached at approximately 200 residues resulting in sub-unit chain molecular weight of ca. 22,000 daltons (Shen and Morr, 1979). Much larger sub-units do not cause an increase in the tensile strength and even can be detrimental to fiber formation (Shen and Morr, 1979; Huang and Rha, 1974).

The presence of protein interactions leading to fiber formation and extended cross-linking was clearly demonstrated in the acid-precipitated, unfrozen protein isolate, as shown in Figure 3. These results strongly depict the occurrence of protein interactions during the acidification of the alkali extract from pH 10.5 to 5.0; needed for the isoelectric precipitation. In our study, the protein samples used for electrophoresis were dissolved in the sample buffer containing 2-mercaptoethanol which showed relatively higher solubility of the protein than in the absence of this reducing agent. Kelley and Pressey (1966) postulated that alkaline conditions favor disulfide bond formation while acidification brings many polypeptide chains close together, favoring hydrogen and ionic bonding. The formation of the disulfide bonds in our protein sample seemed possible, considering the sulfhydryl groups present in the fresh protein isolate (Consolacion, 1986).

A network similar to that in the unfrozen protein was also observed in the freeze texturized, freeze dried protein as shown in Figure 4. It appears that fiber formation and extended cross-linking following the alkali and acid treatments could be preserved and probably promoted during freeze texturization. Hashizume et al. (1971) postulated two events that could take place during this process. First, ice crystal formation during freezing brings the protein close together thereby concentrating the fibers between the parallel-oriented ice crystals. Second, further protein-protein interactions occur at the early stages of the freezing process in the unfrozen concentrated solution. The high degree of protein polymerization as a result of the freeze texturization was confirmed by the poor solubility of the thawed sample (prior to heat setting) in the sample buffer for the SDS-PAGE. Rhee et al. (1981) also employed the SDS-PAGE technique to determine the molecular weight distribution of the extruder-textured protein and indicated that
non-covalent forces (hydrogen, ionic, and hydrophobic forces) and sulfhydryl-disulfide interchange reactions occur during extrusion texturization.

The macroscopic transformation of the protein organization as affected by the freezing technique is illustrated in Figures 5-8. Figure 5 shows the lumpiness or granulated nature of the unfrozen, acid-precipitated protein isolate. Rapid freezing of this sample by direct immersion in liquid nitrogen resulted in a highly porous, and randomly oriented protein mass (Figure 6). The small voids represent the area formerly occupied by the numerous ice nuclei formed by the rapid freezing (Fennema, 1973). In contrast, unidirectional freezing resulted in a very different sheet-like parallel orientation of the fibrous protein mass on the macrolevel (Figures 7 and 8). Similar results were shown by Lugay and Kim (1981) in freeze texturizing soy protein and by Lawrence and Jelen (1982) for poultry protein extracts. As these authors also noted, the sheets of proteins are not completely independent of each other and are joined at sufficient
locations to provide a cohesive fibrous mass (Figure 8a).

A closer analysis of the cross-section of protein sheet structures (Figure 8b) reveals the homogeneity of the protein mass. There is a distinct contrast in appearance of the freeze texturized samples from that of the original material (Figure 5), demonstrating the transformation of the level of organization of proteins as a result of freezing. The fiberization and extended cross-linking and the mechanical compression by the ice crystals had provided a considerable amount of cohesive forces that enabled the texturized protein to maintain its structural integrity during thawing at room temperature and subsequent heat setting.

There is no doubt that the freezing process causes protein concentration, however, the mechanical force exerted by the growing ice crystals may not be enough to align and stretch the randomly oriented protein fibers. There appears to be two levels of orientation exhibited by the freeze texturized protein, namely the distinctly parallel oriented protein sheets and the randomly arranged and totally entangled fibers compressed in these protein sheets, a product characteristic that would differentiate the freeze-alignment process from the other methods for protein texturization.

Conclusions

The slowly growing literature on freeze texturization appears to emphasize the occurrence of protein-protein interactions in the unfrozen liquid part of the protein solution during frozen storage (Hashizume et al., 1971). However, only a few workers demonstrated possible protein polymerization during the pretreatments undergone by the protein materials; in our view, this is an important event prior to the texturization. In our study, the alkali extraction and isoelectric precipitation of proteins provided favorable conditions for fiber formation and cross-linking of the freshly extracted proteins. This protein cross-linking was preserved and probably promoted as a result of freeze concentration. The retention of the structural integrity of the freeze-texturized protein during thawing at room temperature and heat setting provided tentative evidence of the strong chemical bonds formed during protein cross-linking and the compressive force exerted by ice crystals. The parallel, interconnected protein mass, characteristic of the unidirectional freezing technique, was clearly illustrated.

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References


Freeze Texturization of Proteins


THE FINE STRUCTURE OF THE ENDOMYSIUM, PERIMYSIUM AND INTERMYOFIBRILLAR CONNECTIONS IN MUSCLE

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Abstract

Bovine sternomandibularis and rabbit semimembranosus muscles were prepared in buffered hypertonic glutaraldehyde fixative. Freeze substitution methods were utilized to minimize ice crystal damage within the tissues, and samples were further prepared for scanning electron microscopy (SEM) using traditional methods. Using SEM, filamentous material was found joining adjacent myofibrils in both rabbit semimembranosus and bovine sternomandibularis muscles. These myofibrillar interconnections were found to have a periodicity between 2.1 and 2.3 μm within restrained rabbit semimembranosus muscle, and the sarcomere length of this tissue was between 1.9 and 2.3 μm. Internyofibrillar bridges, spaced approximately 1.9 μm apart, were observed within bovine sternomandibularis muscle restrained prior to fixation. Spacing between internyofibrillar bridges was from 1.0 to 1.4 μm within bovine sternomandibularis muscle unrestrained prior to fixation. Using transmission electron microscopy, bridges between adjacent myofibrils were found at or near the Z-lines.

Endomysial connective tissue contained reticular fiber rows arranged either obliquely or perpendicularly to the long axis of myofibers. Reticular fiber arrangement may be dependent upon the degree of muscle shortening. However, irrespective of the degree of restraint, periodicity between reticular fiber rows was found to be approximately 4.0 μm within bovine sternomandibularis muscle and approximately 3.3 μm within rabbit semimembranosus muscle.

Introduction

Interconnections joining adjacent myofibrils have been found at the Z-, M- and N2-lines of sarcomeres (Lazarides, 1980). Vojte (1981) observed filamentous material connecting neighboring myofibrils within bovine longissimus dorsi muscle. Bundles of 10 nm diameter filaments circumventing myofibrils at Z-lines have been observed within mature skeletal muscle cells (Page, 1969). Filaments of 10 nm diameter, termed intermediate filaments, are assembled within postmitotic myoblasts and myotubes. Except for a study carried out by Page (1969), intermediate filaments have never been found within mature myotubes or myofibers (Holtzer et al., 1982; Lazarides et al., 1982), but proteins comprising 10 nm filaments are located within Z-line and 1-band regions of both mature myotubes and myofibers (Kelly, 1969; Holtzer et al., 1982; Lazarides et al., 1982). However, Holtzer et al. (1982) were unable to locate intact 10 nm filaments within mature myotube sarcoplasm. These data indicate that during myotube maturation, cell requirements for 10 nm filaments are lost, and although proteins comprising 10 nm filaments remain associated with structural components of the sarcoplasm, the 10 nm filaments probably disappear gradually as myotubes mature into muscle fibers. Lazarides et al. (1982) proposed that 10 nm filaments function to tie together myofibrils. However, this postulate is not accepted universally (Holtzer et al., 1982).

Recently, Wang and Ramirez-Mitchell (1983) described a model similar to that of Lazarides et al. (1982) depicting internyofibrillar bridges joining adjacent A-bands and Z-lines. Basically, all of the various internyofibrillar bridge models are similar; a doublet disc-like structure or collar composed of desmin sandwiches each Z-line, and small filaments, possibly also composed of desmin, extend from one Z-line collar to parallel Z-line collars of adjacent myofibrils (Robson and Huiatt, 1983; Wang and Ramirez-Mitchell, 1983). Additionally, filamentous connections joining M-line regions of adjacent myofibrils have been observed (Wang and Ramirez-Mitchell, 1983).

Reticular fibers are distinguished morphologically by their extensive branching, and these fibers are easily distinguished from collagen fibers which have little or no branching (Swartland, 1976). Endomysial reticular fibers are arranged approximately perpendicular with respect to the muscle fiber longitudinal axis.
and these fibers are found above yet continuous with the amorphous collagen layer surrounding each muscle cell (Swatland, 1975, 1976). Reticular fibers are shared between myofibers, and are not simply spirally disposed fibrous networks surrounding each myofiber (Swatland, 1976). These networks form structural continuums attaching to the amorphous layer composed of collagen fibrils and endomysial collagen fibers. Reticular fibers are also found within the perimysium (Swatland, 1976).

Each muscle fiber is encased within a sock-like structure, composed of randomly arranged small collagen fibrils of approximately 47 nm diameter (Rowe, 1978), termed the amorphous collagen layer. Presently, it is not known whether the amorphous collagen layer merely surrounds each muscle fiber, or if it connects directly to the sarcolemma at discrete locations. Deep-etching of heart muscle has revealed that the external lamina contains trabecula-like branches which appear to insert into the muscle cell membrane at regular intervals (Frank and Beydler, 1985); similar structures have been observed within skeletal muscle (Bonilla, 1983).

Presently, little is known of native endomysial and perimysial ultrastructures. Further research concerning connective tissue ultrastructure would provide greater understanding of muscle ultrastructure, as recent reports describing myofibrillar interconnections exclusive of the sarco-tubular system require that the sarcomere model be revised to include new structures. The present study is a preliminary examination of muscle endomysium and myofiber ultrastructure. Results obtained from this study provide additional evidence for the existence of internomyofibrillar bridges, and support earlier research regarding endomysial ultrastructure.

Materials and Methods

Preparation of Fresh Rabbit Semimembranosus Muscle for Scanning Electron Microscopy

A New Zealand White rabbit was administered a lethal injection of Cornell Lethal Solution (pentobarbital sodium; Eli Lilly Co.) via cardiac puncture. The semimembranosus muscle was exposed and allowed partial fixation in situ (20 minute duration) in primary fixative (0.18M sucrose, 0.1M sodium cacodylate, 3% glutaraldehyde, 0.05% CaCl2; pH 7.4) prior to removal from tenodesis restraints. During partial fixation, the hind limb was extended laterally with respect to the medial plane of the animal. Following partial fixation, yet while still restrained by tenodesis connections, each muscle strip was tied to an applicator stick, cut parallel to the longitudinal muscle axis into 1 mm diameter strips and placed in fresh primary fixative at 0-4°C for 24 h. Restricted muscle strips were removed from the applicator sticks, cut into 3 to 4 mm lengths, and placed in fresh primary fixative for an additional hour. Following fixation, samples received 3, 10-minute washes in cacodylate buffer (0.1M cacodylate, 0.05% CaCl2; pH 7.4; 0-4°C).

Washed samples were rapidly frozen in Freon 22 and then stored in liquid nitrogen. Frozen samples, fractured parallel to the muscle fiber longitudinal axis using a prechilled razor blade, were equilibrated in an acetone solution containing 4% osmium tetroxide at -78°C for 18 h and at -20°C for an additional 2 h. Final fixation and freeze substitution were accomplished after sample equilibration at 25°C for 2 h.

Samples were washed 5 times, 10 minutes each wash, with 100% acetone. The remaining water and acetone were removed by critical point drying using a DCP-1 Critical Point Drying Apparatus (Denton Vacuum, Inc.). Amyl acetate served as the substitution medium during the critical point drying process. Dried samples were attached to aluminum specimen stubs using silver conducting paint, and a 20 nm thick coat of gold-palladium alloy was applied using a Technics Sputter Coater. Samples were observed and photographed using a JEOL 25S-II Scanning Electron Microscope.

Preparation of Fresh Bovine Sternomandibularis Muscle for Transmission Electron Microscopy

Bovine sternomandibularis muscle preparation as described previously for SEM was equilibrated in 100% propylene oxide for 24 h, and then gradually embedded (1:2, 1:1, 2:1 and 100% resin to propylene oxide; one hour each exchange) with epoxy resin (48.5% LX-H2 (w/w), 25% radic methyl anhydride (w/w), 25% dodecenylsuccinic anhydride (w/w)) and 1.5% (dimethyl amino) phenol (w/w). Samples were equilibrated overnight in 100% resin under vacuum and hardened by heating at 70°C for 2 days. Samples were sectioned, stained using Sato's lead stain (Sato, 1967) and examined using a JEOL 100-S Transmission Electron Microscope.

Preparation of Fresh Bovine Sternomandibularis Muscle for Transmission Electron Microscopy

Bovine sternomandibularis muscles were removed immediately following exsanguination. Muscle strips (1-2 mm diameter) were stretched, restrained and placed in primary fixative (0.08M sodium cacodylate, 3% glutaraldehyde: pH 7.4) for 24 h. Following primary fixation, all samples were washed 3 times in 0.1 M sodium cacodylate (pH 7.4 at 0-4°C) for 10 minutes each, and placed in a secondary fixative (2% osmium tetroxide buffered with 0.1 M sodium cacodylate: pH 7.4, 0.4°C) for 2 h. Samples were then washed 3 times in distilled, deionized H2O (10 min each wash) and placed in 50% ethanol for 10 minutes, followed by embedding in Epon resin (12 ml dodecenyl succinic anhydride, 14 ml natic methyl anhydride and 24 ml Epon 812 with 1.5% (v/v) tri (dimethyl amino methyl) phenol). Tissue blocks were polymerized and hardened by heating at 70°C for 48 h. Embedded tissues were thin sectioned (60-90 nm thick sections), placed on uncoated 400 mesh grids and stained with Sato's lead solution (1 g lead citrate, 1 g lead acetate, 1 g lead nitrate and 2 g sodium citrate dissolved in 82 ml boiled, distilled, deionized H2O, followed by the addition of 18 ml of 4% NaOH) for 30 seconds. Stained sections were washed for 30 seconds using 0.2 M NaOH, followed by two subsequent washes with distilled, deionized H2O. Five samples per treatment in duplicate were observed and photographed using a JEOL JEM-100S Transmission Electron Microscope.

Sampling of Muscles Examined by Scanning Electron Microscopy

Approximately 15 to 20 tissue strips were examined in the rabbit muscle study. Between 10 and 15 tissue strips were examined for each of four bovine sternomandibularis tissue preparations: (1) restrained with hypertonic fixation, (2) restrained with conventional fixation, (3) unrestrained with hyperfixation, and (4) unrestrained with conventional fixation.

Results

Internomyofibrillar Bridges

Myofibrillar shrinkage was evident beneath the sarcolemma.
Fig. 1. Scanning electron micrograph of restrained rabbit semimembranosus muscle. Fixation shrunken myofibrils (M) are found beneath the sarcolemma (S). Intermyofibrillar filaments (I) bridge myofibrils. Spacing between intermyofibrillar bridges is 2.1 μm. Filaments (F) smaller in diameter than those identified as intermyofibrillar filaments or bridges are located between the periodically spaced intermyofibrillar filaments.

Fig. 2. Scanning electron micrograph of restrained bovine sternomandibularis muscle. Beneath the sarcolemma (S), intermyofibrillar bridges (I) were found joining shrunken myofibrils (M). Intermyofibrillar bridge periodicity is approximately 2.0 μm.

Fig. 3. Scanning electron micrograph of unrestrained bovine sternomandibularis muscle. Fixation shrunken myofibrils (M) were found beneath the sarcolemma. Filaments (I) connecting adjacent myofibrils were found throughout the myofibers, and reticular-type fibers (R) were seen joining neighboring myofibers.

of rabbit semimembranosus muscle damaged through preparation in 750mOsm fixative (Figure 1). Tissue processed using primary fixatives of lower osmolarity (approximately 500mOsm or less) generally will not exhibit shrinkage of intracellular material. Rabbit semimembranosus muscle shrinkage allowed exposure of several classes of filamentous bridges joining adjacent myofibrils. Regularly spaced filaments of 2.1 to 2.3 μm periodicity were found joining myofibrils, and between the regularly spaced filaments were intermyofibrillar connections of smaller diameter (Figure 1). Periodicity between these smaller diameter filaments lacked the uniform periodicity of the former filaments. Sarcomere length within rabbit semimembranosus muscle was approximately 2.5 μm, as measured from sarcomere topography, and between 1.9 and 2.1 μm as measured by TEM. This indicated that the regular spacing of the larger diameter filaments was related to sarcomere length.

Within restrained bovine sternomandibularis muscle, intermyofibrillar bridge spacing was 1.9 μm (Figure 2), whereas, sarcomere length ranged from 3.2 to 36 μm, measured by both SEM and TEM. Periodicity between intermyofibrillar bridges was 1.4 μm or less within unrestrained bovine sternomandibularis muscle (Figure 3), and using both SEM and TEM, sarcomere length measured 1.1 μm.

Severe shortening of unrestrained sternomandibularis muscle fibers resulting from muscle contraction during fixation caused severe loss of ultrastructural detail, and intracellular ultrastructure was altered by shrinkage of myofiber filaments and coalescence of various proteinaceous filaments. Similar detail loss was noted within dehydrated restrained bovine sternomandibularis muscle prepared for TEM. However, remnants of Z- and M-lines could be discerned within shrunken myofibrils, and intermyofibrillar bridges were located at the Z-lines within this tissue (Figure 4).

Using TEM, intermyofibrillar connections were found at Z-lines, and bridges smaller in diameter than those located at Z-lines were observed within A-band regions of conventionally prepared sternomandibularis muscle; muscle placed in primary glutaraldehyde fixative (520mOsm) for 24 h (Figure 5). The myofibrillar connections between intermyofibrillar Z-line bridges (the periodically disposed bridges) may be analogous to the smaller diameter filaments located between the periodically
Table 1. Osmolarity of fixatives utilized for scanning electron microscopy of muscle tissue. a

<table>
<thead>
<tr>
<th>Fixative</th>
<th>Osmolarity (mOsm)</th>
<th>Tissue preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 0.18M sucrose</td>
<td>750</td>
<td>a. Bovine Sternomandibularis</td>
</tr>
<tr>
<td>0.1M sodium cacodylate 3% glutaraldehyde</td>
<td>0.05% CaCl₂ pH 7.4</td>
<td>a. Rabbit Semimembranosus</td>
</tr>
<tr>
<td>2. 0.1M sodium cacodylate 3% glutaraldehyde</td>
<td>0.05% CaCl₂ pH 7.4</td>
<td>a. Bovine Sternomandibularis</td>
</tr>
<tr>
<td>3. 0.08M sodium cacodylate 3% glutaraldehyde</td>
<td>520</td>
<td>a. Bovine Sternomandibularis</td>
</tr>
</tbody>
</table>

aOsmolarity determined by calculation from concentrations of buffer contents as described by Segel (1976).

spaced filaments observed in the SEM micrographs. Structural differences between rabbit and bovine muscle were not observed.

Endomysium Ultrastructure

Conventional fixation processes have not exposed the endomysium ultrastructure due to the very tight packing of muscle component structures. Therefore, hypertonic fixation was employed to expose the endomysium. Table 1 lists characteristics of various fixatives utilized for this study and the tissues prepared using the various fixatives. A 350mOsM fixative is considered isotonic; however, for good tissue preservation, approximately 450mOsM fixatives are recommended (Hayat, 1981). Conventional fixative (520mOsM) used in our laboratory provides excellent muscle preservation for TEM, but does not adequately expose connective tissue structures for ideal SEM observations.

Ultrastructure of restrained and unrestrained bovine sternomandibularis muscle was indistinguishable from that of the rabbit semimembranosus muscle. Therefore, unless specified otherwise, micrographs of rabbit semimembranosus tissue will be utilized to describe and discuss the general ultrastructure of both the endomysium and perimysium.

Surrounding each sarcolemma in sock-like fashion was an amorphously constructed sheet containing small diameter collagen fibrils (Figure 6). Continuous with the amorphous collagen fibril sheet, and possibly fused to it, were ordered laminar-like rows of reticular fibers periodically spaced between 3.0 and 3.3 μm (Figure 7). This periodicity was slightly greater (4.0 μm) within bovine sternomandibularis muscle. Moreover, the ordered reticular fiber structure was located only adjacent to the amorphous collagen fibril sheet surrounding each muscle cell (Figure 8). Endomysial connective tissue continuous with the reticular fiber rows formed a lattice network throughout the interstitium between muscle cells (Figure 9). The appearance of the lattice network was either well ordered (Figure 10) or lacked definable structure (Figure 9). Endomysial connective tissue interconnecting adjacent muscle fibers was found as a structural continuum extending throughout the endomysium of each primary fasciculus, and this structural continuum ultimately became confluent with the perimysium (Figure 11).

From restrained bovine sternomandibularis muscle, reticular fiber networks maintaining structural continuity with the perimysium surrounding primary fasciculi were determined to be ultrastructurally similar to reticular fiber arrangements within the endomysium (Figures 12 and 13, respectively). Additionally, within bovine sternomandibularis muscle perimysium, reticular-like fiber rows were associated with collagen fibers and collagen sheets of both primary and secondary fasciculi (Figure 12). Upon close examination, endomysial reticular fibers appeared to attach directly to parallel rows of laminar sheets within the perimysium (Figure 13). Moreover, perimysial laminar sheets were joined to one another by small fibrils or filaments, and within the perimysium, similar small diameter fibrils attached laminar sheets to collagen fibers (Figure 14). These filaments probably help maintain uniform periodicity of both the reticular-type fibers of the endomysium and laminar sheets of the perimysium.

Fig. 4. Transmission electron micrograph of restrained bovine sternomandibularis muscle which had undergone tissue shrinkage during fixation. The M- (M) and Z-lines (Z) are discernible. A severely shrunken A-band (A) appears in the central region of damaged sarcomeres. Sarcomere length ranged from 3.1 to 3.5 μm. Myofibrils are joined at the Z-lines (I) and filaments (F) are found joining A-band regions of adjacent myofibrils.

Fig. 5. Transmission electron micrograph of restrained bovine sternomandibularis muscle placed in 3% glutaraldehyde fixative for 24 hours. Intermyofibrillar bridges (I) join adjacent myofibrils at the Z-lines (Z). The sarcomeres (SA) are extremely short, with Z-lines almost touching each edge of the A-bands (A). The M-line (M) is easily discerned. Filaments (F) join adjacent myofibrils at the A-band region.

Fig. 6. Scanning electron micrograph of rabbit semimembranosus muscle. Enlargement of a fold within a single muscle fiber. The amorphous layer or the portion of the basement membrane composed of small diameter collagen fibrils (C) is found pulled away from the sarcolemma (S).

Fig. 7. Scanning electron micrograph of rabbit semimembranosus muscle. Equidistantly spaced rows of reticular-type fibers (R) are found near the sarcolemma (S) disposed around a muscle fiber and yet continuous with a second myofiber. Periodicity between reticular fiber rows is approximately 3.0 μm. Rows of reticular-type fibers appear continuous with the basement membranes of both muscle fibers.

Fig. 8. Scanning electron micrograph of rabbit semimembranosus muscle. Periodicity between rows of reticular fibers (R) is 3.0 μm. Several collagen fibers (CF) surrounded by the reticular matrix are found running parallel to the longitudinal axis of the muscle fibers. Also the amorphous layer (A) composed of small diameter collagen fibrils lies just above the sarcolemma (S).

Fig. 9. Scanning electron micrograph of rabbit semimembranosus muscle. Reticular fibers (R) are in close association with the amorphous layer. A portion of the amorphous layer, composed of small diameter collagen fibrils (C) is attached to reticular-type fibrils. Collagen fibers (CF) are seen traversing the reticular-type fiber matrix. Periodicity between reticular fiber rows is 3.0 μm. Small diameter filaments (F) appear to connect reticular fiber rows.
Reticular fiber rows of restrained rabbit and bovine muscle were oriented within a plane perpendicular to the muscle fiber longitudinal axis, and divergence from this orientation was not observed (Figure 7). Reticular fiber orientation was altered by the contraction state of the sternomandibularis muscle. The reticular fiber arrangement within unrestrained bovine sternomandibularis muscle was always oblique to the myofiber longitudinal axis (Figure 15). Muscle restrained prior to fixation exhibited reticular fiber rows oriented within a plane slightly oblique or perpendicular with respect to the myofiber longitudinal axis (Figure 16). Periodicity between reticular fiber rows was not changed by the contraction state of the muscle (Figures 15 and 16), periodicity between reticular fiber rows within both restrained and unrestrained bovine sternomandibularis muscle was approximately 4.0 μm.

Reticular fiber attachments to the collagen fibril amorphous sheets were located parallel to A-band regions, Z-lines and A-band/I-band junctions, indicating that the exact point of attachment for reticular-type fibers to the collagen fibril sheets probably is not associated with specific myofibrillar structures (Figure 17). Moreover, reticular fiber connections were always associated with sheets of amorphous collagen fibrils surrounding individual muscle fibers (Figures 17 and 18), and direct reticular fiber attachment to muscle membranes was not observed. It was not possible to determine if a structural continuum existed between muscle intracellular (the myofibrils) and extracellular (the endomysium) structures.

Samples preserved by fixation with 3% glutaraldehyde (bovine sternomandibularis muscle buffered with 0.1M sodium cacodylate; pH 7.4) did not exhibit cellular shrinkage (Figure 19). Additionally, reticular fiber ultrastructure of this tissue appeared flat and less distinct as compared to ultrastructure of similarly prepared tissue using an extremely hypertonic fixative (Figure 18).

Micrographs of two closely associated muscle cells demonstrate that the endomysium is indeed easily distinguished in freeze fractured muscle (Figures 20 and 21). Additionally, the presence of the amorphous collagen layers and adjoining reticular fiber networks further substantiate that the endomysium was exposed using our procedures (Figure 20).

Discussion

Intermyofibrillar Bridges

Using SEM, Sybers and Ashraf (1973) observed structures within heart muscle, similar to the intermyofibrillar bridges observed in the present study, having 10 μm periodicities. Sybers and Ashraf (1973) identified these structures as T-tubules, but these structures also resembled Z-lines. Voyle (1981) found intermyofibrillar bridges within bovine longissimus dorsi muscle which were tentatively identified as T-tubules. In the light of recent evidence, structures previously identified as membraneous T-tubules, may in fact be structural protein serving to covalently link myofibrils to neighboring myofibrils (Bonilla, 1983).

Dense bodies contain the protein α-actinin as a major component (Schollmeier et al., 1976). Membrane-bound dense bodies of smooth muscle cells are analogous to skeletal muscle Z-lines. Intermediate filaments, also called 10 nm filaments, encircle dense bodies (Cooke, 1976). Desmin, thought to be the major protein component of 10 nm filaments (Cooke and Chase, 1971; Cooke, 1976), comprises approximately 5% of the total protein of gizzard muscle and only 0.18% of skeletal muscle proteins (Huiatt et al., 1980). Desmin-containing fibers found within mature skeletal muscle may be remnants of 10 nm filaments (Robson et al., 1981; Holzer et al., 1982; Lazarides et al., 1982). Studies utilizing

Fig. 10. Scanning electron micrograph of rabbit semimembranosus muscle. Reticular fibers (R) bridge adjacent muscle fibers. An ordered structure of reticular fibers is evident, whereby reticular fiber rows attach into or through the amorphous layer (A). Reticular fiber rows have a periodicity of 3.0 μm, and the reticular-type fiber matrix has a honeycomb appearance. A parallel arrangement of reticular fiber rows perpendicular to the axis of the muscle fiber is evident. A bare sarcolemma (SI) (intracellular face) has been exposed and the amorphous layer composed of small diameter collagen fibrils (C) is attached to an extracellular sarcolemmal surface.

Fig. 11. Scanning electron micrograph of bovine sternomandibularis muscle. Muscle fibers (MF) surrounded by endomysium (E) are evident within the micrograph of a cross section of bovine sternomandibularis muscle which had undergone tissue shrinkage during fixation. Perimysium surrounds both primary and secondary muscle bundles. Perimysium surrounding primary muscle bundles (PP) is less complex compared to perimysium surrounding muscle bundles (SP).

Fig. 12. Scanning electron micrograph of bovine sternomandibularis muscle. Reticular fibers (R) similar to those found within the endomysium are found within the perimysium surrounding muscle fiber bundles (MB) and collagen sheets (CF).

Fig. 13. Scanning electron micrograph of rabbit semimembranosus muscle. Bare sarcolemmal membranes (SI) (extracellular surface) are seen on several fibers. Reticular fibers (R), embedded in the amorphous layer (A), are found interconnecting muscle fibers. Reticular fibers seem to be continuous with what appear to be laminar rows (LR) within the perimysium (P; upper left quadrant of micrograph).

Fig. 14. Scanning electron micrograph of rabbit semimembranosus muscle. Rows of laminar material (LR) are oriented parallel to one another within the perimysium. Small diameter filaments (F) join both laminar rows and collagen fibers (CF).

Fig. 15. Scanning electron micrograph of unrestrained bovine sternomandibularis muscle. Reticular fibers (R) joining neighboring muscle fibers are exposed. Shrunk myofibrils (M) and filaments (F) joining adjacent myofibrils are found within one of the myofibers. Filaments (F) join rows of reticular-type fibers. The uniformity of reticular-type fiber distribution is disturbed by the shortened state of the muscle. Where filaments join reticular-type fiber rows, the periodicity between reticular-fiber rows is approximately 4.0 μm.
Intermyofibrillar Connections
immunochemical fluorescence microscopy demonstrated that proteins comprising 10 nm filaments are present at Z-lines and within I-band regions of mature skeletal muscle fibers (Holtzer et al., 1982). However, intact 10 nm filaments, observed using TEM, are absent from mature muscle cells (Holtzer et al., 1982). Progenitor myogenic cells contain 10 nm filaments during early myogenesis, but these are absent in mature skeletal muscle cells (Holtzer et al., 1982). Using TEM, Page (1969) found 10 nm filament groups encircling Z-lines of mature chicken muscle cells.

The present investigation demonstrated that Z-lines of adjacent myofibrils were joined to one another by filaments of between 92 and 147 nm diameters, and many 10 nm filaments may be contained within intermyofibrillar connections. It is possible that structures, composed of many 10 nm filaments or proteins once components of 10 nm filaments during myogenic development, function to connect adjacent myofibrils at each Z-line.

The actual diameter of the intermyofibrillar bridges is unknown as a result of the tissue shrinkage that occurred during sample fixation. The investigation revealed a possible relationship between intermyofibrillar bridge periodicity and sarcomere length, and demonstrated that myofibrillar connections in the Z-lines may be the Z-lines. The interrelationship between sarcomere length and intermyofibrillar cross bridges was investigated further using bovine sternomandibularis muscle, and this study demonstrated that intermyofibrillar filament periodicity changed with degree of muscle stretch. However, direct comparisons between sarcomere length, as measured using TEM, and distance between intermyofibrillar bridges were not always possible. The distance between intermyofibrillar bridges within restrained sternomandibularis muscle was 1.9 μm, indicating that Z-lines should reside at the termini of the A-bands, provided that intermyofibrillar bridges are located at Z-lines. However, from cell surface topography, sarcomere length was found to be between 3.2 and 3.6 μm; similar sarcomere lengths were obtained using TEM. Only one of the restrained bovine sternomandibularis muscle samples cleaved during freeze fracture exposed detail of intracellular structures. This particular muscle cell may not have been restrained during fixation, and possibly had undergone shortening during fixation since both cold temperatures and fixatives are known to elicit shortening of pre rigor muscle. Thus, it is possible that the periodicity between intermyofibrillar bridges within restrained sternomandibularis muscle when determined by SEM does not coincide with measurements obtained using TEM. No definite conclusions could be drawn from the SEM work concerning the myofibrillar location of the intermyofibrillar bridges of restrained sternomandibularis muscle. However, connections between adjacent myofibrils within unrestrained sternomandibularis muscle were disposed with approximately 1 μm periodicities, which was equivalent to the approximate sarcomere length of the tissue. These results demonstrate that intermyofibrillar bridges may be located at Z-lines.

When TEM was used, filaments smaller in diameter than the periodically disposed filaments were found joining neighboring myofibrils. Also, when TEM was used, intermyofibrillar bridges were found between A-bands of adjacent myofibrils. Results obtained from both bovine sternomandibularis and rabbit semimembranosus muscles demonstrate that interconnections between myofibrils are located at the Z-lines, and that smaller diameter filaments connect A-band regions of adjacent sarcomeres.

Endomysium Ultrastructure

The predominant ultrastructural connective tissue restraints found within the endomysium are reticular fibers (Swatland, 1976). Fibers comprising these networks are distinguished morphologically by their extensive branching as compared to that of collagen fibers which have little or no branching and by a unique affinity for silver stain (Swatland, 1976). Connective tissue structures characteristically staining dark or black with silver stain have been considered to contain reticulin, and reticulin-containing structures have been termed reticular fibers.

(Bodwell and McClain, 1971). Currently, new terminology should be devised for the description of reticular fibers to denote an ideologically change with regard to reticular fiber content and structure since recent evidence indicates that reticulin is type III collagen (Sims and Bailey, 1981). The results of the present study and that of Swaland (1976), demonstrate that reticular fibers are arranged in a plane approximately perpendicular to the muscle fiber longitudinal axis, and that reticular fiber orientation is altered through stretching or shortening.

Muscle connective tissue is composed of various collagen species (Swaland, 1975). Type I collagen is the predominant collagen form found within the epimysium, collagen types I and III are found within the perimysium, and types III, IV and V comprise the endomysium (Sims and Bailey, 1981). Sims and Bailey (1981) demonstrated, using immunohistochemical stains in conjunction with electron microscopy, that endomysial reticular fibers are composed of type III collagen. The present investigation revealed the presence of reticular-like fibers not only within the endomysium but within the perimysium as well. Moreover, it was demonstrated that perimysium surrounding primary and secondary fasciculi is composed of laminar-like rows resembling the endomysial reticular fiber structure, and orientation of this reticular tissue appears similar within both the endomysium and the perimysium.

Reticular fiber orientation changed with respect to the degree of stretch or shortening, and the distribution of reticular fiber rows within the unrestrained muscle was less uniform as compared to the restrained muscle. However, when muscle was allowed to shorten, periodicity between reticular fiber rows remained unchanged from that of restrained muscle, suggesting that the reticular fibers do not attach adjacent to specific myofilibrillar structures.

A sock-like structure composed of small diameter collagen fibrils was found encasing each muscle fiber. Rowe (1978) demonstrated that collagen fibrils present within the endomysium were of significantly smaller diameter than collagen fibrils of the perimysium, suggesting that two collagen fibril classes exist within muscle.

Endomysial ultrastructure and endomysial structural associations with the sarcolemma were examined. Sandwiched between two muscle cells are the amorphous collagen layers (components of the basement membrane which also contain significant quantities of mucopolysaccharides). Direct connections exist between the plasma membrane and the basal lamina (Bonilla, 1983). Encircling each myofiber outside or above the collagen fibril sheaths are reticular fiber rows spaced equidistantly from one another, and oriented in a plane approximately perpendicular to the longitudinal axis of each muscle cell. Orientation of reticular fiber rows to their associated muscle fibers appears related to the degree of muscle stretch. Reticular fibers are not attached to the basement membrane adjacent to specific myofilibrillar structures, and based on evidence from the current study, reticular fibers should be considered continuous only with the basement membrane collagen fibrils, and probably do not attach directly to the sarcolemma. The ordered reticular fibers attach to a laminar-like sheet continuum, found within the interstitium between muscle cells, which connects collagen fibers within the endomysium and joins with both laminar structures and collagen fibers within the perimysium. Figure 18 (rabbit semimembranosus muscle) best illustrates the structural arrangements described above.

This study has demonstrated the structural complexity of the endomysium, and has exposed structural interconnections between neighboring myofibrils. It was also revealed that the endomysial structure is more complex, and at the same time more ordered, than was originally assumed.

Presently, a SEM investigation is being carried out using a frozen muscle, placed on a freezing stage within a scanning electron microscope. Since fixation and tissue dehydration are being avoided our research should document native connective tissue ultrastructure.

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Scanning electron microscopy was performed at the Texas A and M University Electron Microscopy Center.

References


Intermyofibrillar Connections


Discussion with Reviewers

R.J. Carroll: Why is the amorphous layer observed in the SEM and not seen in the TEM?

Authors: The amorphous layer has not been observed using TEM possibly because proteins of the amorphous layer do not bind heavy metal ions of the traditional TEM stains. Also, the ground substance of the basement membrane may mask endomysial proteins/collagen fibrils. Using SEM proteins are coated/plated and staining is not dependent upon physical properties of the proteins or protein structures.

R.J. Carroll: Did you observe any differences in tissue structure or preservation using the various fixative combinations employed in this study?

Authors: Yes, when incubation buffer of extreme osmolarity was used in combination with glutaraldehyde fixative extreme tissue shrinkage resulted. Such shrinkage exposed the endomysium and related structures. Without muscle cell shrinkage, muscle cells were densely packed, and there was essentially no space between individual muscle cells.

R.J. Carroll: What differences did you observe between fractured muscle samples frozen in water and those frozen in alcohol?

Authors: All samples were frozen in liquid freon. After freezing, samples were fractured while bathed in chilled (~78°C) alcohol.

R.J. Carroll: What is the rationale for use of the hypertonic fixative?

Authors: Hypertonic fixation caused cell shrinkage such that the endomysial ultrastructure could be exposed.

C.A. Voyce: Please elaborate on the relevance of using fixatives of different osmolalities. It is apparent that a hypertonic fixative more readily exposes the fibrous network by causing myofibrillar shrinkage.

Authors: Buffered fixative solutions of different osmolalities were used to expose muscle endomysium and perimysium.

Osmolality was not calculated or determined. Osmolality was determined mathematically from the known contents of the various buffered fixatives.

The first study involved the use of a fixative having an osmolality that was significantly greater than normally utilized for muscle fixation. Most fixative-buffer combinations used for muscle fixation are in the neighborhood of 500 mOsm. Fixatives of this osmolality generally do not promote tissue shrinkage. Tissue shrinkage was used as a means to expose muscle ultrastructure for examination by SEM. Rabbit semimembranosus muscle was the first tissue tested using 750 mOsm buffered glutaraldehyde fixative. Fortuitously, this tissue provided significant exposure of both the endomysium and perimysium. Beef sternomandibularis muscle was used in a subsequent study to examine muscle prepared using buffered fixative solutions of high osmolality (750 mOsm) and conventional osmolality (570 mOsm). Bovine tissue prepared using the 750 mOsm buffered fixative underwent considerable shrinkage. This opened the perimysium and endomysium for visualization using SEM. Tissue prepared in the 570 mOsm buffered fixative solution did not shrink sufficiently to allow exposure of either the perimysium or the endomysium ultrastructure.
ACTION OF POLYPHOSPHATES IN MEAT PRODUCTS

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Abstract

The action of polyphosphates on meats has been examined in studies using different meats and has been compared with other treatments. The results were monitored by recording cooked yield and observing changes in structure by electron microscopy. Four possible characteristics of polyphosphates are considered: pH, sequestering action, ionic strength and as an ATP (adenosine triphosphate) analogue. In the conditions used in these experiments, the pH-buffering effect of polyphosphates is found as the principal characteristic in meat products.

Some meats were found to be more affected by polyphosphates than others and a hypothesis is developed to explain this. It is proposed that with meats such as pork, which suffer early pH fall post-mortem, there might be a greater association between sarcoplasmic and myofibrillar proteins than with other meats. The association between sarcoplasmic and myofibrillar proteins may be reversed by increasing the pH of the curing brine and this would allow more dispersion of myofibrillar proteins and hence a higher cooked yield.

In most of the meats examined no evidence for specific actomyosin solubilisation by polyphosphates was found except in heart and some higher pH beef meat.

Introduction

Polyphosphates are widely used in meat products to improve binding properties and are claimed to have antimicrobial properties (Elliot et al., 1964). There is no general agreement on the mechanism for polyphosphate action although there is a well-established synergistic action with sodium chloride and the major effect of polyphosphate is seen when meats are cooked (Mahon, 1961).

Particular properties of polyphosphates have been proposed as the main causes of polyphosphate action. Polyphosphates cover a range of compounds; there is some debate as to whether pyrophosphate or longer chain polyphosphates have most effect. Bendall (1954) noted a similarity between the pyrophosphate structure and that of ATP (adenosine triphosphate) and suggested that pyrophosphate could dissociate actomyosin formed in post-rigor muscle. Kotter (1960) extended this idea and proposed that the dissociated actin and myosin could be further dispersed by salt. Electron microscopic observations on cooked salt and polyphosphate-treated pork, however, reveals that the most resistant regions of sarcomere structure are those in which actomyosin is formed (Lewis and Jewell, 1975; Lewis, 1979, 1981; Rahelic and Milon, 1979).

Some work on the action of polyphosphates on raw meat structures has been reported. Working on myofibrils isolated from rabbit, Offer and Trinick (1985) showed that pyrophosphate can produce dissociation of actomyosin. Knight and Parsons (1984) found that beef myofibrils behaved more variable in contact with salt and pyrophosphate solutions. Isolated myofibrils are not necessarily a good model for meat products and to meet this criticism Voylee et al. (1984) repeated the earlier approach of Lewis and Jewell (1975) but examining only uncooked pork samples. They interpreted their micrographs as showing dissociation of actomyosin, although they could not explain a loss of material from either side of the Z-disc. Voylee et al. (1984) supported their case that only actin filaments were still present by reference to the work of Offer and Trinick (1983), in which SDS electrophoresis was used in conjunction with microscopical observations on isolated myofibrils. The electrophoresis was carried out only on myofibrils extracted at pH 5.5, whereas most commercial polyphosphate brines have a rather higher pH than this (generally pH 7.5 to 9.5), and so some care is needed in extrapolating these results. The electrophoresis showed that myosin was the main protein to show increased solubility in the presence of pyrophosphate. However, troponin and tropomyosin, both thought to be structural components of the thin filaments (Ebashi et al., 1969), were also largely
extracted and it would therefore seem unlikely that uncomplexed thin filaments would remain intact during extraction. Vyole et al. (1984) also claimed that the earlier micrographs of Lewis and Jewell (1975) showed evidence of actomyosin dissociation, although they did not offer any alternative interpretation of the micrographs of samples cooked after soaking in salt and polysphosphate brines. These clearly indicate the actomyosin regions as among the most resistant regions of the sarcomere to processing.

In addition to the actomyosin-dissociating capacity of pyrophosphate, Offer and Trinick (1983) also point out that pyrophosphate will depolymerise myosin filaments, especially in the presence of salt. Other properties of polysphosphates claimed to be responsible for their effect on meat are sequestration of metal ions (Ham, 1966), ionic strength, and pH-buffering capacity (Trout and Schmidt, 1984).

In order to test which of these properties produce the structural and functional action of polyphosphates, a range of experiments investigating the effects of meat type, pH (with and without phosphates), sequestering agents, degree of comminution, brine to meat ratio and freezing and thawing have been carried out. This report summarises the findings of the experiments concerning meat type, brine pH and sequestering action.

Methods

Standard Procedure for Soaking and Cooking Meat

Unless stated otherwise, the following method was used to soak and cook the meat pieces.

Cubes of meat, approximately 5 g, were weighed and an equal weight of brine added (4% sodium chloride, 1% sodium pyrophosphate). Samples were stored for 18 hours at 0⁰-5⁰C, then the meat pieces blotted to remove excess liquid and reweighed. Small samples from the outside (1 mm) of the block were taken and prepared for electron microscopy. The meat pieces were reweighed then returned to the brine and the vials placed in a beaker, which was immersed in a 75⁰C water bath for 1 hour. Using a water blanket, the temperature was monitored and found to take 25-30 minutes to reach 71⁰-72⁰C and did not rise above 73⁰C. After cooking, the samples were allowed to cool for 1 hour; the meat pieces were removed from the brine, blotted and any gel removed, then weighed. Samples were taken from the outside of the block for electron microscopy. Samples of the raw meat prior to soaking were also prepared for electron microscopy. The pH of the brine before and after soaking was recorded. The pH of a salt and pyrophosphate brine fell from about 9.4 to about 6.4 during overnight soaking of meat. Brines were prepared using sodium hydroxide to give a similar buffering capacity to pyrophosphate; in this case, the initial pH of the brine was around 12.1 but this fell during soaking overnight to around 6.5. If the pH of a salt brine was adjusted to pH 9.4 with sodium hydroxide the pH fell to a similar level to that for salt only after overnight soaking.

