Microstructural changes in casein supramolecules during acidification of skim milk

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ABSTRACT

Pasteurized skim milk was acidified using different levels of glucono- δ -lactone at 10, 20, 30, and 40°C to give slow, medium, and fast rates of acidification. Milk coagulation was monitored by measuring turbidity and curd firmness, and microstructural changes during acidification were observed on glutaraldehyde-fixed, agar-solidified milk samples using transmission electron microscopy. Rate of acidification had little influence on changes observed during acidification, except at 10°C. At 40°C, the casein supramolecules were spherical throughout acidification, whereas at lower temperatures they became progressively more ragged in appearance. All of the milks gelled at the same pH (pH 4.8), as measured by curd firmness, whereas increases in turbidity, assumed to be brought about by an increase in number of light-scattering particles, were observed to start at about pH 5.2 to 5.4. As the milk was acidified, aggregates of loosely entangled proteins were observed, presumably originating from proteins that had dissociated from the casein supramolecules. These aggregates were often as large as the casein supramolecules, particularly as the pH of the milk approached the isoelectric point of the caseins. Larger aggregates were observed at 40°C than at the lower temperatures, suggesting the involvement of hydrophobic interactions between the proteins. A 3-phase model for acid-induced gelation of milk is proposed in which the first phase involves temperature-dependent dissociation of proteins from the casein supramolecules, with more dissociation occurring as temperature is decreased. Dissociation continues as milk pH is lowered, with the released proteins forming into loosely entangled aggregates, some as large as the casein supramolecules. The second phase of acid gelation of milk occurs between pH 5.3 and pH 4.9 and involves a reassociation of proteins with loosely entangled protein aggregates forming into more-compact colloidal particles or combining with any remaining casein supramolecules. The third and final phase involves rapid

aggregation of the colloidal casein supramolecules into a gel network at about pH 4.8. Different gel structures were formed based on temperature of acidification, with a coarse-stranded gel network formed at 40°C and a fine-stranded gel network at 10°C.

Key words: milk coagulation, casein supramolecule, acid gelation

INTRODUCTION

Acid coagulation of milk is the foundation of many dairy products. It is well recognized that when pH of milk is sufficiently lowered and passes through the isoelectric point of the caseins, their solubility decreases, bringing about an association and subsequent coagulation (Kauzmann, 1959). Formation and physical properties of acid milk gels have been reviewed by Lucey and Singh (1998) and their rheological properties have been extensively studied (Lucey et al., 1998; Lucey, 2001; Tranchant et al., 2001), and mathematical models describing acid coagulation have been developed (Novakovic et al., 2000). Milk can be acidified and coagulated when warm (typically in the range of 30–45°C) or it can be acidified while cold (e.g., 5°C) and then warmed to induce gel formation. Traditionally, milk is acidified by microbial fermentation, but slow chemical acidification using glucono- δ -lactone (**GDL**) is also used, such as in the manufacture of cottage cheese. Depending on the type of product being manufactured, the milk may or may not be given a high-heat treatment before acidification to denature β -lactoglobulin, which also then participates in the gelation process.

It has been observed that different gel properties are obtained based on acidification temperature (Lucey et al., 1997), such as excessive syneresis and a coarser gel texture occurring when the incubation temperature of yogurt is increased (Lucey, 2001). During acidification of milk, calcium phosphate contained in the casein supramolecules is solubilized (Evanhuis and de Vries, 1959; Visser et al., 1986) and proteins dissociate from the casein supramolecules (Roefs et al., 1985; van Hooydonk et al., 1986; Vreeman et al., 1989), although, as stated by Dalglish et al. (2005), it has not been clearly established that lowering pH and solubilizing

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calcium phosphate alters the internal structure of the casein supramolecules. Such protein dissociation is temperature-dependent, with more casein becoming nonsedimentable at 5°C than at higher temperatures (Dalglish and Law, 1988; Singh et al., 1996).

As milk is acidified there is a decrease in magnitude of net negative charge on both individual protein molecules and the overall net charge of casein supramolecules (Heertje et al., 1985), and solubilizing calcium phosphate causes the calcium phosphate nanoclusters that provide an interlocking aspect to the casein supramolecule (McMahon and Oommen, 2008) to be gradually depleted. Any proteins (such as some α_{S1} -, α_{S2} -, and β -casein) that are bound into the supramolecule structure only by the sequestering action of their phosphoserine groups toward the calcium phosphate can dissociate, especially if milk is cold and hydrophobic interactions are minimized.

