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EFFECTS OF PHOSPHATE AND CITRATE ON THE GELATION PROPERTIES OF CASEIN MICELLES IN RENNETED ULTRA-HIGH TEMPERATURE (UHT) STERILIZED CONCENTRATED MILK

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Abstract

Milk was concentrated to 3X (volume reduction) by ultrafiltration. Disodium phosphate and sodium citrate were added, and the milk concentrates were homogenized. The concentrates were then heated at 135°C for 50 s in a laboratory ultra-high temperature (UHT) heating system. Rennet gels were made from heated and unheated milk concentrates and their curd firmness measured using a Formagraph. Gel microstructures were examined by electron microscopy.

When rennet was added to unhomogenized milk concentrate before UHT heating, the resultant gel consisted of a strong protein network that encapsulated the fat globules. Pockets of milk serum were associated with the fat. Homogenization caused the fat droplets to be coated with casein micelles and become tied into the protein network as an integral part of the gel structure.

The microstructure of UHT milk concentrate gels was different from gels made from unheated milk. Gelation of UHT milk proceeded more slowly and the gels were weaker. Much of the casein in such samples had lost their micellar identity and was present as a homogeneous mass around the fat droplets. Large areas in the gel lacked protein network, which weakened the UHT milk gels. Samples with disodium phosphate added did not gel after UHT treatment, even if high concentrations of rennet were added. Samples with sodium citrate added formed only a weak rennet gel after UHT treatment.

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Introduction

Ultra-high temperature (UHT) processing of concentrated milks is of interest to the dairy industry but is impeded by the age gelation that occurs during storage of UHT milk concentrates at room temperature. There have been many studies of age gelation of UHT milk (Harwalkar, 1982) but as yet there is no unifying theory on the mechanism that causes such age gelation in concentrated milks or how it can be prevented. In unconcentrated milk, proteolysis by native and bacterial heat-resistant proteinases has been implicated as the cause of age gelation (Kohlmann *et al.*, 1988). A mechanism involving only physico-chemical reactions has also been suggested (Andrews and Cheeseman, 1971). As milk is concentrated, non-enzymic reactions become of greater importance because the mean free path between casein micelles, and other protein and ionic particles, is reduced.

Various additives to UHT concentrated milk have been studied for their effectiveness in retarding age gelation. These include a variety of compounds that sequester calcium: disodium EDTA, sodium citrate, disodium phosphate and sodium hexametaphosphate. Kocak and Zadow (1985) observed that adding sodium citrate or disodium EDTA to milk accelerated age gelation while adding sodium hexametaphosphate caused a six-fold extension in shelf life of UHT concentrated milk. They suggested that age gelation in UHT concentrated milks was a two-step mechanism because when they added sodium hexametaphosphate to their samples age gelation was delayed but the extent and rate of proteolysis were not affected. Sodium hexametaphosphate thus inhibits the second stage of protein aggregation that occurs during age gelation.

The incentive to manufacture a UHT milk concentrate is that it can be exported to markets that have insufficient indigenous milk production. Considerable cost savings are obtained by transporting a concentrate because of its reduced volume. There are already some frozen milk concentrates being shipped from milk-surplus countries such as Australia to milk-deficient countries along the Pacific rim. However, the full advantage of UHT milk concentrate cannot be taken until the problem of age gelation during storage at ambient temperatures is solved.

Such a concentrate could be reconstituted with water for local use as fluid milk or used for the manufacture of fresh cheese products. If additives, such as polyphosphates, are used to inhibit age gelation, it is essential to know how their use would affect the suitability of UHT milk concentrates as a starting material for cheese production. A critical factor in cheesemaking is the coagulation characteristics of the milk. Successful use of UHT milk concentrates would depend on their having stability against gelation during storage while retaining adequate coagulation properties upon renneting.

In the manufacture of cheese, coagulation of milk is caused by enzymically hydrolyzing a labile peptide bond (Phe_{105}–Met_{106}) in κ-casein. Cheesemaking enzymes (rennets) are chosen for their specificity to hydrolyze this bond (Shaker and Brown, 1985). In contrast, the proteinases that have been implicated in age gelation of UHT milk do not have the same specificity to hydrolyze κ-casein and often cause extensive hydrolysis of other caseins.

When the rennet-sensitive Phe_{105}–Met_{106} bond in κ-casein is hydrolyzed, casein micelle stability is reduced and a gel is formed in the presence of calcium. Enzymic coagulation of milk occurs via a series of overlapping reactions (McMahon and Brown, 1984). Hydrolysis of κ-casein releases a macropeptide and exposes a hydrophobic region of κ-casein on the micelle surface. This initiates the coagulation reaction. The surface of the casein micelles becomes reactive in proportion to the extent of hydrolysed κ-casein and the micelles then aggregate in accordance with the von Smoluchowski mechanism. After sufficient hydrolysis of κ-casein, this aggregation results in gelation and formation of a space network of casein micelles. During gelation, additional casein micelles are incorporated into the gel network and rearrangements of the network structure can occur. If the milk gel is stressed or fractured, part of the serum entrapped within the gel network is expelled and the casein particles fuse and become consolidated into thick strands.

In milk, κ-casein is distributed throughout casein micelles but with a predominance on the micelle surface (Heth and Swaisgood, 1982). Hydrolysis of one κ-casein molecule is not enough to cause aggregation of the micelles but rather sufficient hydrolysis must occur to create reactive zones on the micelle surface through which aggregation between micelles takes place.