Meat was obtained by local purchase; in some cases animal and post-mortem details were well documented whilst in other cases the meats were simply purchased ‘off the shelf’. Reagents used to prepare brines were in most cases food grade; in a few cases standard laboratory reagent grade was used.

Preparation for Transmission Electron Microscopy

Small pieces of meat approximately 1 mm³ were fixed in standard Minsky's fixative in buffer (a commercial preparation available from National Diagnostics Ltd) for 1 hour, washed several times in distilled water then dehydrated through a graded series of ethanol into absolute ethanol. Samples were infiltrated and embedded in LR White resin and thin sections (approx. 80 nm) were cut on a Reichert Ultramicrotome and collected on copper grids. The sections were post-stained in 7% uranyl acetate in absolute methanol for 2 min, washed in methanol and dried. The grids were coated with a layer of carbon and examined in a JEOI 1020 EX operating at 80 kV. Selected samples were also fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer for 1 hour, washed in water, dehydrated using ethanol and treated as above. Glutaraldehyde- and Minsky-fixed samples showed essentially similar structures and so Minsky fixative was used throughout this work.

Sections (1-2 µm) of the resin blocks were also prepared for light microscopy.

Experimental Plan

Effect of Brine pH and Phosphate Type on Pork

All systems were cooked after soaking in 4% salt solution overnight at 50⁰C at 1:1 meat:brine ratio of 1:1. Appropriate additives were added to the brine. Controls containing water only and 4% salt with no additives were also included.

Brine additives investigated were: 1) 1% sodium pyrophosphate (Na₂P₂O₇), initial pH 9.4; 2) 1% sodium hexametaphosphate (NaPO₃)₆, initial pH 5.9; 3) 1% sodium tetrapolyphosphate (Na₄P₂O₁₀), initial pH 7.5; 4) 1% sodiumtripolyphosphate (Na₃P₂O₁₀), initial pH 8.1; 5) 1% sodium pyrophosphate (initial pH adjusted to pH 5.5); 6) 1% sodium pyrophosphate (initial pH adjusted to pH 6.5); 7) 1% trisodium orthophosphate (Na₃PO₄), initial pH 11.5; 8) 1% disodium orthophosphate (Na₂HPO₄), initial pH 8.7; 9) 1% monosodium orthophosphate (NaH₂PO₄), initial pH 4.5; 10) sodium hydroxide to give pH 6.5 in brine after overnight soaking of meat (initial pH 12.1); 11) 0.1M Tris buffer, pH 9.4.

Effect of Calcium Sequestering Agents

Conditions were as above but with the following additives: 12) 1% EDTA; 13) 1% calcium chloride; 14) 1% EDTA plus sodium hydroxide as in 10) above; 15) 1% calcium chloride plus sodium hydroxide as in 10) above.

Effect of Meat Type

Meat samples were soaked in a standard brine containing 4% salt plus 1% sodium pyrophosphate, at a 1:1 brine to meat ratio, overnight at 5⁰C before cooking. Meats used were:

16) pork L. dorsi 24 h post-mortem pH 5.5
17) pork loin commercial retail pH 5.6
18) pork loin commercial retail pH 5.8
19) pork loin commercial retail pH 5.7
20) pork loin 24 h post-mortem pH 6.2
21) beef heart 24 h post-mortem pH 6.3
22) beef flank 24 h post-mortem pH 5.7
23) beef forequarter 24 h post-mortem pH 5.9
24) chicken commercial retail pH 6.0
25) cod commercial retail pH 6.6

The quoted pH values were measured on the meats as received.

Effect of Polyphosphates on Beef

23a) Beef forequarter, water only; 23b) beef forequarter, 4% salt only, initial pH adjusted to pH 9.0; 23c) beef forequarter, 4% salt + 1% pyrophosphate; 23d) beef forequarter, 4% salt + 1% pyrophosphate, adjusted to initial pH 6.5; 23e) beef forequarter, 4% salt + 1% pyrophosphate, adjusted to initial pH
5.5; 26a) beef topside, 4% salt only; 26b) beef topside, 4% salt + 1% tripolyphosphate; 26c) beef topside, 4% salt + 1% commercial polyphosphate blend (Fibrosol C700 instant).

Results

Figures 1 and 2 show light micrographs of raw pork and pork cooked after soaking in salt and polyphosphate brine.

Figures 3 and 4 show TEM micrographs of raw pork and pork soaked in salt and polyphosphate brine. Figures 5 and 6 show electron micrographs of commercial ham products. Figures 7–16 illustrate the range of structures seen in the meat samples examined along with a subjective evaluation of the type of dispersion, coded A, B, C, D and E. The dispersion types are illustrated diagrammatically in Fig. 17 and are interpreted as follows: A) coagulation of proteins with little dispersion other than some loss of thin filaments on cooking (Figs 7 and 8); B) dispersion of material from H-zone as well as loss of thin filaments; M-line and Z-line generally but not always present (Figs 9 and 10); C) obvious dispersion of H-zone and reduction of M-line; Z-line generally but not always dispersed (Figs 11 and 12); D) extensive dispersion of all regions of sarcomere with only A/I overlap regions visible (Figs 13 and 14); E) dispersion of sarcomeres but leaving Z-lines and M-lines visible; some dissociation of actomyosin in A/I bands (Figs 15 and 16).

The predominant type of dispersion found in the systems examined, along with summaries of the cooked yield and post-soaking pH, are presented in Figs 18, 19 and 20.

It should be noted that a wide range of structures could be found in some of the meat samples and these classifications are a subjective evaluation of the main patterns of dispersion of myofibrillar proteins. Dispersion types B to D are considered as progressively more dispersed manifestations of a single mechanism whilst type E is considered to be a quite different mechanism.

Fig. 1. Phase contrast light microscopy of 2-μm section of raw pork showing myofibrillar striations.

Fig. 2. Phase contrast light microscopy of 2-μm section of pork soaked and cooked in salt and polyphosphate brine, showing twin dark bands in each sarcomere.

Fig. 3. Electron microscopy of thin section of pork meat showing features of sarcomere structure.

Fig. 4. Electron microscopy of thin section of pork soaked in salt and polyphosphate brine showing some dispersion of proteins from normal sarcomere structure.

Fig. 5. Electron microscopy of freeze-etched preparation of commercial ham product showing densely packed bands (arrows). (See Lewis and Jewell, 1975, for preparation details.)
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Fig. 6. Electron microscopy of thin section of commercial ham product showing densely staining bands. Similar product to that in Fig. 5. (From Lewis and Jewell, 1975.)

Figures 7–16 are electron micrographs of thin-sectioned preparations of meats cooked after soaking in various brines. Z- and M-lines are indicated on them.

Fig. 7. Beef forequarter soaked and cooked in 4% salt + 1% pyrophosphate adjusted to an initial pH of 6.5; sample 23d. Shows type A dispersion with most myofibrillar proteins heat coagulated in situ. Sarcomere features readily recognisable.

Fig. 8. Pork loin soaked and cooked in 4% salt + 1% monosodium orthophosphate, treatment 9. Shows type A dispersion with only slight extraction of H-zone and readily recognisable sarcomere features.

Fig. 9. Pork loin soaked and cooked in 4% salt + 1M sodium hydroxide to give a post-soaking pH of about 6.5, treatment 10. Shows noticeable dispersion in H-zone but with Z- and M-lines still present. Type B dispersion.

Fig. 10. Pork loin soaked and cooked in 4% salt + 1% sodium hexametaphosphate, treatment 2. Shows marked dispersion of H-zone but Z- and M-lines still present. Type B dispersion.

Fig. 11. Pork loin soaked and cooked in 4% salt + 1% sodium pyrophosphate, sample 18. Shows marked dispersion of H-zone and I-band with loss of Z- and M-lines. Type C dispersion.

Fig. 12. Pork loin soaked and cooked in 4% salt + 1% disodium orthophosphate, treatment 8. Shows marked dispersion of H- and I-bands and loss of Z- and M-lines. Type C dispersion.
Micrographs can often be difficult to interpret even for experienced microscopists. In the case of meats soaked in salt and polyphosphate solutions the interpretation is open to question. Lewis and Jewell (1975) interpret the presence of fibres of similar thickness to natural myosin and actin filaments as indicating that both types of filament are present in soaked meat, whilst Voyle et al. (1984) consider that the thicker filaments are clumped actin filaments. Both views are tenable but in the soaked only samples the filaments are obscured by a colloidal mass of protein, which makes it difficult to decide which of these views is correct.

Interpretation of micrographs must always consider preparation procedures. Voyle et al. (1984) and Offer and Trinick (1983) make much of the relatively minor observations by Lewis and Jewell (1975) that light microscopy of processed and raw meat revealed mainly similar structures with only a few cells in processed meats showing differences. A consideration of the preparation procedures may help to explain this. The light microscopy sections were about 10 \( \mu m \) thick, which means that in the thickness of the section about ten myofibrils will lie on top of each other. The thickness of the sections means that the resolution will rarely be adequate to distinguish sufficiently fine detail to show the changes in the sarcomere. Hence only a few cells might be expected to show differences from the normal structure. Interpretation of changes in density in stained sections must also be carried out carefully. Thus when Voyle et al. (1984) explain changes in density as being due to swelling or extraction they neglect other possibilities, in particular that the charge on the proteins may have been altered, thereby making it more or less capable of taking up stain. Concentrated salt solutions generally decrease the staining of meat. The problems of variable staining between samples is well known to most microscopists. Figure 9 of Voyle et al. (1984) illustrates the problems of uneven staining and points to the dangers of placing too much reliance on changes in density alone.

After consideration of some of the problems of interpretation of micrographs, the present observations can be considered. There is general agreement on the identification of components in the thin-sectioned view of muscle and this is illustrated in Fig. 17. There are various refinements to this simple view of sarcomere structure; in particular, the gap filaments are perhaps the most significant of these. locker (1984) gives an up-to-date account of the gap filaments and explains their possible location and significance in meat behaviour. Where little dispersion of protein occurs before heating (Type A, Fig. 17) the appearance can be simply interpreted with reference to the raw structure, the principal components being coagulated in situ. Type B dispersion of proteins can also be recognised by direct reference to the raw structure. In some cases, it may be difficult to decide which are the remains of M-lines and which are the remains of Z-lines, but often this can be resolved as the I-band is generally more dispersed than the H-zone and in most cases the Z-line is more labile than the M-line.

The problem of interpretation is more difficult with dispersion types C and D, where there are few reference points other than the periodic dark bands and interpretation is based on indirect observations. In type B dispersion, Z- and M-lines are often dispersed to some extent and so it seems unlikely that they would give rise to a constant periodic structure. Examination of the spaces between the dark bands supports this view. In most cases the nature of these spaces alternates, one space
Fig. 17. Sarcomere structure and types of dispersion found in soaked and cooked meats.

Fig. 18. Cooked yields and pH. Summary of the effect of different soaking brines compared with a control system containing 4% salt + 1% sodium pyrophosphate; pork loin meat.

1 All systems contain 4% salt unless stated; results are combined from several experiments; in each experiment the yields are compared with those of the control for that experiment.

2 M-line extracted.

Fig. 19. Comparison of cooked yields, post-soaking brine pH and dispersion type for different meats treated with 4% salt + 1% sodium pyrophosphate brine.

Fig. 20. Cooked yields and pH. Summary of the effect of different soaking brines on beef.
referred to as titin) and it would seem likely that these proteins constitute the dark bands. Little is known about the susceptibility of connectin to salt, polyphosphate and heat, although it is reported (Maruyama, 1983) to be extracted by 0.1M phosphate buffer at pH 6.2–7.0 but not at pH 5.5–5.6, which removes denatured actin. It is extracted from fresh muscle along with myosin by 0.6M potassium chloride (Maruyama, 1985). Connectin can interact with myosin and actin and may enhance super-precipitation of actomyosin, although these interactions are dependent on the ionic conditions and it is not clear how the proteins will behave under meat-processing conditions. Work on overstretched and cooked muscle has produced dark bands in the I-band regions of the sarcomere (Locke et al., 1975). These may be formed from connectin, actin and nebulin; however, they are generally more closely spaced and less densely packed than most of the dark bands found in processed meats. Also, in type B dispersion (e.g. Figs 9 and 10), dark bands can be seen to be within the A-bands, and these dark bands are similar in density and spacing to those seen in type C and D dispersion. On this evidence we conclude that the dark bands derive from the A/I overlap and consist mainly of denatured actomyosin and connectin. The remaining sarcomere proteins, in particular unassociated myosin and actin, are probably dispersed through the meat structure in the form of a water-holding gel. The fate of connectin in meats showing type E structure with substantial dispersion of the A/I junctions is unclear. In these samples, the Z-lines are particularly prominent and it may be that connectin, related to the dispersion of actomyosin, precipitates on the Z-lines. A similar type of precipitation is reported in muscle extracted with potassium iodide (Maruyama, 1985).

Finally, in consideration of artefacts it might be asked whether these dark bands could be produced by the preparation processes used for microscopy. This is unlikely since they can be found in samples examined by light microscopy as well as transmission electron microscopy using different fixatives, and also in unfixed freeze-etched preparations.

Discussion

The results raise a number of interesting points for consideration. Taken with the work of Offer and Trinick (1983), they indicate that phosphates can act in two ways on meat structure. Firstly, they can promote the general dispersion of myofibrillar proteins by salt; in this case the A/I overlap regions, rich in actomyosin, would seem to be most resistant to dispersion. Structure types B, C and D represent varying degrees of this type of action. Secondly, they can promote dissociation of actomyosin specifically and so induce the selective dispersion of myosin by salt. Structure type E represents this type of action. Both these mechanisms have been demonstrated and the main point for consideration is under what circumstances each mechanism applies. The following factors seem to be significant: meat type, condition and history; pH of the system; ratio of extracting medium to meat; extent of protein/protein interaction in the meat; and degree of comminution of the meat. There is little doubt that myofibrils freshly isolated from rabbit poae show dissociation of actomyosin when treated with a great excess of salt and polyphosphate extraction medium at pH 5.5. Equally, from our studies, it seems unlikely that extensive dissociation of actomyosin is the dominant feature when pork meat is cooked after soaking in salt and polyphosphate brine. Beef heart generally shows dissociation of actomyosin; other beef samples have been variable but tend towards limited dispersion of the non-actomyosin regions of the sarcomere. In chicken and cod, the few observations we have made suggest dispersion of the non-actomyosin regions of the sarcomere. One of the features that it is likely to be different in these cases is the state of the sarcoplasmic proteins and their interaction with myofibrillar proteins. In the case of isolated myofibril it may be expected that the sarcoplasmic proteins will have been largely removed and so the polyphosphate can interact directly with the myofibrillar proteins. In the case of pork meat there is a distinct possibility that some post-mortem precipitation of sarcoplasmic proteins may occur around the myofibrils.

Monin and Laborde (1985) have also shown a pH-dependent interaction between myofibrils and sarcoplasmic compounds, which results in higher water-holding capacity at pH values removed from the isoelectric point (i.e. pH 5.5). Thus the interaction between sarcoplasmic and myofibrillar proteins could be reversed by raising (or lowering) the pH. Phosphates are also used in detergents as aids to dispersion by forming soluble complexes, and a similar role in dispersing sarcoplasmic proteins is possible.

Pork has a tendency to produce rapid pH fall post-mortem whilst the meat temperature is high and in extreme cases this can lead to the PSE (pale, soft, exudative) condition, in which large amounts of sarcoplasmic protein are precipitated on to the myofibrillar proteins (Bendall and Wismer-Pedersen, 1962; Scopes and Lawrie, 1963; Voyle, 1979). It is not clear whether any precipitation occurs in non-PSE pork meat but it is possible that it will occur to some extent. Electron micrographs of raw, post-rigor pork generally show some obscuring of the myofilaments (e.g. Fig. 5), compared with freshly excised muscle, which may be caused by sarcoplasmic proteins associating with the myofibrils. In beef, the early pH fall tends to be slower, in extreme cases leading to DFD (dark, firm, dry) meat, where the meat proteins bind their natural water very effectively. It is likely that beef sarcoplasmic proteins will be less prone to associate with the myofibrils. In this respect beef heart had the highest pH and therefore possibly has least association of sarcoplasmic and myofibrillar proteins. In heart, as in isolated myofibrils, actomyosin dissociation is seen as the most noticeable effect of polyphosphate; in both cases it is likely that there is little association between sarcoplasmic and myofibrillar proteins.
between beef and pork is the chemical structure of the myosin and actin. Differences are thought to exist between muscle types (Freeth and others, 1985) and probably between species and between heart muscle and voluntary muscle, and these chemical differences may influence the dispersion of myofibrillar proteins by salt and phosphates.

In pork samples, the pH of the brine after soaking appeared to be the predominating feature in determining the cooked yield; where the brine pH was high the cooked yields were also high. This relationship did not apply to other meats; for example, beef heart and beef forequarter both had high pH values in the soaking brine and yet had lower yields than pork meat, which had lower brine pH values. A possible explanation for this can be found by considering the meat pH. Pork pH values were generally lowest and raised pH brines were more effective in increasing the yield in these meats than in beef. It may be that the lower pH in pork meat has been accompanied by some water loss as drip, and that raising the pH allows this loss to be recovered.

Non-heart beef muscles were more variable in their behaviour than pork; beef flank behaved like some pork samples; beef forequarter was fairly resistant to dispersion of myofibrillar proteins by both salt and phosphates; and beef topside seemed to be able to react in the same way as pork meat or heart. A partial explanation may lie in the post-mortem pH values of the meats; beef heart had the highest pH value — about 6.2 — whilst beef flank had a post-rigor pH value similar to that found in most of the pork samples, i.e., about 5.6. Hence it seems that for beef and pork polyphosphates are most effective in meats having lower pH values. A similar observation was made earlier for meats comminuted with fat (reported by Lewis, 1986). In general terms, meat having a pH value below about 5.9 will show improved cooking yields if the pH of the brine is raised to about 6.1 or more. For meats with a pH greater than 6 the effect of raising the pH or adding phosphates seems to be marginal in terms of cooked yield. The limited observations on chicken and cod suggest that they do not fit directly into the above hypothesis. However, it may be that the general principle applies but that the critical pH values are different. Further work would help to elucidate this.

These observations indicate that pH is the main effect of polyphosphates and that raising brine pH can be most effective in meats which have a lower pH. The work also indicates something of the importance of the other characteristics of polyphosphates which may play a role in meat products.

It seems clear that the sequestering action of polyphosphates is not of great importance in pork since the addition of EDTA and calcium chloride lowered the cooked yield compared with systems in which they were absent.

The series of experiments including 1% pyrophosphate at different pH values indicates that the presence of pyrophosphate (as an ATP analogue) has only a minor effect. Pyrophosphate- and tripolyphosphate-containing systems gave only marginally higher yields than other systems at the same pH.

In these experiments the ionic strength effect of polyphosphates did not seem very significant. At first sight this contradicts the conclusions of Trout and Schmidt (1984), who claim that both pH and ionic strength are significant features of polyphosphate action. In our experiments the level of salt was fairly high (about 2% overall) whilst Trout and Schmidt were considering low-salt products, and at lower salt levels the ionic strength of polyphosphates may be more important. However, previous work (Lewis and Jewell, 1975), using constant ionic strength brines, indicated that both sodium chloride and polyphosphate were necessary to produce optimum effect. This suggests that the chloride ion also has a specific effect and indicates that salt may be only partially replaced by polyphosphate.

A curious feature of meat behaviour on processing is the variable appearance of the M- and Z-lines. This has been observed in our own work and in that of Voyte et al. (1984).

Two small observations in this work may help to explain this phenomenon. Tris buffer produced quite extensive dispersion of myofibrillar proteins but M- and Z-lines could generally be recognised; addition of calcium chloride reduced general myofibrillar dispersion but removed the M-line. Hence it seems that M-line behaviour may be linked to calcium availability whilst Z-line dispersion may be a specific feature of polyphosphates. Previous work (Lewis, 1981) also showed that pork cooked in polyphosphate solution without salt showed extraction of the Z-lines. It is also known that Z-lines in different fibre types have different structures (Gauthier, 1976) and this may be a source of variation. In particular, the dense staining of Z-lines in processed meat probably reflects the different structure of the Z-line in heart. Connectin may also associate around the Z-line and the availability of connectin may depend on the extent to which actin and myosin are dispersed.

Overall, our findings point to pH as the predominant characteristic of polyphosphates in meat products. In our view this is linked to the association between sarcoplasmic myofibrillar and cytoskeletal proteins. Where rapid pH fall occurs post-mortem, we consider that some precipitation of sarcoplasmic proteins may occur; this might be reversed by a high pH brine which would thereby improve cooked yield. If this hypothesis is correct then meat pH can be considered as a principal factor in understanding the variability of meat behaviour.

The nature of the interactions of sarcoplasmic and myofibrillar proteins with the cytoskeletal proteins, in particular connectin, and the influence of pH on these interactions is largely unknown. The next step in understanding polyphosphate and pH effects in meat may well depend on the unravelling of these interactions. It must be emphasised that other differences such as connective tissue organisation, biochemical characteristics of muscle fibres and differences in chemical structure of myosin and actin may also have some bearing on meat behaviour. Control of these features is, however, rather more in the area of genetics, whilst pH adjustment is an operation which can be undertaken in a meat factory.

Conclusions

1. Some meats, generally those with low pH values, are more likely to produce increased yields in the presence of polyphosphates than others.
2. Where polyphosphates are effective in increasing yield, their ability to maintain a high pH seems to be the most important characteristic. Other alkaline materials — sodium hydroxide, trisodium orthophosphate and Tris buffer — are also effective in increasing yields if present at high enough levels.
3. Increased yields are generally associated with increased dispersion of myofibrillar proteins.
4. In most meats the A/I band overlap region is the most resistant to salt and polyphosphate, although evidence for actomyosin dissociation is seen in beef heart muscle and sometimes in other beef muscles.
5. The sequestering action and ATP analogue properties of polyphosphates seem to be of less significance in explaining
Action of Polyphosphates in Meat Products

the behaviour of polyphosphates in meats. The contribution to ionic strength of polyphosphates has little effect in the presence of an overall level of 2% salt.

Acknowledgements

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References


Discussion with Reviewers

R.J. Carroll: Are any data available on the changes in the water-holding capacity of the uncooked product resulting from the various brine treatments? Authors: Weight changes on soaking and cooking were recorded and selected samples were examined by electron microscopy. In our view the microscopy of most of the soaked samples shows dispersion of protein from the H-zone, although the presence of generally dispersed proteins makes interpretation difficult and we have concentrated on the cooked samples. Some examples of soaked yields are as follows: pork in water 101%, pork in 4% salt 107%, pork in 4% salt plus 1% polyphosphate 116%, pork in 4% salt plus 1% trisodium orthophosphate 119%, pork in 4% salt plus 1% pyrophosphate (initial pH to 5.5) 111%, beef heart in 4% salt plus 1% pyrophosphate 105%, and beef forequarter in 4% salt plus 1% pyrophosphate 116%. Note that these figures are results of single experiments and have not been averaged against the controls in the same way as the results in Fig. 18.
S. Cohen and J. Smith: Many researchers indicate that sodium chloride and some phosphates swell the meat microstructure while mechanical action may be required for protein extraction. Would the lack of the mechanical action in your work help to explain the variability you observed in actomyosin dissociation? (Ref. Schmidt GR. (1984). Processing effects on meat product microstructure. Food Microstructure 4, 33-39.)

G.R. Schmidt: Would the application of mechanical energy to meat during its incubation with solutions of different composition have an effect on the amount of structural change taking place?

Authors: Our experience of examining tumbled meats shows that different meats behave variably with respect to actomyosin dissociation even when mechanically treated. This variability seems to depend on intrinsic structural features such as degree of contraction and nature of interstitial connective tissue. Even comminuted meats have shown variable responses to salt solutions. In general, mechanical treatment allows changes to occur more rapidly, but in some cases we have observed that the effect of tumbling in salt-only brines is similar to that of soaking in salt and polyphosphate brine.

S. Cohen and J. Smith: Do you believe that the cook yield data are based on a large enough sample (5 g that is sampled prior to cooking) to represent industrial processing adequately? Additionally, did the length of cooking (1 h at 75°C) possibly negate some yield potential due to over-cooking?

C.A. Voyle: How many samples of each meat type were exposed to the treatment described?

Authors: At least two samples of each meat were used in each treatment. In the case of control samples (i.e. 4% salt plus 1% pyrophosphate), up to twelve samples of each meat were used: the good agreement between these samples leads us to believe that our samples give a reasonable indication of commercial meat performance. There is also a similarity between the structure of our samples and of commercial cooked, cured pork products. Other work carried out in our laboratories suggests that the model system gives a reasonable indication of meat behaviour at pilot-plant scale.

The earlier work of Lewis and Jewell (1975) examined cooking at different temperatures and heating rates. From this work we consider that our process gives a reasonable approximation to a pasteurised product. Higher temperatures (about 90°C and above) produce much greater changes in the connective tissue with consequent changes in cooked yield and texture. The presentation of the results as bar charts rather than as numbers is intended to avoid giving a false impression of precision.

C.A. Voyle: You have referred to ‘dispersion’ of protein rather than extraction or solubilisation. What do you think is the difference, if any?

G.R. Schmidt: Would it have been possible to analyse the incubating media for the presence of proteins extracted from the F-band?

Authors: The reference to ‘dispersion’ is quite deliberate as it describes the effect that we see, i.e. proteins are dispersed from the ordered positions that they occupy in the native meat structure. Extraction would imply complete removal of the protein from the meat and we do not know whether this happens. Solubilisation is more difficult to define, but to us suggests that individual molecules go into solution; we have no way of telling whether the dispersion occurs at a molecular level or at a multi-molecular level. We consider that dispersion covers all these possibilities.

It would probably be possible to analyse the brines for I-band proteins, although we think that there could be problems with the salt and polyphosphate present. Since we are considering dispersion rather than extraction, it may well be that analysing for I-band material would not give too much additional information. However, we consider that our findings and recent work on cytoskeletal proteins open up many possibilities for obtaining a better understanding of meat behaviour and we believe that these will require biochemical and chemical techniques in addition to microscopy.

G.R. Schmidt: Does the fact that the lowest salt concentration utilised in this experiment was 2% preclude making conclusive statements about the role of ionic strength on the changes in microstructure of meat?

Authors: Previous work (Lewis and Jewell, 1975) considers the effect of ionic strength in a little more detail. From this work we conclude that the best salt levels for cooked yield fall between 2% and 5% overall in our system (estimated ionic strength 0.45 to 1.0). At a constant ionic strength (estimated as 0.36), changing the ratio of salt to tripolyphosphate markedly altered the type of structural change observed.

From our past and present work we conclude that the ionic strength of polyphosphates is of minor importance in the presence of 2% salt. Tripolyphosphate present at 0.5% overall in the absence of salt tends to disperse the Z-line on cooking but leaves the other major myofibrillar structures intact and is less effective in improving cooked yield than a mixture of salt and polyphosphates. Tripolyphosphate alone at approximately 1% overall appears to disperse Z-lines and much I-band material, possibly dissociating actomyosin.

G.R. Schmidt: Would knowing more about the origin of the meat have been of any value in interpreting the microstructural changes that take place during incubation with the various solutions?

Authors: Undoubtedly a systematic study of the behaviour of meat from different origins would be of great value. However, to be of real value, it would have to cover a much wider selection of meats and as such was beyond the scope of this project.

G.R. Schmidt: Is there an optimal pH for causing structural changes in muscle?

Authors: Structural changes can occur at all pH values and ‘optimal pH’ depends on the nature of the product required. For most meat products the balance between maintaining structural integrity and obtaining good yield will probably require a pH around 6.0 to 6.5, although this may vary for different meats and microbiological considerations may dictate a lower pH for some products.

Reviewer V: Have you any thoughts on the interaction of meat pH and brine pH during soaking?

Authors: In general, the pH of the soaking brine changes to become closer to the initial pH of the meat during soaking. The extent to which this happens depends largely upon the relative buffering capacities of the meat and brine, although enzymic hydrolysis of polyphosphate may also affect the final pH. In our view it is the buffering capacity of the brine additives rather than their initial pH which is the most important characteristic. On cooking, the pH changed slightly, typically by 0.1 pH unit in either direction.
MICROSCOPICAL OBSERVATIONS ON THE STRUCTURE OF BACON

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Abstract

Commercially processed salt-treated pig longissimus dorsi muscle, in the form of bacon slices, sometimes shows localized variations in the light-scattering properties of the tissue. The phenomenon is described as 'tiger-stripe'. A study of areas of tissue showing such variations, using electron microscopy, has revealed differences in the structure at the myofibrillar level. Areas which appear dark when viewed by incident illumination show ordered myofibrillar structure, whereas areas which appear light under similar viewing conditions appear to be disordered.

Introduction

The traditional Wiltshire process of bacon manufacture, as practised in the United Kingdom, involves the treatment of sides of pig meat with sodium chloride, together with sodium or potassium nitrate and, frequently, sodium or potassium nitrite. Polyphosphates and ascorbates are often used in modern cures. All these substances are incorporated in a solution (brine) which is injected under pressure into the side of meat using a multi-needle injection machine. An informative summary of the processes involved in curing and maturation during the manufacture of bacon is given by Lawrie (1985a). A more detailed account is to be found in the U.K. Bacon and Meat Manufacturers Association (BMMA) Code of Practice (1978).

References in the literature to structural changes occurring in muscle tissue as a result of its conversion to bacon are scarce. However, the microstructure of raw bacon has been briefly described by Cassens et al. (1979), and of raw and cooked bacon by Stanley et al. (1980). Lewis (1974) described variations observed in sections of back bacon. Some myofibrils displayed recognisable features of sarcomeres, such as well-defined Z-lines and an array of filaments. Other myofibrils presented a granular appearance between rather indistinct Z-lines. According to Lewis, these two appearances are similar to those he observed in pork muscle following treatment with 10% salt plus polyphosphate and 10 or 20% salt alone. An additional variable was the brine to meat ratio, a high ratio (10:1) resulting in more structural disturbance than a low ratio (1:1). Lewis (1974, 1979) reported seeing contrasting structures in adjacent cells in commercially produced bacon but pointed out that such variation is not uncommon in any meat product which may be examined microscopically.

In previous work aimed at understanding the structural changes which occur when pieces of muscle are treated with salt, Voyle et al. (1984) reported observations made at the myofibrillar level in a model system. Blocks of pig LD muscle were soaked in a solution containing sodium chloride and sodium pyrophosphate at low (5.5) and high (8.0) pH values. A translucent zone extending to a depth of 3 mm from the surface of
the meat was formed in all the incubating media used. Evidence of swelling of myofibrils with some disruption of the Z-lines was seen in meat treated with either salt alone or salt plus pyrophosphate at pH 5.5. Treatment with salt plus pyrophosphate at both pH values resulted in partial or total removal of myosin from thick filaments close to the surface of the meat block but treatment with salt alone resulted in little or no extraction of thick filaments.

Our attention has been drawn to a phenomenon occurring in commercially produced bacon, in which areas with contrasting light-scattering properties confer a striped appearance on the product. Light and dark areas of varying dimensions occur in the muscle tissue, an appearance which is most conspicuous in the LD muscle. The phenomenon has been referred to as 'tiger-stripe' (M.R.I. Biennial Report 1981-83). A similar phenomenon has been observed in cooked, salt-treated beef. There is considerable interest in this phenomenon because of its possible influence on the acceptability of the product to the consumer. Using information already available regarding changes in myofibrillar structure resulting from salt treatment, it seemed desirable to investigate the structural basis for the difference in light-scattering properties found in the affected areas.

Materials and Methods

Six pouches of vacuum-packed bacon slices, and several loose slices, were obtained from a commercial processor. The pouches and the slices selected displayed a striped appearance consisting of a more or less parallel array of light and dark areas. The slices had been cut from sides of pig meat injected with curing brine using a multi-needle injection machine. The curing brine included sodium chloride, sodium nitrite and sodium nitrate in quantities similar to those recommended in the U.K. BMMA Code of Practice (1978). No polyphosphate was included. In this specification the target figures for the concentrations of the ingredients of the injection brine were:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Sodium chloride</td>
<td>20%</td>
</tr>
<tr>
<td>Sodium nitrite</td>
<td>0.08%</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>0.20%</td>
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The curing process is started within 24 h of slaughter and the brine temperature should be 2-4.5 °C. After injection the sides of pig meat are cured and matured according to the Wiltshire process.

Photographs of affected slices were taken using incident illumination and a black background, or with transmitted light reflected from a white background. Photographs were also taken of the entire contents of a pouch, the slices being arranged serially.

Samples were removed from both a light and a dark area of the LD muscle in one bacon slice from each of the vacuum-packed pouches. These were prepared for examination by transmission electron microscopy. Pieces of tissue measuring 5 x 5 mm were taken from each slice having a thickness of 3 mm. The samples were fixed for 18-24 h in 2.5% glutaraldehyde in 0.1M cacodylate buffer at pH 6.8. Fixation was followed by washing in the same buffer prior to further trimming of the blocks of tissue to final dimensions of 2 x 2 x 3 mm. The tissue blocks were fixed for a further 2h in 1% osmium tetroxide in cacodylate buffer, followed by an overnight wash in the same buffer. Dehydration through graded alcohols from 70% to 100% (absolute) alcohol was followed by impregnation and embedding in LR white resin (London Resin Co., Basingstoke, Hants., UK) and polymerization at 60 °C for 24 h. Six blocks from each sample of tissue were prepared, making a total of thirty six blocks each of tissue from light and dark areas. Sections were cut from a minimum of three blocks from each sample at a thickness of 50-60 nm, using a glass or a diamond knife. The sections were mounted on copper grids and stained with uranyl acetate and lead citrate and examined using a JEOL 1200EX electron microscope.

Results

Macroscopic appearance

Individual slices of bacon showed an uneven distribution of light-scattering properties, particularly in the LD muscle, when viewed under incident illumination. In some slices there was an array of alternate light and dark stripes which were approximately perpendicular to the layer of back fat. In other slices high light-scattering areas occupied the majority of the LD muscle. In slices where stripes were clearly visible, the centre-to-centre spacing between stripes of similar light-scattering properties was about 17 mm. Figure 1 shows odd-numbered slices from a commercial vacuum-packed pouch of twelve slices. A gradual change in the appearance of light-scattering properties in the first slice gave way to increasingly irregular and larger areas of high light-scattering properties so that at the end of the series the majority of the area occupied by the LD muscle in the bacon slice has a light appearance.

Whereas the sites where injection needles entered the meat were not conspicuous in the packed slices of bacon in our sample, some of the loose slices showed regularly spaced marks which were assumed to be caused by the needles. Such marks may be seen in Figure 2a and b (arrows), where they appear to coincide with dark areas in the muscle tissue.

Figures 2a and 2b show the same slice of bacon under incident and transmitted illumination with dark and light backgrounds respectively. Areas which appear dark under incident illumination (Figure 2a), appear light when viewed with transmitted illumination (Figure 2b). This change in appearance indicates a difference in translucency between adjacent areas in the slice of bacon, the areas appearing dark under incident illumination being relatively more translucent than those which appeared light under...
Microstructure of bacon

Figure 1 A series of bacon slices from a commercial vacuum-packed pouch showing change in the shape of light and dark areas. Slices were viewed by incident illumination.

Figure 2a A slice of bacon viewed by incident illumination.

Figure 2b The same slice viewed by transmitted illumination. Areas marked * are examples of the reversed light scattering patterns in the 'striped' areas of the longissimus dorsi (LD) muscle. Arrows indicate injection sites.
the same conditions of illumination.

Microscopic appearance

Sarcomere lengths were found to be in the range of 1.1 to 1.3 μm in our samples. Under these conditions it was difficult to recognise discrete A and I bands. The thin filaments normally seen between the A-band and the Z-line were completely overlapped by the thick filaments of the A-band. Furthermore, there was substantial double-overlap between the thin filaments within a sarcomere. These short sarcomeres may be explained by the fact that while the LD muscle was in the pre-rigor condition following slaughter, the carcass was suspended from the Achilles tendon and the muscle was not under tension. Although this constraint made it difficult to determine the fate of thick and thin filaments with precision, substantial differences were observed in the fine structure of myofibrils from areas of contrasting translucency in the LD muscle.

Dark areas. Sections of tissue removed from areas which appeared dark under incident illumination consistently showed recognisable features of myofibrillar structure, as illustrated in Figure 3. Myofibrils were clearly defined and Z-lines showed the demarcation of sarcomeres. At a higher magnification overlapping thick and thin filaments could be recognised (Figure 4). No evidence of the removal of thick filaments from the A-band was observed, a finding which is consistent with our previous work (Voyle et al. 1984). Discontinuity within some Z-lines was detected however, possibly due to myofibrillar swelling.

Light areas. Thin sections of tissue from areas which displayed high light-scattering power under incident illumination showed a loss of ordered structure. There was an accumulation of amorphous material such that, in general, myofibrils were no longer clearly defined and structural features normally recognised in sarcomeres were no longer apparent. The appearance shown in Figure 5 is representative of the areas of high light-scattering power in the LD muscle.

Gaps were sometimes observed between blocks of amorphous material such as have just been described. These gaps occurred at intervals of about 1 μm, a periodicity which is similar to the sarcomere length in these samples. It is reasonable to suppose that the break has occurred at the junction between the Z-line and thin filaments, since this is the site of similar breaks observed in conditioned or aged meat (Davey and Dickson, 1970).

Discussion

An increasingly familiar phenomenon, known as 'tiger-stripe', has been observed in pre-packed sliced bacon prepared from Wiltshire sides subjected to multi-needle injection of curing brine. About 80% of the production of sweet cure bacon of one producer, and smaller amounts of mild and ordinary Wiltshire bacon have been found to be affected. In addition, a major retail group in the United Kingdom is known to have returned a sizeable batch of back bacon to the processors because of its apparently unacceptable appearance. Observations made in local supermarkets showed that 'tiger-stripe' can be detected readily in packs of sliced bacon in display cabinets. The pattern observed did not always present as a periodic array of stripes, the size and shape of the areas of high light-scattering power being somewhat variable. Figures 1 and 2a and b illustrate this variability. The purpose of this investigation was to seek a structural explanation for the differences in light-scattering power between the light and the dark areas.

Contributory factors associated with the manufacturing process, which we consider are likely to be involved, include the number and location of injection sites, the composition of the brine, and the spread of the brine through the muscle by mechanical movement and diffusion. It is clear from our results that these factors give rise to changes in the final structure of the muscle tissue and that these changes alter the light-scattering properties of the tissue. The low light-scatter in regions close to the injection sites is explained by their ordered structure, while the high light-scatter in the areas in between is explained by their amorphous granular structure.

Number and location of injection sites

In an earlier experiment conducted in this laboratory a brine containing 16% sodium chloride and 4% sodium tripolyphosphate was injected by hand into a block of porcine muscle. Around the injection site there was a marked increase in the translucency of the tissue. Similarly, we have previously reported (Voyle et al. 1984) that a translucent zone was formed at the surface of blocks of pig LD incubated in various salt solutions whether or not these contained polyphosphate. It was interesting, therefore, to observe that the injection sites near the LD muscle included in the slice of bacon illustrated in Figure 2 appear to coincide with the stripes of low light-scattering power. The regular spacing between such stripes seen in some slices supports the view that they result from the introduction of brine into the tissue through the array of needles in the injection machine.

The spacing of the needles is important when considering the distribution of brine through the tissue. It would be expected that the closer the needles are together the more efficient would be the distribution of brine. The U.K. B.B.M.A Code of Practice (1978) does not specify what the distance between needles should be, simply saying that 'the sides should be covered by a regular pattern of injection points'. Pearson and Tauer (1984), describing the curing method used in North America, say that pickle is well distributed through the meat (bellies and hams) and curing is rapid because a large number of needles spaced close together are used. Pockets of brine formed initially around the injection site would be expected to spread mechanically, due to the pressure applied during the injection process. It is to be expected that the main pathway for the...
Microstructure of bacon
distribution of the brine would be longitudinally between muscle fibre bundles. From these pockets the curing agents would spread by diffusion into the fibres.

It has been reported (MRI Biennial Report, 1981-83) that the levels of water and curing salts are similar in the dark and light areas, although in the hand-injected material referred to previously the translucent zone around the injection site had a higher water content than the more remote light zone. It is likely, however, that the structural changes which give rise to the 'tiger-stripped' phenomenon have occurred before homogeneity in salt distribution is attained, i.e. that change in structure depends on the time course of the change in the concentration of salt and, if present, of polyphosphate, in the tissue.

In taking packed bacon slices from the production line of the processing unit it was not possible to choose samples from precisely identified injection sites. It is clear, however, that in further work samples for structural and other forms of analysis should be selected from sites in the proximity of the injection site as well as from positions a known distance away.

Composition of brine

Target values for the percentage composition of injection and immersion brines used in the Wiltshire tank cure method of bacon manufacture have been listed in the U.K. BMMA Code of Practice (1978) and by Wilson (1981) who also discusses the role of the various constituents of the brine. Aspects of product quality affected include preservation, flavour, water retention and colour. In the context of our investigation the role of sodium chloride is most important. The recommended concentration of salt in brine used in the Wiltshire tank cure method lies in the range of 18-22%, with a target figure of 20%. This is very much higher than the final concentration of salt in the meat of about 2-5%.

The influence of salt concentration on water retention and protein solubilization in meat has been discussed at length by a number of workers (Callow, 1929, 1931; Hamm, 1960, 1975; Kotter and Fischer, 1975; Offer and Trinick, 1983; Lawrie, 1985b). Callow (1929, 1931) and Kotter and Fischer (1975) showed that the salt concentration (about 5%) which leads to optimal water uptake is also associated with solubilization of myosin. Offer and Trinick (1983) observed that concentrations of salt of around 3-4% caused substantial swelling of isolated myofibrils, and that this swelling reached a maximum when a substantial part of the A-band had been extracted. Callow (1929, 1931) studied the effect on pig muscle of solutions of sodium chloride over a very large range of concentrations. It is noteworthy that in solutions containing more than 21% salt there was actually a loss of water from the meat and markedly less protein solubilization. It should not be assumed, therefore, that a high concentration of salt, such as would be expected to be present initially around the injection sites, necessarily would cause greater structural
changes than a lower salt concentration. A further factor in determining the translucency of the meat is the pH. It should be appreciated that polyphosphate, when present, may cause this to rise in the region of injection sites due to its high buffering capacity. Changes in the morphology of myofibrils in the presence of concentrated salt solutions, first reported in isolated myofibrils by Hanson and Huxley (1955), may contribute to the changes in appearance of gross tissue, it is probable that extraction of proteins from myofibrils may also play a role. In the experiments reported by Offer and Trinick (1983) and Voyle et al. (1984) there was clear evidence of the removal of protein from the A-band when pyrophosphate was present. In the absence of pyrophosphate A-bands remained intact, an observation which is similar to that made in the present work on bacon cured with salt only. In areas of low light-scattering power thick filaments, and to a lesser extent, thin filaments were visible. The high degree of overlap between the thick and thin filaments limited the visibility of the latter.

The most conspicuous change in the appearance of myofibrils was the amorphous granularity associated with those areas of tissue which displayed a high degree of light-scattering. There are several possible explanations for this. One possibility is that denaturation of films, such as has been observed in cooked meat (Voyle, 1974) may have occurred. Such a change would result in the fusion or destruction of the films normally observed in the sections. Another possibility is that sarcoplasmic proteins have been precipitated, even denatured, and deposited on the myofibrils thus obscuring the detail of their fine structure. A further possible explanation is that sarcoplasmic (soluble) proteins or solubilized myofibrilar proteins have been cross-linked to other structures by the action of the fixing agent, glutaraldehyde. However, this action does not explain the initial variation in light scattering which is clearly seen prior to fixation.

Further investigation is required in order to establish which of these explanations is correct. Further work is also needed to determine how the time course of changes in salt concentration in the presence or absence of polyphosphate, and changes in pH, cause a difference in myofibrillar structure between areas close to injection sites and the intervening areas. These investigations will best be made on a model system initially, before returning to factory-processed material.

C. A. Voyle, P. D. Jolley and G. W. Offer

Acknowledgements

The authors are grateful to Mr. J. Moore (Lucas Ingredients Ltd.) for first drawing their attention to the phenomenon of ‘tiger-stripe’ in processed meats. The authors also wish to thank Mrs Anne Phillips and Richard Angell for their valued assistance.