Dalglish and Law (1988) observed that at 30°C, dissociation of casein upon acidification of milk to pH 5.5 resulted in a constant proportion of α_{S1} -, β -, and κ -casein, suggesting that these proteins may have dissociated as an intact complex. The small size of these particles also agrees with them being enriched in κ -casein and having less α_{S1} -casein than the original casein supramolecules. This would be expected because κ -casein is needed to stabilize the particles because neither β -casein nor α_{S1} -caseins can exist as monomers under these conditions (van Hooydonk et al., 1986). At low temperature there is more dissociation of proteins from the casein supramolecules. Dalglish and Law (1988) reported 30 and 55% being dissociated at 20 and 4°C, respectively, whereas Singh et al. (1996) reported only 7 and 22% dissociation at 22 and 5°C, respectively, probably because they used a higher centrifugal force ($88,000 \times g$ vs. $70,000 \times g$), which would sediment more of the very small casein supramolecules and aggregates.

Maximum protein dissociation occurs at about pH 5.5. Then, as milk is further acidified, the amount of nonsedimentable casein decreases to zero at pH 4.8, at which stage all of the casein is incorporated into the gelled network. In unheated milk, a slight decrease in casein supramolecule particle size has been observed and interpreted as a collapse of a hairy layer on the particle surface that then causes gelation (Dalglish et al., 2005). The model of casein supramolecules proposed by McMahon and Oommen (2008) suggests that such decrease in particle size would result from a global contraction of the supramolecule rather than just the collapse of a hairy layer.

Various models of acid gelation of milk have been developed. Herbert et al. (1999) proposed a 2-phase process involving dissociation of proteins from the casein supramolecules and concomitant swelling of

the casein micelles from pH 6.7 to pH 5.1, followed by reabsorption of protein back into the casein supramolecules and aggregation as pH falls below pH 5.1. Famelart et al. (2004) proposed that a swelling of the casein supramolecules occurs at about pH 5.2, followed by their aggregation at about pH 5.0 and then fusing together as the pH continues to decrease. Heertje et al. (1985) observed casein supramolecules in unheated milk at 20°C down to pH 5.2 that were considered to be depleted of β -casein. Then, as renewed interaction of β -casein with other caseins occurs as the pH decreases below the isoelectric point of β -casein, aggregation of casein supramolecules was observed to commence by pH 5.1.

Even though acid-coagulated milk gels are observed as a network of aggregated colloidal casein supramolecules (Tamime et al., 1984), in all these models it is recognized that the supramolecular structure would change as protein dissociation and reassociation takes place and the charge distribution on the proteins change. At pH 4.8, the protein molecules carry different charges than they do at the native neutral pH of milk, and so their conformational structure, as well as how they interact with each other, would be different. Less calcium bridging and ion-pairing will occur because they will carry fewer negatively charged AA side groups. The casein-casein interactions that do occur will result from hydrophobic liaisons (if the temperature is high enough) and a decrease in electrostatic repulsion. In comparison, during initial mammary gland synthesis of milk, a combination of hydrophobic liaisons and attractive interactions with calcium phosphate nanoclusters orients casein molecules in forming native casein supramolecules (McMahon and Oommen, 2008).

The focus of our paper was to observe the structural changes occurring in casein supramolecules and any related dissociation of protein from the supramolecular structure during acidification of unheated milk (i.e., milk that has not been heat-treated to bring about extensive denaturation and aggregation of whey proteins). This was performed at warm and cold conditions to determine the effect of temperature, as well as rate of acidification, on the gelation process to expand the current model to a wider set of conditions.

MATERIALS AND METHODS

Preparation of Milk

Raw whole cows' milk was obtained from the Gary Haight Richardson Dairy Products Laboratory of Utah State University (Logan), centrifuged (Sorvall RC-5C, Du Pont Company, Mississauga, Ontario, Canada) at $3,000 \times g$ for 60 min at 4°C, and filtered through glass

fiber filter paper under suction. The resultant skim milk was then given a minimal pasteurization treatment of 63°C for 30 min. If the milk was to be chemically acidified, 0.02% (wt/wt) sodium azide (Mallinckrodt, Paris, KY), 0.01% chloramphenicol (Sigma-Aldrich Co., St. Louis, MO), and 0.01% benzylpenicillin potassium salt (Sigma-Aldrich Co.) were added to inhibit bacteria growth. The milk was cooled to 4°C and stored overnight, and then warmed to the required temperature (10, 20, 30, or 40°C) and held for 30 min in a water bath.

Milk Acidification

Microbial acidification with 1.2 g of freeze-dried yogurt starter culture (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*; Danisco USA, New Century, KS) per 100 g of skim milk at 40°C was used to lower the pH to 4.6 in 125 min. Chemical acidification of milk was performed by addition of GDL (Sigma-Aldrich Co.) at levels of 2.0, 3.0, and 4.0 g/100 g at 10°C; 2.0, 2.5, and 3.0 g/100 g at 20 and 30°C; and 1.5, 2.0, and 2.5 g/100 g at 40°C. The addition of 4.0, 2.5, 2.0, and 1.5 g of GDL/100 g of milk at 10, 20, 30, and 40°C, respectively, produced acidification rates such that pH 4.6 was reached in 125 min.