When milk is heated beyond pasteurization conditions (72°C for 15 s), β-lactoglobulin is denatured and forms a complex with κ-casein (Hooydonk, *et al.*, 1987; Zittle *et al.*, 1962). This heat-induced interaction causes the casein micelles to become covered with denatured β-lactoglobulin (and α-lactalbumin) and retards the rennet coagulation of milk. Hydrolysis of κ-casein is inhibited and aggregation of the renneted casein micelles is reduced. Even a mild heat treatment such as 15 s at 95°C, that denatures only 25% of the whey proteins, delays micelle aggregation and reduces the rate of curd-firming. Because of this, it is difficult to make cheese from over-heated milk because the curd is too soft and retains excess moisture.

Milk coagulation is also affected by calcium and phosphate concentrations, pH, ionic strength, and temperature (Yamauchi and Yoneda, 1978). When 0.1% sodium phosphate is added to milk before renneting, its coagulation time is shortened by 10% (McMahon *et al.*, 1984). This occurs even though it would be expected that by sequestering Ca^{2+} the coagulation time would be retarded. It shows that phosphate ions plays an additional role in casein micelle aggregation, possibly through interaction with calcium bound to phosphoester groups of β-casein (Yun *et al.*, 1982; Zittle, 1970). The effects of adding sodium citrate and sodium ortho- and polyphosphates on the rennet coagulation of UHT milk concentrates were the focus of this study.

Materials and Methods

Ultrafiltration

Whole milk was obtained from the Utah State University Dairy Products Laboratory. It was ultrafiltered (UF) to 3X concentration (volume reduction) using an Abcor spiral wound, polysulfone membrane (5 m^2 membrane area, 10,000 nominal molecular mass cutoff, Koch Membrane Systems, Wilmington, MA). To portions of this milk were added 0.1% (w/v) of disodium phosphate (DSP), sodium hexametaphosphate (SHMP) or trisodium citrate (TSC). Each batch of milk concentrate was then homogenized (Model 3DDL Homogenizer, Crepaco Inc, Chicago, IL) at 13.8 MPa with 3.4 MPa second stage pressure before UHT processing. A sample of unhomogenized milk was retained as a control for comparison of renneting properties with the homogenized samples.

Ultra-High Temperature Processing

Milk was heated to 135°C in a laboratory scale UHT processor consisting of 6 mm ID stainless steel tubing based on the design of Wadsworth and Bassette (1985). Milk was placed in a beverage-syrup container and pressurized to 0.41 MPa using nitrogen gas. This prevented vaporization of water from the milk in contact with the hot surface of the tubing immersed in a 160°C oil bath. Flow of milk was maintained at 100 mL/min using a variable speed pump and product line pressure was maintained at approximately 0.55 MPa using an adjustable back pressure valve positioned after the cooling section of the processor. Heating was achieved as milk passed through sections of the tubing immersed in a water bath for preheating and an oil bath for UHT heating. The preheat temperature was 72°C with a 20 s hold time, and the UHT temperature of 135°C was reached over 120 s. An insulated section of tubing provided a UHT hold time of 50 s. A 2°C drop in temperature occurred over this hold time. The next section of tubing was immersed in a cold water bath (10°C) to cool the milk to 30°C before filling into sterile 120 mL plastic containers in a pressurized and sanitized glove box.

Coagulation Measurements

Coagulation properties of the various milk samples were determined using the Formagraph method (McMahon and Brown, 1982). In each sample well, 10.5 g of 3X milk was tempered at 35°C for 30 min. Calf rennet (New Zealand Co-operative Rennet Co., Eltham, New Zealand, 146 Rennin Units (RU)/mL) was diluted with distilled water to 0.5, 1, 2 and 4 RU/mL. At zero time on the Formagraph recorder, 200 μL of diluted rennet was added to the milk, the samples were mixed thoroughly and changes in curd firmness were monitored. Samples of homogenized 3X milk before and after UHT treatment, and unheated unhomogenized 3X milk, were analyzed for comparison of coagulation properties.

Electron Microscopy

Samples of the milk gel (approximately 10 × 1 × 1 mm) were initially fixed for 2 h in 2.5% aqueous glutaraldehyde solution then sent to Ottawa for electron microscopy (Allan-Wojtas, 1984).

For scanning electron microscopy (SEM), the fixed samples were dehydrated in a graded ethanol series. Fat was removed by extraction with chloroform. The samples were then impregnated with absolute ethanol, frozen in Freon 12 at –150°C, and freeze-fractured under liquid nitrogen. The fragments were thawed in ethanol, critical-point dried from carbon dioxide, mounted on SEM stubs, sputter-coated with gold, and examined at 20 kV in an ISI DS-130 scanning electron microscope with an external oscilloscope (Bond and Kaláb, 1988). Micrographs were taken on 35 mm film.

For transmission electron microscopy (TEM), the fixed samples were postfixed in 2% osmium tetroxide in a 0.05 *M* veronal-acetate buffer, pH 6.75. The postfixed samples were embedded in a Spurr's low viscosity medium (J.B. EM Service, Pointe Claire, Dorval, Quebec, Canada). Thin sections were stained with uranyl acetate and lead citrate solutions (Reynolds, 1963) and examined in a Philips EM-300 electron microscope operated at 60 kV.