References


Microstructure of bacon


G.R. Schmidt: Could part of the stripes visualized in the meat product be due to localized pH and ionic strength environments? Could some of the increased disruption of the proteins be due to the physical disruptions supplied by the injecting needle?

Authors: In unpublished trials we have found that striping is more pronounced when polyphosphates are present in the brine, but as in the samples we have described, the phenomenon also occurs in the presence of salt alone. We have drawn attention to the specific effects of polyphosphates as well as to the local rise in pH that will occur near the injection site in the presence of polyphosphate but we do not rule out the effect that this ingredient would have on ionic strength. It is possible that some mechanical disruption may be due to the injecting needles, but we have shown that myofibrillar structure is more, not less ordered in the regions where injection occurs.

H.J. Swatland: Is there any relationship between the periodicity of the 'tiger-stripes' and the fascicular arrangement within the longissimus dorsi (LD) muscle? How are the 'tiger-stripes' arranged in a reconstruction of the muscle from its serial slices?

Authors: Close examination of the surface of bacon slices which include the LD muscle shows bundles of muscle fibres surrounded by perimysial connective tissue. Sometimes made more conspicuous by the presence of intramuscular fat. These bundles range from 0.5 mm to about 4 mm in diameter, this variation clearly representing the fascicular arrangement of the fibres within the muscle. However, the stripes, as observed in Figure 2, appear to run independently of the perimysial network. Our Figure 1 gives some indication of the arrangement of the stripes in a serial reconstruction over a limited distance.

C.E. Carpenter: Why is it likely that structural changes occur before homogeneity in salt distribution is attained? Data on the time course has not been presented.

G.R. Schmidt: Is there any reason to believe that in injected products the material becomes completely homogeneous, even if permitted to stand for a considerable length of time after injection?

Authors: As salt diffuses from the regions near the injection sites the salt concentration will rise gradually. It is known that when a critical salt concentration is reached, extraction of proteins and myofibrillar swelling occur on a relatively rapid time scale. Offer and Trinick (1983-text ref.) demonstrated this to be of the order of a few minutes.

Although the advantages of the multi-needle injection method of introducing brine into meat are said to include more efficient and uniform distribution of brine throughout the tissue, we are not aware of any data demonstrating that complete homogeneity is achieved. However, 'tiger-striped' is readily seen in bacon samples several days, even months, after injection when

Discussion with Reviewers

G.R. Schmidt: Would the pH of the meat at the time of injection have influenced the interaction between the brine and the meat?

Authors: The pH of the meat at the time of injection may well influence between the brine and the meat in that it influences the uptake and retention of the brine. Wilson (1981) states that for curing, pork muscle should be at a pH of 5.6 - 5.8. A low pH (<5.6) results in a low yield. Meat having a high ultimate pH (>6.0) is resistant to the penetration of salt (Callow, 1936). It does, however, display a superior water-holding capacity (Text ref. Hamm, 1975) due to the loosened structure which results from increased electrostatic repelling forces between muscle filaments.

Meat pH is of itself an important determinant of the translucency of the tissue. Since 'tiger-stripe' is a contrast phenomenon, it may be that meat of high pH will exhibit less marked contrast than meat of low pH. The translucency around the injection site in high pH meat being closer to that of the rest of the slice.


salt distribution must be much closer to homogeneity than immediately after injection.

G.R.Scheidt: Does the application of mechanical energy such as tumbling or massaging facilitate the uniform distribution of brine ingredients in a meat product?
Authors: Yes, (text ref. Lawrie, 1985a) but this is more frequently used in the production of cured hams. Mechanical action tends to damage fat and cause it to separate from the lean. It is, therefore, not often used for bacon processing.

C.E.Carpenter: Why should a highly ordered structure show less light scatter? Glass is amorphous yet is almost transparent.
Authors: Highly ordered, crystalline materials do not scatter light appreciably except at their surfaces. This is because scattered radiation from the unit cells in the material interferes destructively. For normal crystals whose unit cells have dimensions very much less than the wavelength of light, constructive interference cannot take place with visible light and thus diffraction, that is scattering at a series of defined angles, does not occur. With other, less well-ordered, materials the degree of scatter depends on the inhomogeneities in the material. What is important here is both the magnitude of the inhomogeneities and the distance scale over which they occur. For appreciable scattering to take place, the inhomogeneities should occur over distances approaching and exceeding the wavelength of light. Although glass, like water, is not uniform at the molecular level, over a scale approaching and exceeding the wavelength of light the fluctuations are small and only a small degree of scatter occurs. By contrast, if a piece of glass is shattered into many small particles very large fluctuations occur and the system is highly scattering.

Muscle is composed of myofibrils in which the repeating unit, the sarcomere, is large enough so that at a few discrete angles of incidence constructive interference can arise and diffraction can be observed. At other angles of incidence this does not occur and the light scatter arises only because of non-periodic features of the muscle structure, e.g., particulate material such as mitochondria, and from the small differences that arise between sarcomeres. Should this regularity of the structure be altered in anyway, either by physical disruption or the creation of particulate material, the inhomogeneity would arise and the light scattering would be expected to increase.

C.E.Carpenter: From the data presented absorbancy, which has no effect on light-scatter, cannot be excluded as a source of translucency differences.
Authors: We agree that this is a possibility.
ACCURACY AND UTILITY OF SARCOMERE LENGTH ASSESSMENT
BY LASER DIFFRACTION

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Abstract

In two experiments the computation of sarcomere length from laser diffraction patterns was tested for accuracy against phase-contrast microscopy. Particular attention was paid to methodological factors such as sampling location and computation formulae. Correlation coefficients between the laser diffraction technique and the microscopical method were high (r= 0.96) in both experiments. However, when computed from a simplified formula, the sarcomere length values, determined by laser diffraction patterns tended to be approximately 0.10 μm lower. It is recommended to use the correct formula in computing the laser diffraction data and to investigate a limited number of fibres (3) in a sufficiently large number (5) of randomly distributed samples.

Introduction

Tenderness of meat has traditionally been considered as being primarily related to the connective tissue content. However, in the past 20 years it has been recognised that myofibrillar proteins play an even more prominent role in determining tenderness. The configuration of these myofilaments appears to be of particular importance (Locker, 1960). The energy released as a result of post mortem glycolysis stimulates thick filaments containing myosin and thin filaments containing actin to interdigitate. Hereby the distance between adjacent Z-lines, commonly referred to as sarcomere length (SL), is shortened. At the onset of rigor mortis this configuration of myofilament remains 'locked' as a result of deficiencies of adenosine triphosphate (ATP). Thus the degree of rigor shortening varies and is reflected by the SL (Locker, 1960).

Locker (1960) was the first to demonstrate the relation of SL and tenderness. He distinguished 4 major contraction groups, each of which corresponded with different tenderness scores. Temperature was shown to have pronounced effects on the degree of shortening and thus on tenderness (Locker and Hagyard, 1963; Marsh and Leet, 1966). Furthermore it was recently suggested that shortening may also have some relation to water retention (Honikel et al., 1981; Smulders et al., 1986). Therefore, when determining these quality traits the assessment of SL is a valuable tool in distinguishing cold shortening as well as other changes in tenderness and water retention.

Traditionally for the measurement of SL, conventional methods were applied such as phase-contrast microscopy and microscopy on longitudinal sections. An advantage of these microscopic methods, apart from their accuracy, is that any aberrant histological condition is easily detected. On the other hand, these methods are time-consuming and consequently are often superseded by a simple technique when conducting meat research experiments.

A rapid method to determine SL is the laser diffraction (LD) technique. This technique originates from experiments of Ranvier (1874),
who found that the striated pattern of skeletal muscle, when exposed to a beam of polychromatic (white) light, functioned as a lattice and consequently effected a diffraction. Several investigators applied this principle in measuring SL by means of optical diffraction patterns (Sandow, 1956; Rome, 1967; Clayworth and Edman, 1969; Kawai and Kuntz, 1973). Voyle (1971) described a practical method for the assessment of SL by using a helium-neon gas laser as the source of monochromatic light. Literature data show that the methodology used since has not always been uniform in terms of sampling, fixatives, number of measurements and computation formulae. Nevertheless, the LD technique, tested against conventional microscopical techniques in chicken pectoralis muscle (Ruddick and Richards, 1981) and beef longissimus muscle (Varcoe and Jones, 1983) was found to be accurate and precise. Variation in the rate of glycogen depletion and in a muscle within a muscle may lead to concomitant differences in degree of contraction (Bendall, 1973). Consequently, it appears relevant to test the value of SL assessment in general and the effects of sampling procedures in particular.

In the present study, the computation of SL from LD patterns using two formulae was tested for accuracy against phase-contrast microscopy measurements in two beef muscles. Furthermore the effect of sampling location as a source of variation was investigated.

Materials and Methods

Two experiments were conducted with beef muscles originating from cattle representing the Dutch Friesian (FH-) breed.

In the first experiment a cow carcass was excised at 24 h post mortem. Two slices, approximately 1 cm thick, were cut transversely at locations near the mandibular and sternal muscle insertion. In addition, two slices were cut from the muscle belly at locations A and B, approximately 4 cm apart from each other. Three cubes, each 1 x 1 x 1 cm, were cut from randomly distributed locations from the central part of the slices. The second experiment involved meat from 16 FH-bulls of approximately one year old. Eight carcasses were stimulated electrically within 5 min post mortem (300 V, 50 Hz, intermittently with 24 bursts of 2 s and 4 s intervals). Eight carcasses served as unstimulated controls. At 24 h post mortem, longissimus muscle samples were excised from the 8-10th rib section. From the 10th rib section a 1 cm thick slice was cut, from which 5 randomly distributed cubes of approximately 1 x 1.5 x 1 cm were sampled from the central part.

In both experiments, the cubes were fixed for approximately 4 h in a 5% glutaraldehyde solution containing 0.1 M NaHPO₄ buffer at pH 7.2 and a temperature of approximately 10°C. Subsequently cubes were washed in a 0.2 M sucrose solution similarly buffered at pH 7.2. Thus the cubes may easily be conserved in the refrigerator for extended periods (Voyle, 1969). Within 4 days of storage 3 fibre bundles were teased from each cube, each consisting of 2-5 fibres. Subsequently these were mounted between a slide and a glass cover-slip in a drop of fresh sucrose solution. SL was measured by LD, and after oil had been placed on the cover-slip, also by microscopy.

For microscopical investigation a phase-contrast microscope (Zeiss) with an oil immersion objective Ph 3, 100/1.30 was used, equipped with a camera lucida. No oil was used on the condenser. In the first experiment a total of 9 groups of 20 adjacent sarcomeres were measured in each cube (3 groups per fibre bundle). The mean SL per location (slice) was computed by averaging the results of 3 cubes. In the second experiment 45 measurements (9 in each of the 5 cubes) of 20 sarcomeres per location were made. Here, the mean SL per location was computed by averaging the result of 5 cubes.

The diffraction pattern was assessed by exposing the same specimen that was used for microscopy, to a 2.0 mW monochromatic beam of light with a wavelength (λ) of 632.8 nm generated by a helium-neon gas laser tube (Spectra Physics). Laser tube, specimen-holding device and a flat measuring screen were mounted on an optical bench (Fig. 1). The entire length of each of the three fibres was imaged on the measuring screen and the highest and lowest diffraction values, S, were read. The mean SL for each cube was calculated from the mean of 6 measurements of S-values. Additionally the mean SL per location was calculated by averaging the results of 3 cubes in the first experiment and 5 cubes in the second experiment. For the computation of SL two formulae were used.

\[
\text{SL} = \frac{\lambda \times D}{S} + \frac{\lambda \times \sqrt{D^2 + S^2}}{S} \tag{1}
\]

The second formula derived from the first one has been reported by Voyle (1971) to be justifiable since small diffraction angles should allow the equation of sine and tangent:

\[
\text{SL} = \frac{\lambda \times D}{S} \tag{2}
\]

* where D = distance between muscle fibre and measuring screen in mm.
* S = distance between 0th - 1st order maximum of the diffraction pattern in mm.

\[
\lambda = \text{wavelength of light generated from the He-Ne laser (632.8 nm).}
\]

Measurements obtained from slices from the muscle belly, in the first experiment, using the microscopical method, were subjected to a random-model design analysis of variance including effects of location, cubes and fibres. Differences between methods of SL assessment and differences between treatments (electrical stimulation and controls) were examined using Student t-tests. Furthermore correlation coefficients between both methods of SL assessment were calculated.
Accuracy of Sarcomere Length Determination

Table 1. The effect of method of assessment on the SL units of a single beef sternomandibularis muscle as assessed in fibre samples from 4 different locations (n = 3).

<table>
<thead>
<tr>
<th>Method of assessment</th>
<th>Mandibular insertion</th>
<th>A (Muscle belly)</th>
<th>B</th>
<th>Sternal insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>microscopically</td>
<td>2.07 ± 0.18abc</td>
<td>1.92 ± 0.23abc</td>
<td>1.79 ± 0.12ab</td>
<td>2.19 ± 0.28abc</td>
</tr>
<tr>
<td>LD formula (1)</td>
<td>2.07 ± 0.15abc</td>
<td>1.95 ± 0.15abc</td>
<td>1.85 ± 0.11abc</td>
<td>2.21 ± 0.23c</td>
</tr>
<tr>
<td>LD formula (2)</td>
<td>1.98 ± 0.15abc</td>
<td>1.84 ± 0.16abc</td>
<td>1.73 ± 0.12a</td>
<td>2.11 ± 0.24abc</td>
</tr>
</tbody>
</table>

abc Figures with superscripts not containing a common letter differ (p<0.05)

Formula (1): $SL = \frac{X \cdot \sqrt{D^2 + S^2}}{S}$
Formula (2): $SL = \frac{X \cdot D}{S}$

Table 2. The effect of method of assessment on the SL (μm) of electrically stimulated (n=8) and control (n=8) beef longissimus muscle excised from the 10th rib.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Formula (1)</th>
<th>Formula (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>electrical stimulation</td>
<td>1.86 ± 0.07a</td>
<td>1.80 ± 0.05a</td>
</tr>
<tr>
<td>control</td>
<td>1.33 ± 0.04c</td>
<td>1.33 ± 0.17cd</td>
</tr>
</tbody>
</table>

a-d Figures with superscripts not containing a common letter differ (p<0.05)

Formula (1): $SL = \frac{X \cdot \sqrt{D^2 + S^2}}{S}$
Formula (2): $SL = \frac{X \cdot D}{S}$

Table 2 includes the results of similar measurements in beef longissimus muscle excised from 16 carcasses, 8 of which had been stimulated electrically. Sarcomere lengths computed with LD formula (1), were not significantly different from those assessed microscopically. With LD formula (2) SL values of stimulated samples were again approximately 0.10 μm lower. The prevention of shortening by electrical stimulation is evident; control samples have contracted to approximately two-thirds of the length of stimulated samples.

In Table 3 the impact of sampling location on the SL of fibres within locations (slices) A and B is shown. Analysis of variance from microscopical data was conducted for three sources of variation being location, cubes and fibres. The contribution of location to the variation was not significant for the locations examined. Within one location cubes were found to contribute significantly more to the variation in

Results

In a pilot experiment 3 muscle fibres of approximately 15 mm length were scanned microscopically and the mean SL computed by averaging the measurements of approximately 22,500 sarcomeres. In addition, the same fibres were subjected to measurements with the LD technique. These measurements yielded virtually identical data ($r = 0.99$) with both methods.

The assessment variations in SL measurements of fibre samples taken from 4 different locations along one single beef sternomandibularis muscle are shown in Table 1. All LD values produced SL's which were similar to values obtained microscopically. Although the correlation between results obtained by the microscopical method and LD was high, whichever formula was used, ($r = 0.96$), the values obtained using formula (1) were closer to those obtained by microscopy than values obtained using formula (2). Formula (2) gave values for SL which were approximately 0.10 μm lower than those obtained with formula (1). The correlation coefficients between the values of SL given by formulae (1) and (2) were 0.964 and 0.960 in the first and 0.957 and 0.956 in the second experiment. Differences between correlation coefficients computed by formulae (1) and (2) were negligible.

When comparing the SL's at different locations in Table 1, the values derived from samples originating from the insertions were generally higher than those from the muscle belly.
P.A. Koolmees, F. Korteknie and F.J.M. Smulders

results than fibres within those cubes \((p < 0.005)\).

**Discussion**

The virtually identical data of the SL's, obtained microscopically and by LD in our pilot experiment, are in agreement with the findings of Paolini et al. (1976). The results of the pilot experiment also indicated that although SL's computed from diffraction angles were not perfect in all respects \((\text{Rüdel and Zite-Ferenczy, 1979})\), representative values for SL were obtained by LD (formula 1), provided a high number of diffraction patterns was measured. The statement of Varcoe and Jones (1983) that LD was only accurate when individual muscle fibres were measured is not substantiated by the results listed. The data of the two experiments described in this paper show that SL assessment by means of LD produces accurate results, particularly when formula (1) is used. Computation from the simplified formula (2) leads to SL's that are lower than the microscopical values. This may be the reason why Varcoe and Jones (1983), in using the laser procedure, found SL's that tended to be lower than those obtained by the microscopical procedure. However, since diffraction values (S) can easily be listed in tables together with the corresponding SL's computed from the correct formula, there is no reason to compromise with respect to the accuracy of the LD method.

Because of the limited contribution of fibres within cubes to the variation in results, it is a prerequisite to compute the mean SL as it was carried out in experiment 2 (i.e., calculation of mean SL per location from an average of the results from 5 cubes). The results of Table 3 also indicate that one should investigate a limited number of fibres (3) in a sufficiently large number of (5) randomly distributed cubes. Hence in meat research experiments cubes, excised from the muscle under investigation, should be considered as the experimental unit.

When the striation pattern of muscle is disturbed, it may be extremely difficult, if not impossible, to assess SL both microscopically and by LD. Such disturbed contraction patterns are found for instance in muscles that have been restrained during the onset of rigor mortis \((\text{van Logtestijn and de Wilde, 1972})\) or in muscles that have been subjected to mechanical stress and low pH's \((\text{Cassens et al., 1963})\). Several authors report that in electrically stimulated muscles, that have been subjected to these conditions, so-called contraction bands are found \((\text{Savell et al., 1978; Voyle, 1981; Sorinmade et al., 1982})\). In our experiments with electrically stimulated beef longissimus, such conditions were not found \((\text{Smulders, 1984})\). Fabiansson and Libelius (1985) suggested that contraction bands are a common artefact when the muscle sample is not properly stretched, especially in cold fixatives. It would be of interest, however, to investigate how this type of contraction pattern would affect the outcome of LD measurements. Possibly an increased width of diffraction maximum is an indication of non-uniformity of SL. Since the manner in which samples are prepared allows the use of the same sample for purposes of microscopical examination, aberrant conditions may easily be detected. In contrast with the findings of Vandendriessche et al. (1984), our second experiment, in which the changes of SL induced by electrical stimulation were measured, showed values which did not differ significantly using both microscopical and LD techniques.

The difference in SL between different locations along the muscle supports the data of Paolini et al. (1976) indicating that considerable differences in degree of contraction may be present within one single muscle. Furthermore, consistently higher standard deviations were found near the insertions. Possibly an increasing amount of connective tissue affects the accuracy of SL assessment.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>F-ratio</th>
<th>Level of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>location</td>
<td>0.102</td>
<td>1</td>
<td>0.102</td>
<td>0.34</td>
<td>N.S.*</td>
</tr>
<tr>
<td>between cubes</td>
<td>1.209</td>
<td>4</td>
<td>0.302</td>
<td>8.88</td>
<td>p&lt;0.005</td>
</tr>
<tr>
<td>within location</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>between fibres</td>
<td>0.404</td>
<td>12</td>
<td>0.034</td>
<td>1.79</td>
<td>N.S.*</td>
</tr>
<tr>
<td>within cubes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>error</td>
<td>0.690</td>
<td>36</td>
<td>0.019</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2.405</td>
<td>53</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* N.S. = not significant \((p > 0.05)\)

Table 3. The effect of sampling location on the SL measured in a single beef sternomandibularis muscle; analysis of variance for three levels of sampling.
tissue near the insertion may have prevented the contraction of myofibrils. To avoid misjudgements resulting from this effect, one may want to excise samples from the muscle belly rather than from muscle insertions. Apart from more extreme conditions such as mechanical stress, other effects, such as the rapidity of pH fall (Cassens et al., 1963) may introduce differences in degree of contraction. Variation resulting from the heterogeneity inherent in biological systems cannot be avoided. As a consequence, occasionally significant differences in SL of adjacent muscle sections may be found in normally contracted muscle. These may be due, for example, to localized differences in rate of ATP depletion. One should take disturbing factors such as these into account. However, since the SL is related to tenderness, an indication of the degree of contraction is a prerequisite in interpreting this quality trait. The laser technique in physiological studies on model systems of muscle fibres may not entirely be conclusive (Rudel and Zite-Ferenczy, 1979). However, large differences in degree of contraction effected by phenomena such as cold shortening will undoubtedly be revealed by measuring the SL. The present data show that the LD technique is suitable for this purpose. Moreover, scanning the entire length of fibres, as pursued by the method described, results in averaging many thousands of sarcomeres. Examining a similar sample by microscopy is impractical in view of the time involved. It is our opinion, therefore, that LD should be adopted as the most appropriate technique to assess SL.

Acknowledgements

The authors thank Dr. J.A.J. Faber and Dr. G.J. Leppink of the Mathematical Institute, Subfaculty of Mathematics, University of Utrecht, for their helpful comment on statistics.

References


**Discussion with Reviewers**

S.H. Cohen: Isn't postmortem pH approximately 5.2 -5.7? If so, what effect did pH of fixative have on sarcomere length?

Authors: In our opinion the degree of contraction of longissimus muscle will not be affected by different pH solutions when the samples are exposed to these as late as 24 h post mortem. In our experiment we fixed the post rigor muscle samples as described by Sabatini et al. (1964). This procedure is not uncommon (Cohen and Trusal, 1980; Jones et al., 1976; Voyle, 1981).

S.H. Cohen: Could you discuss the greater accuracy of SL measurements when the 2nd, 3rd etc. order bands are used?

Authors: We agree that the measurement of the SL is more accurate when the 2nd and 3rd bands are used. However, practice shows that the intensity of the 2nd and 3rd etc. order bands decreases considerably as compared to the first order maximum. Hence the centre of the 2nd and 3rd order bands cannot be determined as accurately as in the case of the first order diffraction. Consequently bands other than the first order bands do not seem appropriate for SL measurements of meat.

C.A. Voyle: Is it likely that the variation in SL found between cubes within sampling locations is a positional effect between the edge and the centre of the slice?

Authors: It is the authors' opinion that such an effect would indeed be relevant had the samples been excised from the peripheral muscle parts. The "heat ring" phenomenon for instance as effected by rapid chilling (Calkins et al., 1980) may possibly reflect a cold shortening condition. Therefore cubes were not taken from locations near the outer edge but, as stated in the Materials and Methods section, rather from the central part of the muscle.

**Additional References**


CRYO-SCANNING ELECTRON MICROSCOPY OF MICROORGANISMS IN A LIQUID FILM ON SPOILED CHICKEN SKIN

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Abstract

Cryo-scanning electron microscopy was used to examine bacteria which had grown in a liquid film on the skin surface of poultry carcasses. Pits or void zones surrounding bacteria on ethanol-dehydrated, critical point dried (CPO) samples were not found on washed or untreated specimens frozen prior to examination by scanning electron microscopy (SEM). However, pits equivalent to those produced during chemical fixation and dehydration were formed when skin tissue was treated with ethanol prior to freezing. We conclude that the pits are dehydration artifacts caused by the chemical preparative methods employed rather than the result of degradation of film proteins by extracellular enzymes of microbial origin.

Introduction

As a consequence of poultry processing procedures involving water immersion, the skin surface of carcasses becomes covered by a liquid film. It consists of a number of serum proteins, amino acids and other suspended or soluble material and may be 50-60 μm thick. The contents of the film originate either from the processing water or by diffusion from damaged skin tissue. Microscopic details of the liquid film were presented by Thomas and McMeekin (1980).

During storage, the amount and content of the film has been shown to increase and it is in this medium that the spoilage flora develops. Spoilage occurred when numbers of bacteria reached 10⁶ cells/cm², while at 10⁷ cells/cm² bacterial 'slime' had formed within the liquid film. Microbial penetration and disruption of skin tissue at refrigeration temperatures has not been observed, but bacteria located at the surface of the film on specimens prepared for SEM were surrounded by pits or cleared zones. In attempts to clarify the cause of these pits, microscopic examination of artificially contaminated specimens showed the test bacteria to be located only on the film surface. Consequently, it was not possible to determine whether the pits resulted from enzymatic degradation of film contents by bacteria, or were an artifact of specimen preparation.

Micrographs illustrating details of the liquid film during refrigerated storage were presented by Thomas and McMeekin (1981).

In this paper we report the use of cryo-SEM to examine microorganisms grown in the liquid film on chicken skin in order to determine the origin of the pits and cleared zones oferved on ethanol-dehydrated, critical point dried (CPO) specimens. Freezing tissue in liquid nitrogen retains the liquid film in situ as does the OsO₄ vapour fixation technique developed by McMeekin et al. (1979) and Thomas and McMeekin (1980, 1981). In addition, liquid nitrogen freezing has the advantage of minimizing shrinkage artifacts caused by chemical dehydration or critical point drying techniques (Boyde, 1972).
Materials and Methods

**Samples**

Commercially processed chicken breast pieces (20 x 15 cm²) were stored in polyethylene bags for up to 10 days at 4°-5°C at which time spoilage odours were evident. Some specimens were also incubated overnight at 25°C to encourage bacterial 'slime' formation on the skin surface.

**Production of samples for cryo-SEM**

Prior to preparation for SEM, samples of skin (4 x 4 cm) were excised from the breast pieces, pinned to dental wax and were either untreated, washed in water, or treated with ethanol. Tissue was washed by agitation in 70% ethanol for up to 5 days or simply treated by stepwise addition of ethanol to the skin surface. Untreated control pieces of skin were included also.

After each treatment, specimens (~1 cm²) were frozen by plunging into nitrogen/argon slush in the specimen freezing chamber of an EM scope sputter coater (EM Scope Instruments, Kent, England) and transferred under argon to the coating chamber which was then evacuated to 0.05-0.03 torr. Specimens were coated with 20 nm of gold and transferred under vacuum to the cryo stage (pre-cooled to -165°C) of a Cambridge Stereoscan 600 SEM (Cambridge Instruments, Cambridge, England). The accelerating voltage used was 15 kV and micrographs were recorded on Kodak Technical Pan 2415 film.

**Preparation of samples for conventional SEM**

Specimens of skin (~1 cm²) were also prepared by solvent dehydration techniques. Samples were fixed in OsO₄ vapour (or by immersion in 2% v/v glutaraldehyde), dehydrated in ethanol and critical point-dried (McMeekin et al., 1979). Specimens were coated with ca. 20 nm of gold and examined in a Cambridge Stereoscan 600 SEM.

**Changes in components of the liquid film during storage**

Numerous of bacteria, glucose concentration and protein were monitored during refrigerated storage of carcasses at 2°C for up to 16 days. Bacteria were enumerated by plating 0.1 ml amounts of suitable dilutions of skin homogenate on the surface of nutrient agar. Skin homogenates were prepared by treating 16 cm² of skin in 100 ml of saline (0.8% w/v NaCl) with a model 400 Colworth Stomacher (A.J. Seward and Co. Ltd., London). Incubated plates of nutrient agar were incubated at 22°C for 3 days and colony counts related to numbers of bacteria contained in the liquid film on 16 cm² of skin surface.

Samples of liquid film were obtained by swabbing 16 cm² or 32 cm² areas of skin with a cotton gauze swab. Swabs were then rinsed in 5 ml water and this solution was used to determine glucose and protein levels. Samples to be examined for glucose were first clarified by centrifugation, then freed of protein by treatment with zinc sulphate and sodium hydroxide, followed by further centrifugation to remove zinc hydroxide and precipitated protein. Glucose was then determined by the glucose oxidase-peroxidase method using blood sugar reagents obtained from Sigma Chemicals Co. Ltd. Protein (as albumin) was estimated by a dye binding method (Toro and Ackerman, 1975). Samples (0.2 ml), or standard bovine serum albumin (0.2 ml of 5 g/100 ml solution) were added to 2 ml of bromocresol green reagent (0.01% w/v in 0.1M succinate buffer, pH 4.0, plus 0.3 ml H₂O), mixed and allowed to stand at room temperature. The absorbance of the resultant solution was read at 630 nm.

**Results and Discussion**

All specimens used in this study were incubated under conditions which allowed spoilage to occur prior to SEM preparation. The bacterial load of samples at this stage was typically 10⁶-10⁷ cells/cm².

Figures 1, 2 and 3 illustrate the typical appearance of pits associated with bacteria in the liquid film on samples of spoiled chicken skin prepared for SEM by conventional procedures. The specimen illustrated in Figure 1 was fixed in OsO₄ vapour, while those shown in Figures 2 and 3 were with immersion in glutaraldehyde solution. This immersion fixation procedure removed much of the film shown in Figure 1, but some portions did remain intact such as that illustrated in Figures 2 and 3. In all specimens where the film remained intact, pits were found associated with bacteria at the film/air interface, but were not evident with those cells just above or below the surface (Figure 3).

Photomicrographs of frozen specimens are shown in Figures 4 and 5. The image shown is the surface of the liquid film, not the skin surface itself. Neither micrograph shows pits associated with the film surface, nor were they discerned in areas of specimen where bacterial colonies were sited just below the film surface (Figure 5). The undulating surfaces evident in Figure 5 represent the outline of bacterial cells growing just underneath the surface. The cause of the larger holes shown is not clear, but they may represent areas of skin devoid of underlying bacteria formed as a result of the coalescence of adjacent colonies. These holes should not be confused with pits.

Washing had little or no effect on the overall appearance of the skin surface of frozen specimens and did not result in production of pits surrounding the cells (Figure 6). The possibility that ice formation obscured the pits seems unlikely, since in several specimens, where this artifact was seen, ice crystals were observed over the entire specimen surface (Figure 6). Similar treatment with 70% ethanol for up to 5 days had little effect on pit formation (Figure 8).
Figure 1 Pits (arrowed) surrounding bacterial cells in liquid film on chicken skin. Specimen was osmium vapour fixed, ethanol dehydrated and critical point dried.

Figure 2 Remains of liquid film on glutaraldehyde immersion fixed specimen. Pits (arrowed) can be seen surrounding cells at film/air interface.

Figure 3 Higher magnification of liquid film area in Figure 2. Pits (arrowed) occur around cells at film/air interface but not on those just underneath the surface.

Figure 4 General appearance of the surface of the liquid film on a frozen specimen. No bacteria or pits are evident.

Figure 5 Liquid film overlying a heavily colonized area on a frozen specimen. The undulations shown represent the outline of bacterial cells growing in a colony beneath the surface of the liquid film. Note the larger "holes" (arrowed) which should not be confused with "pits".
Figure 6 Surface of the liquid film in a specimen washed prior to freezing. No pits are evident but outlines of bacterial cells (arrowed) can be seen under the film surface.

Figure 7 Ice crystal formation on the surface of a frozen specimen. The artifact is obvious.

Figure 8 Specimen treated in 70% ethanol prior to freezing. No pits are evident, but bacterial outlines (arrowed) can be seen under the surface of the liquid film.

Figure 9 Specimen treated by dropwise addition of 100% ethanol prior to freezing. Note development of pits (arrowed) surrounding the cells in the liquid film.

Figure 10 Specimen treated by immersion in 100% ethanol prior to freezing. Film shrinkage and pits (arrowed) surrounding individual cells are evident.
However, dropwise addition of 100% ethanol prior to freezing caused channel formation around cells (Figure 9) and immersion for 2 hours or more produced pits equivalent to those formed by cryo-techniques (Figures 10, 11).

These results demonstrate positively that pits observed around bacteria growing in a proteinaceous film can be induced by treatment of the sample with ethanol. This is consistent with the hypothesis that these features are artifacts of the methods of preparation rather than the result of bacterial activity.

Other evidence is available to support this conclusion. For example, pits associated with microbial cells in the film on skin from carcasses stored 4 days at 2°C have the same dimensions as those on spoiled samples incubated 16 days at 2°C. This observation is not consistent with ongoing proteolytic degradation (Thomas and McMeekin, 1981). Also, protein degradation by bacteria prior to spoilage is not consistent with accepted biochemical events associated with spoilage, in which proteases are repressed in the presence of small molecular weight carbohydrates and amino acids (Dainty et al., 1975; Gill, 1976). We have demonstrated an increase in glucose content of the surface film on skin for up to 8 days at 2°C. This occurs apparently as a result of diffusion of film components from underlying tissues and net utilization of glucose occurred only after this time (Table 1). Protein levels remained constant over a 16 day sampling period at 2°C. Furthermore, the predominant type of bacteria present in the film is actively motile Pseudomonas spp. Since the majority of these bacteria are not associated with the skin surface but are located within the liquid film, they would be unlikely to elaborate discrete, individual zones of film degradation (Thomas and McMeekin, 1981).

Specimen shrinkage is known to result both from ethanol dehydration prior to critical point drying (Boyde, 1972, 1978) and the critical point drying process itself (Gusnard and Kirschener, 1977; Schneider, 1976). In this study we have not distinguished quantitatively between the effects of solvent dehydration and critical point drying. However, it was evident that pits can be initiated by ethanol treatment and that elimination of solvent dehydration procedures by cryo-techniques prevented pit formation.

Other reports of SEM observations of food associated microorganisms have demonstrated structures similar to the pits/void spaces described in this paper. 'Pockets' were noted by Kalab (1978; 1979) surrounding lactic acid bacteria in SEM preparations of yogurt and cottage cheese. These were considered to be the result of bacterial enzyme action on the casein micelle and Kalab et al. (1983) have presented further results to support the initial findings.

The results presented in this paper demonstrate a situation different to that in milk gels and indicate pits in the liquid film on spoiled chicken skin prepared for SEM are an artifact of specimen preparation rather than the result of bacterial enzymatic activity. In view of these results, we suggest a re-evaluation of the cause of 'erosion troughs' associated with Pseudomonas putrefaciens observed on Oso 4 vapour-fixed samples of pork skin (Butler et al. 1979) may be necessary.

Table 1. Changes in numbers of bacteria, glucose and protein levels in breast skin washings of chicken carcasses stored at 2°C.

<table>
<thead>
<tr>
<th>Storage interval (days)</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log bacteria per 16 cm² skin</td>
<td>4.81</td>
<td>4.91</td>
<td>6.00</td>
<td>7.56</td>
<td>9.58</td>
</tr>
<tr>
<td>Glucose mg/32 cm²</td>
<td>370</td>
<td>450</td>
<td>882</td>
<td>475</td>
<td>116</td>
</tr>
<tr>
<td>Protein (as albumin) mg/32 cm²</td>
<td>9.96</td>
<td>10.53</td>
<td>9.10</td>
<td>10.26</td>
<td>12.43</td>
</tr>
</tbody>
</table>

Each value represents the mean of 3 estimates.

References


T.A. McMEKIN, D. McCALL and C.J. THOMAS


McMEEKIN TA, KALAB M (1983). Development of microstructure in the skin of broiler carcass skin during processing conditions. Again it is important to note that the film surface is not the skin surface. This is very unlikely. If the products of protein degradation were washed away by ethanol treatment, they would also be eluted by the severe aqueous washes described.

S. Humphreys: Rather than creating an artifact due to shrinkage, is it not possible that the ethanol washed away components of the liquid film around the bacterial cells while the intact components of the liquid film farther removed from the bacterial cells were not washed away by the ethanol and remained in place?

Authors: This is very unlikely. If the products of protein degradation were washed away by ethanol treatment, they would also be eluted by the severe aqueous washes described.

B. Skura: Are there special precautions that should be taken when freezing chicken skin in liquid nitrogen for cryo-scanning electron microscopy?

Authors: The major concern was to preserve the liquid film in situ on the skin surface. This means that minimum disturbance prior to freezing is important. During freezing, coating and observation, it is important to monitor temperature, vacuum and argon flushing procedures at various stages.

Discussion with Reviewers

Authors: No particular precautions were taken to dry the skin before freezing. Excess water was not removed as we were concerned to maintain the film intact with minimal disturbance of the film and bacteria. The possibility that ice formation obscured the pits seems unlikely since in several specimens where this artifact was seen ice crystals were observed over the entire specimen surface (see Figure 7).

S. Humphreys: What do these pits look like in sections?


M. Kalab: I was surprised to learn that to study the effect of dehydration, unfixed samples (of chicken skin) were immersed (directly) in 70 or 100% ethanol. I wonder why no attempts had been made to fix parallel samples before subjecting them to dehydration in ethanol?

Authors: In all our previous work with conventional SEM preparations, pits were observed after chemical fixation and dehydration. Therefore it seemed unlikely that prior fixation would prevent pit formation. Our intention was to fix by liquid nitrogen freezing with or without ethanol treatments.

M. Kalab: Is the liquid film already some kind of a degradation product?

Authors: The liquid film is not a degradation product. It results from the processing procedures used and is the substrate for bacterial growth and activity. There is very little bacterial action associated with the actual skin compared to that in the overlying film.

B. Skura: Rather than creating an artifact due to shrinkage, is it not possible that the ethanol washed away components of the liquid film around the bacterial cells while the intact components of the liquid film farther removed from the bacterial cells were not washed away by the ethanol and remained in place?

Authors: This is very unlikely. If the products of protein degradation were washed away by ethanol treatment, they would also be eluted by the severe aqueous washes described.

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TEXTURE AND MICROSTRUCTURE OF SOYBEAN CURD (TOFU) AS AFFECTED BY DIFFERENT COAGULANTS

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Abstract

The coagulating properties of five coagulants and the nature of the curd obtained from soymilk was investigated. Viscosity changes during coagulation were studied using a NAMETE R Vibrating Sphere Viscometer and texture measurements were made by compression and computer assisted analysis. pH and amount of solids in the whey were determined. The microstructure of the tofu was examined by scanning electron microscopy. It was observed that CaCl₂·2H₂O and MgCl₂·6H₂O coagulated the milk instantly while CaSO₄·1/2H₂O, glucono delta lactone (GDL) and MgSO₄·7H₂O acted comparatively slowly. The texture of the curd was greatly influenced by type and concentration of coagulant. Curd obtained with CaCl₂·2H₂O and MgCl₂·6H₂O was coarse, granular and hard, whereas CaSO₄·1/2H₂O and GDL (fresh solution) gave a very smooth, soft and uniform curd. Among the five coagulants studied, 0.75% CaSO₄ and 0.4% GDL (fresh solution) appeared to be most suitable for making tofu of high bulk weight and smooth texture.

Introduction

Tofu, a soybean derived curd, is a low-cost, high protein product which has been widely used in the Orient. In a study by Muto et al. (1963), tofu was judged to be nutritionally equivalent to the protein derived from a mixture of eggs, fish and liver. Depending on the kind and concentration of coagulant used, as well as stirring during coagulation and pressure applied to the curd, tofu ranges in hardness from soft to firm with a moisture content of 70 to 90% and protein content of 5 to 16%.

Making tofu is a relatively simple process but due to its bland nature, its textural properties play a big role in influencing quality and consumer acceptability. Shurtleff and Aoyagi (1984) presented a good review on the manufacturing of tofu. The variety of soybeans used may affect the quality of the tofu (Kamel and deMan, 1982; Skurray et al., 1980) and this is considered to be due to differences in protein content of the soybeans and the ratio of 7S and 11S proteins. Saio (1979) reported that higher solids in soy milk correlated with harder tofu and increasing coagulating temperature with increased hardness of tofu. Recently, Wang and Hesselhine (1982) investigated some of the coagulating conditions in tofu processing and reported that to obtain a good curd, the concentration required for commonly used salts was in the range of 0.01 to 0.1M.

One of the most important factors in determining the texture of tofu is the selection and addition of a coagulant at the proper concentration. This study was conducted to get more detailed information on the coagulating properties of different coagulants and the nature of the curd obtained under different conditions. The curds prepared in the laboratory were compared with some commercial tofu samples.

Materials and Methods

Preparation of tofu

The yellow hilum Ontario soybeans used in this study contained 10% moisture, 16.8% fat and 36.6% protein. The soybeans were made into milk by the following procedure: 300 g of beans were soaked overnight at 20°C. The soaked beans were drained, rinsed and blended for 4 min at high speed in a Waring blender with 750 ml of water. The resultant slurry was mixed with 800 ml of boiling water and strained through a filter cloth. The soymilk contained 10% total solids with 4.7% protein and 2.5% fat. Fresh soymilk was used to make tofu.

Key Words: Soybean curd, tofu, texture, viscosity, microstructure, coagulation, scanning electron microscopy, calcium salts, magnesium salts, glucono-δ-lactone.
The following coagulants were used: CaSO₄.1/2H₂O or plaster of Paris; CaCl₂.2H₂O; MgCl₂.6H₂O; MgSO₄.7H₂O and glucono-δ-lactone (GDL). To make tofu, 300 ml of fresh soy milk was heated to near boiling and the required amount of coagulant dissolved or suspended in 7.5 ml of water. The hot soymilk and coagulant were poured simultaneously into a glass container ensuring good mixing without stirring. The curd was left to set for 15 min and then transferred to a perforated plastic container with a diameter of 9 cm and lined with a filter cloth. The curd was pressed by applying weights (31.4 g/cm²) for 15 min. After pressing, the curd was left in running water for 1 h and then stored in a refrigerator.

**Viscosity**

A Nametre vibrating sphere viscometer (Nametre Co., Edison, N.J.) was used to follow changes in viscosity. The hot soymilk and coagulant were poured simultaneously into a glass container ensuring good mixing, and the vibrating sphere was immediately immersed to the mark. The kinematic viscosity was measured as a function of time. This provides a non-destructive method of measuring changes in viscosity.

**Texture**

For texture evaluation, the mechanical part of an Instron Universal Testing Machine was used. The original load sensing mechanism was replaced with a Daytronic load cell (cap. 12 kg) and a Daytronic 9000 strain gage amplifier-indicator (Daytronic Corp., Miamisburg, OH). The signal voltage was fed to an A-D converter (All3 Interactive Structures, Bala Cynwyd, PA) and from there to an Apple IIe computer. The instrument output was stored on floppy disks and analyzed using a program developed by the Statistical and Engineering Research Institute, Agriculture Canada, Ottawa, Ont. (Buckley et al., 1984).

The information obtained by this system included: peak force (N), time to peak (s), deformation to peak (mm), firmness (N/mm), and force at different points (N). Cylindrical samples were pressed from the curd with a boring tube and wire cutter, sample dimensions were 20 mm diameter and 20 mm height. Samples were compressed by a flat plate to 50% deformation using a crosshead speed of 10 mm/min. Peak force at 50% compression was measured as well as force at 25% deformation.

**Moisture**

For moisture determination about 100g of tofu was homogenized in a blender and 3-5g dried on a steam bath for 15 min followed by forced air oven drying at 98°C-100°C overnight. Total solids in the whey was determined by drying for 15 min on a steam bath and 3 h in the oven at 98°C-100°C.

**pH**

pH of the whey was measured using a Fisher Accumet pH meter model 825 MP.

**Scanning Electron Microscope Observations**

A scanning electron microscope (ETEC Autoscan) was used to examine the fine structure of tofu coagulated with different coagulants. The procedure used for sample preparation was that of Saio (1981) with some modifications. Small pieces of (< 2 mm cube) were fixed at room temperature with 5% glutaraldehyde in 0.1M phosphate buffer (pH 6.7) for 90 min. After five washes in 0.1M phosphate buffer (pH 6.7) at 10 min intervals, they were postfixed in 1% osmium tetroxide in the same buffer for 90 min at room temperature. The fixed samples were rinsed five times with phosphate buffer at 10 min intervals. Dehydration was done using a 10% incremental ethanol series, leaving samples at each concentration for 15 min followed by three rinses with 100% ethanol. The samples were then rinsed three times with chloroform. Critical point drying (CPD) was conducted using CO₂.

For freeze drying, the samples were dehydrated using the ethanol series, frozen in liquid nitrogen and transferred to a Polaron E5300 freeze drier and dried for 24 h. All of the samples were mounted on stubs and sputter coated with 20-30 nm of gold palladium (60:40) using a Technics Hummer V Sputter Coater. The observations were made at 10 kV.