Electron Microscopy

Before and during the acidification process, 5-mL samples were taken and fixed by the addition of 0.2 mL of a 50% glutaraldehyde solution (Electron Microscopy Sciences, Fort Washington, PA). After 5 min, the milk was solidified by mixing with an equal volume of warm (50°C) 3% (wt/wt) agar (Bacto-Agar, Difco Laboratories, Detroit, MI). The samples were cut into small strips (1 × 1 × 10 mm) and preserved in 0.1 M phosphate buffer solution, pH 6.8, containing 2% (wt/wt) glutaraldehyde and stored at 4°C.

The glutaraldehyde-fixed, agar-solidified samples were reduced in size to 1-mm³ cubes, rinsed with 0.1 M phosphate buffer, and post-fixed in 2% (wt/wt) osmium tetroxide (Electron Microscopy Sciences) in the phosphate buffer. Dehydration was performed by transferring samples through an increasing concentration of graded ethanol (30, 50, 70, 95, and 100%). After dehydration, the samples were transitioned into propylene oxide and mixed in varying proportions ranging from 25% ethanol in propylene oxide to 100% propylene oxide alone in 4 steps. The cubes were infiltrated with a solution containing propylene oxide and epoxy resin (Electron Microscopy Sciences) stepwise in various proportions ranging from 25% propylene oxide in epoxy resin to 100% epoxy resin. The infiltrated

samples were embedded in Beem capsules (Electron Microscopy Sciences) and incubated at 45°C followed by 60°C for 24 h each. The hardened epoxy resin was removed from the Beem capsule and excess epoxy was trimmed to expose the sample. Ultrathin sections were cut using an Ultracut E ultramicrotome (Leica Inc., Gilroy, CA) and collected on 300 hex grids (Electron Microscopy Sciences). Sections were post-stained with uranyl acetate and lead citrate and examined using a Zeiss CEM 902 transmission electron microscope (Zeiss Inc., Thornwood, NY) at 80 kV. Images were recorded on Kodak electron image film SO-163 (Eastman Kodak Co., Rochester, NY).

Turbidity

Turbidity changes brought about by changes in particle size, aggregation, and gelation were monitored using a single beam spectrophotometer (model DU-8B; Beckman Instruments Inc., Fullerton, CA) as described by McMahon et al. (1984). Milk was warmed to 10, 20, 30, or 40°C for 30 min, and a 3-mL portion was poured into a 1-cm-pathlength cuvette and placed into the spectrophotometer. Turbidity of the milk (typically >2 absorbance units when measured at 600 nm) was set to zero before acidification so that only the change in turbidity was recorded. This sample was then removed from the cuvette holder and, after appropriate amounts of GDL (or starter culture) had been added, aliquots of milk were poured into duplicate 3-mL cuvettes, placed in the cuvette holder, and maintained at 10, 20, 30, or 40°C. Changes in turbidity at 600 nm were then measured using one cuvette while the other was used to monitor changes in pH using a glass microelectrode (Microelectrodes Inc., Londonderry, NH). Measurements were performed in triplicate.

Gelation

The pH at which gelation occurred was determined in triplicate using a Formagraph (Dicky-John Corp., Fishkill, NY) with a modification of the method described by McMahon and Brown (1982). Milk was warmed to the appropriate temperature (10, 20, 30, or 40°C) for 30 min and the Formagraph 10-well sample holder was set to the same temperatures. For each temperature, the required amount of GDL or starter culture was added to the milk at the same time that the Formagraph recorder module was started. About 10 mL of milk was then deposited in each sample well and the sample holder transferred to the recorder module. Detection of curd formation was defined as the point where the baseline of firmness versus time diagram began to increase in width. Changes in pH of the milk samples were recorded using a glass microelectrode.

Figure 1. Transmission electron micrographs of skim milk, glutaraldehyde-fixed and agar-solidified at (A) 40°C, (B) 30°C, (C) 20°C, and (D) 10°C (short arrows = agarose fibers; d = dissociated protein; bar = 500 nm).

RESULTS

Prior to Acidification

The measured pH of the milks at 10, 20, 30, and 40°C was pH 6.96, 6.75, 6.64, and 6.55, respectively. This shift in pH as milk is cooled is related to uptake of protons as calcium phosphate present in nanoclusters in the casein supramolecule is solubilized upon cooling and their release when milk is warmed and calcium phosphate precipitates. The microstructure of the casein supramolecules that was observed (Figure 1) was similar to that which has long been reported using transmission electron microscopy to examine glutaraldehyde-fixed milk (e.g., Kalab et al., 1976). Other methods of imaging casein supramolecules (McMahon and McManus, 1998; McMahon and Oommen, 2008) have shown that

casein supramolecules have a more open structure than previously observed. However, to capture the spatial arrangement of protein aggregates during acidification but before gelation, we solidified the milk by mixing it with agar, which necessitated that we first fixed the proteins with glutaraldehyde. There were strong interactions between the casein supramolecules and agar that caused structural artifacts if acidified milk was mixed with the warm agar without prior glutaraldehyde fixation (data not shown). Strands of protein that appeared to radiate from the casein supramolecules were observed in non-glutaraldehyde-fixed acidified milks similar to those observed by Kalab et al. (1976). Thus, whereas the fine internal supramolecular structure of the casein supramolecules could not be retained, the dissociation of material from the casein supramolecules that occurred

when milk was cooled or acidified could be observed. Thin, fibrous strands observed in the micrographs were designated as artifacts relating to solidifying the milk in agar and were assumed to be agarose fibers (Alleyne et al., 1993).