Results and Discussion

Effect of Homogenization of Milk Fat Globules

In milk, the casein micelles and the fat globules co-exist independently of each other (Henstra and Schmidt, 1970). This is no longer the case when milk is homogenized. The size of the fat globules is reduced, because of the intense turbulence and cavitation that occurs during homogenization, from an average diameter of 5–6 μm in native milk to <2 μm after homogenization (Jackson and Brunner, 1960). This increases the fat interfacial surface by a factor of 5–6. Simultaneously, fat globules become coated with a layer of casein and other milk proteins creating a new membrane at the interface between fat droplets and the serum phase of milk (Fenwick, 1971). This interfacial membrane consists of a protein composite containing casein micelles, casein micelle sub-units, and non-micellar protein (Darling and Butcher, 1978)

The strength of casein binding to the fat–water interface is greater than that of β-lactoglobulin and α-lactalbumin, these whey proteins are only bound to the interface if milk is heated after homogenization. The caseins are relatively flexible, open-structured molecules whereas undenatured whey proteins are globular and exhibit highly ordered structure. In an aqueous environment, the lipophilic domains of the caseins are free to interact with any exposed fat surface. So, when a new fat–serum interface is formed, the binding of flexible caseins to the fat surface is stronger than that of the inflexible undenatured whey proteins.

The way in which the casein micelles cover the surfaces of fat globules suggests that they spread during homogenization (Henstra and Schmidt, 1970). Some of the micelles are dissociated and the fat globules are partly covered by micellar sub-units (Walstra, 1980). After homogenization, the presence of these micellar sub-units at the interface implies that creation of the new interface disrupts casein micelles into sub-units. This is caused not by the pressure drop that milk undergoes during homogenization but rather by the interfacial forces generated by creation of the new interface. These interfacial forces are sufficient to disrupt micelles into sub-units. The sub-units, however, appear to stay intact on adsorption onto the interface (Darling and Butcher, 1978).

Thus, the consequence of homogenization is that fat globules acquire surface properties characteristic of casein micelles (Dalgleish, 1984). For example, native fat globules have ζ-potentials of –10 mV while homogenized fat globules have electrophoretic mobilities equivalent to casein micelles with ζ-potentials in the range –17 to –13 mV. The role of fat globules during milk coagulation is thus different when homogenized milk is renneted compared to unhomogenized milk.

Effect of Homogenization on Coagulation

When rennet was added to homogenized 3X UF milk, the milk coagulated more quickly than a corresponding non-homogenized milk concentrate (Fig. 1). Robson and Dalgleish (1984) reported that coagulation of homogenized milk is controlled in general by the same factors as skim milk even though coagulation time is shorter for homogenized milk than skim milk. They found that homogenization does not make κ-casein more susceptible to enzyme hydrolysis and the maximal rate of aggregation for both skim and homogenized milks is achieved after about the same exposure to rennet. Differences in coagulation times were attributed to a smaller critical extent of hydrolysis being needed to start coagulation of homogenized milk compared to unhomogenized milk. The reason is that κ-casein spreads over the surface of homogenized fat particles. And, whereas one κ-casein molecule covers ~40 nm^2 of the surface of native casein micelles, the surface of homogenized fat particles covered

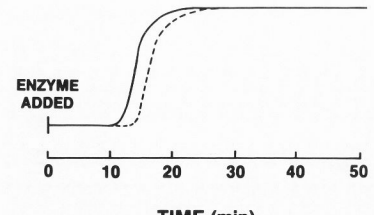


Figure 1. Diagram of curd firming of unhomogenized and homogenized 3X UF milk concentrates (non-UHT heated) at 35°C. Curd firmness (y axis) was recorded with a Formagraph as a function of time after adding 0.02 RU/g of rennet; —— homogenized, – – – unhomogenized.

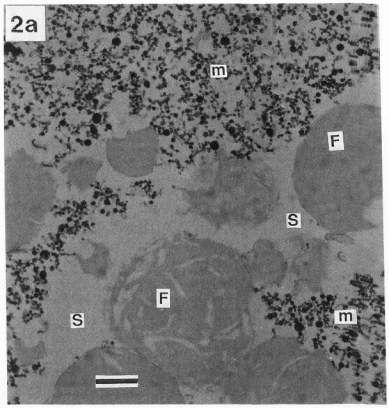
with one κ-casein molecule is almost twice as large (~80 nm^2). As κ-casein is spread more thinly, its stabilizing power is reduced because a larger particle area is deprived of this stabilizing protein when κ-casein is hydrolyzed.

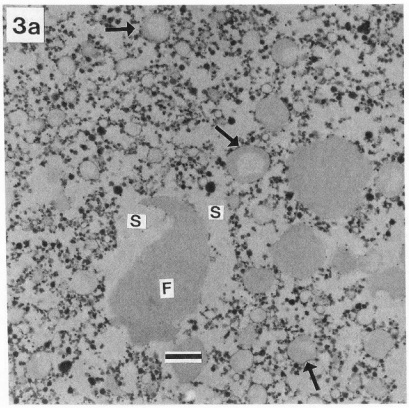
Green *et al.* (1983) observed that the rate of casein micelle aggregation was reduced in homogenized milk treated with calf rennet. Robson and Dalgleish (1984) calculated that the von Smoluchowski rate constant for milk coagulation is two orders of magnitude smaller for homogenized milk than for skim milk. This is because the interactions between fat and casein in homogenized milk cause the fat to become part of the casein network that forms the milk gel. This increases the volume of the network relative to that of the serum interstices and reduces the ease of movement of the network strands. Therefore, whey loss from cheese curd made from homogenized milk occurs at a slower rate than that made from unhomogenized milk(Green *et al.*, 1983). More moisture is retained in the cheese and a softer, smoother and more elastic cheese is obtained. Some of these characteristics of cheese made from homogenized milk are undesirable but there is potential that they may be improved by using concentrated milk.