**Making of commercial tofu**

Tofu was made in a commercial tofu plant (Victor Food Products Ltd., Toronto, Ont.) by a semiautomatic process using the optimum concentration of coagulants based on laboratory experience.

**Results and Discussion**

Results are reported only for those concentrations of coagulants which gave curds with clear or nearly clear whey. The minimum coagulant concentration required was 0.5% CaSO₄.1/2H₂O, 0.15% CaCl₂.2H₂O, 0.3% MgSO₄.7H₂O, 0.2% MgCl₂.6H₂O or 0.3% GDL.

**Viscosity**

The coagulation rates as measured with the Nametre vibrating sphere viscometer are shown in Table 1. The coagulation rate was very rapid with CaCl₂.2H₂O and MgCl₂.6H₂O and visible whey separation occurred at an early stage. A more gradual increase in viscometer readings was obtained with CaSO₄.1/2H₂O, MgSO₄.7H₂O and GDL. With these coagulants up to 10 min were required for curd formation and no whey separation was observed. The recordings of viscosity when using different concentrations of CaSO₄ are presented in Fig. 1.

Saio (1979) reported that GDL only coagulates soymilk when heated. GDL activity is influenced by temperature and time of preparation. Curd obtained by addition of fresh cold GDL solution to hot soymilk (near boiling) was very smooth and similar to curd obtained with CaSO₄. When the GDL solution was left at room temperature for 30 min, a very hard curd was obtained and even harder curd resulted with a hot (90-95°C) GDL solution. In the latter case, the curd was grainy, less cohesive and similar to curd made with MgSO₄. The active coagulant in GDL is gluconic acid and when a freshly prepared GDL solution is aged more gluconic acid is formed. The pH of a 0.4% GDL solution dropped from 3.0 after 10 min to 1.7 after 4 h. GDL has been reported to recover more protein in the tofu (Shurtleff and Anyagi, 1984) and is used for making silken tofu.

**Texture**

Results of textural evaluation are presented in Table 2. Reported values are means of six replicates. Peak force values for CaSO₄.1/2H₂O produced curd are not reported because the samples fell apart before reaching 50% compression. The hardest curd was obtained with 0.5% MgCl₂.6H₂O. It appears from these data that curd firmness can be affected by using various coagulants at different concentrations.

**pH**

According to Lu et al. (1980) pH, not the calcium ion concentration, is by far the most important factor in the precipitation of soy protein. These authors reported that protein starts to coagulate when the pH drops to about 6.0, therefore, according to Lu et al. (1980), the addition of salt should be stopped when the pH approaches 6.0. In this study, with all the coagulants except GDL, the pH of whey was in the range of 5.89 to 6.25.
**Texture and Microstructure of Soybean Curd**

![Graph showing development of kinematic viscosity with time of soymilk with 0.5%, 0.75% and 1.0% added CaSO₄·1/2H₂O.](image)

*Fig. 1. Development of kinematic viscosity with time of soymilk with 0.5%, 0.75% and 1.0% added CaSO₄·1/2H₂O.*

**Table 1. Effect of coagulant type and concentration on viscosity of soymilk.**

<table>
<thead>
<tr>
<th>Coagulant</th>
<th>Concentration (g/L) at time (min)</th>
<th>Kinematic viscosity (cP.g/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaSO₄·1/2H₂O</td>
<td>0.50 0.034 0.75 0.052 1.00 0.059</td>
<td>7.3 8.5 9.3 20.9 23.5 45.0</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.15 0.010 0.20 0.014 0.30 0.012</td>
<td>20.9 19.6 22.0 27.8 41.8 45.0</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.30 0.012 0.40 0.016 0.50 0.025</td>
<td>27.8 41.8 83.5 105.0 153.0 213.5</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0.20 0.010 0.30 0.015 0.50 0.025</td>
<td>12.2 31.2 40.3 46.5 59.3 91.0</td>
</tr>
<tr>
<td>GDL (heated)</td>
<td>0.30 0.017 0.40 0.022 0.50 0.025</td>
<td>12.0 13.0 20.0 28.3 40.0 50.0</td>
</tr>
<tr>
<td>GDL (fresh solution)</td>
<td>0.40 0.022 0.50 0.025 0.60 0.025</td>
<td>3.9 13.0 20.0 28.3 40.0 50.0</td>
</tr>
</tbody>
</table>

*(Table 3) and even with the same pH of whey, the coagulants behaved differently. For example, the curd obtained with 0.3% MgCl₂·6H₂O was three times harder than the curd obtained with 0.5% CaSO₄·1/2H₂O although the pH of whey was 6.03 in both cases.*

**Moisture content of tofu and solids in whey**

Results in Table 3 show that the coagulant used affects the amount of whey liberated and, therefore, the weight and moisture content of the final product. With increase in coagulant concentration, there was a decrease in moisture content of the tofu. With increase in coagulant concentration, the structure of tofu became more porous separating more whey and leaving less moisture in the tofu.

There was a general trend towards decrease in solids in the whey with increase in coagulant concentration. However, the difference was not significant. The solids content of the whey increased dramatically when the coagulant concentration used was lower than the minimum concentration listed in Table 3.

**Scanning electron microscopy**

Figures 2a and 2b are micrographs of the critical-point dried (CPD) and freeze-dried (FD) tofu coagulated with 0.4% fresh GDL. The network structure appeared to be similar in the two pictures, although the CPD sample seemed to have shrunken considerably. CPD has been shown to cause shrinkage (Cohen, 1977). Freeze drying appeared to be more appropriate for observing tofu structure. In Figures 3 and 4, SEM micrographs of tofu coagulated with different coagulants show clearly different fine structures. The microstructures as indicated in these pictures can be easily related to the visually observed texture. Tofu obtained with GDL (fresh solution) was judged best in texture on the basis of smoothness, and the micrograph showed a fine and uniform honeycomblike structure (Fig. 3a). The structure was very uniform with smaller holes than those prepared

<table>
<thead>
<tr>
<th>Coagulant</th>
<th>Conc. %</th>
<th>Peak force (N)</th>
<th>Force at 25% compression (N)</th>
<th>Firmness (N/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaSO₄·1/2H₂O</td>
<td>0.50</td>
<td>6.04</td>
<td>3.1</td>
<td>16.17</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>5.97</td>
<td>3.2</td>
<td>17.50</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>5.94</td>
<td>3.0</td>
<td>18.93</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.15</td>
<td>5.98</td>
<td>3.3</td>
<td>35.77</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>5.94</td>
<td>3.3</td>
<td>45.33</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.30</td>
<td>6.09</td>
<td>3.2</td>
<td>33.11</td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>6.07</td>
<td>3.2</td>
<td>47.83</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
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<td>6.25</td>
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</tr>
<tr>
<td></td>
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<td>6.03</td>
<td>3.1</td>
<td>56.23</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>5.89</td>
<td>2.7</td>
<td>59.67</td>
</tr>
<tr>
<td>GDL (heated)</td>
<td>0.30</td>
<td>5.52</td>
<td>3.5</td>
<td>42.10</td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>5.27</td>
<td>3.5</td>
<td>49.33</td>
</tr>
<tr>
<td>GDL (fresh solution)</td>
<td>0.40</td>
<td>5.41</td>
<td>3.3</td>
<td>19.43</td>
</tr>
</tbody>
</table>

*Samples disintegrated before 50% compression was reached.*

**Table 2. Effect of coagulant type and concentration on texture of curd.**

<table>
<thead>
<tr>
<th>Coagulant</th>
<th>Conc. %</th>
<th>pH of whey</th>
<th>Solids in whey %</th>
<th>Whey %</th>
<th>Moisture of tofu %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaSO₄·1/2H₂O</td>
<td>0.50</td>
<td>6.04</td>
<td>3.1</td>
<td>16.17</td>
<td>91.0</td>
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<tr>
<td></td>
<td>0.75</td>
<td>5.97</td>
<td>3.2</td>
<td>17.50</td>
<td>89.4</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>5.94</td>
<td>3.0</td>
<td>18.93</td>
<td>89.1</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
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<td>5.98</td>
<td>3.3</td>
<td>35.77</td>
<td>87.4</td>
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<td></td>
<td>0.20</td>
<td>5.94</td>
<td>3.3</td>
<td>45.33</td>
<td>86.4</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
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<td>6.09</td>
<td>3.2</td>
<td>33.11</td>
<td>88.2</td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>6.07</td>
<td>3.2</td>
<td>47.83</td>
<td>86.4</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0.20</td>
<td>6.25</td>
<td>3.2</td>
<td>40.00</td>
<td>88.4</td>
</tr>
<tr>
<td></td>
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<td>3.1</td>
<td>56.23</td>
<td>85.0</td>
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<tr>
<td></td>
<td>0.50</td>
<td>5.89</td>
<td>2.7</td>
<td>59.67</td>
<td>82.8</td>
</tr>
<tr>
<td>GDL (heated)</td>
<td>0.30</td>
<td>5.52</td>
<td>3.5</td>
<td>42.10</td>
<td>89.2</td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>5.27</td>
<td>3.5</td>
<td>49.33</td>
<td>84.8</td>
</tr>
<tr>
<td>GDL (fresh solution)</td>
<td>0.40</td>
<td>5.41</td>
<td>3.3</td>
<td>19.43</td>
<td>88.5</td>
</tr>
</tbody>
</table>

*(Table 3)*
Comparison of critical point drying (a) and freeze drying (b) of tofu coagulated with 0.40% GDL (fresh solution) (Bar = 5 μm).

with CaCl₂·2H₂O, MgCl₂·6H₂O and MgSO₄·7H₂O. Coagulation with CaSO₄·1/2H₂O gave a structure similar to that obtained with GDL but less uniform. The SEM pictures taken at higher magnification (Fig. 4) of curd made with MgCl₂·6H₂O and CaCl₂·2H₂O appear to show similar structures. The networks of these samples were not as fine and continuous as those obtained with GDL and CaSO₄·1/2H₂O. MgSO₄·7H₂O gave a more continuous and uniform structure than curd prepared with CaCl₂·2H₂O and MgCl₂·6H₂O.

**Commercial tofu**

The process used in the plant was basically the same as used in the laboratory. However, in the plant the curd is broken up during the transfer to the press. Due to this difference, the moisture content of tofu made in the plant was slightly lower than tofu made in the laboratory and turned out to be harder (Table 2 and Table 4).

For good curd production and clear whey formation, the concentration of coagulants on a molarity basis ranged from 0.01 to 0.02M except for CaSO₄·1/2H₂O which required a minimum of 0.03M. It is not feasible to decide on a common optimum concentration for all of the coagulants as has been pointed out in some other studies (Wang and Hesseltine, 1982; Tsai et al., 1981). Tsai et al. (1981) noticed a dramatic change in the texture of tofu when increasing the concentration of coagulants above 0.03N (0.015M). The present study reemphasizes the different behaviour of various coagulants.

Table 4 lists results of texture and moisture analyses of some commercial tofu samples. Texture and moisture content of samples A, B, C and D fall in the range obtained with the laboratory made tofu.

It has been suggested that the coagulation of soymilk is due to the crosslinking between protein molecules by divalent cations (Saio et al., 1967). However, the site of crosslinking is still under debate. Saio et al. (1967) suggested that the free carboxyl group of soybean protein is the major site of calcium binding and phytic acid also acts as a binding site. According to Appurao and Narasimga Rao (1975) a probable binding site on the protein molecules is the imidazole group. In addition to uncertainties about the binding sites of the soy proteins, there is a lack of understanding of the mechanism of coagulation with GDL.

**Acknowledgements**

Financial support was provided by the Natural Sciences and Engineering Research Council of Canada and the Ontario Ministry of Agriculture and Food. Technical support for the SEM work was supplied by A. Smith. Software for the computer assisted texture analysis was supplied by Statistical and Engineering Research Service, Agriculture Canada, Ottawa, Ontario. Mr. Stephen Yu of Victor Food Products Ltd., Scarborough, Ont. gave helpful advice and provided use of manufacturing facilities.

**References**


Fig. 3. SEM-images of freeze dried tofu prepared with different coagulants. (Bar = 20 μm).

a) - 0.40% GDL (fresh solution)
b) - 0.75% CaSO₄·1/2H₂O
c) - 0.30% MgCl₂·6H₂O
d) - 0.15% CaCl₂·2H₂O
e) - 0.30% MgSO₄·7H₂O
Fig. 4. SEM-images of freeze dried tofu prepared with different coagulants. (Bar = 5 μm).

- a) - 0.75% CaSO₄·1/2H₂O
- b) - 0.30% MgCl₂·6H₂O
- c) - 0.15% CaCl₂·2H₂O
- d) - 0.30% MgSO₄·7H₂O

Texture profile analysis with curve smoothing using a personal computer system. J. Texture Studies. 15, 247-261.


Discussion with Reviewers

W.J. Wolf: Is pH a factor in the coagulation of tofu with GDL? The pH values in Table 3 for GDL are all significantly lower
Texture and Microstructure of Soybean Curd

than for any of the other coagulants. Moreover, pH values of whey obtained by GDL coagulation are all below 6 and are approaching the isoelectric range for the proteins.

Authors: GDL acts by opening of the lactone ring to form gluconic acid. This occurs when GDL is dissolved in water even at room temperature as is demonstrated in the paper by monitoring the pH of a GDL solution at room temperature. Saio (1979) reported that GDL solution should be added to cold soymilk and then reheated. In industry this means cooling of the soymilk and then reheating with GDL solution. It is better to dissolve the required amount of GDL in water just before addition to a batch of hot soymilk coming from the production line as is done with the other coagulants. The release of gluconic acid at that temperature results in a very uniform curd as shown in the SEM photograph. When the GDL solution is left at room temperature for a longer time the reaction with hot soymilk is like acid precipitation and produces a coarse curd.

Reviewer III: How do you define texture and what proof is there that “peak force,” “force at 25% compression” and “firmness” measure texture?

Authors: Texture can be defined as “The way in which the various constituents and structural elements are arranged and combined into a micro- and macrostructure and the external manifestations of this structure in terms of flow and deformation.” Instrumental analysis of texture involves measurement of mechanical properties such as resistance to deformation, in this case peak force and force at 25% compression and also the stress/strain ratio which is defined as firmness.

K. Saio: As shown in Fig. 4 the hardness of tofu is influenced by the concentration and kinds of coagulants. Japanese like tofu because of its texture and bland flavor. Different coagulants are used for various tofu types, e.g., CaCl₂ for kori tofu, CaSO₄ or MgSO₄ or MgCl₂ (with phosphoric or citric acid) for hard tofu and GDL and CaSO₄ (alone or with GDL) for silken tofu. It was mentioned in the paper that tofu coagulated with GDL had the best smooth, soft and uniform texture but do you think North Americans prefer such silken tofu to the hard kind?

Authors: From our experience it appears that North Americans prefer the firmer styles of tofu. These seem to be more suitable for western style cooking and are preferred in salads.

K. Saio: Is it possible to distinguish the differences of coagulation state among soybean varieties with a Natemt vibratirg sphere viscometer?

Authors: We have tested this on 17 different varieties of soybeans grown in Ontario, and found no significant differences between them. However, these soybeans were harvested at the same time and stored under identical conditions. We suspect that storage conditions have a greater effect than varietal differences and this is now being investigated.
OBSERVATIONS ON THE MICROSTRUCTURE AND RHEOLOGY OF OVALBUMIN GELS

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3130 AC Vlaardingen, The Netherlands

Abstract

Understanding the gelation behaviour of proteins is of importance in order to be able to influence the properties of many food systems and it may lead to proposals for product or process improvement. In this context the formation of heat-set ovalbumin gels, in different media, has been studied by microstructural, rheological and conformational observations. An ovalbumin/water gel, prepared at pH 5, shows a granular, inhomogeneous microstructure. At this pH there are both many inter- and intramolecular interactions, and network formation occurs via aggregates of folded, globular protein chains. On the other hand, gels prepared at pH 10 or in urea solution (6 or 8 mol/l), show a uniform, homogeneous microstructure. Under these conditions, network formation occurs via flexible, unfolded protein chains. The ultimate properties of the gels are well in accordance with these microstructural observations: with respect to the deformation at break it is found that the network composed of flexible unfolded protein chains (pH 10, urea) can be extended further without breaking, leading to a higher breaking stress, than the network composed of spherical aggregates of strongly interacting protein molecules (pH 5). In the latter case the regions of low protein concentration will act as weak points and consequently these gels will break at a lower stress than the gels with a more homogeneous microstructure. A study to determine whether glutaraldehyde acts as a proper fixative revealed that this compound very effectively maintains the original gel structure.

Introduction

Proteins play an important role in many food systems. Insight into structure formation and into the relationship between molecular properties, microstructure and macroscopic properties of these structured materials, may stimulate the proper use of proteins and lead to proposals for product or process improvement.

From time immemorial, ovalbumin has been known to form an irreversible gel on heating in aqueous solution to 100°C and subsequent cooling to room temperature (boiled egg white). An investigation has now been made into the heat induced gelation of ovalbumin under different conditions of pH and solvent. These parameters induce conformational changes in the protein molecules and consequently influence the intra- and intermolecular forces between the protein molecules and thus the rheology and the microstructure of the gelled material. In this context urea solutions were used in order to distinguish between formation of covalent and non-covalent crosslinks during gelation. It was envisaged that in the presence of urea, the protein molecules behave like randomly coiled polymers that show little if any intra- and thus intermolecular interaction such as hydrogen bonding or hydrophobic interaction. Consequently if a protein/urea solution shows thermally induced gelation it is clear that gelation is caused by the formation of covalent crosslinks.

In the present work ovalbumin gels heat-set at 100°C were examined by scanning and transmission electron microscopy and rheologically characterized by tensile experiments, to determine the ultimate (breaking) properties. 1H-NMR was used to measure conformational changes in the protein molecules.

As during the sample preparation for electron microscopy, glutaraldehyde is used as a fixative, it has also been investigated how effective this compound is in fixing properly the ovalbumin structure in the different media.

Materials and Methods

Materials

Pure ovalbumin (grade V ex Sigma) was added with stirring as a dry powder to distilled water and to urea solution (6 or 8 mol/l) to a final concentration of 20 g/100 g. The pH was adjusted by adding dropwise aqueous NaOH (0.1 mol/l) or HCl (0.1 mol/l) until the required pH was obtained. The solutions were clarified by centrifugation and de-aerated prior to heat-setting.
Rheological measurements

Tensile experiments were carried out on an Instron Universal Testing Instrument 1122. Ovalbumin solution was poured between two parallel polyester coated copper plates which were placed in a boiling water bath for 20 min. The sheet of gel so formed was removed from between the two plates. The thickness of a typical gel sheet was about 3 mm. Tensile strips were made from the sheet with the aid of a Zwick Stanzpresse using a DIN 53504 S3 blade module. The strips were fixed between the clamps of the Instron, the strips being wetted with liquid paraffin in order to prevent evaporation. The original length of the tensile strip was 2.75 cm and the width 4 mm. Results of the tensile experiments are expressed in terms of the tensile modulus of the gel sheet from the clamps of the Instron. Therefore the mechanical properties of ovalbumin/urea gels were characterized at large deformations, by means of simple shear using the Weissenberg Rheogoniometer model R8B. Detailed information on these rheological measurements can be found in Ref. 9.

Electron microscopy

The gel slices prepared for tensile measurements were also used for the microstructural observations by electron microscopy. A three-dimensional impression of gel structures can best be obtained using scanning electron microscopy (SEM). To this end the gel was sliced into small pieces by a rotor blade and fixed in glutaraldehyde solution (15 or 30 g/l) for 1 h at room temperature in cacodylate (0.1 mol/l) buffer at pH 6.6. After fixation, the gel slices were washed with distilled water to remove all water soluble substances, such as urea in the case of urea treated gels. Excess water was removed with filter paper. The gel samples were rapidly frozen in melting nitrogen slush and subsequently freeze-dried in a Balzers freeze-etching unit, avoiding any temperature rise above -80°C. Alternatively critical point drying was used. After fixation, the gel was dried in a graded series of water/ethanol mixtures and finally with carbon dioxide in a Balzers critical point dryer. After the drying procedure (freeze drying as well as critical point drying) fresh fracture surfaces were obtained by fracturing the dried material. The dried samples were mounted on a copper specimen plate with silver paint and coated with 0.05 µm platinum. Photographs were taken using the scanning device of a JEOL 100C Temscan in the secondary electron image mode at 40 kV.

Samples for transmission electron microscopy (TEM) were prepared using the thin sectioning technique. Small gel pieces (volume about 1×10⁻⁹ m³) were fixed by successive immersion in buffered solutions of glutaraldehyde (15 g/l) and osmium tetroxide (10 g/l). This treatment was followed by staining in aqueous uranyl acetate (10 g/l) for 2 h. Samples were then washed and dehydrated with ethanol and propylene oxide and embedded in Epon. Thin sections of about 60 nm were cut and collected on electron microscope grids. The sections were poststained with lead tartrate solution for 90 seconds and viewed in a JEOL 100C transmission electron microscope, operating at 80 kV.

Fig. 1. TEM micrograph of ovalbumin gel (20 g/100 g), pH 10. Dark area represents protein.

NMR measurements

NMR spectra of ovalbumin in D₂O (1 g/100 g) were measured in 5 mm sample tubes at 70, 80 and 90°C. Chemical shifts were referenced to the 1H-resonance of TSS (2.2,3,3-tetradeutero-3-trimethylsilyl-propionic acid sodium salt). NMR spectra were recorded on a Bruker WP 200 (200 MHz) spectrometer. The resonance from residual HDO was suppressed by the gated decoupling technique. The pH values were obtained by adding 0.1 N NaOH to the normal pH meter reading. The aliphatic proton intensity (I) (indication of the mobility of the hydrophobic groups) was determined from the ratio between the integrated signal from about 3-0.5 ppm and that of the external TSS standard.

Results

The results on water gels prepared at pH 5, pH 10 and in urea solution can be seen as extremes: at pH 5 the ovalbumin molecule is very near to its IEP and thus maximally globular, at pH 10 the most elastic gel is formed without protein denaturation. Electron micrographs were only taken at these pH values. Rheological characterizations were carried out at other pH values as well (9).

Microstructure

The microstructure of heat-set gels prepared in water at pH 10 and pH 5, observed by TEM, are given in Figs. 1 and 2. Similar types of network structures of protein gels have been reported (1). Observations by SEM of gels at pH 10, pH 5 and in urea (8 mol/l) are shown in Figs. 3-5. As is evident from the TEM pictures (Figs. 1, 2), the homogeneity of the protein distribution is an important parameter. At pH 10 a homogeneous

Fig. 2. TEM micrograph of ovalbumin gel (20 g/100 g), pH 5. Dark area represents protein.

Fig. 3. SEM micrographs of ovalbumin gel (20 g/100 g), pH 10. a: freeze-drying, b: critical point drying.

Fig. 4. SEM micrographs of ovalbumin gel (20 g/100 g), pH 5. a: freeze-drying, b: critical point drying.

Fig. 5. SEM micrograph of ovalbumin/urea (8 mol/l) gel (20 g/100 g). Freeze drying.
Microstructure and Rheology of Ovalbumin Gels
distribution of protein filaments is found, whereas at pH 5 an inhomogeneous distribution is observed.

The SEM pictures, obtained via freeze-drying, show a granular structure of pH 5 gels (Fig. 4a), whereas a cellular type of structure is observed at pH 10 (Fig. 3a) and in the urea gel (Fig. 5). Comparing the TEM results with those of the SEM it is most likely that the cellular type of structure is induced by ice crystal formation during the freezing step in the sample preparation (2, 7, 11). The granular structure observed at pH 5 is indicative of an inhomogeneous distribution of protein filaments. Apparently ice crystals also cause the tiny filaments visible in the TEM micrograph (Fig. 2) to be concentrated onto the dense protein regions, so that they are no longer visible in the SEM micrograph. The cellular structure at pH 10 and in urea is considered to indicate a homogeneous distribution. This view is further confirmed by comparing the results of freeze-drying (Figs. 3a and 4a) to those of critical point drying (Figs. 3b and 4b). At pH 5 the same type of granular structure is observed in both cases, whereas at pH 10 critical point drying leads to an amorphous, homogeneous, non cellular structure. Gelation of ovalbumin in water at pH 5 apparently proceeds via the formation of relatively large aggregates, which form a network. Gelation at pH 10 and in urea proceeds via interaction of tiny protein filaments.

When a gel made in urea was immersed for 24 h in water which was changed several times to ensure complete exchange of urea by water, the microstructure of the resulting gel was similar to that of the original gel. However, when the experiment was carried out in the opposite way, i.e., if a water pH 5 gel was swollen in urea solution (8 mol/l), the microstructure of the swollen gel was that of a urea gel. Thus, an ovalbumin/urea gel retains its homogeneous (cellular after freeze-drying) microstructure when urea is exchanged by water, but the microstructure of an ovalbumin/water pH 5 gel changes from granular to homogeneous when the water in the gel is exchanged by urea.

Rheology

Typical results of tensile measurements are shown in Fig. 6. The ultimate properties of the gels, as expressed by the breaking stress (\( \tau_b \)) and the deformation at break (\( \lambda_b \)) are given in Table I. The breaking stress for the pH 10 gels is considerably larger (about 30 times) than that for the pH 5 gels. The deformation at break differs by a factor of two.

The rheological properties of the pH 10 gels are very similar to those of the gels made in urea (8,9) and both differ considerably from those of the pH 5 gels. The difference in ultimate properties between a urea (6 mol/l) gel and a pH 5 gel is demonstrated in Fig. 7, showing the shear stress-strain behaviour: the deformability and the stress at breaking of the urea gel is much higher than that of the pH 5 gel. A more detailed discussion of the rheological aspects of the heat induced gelation of proteins is given in Ref. 9.

NMR measurements

Fig. 8 shows the \(^1H\)-NMR spectra of ovalbumin/D\(_2\)O solutions. The integrated intensity of the NMR signal from 3.0 to 0.5 ppm of a fully unfolded ovalbumin molecule (i.e., in urea solution of 8 mol/l at 60°C) was taken as 100%. At room temperature, the intensity is about 30–50%, indicating a low mobility of the hydrophobic (aliphatic) groups of the protein. Up to 70°C, the behaviour of the pH 5 and pH 10 solutions is the same: intensity and resolution increase. From 70 to 90°C, the pH 10 solution shows further increase of intensity and resolution, whereas the pH 5 solution shows constant I and increase of resolution. At pH 10, a 10 g/l ovalbumin gel unfolds fully at increasing temperature. At pH 5, this gel unfolds partly up to 70°C; at higher temperature, the constancy of I indicates that the partly unfolded protein aggregates.

Glutaraldehyde as a fixative

During sample preparation for electron microscopy all ovalbumin gels were fixed in an aqueous solution of glutaraldehyde. In particular for the ovalbumin/urea and the ovalbumin/water pH 10 gels, the nature of the solvent during fixation was different from that in which the protein was dissolved and heat-set. As the nature of the solvent considerably influences the gel properties, it may be questioned whether under these circumstances glutaraldehyde fixes the gel structure in its original state. Therefore the effect of glutaraldehyde on the microstructure and the swelling properties of the gels was investigated.

Aqueous gels at pH 5 were subsequently immersed in different media (Table 2). The degree of swelling (S) is defined as the ratio of the weight at a certain time and the original weight

<table>
<thead>
<tr>
<th>Medium</th>
<th>Microstructure</th>
<th>S</th>
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</thead>
<tbody>
<tr>
<td>Distilled water + 3% GA</td>
<td>granular structure typical of pH 5 gel</td>
<td>1.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>Urea for 20 h followed by 3% GA/urea for 1 h</td>
<td></td>
<td>5.5</td>
</tr>
<tr>
<td>3% GA/water followed by urea for 20 h and by 3% GA/urea for 1 h</td>
<td></td>
<td>5.0</td>
</tr>
<tr>
<td>3% GA/urea for 20 h</td>
<td></td>
<td>1.2</td>
</tr>
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</table>

*Since the measured values of \( \lambda_b \) were very similar and the number of successful experiments was rather low, an average value for the three crosshead speeds is given.

<table>
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<tr>
<th>Cross head speed (cm/min)</th>
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<th>pH 10</th>
</tr>
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<tbody>
<tr>
<td>( \lambda_b )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \tau_b/10^4 \text{Pa} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>1.31</td>
<td>±0.12</td>
</tr>
<tr>
<td></td>
<td>2.06</td>
<td>±0.98</td>
</tr>
<tr>
<td>1</td>
<td>1.32</td>
<td>±0.06</td>
</tr>
<tr>
<td></td>
<td>2.20</td>
<td>±0.41</td>
</tr>
<tr>
<td>10</td>
<td>1.28</td>
<td>±0.04</td>
</tr>
<tr>
<td></td>
<td>2.24</td>
<td>±0.37</td>
</tr>
<tr>
<td>100</td>
<td>1.26</td>
<td>±0.07</td>
</tr>
<tr>
<td></td>
<td>2.62</td>
<td>±0.58</td>
</tr>
</tbody>
</table>

| Table 1. Comparison of fracture properties of pH 5 and pH 10 ovalbumin gels (20 g/100 g); \( \lambda_b \): deformation at break; \( \tau_b \): breaking stress (average values ± standard deviation). |

| Table 2. Microstructure (Scanning EM, freeze-drying) and degree of swelling (S) of ovalbumin pH 5 gel after immersion in various media. Urea concentration 8 mol/l; GA: glutaraldehyde. |
Microstructure and Rheology of Ovalbumin Gels

![Graph showing true stress versus extension for pH 5 and pH 10 samples.](image)

**Fig. 6.** Tensile curves of ovalbumin gels (20 g/100 g) at pH 5 and pH 10. Cross-head speed 10 cm/min; 20°C.

**Fig. 7.** Shear stress-strain curves of ovalbumin/urea (6 mol/l) gel and pH 5 gel (10 g/100 g). *“Egg white protein,”* Baker.

Discussion

As generally assumed, protein gelation involves the thermal denaturation of protein molecules followed by aggregation into a network (1, 4, 5, 6, 8, 9). The nature of the gelation represents a rather delicate balance between chain-solvent and chain-chain forces (3). When there is much chain-chain interaction, a coarse network is obtained. Due to this localized structuring and the resulting large pores, such a gel has a high opacity and shows syneresis (contraction accompanied by water exudation). When there are no strong interactions, but only the possibility of isolated points of contact between the protein molecules, a fine network and a clear gel with no syneresis is obtained (3). Therefore, the forces between the denatured protein molecules determine the final gel structure and gel properties. The types of forces between the protein chains are those normally encountered in protein chemistry: electrostatic, hydrogen bond, hydrophobic and covalent (S-S) interactions. In denaturing agents such as concentrated solutions of urea, proteins can be regarded as unfolded, flexible molecules, having random coil conformation, without residual non-covalent structure (10, 13). The mechanical properties of ovalbumin/urea gels approximate those of an ideal rubber (8). In a recent, mainly rheological, study (12) it is shown that textural characteristics of egg white can be significantly altered by means of chemical modification.

In the present work both the microstructure and the rheological properties of the ovalbumin/urea and the pH 10 gels appeared to be quite similar, whereas the pH 5 gels showed quite different microstructures and rheological properties. Furthermore, the gels heat-set at pH 5 were inhomogeneous, opaque and showed water exudation, whereas the urea and pH 10 gels were homogeneous, transparent and did not show syneresis. The NMR measurements (Fig. 8) show that when an ovalbumin solution is heated at pH 10, complete unfolding takes place prior to network formation, whereas at pH 5 some unfolding occurs followed by aggregation of the only partly unfolded protein. So both for the pH 10 and the urea gel, network formation occurs via flexible unfolded protein chains, whereas at pH 5 this formation occurs via aggregates forming a network. This is not

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of the gel. Only when the gel is first immersed in aqueous urea (8 mol/l), is the original microstructure completely changed, which is accompanied by considerable swelling. However, when the gel is directly immersed in a mixture of aqueous glutaraldehyde (30 g/l) and urea (8 mol/l), the microstructure of the original pH 5 gel is retained and swelling is very limited. These results indicate that glutaraldehyde has a rapid and effective fixative capacity which largely counteracts the action of urea as a swelling agent. Apparently, glutaraldehyde is effective in fixing the structure of the original gel.
surprising: at the low pH, near the isoelectric point of ovalbumin (pH 4.5), the protein conformation is strongly stabilized by hydrogen bonds and hydrophobic interactions and gelation will take place between these globular entities. At the high pH, the protein molecule is negatively charged, which leads to electrostatic repulsions and at elevated temperatures to flexible unfolded protein chains, without much interaction. Indeed the microstructure observed is in good harmony with this picture: when there are strong inter and intramolecular interactions (pH 5), a granular inhomogeneous microstructure (Figs 2 and 4) is observed, whereas at high pH and in urea, with small interaction forces between the largely unfolded protein molecules, a homogeneous, uniform distribution of protein over the gel volume is found (Figs 1, 3 and 5).

The ultimate rheological properties (7), Table 1) of the gels are well in accordance with this type of microstructure and with the character of the forces between the protein molecules. The structure which is composed of flexible, unfolded not strongly interacting protein chains (pH 10, urea) can be extended further without breaking, than the aggregated structure composed of compact, strongly interacting protein molecules (pH 5). The regions of low protein concentration in the inhomogeneous gels (pH 5), will act as weak points, resulting in a low breaking stress. A summary of the properties of the different ovalbumin gels is given in Table 3.

Conclusions

To fully understand the behaviour of proteinaceous gels, information on all levels of structural organisation is needed (3). Microstructural information can give insight into the attractive and repulsive molecular forces in the structure forming process and in macroscopic properties such as mechanical behaviour and water binding. These elements are necessary ingredients in the optimal use of proteins and modified proteins in food and food processing.

References


Discussion with Reviewers

V.E. Colombo: Can you explain how the use of urea can give insight into the structure formation and how this may lead to proposals for product or process improvement?

H.A. Morris: How can the information of this paper lead to proposals for product or process improvement?

Authors: It is true that for food products as such, urea cannot be applied. Urea was used as a model to distinguish between covalent and non-covalent interaction and it was found that a similar distinction could be made when using high pH aqueous solutions. We feel that it is relevant to have information on the effect of solvents and pH on molecular interactions since it is shown in this paper that the nature of the molecular interactions has an effect on the mechanical properties of the gels. This knowledge can now be applied in food technology for introducing in food products the desired rheological, and thus tactile and organoleptic properties.

V.E. Colombo: Could you comment on your statement that the homogeneity of the protein distribution would be an important parameter?

Authors: When in a gel the proteins are distributed evenly over the volume, each cross section will have the same average protein concentration. With an inhomogeneous distribution there will be areas with more and with less than the average protein concentration. Assuming that the breaking stress is related to the weakest points, it will be clear that the more inhomogeneous the protein distribution is, the larger the number of weak points will be. The highest strength is thus obtained with a completely homogeneous distribution.

V.E. Colombo: You attribute cellular types of gel structures to ice crystals originating during the freezing step by means of nitrogen slug. Could you comment on whether any improvements could be achieved by using freezing agents with higher freezing rates, such as propane or various kinds of Freon?

Authors: The freezing rate of a sample is not only determined by the properties of the coolant but also by the sample size. To
Microstructure and Rheology of Ovalbumin Gels

Table 3. Summary of properties of ovalbumin gels in different media

<table>
<thead>
<tr>
<th></th>
<th>pH 5</th>
<th>pH 10</th>
<th>Urea (8 mol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microstructure</strong></td>
<td>inhomogeneous, granular; network of aggregated protein</td>
<td>homogeneous, uniform; network of protein filaments</td>
<td>homogeneous, uniform, network of protein filaments</td>
</tr>
<tr>
<td><strong>Transparency</strong></td>
<td>opaque</td>
<td>transparent</td>
<td>transparent</td>
</tr>
<tr>
<td><strong>Rheology</strong></td>
<td>low breaking stress</td>
<td>high breaking stress</td>
<td>high breaking stress</td>
</tr>
<tr>
<td><strong>Conformation (NMR)</strong></td>
<td>rigid globular protein chain</td>
<td>flexible unfolded protein chain</td>
<td>flexible unfolded protein chain</td>
</tr>
<tr>
<td><strong>Protein-Protein interaction, (hydrophobic, hydrogen bond)</strong></td>
<td>strong</td>
<td>weak</td>
<td>weak</td>
</tr>
<tr>
<td><strong>Protein-water interaction</strong></td>
<td>weak</td>
<td>strong</td>
<td>strong</td>
</tr>
<tr>
<td><strong>Water release</strong></td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
</tbody>
</table>

We feel however that this also takes place, albeit to a lesser extent, for the pH 5 gels. As is indicated by the standard deviations given in Table 4, the reproducibility of the measurements is rather poor and the number of (successful) experiments is too low to draw firm conclusions about the effect of crosshead speed on breaking stress. But, as said before, we agree there is a trend (for both pH 5 and pH 10 gels) that $\tau_b$ increases with increasing crosshead speed. This behaviour is common for viscoelastic materials, in which stress relaxation during extension can take place.

A.M. Hermansson: It is concluded that flexible unfolded protein molecules are present in urea and at pH 10. Have the authors any ideas on how these molecules interact to form chains in a continuous network structure?

Authors: It has not been our intention to suggest that flexible unfolded protein molecules present in urea and at pH 10 (at high temperatures) form chains in a continuous network. When we speak about chains, we mean protein chains. How the flexible chains are structured in the final gel network cannot be concluded from our rheological and microstructural analysis.

S.S.H. Rizvi: I agree with the conclusion that cellular structure observed in freeze-dried gels at pH 10 (Fig. 3a) and urea heated gels (Fig. 5) is an artefact induced by ice crystal formation during sample preparation. However, it has also been observed (Ziegler GR. (1982). The heat-induced polymerization and gelation of beef natural actomyosin. M.S. Thesis, Clemson University; and Woodward SA, Cotterill OI. (1985). Preparation of cooked egg white, egg yolks and whole egg gels for scanning electron microscopy. J. Food Sci. 50(6): 1624) that gels fixed with glutaraldehyde alone shrank by as much as 50% during critical point drying. Can the interpretations made and conclusions drawn about protein interactions be supported, given the fact that major artefacts are introduced by either preparation technique?

Authors: We agree with the criticism on both the freeze drying and critical point drying techniques. Therefore it should always be recommended to use other independent preparation techniques. We therefore applied, apart from the SEM techniques, a thin sectioning TEM preparation technique. We feel...
as we have discussed in our paper, that considering the possible artefacts, there exists a good agreement between the different preparation techniques. Certainly in as far as we have concluded that an aggregated structure is present at pH 5 and a non-aggregated structure at pH 10 and in urea solution. Consequently we are of the opinion that our conclusions about the character of the protein interactions are justified.
THE MICROSTRUCTURE OF THE HEN'S EGG SHELL - A SHORT REVIEW¹

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Abstract

The structure of the hen's egg shell can be divided into five separate layers. The innermost layer consists of two distinct membranes known as the inner and outer shell membranes. These membranes are composed of networks of protein/polysaccharide fibres and are ≈70 μm thick. Attached to the outer fibres of the outer membrane are polycrystals of calcite (calcium carbonate) which extend outward in an inverse conical manner until the cones from several sites of crystal initiation fuse together. The fibre/crystal attachment sites, known as basal caps, and the cones form the mammillary knobs layer, which is ≈100-110 μm thick. After the cones fuse with each other, continuing calcite deposition produces columnar crystals 10-30 μm in diameter and ≈200 μm in length. These crystals form the palisade layer and are intermingled with a protein/polysaccharide matrix that differs in composition from the shell membranes. Over the columnar crystals is a thin layer (≈5-8 μm thick), known as the vertical crystal layer, of small calcite crystals that are orientated perpendicular to the shell's surface. The cuticle is the outermost layer of the shell; it is ≈10 μm thick and contains predominantly protein. Passing vertically through the palisade layer of the shell from "valleys" between the mammillary knobs to the surface of the vertical crystal layer are funnel-shaped, unbranched pores. These pores are capped by the cuticle which is cracked and thus allows the diffusion of gases between the contents of the egg and its environment. The geometrical configuration of the cones in the mammillary knobs layer is related to the thickness of shell. Specific amino acids in the membrane fibres of the basal caps influence shell strength.

KEY WORDS: Egg shell, microstructure, shell membranes, mammillary knobs, palisade/matrix, cuticle.

Introduction

The hen's egg shell surrounds the biological material essential to the reproduction of the species (Fig. 1A). Besides providing mechanical protection to the egg's contents or the developing embryo, the shell is a barrier to microorganisms, a reservoir of calcium for the embryo, a permeable membrane that facilitates the exchange of respiratory gases between the egg and its environment.

Fig. 1. Schematic diagrams of the principal components of the hen's egg (A) and of the layers that constitute the shell (B). These diagrams are not drawn to scale. Figure B based on diagrams from Schmidt (1962), Simkiss (1968), Tyler (1969a) and Simons (1971b).

¹Contribution #: 1384 - Animal Research Centre
environment without excessive dehydration and is, for wild birds, a camouflage (Board, 1982).

Interest in the egg shell dates back to at least the time of Aristotle (384-322 BC). According to Tyler (1969a), Purkinje published one of the first detailed studies of the egg shell in 1825. In a series of over 30 classical papers from work done between 1821 and 1899 as a hobby, the industrialist von Nathusius demonstrated clearly that the avian egg shell is a complex, highly ordered structure composed of a calcified matrix superimposed on fibrous membranes. Even though von Nathusius had only a simple microscope, his drawings rival in detail photomicrographs obtained with modern light and electron microscopic techniques. The original papers of von Nathusius have been translated into English and edited by Tyler (1964) and include detailed line drawings of the shell structure of eggs from about 130 species, including the domestic hen. More recently, Simons (1971a) published the results of a comprehensive study of the hen's egg shell and membranes involving light, and transmission and scanning electron microscopy. This monograph also contains an extensive review of previously published data.

Reviews on the morphological aspects of the hen's egg shells have been published by Stewart (1935), Burmester (1940), Simkiss (1968), Tyler (1969a) and Parsons (1982). Microscopic studies of egg shell formation have been reported by, among others, Simons (1971a), Hoffer (1971), Draper et al. (1972), Creger et al. (1976), and Makita (1981). In addition, reviews have been published on shell calcification (Wilbur and Simkiss, 1968; Eastin and Spaziani, 1978a,b), organic components of the shell (Kramplitz and Witt, 1978; Kramplitz, 1982; Leach, 1982) and the relationship between shell structure and egg shell breakage (Parsons, 1982; Washburn, 1982).

It is now generally accepted that the shell of the hen's egg (Fig. 2) contains at least five distinct layers as indicated schematically in Figure 1B. These layers are 1) inner and outer shell membranes, 2) mammillary knobs, 3) palisade/shell matrix, 4) vertical crystals, and 5) the cuticle (Simons, 1971a). Because this presentation is a tutorial, the discussion will be limited to the major structural and biochemical aspects of these layers. Little evidence will be presented to confirm these aspects since, in some cases, the data is extensive.

Egg and Shell Formation

The following brief description of egg and shell formation is given to indicate the specific area of the hen's reproductive tract and the time required for the synthesis of these layers. The size of the yolk (Fig. 1A) present in the ovary of the hen increases rapidly over a period of 10-14 days before ovulation. During this time its weight increases from about 200 mg to 150 g through the incorporation of proteins and lipids that are synthesized mainly in the liver (Redshaw and Follett, 1972). After the yolk or ovum is mature, it is released from the ovary (ovulated) and begins its descent of the hen's reproductive tract where the albumen and shell
are formed. The average time between successive eggs is 24-25 hours for high-producing birds.

The reproductive tract consists of at least five distinct regions, namely: 1) infundibulum, 2) magnum, 3) isthmus, 4) uterus or shell gland, and 5) vagina. The time taken and the component added to the egg during passage through the reproductive tract of the hen are summarized in Table 1. Shell color pigments are incorporated within the last 5 hours of shell deposition (Warren and Conrad, 1942). The brown pigment that colors the eggs from some hens is porphyrin synthesized by the shell gland from 6-amino levulinic acid (Gebel, 1952). Brown coloration may be obtained from Robinson (1972) and Gilbert (1971) for albumen synthesis and Simkiss and Taylor (1971); Creger and Reed (1976) and Reed and Yurkiewicz (1982) for shell formation.