At 40°C, the casein supramolecules were observed to have predominantly smooth spherical surfaces and had a range of sizes. There appeared to be very few casein supramolecules <80 nm in diameter. Some casein supramolecules had appendages, but the majority of the electron-dense areas in the micrographs were the colloidal casein supramolecules, with little nonparticulate protein being observed (Figure 1A). Individual protein molecules and small oligomers are too small to be observed against the micrograph background or retained in the samples during sample preparation, and so whey proteins present in the original milks were not observed in the micrographs. At lower temperatures there appeared to be more electron dense-material that was only loosely attached to the casein supramolecules with no extensive dissociation of the casein supramolecules, except for milk at 10°C.

At 30°C (Figure 1B), the casein supramolecules were spherical but many of them had surface protuberances similar to that reported using scanning electron microscopy (Kalab et al., 1982; Dalglish et al., 2004b). In some cases, the surface material seemed only loosely attached to the rest of the colloidal particle. At 20°C (Figure 1C), the casein supramolecule surface had a more tendrillar appearance, with electron-dense areas protruding from the particle surface.

At 10°C (Figure 1D), the casein supramolecules appeared close together and grouped in clumps; however, this was attributed to rapid solidification of the warm agar before it could be well dispersed throughout the cold milk. The casein supramolecules had a more ragged structure and there was a greater proportion of smaller particles, including some that were no longer spherical in appearance. Also, there were some particles that had an absence of material in their core. Compared with casein supramolecules at 40°C, the casein supramolecules at 10°C were less electron-dense (i.e., there was less heavy-metal staining) and their peripheral edges were less distinct with a relatively open structure. There was also more protein material dispersed as loose aggregates among the casein supramolecules at 10°C than at higher temperatures.

Acidification Rate

Microbial acidification of milk at 40°C using starter culture began slowly (apart from a small decrease caused by adding the starter culture itself). After approximately 15 min the rate increased as the starter

Figure 2. Reduction of pH in skim milk as a function of temperature based on (A) amount of glucono- δ -lactone (GDL) added and (B) addition of 1.2% freeze-dried starter culture.

culture moved into log phase and began to multiply, generating lactic acid (Figure 2A). Then the pH gradually decreased, with a pH of 4.6 being reached after 125 min. In contrast, during chemical acidification there was an initial rapid decrease in pH as GDL was hydrolyzed to gluconic acid, then acidification rate slowed as the concentration of remaining GDL decreased (Figure 2B).

Microstructure of Warm Acidified Milk

GDL-Acidified Milk. In 40°C milk, microstructural changes in the casein supramolecule structure were observed during acidification by GDL (Figure 3). At pH 5.5 (Figure 3A) there were numerous small casein supramolecules, many as small as 30 nm in diameter. Casein supramolecules up to 200 nm diameter were still present, although on a number basis the smaller casein supramolecules were predominant. There were no protuberances on the periphery of the casein supramolecules, which appear more spherical than before acidification. As acidification continued to pH 5.2 (Figure 3B), the very small casein supramolecules (30–40 nm diameter) were no longer present, being replaced by loosely entangled proteins dispersed among the larger spherical casein supramolecules. Some of these loosely

Figure 3. Transmission electron micrographs during warm acidification at 40°C of skim milk by 1.5% glucono- δ -lactone, glutaraldehyde-fixed and agar-solidified at (A) pH 5.5, (B) pH 5.2, (C) pH 4.95, and (D) pH 4.8 (d = loosely entangled protein; short arrows = agarose fibers; bar = 500 nm).

entangled proteins were present in clumps of up to 100 to 200 nm in size.

At pH 4.95 (Figure 3C), the dissolution of the casein supramolecules continued, with only casein supramolecules >100 nm in diameter remaining. Casein supramolecules were present that had only low electron density (gray spherical particles) and were mainly in the size range of 100 to 200 nm diameter. Larger, denser casein supramolecules (black spherical particles) were also present, ranging from 150 to 250 nm in diameter. In addition to the spherical casein supramolecules there were clumps of loosely entangled protein dispersed throughout the sample. Compared with the milk at pH 5.2, this loosely entangled protein at pH 4.95 tended to be present as very low electron density particle-like aggregates.