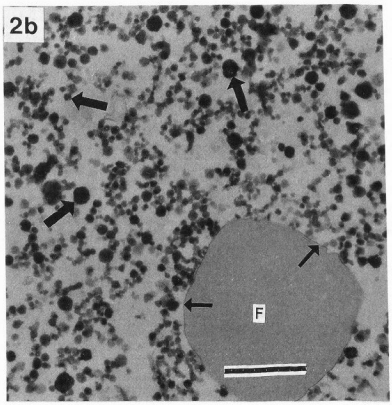
No differences in the firmness of curd made from unhomogenized and homogenized 3X milk were observed (Fig. 1). This is in contrast to a much weaker curd produced by renneting homogenized milk. The increased solids content of 3X milk produces a gel network of much greater volume density so that any further changes because of incorporation of fat into the network are not significant in increasing curd firmness.

After renneting, the casein micelles of unhomogenized 3X milk formed a dense, uniform gel network interspersed with fat globules and void spaces containing milk serum (Figs. 2a and 2b). Chains of micelles were close to the fat globules but had not penetrated onto the fat–water interface. The fat globules remained coated with intact fat globule membrane (Fig. 2b) and were often clustered together and associated with pockets of milk serum as reported by Gavaric *et al.* (1989). Frequently, the fat globules were separated from the casein micelle network and surrounded by serum. It is the presence of these pockets of serum and fat that are proposed to be responsible for the high fat losses that occur during syneresis of UF milk gels. As stress is applied to such curd during cheesemaking, the loss of fat in whey is high compared to curd made from normal strength milk in which the fat is more fully encapsulated within the protein gel structure.

In contrast,the gel microstructure of homogenized 3X







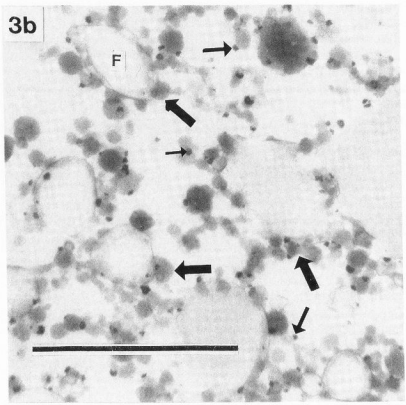
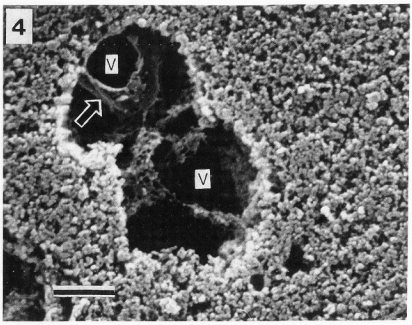


Figure 2. *Transmission electron micrographs of gel made from unhomogenized 3X milk concentrate (non-UHT heated).* ***Fig. 2a.*** *Large pockets of milk serum (S) surround the fat droplets (F) and separate many of them from the network of casein micelles (m).* ***Fig. 2b.*** *The intact fat globule membrane (small arrows) clearly marks the boundary between the fat globule (F) and the micelles. Individual casein micelles (large arrows) can be easily identified in the gel network.. Bars = 1 μm.*

Figure 3. *Transmission electron micrographs of gel made from homogenized 3X milk concentrate (non-UHT heated).* ***Fig. 3a.*** *Some fat droplets (F) were still surrounded by serum (S) but those that have been reduced in size by homogenization were tied into the micelle network (arrows).* ***Fig. 3b.*** *Homogenized fat particles (F) had adsorbed casein micelles on their surfaces (large arrows). The small black particles (small arrows) were artifacts (Parnell-Clunies et al. 1986). Bars = 1 μm.*

milk (Figs. 3a and 3b), was different from that made from unhomogenized 3X milk. Adsorption of casein micelles at the fat–water interface during homogenization reduced the number of micelles 'free' in the water phase. So that after renneting, the gel network between fat droplets was less dense compared to unhomogenized 3X milk even though its overall volume had been increased by incorporation of the fat. Also there were no large pockets of milk serum surrounding the fat droplets. The small electron-dense particles observed in Fig. 3b were assumed to be difficult-to-avoid artefacts caused by formation of a glutaraldehyde–osmium tetroxide complex during TEM sample preparation (Parnell-