Gross Composition and Physical Characteristics of Eggs

The hen's egg contains about 58% albumen, 32% yolk and 10% shell by weight, with the first two components containing approximately 76 and 24% water, respectively (Gilbert, 1971). About 96% of the material in the shell is inorganic and 4% organic (Gelger et al., 1974); the latter is composed of a number of discrete proteins, glycoproteins and glycosaminoglycans. Calcium carbonate constitutes 98% of the inorganic material while magnesium carbonate and calcium phosphate contribute equally to the remainder; trace amounts of other inorganic ions are present.

Numerous factors influence egg size (weight) such as the genotype of the hen, age of hen, position of the egg in a sequence, rate of lay, environmental temperature, diet and disease status (Gilbert, 1971). The data presented in Table 2 show the means and variability in egg characteristics and shell characteristics for white- and brown-shell eggs laid by mature hens (Thompson et al., 1981). Characteristics such as shell thickness, compression fracture strength and deformation vary among eggs at a particular site (Table 2) and also among sites on a single egg. For example, the shell is thickest at the equator and thinnest at the narrow pole; thickness at the blunt end is intermediate between the narrow pole and equator (Tyler, 1961; Tyler and Geake, 1965).

Shell Membranes

Even though the membranes located on the inner surface of the egg shell appear to be a single layer (Fig. 3), they can be divided, by careful manipulation, into two distinct layers of fibrous material. They adhere closely to each other except at the blunt end of the egg where they are separated by the air cell (Fig. 18). One layer surrounds the albumen while the other is attached to the "tips" of the calcified material of the shell (Fig. 3); these layers are known as the inner and outer shell membranes, respectively.

Microscopic examination of these layers reveals an interwoven meshwork of fibres (Fig. 4) arranged in a random manner (Stewart, 1935; Moran and Hale, 1936; Kaplan and Stiegemsund, 1973; Creger et al., 1976; Wong et al., 1984). The fibres of the inner membrane are about one half as thick as those in the outer membrane (22 vs 48 mm; Simons, 1971a). Thickness of the shell membranes vary within an egg and among eggs (Balch and Tyler, 1964). Electron microscopy studies of Masshoff and Stolpmann (1961) showed that each fibre of the membranes had a central core that was fine and fibrillar in structure and this core was surrounded by a sheath. Delicate strands of material spanned the gaps present between the core and the sheath. Fibres from the inner and outer membranes were similar in structure, except the former were...
thinner. Draper et al. (1972) described the material in the core as "electron dense" and that in the sheath as "less dense" substance. Simons and Wiertz (1962) and Hoff (1971) reported similar observations for membranes from hen's eggs and Japanese quail eggs, respectively. Frequently the mantle of adjacent fibres coalesces, giving the appearance of extensive branching of the fibres (Masshoff and Stolpmann, 1961; Fig. 4). Also, evident on these fibres are small protuberances (see Creger et al., 1976; Leach, 1982; Fig. 4), which can be useful when studying the relationship of composition to shell structure such as occurs with dietary copper and manganese deficiencies (Baumgartner et al., 1978; Leach and Gross, 1983; respectively).

Chemical analyses indicate that the shell membranes in their native state contain about 20% water, 75-76% proteinaceous material and 4-5% carbohydrate (Gilbert, 1971). The nature of the proteinaceous material of the shell membranes remains to be resolved. Some researchers have concluded that these membranes contain keratin or ovokeratin (Simkiss and Taylor, 1971) whereas others consider the proteins as collagen-like due to the presence of hydroxyproline (Balch and Cooke, 1970) and hydroxylysine (Candlish and Scougall, 1969). An elastin-like protein has been suggested also to occur in the shell membranes (Simkiss and Tyler, 1957; Harris et al., 1980). The keratinous nature of the membrane proteins has been questioned because of differences in structure, amino acid content, and protein solubility of soft and hard keratins and the proteinaceous material from shell membranes (Wedral et al., 1974; Vadehra et al., 1971). Similarly, results from amino acid analysis, radiolimnnoassay and enzymatic hydrolysis led to the conclusion that elastin is not present in the shell membranes (Starcher and King, 1980; Leach et al., 1981; Crombie et al., 1981) even though desmosine and isodesmosine, lysine-derived molecular crosslinks found in elastin, occur in the hydrolysates of the membranes (Baumgartner et al., 1978; Harris et al., 1980; Leach et al., 1981). The presence of both membranes of two forms of collagen-like proteins, similar to collagen I and V, in the ratio of approximately 100:1. Immunofluorescence microscopy indicated that within each membrane, collagen I was associated predominantly with the large, coarse fibres (>2.5 μm diameter) and the collagen V with the delicate, narrow fibres (0.6 μm diameter). At the electron microscopic level, the normal 67-nm banding usually seen with type I collagen was not present in either the large, coarse, or the delicate, narrow fibres.

The simple sugars represent 70-80% of the carbohydrate present in the shell membranes (Balch and Cooke, 1970). Glucose, galactose, and mannose were reported by both Balch and Cooke (1970) and Wedral et al. (1974). However, the former also reported the presence of fructose and the latter xylose; the reason for the discrepancy is not evident. Both research groups found sialic acid in small amounts. Balch and Cooke (1970) also found small quantities of glucosamine and galactosamine, but due to the absence of uronic acid they proposed that the galactosamine was not associated with chondroitin sulphate. Abatangelo et al. (1978), however, reported the presence of 0.5% uronic acid in the shell membranes.

Lipids have been isolated in small quantities from the shell membranes by Haslak et al. (1970a,b) and Britton (1971). These included mono, di and triglycerides, sodium fatty acids, cholesterol and its esters, lecithin, lysolecithin, cephalin and sphingomyelin (Haslak et al., 1970a,b). Trace amounts of sodium, potassium, manganese, zinc, copper, boron and aluminium were found to be present in both the inner and outer shell membranes by Wedral et al. (1974). Calcium which was also observed was thought to have a structural role.

**Mammillar Knob Layer**

In the mammillary knob layer, the calcified portion of the egg shell connects with the fibres of the outer portion of the outer shell membrane (Fig. 1B and 3). The part of the mammillary knob layer that is embedded in the outer membrane fibres is termed the basal caps while the portion above and between these membrane fibres and the palisade layer is known as the cone layer (Tyler, 1965). The membrane fibres penetrate at least 70 μm into the palisade layer (Simons, 1971a). Removal of the membrane fibres results in the shell membranes with about 5% w/v or protease digestion revealed microscopic tracts as evidence of attachment of the mammillary knobs in the fibres (Kaplan and Siegesmund, 1973; Stevenson, 1980; respectively). The appearance of the mammillary knobs after the removal of the membrane fibres is sometimes described as "rose-bud". About one-third of the shell thickness is accounted for by the mammillary knob layer (Burmester, 1940).

The calcified portion of the shell is composed of small individual crystallites (Simons, 1971a) of calcium carbonate in the form of calcite (Terepka, 1963a). The individual crystallites demonstrate in almost all directions the centre of the mammillary core where they attach to the membrane fibre. According to Schmidt (1965), growth of individual crystallites, sometimes known as spherulites (Wilbur and Simkiss, 1968), inwards to the outer membrane is impeded by the resistance of the outer membrane fibres. Their growth is further decreased by a reduced flow of calcifying material from the uterine tissue due to more rapid growth of crystals immediately adjacent to that tissue. As the crystals grow outward from the basal caps, they form irregular polygonal cones and eventually fuse with those from adjacent formations to complete the cones of the mammillary knob layer. The crystallites that grow into membranes are also known as eosospherites and those that grow outward from the membranes as exospherites (Tyler and Fowler, 1978).

Electron microscopic studies indicate that the structure and spatial configuration of the mammillary knob layer is an important determi-
Egg Shell Microstructure

nant of shell strength. El-Boushy et al. (1968), Robinson and King (1970), King and Robinson (1980; 1982), Terepka et al. (1981) observed and the mammillary layer was frequently disorganized in shells of low strength compared to those of high strength. The number of mammillary knobs per square centimeter was higher for shells of low breaking strength than those of high strength, the former shells were thinner than the latter (307 vs 356 w/m²), respectively. These results support the findings of Tyler and Fowler (1978) that the shell thickness of eggs from wild birds was highly correlated (r = 0.94) to the "nearest neighbour" distance between mammillary cones. Leach and Gross (1983), however, found that although the shell impact strength of eggs from manganese deficient hens was lower than for those from non-deficient hens, the former shells had fewer and wider mammillary knobs per unit area than the latter.

Simkiss and Tyler (1957) showed, by histochemical techniques, that the concentration of organic material was higher in the mammillary cones than elsewhere in the mammillary knob layer. Subsequently, Robinson and King (1968) demonstrated that the organic portion of the mammillary cones contained mainly neutral mucopolysaccharides. The results of Cooke and Balch (1970a) confirm the histochemical observations of Robinson and King (1968) and provide quantitative data that about one half of the carbohydrate material present in the mammillary cones was glucosamine.

The chemical composition of the fibres in the mammillary cones also affect shell strength. The amino acid content of the outer portion of the outer membrane influences the shell strength of eggs from young and old hens (Britton and Hale 1977; Blake et al. 1985). Methionine, serine, phenylalanine and aspartic acid along with lysine (Britton and Hale, 1977; Blake et al., 1985) were found to influence shell deformati and egg specific gravity; (Britton and Hale, 1977; Blake et al., 1985) were found to influence shell deformation and egg specific gravity; defomaition and specific gravity are two methods commonly used to estimate egg shell strength (Schmidt, 1964).

Detailed studies of the crystalline structure of the egg shell have shown that the palisade layer contains individual column crystals of calcium carbonate as rhombohedral calcite (Schmidt, 1962; Terepka, 1963a). Each column of calcite consists of crystallites with reported diameters of 10-15 μm (Terepka, 1963a) to 20-30 μm (Perrott et al., 1981). Both Terepka (1963a) and Schmidt (1964) concluded from microscopic studies with polarized light that the orientation of c-axis of the hexagonal unit cell of the crystallites in the palisade layer was perpendicular to the surface of the egg shell. On the other hand, Cain and Heyn (1964) reported that the c-axis of the calcite crystals in the palisade layer was inclined 28° ± 16° from the perpendicular to the shell surface in X-ray diffraction studies. From a limited X-ray diffraction study, Favejee et al. (1965) observed that the c-axis of calcite in egg shell was randomly arranged and on the average was inclined 45° to the shell's surface.

Sharp and Silyn-Roberts (1984) point out that c-axis angles reported by Cain and Heyn (1964) are improbable because of the manner in which they were determined. In contrast, Perrott et al. (1981) found that, while the c-axes of the crystallites were inclined at angles between 12° and 48° from the perpendicular to the surface within a crystal column, there was no evidence from chemically thinned specimens of a preferred orientation among the columns in the palisade layer as a whole. However, when specimens were thinned with an ion beam some preferred orientation within a single crystal was observed, such that the c-axis lay within a range of 30±18° from the perpendicular to the surface. Sharp and Silyn-Roberts (1984) showed by x-ray diffraction that a preferred orientation developed gradually throughout the shell of hen's eggs, beginning at the shell membranes and reaching a maximum at the exterior surface. Two of 20 shells examined exhibited a single preferred orientation in which the pole of the (001) plane was perpendicular to the shell's surface; whereas, a second preferred orientation, (104) plane, occurred simultaneously with the (001) plane in the remaining eggs. This second orientation occurred only in the outer half of the shell where the (104) pole was perpendicular to the surface. The data reported by Favejee et al. (1965) was interpreted by Sharp and Silyn-Roberts (1984) also to indicate the presence of crystals in two preferred orientations. In another study by Silyn-Roberts and Sharp (1985a) found that the (001) preferred orientation was characteristic of the shells of eggs from ratite and tinamou species of birds and the (104) orientation appeared in the shells of only a small proportion of the species and even only as a sec-
ondary development. This phenomenon was found also to occur in the shells of reptilian eggs by Simons (1971a,b) and Pooley (1979) observed that the calcite crystals in the vertical crystal layer were small with their greatest dimension at approximately right angles to the shell’s surface. Perrott et al. (1981) also found that the preferred orientation of the crystals in the vertical crystal layer was parallel to the surface.

Holes with diameters of about 0.4 μm are present in the decalcified preparations of the true shell. These holes, known as vestigial holes (Simons, 1971a,b), are apparently more numerous in the mammillary knobs than in the palisade layer. Sometimes minute crystals lie close to the holes (Simons, 1971a; Heyn, 1963) which were described by Cooke (1909). The organic material that constitutes the matrix of the calcified portion of the shell consists of a protein/polysaccharide complex (Simkiss and Tyler, 1958; Baker and Balch, 1962; Cooke and Balch, 1970a,b; Heaney and Robinson, 1976). This complex contains at least 70% protein and 11% polysaccharide along with a small amount of fat (Baker and Balch, 1962). The matrix protein is characterized by the absence of hydroxyproline (Baker and Balch, 1962; Frank et al., 1965), a low content of aromatic and sulphur amino acids and a ratio of about 2-to-1 for dicarboxylic amino acids to basic amino acids (Baker and Balch, 1962). Frank et al. (1965) reported a moderate correlation (r = 0.88) between the amino acid composition of the shell matrix and that of non-collagenous protein isolated from porcine hyaline cartilage. Chondroitin sulphates A and B, and hyaluronic acid that contained equal molar amounts of glucosamine and galactosamine, were identified in the shell matrix by Balch (1962, 1968), Cooke and Balch (1970b) and Heaney and Robinson (1976), respectively. Small amounts of sialic acid were reported by Frank et al. (1965) and Cooke and Balch (1970b), but when the shell matrix material was analyzed directly, no measurable sialic acid was found in the water soluble residue obtained from the matrix (Abatangelo et al., 1978; and Cortivo et al., 1982). Other carbohydrates reported to be present in the shell matrix include mannose, glucose, fucose and xylose (Cooke and Balch, 1970b).

Cooke and Balch (1970b) demonstrated an uneven distribution of the organic matrix material within and among the crystalline material of the shell. They found its concentration increased to maximum in a region about two-thirds of the distance from the inner membrane and then rapidly decreased towards the shell’s surface. These results do not support the postulate of Tyler and Geake (1958) or the conclusion of Carter (1969) that the organic content of the “true shell” or “incremental shell”, respectively, is constant.

It is interesting to note the decrease in the acid solubility of the calcified material in the region of the true shell observed by Cooke and Balch (1970b) occurred in approximately the same region Sharp and Silyns-Roberts (1984) found the second preferred orientation in the crystalline structure started to develop in the majority of the shells they examined. Simons (1971b) observed, by electron microscopy, that calcium in the egg shell follow the layers of organic material in the palisade layer; consequently, the presence of both radial and tangential fractures in its photomicrographs may be due to the influence of the organic matrix material on the preferred orientation of crystals in the palisade layer. Both Simkiss and Tyler (1958) and Simons (1971a) postulate that the deposition of organic material precedes that of the inorganic material. Frank et al. (1965) found little evidence that the amino acid content of the shell matrix influences shell breaking strength.

Terepka (1963b) suggested an inverse relationship between the concentration of shell matrix material and mineralization. Simkiss and Tyler (1958) hypothesized that the shell matrix acts as a chelating agent probably due to the mucopolysaccharides, or hyaluronic acid as it is presently known. Abatangelo et al. (1978), however, demonstrated that the egg shell matrix could bind calcium ions only if carboxylic side chains of aspartic and glutamic acids were available. Subsequently, Cortivo et al. (1982) isolated a peptide that was resistant to alkaline hydrolysis. About 50% of the amino acid residues in this peptide were aspartic and glutamic acids which were found to be important in the binding of calcium ions. These researchers found no y-carboxyglutamic acid present in the matrix. Krampitz et al. (1980) isolated a peptide from porcine hyaline cartilage. Blake and Kling (1984) demonstrated that in vivo decarboxylation of y-carboxyglutamic acid had no significant effect on shell strength when measured by specific gravity.

Histochemical staining of the shell matrix indicates the presence of the enzyme carbonic anhydrase (Robinson, 1970) which has subsequently been isolated (Krampitz, 1982). This enzyme catalyzes the reversible reaction:

$$\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{HCO}_3^-$$

(1)

(Robinson and King, 1963) and is considered to be involved in shell calcification of the hen’s egg (Krampitz and Witt, 1978).

In addition to calcium carbonate as calcite in the true shell, phosphate and magnesium occur in the outer part of the shell (Itoh and Hatano, 1964; Simons, 1971a). Small amounts of potassium, sodium, iron, copper, manganese, sulphur and zinc are present in the shell but their location and purpose is not known (Simons, 1971a). The magnesium content in the shell has been demonstrated to increase from the membranes to the shell’s surface (Brooke and Hale, 1955; Simons, 1971a). However, Board and Love (1983) found, from electron probe microanalysis across the radial axis of the egg shell, the occurrence of a narrow band of magnesium-rich shell at the mammillar knobs layer and, after an initial decrease, a progressive increase in magnesium concentration to a maximum at the outer surface of the shell. According to the results of Smith
et al. (1954) most of the phosphate(s) present in the shell was also located in the outer part.

Cuticle

Cuticle is the term used to identify the layer of organic material that is deposited over the vertical crystal layer of the egg shell (Simons, 1971a,b); it is sometimes called the "bloom". The thickness of the cuticle was found by Simons (1971a) to vary between 0.5 and 12.8 μm among eggs and at different sites on the same egg. Many star-shaped crack systems are apparent with scanning electron microscopy (Simons, 1971a,b); these cracks in the cuticle contain vesicles up to about 1 μm. Large crack systems tend to be oval in shape and have clearly defined edges (Simons, 1971a); they are considered to represent the surfaces of oval pore plaques. Small crack systems also occur in the cuticular material on the surface of the egg's shell. The microscopic appearance of the cuticular material changes with time due possibly to drying and oxidation of sulphhydryl (-SH) and disulphide (-S-S-) groups present in the protein(s) of the cuticle. At the time the egg is laid, the cuticle may be easily disrupted mechanically, especially if it comes into contact with the wire floor of a cage, or the claws or beak of the hen (Denison, 1967; Tyler and Standen, 1969; Talbot and Tyler, 1974). The areas where the cuticle has been disrupted are translucent in appearance, contain more water and are weaker than the surrounding shell material (Tyler and Geake, 1964). The presence of translucent areas on the egg shell is known as mottling and is more evident in eggs that have been stored possibly because of shrinkage of the cuticle over time.

The cuticle of the shell is considered to be important in the waterproofing of the avian egg (Board and Halls, 1973). Studies by researchers found that of 453 brown shelled eggs examined, 3.5% had no demonstrable cuticle and another 8% had no cuticle at either the pointed or broad end of the egg. Belyavin and Boorman (1980) found that removal of the cuticle from the shell with EDTA significantly reduced their specific gravity, which is considered to be a factor associated with shell strength. Chemical analysis indicates that the cuticle contains 85-90% protein (Wedral et al., 1974; Baker and Balch, 1962) most of which is insoluble in water or potassium chloride solution (1% (w/v); Wedral et al., 1974), 4-5% carbohydrate (Wedral et al., 1974; Cooke and Balch, 1970a), 2.5-3.5% lipid (Wedral et al., 1974) and 3-3.5% ash (Wedral et al., 1974; Baker and Balch, 1962). Based on electron microscopy, cuticles of eggs removed directly from the oviduct contain no fibrous or crystalline material in their lumen (Tullett et al., 1975). Simkiss (1961) found that the pores of eggs removed from the uterus that had no demonstrable cuticle were filled with sulphur-containing protein. The opening at the shell surface is plugged with cuticular material that contains cracks which allow the diffusion of respiratory gases.

Egg Shell Microstructure

Passing vertically through the palisade layer of the shell from the valleys between the mammillary knobs (Board et al., 1977) to the surface of the vertical crystal layer are funnel-shaped pores (Fig. 18; Tyler, 1956). These pores in the shells of eggs from domesticated hens are unbranched and capped by the organic material of the cuticle (Board et al., 1977). Each pore varies in width from 15 to 65 μm at the surface to between 6 to 23 μm at the inner levels of the shell (Tyler, 1956). Scanning electron microscopy indicated that the walls of the pores are rough but without a definite ultrastructure and contain no fibrous or crystalline material in their lumen (Tullett et al., 1975). It is estimated that there are between 100 and 300 pores/cm² of shell surface (Gilbert, 1971). There is a strong positive linear correlation (r = 0.92) between the number of pores/cm² and mammillary knobs/0.25 mm² which Tullett (1975) speculated may be due to the distribution of cells in the uterus that secrete the sites on which calcification is subsequently initiated. Tyler and Fowler (1978) postulated that when three or more cones having approximately circular cross sections meet at the beginning of the palisade layer a "channel" will be formed between them and this channel might, under certain circumstances, continue through the palisade layer to form a pore. In addition, Tyler (1956) speculates that liquid might pass through these "channels" of the forming shell into the egg, but gradually some of the channels become blocked by the developing crystals until shell deposition is completed.
As a result, only a relatively small proportion of the initial channels would remain as pores when the egg is laid.

**Future Research**

Even though the hen's egg shell is less than 400 µm in thickness, it has a complex microstructure that consists of a "composite" material. Further research is needed to establish the interrelationships among the inorganic and organic constituents that form this composite material, the preferred orientation of the crystalline material. Further research is needed to establish the interrelationships among the inorganic and organic constituents that form this composite material, the preferred orientation of the crystals of the shell and the strength of an egg's shell, especially at the microstructure level. Composition of the organic matrix material present in the palisade and vertical crystal layers should be reinvestigated with modern analytical techniques. The mechanism involved when the egg shell fractures due to the application of forces slowly or rapidly also requires elucidation. Additional studies are required to increase our understanding of the "biology of egg shell formations" so that it can be explained in terms of biochemical and physiological processes. In order to resolve this problem, the expertise of such groups as electron microscopists, biochemists, material science engineers and poultry scientists are needed.

**Acknowledgements**

The author thanks A.F. Yang, Electron Microscopy Section, Chemistry and Biology Research Institute, Agriculture Canada, Ottawa for the electron photomicrographs of egg shells. The figures were prepared by Research Program Services, Research Branch, Agriculture Canada, Ottawa.

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Egg Shell Microstructure

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Discussion with Reviewers

A.S. Pooley: Does the structure of the eggshell have any influence on the egg as a food material?

Author: No, eggshell structure has no direct influence on the egg's contents. However, if
thickness, a structural property of the shell, is considered, then shell structure may be considered to have an indirect effect because thin shelled eggs will be more susceptible to moisture loss and/or microbial contamination of their content during storage.

A.S. Pooley: Is anything that has been learned about eggshells in the past 30 years likely to lead to breeding selection that will provide increased resistance to breakage of eggs?

Author: Yes, research on the measurement of eggshell strength has provided techniques that are routinely used in selection programs by the primary breeder to produce the commercial stocks used for egg production. In addition, information from this research, such as cool eggshells are stronger than warm ones, has been discussed with egg producers for use in their operations.
Ultrastructure of Cooked Spaghetti

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Abstract

Several electron microscopy (EM) complementary techniques (scanning electron microscopy, freeze-fracturing and thin-sectioning) have been applied in the ultrastructural study of spaghetti.

Experimental spaghetti have been produced starting from two semolinas from the same wheat cultivar and using a low temperature (LT) and very high temperature (VHT) drying schemes.

Cooking quality of these products was not related to the quantity of the main components present in semolina. However, the drying conditions (temperature and humidity) and the nature of the cooking water greatly influenced cooking characteristics.

The three EM techniques were used to detect differences in protein and starch organization in relationship to spaghetti quality. Structural differences present in the uncooked product were more evident after cooking. In particular, in each high quality spaghetti, interesting macromolecular arrangements were always found inside starch granules. These new structures, which were dramatically promoted by VHT drying, exhibited an exceptional resistance to alpha-amylase digestion.

Introduction

The characteristics and texture of cooked pasta are strongly affected by semolina protein quality and quantity (D'Egidio et al., 1979; Frey and Holliger, 1972; Grzybowski and Vonnely, 1979; Matsuo et al., 1972; Wasik and Bushuk, 1975), drying conditions (Dexter et al., 1981 a and b; Manser, 1979; Resmini and Pagani, 1983; Wyland and D'Appolonia, 1982), and by the nature of the cooking water (Alary et al., 1979; D'Egidio et al., 1981; Dexter et al., 1983).

Starch gelatinization and swelling and protein coagulation occur during pasta cooking (Resmini and Pagani, 1983). These phenomena occur at approximately the same conditions of temperature and moisture and are competitive and antagonistic (Frey and Holliger, 1972; Resmini and Pagani, 1983). At present we do not have a complete understanding of the biochemical and biophysical factors affecting protein coagulation or starch swelling.

Electron microscopy (EM) techniques allow one to observe the organization of pasta components. Thus, they represent good tools to obtain informations about protein and starch changes which are promoted by different pasta-making technologies. Also, EM helps to evaluate interactions that occur between the two components during pasta cooking.

Materials and Methods

Semolina composition

Protein content was determined by the Kjeldal method (N x 5.7). Starch content was evaluated according to Thivet et al. (1972); amylose determination was carried out by amperometric titration (Larson et al., 1953). Ethanol-soluble carbohydrates (80% aqueous ethanol) were measured as proposed by Mercier and Feillet (1975); total lipid content according to Drapron (1975).

Spaghetti production

All spaghetti samples were produced at the "Laboratoire de Technologie des Céréales", I.N.R.A., in Montpellier (France), starting from French semolina of Hondur cultivar grown in two different places (Montpellier and Niverville).
Fig. 1. Commercial uncooked spaghetti. a) SEM image; b) FF image; c) thin-section (uranyl acetate/lead citrate staining); d) thin-section (PATAg staining). Small arrows indicate artifacts due to the fracture (Fig. 1b) and to folds (Fig. 1d). (S) starch granule; (p) protein matrix; (l) lipid inclusions; (w) water; (em) embedding medium.
Ultrastructure of cooked spaghetti

Fig. 2. Commercial cooked spaghetti. a) SEM image; b) FF image; c) thin-section (uranyl acetate/lead citrate staining); d) thin-section (PATAg staining). (gs) gelatinized starch; (pn) protein network; white arrows (Fig. 2c) indicate alveoles.
Spaghetti was prepared in a Demaco scale laboratory press and dried by a low temperature (LT) drying method in a laboratory scale drying cell equipped with an automatic system for ventilation and resting. Spaghetti Mondur Niverville was also dried at very high temperature (VHT), a few hours over laboratory press and dried by a low temperature (LT) drying method in a laboratory scale drying system. This spaghetti resulted in changes in plasticity and resting properties that its ultrastructural studies seemed quite advisable.

**Spaghetti cooking test**

5 g of spaghetti (strands of 2 cm) were plunged into 150 ml of boiling mineral water (Evian water, pH 7.2) (Alary et al., 1979) and stirred for the first 3 minutes. After 5 minutes of cooking, few strands were taken out and drained; the rest of the spaghetti was cooked to the optimal cooking time (Dexter et al., 1981b). Cooking quality was evaluated as proposed by Alary et al. (1979) taking into consideration stickiness, aspect and viscoelasticity properties. LT Mondur Niverville spaghetti was also cooked in boiling distilled water (pH 5.5) (Alary et al., 1979) to assess the cooking quality improvement observed by several authors (Alary et al., 1979; D'Egidio et al., 1981; Dexter et al., 1983).

**Ultrastructure studies**

All the uncooked spaghetti was soaked for 12 hours in aqueous media prior to each EM preparation. Soaking was carried out in 30% glycerol-water for freeze-fracturing (FF) and in mineral water for thin-sectioning and SEM.

**SEM specimen preparation.** Uncooked and cooked spaghetti were examined both at the surface and in transversal section. Samples were fixed one hour in 6% and one hour in 3% glutaraldehyde solutions in 0.1M cacodylate buffer (pH 7), dehydrated in graded acetone series, critical point dried, mounted on stubs and shadowed with gold (ion sputtering J EOL JFC 1100). The thin-sections were exposed to a gold layer: 40 nm. Samples were observed in a JEOL 50A scanning electron microscope at an acceleration voltage of 20 keV.

**TEM specimen preparation.**

Sections of uncooked and cooked spaghetti were fixed differently according to the staining technique and carbohydrate staining. Samples were fixed one hour in 6% and one hour in 3% glutaraldehyde solutions in 0.1M Na cacodylate buffer (pH 7). For protein staining, after the fixation in glutaraldehyde solutions as described above, the specimen were post-fixed one hour in 1% buffered OSO₄ solution in order to obtain better contrast for protein and lipid.

The fixed specimens were dehydrated in graded acetone solutions, embedded in Epon and sectioned using a diamond knife in a JEOL JUM 7 ultramicrotome (thickness of specimen 70-100nm). The thin-sections were placed either on copper or gold grids supported with a collodion membrane and stained. The staining with uranyl acetate and lead citrate solutions (Bechtel et al., 1978; Frey and Holliger, 1972) is selective for protein and lipid which appear dark grey or black. With PTAg staining technique (Gallant, 1974; Gallant and Gutibot, 1969) starch and other carbohydrates appear black. The stained thin-sections were observed in a JEOL 100S transmission electron microscope at 80 or 100 keV.

**Freeze-fracturing.** Replicas were prepared in the conditions recently proposed by Resmini and Pagani (1983). Spaghetti samples were frozen in super-cooled liquid nitrogen, transferred into a BALZERS FF unit (BAF 301) heated to -95°C, fractured at -195°C and shadowed with Pt/C film immediately after the fracture. Replicas, after suitable cleaning in H₂SO₄ solutions, distilled water, acetone and bidistilled water, were observed in a JEOL 100S transmission electron microscope at 60 or 80 keV.

**Enzymatic studies.**

To understand the nature of the particular structures, the thin-sections were treated with enzymes and stained as described above to identify proteins and carbohydrates.

Alpha-amylase hydrolysis. The grid with the section was exposed to a 0.1% buffered amylase solution (alpha-amylase from Bacillus stearothermophilus, Boehringer; 0.1M phosphate buffer, pH 7.2). The amylolysis was carried out for 15 minutes at room temperature.

Proteolytic hydrolysis. The grid with the section was exposed to a 0.2% buffered pronase solution (pronase from Streptomyces griseus, Boehringer; 0.1M phosphate buffer, pH 7.5). The proteolysis was carried out for 30 minutes at 40°C.

**Results and Discussion**

Advantages and disadvantages of the different techniques used in EM

Each EM technique offers advantages and disadvantages in the ultrastructural study of spaghetti. Preparations are generally simple in scanning electron microscopy (SEM). Before cooking, starch granules (size ranging from 2 to 40 μm) can be clearly seen below the protein matrix (Fig. 1a). The protein, because of hydration (Chabot, 1979; Dexter and Matsuo, 1977; Dexter et al., 1978), appears organized in a fibrillar network (the gluten) which surrounds the starch granules. But SEM shows only surface morphology (Angold, 1979; Davis and Gordon, 1983; Hsieh et al., 1981) and the three dimensional organization. Furthermore SEM preparation methods produce artifacts due to chemical fixation and dehydration (Chabot, 1979; Davis and Gordon, 1983; Varriano-Marston, 1981).

Transmission electron microscopy (TEM), methods such as freeze-fracturing (FF) and thin-sectioning techniques, allow the investigation of internal fine structures. In a traditional spaghetti before cooking, we can observe by FF (Fig. 1b) the compact spherulitic structure of the starch granules, the protein matrix organized into subunits, and the uniformly dispersed protein among the starch granules and containing numerous lipid inclusions. The information given by this technique is of extreme interest. FF is recognized as one of the most suitable techniques in food ultrastructure investigations since it does not introduce artifacts due to chemical fixation and/or dehydration (Chabot, 1979; Davis and Gordon, 1983; Freitzdorff et al., 1982a; Resmini and Pagani, 1983). On the other hand, information and interpretation of past FF images require considerable experience. The identification of starch and proteins is made easily after
Ultrastructure of cooked spaghetti

Fig. 3. SEM images of experimental uncooked spaghetti. a), b) before hydration; c), d) after hydration. a), c) MM, good quality spaghetti; b), d) MN, poor quality spaghetti. (p) protein matrix; (S) starch granule.
Ultrastructure of cooked spaghetti

Fig. 4. FF images of experimental uncooked spaghetti. a) MM, good quality spaghetti; b) MN, poor quality spaghetti. (l) lipid inclusions; (p) protein matrix; (S) starch granule; (w) water.

Fig. 5. Uncooked MN-VHT, high quality spaghetti. a) FF image; b) thin-section (uranyl acetate/lead citrate staining); c) SEM image. (p) protein matrix; (S) starch granule.
a careful study of the isolated components, before and after cooking (Fretzdorff et al., 1982a; Resmini and Pagani, 1983).

TEM which involves thin-sectioning and specific staining for proteins and starch, provides immediate identification of these components. The staining with uranyl acetate/lead citrate is selective for proteins and lipids and appears as markedly grey (Fig. 1c) (Gallant, 1974; Bechtel et al., 1978; Fretzdorff et al., 1982b; Hood and Liboff, 1983). In the staining with silver salts (PATAg technique, Fig. 1d) identifies starch granules and all other carbohydrate components, giving them the black, granules and all other carbohydrate components. The staining with silver salts (PATAg technique, Fig. 1d) identifies starch granules and all other carbohydrate components, giving them the black, characteristic, punctuated image (Gallant, 1974; Gallant and Guilbot, 1969; Duprat et al., 1980; Gallant and Sterling, 1976; Fretzdorff et al., 1982b). However thin-sectioning methods may cause artifacts due to the indispensable chemical fixation and dehydration steps as with SEM. It is impossible to determine the actual water distribution in thin-sections and by SEM (Fretzdorff et al., 1982a). Another disadvantage of thin-sectioning, also common to FF techniques, is the difficult and the tedious specimen preparation, especially for cereal products (Hsieh et al., 1981).

Some comments may be extended to the spaghettli ultrastructures observed after cooking by the different techniques. Morphologic changes of starch granules as a consequence of their water absorption and swelling can be observed by SEM (Figs. 2a). But it is important to keep in mind the artifacts introduced by the specimen preparation (e.g., in our case dehydration by critical point drying). The more the granules are swollen, the greater will be their deformation. In fact, dehydration always produces granule shrinkage (Chabot et al., 1979; Christianson et al., 1982; Hood and Liboff, 1983). A network consisting of coagulated proteins surrounds the swollen starch granules.

TEM provides informations on fine component modifications. Thin-sectioning shows (Fig. 2c, 2d) the developing of alveoles containing solubilized material inside the swelling granules. However, it is impossible to follow protein changes. Using TEM in fact, protein appear as a smooth compact matrix, both in uncooked and cooked products. Conversely, the examination of FF ultrastructure of cooked spaghettli reveals interesting details (Fig. 2b). Starch swelling and gelatinization show the native spherulites. The native protein subunits interact and coagulate into a network which, if continuous, may retain and contain the swelling granules (Resmini and Pagani, 1983). Pasta drying and/or cooking produce changes in the three-dimensional arrangement and in the internal organization of proteins and starch. Integration of the microstructural methods (SEM, FF and thin-sectioning) provides important information to scientists engaged in this field of study. Furthermore, the possibility of comparing the microstructure of the same sample using several techniques makes it possible to detect artifacts in order to avoid misinterpretations.

Table 1. Cooking characteristics of experimental spaghettli.

<table>
<thead>
<tr>
<th>SEMOLINA</th>
<th>DRYING</th>
<th>COOKING</th>
<th>OPTIMAL COOKING</th>
<th>COOKING LIQUID</th>
<th>TIME (min)</th>
<th>QUALITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>MONDUR</td>
<td>LT</td>
<td>Mineral</td>
<td>10</td>
<td>Poor</td>
<td>Good</td>
<td></td>
</tr>
<tr>
<td>Montpellier</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MONDUR</td>
<td>LT</td>
<td>Distilled</td>
<td>10</td>
<td>Good</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Niverville</td>
<td>LT</td>
<td>VHT</td>
<td>18</td>
<td>High</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Therefore, the purpose of this work was only to investigate the ultrastructure of experimental spaghettli of different cooking quality by FF, thin-sectioning and SEM techniques and to relate some spaghettli microstructured observations with cooking behaviour.

Characteristics of experimental spaghettli

As shown in Table 1, the experimental spaghettli shows different cooking qualities. Even if the quantity of the main components present in semolina is recognized as an important factor in determining pasta cooking characteristics (Grzybowski and Donnelly, 1979; Matsuo and Irvine, 1970; Matsuo et al., 1972), the differences in quality tested on our samples after cooking are not related to the quantitative composition. Protein, starch and lipids contents were quite similar (Table 2) in the two semolinas.

Good cooking properties as determined by a sensory panel may provide by the use of good semolina (Mondur Montpellier-low temperature-MM) but, if not the case (Mondur Niverville-low temperature = MN), to improve it by inducing water acidity (MN-distilled water = MN-dw) or the use of very high temperature (MN-VHT) during spaghettli drying schemes.

Ultrastructure of experimental spaghettli before cooking

When we examined surface of dry spaghettli products by SEM, one observed the starch granules embedded into protein matrix (MM, Fig. 3a), the poor quality showing more pasty aspect (MN, fig. 3b). As stated by Angold (1979) starch and protein components are difficult to identify, a continuous film, probably of proteic nature enveloping and hiding the starch granules (Dexter et al., 1978).

Hydration of uncooked samples may introduce artifacts, especially in protein organizing, but this preparation step allows a better correlation with FF images (Chabot, 1979) and provides a clearer identification of spaghettli components (Resmini and Pagani, 1983). Hydration of uncooked spaghettli did not cause changes in starch ultrastructure (Matsuo et al., 1978; Resmini and Pagani, 1983) but promoted a compact protein structure in the good quality spaghettli (Fig. 3c) and porous protein structure in the poor quality one (Fig. 3d). A protein matrix showing the characteristics of the latter sample may certainly promote a quicker water absorption at the beginning of cooking.

Fig. 6. FF ultrastructure of uncooked MN poor quality spaghettli. a) after VHT drying; b) after LT drying. Arrows indicate probable starch-protein interactions (Fig. 6a) and water coat (Fig. 6b). (I) lipid inclusions; (P) protein matrix; (S) starch granule.
Fig. 7. Starch damage in MN, poor quality spaghetti. a) FF image; b) thin-section (uranyl acetate/lead citrate staining). (em) embedding medium; (p) protein matrix; (S) starch granule; (w) water.

Fig. 8 SEM images of experimental spaghetti after partial cooking. a) MN, poor quality spaghetti; b) MM, good quality spaghetti; c) MN-dw, good quality spaghetti; d) MN-VHT, high quality spaghetti. (p) protein matrix; (S) starch granule.
SOLUBLE LIPID CARBOHYDRATES

of good (MM) and poor (MN) spaghetti quality were less impressive than detect as well the interactions among protein subunits which may have formed during semolina kneading (Fig. 4). These images observed in FF micrographs (Fig. 4a). We could correlate these protein patterns with the SEM protein structures observed by Matsuo et al. (1978) in doughs of the same semolina kneaded with different water contents. Based on the images presented by these authors, we can assume that in poor sample (MN) the quantity of water normally used in kneading (about 30%) was insufficient for a complete gluten development, as opposed to what occurred in good quality products.

The exceptional cooking properties of MN-VHT spaghetti (Table 1) are related to a particular protein features (Fig. 5). As stated in the recent study of Resmini and Pagani (1983), high temperature drying promotes the coagulation of protein fractions into a continuous network, clearly visible in FF (Fig. 5a), TEM (Fig. 5b) and SEM (Fig. 5c) micrographs, and that prevents excessive starch swelling and subunits scattering during cooking. All three EM techniques pointed out the substantial compactness of MN-VHT spaghetti as well as a slower water penetration and a longer optimal cooking time (Table 1) are required. In FF micrographs (Figs. 5a and 6a) proteins took up the area where a water coat (at least 30 nm wide) generally surrounded the starch granules in a spaghetti dried at low temperature (Fig. 6a). These structures may represent the starch-protein interactions observed by Resmini and Pagani (1983) in spaghetti cooked after an oven treatment. The effects of this VHT diagram on cooking quality and ultrastructure and by Resmini's oven treatment were quite similar.

Cooking quality is also affected by starch behaviour (D'Egidio et al., 1983; Dexter and Matsuo, 1979; Resmini and Pagani, 1983). Enzymatic and/or mechanical damage to starch granules during pasta making induce negative cooking characteristics (Lintas and D'Appolonia, 1973; Matsuo et al., 1982). Therefore, the poor quality of MN spaghetti may be also related to damaged starch granules as suggested in FF (Fig. 7a) and thin-sectioning (Fig. 7b) micrographs when appear to have a solubilized inner fraction, the center part representing the most susceptible area for enzymatic digestion (Chabot, 1979; Gallant, 1974; Gallant and Guilbot, 1969).

**Table 2. Semolina composition (g/100 g d.m.)**

<table>
<thead>
<tr>
<th>SEMOLINA</th>
<th>PROTEIN (N x 5.7)</th>
<th>STARCH</th>
<th>AMYLASE (*)</th>
<th>ETHANOL-SOLUBLE CARBOHYDRATES</th>
<th>LIPID</th>
</tr>
</thead>
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(*) g/100 g of starch.

The same differences in the protein pattern of good (MM) and poor (MN) spaghetti quality were observed in FF micrographs (Fig. 4). These images were less impressive than SEM ones but we could detect as well the interactions among protein subunits which may have formed during semolina kneading. These interactions resulted much more extensively in the good quality product (MM, Fig. 4a). We could correlate these protein patterns with the SEM protein structures observed by Matsuo et al. (1978) in doughs of the same semolina kneaded with different water contents. Based on the images presented by these authors, we can assume that in poor sample (MN) the quantity of water normally used in kneading (about 30%) was insufficient for a complete gluten development, as opposed to what occurred in good quality products.

**Ultrastructure of experimental spaghetti after partial cooking**

Because of the physical competition between starch gelatinization and protein coagulation during cooking (Resmini and Pagani, 1983), shorter cooking times may give important information about the kinetics of these phenomena and may explain the cooking results. As shown by SEM in Fig. 8a, in the Inner part of poor quality spaghetti (MN) after only 5 minutes of cooking, the larger starch granules, which are believed to gelatinize at a lower temperature, already swollen while the protein matrix is not yet completely coagulated.

In all the good (MM, Fig. 8b; MN-dw, Fig. 8c) and high (MN-VHT, Fig. 8d) quality spaghetti, coagulated protein, promoted by native protein characteristics (Fig. 8b) or by high temperature treatment (Fig. 8d) or induced by water acidity (Fig. 8c), prevailed over gelatinized starch.

**Ultrastructure of experimental spaghetti after optimal cooking**

Cooking to the optimal cooking time clearly shows that there is competition for water to gelatinize starch and coagulate protein. Stickiness and absence of firmness exhibited by poor quality MN spaghetti (Fig. 9) are the consequences of starch swelling preferentially and not being prevented during continuous protein network formation as well as a slower water penetration and a longer optimal cooking time (Table 1) are required. In FF micrographs (Figs. 9a and 9b) proteins took up the area where a water coat (at least 30 nm wide) generally surrounded the starch granules in a spaghetti dried at low temperature (Fig. 9a). These structures may represent the starch-protein interactions observed by Resmini and Pagani (1983) in spaghetti cooked after an oven treatment. The effects of this VHT diagram on cooking quality and ultrastructure and by Resmini's oven treatment were quite similar.