When the 40°C milk had been acidified to pH 4.8 (Figure 3d), the casein protein appeared as an interconnecting colloidal mass, indicative of a 3-dimensional gel network being formed. When using transmission electron microscopy to examine thin sections of a gel, only a cross-sectional view is obtained, which underestimates the extent of strand formation that makes up the gel network (Kalab et al., 1976). Even what appears to be individual colloidal particles would most likely be a cross-section of a strand that is traversing the thin section at a perpendicular angle. The strands of the gel network appeared to be about 180 to 300 nm in thickness. Some clumps of loosely entangled protein were also evident, but the majority of this protein had become associated with the casein supramolecules and could be observed as filamentous material on the pe-

Figure 4. Transmission electron micrographs during warm acidification at 40°C of skim milk by addition of 1.2% starter culture, glutaraldehyde-fixed and agar-solidified at (A) pH 5.4, (B) pH 5.0, (C) pH 4.75, and (D) pH 4.6 (d = loosely entangled protein; short arrows = agarose fibers; bar = 500 nm).

riphery of the gel network strands. Heertje et al. (1985) had observed that in warm acidified milk, the coagulum that formed consisted of clusters of aggregated micelles that had joined together to form a 3-dimensional network.

Microbially Acidified Milk. Changes in microstructure during microbial acidification of warm milk (Figure 4) were similar to that observed when using GDL (Figure 3). The only difference was that dissociated of protein from the casein supramolecules seemed to occur earlier. By pH 5.4 (Figure 4A), there was an increase in number of small (40–60 nm diameter) casein supramolecules and a considerable amount of loosely entangled protein (whereas in the GDL-acidified milk this was not apparent until pH 5.2). At pH 5.0 (Figure 4B),

there were no longer any intact casein supramolecules <70 nm diameter. As was observed with GDL-acidified milk, the smaller casein supramolecules tended to be less electron-dense than the larger casein supramolecules. The loosely entangled protein was more interconnected than was observed at higher pH.

Formation of large network strands was slightly delayed, although by pH 4.75 considerable reassociation had occurred with virtually no colloidal particles being less than 100 nm diameter, with some clumps of loosely entangled proteins still present. The large network strands developed upon further acidification with the gel network formed (Figure 4D), being similar in appearance to the gel formed in warm GDL-acidified milk (Figure 3D). The loosely entangled protein and

Figure 5. Transmission electron micrographs during cold acidification at 10°C of skim milk by 4.0% glucono- δ -lactone, glutaraldehyde-fixed and agar-solidified at (A) pH 5.9, (B) pH 5.3, (C) pH 5.0, and (D) pH 4.8 (d = loosely entangled protein; short arrows = agarose fibers; bar = 500 nm).

the smaller casein supramolecules present at this pH were observed mostly to be fused to the periphery of the strands making up the gel network.

Microstructure of Cold Acidified Milk

When cold (10°C) milk was acidified, there was a large increase in the amount of loosely entangled protein present, even at pH 5.9 (Figure 5A). The same artifact described above for addition of warm agar to cold milk samples was also present in the micrographs, with the agar solidifying before complete mixing with the milk. Increased dissociation of the casein supramolecules was not unexpected because there was more dissociation observed in cold milk before acidification. At pH 5.3 the proportion of loosely entangled protein appeared to have decreased (Figure 5B), suggesting that a reas-

sociation of proteins back into colloidal particles had already commenced.

Cold acidified milk at pH 5.0 (Figure 5C) was similar to that at pH 5.3, with the colloidal particles having numerous appendages on their peripheral surface with some small chains and clumps of particles. At pH 4.8 (Figure 5D), virtually all the protein had become associated back into spherical particulate form and incorporated into a fine-stranded gel network. There was no evidence of the very large and electron-dense colloidal particles observed when cooled milk was acid coagulated. Differences in size of the colloidal particles forming the aggregated protein network as temperature was lowered to pH 4.8 were more obvious when the gelled milks were examined at lower magnification (Figure 6). When coagulated at 10 and 20°C, the gel network was made up of strands of aggregated colloidal particles that

Figure 6. Transmission electron micrographs of acid milk gels at pH 4.8 formed after acidification of skim milk by glucono- δ -lactone at (A) 40°C, (B) 30°C, (C) 20°C, and (D) 10°C (bar = 1 μ m).

were mostly 100 nm or less in diameter. At 30°C the colloidal particles of the network strands were slightly larger and about 100 to 200 nm diameter, whereas at 40°C they were in the range of 300 to 400 nm diameter. Similarly, the spacing between the network strands decreased as temperature was lowered, with spacings of 1 to 2 μ m regularly occurring at 40°C and spacings of a few hundred nanometers at 10 and 20°C. At the lower temperatures the network strands appeared to be made of clusters of small particles rather than being a single linear strand as implied by the cross-sectional view of the gel network at 40°C.