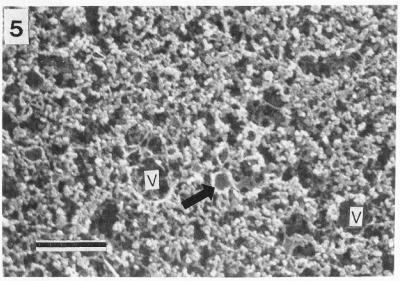


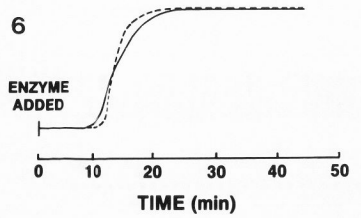
Figure 4. *Scanning electron micrograph of gel made from unhomogenized 3X milk concentrate (non-UHT heated). Void spaces (V) were present in the gel because of fat being extracted during sample preparation. Fragments of residual fat globule membrane were evident (arrow). Bar = 2 μm.*

Figure 5. *Scanning electron micrograph of gel made from homogenized 3X milk concentrate (non-UHT heated). Void spaces (V) were smaller but occurred with greater frequency. Protein adsorbed at the fat–water interface of the fat droplets remained integrated in the gel matrix (arrow). Bar = 2 μm.*

Clunies *et al.*, 1986).

In the homogenized 3X milk gel, portions of the fat–water interface were still composed of intact fat globule membrane, but in areas where casein micelles had been adsorbed onto the interface, the fat droplets were tied into the gel network. The pockets of serum, that characteristically surrounded fat globules in unhomogenized 3X milk gels, were only evident in areas where there had been no adsorption of casein micelles onto the fat–water interface (that is, where intact fat globule membrane remained). Where there had been protein adsorption, the fat droplets became incorporated into the gel structure.

The dense protein network of gels made from unhomogenized 3X milk can be observed in Fig. 4. Fat globule membrane was visible as material spanning the spaces from which fat was extracted during SEM sample preparation. Because native fat globule membrane does not become integrated in the gel structure, it collapses when the fat is removed. In contrast, protein adsorbed onto fat droplets during homogenization becomes an integral part of the gel network and retains its shape when fat is extracted. This was observed in SEM micrographs of homogenized 3X milk gels as smooth hemispherical cavities on the freeze-fractured surface (Fig. 5). The increased openness of the gel network of homogenized concentrate can also be seen when Figs. 4 and 5 are compared.



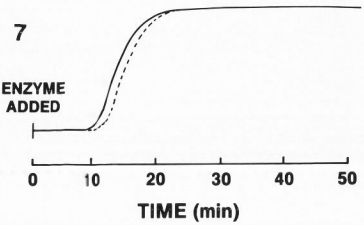


Figure 6. *Diagram of curd firming of homogenized 3X UF milk concentrate (non-UHT heated) containing 0.1% DSP. Curd firmness (y axis) was recorded with a Formagraph as a function of time after adding 0.02 RU/g of rennet at 35°C; —— DSP, – – – control (no additives).*

Figure 7. *Diagram of curd firming of homogenized 3X UF milk concentrate (non-UHT heated) containing 0.1% TSC or 0.1% DSP. Curd firmness (y axis) was recorded with a Formagraph as a function of time after adding 0.02 RU/g of rennet at 35°C; —— DSP, – – – TSC.*

Effect of Calcium Sequestering Agents

Addition of DSP to homogenized 3X UF milk hastened coagulation but retarded the rate of gel firming (Fig. 6). In contrast, adding TSC did not hasten coagulation yet gel firming rate was retarded (Fig. 7). Calcium has been shown to be involved in both micelle aggregation and gel firming reactions (McMahon *et al.*, 1984). Both DSP and TSC sequester calcium so their different affects on milk coagulation suggests that phosphate anions are involved in micelle aggregation but are not involved in subsequent changes in the gel network that cause the gel to become firmer. Such rearrangements were observed in a TEM study of Cheddar cheese made by Kimber *et al.* (1974). They observed that in the protein gel of cheese curd, the casein micelles were linked together into an irregular space network, often with contact areas between micelles involving large areas of their surfaces. Then as the curd was stressed mechanically and thermally, a process of chain shrinkage and rearrangement occurred. Although the network structure was retained, the casein micelles became fused together to the extent that individual micelle identity was lost. It is this chain rearrangement that causes gel syneresis.

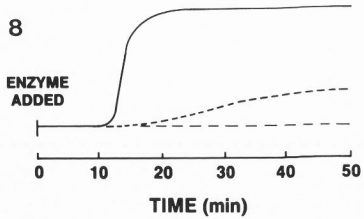


Figure 8. Diagram of curd firming of UHT heated, homogenized 3X UF milk concentrate compared to unheated milk concentrate. Curd firmness (y axis) was recorded with a Formagraph as a function of time after adding 0.02 RU/g of rennet at 35°C; —— non-UHT, - - - UHT, — — — no enzyme added.

Addition of DSP or TSC to milk reduces serum ionic calcium concentration. This causes colloidal calcium phosphate to be solubilized from the micelles to preserve calcium equilibrium. Based on either a submicelle model or a continuous network model of casein micelle structure such a loss of colloidal calcium phosphate would cause a partial disintegration of the micelles. Visser *et al.* (1986) observed that when TSC is added to milk there is a sharp increase in the number of small particles in the milk system. They concluded that these are β- and κ-caseins aggregates released from micelles. Such submicellar casein particles would also take part in rennet coagulation and be observed as an accumulation of material around other intact micelles.