Cooking quality is also affected by starch behaviour (D'Egidio et al., 1983; Dexter and Matsuo, 1979; Resmini and Pagani, 1983). Enzymatic and/or mechanical damage to starch granules during pasta making induce negative cooking characteristics (Lintas and D'Appolonia, 1973; Matsuo et al., 1982). Therefore, the poor quality of MN spaghetti may be also related to damaged starch granules as suggested in FF (Fig. 7a) and thin-sectioning (Fig. 7b) micrographs when appear to have a solubilized inner fraction, the center part representing the most susceptible area for enzymatic digestion (Chabot, 1979; Gallant, 1974; Gallant and Guilbot, 1969).
Ultrastructure of cooked spaghetti

Fig. 9. MN, poor quality spaghetti cooked in mineral water to the optimal cooking time. a) SEM image; b) FF image; c) thin-section (uranyl acetate/lead citrate staining); d), e) thin-sections (PATAg staining). At high magnification, alveoles (a) are seen in the swollen starch granule (Fig. 9d). When gelatinized starch is completely hydrolysed after alpha-amylolysis of the thin-section (Fig. 9e) it only remain grey alveoles-like structure (black arrows) which correspond to the embedding material that had penetrated the swollen granules. (em) embedding medium; (gs) gelatinized starch granule; (pn) protein network.
Fig. 10. TEM micrographs of experimental spaghetti cooked to the optimal cooking time. a) thin-section of MM good quality spaghetti; b) thin-section of MN-dw, good quality spaghetti; c) thin-section of MN-VHT, high quality spaghetti; d) FF image of MN-VHT, high quality spaghetti. In this kind of product we observe no alveoles rising (see Fig. 2c). On the contrary particular groupings and macromolecular arrangements are present inside each starch granule (arrows). All the thin-sections are stained with uranyl acetate/lead citrate. (em) embedding medium; (gs) gelatinized starch granules; (p) protein.
Ultrastructure of cooked spaghetti

Fig. 11 Thin-sections of cooked MN-VHT, high quality spaghetti after PATAg staining. a), b) show the starchy nature of the macromolecular arrangements. These groupings, despite contrasted as proteins in Fig. 10, are kept intact by pronase (c) and present a strong resistance to alpha-amylolysis (d). (gs) gelatinized starch granules; (pn) protein network.
thin-sections the nature of starch in these samples was dubious because staining revealed protein structure of similar contrast (Fig. 10a, 10b and 10c). Enzymatic hydrolysis using pronase (Fig. 11c) or alpha-amylose (Fig. 11d) clarified the origin of the macromolecular forms. Pronase caused a complete hydrolysis of the protein network (Fig. 11c, white area) leaving the starch granules intact. The starch exhibited an exceptional resistance to alpha-amylose digestion (Fig. 11d), typical of retrograded starch (Banks and Greenwood, 1975; Collison, 1968). Therefore, in all the good and high quality samples, starch had undergone some retrogradation, particularly after ultra high temperature treatment (Fig. 11d). Domovan et al. (1983) also observed new physico-chemical properties in starch after heat-moisture treatments and suggested the formation of new crystalline structures, even though they underwent no processing treatments.

**Conclusion**

Pasta quality can not be ascertained by determining the chemical composition or the ultrastructure of the uncooked product. Nevertheless the spaghetti ultrastructure before cooking is useful in determining changes occurring as a result of processing that would have a positive or negative effect on cooking quality (e.g. protein coagulation during high temperature drying, or starch damage during kneading and drying). Freeze-fracture techniques are useful in such studies. Thin-sectioning methods, even if interesting, may produce artifacts due to chemical fixation.

After cooking, pasta quality is characterized by two different ultrastructural models. In a sticky product, starch gelatinization overcomes protein reticulation. In contrast, in a firm spaghetti, proteins coagulate forming a continuous network around each starch granule. The SEM images allow one to observe growth of component changes. But the fine modifications of protein and starch during cooking, which are strictly related to pasta quality, can be appreciated only by a TEM technique, for example in studying the macromolecular aggregation inside the starch granules in good spaghetti detected by FF.

EM observations supply ideas of factors affecting pasta quality. However, in order to truly understand the relationship to component characteristics, one must also relate them to chemical, physico-chemical and physical evaluations.

**Acknowledgments**

The authors thank Dr. P. Feillet and J. Abecassis for samples availability, Dr. C. Mercier, Dr. P. Colonna and Dr. J.L. Doulbiere for their kind assistance in the biochemical and rheological data as reported in discussions with reviewers, and Mrs Chapeau for typing this paper.

**References**


**Ultrastructure of cooked spaghetti**


**Hansen LP, Jones FT. (1977).** A microscopic view of thermal processed wheat flour. J. Food Sci. 42, 1236-1242.


**Manser J. (1979).** High temperature drying of pasta products. Macaroni J. 60 (9), 30-32.


Discussion with Reviewers

E.A. Davis: Were the starch granules sensitive to electron beam damage for SEM samples, when using an accelerating voltage of 20 keV?

Authors: When accelerating voltage of 20 keV is used with very low current (as $10^{-12}$ to $10^{-13}$ ampere) starch granules are not sensitive to electron beam damage.

R.R. Matsuo: Since the chemical composition of the two samples is essentially the same, differences in cooking quality must be attributable to environmental effects. That is to say, the sample from Niverville must have been damaged, e.g. weathering, sprouting, damage by insects or by microorganisms. The quality characteristics of a pure variety do not change when grown under comparable conditions.

Why did the authors not choose a variety like Tomclair to represent a poor quality durum and a variety like Arcour or Agathe to represent a good quality durum?

Authors: The aim of this work was not to determine the wheat durum variety giving the best cooking quality or the best conditions of manufacturing such spaghetti. The aim of this work was only to correlate, using different and complementary microscopic techniques, the differences in the cooking quality of spaghetti as tested by sensory panels according to some differences observed in the organization of their components. We have chosen the spaghetti manufactured from only one wheat variety to be sure that the structural differences we observed were only due to parameters of production (low or high drying temperature) or to cooking parameters (pH of cooking water).

Apparently no change occurred in the chemical composition of wheat semolina from Montpellier and Niverville but starch granules of sample from Niverville were more damaged, as seen in figure 7, which could be due to environmental effect as weathering. For example, it was shown (Al Salen and Gallant, 1985) that there is significant difference between the vitreosity value of the same variety of durum wheat cultivated in different geographical regions. As a consequence, there is more chance that starch granules mechanically damaged in milling are due to that factor rather than sprouting or damage by insects or by microorganisms.

J. Jacobs: The drying schedule is not given for HT cycle and should be. Color and flavor can be very negatively affected. Just drying a product is not enough if end-product is unpalatable.

V.L. Youngs: What temperature were used in drying? The effects of VHT drying seem extreme.

R.R. Matsuo: The change in taste and color of the VHT spaghetti suggest a significant denaturation of components brought about by the uncontrolled high temperature.

Authors: Regarding VHT drying, the authors fully recognize that this kind of treatment cannot be used without further studies, particularly in the spaghetti processing. Although certain organoleptic properties such as color and taste were altered in the VHT spaghetti, this sample showed some exceptional rheological properties. For example, the index of General Viscoelasticity (IGV) as used by Alary et al. (1979) has also been determined on our samples: wheats Miradur-Niverville and Mondur-Niverville dried at 40°C and cooked in mineral water presented bad cooking quality and very low IGV, 1.4 and 2.8 respectively; on the contrary, Tomclair-Niverville and Mondur-Montpellier dried at 40°C and cooked in mineral water presented good cooking quality and higher IGV, 7.9 and 7.7 respectively. Exceptionally, Mondur-Niverville dried at VHT (higher than 100°C) and cooked in mineral water showed the best cooking quality and the best IGV (better than 10.0). According to these results, the authors thought that the texture of spaghetti MN-VHT might be bound to a special organization of its components and they decided to study the ultrastructure of one sample presenting such characteristics.

J. Jacobs: Table 1. Difference in cooking quality due to mineral vs distilled water seems extreme.

V.L. Youngs: Are data available on use of distilled water for cooking the MM as well as the MN samples? The effects of using distilled water on the MN samples seem extreme.

R.R. Matsuo: Why was the sample from Montpellier not also dried at VHT? Why was the Montpellier sample only cooked in mineral water? Is the Mondur-Niverville sample cooked in distilled water as good as the Mondur-Montpellier sample cooked in mineral water?

Authors: VHT drying is unusual. Therefore, it was not applied to all samples.

Cooking in distilled water, as demonstrated by Alary et al. (1979) and D'Egidio et al. (1981) improves the cooking quality of pasta but biochemical mechanisms of such changes are not yet completely known. They could be bound to changes in the acidity or ionic strength. This improvement is more evident when the quality of pasta is poor. For that reason we only cooked the poor quality sample (MN) in distilled water.

Mondur-Niverville (MM) sample cooked in distilled water was slightly stickier than Mondur-Montpellier (MM) sample cooked in mineral water and denotes a little higher porosity. Mondur-Montpellier sample dried at VHT (MM) is even stickier too. Note that pH of cooking water before and after cooking was 7.2 and 9.4 respectively for both MM and MN in mineral water, but only 5.5 and 6.5 respectively for MN in distilled water.

V.L. Youngs: The authors state the poor quality of MN may be related to damaged starch granules that appear. Why is there a difference? Do the authors have Falling Number data to indicate alpha-amylase activity in the original samples? This would be useful. Data from other objective measurements also should be included, such as firmness scores, cooking loss.

R.R. Matsuo: The implication of starch damage is questionable. Normal semolina contains a low level of mechanically damaged starch, about 10 Farrand units, higher if the level of fine particles is higher. If, as the authors claim, the level of damaged starch is high in the MN sample, it must arise from alpha-amylase degradation. If
Ultrastructure of cooked spaghetti

can is so then the wheat must have been moderately or severely damaged by sprouting. Information on the level of starch damage, amylograph viscosity or Falling Number would be most useful.

Authors: Amyloviscograms of semolina from MN were different according to the water (mineral or distilled) used. In distilled water at 96°C, consistency was higher (438 Brabender Units) than in mineral water (400 BU). But temperature, from which consistency starts to increase, was the same in both cases (87.5 °C).

Solubility of the MN starch granules in boiling water was lower (38.5%) in distilled water than in mineral water (43.6%).

Susceptibility of the starch granules to alpha-amylolysis showed also differences between MM and MN samples. The starch fractions easily digested resulting from the alpha-amylolysis curves was 9 and 13% for MM and MN semolinas respectively.

The starch fraction easily digested in spaghetti was 27.5% for MM, 40.0 and 48.0% for MN, respectively low temperature and VHT.

It is right that Falling Number would have been useful but has not been done.

R. R. Matsuo: No information on commercial sample is given, i.e. whether it was processed by low temperature or high temperature, whether the cooking quality is good or poor.

Authors: We are not commercial and the technology used is usually not labelled on manufactured products. What the microscopists can say concerning spaghetti in figure 1 is that structure of freeze-fractured proteins correspond to a pasta dried at low temperature.

J. Jacobs: Is not the cooking quality related to quantity of main components? How about protein quality? This point is not stressed. There is very limited data to draw such a conclusion.

Authors: Cooking quality is bound to numerous parameters, amongst them the protein rate. Below 10% proteins, characteristics of pasta are generally not good. But between 10 to 15% proteins, characteristics of pasta can be either good or bad. As in our case. Protein quality is also an important factor and is reliable to the variety of the cultivar. The best method to study such parameter is the electrophoretic analysis.

In our study all samples were prepared from the same variety. Electrophoretic outline must be the same for all the samples. But there are probably other parameters, unfortunately unknown (presence of specific groups in proteins) which act and give differences in cooking quality.

J. Jacobs: Extremely long cooking optimum (+ 80%) for HT dried sample makes one wonder about the severity of the drying cycle conditions.

Authors: Longer cooking time for VHT spaghetti is a consequence of modifications which occur during VHT drying, either at the level of the components or at level of their water content.
MICROSTRUCTURE OF MEALY AND VITREOUS WHEAT ENDOSPERMS (Triticum durum L.)
WITH SPECIAL EMPHASIS ON LOCATION AND POLYMORPHIC BEHAVIOUR OF LIPIDS

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Abstract

Dark inclusions observed in osmiophilic zones, already described in mature wheat endosperm using transmission electron microscopy, were confirmed by freeze-fracture electron microscopy to be lipids. The polar lipids (glycolipids, phospholipids, free fatty acids, monoglycerides) were organized in a liquid crystalline phase. The reversed hexagonal or HII phase should be the main lattice which might arise from the transition of lipids present in membranous structures as a lamellar phase. This transition was caused by dehydration occurring during maturation. It is suggested that the water-dependent lamellar hexagonal phase transitions are of considerable importance in cereal food technology.

Introduction

Wheat lipids, though present at a low level (about 2% on a dry basis), are known to play an important role in cereal food technology such as breadmaking (MacRitchie and Gras, 1973; Chung et al., 1978).

The determination of location and structure of lipids in mature wheat endosperm is of considerable importance to explain their roles in the functional properties of wheat flour doughs and gluten.

In regard to its resolving power, scanning electron microscopy (SEM) is not sufficient to study lipid structures in the endosperm, so that transmission electron microscopy (TEM) is the best available method for this purpose.

In fact, TEM investigations of mature wheat endosperm are very limited because of major technical difficulties. Graham et al. (1963) and Jennings et al. (1963) did not examine any material more advanced in maturity than the 25th day after anthesis. To improve resin embedding, Simmonds (1972) infiltrated samples up to several weeks in glycol methacrylate and Parker (1980) infiltrated for two weeks with Spurr resin, endosperms aged up to 40th day after anthesis.

In TEM, lipids are viewed as osmiophilic inclusions and bilayer structures (Seckinger and Wolf, 1967; Simmonds, 1972; Bechtel et al., 1982) but long infiltration time and other treatments might form artifacts (Crozet, 1977).

Numerous works in the area of biomembrane research have shown that freeze-fracture electron microscopy (FFEM) is the best method to observe native lipid structures (Cullis and de Kruijff, 1979; Quinn and Williams, 1983) inasmuch as no chemical fixations are needed. Fretzdorff et al. (1982) have used FFEM with success to describe the ultrastructures present in wheat flour, dough, gluten and bread.

In the work reported here, FFEM was used to investigate for the first time lipid structures in mealy and vitreous endosperms. In order to compare our results with previous works, the organization of storage components was also determined by SEM and TEM. The latter method was improved in regard to the infiltration time.

Key words: Freeze-fracture, lamellar hexagonal phase transition, lipids, protein matrix, scanning electron microscopy, transmission electron microscopy, vitreosity, wheat.
Materials and Methods

Wheat sampling

Two wheat varieties (Triticum durum L.), Wad 6292 and Mondur 1091 cultivated in the south of France and harvested in 1982 were studied. Wad 6292 is a vitreous variety (95 % vitreosity), and Mondur 1091 a mealy variety (47 % vitreosity) as determined by the method of Mauzé et al. (1972) modified by Al-Saleh and Gallant (1985). Water content 10% (dry basis) was measured according to the procedure of Multz et al. (1980). Samples for protein and lipid analysis were conditioned to 16% water content and milled to approximately 65% extraction on a Brabender Junior FL 1/4 ml.

Scanning electron microscopy

Grains were fractured transversally, mounted on copper stubs, and coated with a layer of gold (40nm) before observation in a JEOL JSM-50 A at 20 keV, absorbed current being less than 10-13 mA.

Embedding procedure and sectioning for TEM

Endosperm pieces measuring approximately 0.3 mm were cut from mature wheat grains with a razor blade under a zoom stereomicroscope. Samples were fixed, at room temperature, in 6% and 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h respectively. Then, they were post fixed for 1 h in the dark under low pressure (1 to 2 torrs) in 1% osmium tetroxide in 0.1 M cacodylate buffer. Samples were dehydrated in graded acetone series, transferred successively into pure propylene oxide, propylene oxide-Epon 812 (40nm) and finally into pure Epon 812 for infiltration. The infiltration lasted for 24 h. Samples were sectioned with a glass knife on a JEOL JUM 7 ultramicrotome. Silver sections were stained for 30 min in 2.8% uranyl acetate in 50% methanol at 48°C and for 4 min in lead citrate (pH 13). Sections were viewed in a JEOL 100S at 80 keV.

Freeze Fracture

Small samples of wheat endosperm (about 0.3 mm) were soaked in 90% glycerol-water solution (v/v) overnight. They were then freeze fractured transversally, mounted and studied in a Reichert cryo fracture apparatus, at -150°C and at 10-7 torr. Fractured samples were shadowed with platinum from a 45° angle and carbon from a 90° angle. The replicas were cleaned in 50% chromic acid overnight, washed in distilled water, picked up on 400 mesh copper grids and viewed in a JEOL 100S microscope at 80 keV.

Lipid analysis

Nonstarch lipids from 15.0 g flour were extracted twice with chloroform:methanol (2:1 v/v) and finally with cold water-saturated n-butanol (65:35 v/v) at room temperature. The crude extracts were washed according to the procedure described by Folch et al. (1957). Lipids were dried, weighed and separated by silicic acid column chromatography into neutral lipids, glycolipids and phospholipids by eluting with chloroform, acetone and methanol, respectively (Rouser et al., 1967). Neutral lipids were separated by analytical thin layer chromatography (TLC) on 0.2 mm thick silica gel 6 plates (Merck), and were viewed by spraying with 3% copper acetate in 8% orthophosphoric acid and charred at 180°C. Spot intensities were recorded with a Vernon densitometer. Glycolipid and phospholipid classes were separated by high performance liquid chromatography (HPLC) into their individual lipid classes according to the method described by Marion et al. (1984).

Protein analysis

Flour proteins were fractionated by Osborne's procedure into albumins and globulins, which were soluble in salt water (0.04 M NaCl); gliadin, soluble in 70% ethanol; and glutenin, the alcohol insoluble component. Protein contents were determined by the Kjeldahl method using a factor N x 5.7.

Results and Discussion

Organization of storage components in vitreous and mealy mature endosperms

Scanning electron micrographs of vitreous endosperm showed a continuous protein matrix covering the starch granules and giving a coherent physical structure to the endosperm (Fig. 1, arrowheads). On the contrary, the mealy endosperm exhibited a fragmented protein matrix with numerous air spaces (Fig. 2, arrowheads). These observations were confirmed by TEM of vitreous (Fig. 3) and mealy endosperm (Fig. 4). Especially in the case of mealy endosperm, large spaces were observed between starch granules and proteins (Fig. 4). Thus, vitreous endosperm was characterized by the compactness of the main components (starch, protein) which resulted from the continuity of the protein network. Our results were in accordance with those obtained with TEM by Stenvert and Kingswood (1977).

Figure 1. SEM of vitreous endosperm (Wad 6292), showing continuous protein matrix. Pm: protein matrix; S: starch.

Figure 2. SEM of mealy endosperm (Mondur 1091), showing fragmented protein matrix. Cw: cell wall; Pn: protein network; S: starch.

Figure 3. TEM of vitreous endosperm (Wad 6292), showing continuous protein network and osmiophilic zones. Cw: cell wall; Oz: osmiophilic zone; Pn: protein network; S: starch.

Figure 4. TEM of mealy endosperm (Mondur 1091), showing fragmented protein network and osmiophilic zones. Cw: cell wall; Oz: osmiophilic zone; Pn: protein network; S: starch.

Figure 5. TEM of vitreous endosperm (Wad 6292), showing vestige of endoplasmic reticulum. Cw: cell wall; Er: endoplasmic reticulum; Oz: osmiophilic zone; Pn: protein network.

Figure 6. TEM of vitreous endosperm (Wad 6292), showing stage of protein body formation. Pn: protein body; Er: endoplasmic reticulum.

Figure 7. TEM of vitreous endosperm (Wad 6292), showing protein body to which ribosomes were attached. Pn: protein body; R: ribosomes.

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Continuity of the protein matrix should be related to protein content; vitreous endosperm having a 15% protein content, whereas mealy endosperm has only 10% protein (Table 1). This relation has already been suggested by Stenvert and Kingswood (1977). The higher protein content in vitreous endosperm was mainly due to a higher gliadin content than in the mealy variety. Our protein compositions were quite different from those obtained by Nierle and Elbaya (1978) using other vitreous and mealy varieties which make it difficult to relate protein matrix continuity to protein composition.

Osmiophilic zones observed with TEM in the protein matrix were less numerous in the mealy endosperm than in the vitreous one. Rough endoplasmic reticulum (RER) and polysomes were observed in these osmiophilic zones (Figs. 5 and 6). For the first time, stages of protein body formation in mature vitreous wheat grains were highlighted such as: i) protein bodies enclosed in a spiral rough endoplasmic reticulum (Fig. 7), corresponding to cellular organelles similar to those already described by Bechtel and Juliano (1980) for the early stage of protein bodies formation in rice endosperm 7 days after anthesis; ii) protein bodies surrounded by a single membrane to which ribosomes were attached (Fig. 8); and iii) non-fused mature protein bodies (Fig. 9). A large number of polysomes and RER in vitreous endosperm suggested that an intensive protein biosynthetic activity had taken place during maturation, which might explain the higher protein content in the vitreous variety than in the mealy one. Non-fused protein bodies might be explained by grain dehydration which stopped coalescence of endosperm protein bodies. In addition to the cytoplasmic remnants, dark inclusions were observed (Figs. 3, 4 and 5) which have been suspected by many authors to be lipids (Seckinger and Wolf, 1967; Simmonds, 1972; Crozet et al., 1974; Crozet, 1977; Parker, 1980).

To our knowledge, no detailed work has been carried out on lipids in mature wheat endosperm, so that we have chosen to focus our investigations on the location and structure of these osmiophilic inclusions using FFEM.

Location and polymorphic behaviour of lipids in wheat endosperm

Photomicrographs of replicas obtained from freeze-fractured wheat endosperm showed different nonprotein structures located in large water areas or included in the protein matrix. Structures observed exhibited globular or tubular shapes (Figs. 10, 11 and 12). According to work on biomembranes (Cullis and de Kruifff, 1979; Quinn and Williams, 1983), surfactants (Krog, 1981) and fats (Rigler et al., 1983; Timms, 1984) it was obvious that nonprotein inclusions were formed by lipids. The structures composed of stacked tubes, the mean diameter of which was 6 nm (Fig. 12), were in accordance with those of amphiphilic lipids (glycolipids, phospholipids, free fatty acids, monoglycerides) organized in a hexagonal liquid-crystalline phase (Deamer et al., 1970; Van Venetie and Verkleij, 1981; Borovjagin et al., 1982). According to the work of Carlson et al. (1976) on the phase equilibria of extracted wheat flour lipids, this hexagonal structure was of reversed type (HII). In these structures a water channel was contained in each tube, with lipid arranged around this channel so that the polar head groups surrounded the water and the fatty backbone extended radially from the tube axis. The globular structures exhibited different kinds of fractures. In most replicas, only smooth vesicles measuring 20 to 300 nm were observed (Fig. 13), but in some cases, vesicles

<table>
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Figure 9. TEM of vitreous endosperm (Wad 6292), showing non fused protein body. Pb: protein body; Pn: protein network; S: starch.

Figure 10. Freeze fracture of vitreous endosperm (Wad 6292), showing lipid vesicles. L : lipid lamellar vesicle; Mlv: lipid multilamellar vesicle; V: lipid vesicle; S: starch; W: water.

Figure 11. Freeze fracture of vitreous endosperm (Wad 6292). H: lipid hexagonal phase; Pn: protein network; S: starch.

Figure 12. Freeze fracture of vitreous endosperm (Wad 6292). H: lipid hexagonal phase; T : tubes (arrows); V: lipid vesicle.

Figure 13. Freeze fracture of vitreous endosperm (Wad 6292). H: lipid hexagonal phase; S : starch; V: lipid vesicle.

Figure 14. Freeze fracture of mealy endosperm (Mondur 1091). Mlv: lipid multilamellar vesicle; S: starch; W: water.

Figure 15 a. Freeze fracture of mealy endosperm (Mondur 1091). Ga: lipid granular aggregate; Mlv: lipid multilamellar vesicle; V: lipid vesicle; W: water. b. Magnification of multilamellar vesicle of figure 15a.

Figure 16. Freeze fracture of mealy endosperm (Mondur 1091). Mlv: lipid multilamellar vesicle; W: water.
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showed laminations (Fig. 14) and multilamellar structure was noted in fractured vesicles (Fig. 15 a,b). The lamellar repeat distance was about 5 nm (Fig. 16) which was similar to that obtained for polar lipids organized in a lamellar liquid-crystalline phase (Deamer et al., 1970; Shipley et al., 1973; Hui et al., 1981). The vesicles which did not show lamellar structure might be oil droplets stabilized by mono- or multilayers of polar lipids. These vesicles might also be composed of polar lipids arranged in unilamellar or multilamellar structures.

It was noteworthy that vesicles were mainly observed in large water areas (Fig. 10), while tubes were located in protein matrix closely bound to starch granules, in a zone of low water content (Fig. 12). Moreover, the vitreous endosperm exhibited a structure more tubular than the mealy one. Mealy endosperm replicas, in the main, showed only lipid vesicular structures (Fig. 17 thick arrows). The composition of the main lipid classes did not highlight differences between vitreous and mealy varieties (Table 2). Moreover, TLC and HPLC chromatograms of mealy and vitreous endosperm lipids were quite similar. These results implied that lipid composition could not be responsible for the different polymorphic behavior of lipids in vitreous and mealy endosperms.

In fact, tubular structures were mainly observed in the central zone of replicas. Apparently the ratio of tubular to globular lipid structures decreased from the center to the outer area of the fracture face, so that only vesicles were observed in the outer area of vitreous endosperm replicas (Fig. 18). It is important to take into account the fact that 10% water was present in the cryoprotective medium which could diffuse into samples before cryofixation. Water diffusion was suggested by the fragmentation of the protein network near the outer area of the fracture face (Fig. 18, arrows) in comparison to the compactness of the protein network in the central zone (Fig. 11). Thus, the difference in the polymorphic behavior of lipids in vitreous and mealy endosperms may be related to the difference in the speed of water diffusion. The low diffusibility of water in vitreous endosperm might be due to the compactness of the protein matrix.

We believe that the non-hydrated mealy endosperm exhibited many more tubular structures than were observed in the rehydrated one. Attempts to decrease water in the cryoprotective medium did not allow us to obtain fractures of endosperms with the cryofracture device used in this work.

Thus the diffusion of water induced a transition of polar lipids from hexagonal to lamellar phases. Our conclusions are in accordance with the work of Carlson et al. (1978) which showed by X-ray diffraction that extracted wheat flour lipids exhibited a tubular structure below 15% water content and a lamellar structure between 15 and 50% water content.

The transition from hexagonal to lamellar phase has been studied by Vail and Stollery (1979). Many of their freeze-fracture photographs concerning the intermediate steps of transition were quite similar to what was observed in vitreous and mealy replicas, especially the granular aggregates surrounded by vesicles (Fig. 19). They suggested that the granular aggregates corresponding to an intermediate phase in the hexagonal to lamellar transition, were invaginations in lamellae, fusion of which produced the unilamellar vesicles covering the aggregates (Fig. 19). An association between a multilamellar vesicle, granular structure and smooth vesicles, which was not observed by Vail and Stollery (1979) during lipid phase transition, could be noted (Fig. 20). Water could be released into the aqueous phase from the hexagonal structure observed in both vitreous (Fig. 20) and mealy endosperm (Fig. 21). In some cases, all hexagonal to lamellar transition steps were observed (Fig. 22).

Thus, the dehydration and rehydration of wheat endosperm could produce morphological changes in lipids by inducing lamellar-hexagonal phase transitions. Such events were recently shown in model membrane systems (Crowe and Crowe, 1982; Gordon-Kamm and Steponkus, 1984). It was obvious that similar transitions occurred during certain physiological or technological processes. Water loss during maturation, in particular, might induce a lamellar to hexagonal phase transition, as suggested in lettuce seed (Toivio-Kinnucan and Stushnoff, 1981) whilst a hexagonal to lamellar phase transition might occur as seeds are soaked in water for germination. The lamellar structures observed by Simmonds (1972) in mature endosperm and by Bechtel et al. (1982) in endosperm, 28 days after anthesis, might arise from the hexagonal phases during the fixation steps carried out in an aqueous medium.

Table 2. Composition of lipids in vitreous and mealy endosperms (% dry basis and % total lipids in brackets)

<table>
<thead>
<tr>
<th>Variety</th>
<th>Total lipids</th>
<th>Neutral lipids</th>
<th>Glycolipids</th>
<th>Phospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wad 6292</td>
<td>1.84</td>
<td>1.14 (61.9)</td>
<td>0.47 (25.4)</td>
<td>0.23 (12.7)</td>
</tr>
<tr>
<td>Mondur 1091</td>
<td>1.59</td>
<td>1.00 (63.4)</td>
<td>0.40 (25.4)</td>
<td>0.18 (11.2)</td>
</tr>
</tbody>
</table>
Lipid Polymorphism in Wheat Endosperm

Figure 17. Freeze fracture of mealy endosperm (Mondur T091). Pn: protein network; V: lipid vesicles; W: water.

Figure 18. Freeze fracture of vitreous endosperm (Wad 6292), showing fragmented protein network. Pn: protein network; S: starch; V: lipid vesicle; W: water.

Figure 19. Freeze fracture of mealy endosperm (Mondur T091). Ga: lipid granular aggregate; V: lipid vesicle; W: water.

Figure 20a. Freeze fracture of vitreous endosperm (Wad 6292). H: hexagonal phase; Mlv: multilamellar phase; S: starch; V: lipid vesicle; W: water. b. Magnification of hexagonal phase of figure a.

Figure 21a. Freeze fracture of mealy endosperm (Mondur T091). H: hexagonal phase; Lv: lipid lamellar phase; Mlv: lipid multilamellar phase; V: lipid vesicle; W: water. b. Magnification of hexagonal phase of figure a.

Figure 22. Freeze fracture of vitreous endosperm (Wad 6292), showing lipid phase transition. Ga: lipid granular aggregate; H: hexagonal phase; Pn: protein network; S: starch; V: lipid vesicle; W: water.
Furthermore, such phase transitions might occur during dough development and in other steps of breadmaking technology. Kinetics of these transitions during dehydration or rehydration would bring new ideas on the role of lipids in physiological and technological processes.

Acknowledgements

The authors thank Dr. Y. Popineau (INRA, Nantes) for helpful advice and assistance throughout the analysis of wheat proteins. Many thanks are due to the Laboratory of Phytopathology (Faculty of Sciences, Nantes University) for assistance in freeze-fracturing. Gratitude is expressed to Mrs. A. Scherer and Mrs. M. Chapeau for typing this paper.

References


Lipid Polymorphism in Wheat Endosperm


Discussion with Reviewers

W.J. Wolf: Description of water content measurement is not clear. What is the significance of "10%"? Could it be deleted?

Authors: 10% is the water content of wheat samples before conditioning and is obtained by weighing wheat sample after heating at 133°C for 90 min. in an isothermal oven (French standard V 03-707).

W.J. Wolf: You state that the cryoprotectant contained 10% water but what was the water content of the wheat before and after exposure to the cryoprotectant? I suppose that it would be difficult to determine the water content after exposure to the cryoprotectant, but it might be interesting to start with wheat samples of varying moisture contents and see whether there is a moisture content that is in equilibrium with a 10% water content in the cryoprotectant. At equilibrium you should have a uniform structure from the exterior to the interior of the samples if your explanation of the effects of water diffusion is correct.

Authors: It would be effectively important to determine water and also glycerol content of wheat after exposure to the cryoprotectant but these determinations technically difficult in regard to the small size of endosperm samples used for freeze-fracture, should not allow to appreciate the water gradient from the exterior to the interior of the endosperm specimen. We agree with you that equilibrium studies of water between cryoprotectant medium and wheat sample would allow to assess our hypothesis on the effects of water diffusion. However our first aim is to improve our freeze-fracture device in order to get replicas from noncryoprotected material. As a matter of fact it should be mentioned that glycerol is used only as a binding medium between sample and copper sheets to ensure a good fracture. In this low water sample cryoprotectant is not necessary because free water is not present and consequently, ice crystals cannot grow. Without use of cryoprotectant, it will be possible to assess our hypothesis on the effects of water diffusion and also to appreciate the ratio between lamellar and hexagonal structures in the native state.

D.D. Christianson: Is the total lipid extraction of the vitreous endosperm complete?

Authors: Our procedure is only able to extract nonstarch lipids so that total lipid extraction of wheat endosperm is not complete. However, concerning nonstarch lipids it is our own experience on freeze-dried gluten that this procedure allows extraction of at least 95% of total lipids on a fatty acid methyl ester basis.

D.D. Christianson: Are both these wheat varieties of breadmaking quality?

Authors: These varieties are not used in breadmaking technology but only in pasta and semolina technology.

D.D. Christianson: What moisture level can be handled in the sample and still accomplish FFEM?

Authors: Theoretically FFEM may be carried out on samples whatever the moisture content but some technical difficulties arise at low water content (5 to 15%). Above 20% cryoprotectant must be added to avoid ice crystal growth.

D.B. Bechtel: Glycerol, being polar, will have a pronounced effect on endosperm lipids. How do you know that the structures you are looking at are not glycerol derived?

Authors: Concerning the hexagonal structures they are mainly observed in zones in which water has not diffused and consequently in zones without glycerol. Moreover it is known that glycerol and polyol sugars stabilize lamellar structure in medium of low water activity (Crowe et al., 1984 Biochim. Biophys. Acta. 769, 141-150.) so that we think that hexagonal structures are not glycerol derived.
Concerning the transition from the hexagonal to the lamellar phase we obtained the same FFEM micrographs than those of Vail and Stollery (1979) who did not use glycerol. Moreover kinetics of this transition during hydration of wheat flour were recently carried out in our laboratory by one of us (D. Marion, personal communication) and show that, during the first hours, hexagonal structures disappear while granular aggregates and lamellar structure (mainly vesicles) appear. After 4 hours only vesicles are seen. In this experiment, glycerol was added only just before cryofixation so that we think that water was mainly responsible for this transition and not the glycerol.

D.B. Bechtel: How can you identify large water areas depicted in Figs. 10, 14, 15a, 16, 17, 18, 19, 20a, 21a and 22 as such when water content is 10% and no etching was conducted ? I believe what you identified as water is in actuality storage protein matrix. In addition, the vesicles you describe are not true vesicles but protein matrix inclusions.

Authors: Water areas are identified by comparison with the replicas of the cryoprotective medium. The water areas are due to the diffusion of the water contained in the cryoprotectant leading to the disruption of the continuity between starch and protein matrix and between lipids and proteins. These water areas are not present in the native state at low water content (10%). During this water diffusion some vesicles embedded in the protein matrix have been excluded in these water areas. This phenomenon has been observed during hydration of wheat flour and extraction by handwashing of wheat gluten (D. Marion, to be published).

D.B. Bechtel: The most glaring problem with the interpretations is the assignment of the various classes (types) of lipid to various structures. For example, Ga: Lipid granular aggregate, Mlv: Lipid multilamellar vesicle, V: lipid vesicle. As these have not been isolated and characterized either by chemical and/or cytochemical methods, there can be no basis for such conclusions.

Authors: These lipid structures have been described by many authors in native and model membrane systems (see the review of Cullis and De Kruijff (1979) and of Quinn and Williams (1983); the original work of Gulik-Krzywicki et al. (1984) in: Surfactants in Solution (K.L. Mittal and B. Lindman, Eds), Plenum Press, New York, 1, 237-258). The authors have additional EM information showing if the periodicity of this phase always is the same as in Fig.19 or if the repeat distance can vary?

Authors: The periodicity of this phase is almost the same in these granular aggregates and the repeat distance is comprised between 12 and 13 nm, value consistent with a cubic phase such as this one observed by Gulik-Krzywicki et al. (1984).

E.A. Davis: Do you believe that the functional lipid of certain flours may be affected by the participation of water in lipid phase changes in preference to hydrating the starch and wheat proteins of flour?

Authors: To our knowledge this problem has not yet received attention and will have to be considered in the future.
ULTRASTRUCTURE OF MAIZE STARCH GRANULES. A REVIEW
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Abstract
History of starch granule ultrastructure and the principal data obtained on maize starch
granules are analyzed. New results are developed: i) growth and development of the maize starch
granules during maturation depend on the maize varieties and the tissue site in the kernel,
especially the horny and floury endosperms; ii) cytochemical studies of the starch granules differ­
ing from their amylose/amylopectin ratio show important differences in the distribution of
their crystalline and amorphous zones that explain their behaviour under some hydrolytic
reagents; iii) complexing between colloidal gold labelled Concanavalin-A and starch
(amylo­
pectin) permits new and greater specificity to ultrastructural study of the starch granule.

History of the starch granule ultrastructure
Historically, the understanding of starch granule ultrastructure is deeply connected to the
development of electron microscopy techniques and microscopes (Gallant, 1974). Studies and results
which are summarized in some papers (Gallant, 1974; Gallant and Sterling, 1976; Nikuni, 1978;
Hood and Liboff, 1982; French, 1984) can be divided into three principal periods:
1) The replica period: From 1950 to 1960 most of
experiments on the starch granule ultrastructure
were limited to making surface replicas of com­
cmercially purified starch (Kore-Eda and Nikuni,
1955; Whistler et al., 1955) or internal starch
granule replicas after sectioning (Whistler and
Turner, 1955; Nikuni and Hizukuri, 1957; Whistler
and Thornburg, 1957; Whistler et al., 1958). The
starch granule surface basically appeared smooth
with occasional indentations of small starch
granules or protein bodies in the hard endosperm.
Internally, very fine granular structures (micro­
granules of 20 to 30 nm diameter) with occasional
central cracks were seen. Using similar techni­
quies, Sterling and Spit (1957) provided evidence
for the structural compositions of the intercellular
fibrils and the surface papillae (about 20 nm
diameter) which were believed to be the surface
end of the internal microfibrils.
2) The sectioning and contrasting period: From
1960 to 1980 workers used the degradative treat­
ments introduced by Buttrose (1960) in the study
of the starch granule with mild acid or enzymatic
(germination) treatments. These treatments were
used to improve the in vivo or in vitro starch
granule susceptibility and ultrathin sections of
the degraded starch granules were studied. Nei­
ther Buttrose (1960), nor Mussulman and Wagoner
(1968) used carbohydrate contrasting. Although
the general results were of some interest, the
resolution seen in the micrographs remained very
poor. It was, in fact, impossible to discern the
ultrafine structure of the starch granule except
for what remained after hydrolysis.
Elsewhere, acid treatment was combined with
the use of potassium permanganate (Innamorati,
1966), or chromic acid (Hülzl and Bancher, 1965).
Potassium permanganate at low pH, or chronic acid
reacted as oxidants of the primary alcohol of
alpha-D-anhydroglucose units and the precipi­

Key words: Starch granule ultrastructure, amylomaize, waxy and normal maize, corn, scanning
electron microscopy, transmission electron micro­
scopy, histochemistry, lectins, Concanavalin-A.
tation of Mn or Cr gave rise to a high contrast in the residual fraction of the hydrolyzed starch granules.

During this period, the chemical procedures for chelating oxidized cellulose were introduced by Edel (1962) in the textile field. Radioactive metals fixed by the alpha-glycols were chelated stoichiometrically and analyzed quantitatively, after specific oxidation of the C2-C3 secondary alcohols and thiosemicarbazide fixation. Such procedures were developed for electron microscopy of carbohydrates and the starch granules under the periodic acid-thiosemicarbazide-silver reaction (PATAG) which used silver nitrate as the contrasting reactant (Gallant, 1974; Gallant et al., 1972, 1973; Gallant and Guilbot, 1969a, b and 1973; Gallant and Sterling, 1976; Kassenbeck, 1975, 1978).

Also, chemical reactions were used to locate carbohydrates and starch granules on ultrathin sections and were developed by: i) the fixation of plumbite HPD0, (Gallant, 1974) via hydrogen bonding according to Karnovski (1961) in very alkaline medium; ii) the fixation using cesium butylate (Gallant et al., 1967; Gallant, 1974) according to Hagège (1967) via the free secondary alcohols R-OH of alpha-D-anhydroglucose units being substituted with cesium butylate (C6H13OCs). Such reaction with alkaline alcohohates was confirmed by Mentrè (1972) on starch granules and other polysaccharides using the thallium ethanolate C2H5OTl as a reactant; iii) the fixation of iodine as insoluble lead iodine (PbI2) for starch granule studies. The starch granules appear to staining. The starch granules appear to con­trast in TEM i) several ways can be used for nonspecific and specific detection of starch granules and other polysaccharides on ultrathin sections; ii) ultrastructural studies of starch granules require acid, enzymatic or/and oxidative treatments of the whole granules prior to staining. The starch granules appear to consist of alternate rings of crystalline and amorphous materials; iii) the macromolecular structure is controversial with some workers convinced of the granular-like units (Gallant, 1974; Duprat et al., 1980) and others (Kassenbeck, 1978; Yamaguchi et al., 1979; French, 1984) suggesting that the fine structure is a periodic organisation of superimposed clusters (amylopectin) radially oriented according to the model of amyllopectin hypothesized by biochemists (French, 1975, 1984; Robin, 1976).

3) The scanning electron microscopy period: Since 1970 and the Interesting paper series of Hall and Sayre (1969, 1970a, b, 1971a, b, 1973), Evers (1969, 1970, 1971), Evers and Molcereff (1970), and Evers et al. (1971), numerous papers on the SEM of starch granules have been published on cereal (wheat, barley, maize, sorghum, and rice), tuber and legume starches, especially after enzymatic, physical, chemical and hydrothermical treatments. These studies have shown: i) a good correlation between SEM micrographs of the starch granule surfaces and micrographs of native granule replicas; ii) very useful three-dimensional views of the degradation by canals of corrosion; iii) very important structural differences between starch of different origins; and iv) very close correlation between SEM and TEM studies (Gallant, 1974; Gallant and Guilbot, 1973; Gallant et al., 1973).

Maize starch granule morphology and development

The structures of starch and the protein matrix were studied in the soft and hard endosperms of normal and opaque-2 maize kernels with SEM using a modified, natural drying process (Robutti et al., 1974). Starch from the floury and horny endosperm differed in shape; polygonal and tightly packed starch granules showed indentations made by zein bodies embedded in the protein matrix of the hard normal endosperm whereas soft endosperm contained intergranular air spaces, particularly in opaque-2 maize kernels and round starch granules.

These fundamental differences can be generally observed in all cereal seeds containing hard or soft endosperms, as shown in Figure 1 on waxy, normal maize and amylo maize. The differences are demonstrated by the heterogeneous shape and the smaller size of amylo maize starch granules and, in particular, by the weakness of the larger waxy maize starch granules in horny endosperm.

Physico-chemical characteristics of normal (24% amylose) and amylo maize (38 and 64% amylose) starch granules, A76, A62, and A36, respectively, have been followed during their formation and maturation (Mercier et al., 1970). Amylose content increased from the beginning of grain formation up to the 35th day after anthesis. Two weeks after anthesis, both A76 and A62 exhibited type A X-rays spectra, but A36 was typical of type B spectra. Amylomace A62 shifted to type B spectra around the 22nd day after anthesis. Starch granule shape and size from floury endosperm was studied too. Normal maize starch granules increased in a regular manner in size and a new population of small granules appeared on the 72nd day after anthesis. Amylomazes (A62 and A36) showed two populations of starch granules, spherical and filamentous.

We observed that in commercial amylo maize, the greater the amylose content in the starch, the greater the number of filamentous granules. Starch formation was studied during maturation (Mercier et al., 1970). The percentage of irregular starch granules in the 38% amylose increased between the 22nd and 29th day after anthesis and decreased by the 72nd day after anthesis. Inversely, the percentage of irregularly shaped starch granules in the 64% amylose, mostly filamentous in shape, increased continuously up to the 72nd day after anthesis (Fig. 2).

A comparison between the size and shape of starch granules in hard and soft endosperms was studied by Gallant (1974). As seen in Figure 3, 6
Ultrastructure of maize starch granules

Fig. 1. SEM of maize starch granules. a) waxy maize in floury and b) in horny endosperms; c) normal maize in floury and d) in horny endosperms; e) amylomaize in floury and f) in horny endosperms. cw: cell walls; fs: filamentous starch granules; i: zein bodies Indentations; zb: zein bodies.
**Fig. 2.** Morphological evolution of maize starch granules in floury endosperms of normal maize (A76) and amylomaizes (A62 and A36) during maturation from 15th to 72nd day after anthesis as seen under polarized light. Note that filamentous starch granules of the amylomaizes show birefringency (Maltese cross) only from part to part in some nucleations. Percents are amylose contents during maturation.
Fig. 3. Histograms of the starch granule size in horny (black) and floury (white) endosperms during maturation. a) normal maize (24% amylose); b) amylomaize A62 (38% amylose); c) amylomaize A36 (64% amylose). Class interval: 2.2 μm. Heavy numbers outside histograms: kernel hydration. Irregular filamentous starch granule content is indicated by numbers inside histograms. f: frequency.

Maize starch granule ultrastructure

Approximately ten years ago, SEM confirmed that in vitro alpha-amylolysis of commercial starch granules led to somewhat damaged granules and that the pattern of degradation was characteristic of each species (Gallant et al., 1973). SEM also was used for nutritional studies. It was used to study breakdown of maize starch granules by microflora in the digestive tract of chicken (Champ et al., 1981). In vitro evidence showed that Lactobacillus strain 207 hydrolyzed normal maize starch granules less than strain 220 did. A more extensive degradation (pin holes and internal cavities) was seen when starch was included in the feed indicating some mechanical damages due to processing (pelleting). No damage appeared in starch granules obtained from the crop of axenic (germ free) chicken. Maize starch granules drawn from the crop of holoxenic (conventional) and monoxenic (only one microorganism) chicken were scarcely damaged and erosion was always superficial.
When treated with porcine (hog) pancreatic juice (Gallant et al., 1973), normal and waxy maize starch granules showed numerous rapidly developing pin holes randomly distributed on the starch granule surface, giving internal saw-tooth patterns inside the granules. Differences were observed mainly in the hydrolysis rate. Normal maize starch granules showed random endocorrosion, the radial rate of degradation being faster than the tangential one. Waxy maize starch granules showed random endocorrosion too; however, the radial degradation rate was slower than the tangential one. Starch granules stained after mild periodic oxidation were studied by TEM showing that in both cases a good correlation exists between zones which are most susceptible to amylolysis and those that are the less crystalline layers.