Turbidity

Although none of the treated milks formed a firm coagulum until pH 4.8, changes taking place as described above were also detected by monitoring turbidity (Fig-

ures 7 and 8). For warm milk (30 and 40°C) the turbidity remained constant down to pH 6.2, after which turbidity gradually increased by approximately 0.02 absorbance units. This was followed by a rapid turbidity increase at pH 5.3 and 5.5, respectively (Figure 7). In contrast, for cooler milk (10 and 20°C) the increase in turbidity commenced sooner at approximately pH 6.5 and followed the same general pattern as above, continuing to a maximum at pH 5.9 and 5.6, respectively.

Directly after acidification, increase in turbidity was more pronounced at 10°C. As acidification proceeded, the turbidity at 10°C decreased below its original level with a minimum occurring at about pH 5.3 (Figure 7). The depth of this turbidity minimum in the 10°C milk was larger if the acidification rate was slower (Figure 8a). No such turbidity decrease was seen for the other temperatures (data not shown). After further acidifica-

Figure 7. Influence of temperature on changes in turbidity upon acidification of skim milk by 4.0%, 2.5%, 2.0%, and 1.5% (wt/wt) glucono- δ -lactone (GDL) at 10, 20, 30, and 40°C, respectively.

tion, a rapid increase in turbidity was observed in all samples. At 40°C, after this increase the turbidity decreased from its maximum, starting at pH 5.0 and leveling off at pH 4.8. Similar changes in turbidity of warm milk during acidification had earlier been reported by Bringe and Kinsella (1990) at 25°C, and Banon and Hardy (1991, 1992) at 30 and 42°C. Recently, Alexander and Dalgleish (2004) and Dalgleish et al. (2004a), using diffusing wave spectroscopy to monitor GDL acidification of 30°C milk, reported changes in inverse photon free path ($1/l^*$) of unheated milk that had the same profile as our turbidity measurements. The value for $1/l^*$ stayed virtually constant until pH 5.5 and then rapidly increased before an increase in particle radius occurred at pH 5.0, at which point a decrease in $1/l^*$ occurred before reaching a steady value, as we observed with turbidity. The change that occurs before gelation is that the average distance between light-scattering events (i.e., l^*) decreases, implying that the number of colloidal particles increases (Alexander and Dalgleish, 2004).

If the inflection point of the turbidity curve is used as a measure of the pH at which the increase in particle number increases, then such an increase occurred at a higher pH as temperature was increased. The average turbidity inflection point for milks acidified using GDL was calculated at pH 4.96, 4.98, 5.05, and 5.20 at 10, 20, 30, and 40°C, respectively. Kim and Kinsella (1989) similarly observed that turbidity changes during acidification of pasteurized skim milk started between pH 5.1 and 5.2 at 35 to 50°C and increased to pH 5.6 at 55°C. The pH at which turbidity increased was not influenced by acidification rate at any of the temperatures (Figure 8A). Similar observations were made by Alexander and Dalgleish (2004), who observed that changes in apparent particle size during acidification of skim milk (at 30°C) were dependent on the pH of the milk, with a sharp increase in apparent radius occurring at pH 5.0.

When milk at 40°C was acidified using starter culture, similar turbidity changes were observed except that, starting at pH 5.5, the rate at which turbidity increased was slower (Figure 3B). Thus, the turbidity infection point occurred at pH 5.0 rather than pH 5.2 when the milk was acidified using GDL (Figure 8B), even though gelation pH as determined by measuring curd firmness was the same for both.

DISCUSSION

If the casein supramolecules in milk at 40°C before pH adjustment are considered to be in their native supramolecular form, then at lower temperatures or at lower pH they should be considered as having a non-native structure. In general, this is seen as an increase in the number of small colloidal particles and the presence of loosely formed, nonspherical protein aggregates. Throughout the acidification process, differences in size and physical form of the casein supramolecules and aggregates occur depending on temperature (through its influence on hydrophobic interactions) and pH (through its influence on protein charge and calcium phosphate solubility). However, the actual physical gelation of the milk appears to override these differences because gelation occurs at the same pH (pH 4.8) at all

Figure 8. Change in turbidity of skim milk as a function of slow, medium, and fast acidification rates using glucono- δ -lactone (GDL) at (A) 10°C and (B) 40°C, as well as by using 1.2% starter culture at 40°C (B).

Figure 9. Microstructural changes occurring during chemical acidification of milk as a function of temperature and pH. Images derived from transmission electron micrographs, with colloidal casein supramolecules depicted in black and loosely entangled protein aggregates depicted in gray, and all background digitally converted to white.

temperatures tested, regardless of the differences in the gel networks.