Effect of UHT Processing on Coagulation

When milk is heated above 70°C, β-lactoglobulin is denatured and complexes with κ-casein through hydrophobic and covalent interactions (Haque and Kinsella, 1988). Because most of the κ-casein is located on the surface of the casein micelles, the presence of this complex severely retards rennet coagulation of milk. Consequently, milk that has been heated beyond pasteurization is not usually suitable for making cheese because it forms a soft curd with poor syneresis properties. When we processed 3X UF milk through our laboratory-scale UHT system we observed this adverse effect of heating on milk's coagulation properties (Fig. 8). Coagulation time was retarded and only a weak gel was formed. When the UHT 3X milks containing DSP or SHMP were renneted, they did not gel. In contrast, the UHT sample containing TSC coagulated faster than the control UHT sample but formed only a weak gel. This gel was so soft that it resembled a viscous liquid fluid more than a gel.

The UHT gels from this study were difficult to freeze-fracture during sample preparation for SEM analysis. The weakness of these gels was evident from the areas in the gel microstucture void of a continuous protein network. This was observed using both SEM (Fig. 9) and TEM (Fig. 10) to examine the UHT 3X milk gels. Whereas the fat droplets in gels made from unheated 3X milk were encapsulated within the protein network, in gels made from the UHT treated milk the fat droplets were clustered together. In these gels made from UHT treated milk, there was little evidence of any casein micelle structure in the gel network. Instead the fat droplets were surrounded by a dense, homogeneous protein mass. When TSC was added to milk prior to UHT heating, strings of non-micellar protein material were observed as part of the network of the milk gel (Fig. 11). The phenomenon of clumping of fat droplets together with the homogeneous protein mass was also accentuated (Fig. 12).

In gels made from unheated 3X milk (Figs. 2 and 3), the individual casein micelles could be easily identified. However, after UHT heating, the micelles had been altered so much that upon renneting, a homogeneous mass of protein material formed around the fat droplets. Some fat droplets had also fused together. This amassing of protein and fat into large clumps reduced the effective volume of the gel network (Fig. 12) and prevented the formation of particle chains that is essential for a gel to be produced.

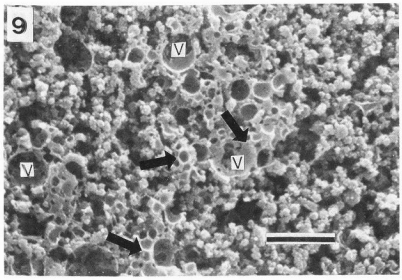
Effect of Cluster Formation on Gelation

When particles of colloidal size, such as casein micelles, aggregate they frequently form chains through collision of clusters of particles (McMahon and Brown, 1984). It is the size and shape of these clusters rather than that of the casein micelles that influence the behavior of milk as it coagulates. Formation of a milk gel relies on rigid bonds being formed between casein micelles to maintain an open network structure. A gel is formed when the particle size and solids concentration are such that the space network produced by cluster-addition growth fills the whole of the suspension space before breakdown can occur by disruptive forces. Particle chain flexibility and inter-particle fusion during aggregate growth will then cause rearrangement of particles in the final gel structure.

If renneting of casein micelles is considered as producing reactive zones on the micelle surface, then aggregation occurs because of a build up of polymer functionality on the micelle surfaces. As the micelles aggregate into clusters there is a rapid increase of functionality with the clusters containing many unreacted sites. In unheated milk, collisions of renneted micelles produce sufficiently rigid bonds between their reactive zones to provide a large energy barrier against subsequent separation of the micelles. This build up of functionality on the micelle clusters is, thus, not significantly affected by rennet concentration and the final curd firmness is relatively independent of coagulation time. However, this is not the case for UHT treated milks.

If milk (unconcentrated) is UHT treated and then renneted, there is no observable gel formed even though its initial particle concentration is sufficient for rennet coagulation to occur as it does in unheated or pasteurized milk. This suggests that the shape of the particle clusters is not inducive to chain formation. When milk is concentrated to 3X, a UHT milk gel can be formed although it is not as firm as a gel made from unheated milk (Fig. 8). When β-lactoglobulin is complexed onto the surface of the casein micelles, bond formation between colliding micelles is sterically hindered. The para-κ-casein regions on the micelles become physically separated by β-lactoglobulin so that fewer hydrophobic or electrostatic interactions can occur between them. This means that the bonds between micelles will be less rigid than in unheated milk. And, because formation of an open coagulum is dependent upon rigid bonds between aggregating particles, UHT heated micelles will tend to form clumps rather than chains. Consequently, gel formation will be hindered.

In the enzymic coagulation of normal strength milk, enzyme concentration does not affect the final strength of the renneted milk gel. During these experiments it was observed, however, that rennet concentration did affect the type of gel produced upon renneting of UHT concentrated milks. If the rennet concentration is increased a firmer milk gel is formed (Fig. 13). We suggest that increasing enzyme concentration has this affect because it controls the rate at which functionality develops on the casein micelle surfaces. At low rennet levels, the rate of aggregation is so slow that rearrangement of the particles occurs before the clusters have grown to sufficient size to form a space-filling gel network. Large