Amylomaize (amylose extender) starch granules treated with porcine pancreatic juice did not show such structure (Gallant et al., 1973). Using SEM, no evidence of exocorrosion occurred with granule surfaces remaining almost completely smooth except in some points where the enzyme had penetrated. These points were seen to have narrow pin holes surrounded by circular protuberances, crater-like. Evidence of internal hydrolysis having taken place could be seen only by TEM. Such SEM observations were in agreement with Knutson et al. (1982) who noted that the mode of attack by hog pancreas alpha-amylase on amylomaize V and VII was quite different from that on dent corn starch. For example, instead of typical corrosion type effects in dent corn, they only observed roughening of amylomaize granule surfaces. Beneath the surface protuberances, amyllose susceptible material was more or less digestible with the radial degradation being greater than the tangential degradation (Gallant et al., 1973). Nevertheless, this effect was not considered similar to normal and waxy maize starches with the shell organization being less pronounced or absent in case of amylomaize.

SEM of normal, waxy and high-amyllose maize starch granules was studied by Takaya et al. (1978) using various strains of alpha-amylase (crude powder of Streptomyces hygroscopicus, Aspergillus oryzae Alpha-amylase, Rhizopus anagaskiensis glucoamylase and Streptomyces hygroscopicus alpha-amylase). Normal maize starch granules were also studied after treatment with Streptomyces precox alpha-amylase (Takaya et al., 1979). As previously reported (Evers and McDermott, 1970; Gallant et al., 1973; Gallant, 1974), amylase attack started with small pits on the granule surface. With time the pits increased in number and size, and pores penetrated toward the center of the granules. Streptomyces precox alpha-amylase seemed to hydrolyze starch granules better than other alpha-amylases (Takaya et al., 1979).

Using Rhizopus glucoamylase or crystalline bacterial alpha-amylase, Fuwa et al. (1978a, b) found a higher resistance in the granule of high-amyllose maize starches showing shapes and surface similar to the native granules, but other maize starch granules presented the same pin holes already described.

On the other hand, Fuwa et al. (1978a) studied comparative susceptibility of several single endosperm mutants and their double-mutant combinations with opaque-2 in four inbred lines of maize, giving internal hydrolysis having taken place could be seen only by TEM. Such SEM observations were in agreement with Knutson et al. (1982) who noted that the mode of attack by hog pancreas alpha-amylase on amylomaize V and VII was quite different from that on dent corn starch. For example, instead of typical corrosion type effects in dent corn, they only observed roughening of amylomaize granule surfaces. Beneath the surface protuberances, amyllose susceptible material was more or less digestible with the radial degradation being greater than the tangential degradation (Gallant et al., 1973). Nevertheless, this effect was not considered similar to normal and waxy maize starches with the shell organization being less pronounced or absent in case of amylomaize.

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D.J. Gallant and B. Bouchet
Ultrastructure of maize starch granules

Assuming that the stains proposed by Kassenbeck (1978) were not really specific for amyllose and amylopectin, preliminary evaluations by Miller et al. (1984) and Bouchet et al. (1984) suggest that using lectins as a tool will soon permit greater specificity to such studies.

Miller et al. (1984) treated glycol methacrylate sections of cereal grains with *Lens culinaris* agglutinin (LCA) labelled with fluorescein isothiocyanate (FITC), and observed them under UV illumination (450 - 490 nm/520 nm). When applied to oat grain sections, LCA-FITC stained only the compound starch granules. When applied to wheat starch sections, the shell organization of lenticular starch granules was clearly seen and an intense fluorescence of the central core of the smaller starch granules was observed.

Bouchet et al. (1984) studied the specificity of *Canavalia ensiformis* agglutinin, namely Concanavalin-A (Con-A), towards maize starch granules (amylo maize, normal and waxy maize with an amylopectin content of 30, 76 and 99%, respectively). Con-A interacts (Fig. 5) only with the non-reducing, end chain groups of alpha-D-glucosides, alpha-D-mannosides, alpha and beta-D-fructofuranosides, by coupling with the C3, C4 and C6 hydroxyl groups; and with the C2, C3, C5 hydroxyl groups of alpha and beta-D-arabinofuranosides (Goldstein, 1972, 1976). Reaction occurs only when Con-A is in its tetrameric form. Complexing was achieved for light microscopy with dichlorotriazinyl-amino-fluorescein, the starch suspension being observed under blue light illumination (400/530 nm). For TEM, colloidal gold labelled Con-A according to Hörisberger and Rosset (1977) and Hörisberger (1979) has been used. The following are new TEM results:

New approach on starch granule ultrastructure
Fig. 5. A1: diagram of the linear and helicoidal amylose macromolecule (after Nikuni, 1978); A2: the amylose macromolecule which contains about 2,000 anhydroglucose units shows one reducing end chain group (c, Fig. A1) and only one non-reducing end chain group (e, Fig. A1); B1: diagram of the grape-like clustering of branch points amylopectin macromolecule (after French, 1972); B2: the amylopectin macromolecule which contains 100,000 to 1 billion anhydroglucose units, shows only 1 reducing end chain group (c, Fig. B1) but 10,000 to several million non-reducing end chain groups (e, Fig. B1). Note that the molecular configuration with the 3 hydroxy-groups allowing Con-A coupling is only shown by the non reducing end chain groups (amylose and/or amylopectin).

In the inset are the oligosaccharides (hexapyranosides and furanosides) reacting with Con-A. C1: alpha-D-glucose; C2: alpha-D-mannose; C3: alpha-D-arabinofuranose; C4: beta-D-arabinofuranose; C5: alpha-D-fructofuranose; C6: beta-D-fructofuranose. Specificity of Con-A is shown too for the non reducing end chain groups of their polymers.

Inset symbols represent: □ (CH2), ○ (OH groups) not involved and ● (OH groups) involved in the coupling reaction with Con-A.

i) Colloidal gold labelled Con-A can be used specifically to reveal Con-A binding sites on the whole starch granule but after chemical or enzymatic treatments only (Bouchet et al., 1984).

ii) Contrary to LCA binding as shown by Miller et al. (1984), the reaction between Con-A and the whole starch granule was negative (Bouchet et al., 1984), possibly because the starch granule surface does not have free non-reducing end chains. It must be remembered that Miller and co-workers treated thin-sections with C LA and not the whole granules. That is confirmed by the strong surface reaction we obtained after mild periodic oxidation of the whole granule (Figs. 6a, 9b and 10b). Such release of the non-reducing end chains was also observed after alpha-amyolysis of the whole granules Figs. 7, 8a, 9a and 10a) but the intensity of coupling was always lower than that after periodic oxidation.

iii) Whole amylomaize starch granules always showed weak coupling. Their high amylose content confirmed interactions between Con-A tetramers and non-reducing end chains groups. Theoretically, as shown Figure 5, there was single coupling in the case of the amylose macromolecule (DP around 2,000 anhydroglucose units), but possibly ten thousand to several million couplings in case of the amylopectin macromolecule (DP reaching 100,000 to one billion anhydroglucose units). Such giant macromolecules which are of a crystalline, structured conformation, then must be fully closed; their external chains which may react with lectins are consequently those which can be released after mild treatments.

iv) Surface oxidized amylomaize starch granule reacted with Con-A, although the reaction was weak. The resistant surface membrane previously described (Gallant et al., 1973) contains sites of alpha-D-glucose which may be sites penetrable by alpha-amylase.

Fig. 6. ConA-Au coupling of waxy maize A99 (99% amylopectin) starch granule after mild periodic oxidation of the whole granule. a) very dense reaction on starch granule surface showing gold particles (arrows); b) check sample of same starch after saturation of Con-A solution with 0.1M aqueous glucose solution. em: embedding medium; s: starch granule; arrows: gold particles.

v) After mild periodic oxidation of whole normal and waxy maize starch granules, coupling did not occur inside the granule (Bouchet et al. 1984). Loss of the crystalline structure on macromolecules at the granule surface may allow better accessibility of the amylopectin non-reducing end chains to Con-A.

vi) Binding can be achieved with thin sections (Fig. 7, 8b, 9c and 10c) of starch granules which were treated with alpha-amylase. We observed gold labelling on the whole sections of waxy (Fig. 7 and 8b), normal (Fig. 9c) maize, and amylomaize (Fig. 10c) starch granules, and stronger reaction at levels of less crystalline layers (Fig. 7). It is interesting to note that waxy and normal varieties reacted as well as amylomaize. As for histochemical reactions, variations in binding seem more selective on the whole granule than that on thin sections of the starch granules.
D.J. Gallant and B. Bouchet

Fig. 7. ConA-Au coupling achieved with thin section of waxy maize starch granule which was treated with alpha-amylase.

Fig. 8. ConA-Au coupling of waxy maize A99 (99% amylopectin) starch granule. a) coupling of the whole granule after partial alpha-amylolysis; b) coupling of thin section of granule partly digested by alpha-amylase. Same abbreviations as Fig. 6.

Fig. 9. ConA-Au coupling of normal maize A76 (76% amylopectin) starch granule. a) coupling of the whole granule after partial alpha-amylolysis; b) after mild periodic oxidation of the whole granule; c) coupling of thin section after partial alpha-amylolysis of the whole granule. Same abbreviations as Fig. 6.
Ultrastructure of maize starch granules

Fig. 10. ConA-Au coupling of amylo maize A30 (30% amyopectin) starch granule. a) coupling of the whole granule after alpha-amyolysis. From stars to periphery there is the peripheral part resistant to amyolysis (after Gallant et al., 1973); b) after periodic oxidation of the whole granule; c) coupling of thin section after partial alpha-amyolysis of the whole granule. Same abbreviations as Fig. 6.

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Ultrastructure of maize starch granules


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**Acknowledgments**

Many thanks are due to Mrs Martine Chapeau for typing this paper.

**Discussion with Reviewers**

P. Resmini: Do the authors include in the term "Replica" also the freeze-fracturing preparations?

Authors: In the term "replica" we only included replicas of whole granule surface as usually performed in the years before 1960 (see Gallant, 1974; Gallant and Sterling, 1976). A collodion or plastic replica was made first, and then was shadowed with heavy metal, either before or after removing the biological material, according to the different workers.

Freeze-fracturing methods were developed mainly after 1960 and used first by Mühlethaler (1965) for ultrastructural study of the starch granule.

P. Resmini: Does the amylomaize starch exhibit crystalline or amorphous properties?

Authors: As noted in this paper, and contrary to normal and waxy maize starch granules which show A-type spectra X-rays, mature amylomaize starch granules show B-type spectrum X-rays (Mercier et al., 1970).

Nevertheless, under polarized light, round amylomaize starch granules exhibited very clear Maltese crosses, whereas the filamentous ones never exhibited such birefringency (see Fig. 2).

P. Resmini: What were the conditions for alpha-amylolysis?

Authors: Ultrathin sections were incubated 24h (37°C) with a 0.01% solution bacterial alpha-amylase (from Bacillus subtilis, Sigma) in saline phosphate buffer (PBS) 0.01M (pH 7.2) containing 0.01% NaN3.

P. Resmini: In some Figures (8b, 9c and 10c), clumps of gold particles are visible. Do these areas correspond to particular aggregates of starch material (perhaps areas with high density of amylpectin molecules)?

Authors: We cannot say what the clumping of gold particles means as it was seen on previous photomicrographs. Since the review, we improved the coupling technique we used. Photomicrographs 7, 8a, 9c and 10c are new micrographs, showing better, more intense couplings than the ones which were originally submitted to the reviewers.

Clumping of gold particles may be areas showing higher density of amylpectin macromolecules, but may also be related to more numerous free non-reducing end chains. Now we are working on this reaction in order to understand better what happens at level of the starch granule section.

P. Resmini: Did the authors study the labelling with lectins on gelatinized starch granules and if so, is this possible without amylolysis or periodic oxidation?

Authors: As indicated in our paper (Bouchet et al., 1984), such a study was planned but has not yet been completed using colloidal gold labelled Concanavalin-A. We only tried to label swollen maize starch granules (normal, waxy maize and amylomaize) using the fluorescent (FITC) labelled Concanavalin-A. Reaction was negative with all these swollen starches.

S.H. Yiu: Con-A binds materials that contain free alpha-D-glycosyl and/or alpha-D-mannosyl side chains. In your opinion, can the Con-A-gold conjugate be used as marker for intact starch granules?

Authors: Theoretically, when intact starch granules contain free alpha-D-glycosyl side chains, coupling might occur. Unfortunately that has never been seen in our experiments.

We can consider the "grape-like clustering of branch points with long and short chain portions" which has been proposed by biochemists (Rolli ns, 1985) as the most probable model for amylpectin. However, because granules did not react without additional treatment, such macromolecular conformations must be very close at the surface of native starch granules. Then, the "blocklets", already shown by electron microscopists as real structural units could explain this lack of reactivity and must be taken into account in any serious hypothesis of the native starch granule ultrastructure.

Only some treatments are able to increase the porosity of the starch granule, thereby exposing the structural units, producing the release of non-reducing end chains and increasing number of end chain groups being able to react. Thus, in our opinion, Con-A-gold conjugate cannot be used as EM marker for intact starch granules.

Experiments have shown that coupling might occur in thin-sectioning starch granules because, when such closed structures are cut and then opened, more non-reducing side chains are released and more non reducing side chain groups are stoichiometrically free.
S.H. Yiu: Your findings show that oxidation or enzymatic hydrolysis is required for the binding between Con-A-gold and starch to take place. Have you observed binding of Con-A-gold, to mechanically damaged (due to processing) starch granules?

Authors: Although not yet investigated, it seems likely that damaged starch granules could react, at internal fissures due to processing, as well as with sectioned starch granules.

S.H. Yiu: How do you relate your findings to the ultrastructural organization of maize starch granules?

Authors: It is somewhat premature to discuss ultrastructural organization of maize starch granules in relation to the new findings using Con-A-gold. Multiple treatment combinations are now under investigation and results appear partly contradictory with what we knew before the experiments about starch organization.
MUCILAGE IN YELLOW MUSTARD (BRASSICA HIRTA) SEEDS

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Abstract

Release of mucilage from yellow mustard (Brassica hirta, also known as Sinapis alba) seed coats (hulls) was studied by optical and scanning electron microscopy. Micrographs were obtained of the mucilage which had exuded from briefly moistened seeds and dried subsequently in the form of small droplets on the seed surface.

The mucilage collected from the seed surface and mucilage isolated on a larger scale from seed hulls was hydrolyzed with sulfuric acid and the hydrolyzates were analyzed for sugar composition. Galactose, glucose, and galacturonic acid were found to be major components and mannose, arabinose, xylose, and rhamnose were minor components. Individual neutral monosaccharides were identified by paper chromatography, and paper electrophoresis, and finally quantitated by gas-liquid chromatography and characterized by combined gas-liquid chromatography chemical-ionization mass-spectrometry of the derived alditol acetates. Mucilage from both sources was found to be identical.

Introduction

Seeds of the genus Brassica are known to contain varying amounts of mucilage. The mucilage is of particular importance in mustard seeds because it contributes to the consistency of prepared mustard (Weber et al., 1974). Of several mustard varieties, yellow mustard (B. hirta) is particularly rich in mucilage and contains approximately 2% of it (Bailey and Norris, 1932; Woods and Downy, 1980), and for that reason, the seed is an important commercial commodity. A similar mucilage is found in rapeseed (Van Caeseele et al.; 1981, Yiu et al., 1982; Van Caeseele and Mills, 1983).

In some stored mustard seeds, a small amount of a whitish substance was found on the seed surface. Seeds, which had been moistened and immediately dried, were found to be glued together by a similar substance, showing thereby that the mucilage in the seeds rapidly exuded following exposure of the seeds to moisture. The release of mucilage from seeds immersed in water was used as an indicator of the presence of the mucilage in rapeseed cultivars (Van Caeseele and Mills, 1983).

Previous cytological studies, which were carried out using light microscopy of seed coats (hulls) in an aqueous medium and using electron microscopy of hulls embedded in a resin revealed that in rapeseed the mucilage developed between the plasmalemma and the outer tangential wall of the epidermal cells and that as maturity, the seed epidermal cells were totally devoid of cytoplasm and engorged with mucilage (Van Caeseele et al., 1981).

The present study was designed to examine the development of the efflorescence on the seeds by optical microscopy and scanning electron microscopy and to compare its composition to the mucilage isolated from the seed hulls. The polysaccharide components of the mucilage in mustard seed have been known and contain an arabian (Mirst et al., 1965; Rees and Richardson, 1966; Aspinall and Cottrell, 1971), a xylglucan (Gould et al., 1971 (amyloid), and pectic materials (Rees and Wight, 1969). Results of analyses such as chemical ionization and mass spectrometry are reported in this paper.

Materials and Methods

Seeds. Seeds of yellow mustard (B. hirta) were obtained from the 1980 harvest from Outlook Isolation (Ref. 80-7500040-01). The seeds were examined intact; a part of the sample was gently ground in a Krupp 75 coffee mill so as to break the hulls away from the seeds and retain the integrity of the hulls at the same time.
Extraction of mucilage from mustard hulls. Yellow mustard hulls (Batch FR-71-29), were extracted with boiling water (1:16, w/v) for 35 min and centrifuged at 2000 rpm for 20 min, yielding a viscous aqueous solution (Weber et al., 1974). Isopropanol was added to the filtrate to a final concentration of 70% (v/v) and the resulting precipitate (mucilage A) was separated using an organdie cloth, washed with 70% isopropanol, air-dried, and pulverized.

Isolation of mucilage from hulls of intact mustard seeds. The yellow mustard seeds were moistened with water and transferred by gently rubbing the seeds on the surface of a glass plate. The dried material was isolated by scraping the glass surface, dissolved in water, and recovered by precipitation in 70% isopropanol (mucilage B).

Chemical analyses. Gas-liquid chromatography (GLC) was performed with a Varian Vista 6000 Gas Chromatograph, with flame-ionization detectors, glass column (1524 x 3.18 mm i.e. 5 ft x 0.125 in) packed with 5% OV-1 on Chromosorb WHP (80-100 mesh), a temperature program 100 → 230°C at a rate of 2.5°C/min and a nitrogen flow rate of 30 mL/min. Peak areas were evaluated with a Pye-unicam CPD1 Computing Integrator. Combined gas-liquid chromatography chemical-ionization mass-spectrometry (GLC-CI-MS) (Horton et al., 1974) was performed with a Finnigan Inc. 3121 system with butane as the reagent gas, operating with CI ion source temperature 160°C, 250 eV, filament emission 0-5 mA, accelerating voltage 3 kV, electron multiplier 2 kV, scanning range 60-500 at 2 s/scan, and a column (1829 x 6.35 mm i.e. 6 ft x 0.25 in) of OV-225 on Chromosorb WHP (80-100 mesh), a temperature program 120 → 230°C at a rate of 5°C/min and a helium flow rate of 30 mL/min. Optical rotations were measured on a Perkin-Elmer 141 polarimeter.

Descending paper chromatography was performed on Whatman No. 1 paper using organic phases of an ethyl acetate-pyridine-water (8:2:1) or a 1-butanol-acetic acid-water (4:1:5) system.

Paper electrophoresis (Haug and Larsen, 1961) was performed on Whatman No. 3 MM paper in a borate-calcium chloride buffer (pH 9.2) at a potential gradient of 14 V/cm. Sugars were detected with aniline hydrogen phthalate. Concentrations were carried out at 35°C in a rotary evaporator.

Hydrolysis of the mucilage samples (2-3 mg) was performed with 1 M sulfuric acid (0.5 mL) for 3 h at 100°C or with aqueous 72% sulfuric acid (0.12 mL) at 5°C for 3 h followed by dilution to 1 M acid and heating for 3 h at 100°C. The hydrolyzates were neutralized with barium carbonate. The sugar samples (3 mg or less) were reduced with sodium borohydride, and the alditol acetates were acetylated essentially as described by Bjorndal et al. (1967) except that the acetylation was carried out at 100°C for 1 h followed by storage overnight at 22°C. Portions (2-3 mg) of the hydrolyzed sugars were fractionated on columns (70 x 5 mm) of Dowex 1x2 (CO₃⁻). Elution with water (5 mL) yielded neutral fractions which were concentrated, reduced with sodium borohydride, acetylated, and examined by GLC and GLC-CI-MS.

Light microscopy. Mature yellow mustard seeds were fixed in 5% glutaraldehyde in 0.01 M potassium phosphate buffer, pH 7.2 at 4°C for 48 h. After fixation, the seeds were mounted on cold object discs and fixed in Histo Prep medium (Fisher Scientific Co., Fair Lawn, N. J.) at -20°C. Frozen sections, 6-8 μm thick, were cut from the seeds using a Reichert-Jung Cryo-cut E microtome. Alternatively, the fixed seeds were embedded in glycol methacrylate (GMA) resin as described by Yiu et al. (1982). They were dehydrated in an alcohol series in the order of methyl cellosolve, ethanol, n-propanol, and n-butanol. The dehydrated seeds were then infiltrated with GMA monomer for 3-5 days prior to polymerization at 60°C in gelatin capsules. Sections 2 μm thick were cut using glass knives in a Sorvall Porter-Blum microtome, affixed to glass slides, and stained with one of the following dyes:

Toluidine Blue 0, 0.05% (w/v) in 0.1 M potassium phosphate buffer, pH 6.5, for 1-2 min. After staining, the sections were briefly rinsed in distilled water, air-dried, mounted in immersion oil, and examined using brightfield illumination.

Calciofluor White, 0.01% (w/v) aqueous solution, for 1-2 min. The sections were rinsed in water, air-dried, mounted in non-fluorescent immersion oil, and examined for fluorescence using filter system FC I (see below).

Congo Red, 0.01% (w/v) aqueous solution, for 1-2 min. The sections were briefly rinsed in water, dried, mounted in non-fluorescent immersion oil, and examined for fluorescence using filter system FC III (see below).

Fluoresceinated wheat germ agglutinin. The sections were incubated with 1.2 mg/mL of fluorescein-labelled wheat germ agglutinin (Cedarlane Lab. Ltd., Hornby, Ont., Canada) in 0.01 M sodium phosphate buffer, pH 7, at 22°C for 1 h. The sections were rinsed thoroughly with distilled water, air-dried, mounted in oil, and examined under the microscope using filter system FC II (see below).

The sections were examined using a Zeiss Universal Research Photomicroscope equipped with both a conventional brightfield illuminating system and a III RS epifluorescence condenser combined with an HBO 100 W mercury lamp for fluorescence analysis. Three fluorescence filter combinations, each with a dichromatic beam splitter and an exciter/barrier filter set for maximum transmission at 365 nm/ >418 nm (FC I), 450-490 nm/ >520 nm (FC II), and 546 nm/ >590 nm (FC III), were used for fluorescence examination. Photographs were taken on Kodak Tri-X pan film.

Scanning electron microscopy (SEM). Dry yellow mustard seeds were examined intact. They were also cut in half and the hulls were separated and mounted for examination of their outer and inner surfaces. In parallel experiments, seed hulls were extracted with 3 changes of boiling water for the total time of 3 h. The extracted hulls were dried in air and prepared for SEM by mounting on aluminum stubs using silver cement and coating with gold (approx. 20 nm) by vacuum evaporation. The seeds and hulls were examined in a Cambridge Stereoscan Mark II scanning electron microscope operated at 20 kV.

Results and Discussion

One of the simplest methods of detecting the presence of mucilage in mustard seeds is by wetting the seeds with water and examining them under a stereomicroscope several minutes later. The appearance of a gelatinous halo surrounding the wetted seed indicates the presence of mucilage on the seed surface. More elaborate microscopic techniques were required in order to examine
the structure of mucilage in detail. Several microscopic methods were used for the detection of yellow mustard mucilage in this study.

Toluidine Blue O, a metachromatic dye which was found to be useful for staining various plant structures (O'Brien et al., 1964, and Yu et al., 1983) including mucilage in rapeseed, B. campestris cv. Candle (Van Cuesto et al., 1981), was used for staining GHA sections of the yellow mustard seeds. Microscopic examination under brightfield illumination revealed a swollen epidermis, the outer seed coat layer of the seed, with visible pink striated contents and thickened cell walls (Fig. 1) indicating the presence of mucilage. Fluorescence microscopy was used to achieve a higher resolution of the structure. Several fluorescent dyes or reagents (Calcofluor White, Congo Red, and fluoresceinated wheat germ agglutinin) that have known affinities for specific polysaccharides (Yu et al., 1982, Miller et al., 1984) were employed in this study. While all three reagents were useful in detecting the presence of mucilage in the frozen sections of the mustard seeds, Calcofluor White was the only effective fluorescent marker for the GMA sections. One such section stained with Calcofluor White is demonstrated in Fig. 2. It shows the structural relationship between mucilage and the rest of the seed coat layers. The structure of the mustard seed has already been described elsewhere (Winton and Winton, 1932; Vaughan et al., 1976) and will not be repeated here. Briefly, the mustard seed coat (hull) consisted of an epidermal layer where mucilage was detected, a sub-epidermis, a palisade layer, and a pigment layer. The aleurone layer of the endosperm remained associated with the seed coat during dehulling. Although Congo Red had been shown to stain mucilage well in GMA sections of both rapeseed (Yu et al., 1982) and oriental mustard seeds (Holley et al., 1983), it failed to stain the GMA sections of yellow mustard seeds. It is not certain whether this finding reflects any chemical or dye-affinity differences between the mucilage of yellow mustard and mucilage of rapeseed and oriental mustard. More studies are required in order to understand the mechanism of interaction between Congo Red and different mucilages. On the other hand, it could be speculated that owing to the relatively large molecular size of fluoresceinated wheat germ agglutinin, the lectin was not able to penetrate the GMA resin easily and, consequently, did not stain the mucilage as well as it did in the frozen sections. The findings were similar to results obtained with rapeseed sections which were used as controls. Although the use of fluoresceinated wheat germ agglutinin was thus limited to frozen sections, which did not reveal structural details because of their thickness, the lectin served as a specific probe staining no other seed coat structures but the mucilage (Fig. 3). Besides, informative results can be obtained from frozen sections within a relatively short time (1-2 days) as compared with GMA sections which require more than a week to complete the sample preparation procedures. In spite of being relatively rapid and simple to perform, the techniques of light microscopy do have some disadvantages. Aqueous fixation is usually required for sample preparation. Fixation using glutaraldehyde vapour is possible but is not as effective as glutaraldehyde solution. In addition, most of the staining methods use aqueous solutions. The presence of water may lead to the emergence of mucilage at the seed surface that does not occur in dry seeds and this can be regarded as an artefact.

Scanning electron microscopy (SEM) was used to
Fig. 4. Mucilage emerged from the hull of a seed moistened for 1 h and subsequently dried. The mucilage is in the form of individual minute droplets (arrows) or in the form of droplets coalesced into a stratified cover (C). In this stereo pair of micrographs (12° angular separation), two dots have been provided to facilitate focussing of the eyes.

Fig. 5. Mucilage (arrow) cementing two yellow mustard seeds (asterisks) following their exposure to moisture for 4 h and drying.

Fig. 6. Internal surface of an intact seed hull. Regular depressions are marked with asterisks. Arrows point to fine wrinkling of the surface.

Fig. 7. Mechanical removal of the inner lining from the internal surface of a seed hull reveals broken cells (arrows).

Fig. 8. Internal surface of a seed hull extracted with boiling water for 3 h and subsequently air-dried. Depressions (asterisks) in the surface and fine wrinkling (arrows) are similar to those in an intact hull.
record the exudation of mucilage from a seed exposed to moisture for 1 h and the result is shown in a stereo pair of micrographs (Fig. 4). This treatment altered the seed surface compared to the images of untreated seeds shown by Mulligan and Bailey (1976). In some places on the seed surface, small droplets of the mucilage had already started to coalesce and form a stratified mucilage cover; coalescence of the mucilage droplets was common to seeds exposed to moisture for periods longer than 1 h. The emergence of the mucilage from isolated hulls in water was observed and resembled the emergence of mucilage from rapeseed as documented by Van Caeseele and Mills (1983). A compact layer of mucilage dried on the seed surface (4 h exposure to moisture) is shown in Fig. 5. It was this layer of mucilage, which was the subject of the present study. The mucilage was initially isolated by gently scraping the dried seeds but later a more efficient technique was developed whereby wet seeds were rolled on a glass plate and the mucilage was transferred to the glass plate and scraped off for analysis.

The inner surface of the hulls was also examined by SEM. The surface of an intact hull is shown in Fig. 6. There are depressions in the surface which appears as if coated with a finely wrinkled film. This film can be observed by a naked eye as a whitish material and can be easily removed with a pair of tweezers. Its mechanical removal exposed the underlying cells (Fig. 7). Exposure of the hull to boiling water for 3 h and consecutive air-drying did not alter this surface to any considerable extent (Fig. 8) except that the depressions are somewhat deeper. The emergence of the mucilage from hulls immersed in water was observed only on the outer hull surface and was not observed on the inner surface. Results of chemical analyses following hydrolysis of the mucilage with 1 M as well as 72% sulfuric acid, paper chromatography, and paper electrophoresis are presented in Table 1. The results indicate that the materials were free from low molecular weight sugars and were essentially free from protein contaminants. The analytical data, [α]D values, and the hydrolysis (GLC-Cl-MS of the derived alditol acetates) confirmed the identity of the component sugars and indicated the molar proportions of the parent neutral sugars, constituting samples A and B, to be very close. A typical gas chromatogram of the alditol acetates from mucilages A and B is shown in Fig. 9 and data are summarized in Table 2. This evidence demonstrates that the two samples were identical except that mucilage B had significantly lower pectin and protein contents (Table 1).

The hydrolysis data further revealed certain aspects of the polysaccharide composition of the mucilage. The presence of uronic acids, and rhamnose residue, and possibly an aldobiuronic acid, probably the commonly occurring (1→2)-linked (galactosyluronic acid)-rhamnose, showed that a major portion of the mucilage is composed of polysaccharides of the pectic type. The presence of glucose, galactose, and xylose residues and the different galactose and glucose ratios (1:0.23, M H2SO4; 1:0.62, 72% H2SO4, mucilage A) and (1:0.31, M H2SO4; 1:0.72, 72% H2SO4, mucilage B) were indicative of the presence of a xylglucan (amylod). These differences originate from different degrees of hydrolysis of the cellulose backbone in amylod. Both polysaccharides have been reported in mustard (Gould et al., 1971; Rees and Wight, 1969).

The present results could not demonstrate unequivocally the presence of an arabinan component but the presence of arabinose residues in conjunction with literature reports (Aspinall and Rees, 1965, Rees and Richardson, 1966) strongly suggest that the araban was also present.

Table 1

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Optical rotation [α]D (0.1 M NaOH)</th>
<th>Hydrolysis results*</th>
<th>Analytical data (%)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Ash</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Moisture</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0 to -5°</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0 to 5°</td>
<td>0.22</td>
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<tr>
<td>Galactose</td>
<td>major</td>
<td>major</td>
<td></td>
</tr>
<tr>
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<td>major</td>
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<tr>
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<td>minor</td>
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</tr>
<tr>
<td>Arabinose</td>
<td>minor</td>
<td>minor</td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td>minor</td>
<td>minor</td>
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</tr>
<tr>
<td>Rhamnose</td>
<td>minor</td>
<td>minor</td>
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</tr>
<tr>
<td>Galacturonic acid</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aldoburonic acid</td>
<td>+</td>
<td></td>
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</tbody>
</table>

Fig. 9. Gas-liquid chromatograms of neutral sugars, in the form of their alditol acetates, as obtained after hydrolysis with 72% H2SO4. Relative retention times are listed in Table 2. A = yellow mustard hull mucilage: B = yellow mustard mucilage scraped from the seed surface.

* Obtained by paper chromatography and paper electrophoresis.
Acknowledgments
The authors thank Dr. Ian McGregor, Agriculture Canada, Saskatoon, Saskatchewan, for the donation of yellow mustard seeds, and Dr. R. G. Fulcher for useful suggestions. Skillful technical assistance provided by Mrs. S. Phipps-Todd and by Messrs. J. Emery and J.A.G. Larose is acknowledged. Electron Microscope Research Branch, Agriculture Canada in Ottawa provided facilities. Contribution 680 from the Food Research Institute.

References

Table 2
ANALYTICAL AND COMBINED GAS-LIQUID CHROMATOGRAPHY CHEMICAL IONIZATION MASS SPECTROMETRY (GLC-CI-MS) DATA FOR SUGARS FOLLOWING HYDROLYSIS (72% H₂SO₄), REDUCTION, AND ACETYLYATION

<table>
<thead>
<tr>
<th>Acetate of</th>
<th>Retention time relative to galactitol</th>
<th>Molar ratio A</th>
<th>Molar ratio B</th>
<th>CI-MS data: m/z [relative abundance, (%)]</th>
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<tr>
<td>Glucitol</td>
<td>1.02</td>
<td>0.62</td>
<td>0.72</td>
<td>[MH]⁺ 375 (100), 257 (15), 376 (15)</td>
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<td>Galactitol</td>
<td>1.60</td>
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<td>1.00</td>
<td>[MH]⁺ 375 (100), 257 (15), 376 (15)</td>
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<td>Mannitol</td>
<td>0.08</td>
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<td>0.27</td>
<td>[MH]⁺ 303 (100), 185 (15), 304 (15)</td>
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<td>Xyloitol</td>
<td>0.85</td>
<td>0.29</td>
<td>0.27</td>
<td>[MH]⁺ 303 (100), 185 (15), 304 (15)</td>
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<td>Arabinitol</td>
<td>0.80</td>
<td>0.29</td>
<td>0.27</td>
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<td>Rhamnitol</td>
<td>0.74</td>
<td>0.20</td>
<td>0.26</td>
<td>[MH]⁺ 317 (100), 199 (15), 318 (15)</td>
</tr>
</tbody>
</table>

RHEOLOGICAL AND PARTICLE SIZE CHANGES IN CORN OIL-IN-WATER EMULSIONS STABILIZED BY 7S SOYBEAN PROTEINS

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Abstract
The viscoelastic properties and mean drop size ($D_\text{m}$) of corn oil-in-water emulsions stabilized by 0.5-1.5% (wt/wt) 7S soybean proteins have been examined at various pH's. Changes in these parameters when the emulsions were stored at 4-5°C were measured also. Viscoelasticity parameters were derived from time-dependent strain behaviour at a constant low shear stress of 41.7 dyne cm$^{-2}$. Although each emulsion showed a continuous increase in $D_\text{m}$ during storage, due to drop coalescence, its instantaneous elastic modulus ($G_\text{s}$) rose initially over several days to an optimum value and then subsequently decreased. The trends in $D_\text{m}$ and $G_\text{s}$, which indicated that the dominant process during early storage was drop flocculation and drop coalescence at longer times, were used to deduce the structure of the flocculated drop networks.

Introduction
Rheological methods have been used for a long time to evaluate the flow properties of fluid and semi-solid foods. This involves the measurement of viscosity over a wide range of shear conditions. Foods exhibiting non-Newtonian flow, and this includes the majority of fluid and semi-solid foods, are usually shear thinning, thus, the viscosity decreases as the shear rate is increased, because there is a progressive breakdown of the internal two, or more, phase structure. More recently rheometers have been developed which permit these foods to be studied at very low shear stress which minimize the degree of structural breakdown in the test situation. Under these conditions, the internal structure is not broken down to any significant extent and the procedure offers an alternative, and sensitive, method for its study. This approach has distinct advantages when studying foods which have an emulsion based structure. It can help to identify structural changes long before there is any visible oil or water separation, or obvious changes in consistency.

The procedure has been applied recently to the study of oil-in-water (o/w) emulsions stabilised by the polyelectrolyte polysaccharide mesquite gum (Vernon Carter and Sherman, 1980), mayonnaise (Kiosseoglou and Sherman, 1983), corn oil-in-water emulsions stabilised with milk proteins and mono- and di-glycerides (Doxastakis and Sherman, 1983).

Because of the interest in using soybean proteins to manufacture simulated dairy-type food products a study is in progress to establish the influence of soybean proteins, when used in conjunction with mono- and di-glycerides, on the internal structure, rheological properties and long term stability of corn oil-in-water emulsions. This paper reports on part of this study which relates to the effect exerted by increasing concentration (0.5-1.5% wt/wt) of the 7S soybean protein fraction.

Materials and Methods

Materials
Water used to prepare the emulsions was distilled from an all Pyrex apparatus. Pure corn oil (Mazola, CPC U.K. Ltd., Esher, Surrey England) was used as the oil phase. It had a relative density
of 0.919 gm/ml at 20°C. All the chemicals used were of Analytical Reagent grade.

Isolation of 7S Soybean Protein

Cypressa soybeans (Katsouris Bros. Ltd., Drayton Park, London, England) were used. The variety was named H, Canada grown white, 1983 harvest. The beans were stored at 4°C until they were required.

Soybeans were cracked, dehulled, the flour isolated and defatted as described elsewhere (Rivas and Sherman, 1983). The coarsely ground material was reground in a Gruphon grinder (Brook Motors, England) fitted with a 60 mesh screen. The resultant flour was defatted three times with hexane, air dried and stored in glass jars at 5°C until required. Fractionation of the flour into 7S and 11S protein fractions utilized the procedure of Saio et al. (1973).

Emulsion preparation

O/W emulsions were prepared using a 60/40 weight ratio of corn oil and aqueous phase. The aqueous phase contained the appropriate concentration of 7S protein.

The aqueous solution of protein was prepared by dissolving the 7S protein in water heated to 45°C and stirring continuously with a magnetic stirrer for 1 h. The vessel was covered with a foil lid during this time. At the end of 1 h, the aqueous solution had a pH of 4.5, and this was adjusted to pH 7.0 with 0.5 M NaOH. Vigorous stirring was then applied for 2 h, while maintaining a temperature of 45°C, and this produced a "milky" solution. The solution was filtered through fine muslin, cooled to room temperature, 0.01% (wt/wt) Penicillin G, or potassium sorbate, was introduced as a preservative and the solution was maintained at 4°C until it was required.

Corn oil was added slowly to the aqueous phase and dispersed therein with the aid of a magnetic stirrer. The coarse emulsion produced in this way was then homogenized, thereby reducing the mean drop size and narrowing the drop size distribution, by passing through a Rannie homogenizer at a homogenizing pressure of 3400 kPa. Each homogenized emulsion was divided into four parts and the pH of each part was adjusted with 3M HCl or 3M NaOH. The pH values selected were 7.5, 5.5, 3.5, and 2.5, thus providing pH values on either side of the isoelectric point (pH 4.5) of the 7S protein. The emulsions were stored at 4°C-5°C in a refrigerator.

Rheological evaluation of the o/w emulsions

The viscoelastic properties of each emulsion were examined, at 21.0° ± 0.1°C, at regular intervals during storage by means of its creep compliance-time response characteristic of viscoelastic behaviour. The creep compliance-time response with time, \( J(t) \), of each emulsion could be represented by

\[
J(t) = J_0 + J_1 (1 - \exp(-t/\tau_1)) + J_2 (1 - \exp(-t/\tau_2)) + \frac{\Delta \eta}{\eta_0} \tag{1}
\]

where, \( J_0 \), is the instantaneous elastic compliance, \( J_1 \) and \( J_2 \) are the first and second retarded elastic compliances, \( \tau_1 \) and \( \tau_2 \) are the first and second retardation times, and \( \Delta \eta \) is a Newtonian compliance.

From Eq. (1) six parameters are derived using the following relationships.

\[
G_0 = \frac{1}{J_0} \tag{2}
\]

where, \( G_0 \) is the instantaneous elastic modulus

\[
G_1 = \frac{1}{J_1} \quad \text{and} \quad G_2 = \frac{1}{J_2} \tag{3}
\]

where, \( G_1 \) and \( G_2 \) are the first and second retarded elastic moduli, and

\[
\tau_1 = \frac{J_1 \eta_1}{\Delta \eta} \quad \text{and} \quad \tau_2 = \frac{J_2 \eta_2}{\Delta \eta} \tag{4}
\]

where, \( \eta_1 \) and \( \eta_2 \) are viscosities associated with \( J_1 \) and \( J_2 \).

When \( t \to 0 \), Eq. (1) reduces to

\[
J(t) = J_0 \tag{5}
\]

so that at very short times after the small, constant, shear stress has been applied to each emulsion it behaves like a solid in that its response to the stress applied is characterised by
generally continued

There was an initial increase in G which generally continued through the first 5 days of storage, to a maximum value and then it decreased. At pH 7.5 or 2.5, the initial value of G was lower than at pH 5.5 or 3.5, for a given 7S protein concentration, the only exception being the emulsion containing 0.5% protein at pH 3.5. In addition, G changes in stored emulsions were less marked at pH 7.5 and 2.5 than at pH 5.5 and 3.5. At pH 5.5 and 3.5 the optimum value of G was higher than at pH 7.5 or 2.5 the difference becoming more pronounced as the 7S protein concentration increased. For emulsions containing 1.0% or 1.5% 7S protein higher optimum values of G were achieved at pH 5.5 than at pH 3.5 when stored. However, for emulsions containing 0.5% 7S protein the reverse applied, but the difference was relatively small, and it was probably not significant. At any pH and aging time selected G increased with increasing concentration of 7S protein.

The G values calculated for emulsions incorporating 0.5, 1.0 and 1.5% 7S protein are quoted in Tables 1, 2 and 3, respectively along with their mean volume diameters. Only the G data are presented because they reflect the properties of the intact emulsion structure prior to any breakdown, and it is, therefore, the most useful parameter from which to draw conclusions relating to the internal structure.

The data in Tables 1-3 indicate that, irrespective of pH and 7S protein concentration, the internal structure of each emulsion underwent a distinctive pattern of change when it was stored. There was an initial increase in G, which generally continued through the first 5 days of storage, to a maximum value and then it decreased. At pH 7.5 or 2.5, the initial value of G was lower than at pH 5.5 or 3.5, for a given 7S protein concentration, the only exception being the emulsion containing 0.5% protein at pH 3.5. In addition, G changes in stored emulsions were less marked at pH 7.5 and 2.5 than at pH 5.5 and 3.5. At pH 5.5 and 3.5 the optimum value of G was higher than at pH 7.5 or 2.5 the difference becoming more pronounced as the 7S protein concentration increased. For emulsions containing 1.0% or 1.5% 7S protein higher optimum values of G were achieved at pH 5.5 than at pH 3.5 when stored. However, for emulsions containing 0.5% 7S protein the reverse applied, but the difference was relatively small, and it was probably not significant. At any pH and aging time selected G increased with increasing concentration of 7S protein.

### TABLE 1

<table>
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<tr>
<th>pH</th>
<th>Aging Time (days)</th>
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Influence of pH and storage time on the instantaneous elastic modulus and mean drop volume of corn oil-in-water emulsions incorporating 1.5% 7S soybean proteins.

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Discussion

An emulsion is not a thermodynamically stable system. Following its preparation the drops move closer together by the process of flocculation and they form aggregates. These aggregates grow larger during storage due to the amalgamation of similar aggregates, or due to individual drops combining with the aggregates. The surfaces of the drops within the aggregates are not in direct contact. They are separated by a thin film of continuous (external) phase fluid, and the precise thickness of this film of immobilised fluid depends on the configuration of the film of emulsifier adsorbed around the surface of each drop and on its electrical charge. These two factors also determine the nature of the interaction between the drops and, consequently, the degree of viscoelasticity exhibited by the emulsions. Drop flocculation, and the resulting interaction between drops, increases.