Temperature-Induced Changes

Reducing hydrophobic interactions by lowering temperature allows some casein molecules (especially β -casein), calcium, and phosphate to dissociate from the supramolecules (Qvist, 1979; Pierre and Brule, 1981; Dalgleish and Law, 1989) and for the caseins to be less tightly bound to each other. This appears to bring about loosening of the supramolecular structure as milk temperature is lowered from 40 to 20°C. At 10°C, a large number of smaller, less electron-dense and loosely entangled protein particles are present. When the micrographs were digitally modified through pixel binning to remove artifacts caused by agar solidification of the milk during sample preparation, to show only electron-dense casein supramolecules (black) and

less electron-dense (gray) entangled protein aggregates (Figure 9), these changes become more apparent.

At 40°C (which is the closest temperature to their native state), most of the casein supramolecules were spherical and electron-dense, whereas at 10°C they were smaller, more ragged in appearance, and less electron-dense. The casein proteins were still predominantly present as colloidal particles, although some had tendrils and appendages extending from the particle periphery. This would be expected as Dalgleish and Law (1988) showed that as milk is cooled from 30 to 5°C, the fraction of caseins (or casein aggregates) that are too small to sediment at $70,000 \times g$ increases only slightly, from 5 to 13%. This temperature-dependent difference of casein supramolecules needs to be taken into account when developing a model of acid-induced gelation of milk, as well as the differences in light-scattering properties of the loosely entangled protein aggregates compared with the intact supramolecules.

Acid-Induced Changes

Dissociation of Protein from Casein Supramolecules. The first phase of acid gelation of milk involves a temperature-dependent dissociation of proteins from the casein supramolecules with less protein being released when the milk is warm (40°C). At low temperatures (e.g., 10°C), the dissociated proteins can be present as loosely entangled aggregates. At 40°C, there appears to be sufficient hydrophobic interactions to maintain the proteins as (small) spherical colloidal particles (many with diameters <50 nm) as shown in Figure 9. This hydrophobic interaction would require a rearrangement and consolidation of the supramolecule interior structure with a predominance of κ -casein remaining on their periphery. Casein molecules can also be bound into the supramolecule structure via Ca-mediated salt bridges between carboxyl or phosphoserine side groups. These would remain so until the negative charge of the proteins is neutralized as pH is lowered. About 3.5 mM of calcium in milk is directly bound to the caseins (Singh et al., 1996) and this represents an average of 3 Ca^{++} ions bound per protein molecule.

As pH is lowered, some loosely entangled, nonspherical aggregates were observed to be as large as the native casein supramolecules. Therefore, even though average particle size has been reported to remain relatively constant until the rapid aggregation stage (Singh et al., 1996; Dalgleish et al., 2004a), there are major changes taking place to the casein supramolecule particles and it does not appear that the structural framework of the casein supramolecules is maintained as earlier postulated. As shown in Figure 9, at 40°C there appears to be a decrease in size of the casein supramolecules, with many more small casein supramolecules being present at pH 5.7. This corresponds to the pH at which Dalgleish and Law (1988) observed maximum dissociation of casein from the supramolecules at 30°C. However, the quantity of dissociated casein at 30°C they measured was still less than 10% of the total casein in milk, which supports our observation that at 30°C there are loosely entangled protein aggregates present at pH 5.7 as well as many casein supramolecules in the 50 to 100 nm range. At 40°C and this pH, the loosely entangled protein aggregates were not evident but there were many very small casein supramolecules in the 30 to 50 nm range.

It was apparent from our microstructural observations that, even at temperatures above 25°C, there was dissociation of proteins from the casein supramolecules during acidification, but the dissociated proteins also existed in an aggregate or particulate form that would allow them to sediment during centrifugation. Thus, between pH 6.7 and pH 5.5 the dominant influences on

protein dissociation are hydrophobic effects (temperature dependent) and calcium phosphate solubilization (which is both pH and temperature dependent).

Loosely Entangled Protein Aggregates. As further acidification occurs, there is more dissociation of proteins that appear to form into large, loosely entangled protein aggregates. Such aggregates are nonspherical and do not have the compact nature observed for casein supramolecules, although some are as large as the casein supramolecules and occasionally are attached to the casein supramolecules. The second phase of acid gelation occurs between about pH 5.3 and pH 4.9 and involves these loosely entangled proteins forming into more compact colloidal particles. At 40°C, the loosely entangled aggregates become quite large (up to 200–300 nm), whereas at 10°C they are much smaller and have already started to reassociate with the casein supramolecule remnants. As β -casein develops a net positive charge in this pH region it can act as a polymerizing agent with the negatively charged α_{S1} - and α_{S2} -caseins.

This dissociated material was prevalent in the 10°C milk before and during the initial acidification and could account for the observed turbidity increase in cold milk (Figure 6), especially with slower acidification rates (Figure 7) that would allow more time for dissociation to occur. At cold temperatures, the dissociation of α_{S1} -, β -, and κ -caseins from casein supramolecules are independent of each other and, down to 5.2, the proportion of β -casein in the nonsedimentable fraction decreases, whereas that of α_{S1} -casein increases (Dalgleish and Law, 1988). Dalgleish and Law (1988) suggest this difference in behavior may be the greater hydrophobicity of β -casein or its ability to form larger aggregates as temperature is increased that could sediment when centrifuged. This explains why it was thought that no dissociation of proteins from the casein supramolecule occurs during acidification of milk as long as the temperature is above 25°C.