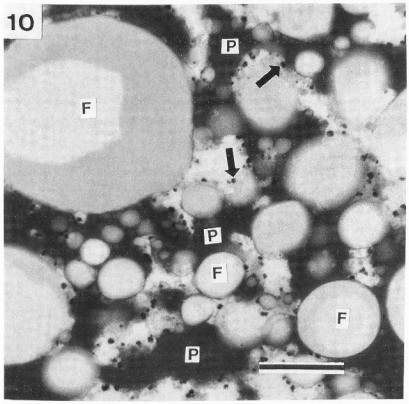
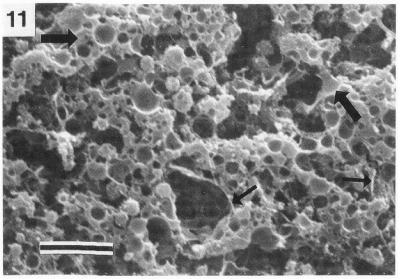


Figure 9. *Scanning electron micrograph of gel made from UHT homogenized 3X milk concentrate. As evidenced by the hemispherical voids (V) on the fractured surface, this gel had contained fat droplets that were dispersed within a homogeneous mass of protein. (arrows). Bar = 2 μm.*

Figure 10. *Transmission electron micrograph of gel made from UHT homogenized 3X milk concentrate. The loss of micellar structure can be seen by the dark stained mass of protein (P) surrounding the fat droplets (F). The same artifacts (arrows) as in Fig. 3 were also observed. Bar = 1 μm.*

spaces without any gel structure would therefore be expected (Fig. 12). Also, rotational or translational movement of micelles that can occur when micelles are held together only by weak interactions, would tend to produce clusters of casein micelles that are closely packed together into clumps (Fig. 10). In contrast, chain formation would predominate in a system in which the micelles are tightly bound together, as happens with unheated milk, and an open, space-filling network of interconnected casein micelles would form (Fig. 2b).



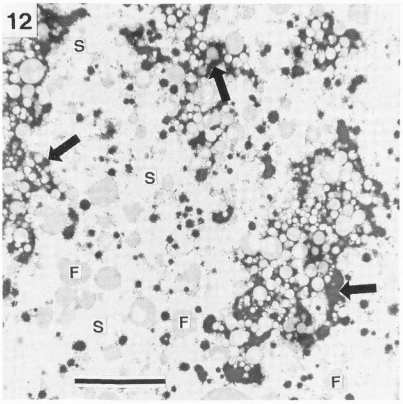


Figure 11. *Scanning electron micrograph of gel made from UHT homogenized 3X milk concentrate containing 0.1% TSC. The homogeneous protein mass (large arrows) around the fat droplets was evident in the void spaces from which the fat had been extracted . Strings of protein material were observed between some of the fat droplets (small arrows). Bar = 2 μm.*

Figure 12. *Transmission electron micrograph of gel made from UHT homogenized 3X milk concentrate containing 0.1% TSC. Much of the gel consisted of an agglomeration of fat droplets with a homogeneous mass of protein material (large arrows). Fat droplets (F) that had not clustered had no protein attached to their surfaces (small arrows). These fat globules were present in areas that were devoid of any protein network (S). Bar = 5 μm.*

Conclusion

The difference in curd firmness that occurs when milk is heated beyond pasteurization conditions is well known in the cheese industry. The interaction of denatured β-lactoglobulin with κ-casein on the casein micelle surfaces interferes with aggregation of renneted casein micelles and prevents adequate syneresis of the cheese curd. Heating milk beyond

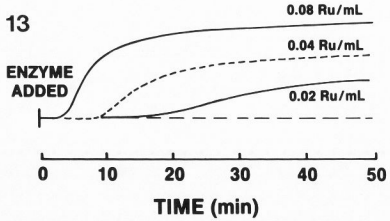


Figure 13. Diagram of curd firming of UHT treated, homogenized 3X UF milk concentrate. Curd firmness (y axis) was recorded with a Formagraph as a function of time after adding 0.02, 0.04 or 0.08 RU/g of rennet at 35°C; — — — no enzyme added.

pasteurization has been used as a means to increase cheese yield, but because of the weaker curd produced from overheated milk, it is not commonly used.

In this study, gels made from renneted UHT 3X milk were weaker than those from unheated milk concentrate. However, the firmness of such gels was increased when rennet concentration was increased. Therefore, while UHT treatment of normal milk severely hindered its suitability for making cheese it should be feasible to design a UHT milk concentrate that would perform satisfactorily for cheese making.

However, when DSP or SHMP was added to 3X UF milk, no milk gel was formed when the milk was renneted after UHT treatment. Adding TSC also prevented UHT 3X milk from coagulating into a firm gel. This suggests that the use of these additives to prevent age gelation of UHT 3X milk would not allow the subsequent use of that milk in cheese-making. Similar work should be conducted on commercial UHT equipment to confirm these results.

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Discussion with Reviewers

D. Dalgleish: Comparing Figs. 5 and 9, the differences appear to the untutored eye to be quite small. Are they sufficient to explain the weakness of the gel in the heated milk?

Authors: Both micrographs were obtained at the same magnification. In Fig. 5 (non-UHT 3X milk gel) the casein micelles are part of a continuous three dimensional network which would give elasticity and resilience to the gel. In contrast, renneting of UHT 3X milk (Fig. 9) produced a gel that could be described as having 'phase separation.' The protein is no longer in the form of strands of micelles but is concentrated into an amorphous mass around the fat particles. The consequence of this is there are weak areas within the UHT 3X milk gel. On a micro-scale the protein is more tightly packed together but on a macro-scale there are only limited amounts of protein connecting those micro-scale areas of protein together. So, greater internal movement (or even collapse) of the gel can occur when stress is applied. This is observed as a weaker gel.