With regard to the structure of the flocculated drop aggregates, especially in the vicinity of their interacting surfaces, certain deductions can be made from the rheological data, the influence of pH thereon, and from the way in which proteins adsorb at an oil-in-water interface. Current theories relating to the adsorption of polymer molecules at interfaces are used for this purpose. The 7S protein fraction has a molecular weight of about 193,000 with 7S globulin, which has a molecular weight range 180,000-210,000 constituting more than 50% of the fraction. Lower molecular weight constituents are hemagglutinins, lipoperoxidase, and B-amylase (Sosulki, 1977). Following adsorption the behaviour of globular proteins, such as 7S soybean fraction, at an oil-in-water interface is not very different from that of adsorbed flexible proteins (Graham and Phillips, 1979). Oil molecules reduce the van der Waal’s cohesion between apolar side chains in the protein molecules so that they can unfold to a greater extent and develop a configuration resembling the loop and train structure of flexible proteins (Graham and Phillips, 1979). Provided the concentration of protein adsorbed on to the drop surfaces is not very low only portions of the protein molecules are actually adsorbed on to the drop surfaces (trains), while other segments (loops) project outwards from the surfaces.

The number of loops per unit area of surface increases with increasing adsorption of protein, which, in turn, depends on the concentration of protein incorporated in the emulsion. The pH also exerts an effect. At the isoelectric point of the protein the adsorbed
molecules are in their most compact configuration and there are more loops per unit area of drop surface than at higher or lower pH values, when the more expanded configuration is adopted. The longest loops projecting from drops in close proximity link up to form a network (Van Vliet et al., 1976; Sonntag et al., 1982). It is this network which makes the major contribution during flocculation and it confers a weak gel-like structure on the thin film of continuous phase between adjacent drops. G increases as the number of interlinked loops increases.

During storage of the emulsions the adsorbed protein loops on the oil drops are either compressed and flattened, or they overlap (Napper, 1977). At present it is not known which of these two processes occurs, but either would increase the degree of contact between loops. G would then increase provided that no other changes which had a more profound opposite effect were proceeding at the same time. The rheological data indicate that one of these two processes exerts the major effect during the first few days of storage. Since G increases most at pH values close to the isoelectric point, i.e. when it would be more difficult for loops to overlap than at higher or lower pH values due to the more compact protein configuration, it would appear that the protein loops are more likely to be flattened and compressed.

Concurrent with flocculation drops are coalescing, a process which reduces G, since G is inversely proportional to the third power of mean drop size (Sherman, 1970). Therefore drop flocculation and aggregation is the dominant process only during the early days of storage and then drop coalescence becomes the major factor. Protein concentration and pH influence the physical properties of the protein film around each drop through their effect on molecular concentration and configuration, as discussed previously. The more compact the configuration, and the slower the drainage rate of liquid from between the droplets the more resistant will the protein film be to rupture. Both of these processes must precede drop coalescence.

Conclusions

Oil-in-water emulsions stabilised by 7% soybean protein fraction have a complex internal structure. When stored the oil drops develop a randomly close-packed configuration and they are linked together by long loops of protein which project from the surfaces of adjacent oil drops. Collectively, they form a weak gel structure within the thin films of aqueous phase between adjacent oil drops. The number of linkages, and their number per unit area of drop surfaces, is influenced by pH and the protein concentration incorporated in the emulsion. The flocculated emulsions exhibit viscoelasticity, and an elastic modulus, indicative of solid-like behaviour, can be derived from the flocculated drop structure from their strain response at very short times after the imposition of a constant low shear stress. This structure is not static. When the emulsions are stored loops are compressed and flattened and, simultaneously, drops coalesce. During the initial days of storage the first process is dominant whereas later the second process is dominant.

Acknowledgement

The authors are grateful to the U.K. Ministry of Agriculture, Food and Fisheries for the financial assistance to one of the authors (M.R.) which made this study possible.

References


The aqueous dispersions of protein were prepared with the pH adjusted to 7.0 and these were combined with oil and homogenized to form emulsions. Portions of these emulsions were then treated to adjust pH to 7.5, 5.5, 3.5 and 2.5. Would you expect the nature and disposition of proteins in these emulsions to be similar to emulsions prepared by first adjusting the pH of the protein dispersion prior to emulsion formation?

Authors: Adjusting the pH of the protein dispersion prior to emulsion formation would alter the configuration of the protein and, consequently, its rate of diffusion to, and rate of adsorption at, the oil-water interface. The ultimate configuration of the adsorbed protein could also be affected. All of these effects could produce differences in the viscoelasticity parameters, and the influence of aging time thereon, as compared with emulsions prepared as described in the paper.
MECHANICAL PROPERTIES OF CHEESE, CHEESE ANALOGUES AND PROTEIN GELS IN RELATION TO COMPOSITION AND MICROSTRUCTURE

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²Biomechanics Group, University of Reading, Whiteknights,
Reading, RG6 2AJ, UK

Abstract

Samples of commercial Cheddar cheese, experimental Cheddar cheeses made from heated, ultrafiltration-concentrated milks, processed cheese analogues and whey protein gels of defined composition were examined microscopically and some fracture and deformation properties were determined. Surfaces of cheese prepared by critical point drying and those examined frozen were comparable in microstructure. As the concentration factor of the milk used for experimental cheesemaking increased, the cheese became more resistant to reversible deformation, the work required to cut with a wire or break with a hammer increased and the microstructure showed that the protein matrix was coarser. The force to deform or work to cut cheese analogues depended on the composition. The work to deform and break whey protein gels depended on the composition, microstructure and testing direction. In general, gels containing more β-lactoglobulin were less easy to break and formed tighter gels. Microscopy of surfaces formed by impact fracture indicated that the combination of cutting and cracking may vary between samples.

Introduction

Food texture broadly describes the consumer's mouthfeel. The handling properties, especially the ease of cutting, packaging and transport, are also important in practice. The texture of a food must result from its composition and microstructure.

The various components of the physical properties of a food can be estimated directly by subjective sensory means, but instrumental measurements are preferable as they are easier to standardize and reproduce and require fewer trained people. Instrumental measurements are also potentially more useful as they can generate absolute rheological data provided care is taken to understand the types of forces exerted and the outputs obtained. Such data can be used to interpret the mechanics of the structures.

Other types of instrumental measurement are really useful only if they can be directly correlated with some sensory or structural feature of the food. This applies to a number of empirical measurements of individual foods (Bourne, 1982). However, to be of general application, measurements should probably reflect the actual circumstances of consumption. This may involve cutting and certainly involves mastication, in which food is usually deformed rapidly under a high force and to beyond the point of failure. In such instances, the texture may depend on the fracture mechanism (Jowitt, 1979; Hamann, 1983).

The deformation of three types of cheeses under equilibrium conditions has been studied by applying an oscillating force of known frequency (Taneya et al. 1979). The relaxation times became larger as the cheeses matured, reflecting the formation of a rigid structure. Processed cheeses had a relaxation time distribution extending to shorter times, reflecting the weaker network structure. As heat-denatured protein gels are less brittle than cheese, conventional compression measurements are often made under equilibrium conditions, so providing useful structural data. More rapid aggregation of the protein tends to result in microstructures in which the strands and pores of the network are

Key Words: Cheese, cheese analogues, whey protein gels, scanning electron microscopy, deformation, fracture
coarser (Tombs, 1974) and firmer, less elastic textures in compression (Hermansson, 1982).

For soy protein gels, the peak force measured by compression could be related to the number of linkages and the evenness of the gel observed by SEM (Furukawa et al., 1979).

When hard cheese is compressed to the point of failure, the sample cracks vertically (Culloli and Sherman, 1976) or obliquely (Green et al., 1985). However, the mechanism of fracture of Cheddar and Cheshire cheeses in compression is clearly different from that in cutting or biting (Green et al., 1985). The cracks occur between the grains in compression, but are mostly through the grains when the cheese is cut or bitten.

The present paper describes preliminary work extending that of Green et al. (1985). Both fracture and reversible deformation of cheeses, cheese analogues and protein gels have been considered in relation to their compositions and microstructures. The work has included microscopic examination of fracture surfaces.

Materials and Methods

Samples

Cheddar cheese used for the examination of fracture surfaces was mild Irish Cheddar obtained from a commercial source, and probably matured for less than 6 months. Experimental Cheddar cheeses were made from milk ultrafiltered (UF) at 50°C at their natural pH to increase the concentration of fat and protein by 2.67- and 3.14-fold, and then heated in a flow-through plant at 90°C for 15 s. Conventional cheesemaking methods were used, except that the starter and rennet levels were adjusted to give a pH decrease and a coagulation time equal to the control. The cheeses were ripened for 5 months at 13°C before assessment. Processed cheese analogues were made by mixing skimmed milk powder (SMP), sodium caseinate and lactic acid to a final pH value of 5.7 with an emulsion of butteroil in water stabilised by Tween 80 at 45-60°C. Air was removed by vacuum treatment and the mixture poured into moulds. These were placed at 30°C after 30 min and held overnight before sampling. The components of the mixture were adjusted so that only the fat and water concentrations varied significantly during the experiment. Heat-denatured protein gels were prepared from whey protein powders, produced by ion exchange (Skudder, 1983). These were dissolved in water at pH 6.5, poured into 15 mm (i.d.) glass tubes and heated at 80°C for 10 min. The gels were cooled to room temperature prior to testing.

Determination of chemical compositions

Fat, moisture, salt and pH in cheeses and cheese analogues were determined as described by Green (1985). The protein compositions of whey powders were determined as described by Langley et al. (1986).

Scanning electron microscopy (SEM)

Samples which were prepared conventionally for SEM were fixed for 2 h in 3% glutaraldehyde adjusted to pH 7.2 with 0.1M phosphate buffer. They were washed several times in phosphate buffer and post-fixed for 1.5h in 1% OsO₄ at pH 7.0. After dehydration in acetone, specimens were critical point dried, attached to aluminium stubs and coated with gold in an Edwards S150 sputter coater. They were then examined in a Hitachi S 570 scanning electron microscope.

Some samples were examined by SEM whilst still hydrated, using an EMscope cryopreparation unit and a low temperature microscope specimen stage. Specimens were frozen by rapidly plunging into nitrogen slush which had been prepared by evacuating a closed container of liquid nitrogen. They were then sputtered with gold and examined in the electron microscope using an accelerating voltage of 1-5kV. Where indicated, uncoated frozen samples were etched at -40°C using a specimen stage heater whilst observed with the microscope. When sufficient water had been sublimed, the specimen was cooled to liquid nitrogen temperature, coated with gold and re-examined.

Since the electron scattering properties were poor compared with their secondary electron emission, TEM of whey protein gels did not provide adequate information and thin sections of these specimens were examined by SEM. Gels were fixed as described above and were then embedded in Araldite. Sections (1 μm thick) were cut using a glass knife and a Reichert Omu2 ultramicrotome, mounted on coverslips and the Araldite removed using ethyl alcohol saturated with sodium hydroxide as described by Brooker and Wells (1984). Sections were critical point dried, mounted on stubs and coated with gold before examination in the microscope.

Light microscopy and transmission electron microscopy

Light micrographs, magnification ×320, were prepared from rapidly frozen cheese samples sectioned and stained with carbolfuchsin. The mean coarseness of the matrix of pairs of micrographs was determined by counting the number of times any line on a 28 x 19 rectangular grid was visible through any hole in the network (Green et al., 1981). Transmission electron micrographs, magnification ×7800, were prepared from 60-90nm sections of cheese samples fixed in glutaraldehyde, post-fixed in 1% OsO₄, stained in 1% uranyl acetate, dehydrated and embedded in Araldite. The fat/protein interfacial area was determined stereologically as the mean from 3 micrographs using a 16 x 11 line rectangular grid (Green et al., 1981).

Determination of mechanical and sensory properties

Impact testing was carried out at 10 ± 3°C using the Charpy pendulum method with a wedge-shaped hammer. For the hammer energy required to fracture the sample was determined. For gels, the average velocity of the hammer on contact with the sample was
Mechanical properties, composition and microstructure

about 0.7 m s⁻¹. The impact strength was determined by calculating the energy absorbed per unit distance of penetration of the sample. All other mechanical tests used an Instron food tester (Table Model 1140 at IFR or 4202 in Biomechanics Group). The stiffness of cheese was determined by compressing 15 mm cubes in triplicate to a point where linearity of response just disappeared (i.e., the onset of a yield) then recording the displacement between that point and zero until the results were repeatable (i.e., all plastic deformation had disappeared). Some 50% hysteresis remained. The strain to this yield was about 22% at 0.033 mm s⁻¹. The compressive Young's modulus was estimated from the initial slope of the stable loading curve. The work of fracture of the experimental cheeses was determined in triplicate as the area under the force/distance curve when 10 mm cubes were cut with a 25 μm diameter tungsten wire at 0.083 mm s⁻¹. For cheese analogues, the work of fracture was determined using 20 mm wide blocks in quintuplicate cut 0.083 mm s⁻¹ by tungsten wires 25-100 μm diameter tensioned to constant stress. The Young's modulus and stress at fracture were estimated from the initial slope and the first peak of force/distance curves of axial compression of individual cylinders between flat plates at 0.83 mm s⁻¹. A total of 16 cylinders (17 mm diameter × 15 mm high), 8 cut so the cylinder axis would have been vertical in the moulded sample and 8 cut so the cylinder axis would have been horizontal were tested. For compression testing, whey protein gel cylinders were cut into 15 mm lengths and compressed between plates coated with fine grade emery cloth at 0.83 mm s⁻¹ by tungsten wires 0.83 mm s⁻¹ by tungsten wires 25-100 μm diameter tensioned to constant stress. The Young's modulus and stress at fracture were determined as for the cheese analogues. Tensile testing was carried out as described by Langley et al. (1986).

The elastic moduli of whey protein gels compressed along or perpendicularly to the cylinder axis were determined from the equations:

Along the axis:

\[
\frac{h(L-h)}{L} = \frac{h_0}{E_0}a_0^2 - \frac{h}{E_0}a^2
\]  

(1)

Perpendicular to the axis:

\[
\Delta d = \frac{4p}{(1-v^2)/\pi E + 4p[(1-v^2)/\pi E]\ln[(DE)^{1/2}(1.079p)^{1/2}]}
\]

where \(h_0\) is the initial length, \(h\) is the vertical displacement at an applied load, \(a_0\) is the initial radius, \(E\) is the elastic modulus, \(D\) is the initial diameter, \(\Delta d\) is the change in diameter, \(P\) is the applied force per unit length of cylinder and \(v\) is Poisson's ratio. Equation (2) is taken from Roark (1965) and \(\Delta d\) was determined from photographs during compression.

Sensory firmness and graininess of cheeses were assessed by panels as described by Green (1985).

Results

Cheddar cheese fracture surfaces

Green et al. (1985) used critical point dried specimens to examine the surfaces formed when Cheddar cheese was fractured in various ways. Here the two parts formed when the sample was fractured have been prepared for microscopy in different ways, so as to compare the structures observed on the complementary surfaces. One surface was frozen and etched and the other critical point dried. Some fat globules can be differentiated on the critical point dried surface but not on the freeze surface (Fig. 1). In 4-point manual breaking, in which fracture results in irregular surfaces with tearing and stepped cleavage planes, these are observed using both preparation methods. Conversely when the sample was cut, the surfaces prepared by both methods were relatively smooth.

Cheddar cheeses made from heated UF-concentrated milks

Cheddar cheeses were made from milks which had been concentrated by UF then heated at 90° C/15 s. As the concentration factor (CF) was increased the moisture-in-non-fat solids and fat-in-dry matter content of the cheeses decreased (Table 1). At 5 months of age, the degree of proteolysis in the experimental cheeses was comparable with the control. The microstructures differed from the control in that the protein networks increased in coarseness (Fig. 2), with a corresponding decrease in the fat/protein interfacial area, as the CF increased (Table 1). Comparison of the changes in stiffness and sensory firmness with CF indicate that these measurements are related to each other but not directly to composition. The energy required to cut the cheese with a wire, the work of fracture, and the force required to break a cylinder, the impact force, both increased with CF. It seems probable that these measurements do relate to the microstructure, and possibly also the composition of the cheese. The requirement for more energy to fracture a coarser network is in accord with general fracture mechanics theory (Kinloch and Young, 1983).

However, the impact fracture surfaces of the 3 cheeses appear to be very different (Fig. 3). The control cheese was indented by the hammer for some 7 mm into the sample. Beyond this, the sample was cracked, with an irregular surface. The cheese from the 2.67-fold concentrated milk was indented for about the first 6 mm of penetration, though some pieces may also have been knocked from the surface. The further 2 mm penetrated by the hammer appeared to cause irregular cracking and loss of material. Beyond this, the sample showed a very irregular surface suggesting that cracking had occurred both round and through cheese grains. The cheese from the 3.14-fold concentrated milk was possibly cut for about the first 5 mm of penetration (not shown in Fig. 3). The hammer penetrated for about a further 1 mm, and in this section and beyond it...
Fig. 1. Comparison of critical point drying and etching after freezing for examination of Cheddar cheese fracture surfaces. a, c, critical point dried; b, d, etched after freezing; a, b, broken manually; c, d, cut. In each instance the fracture was from top to bottom of the micrograph.

appeared that the sample was mostly cracked along grain boundaries rather than through the grains.

Cheese analogues

Processed cheese analogues with 5-20% fat and 50-60% moisture in non-fat solids (MNFS) have been prepared by mixing SMP and sodium caseinate into a butteroil emulsion. The structures were not stable to fixing by glutaraldehyde followed by critical point drying, so SEM samples were examined frozen with etching to a constant appearance. The microstructure of a surface of the 5% fat analogue prepared by cutting with a wire is shown in Fig. 4. The surface was fairly smooth, comparable to that in Cheddar cheese prepared in a similar way.

The Young's modulus and the stress at fracture of analogues varying in composition were measured by compression applied at a medium rate. Both properties were highly correlated with a model containing both fat and MNFS contents ($P<0.001$, >80% of variance accounted for). The effects of composition are shown in 3-dimensional graphs (Figs. 5 and 6). The Young's modulus decreased as the fat content was increased at low MNFS (Fig. 5). However, at low fat levels, increasing MNFS caused a steady decline in Young's modulus. There was a central region, 13-20% fat and 50-60% MNFS, where the Young's modulus hardly altered. The stress at fracture was high at low fat and MNFS and decreased as either parameter increased (Fig. 6). However, above 10% fat and 55% MNFS, further change in composition had little effect on the stress at fracture.

For cheese analogues, the work to fracture was determined using wires of a range of diameters. The work done increased linearly
Mechanical properties, composition and microstructure

Table 1

Mechanical and structural properties of 5 month-old Cheddar cheeses made from heated, UF-concentrated milks

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Concentrates heated 90°C/15 s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x 2.67</td>
<td>x 3.14</td>
</tr>
<tr>
<td><strong>Composition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Moisture in non-fat solids</td>
<td>57.3</td>
<td>56.6</td>
</tr>
<tr>
<td>% Fat in dry matter</td>
<td>56.9</td>
<td>49.1</td>
</tr>
<tr>
<td>% Salt in moisture</td>
<td>4.2</td>
<td>4.6</td>
</tr>
<tr>
<td>pH</td>
<td>5.1</td>
<td>5.4</td>
</tr>
<tr>
<td><strong>Microstructure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat/protein interfacial area/unit vol., $\mu$m$^{-1} \times 10^{3}$</td>
<td>9.1$^a$</td>
<td>6.7$^a$</td>
</tr>
<tr>
<td>Coarseness of protein network</td>
<td>2.5$^c$</td>
<td>3.8$^d$</td>
</tr>
<tr>
<td><strong>Sensory properties</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Firmness</td>
<td>-0.2$^e$</td>
<td>-0.4$^e$</td>
</tr>
<tr>
<td>Graininess</td>
<td>-0.1$^g$</td>
<td>1.3$^h$</td>
</tr>
<tr>
<td><strong>Mechanical properties</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stiffness at 18°C, kNm$^{-2}$</td>
<td>640</td>
<td>600</td>
</tr>
<tr>
<td>Stiffness at 24°C, kNm$^{-2}$</td>
<td>370</td>
<td>320</td>
</tr>
<tr>
<td>Work of fracture, Jm$^{-2}$</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>Impact energy to break, J</td>
<td>0.33±0.06</td>
<td>0.57±0.08</td>
</tr>
</tbody>
</table>

Superscripts: Values for microstructure and sensory properties having different superscripts differ with a probability of at least 5%.

Fig. 2. Light micrographs of 5 month-old Cheddar cheeses made from heated, UF-concentrated milks stained for protein. a, control; b, milk concentrated x 2.67 and heated 90°C/15 s; c, milk concentrated x 3.14 and heated 90°C/15 s.
Fig. 3. Scanning electron micrographs of critical-point dried samples of 5 month-old Cheddar cheeses made from heated, UF-concentrated milks showing part of surfaces fractured in impact tests, where the fracture travelled from bottom to top. a, control; b, milk concentrated x 2.67 and heated 90°C/15 s; c, milk concentrated x 3.14 and heated 90°C/15 s; →, level of penetration of hammer.

Fig. 4. Scanning electron micrograph of surface of 5% fat cheese analogue cut with a wire. The sample was frozen and etched. The sample was cut from top to bottom.

Fig. 5. Three-dimensional graph of the variation of the Young's modulus in compression with the composition of cheese analogues.

Fig. 6. Three-dimensional graph of the variation of the stress at fracture in compression with the composition of cheese analogues.
Mechanical properties, composition and microstructure

Table 2: Energy to cut cheese analogues containing 50% MNFS but varying in butterfat content

<table>
<thead>
<tr>
<th>Fat content</th>
<th>Work of cutting Jm⁻²</th>
<th>Work of deformation Jm⁻³ x 10⁻⁸</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>7.5</td>
<td>0.17</td>
</tr>
<tr>
<td>12</td>
<td>4.4</td>
<td>0.12</td>
</tr>
<tr>
<td>20</td>
<td>2.0</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Fig. 7. Scanning electron micrograph of the concave fracture surface of a whey protein gel failed in tension.

With wire diameter. This is consistent with a model where the work to fracture is made up of 2 terms, the work used in deforming the sample and the work involved in creating the two new surfaces on either side of the cut (Atkins and Vincent, 1984). The first term would be some function of wire diameter and the second would be a parameter of the material. Thus, the work to make an infinitely thin cut through the cheese could be determined by projection to zero wire diameter. Using such a projection, it was found that both the work to cut a sample and the work to deform it decreased as the fat content increased (Table 2). This is presumably because the fat is softer than the aqueous matrix.

Heat-denatured protein gels

Whey protein gels, formed as cylinders, were tested to failure in compression and tension. In compression, strong samples failed by cracking obliquely forming a shear fracture. In tension, failure occurred across the sample forming a convex and a concave surface. The irregular stepped fracture surface (Fig. 7) indicated that failure occurred across a weak part of the protein network (Beachem, 1968).

The composition of the whey protein powder determined both the microstructure (Fig. 8) and the mechanical properties of the heat-denatured protein gel (Table 3). The cracks and voids in the microstructure are probably artefacts of the preparation. There were relatively few in Gel 35 compared with the other gels, presumably reflecting the high tensile strength. The tight, homogeneous matrix of Gel 12 could have been the cause of the relatively high resistance to compression. Gel 19 was stronger in compression than Gel 35, presumably because part of the matrix was tight and compact.

For powders containing less than 25 α-lactalbumin, the tensile strength and impact force to fracture increased with β-lactoglobulin concentration (Fig. 9). The fracture surfaces resulting from impact differed between gels of different composition and across the gels (Fig. 10). Both surfaces were irregular, without evidence of tearing indicating that they were formed as a result of
Table 3: Composition and mechanical properties of 15% gels formed by heating whey protein solutions

<table>
<thead>
<tr>
<th>Powder</th>
<th>12</th>
<th>19</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-lactalbumin</td>
<td>0</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>(\beta)-lactoglobulin</td>
<td>70</td>
<td>40</td>
<td>55</td>
</tr>
<tr>
<td>Casein-derived protein</td>
<td>30</td>
<td>46</td>
<td>36</td>
</tr>
</tbody>
</table>

**Mechanical properties, kN m\(^{-2}\)**

<table>
<thead>
<tr>
<th></th>
<th>12</th>
<th>19</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tensile strength</td>
<td>9.5 \pm 1.5</td>
<td>9.4 \pm 1.6</td>
<td>20.3 \pm 1.2</td>
</tr>
<tr>
<td>Stress at fracture in compression</td>
<td>91.2 \pm 14.3</td>
<td>43.2 \pm 1.3</td>
<td>27.5 \pm 1.4</td>
</tr>
<tr>
<td>Young's modulus in compression</td>
<td>24.0 \pm 1.2</td>
<td>10.0 \pm 0.6</td>
<td>3.0 \pm 0.4</td>
</tr>
</tbody>
</table>

Fig. 9. Tensile strength (•) and impact force to fracture (○) of 15% gels made from whey protein solutions containing \(\beta\)-lactoglobulin and casein derived protein only.

shown by the difference between the elastic moduli when they are compressed along or perpendicularly to the cylinder axis (Fig. 11). This is consistent with a tighter, more rigid structure on the outside of the gel cylinder, such as observed on a radial motor tyre.

**Discussion**

A well-known problem in many kinds of microscopy is that specimen preparation methods may generate artefacts. This could well apply to the examination of surfaces formed in a hydrated state and observed after drying, as was carried out by Green et al. (1985) on fractured cheeses. In the present work, similar surfaces were examined in the hydrated state by a cryo technique. Although this did not give as much detail as the dried specimens, the general structure and regularity of the complementary surfaces was comparable, indicating that critical point drying did not generate excessive artefacts.

Further, the cryo technique was essential for the examination of fracture surfaces in cheese analogues, since the specimens were not stable to fixing and drying. The technique is also likely to be useful for delicate, highly hydrated specimens such as protein gels, although attention will need to be paid to the freezing rate to avoid distortion of the network (Hermansson and Buchheim, 1981).

When Cheddar cheeses were subjected to impact testing, the surfaces formed suggested that the structures had failed in different ways. Thus, the fracture mechanisms varied across the range of samples, so the impact energies were not strictly comparable and could not be used to give information on the variations in microstructure. Further, failure must occur at a weak plane, which can be through or round the grains in the case of cheese. Thus, the surface formed is not necessarily typical of the material as a whole. These observations suggest that it is important to take account of the method used to measure fracture properties when the results of failure tests on different materials are...
Mechanical properties, composition and microstructure

Fig. 10. Scanning electron micrographs of impact fracture surface of 15% whey protein gels at 2 points across the surface. The whey protein powder for Gel A contained 83% \(\beta\)-lactoglobulin + 17% casein-derived protein and that for Gel D contained 44% \(\beta\)-lactoglobulin + 56% casein-derived protein. The direction of fracture was from left to right. →, crater. Surface locations: 1, at edge where impact occurred; 2, in centre.

The results obtained here suggest that controlled cutting of a sample with a wire may be a useful mechanical property to study. The morphology of the surface created confirms that the plane of cutting was not dependent on weak planes within the sample. The work with cheese analogues reported here and that of Emmons et al. (1980) with experimental cheeses suggest that the results are simple to interpret and can be related to the composition of the sample. However, only rather slow rates of cutting appear to have been used so far in work with foods. This may be useful in relation to

Fig. 11. Elastic moduli of gel cylinders of the concentration of whey protein shown compressed along (♀) and perpendicular to (○) axis of cylinder.
handling of the materials, but is unlikely to provide good simulation of biting. For this, cutting rates of the order of 20 mm s\(^{-1}\) are likely to be required (Bourne, 1982), which could raise problems not found at lower rates. Provided there is no failure of the specimen, equilibrium deformation properties would be expected to be closely related to both the composition and the microstructure. However, the results with all the types of samples tested here indicates that the relationships were not simple, though they tended to follow the expected trends. Hermansson (1982) made a similar observation finding that whey protein gels having a denser, more aggregated structure tended to be firmer.

Acknowledgements

The authors thank Dr A.C. Smith of AFRC Institute of Food Research, Norwich Laboratory, for assistance with the use of his impact tester, Misses R. Oakman and E.C. Pope and Messrs J.C.D. Taylor and P. Dunthorne for skilled technical assistance and Mr E. Florence and his staff for gross chemical analyses.

References


Discussion with Reviewers

Reviewer 1: The authors discuss surfaces of cheese, cheese analogues and whey protein gels. If the surfaces were created prior to preparation by critical point drying and freezing the question arises how the surface structure was affected by the various steps during preparation?

Authors: We have no definitive answer. However, the complementary fracture surfaces prepared in the two ways and shown in Fig. 1 are recognizably similar, suggesting that the structures were not too much affected by the preparation.

Reviewer 2: The results mainly indicate that impact fracture could result in a combination of cutting and cracking due to the conditions during fracture as well as to the sample tested. Will it be possible to explain the relationships between the different mechanical properties of the samples and variations in their microstructure by work at higher magnifications?

Authors: We have not found this very useful. A general appearance of the whole fracture...
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surface has enabled us to detect the various characteristics of the different types of failure. At higher magnification the details of the structure tend to obscure the major features. However, there is obviously the possibility of gaining information about specific features by examination at a range of magnifications.

D. E. Carpenter: You mention that the degree of proteolysis was the same in control and experimental cheese. How was this assessed? Since less coagulant is added for the concentrated milk cheese, isn't this unexpected? (Green, 1985).

Authors: Proteolysis was assessed by polyacrylamide gel electrophoresis, measuring casein disappearance, and by concentration of trichloroacetic acid soluble N, measuring peptides and amino acids. Less coagulant was used for concentrated milk cheese than for controls, but more than for unheated concentrates. As the proportion of cheese: whey is increased by the use of concentrates, a higher proportion of coagulant may be retained in the curd than for the controls. It seems that the differences approximately balanced out in the present instance.

D. E. Carpenter: Could microstructural coarseness be quantified with units for frequency of troughs/peaks so that data could be related from various papers?

Authors: Yes, but what constitutes a peak or a trough, since there are variations in the background stain level across light micrographs? This method has been tried (Green et al., 1981) but is no quicker and less widely applicable than the method used in the present paper. Neither method is absolute, so the data are necessarily comparative. However, the ratios of two values should be the same regardless of the quantitation method used.

D. E. Carpenter: The composition and pH of cheese made from concentrated milk must play a large role in texture, i.e., x 2.67 cheese has a pH of 5.4, and the x 3.14 cheese has a low fat content which must contribute to its firmness.

Authors: Yes. In cheesemaking experiments it is difficult to precisely control composition, especially when using modified milks. The main purpose of using cheese analogues was to control composition and reduce the variables resulting from the use of biological materials.

D. E. Carpenter: What effect would the 90°C, 15s milk heat treatment have on the textural properties of the experimental cheese? One would expect some whey protein denaturation at this temperature with resulting textural/microstructural changes.

Authors: This is under investigation currently.

D. D. Hamann: Many of us are not very experienced with the interpretation of SEM photographs of foods as related to texture. Has the field developed to the point that a systematic approach of what to look for has been established for specific foods such as Cheddar cheese? If not can the start of such a system be based on the results of this paper?

Authors: We now have considerable information on some of the structural influences on the texture of cheese. The distribution of fat and protein clearly affects some aspects of mouthfeel (Green et al., 1981), and their orientations influence some rheological properties (Taran et al., M. V., Wan, P.J., Chen, S.I., Rhee, K.C. (1979) Scanning Electron Microsc. 1979; III: 273-278). Complete fusion of the protein matrix appears to cause crumbliness in Cheddar cheese (Marshall, R.J. (1986) J. Dairy Res. 53: 313-322). The degree of interaction between curd particles at curd junctions, which probably relates to the presence of cracks and voids, influences the uniformity of Cheddar cheese body (Lowrie, R.J., Kalab, W, Nicholas, D. (1982) J. Dairy Sci. 65: 1122-1129). However, most of this information is qualitative, and we really have no means yet of assessing the degree of importance of different structural factors. The problem is exacerbated by the difficulty of making useful measurements of cheese texture.

M.A. Tung: In the small strain compressive tests of Cheddar cheese cubes the loading curves were said to be linear while repeated loading and unloading curves exhibited about 50% hysteresis which implies that the curves are not closed. However, the degree of interaction between curd particles at curd junctions, which probably relates to the presence of cracks and voids, influences the uniformity of Cheddar cheese body (Marshall, R.J. (1986) J. Dairy Res. 53: 313-322). The degree of interaction between curd particles at curd junctions, which probably relates to the presence of cracks and voids, influences the uniformity of Cheddar cheese body (Marshall, R.J. (1986) J. Dairy Res. 53: 313-322). The degree of interaction between curd particles at curd junctions, which probably relates to the presence of cracks and voids, influences the uniformity of Cheddar cheese body (Marshall, R.J. (1986) J. Dairy Res. 53: 313-322).

Reviewer 1: In the "Materials and Methods" section the authors state that "Since the electron scattering properties were poor...TEM of whey protein gels did not provide adequate information and thin sections of these specimens were examined by SEM." It is difficult to understand why primary transmitted electrons did not give adequate information with regard to whey protein gels. Thin sections of whey protein gels generally give good contrast and these sentences are therefore better omitted.

Authors: Sections do give good contrast but the images obtained are probably from only part of the total organic matrix. The scattering power depends on the attachment of electron dense atoms to protein molecules. There is no guarantee that this will occur in such a way as to render the entire molecule visible. TEM of protein gels suffers from this. The secondary electron emission, especially after coating with gold, is from the entire organic matrix.
as in the original cream sample. In contrast, a cream having a coffee stability of 55°C (cream sample No. 4 after 2 months of storage) showed a pronounced aggregation of fat globules when added to a coffee solution of 45°C (Fig. 8).

Further information on the fine structures of large floccules of cream is obtained by applying the freeze-deep-etching technique. This preparatory modification requires, however, the isolation of cream floccules and the removal of soluble constituents (e.g., by washing with distilled water). Figs. 9a and 10a give representative views of parts of large floccules which formed in a coffee solution of 90°C with those 2 cream samples which have already been compared above at 45°C, i.e., sample No. 6 (Fig. 9) and No. 4 (Fig. 10). Although both samples differ in their coffee stability values (No. 6: 80°C and No. 4: 55°C) the overall appearance of the fine structure of the floccules is similar. It is a network of strongly aggregated fat globules consisting of strands of globules of approx. 1 μm in thickness. It appears as if the individual globules have largely retained their original size, i.e., that destabilization phenomena occur at least rarely. At higher magnification the fine structure of the interfacial protein layer can be examined in more detail (Figs. 9b and 10b). The globules appear to be predominantly covered, similar to the original cream, by a smooth and very thin protein film to which occasionally somewhat larger protein aggregates (casein micelles) are attached. Pronounced differences between the 2 samples under study could not be detected with the experimental conditions used.

**Creaming phenomena**

The formation of compact layers (plugs) at the top of a cream sample during storage is an only rarely occurring phenomenon. Such layers are highly undesirable because they will generally not disperse when the cream is added to a coffee solution. Electron microscopic examination of such cream plugs of one cream sample revealed that they consisted of very densely aggregated fat globules (Fig. 11) and even contained larger particles of destabilized fat (Fig. 12). The cream sample showing this peculiar phenomenon exhibited a major degree of clustering of fat globules (Fig. 13) although the average size of individual globules was typical for a well homogenized product.

A pronounced clustering of fat globules in a homogenized cream may be a result of inadequate homogenizing conditions such as a non-optimum temperature or an imperfectly operating homogenizing valve. Furthermore the formation of cream layers during storage is facilitated by a low degree of homogenization, i.e., (volume moment average) fat globule diameters (d₄₃) higher than ca. 0.7 μm.

**Sediment formation**

The formation of a rather compact sediment layer is another undesired phenomenon which may occur, although rarely, in UHT-treated coffee creams. According to our experience such sediments develop slowly and may not become visible until 1-2 months of storage but then increase during further storage. The sediment may consist of a solid layer, up to approx. 1 mm in thickness, which will not dissolve when the cream is added to hot coffee. In water (at room temperature) small pieces of such a sediment may remain intact even after intensive stirring.

**Fig. 7.** Freeze-fracture preparation of a mixture of cream (No. 6; coffee stability value: 80°C) with a coffee solution of 45°C showing very little aggregation of fat globules (f); p: protein particles.

**Fig. 8.** Freeze-fracture preparation of a mixture of cream (No. 4; coffee stability value: 55°C) with a coffee solution of 45°C showing a pronounced, but visually not detectable aggregation of fat globules (f).
Ultrastructure of coffee cream

Fig. 9. Freeze-deep-etched floccules from a cream (No. 6) after feathering. a: general views of the network of aggregated fat globules. b: detailed views showing the interfacial protein layers (ip). Sometimes the fat phase becomes partially exposed as a result of local cleavage during freeze-fracturing (f).

Fig. 10. Freeze-deep-etched floccules from a cream (No. 4) after feathering.

Fig. 11. Aggregated complexes of fat (f) and protein (p) of a cream plug.

Fig. 12. Destabilized fat within a cream plug.
The coffee cream sample which showed this peculiar sedimentation phenomenon had an elevated pH-value (pH 7.05). According to the information which could be obtained from the manufacturer, stabilizing salts (a phosphate-citrate mixture) had been added continuously to the cream immediately before it underwent the UHT-treatment (homogenization was done after the thermal treatment under aseptic conditions). Possibly such processing conditions favour the formation of large protein aggregates at the high temperatures (ca 140°C) occurring in the UHT plant. A direct injection of a highly concentrated solution of basic salts immediately before the heating step will probably not allow an equilibrium state of all milk salts to build up and may furthermore result in an uneven distribution of salts (and pH-value) within the cream flow in the heating section.

Gelation

It is well known that UHT-treated, homogenized cream as well as UHT milk show gelation phenomena after prolonged storage. These phenomena are generally ascribed to the action of proteolytic enzymes which have not completely been inactivated during the heat treatment, and also to purely chemical mechanisms, such as the Maillard type of reaction (3, 9).

The viscosity measurements made from the 7 commercial coffee cream samples (Fig. 1) demonstrate that the pH-value of the cream appears to be an additional factor leading to a quicker gelation at higher pH-values.

Electron microscopical studies were made on two gelled samples of UHT-treated coffee cream (12 % fat) after a storage period of 10 months at room temperatures. Fig. 15 is from a product made without any stabilizing salts having a pH-value of 6.72. The gel consisted primarily of chains and loose aggregates of fat-protein complexes. Fig. 16 is from a product to which basic phosphates and citrates had been added resulting in a pH-value of 7.05. The fine structure of the gel is distinctly different, i.e. it appears to consist mainly of interconnected protein particles, smaller protein aggregates and, to a lesser extent, fat globules (fat-protein complexes). In some parts of Fig. 16 the small protein particles (subunits) appear to be arranged in small rows indicating that the formation of thin protein strands (fibres) play a major structural role for the gel network. The decisive differences between the gel structures of both cream samples may be explained by the differences in the protein-fat interaction. Whereas in the cream without stabilizing salts most of the protein (casein and denatured whey protein) is generally adsorbed to fat globules (compare to Fig. 2) the addition of pH-elevating stabilizing salts results in a disintegration of protein aggregates (casein micelles), a distinct decrease of protein adsorption and, consequently, an increase in 'free' (i.e. non-adsorbed) protein in the serum (compare to Fig. 3). This may be the reason that in the cream sample with the higher pH-value these 'free' protein particles are the major constituents of the gel network whereas in the other cream sample, in which 'free' protein particles are comparatively rare, the fat-protein complexes interact and constitute the network structure.

Conclusion

The results of the comparative studies on commercial UHT-treated coffee creams have shown that this type of product requires further research in order to develop optimum processing conditions. The resistance against feathering in hot coffee solutions appears to be still a major problem.

There is no doubt that various compositional factors (e.g. fat content, fat/protein ratio, mineral balance) and processing parameters (e.g. homogenization and heating conditions) influence the final physico-chemical properties. From the results obtained it appears the composition and structure of the interfacial layers of fat globules are mainly responsible for the extent of flocculation in hot coffee solutions. Therefore special attention will be given to this aspect during further studies.

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Ultrastructure of coffee cream

Fig. 13. Fat globule cluster in a cream sample which had formed a cream plug.

Fig. 14. Sediment layer from a coffee cream sample (pH 7.05) showing the presence of larger and obviously interconnected protein aggregates (p).

Fig. 15. Gelled coffee cream sample (pH 6.72). The network appears to be formed by loosely aggregated complexes of fat globules (f) and protein (p).

Fig. 16. Gelled coffee cream sample (pH 7.05). The network appears to consist primarily of loosely aggregated protein (p) and occasionally fat globules (f).


Discussion with Reviewers

B.E. Brooker: As a result of these observations, I wonder if you would care to comment on possible mechanisms to explain the heat instability of the fat-casein complex in UHT cream. This appears to be one of the central questions in this problem.
Authors: Because of the numerous factors which have been found to be related to the resistance of a homogenized, UHT-treated cream against feathering it is still very difficult to give a satisfying explanation of this phenomenon.

I. Heerdtje: Is there not a danger that under the influence of the washing regime in the deep-etching technique a redistribution of protein occurs, leading to wrong interpretations? It may be argued that in particular larger aggregates attached to the interface layer have a chance to be removed (Ca concentration during washing is 0).

Authors: It is justified to suggest that the washing in distilled water might eventually lead to a redistribution of protein. We are therefore trying to improve the washing procedure, e.g. by using at first buffered solutions and also to introduce a chemical fixation of the protein (by glutaraldehyde).

I. Heerdtje: Sediment formation and gelation appear to be related phenomena. Should you not consider the possibility that the difference in behaviour between the pH 7.0 samples should be ascribed to the difference in the heat induced association of whey proteins (in particular beta-lactoglobulin) and casein micelles. At pH 7.05 there will be no interaction, whereas at pH 6.5 the interaction is nearly complete. This may lead to an enhanced possibility for interaction of caseins with itself at high pH and perhaps to an enhanced possibility for interaction of the beta-lactoglobulin-casein complex with the interfacial layer at lower pH. What is your comment?

Authors: It remains questionable whether sediment formation and gelation are related phenomena. It should be pointed out that the formation of unusually large protein aggregates which sedimented during storage (see Fig. 14) took place only irregularly at one manufacturer who added pH-elevating salts to his product. Normally, aggregated protein (casein micelles) disintegrates as a result of calcium complexing salts at pH-values above ca 6.9. The pH-dependent differences in the heat-induced association of whey proteins and caseins at low and high pH-values (6.5 v. 7.0) might well explain the observed differences in the rate of gelation.

W.G. Soucie: What was the time allowed for the coffee stability testing? Are you assuming that a lower temperature will slow aggregation but not stop it?

Authors: When a hot coffee solution is poured over the cream (or vice versa) the phenomenon of feathering occurs almost immediately. A pronounced increase in the average size of flocules does not take place in the mixture of coffee and cream except when larger flocules rise up to the surface (see Fig. 6). At a temperature lower or equal to the 'coffee stability' value visible floculation will generally not occur also after a longer time.

W.G. Soucie: How did you determine that 15-20 min is long enough for glycerol to diffuse into the fine structure areas of the aggregated samples?

Authors: It is common experience that the diffusion of glycerol into small volumes of such types of samples is completed within such a period. An insufficient penetration of the cryoprotectant would result in freeze-artifacts, i.e., ice crystals which are easily detectable on the micrographs.

W.G. Soucie: The 'free' salts and protein are in equilibrium with the fat globule membrane. With the washing procedure affect the fat globule membrane and was there any evidence of fat release?

Authors: There was no evidence that the washing procedure used resulted in a destabilization of fat globules. It appears, however, necessary to study the effects of washing on the structure of interfacial layers systematically in order to avoid misinterpretations.

W.G. Soucie: In your particle size determination how did you account for the fact that the fracture lines will slice through the spheres at different levels and not only at the maximum diameter? How do your fat globule sizes compare to published results using similar homogenization conditions?

Authors: The conversion of apparent (two-dimensional) size distributions into true (three-dimensional) size distributions is described in the literature (see e.g. Ref. 13). The fat globule sizes (e.g. d_{32} and d_{43}) which have been found for the various cream samples compare well with published results for commonly used homogenization conditions (ca 200 bar).

W.G. Soucie: Addition of salts, changes in pH and temperature all affect the zeta potential of particles and consequently stability to aggregation. How might your results be interpreted when considering the effect of these factors on the zeta potential of the fat globule surfaces?

Authors: It appears as if the composition and structure of the interfacial protein layers, i.e. complexes formed by casein, denatured whey protein and calcium phosphate, are mainly responsible for the degree of stability of the homogenized creams under the acidic conditions of hot coffee solutions. Hence, the zeta potential of the fat globule surfaces might be an important factor. So far as I know zetapotential measurements of such systems have not yet been published.

W. Buchheim, G. Falk, and A. Hinz
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