Colloidal Protein Reassociations. As discussed by Heertje et al. (1985) and Dalgleish and Law (1988), when pH of milk approaches pH 5.2, the isoelectric precipitation of β -casein can occur. Above this pH it would be expected that β -casein would be readily released from the casein supramolecules at low temperatures but would then begin to reassociate with the casein supramolecules or aggregate into large polymers and precipitate. Thus, as temperature increases (and there is a corresponding increase in importance of hydrophobic interactions), the return of nonsedimentable casein into large aggregates would occur at higher pH. This is what we observed with larger aggregates of loosely entangled protein occurring at pH 5.3 when the milk was acidified at 40°C than at 20 and 30°C.

At low temperatures (10°C) and slow acidification (using 2.0% GDL, in which it required 175 min to reach pH 5.3 and 265 min to reach pH 5.0), the protein dissociation as shown by the turbidity increase occurred well before aggregation. The turbidity minimum appeared to coincide with a reduction in the loosely entangled protein aggregates and reincorporation of protein back into the casein supramolecules. The turbidity change was smaller with rapid acidification (using 4.0% GDL in which it required only 60 min to reach pH 5.3 and 85 min to reach pH 5.0), suggesting that dissociation from the casein supramolecules is relatively slow and that, as the pH passes through the β -casein isoelectric point, reassociation is induced before dissociation is completed.

Gel Formation. In the 40°C milk, large spherical colloidal particles were observed throughout the acidification process (see Figures 3 and 8), showing the importance of hydrophobic interactions in maintaining a supramolecular structure (McMahon and Oommen, 2008) even in the absence of calcium phosphate nanoclusters. By pH 5.0, the dissociated proteins appeared to be forming into more densely packed aggregates. Then, by pH 4.8 when a gel had formed, all of the caseins had either reassociated back into the remaining casein supramolecules or coaggregated as part of the 3-dimensional protein network that forms as the milk coagulates. It is, however, unlikely that this reassociation would occur in the same locations from which they dissociated.

Phase 3 of acid gelation of milk thus involves the rapid aggregation of the colloidal casein supramolecules into a gel network as the pH decreases to 4.8 and lower. This gel network consists of chains of compact spherical particles made of casein supramolecule remnants that remained in colloidal form during acidification, as well as those formed during reassociation of the casein molecules. The nature of the gel is dependent on temperature, with gels at low temperature being composed of smaller particles that form thinner strands and have more crosslinking. At 40°C, the large size (~200 nm diameter) of the aggregating colloidal casein particles (Figure 8A) resulted in a coarse-stranded gel being formed, which is in agreement with Lucey et al. (1997). The importance of hydrophobic liaisons in forming large colloidal particles below pH 5.1 has been shown by Herbert et al. (1999) and consequently, at 10 and 20°C (Figure 8C and 8D), the aggregating particles were much smaller (<100 nm) and a fine-stranded gel was formed.

CONCLUSIONS

Based on microstructural observations of milk acidified at 10, 20, 30, and 40°C, we propose a 3-phase model

for acid-induced gelation of unheated milk that is temperature-dependent and helps explain the formation of coarse-stranded gels at higher temperatures. Phase 1 involves a temperature-dependent dissociation of proteins from the casein supramolecules, with less protein being released when the milk is warm (40°C). At low temperatures (e.g., 10°C), these dissociated proteins are present as loosely entangled aggregates. At 40°C, there appears to be sufficient hydrophobic interactions to maintain the proteins as (small) spherical colloidal particles (many with diameters <50 nm). As acidification occurs, this dissociation continues, with proteins forming into large, loosely entangled protein aggregates. Such aggregates are nonspherical and do not have the compact nature observed for casein supramolecules, although some are as large as the casein supramolecules and are occasionally attached to the casein supramolecules. Phase 2 of acid gelation occurs between about pH 5.3 and pH 4.9 and involves the loosely entangled proteins forming into more compact colloidal particles. At 40°C, the loosely entangled aggregates become quite large (up to 200–300 nm) whereas, at 10°C, they are much smaller and have already started to reassociate with the casein supramolecule remnants. Phase 3 involves rapid aggregation of the colloidal casein supramolecules into a gel network as the pH decreases to 4.8 and lower. This gel network consists of chains of compact spherical particles made of casein supramolecule remnants that remained in colloidal form during acidification, as well as those formed during reassociation of the casein molecules. The nature of the gel is dependent on temperature, with the gel at low temperature being composed of smaller particles that form thinner strands and have more crosslinking. Acid coagulating milk at 10°C forms a fine-stranded gel, whereas at 40°C a coarse-stranded gel is formed.

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