D. Dalgleish: In the reference to submicelles, it is assumed that (a) such entities exist and (b) that they will necessarily coagulate. It seems that submicelles come in different forms, with some being devoid of κ-casein: in this case would they necessarily re-associate at all, if they have been released by dissolution of micellar calcium phosphate?

Authors: Re-association of sub-micellar material can be assumed based on either the submicelle or framework model of casein micelle. In observations by Visser *et al.* (1986), from which a framework structure was proposed, the material released from casein micelles by sequestering calcium was present as particles. Once the macropeptide domain of κ-casein is removed by renneting these particles would be free to re-associate through hydrophobic interactions or ionic interactions between the negatively charged phosphoserine groups on α- and β-caseins and positively charged regions on para-κ-casein, regardless of whether the sub-micellar particles contain κ-casein. These same phosphoserine groups would have been bound to colloidal calcium phosphate within the casein micelle before addition of the calcium sequestering agent. (Note: Our discussion about casein micelle structure was rewritten to accommodate both models of casein micelle structure.)

D. Dalgleish: Can serum protein be identified as having a specific function in the networks formed from heated milk?

Authors: This cannot be ruled out. Evidence of such non-micellar protein as strands was observed in the UHT 3X milk gel that contained DSP. The origin of this material was not determined. Although because it was not observed in other samples it could be concluded that it was protein that had been dissociated from micelles.

D. Dalgleish: Increasing rennet gives increased curd firmness in heated milks. What insight does this give into the mechanism of renneting in these cases? One might expect that the gels formed would eventually have a strength independent of rennet coagulation, but this seems not to be so. Why should this be?

Authors: One factor affecting the strength of renneted milk gels is the rigidity of the bonds that form between individual casein micelles in the gel network. The complexing of β-lactoglobulin onto the surface of casein micelles will affect the rigidity of bonding between micelles. A discussion of this has been included in the text of this paper.

Y. Kakuda: Does not the observation that higher levels of rennet increased curd firmness and shortened coagulation time imply that denatured β-lactoglobulin interferes with the hydrolysis of κ-casein rather than the aggregation step?

Authors: It has been shown that this complexing of β-lactoglobulin with κ-casein does interfere with κ-casein hydrolysis by rennet (Haque and Kinsella, 1988). This is a reason why more rennet had to be added to the UHT 3X milk to form a gel, compared with unheated 3X milk. Our observations on the dependence of curd firmness on rennet concentration, however, show that the aggregation process itself is affected by the rate at which the 'surface' of the casein micelles are made reactive by renneting. It should also be remembered that this 'surface' κ-casein is buried under a mat of denatured β-lactoglobulin and so the interactions that occur when two micelles collide are different for heated and unheated milks. It could be concluded that in concentrated milk, the associations among micelles occur before the micelles are "ready" which results in a weaker gel being formed.

Y. Kakuda: Will the authors more fully explain the statement "...phosphate anions affect the tertiary rearrangements of the milk gel required to increase its firmness." What sort of rearrangements does the gel have to undergo to increase firmness? Is it a rearrangement of the gel or do phosphate anions interfere with the initial formation of the gel?

Authors: The initial gel network that is formed upon renneting incorporates only a fraction of the total number of casein micelles in milk. A strong gel is only produced upon addition of further micelles into the protein network. Rearrangements in the gel network occur because of applied stress. In cheesemaking, this can be applied thermally (by heating), mechanically (by stirring and Cheddaring), or chemically (by lowering pH). Even without these added factors, the gel network is still subjected to stress from Brownian and rotational motions of particles as well as gravity. Thus, marginal interactions between micelles are 'rearranged' to relieve stress and at the same time build up the strength of the gel. The phosphoserine residues of β-casein are involved in aggregation of casein micelles. This is thought to occur through calcium–phosphate linkages between micelles. Consequently, adding DSP to milk affects micelle aggregation by sequestering calcium.

N. Carrell: Since the gels from UHT heated concentrate were so weak, is it possible that syneresis occurred and structures collapsed during sample processing, creating voids in the network around the fat globules?

Authors: This cannot be disregarded. The creation of artefacts during sample preparation must constantly be considered. However, renneted milk gels made from ultra-heated milk have poor syneresis properties and so it is unlikely that enough syneresis would occur during fixation to create these voids.

N. Carrell: Are the effects of DSP and TSC on the gelation properties of milk, UHT treated milk and homogenized milk the same as on the concentrated milk?
Authors: No comparative testing of nonconcentrated milk was done during this study but in earlier work we had observed that adding up to 10 m*M* phosphate to milk does shorten coagulation time (McMahon *et al.*, 1984).

N. Carrell: During cheesemaking, ionic calcium increases during fermentation. Would you expect the effects of DSP and TSC on gelation to be as severe if the pH of the concentrate were adjusted down? Based on the findings in this study, how would you propose overcoming the effects of DSP and TSC on curd firmness?
Authors: The effects of adding DSP or TSC to milk do not drastically affect gelation unless the milk is subsequently heated. It was the combination of UHT treatment and adding DSP and TSC that severely affected gelation. With the effect acidification of milk has on speeding up coagulation (primarily because of increased ionic calcium) it would be expected that acidification would also counter some of the detrimental effects of UHT heating of milks treated with DSP or TSC.