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Rheological Properties of Acid Gels Prepared from Heated pH-Adjusted Skim Milk

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Reconstituted skim milk was adjusted to pH values between 6.5 and 7.1 and heated (90 °C) for up to 30 min. The skim milk samples were then readjusted to pH 6.7. Acid gels prepared from heated milk had markedly higher *G′* values, a reduced gelation time, and an increased gelation pH than those prepared from unheated milk. An increased pH at heating decreased the gelation time, increased the gelation pH, and increased the final *G′* of acid set gels prepared from the heated milk samples. There were only small differences in the level of whey protein denaturation in the samples at different pH values, and these differences could not account for the differences in the *G′* of the acid gels. The levels of denatured whey protein associated with the casein micelles decreased and the levels of soluble denatured whey proteins increased as the pH at heating was increased. The results indicated that the soluble denatured whey proteins had a greater effect on the final *G′* of the acid gels than the denatured whey proteins associated with the casein micelles.

KEYWORDS: Milk; denatured whey proteins; casein micelles; rheology, acid gelation; heating; pH

INTRODUCTION

The heat treatment of milk at temperatures above ∼70 °C results in the denaturation of the whey proteins (*1*, *2*). These denatured whey proteins can undergo a complex series of aggregation reactions with other denatured whey proteins and with the casein micelles (*3*−*13*). The major reaction process appears to involve thiol−disulfide exchange reactions between protein species with free thiol groups and those with disulfide bonds (*3*, *6*, *14*), although ionic and/or hydrophobic interactions may be involved in the early stages of the reaction (*4*, *5*).

Heat treatments of milk under conditions where the whey proteins are denatured are used to modify the texture and consistency of acid gels (*15*−*19*). Increasing levels of denaturation in heated milk result in set gels with increased firmness and reduced levels of syneresis (*20*−*22*). Rheological investigations have shown that the heat treatment of milk reduces the gelation time, increases the gelation pH on subsequent acidification, and produces acid gels with markedly higher *G′* values than those prepared from unheated milks (*23*−*25*).

Native whey proteins are soluble at their isoelectric point (pH ∼5.3), whereas denatured whey proteins are insoluble. The effects of heating milk on the subsequent properties of acid gels have been attributed to the involvement of the denatured whey proteins in the acid gel structure by bridging casein micelles and increasing the number and strength of contact points in the subsequent gel network (*23*−*25*). It has also been shown that the interactions of denatured whey proteins with the casein micelles are important in the structure formation of acid gels. The *G′* values of acid gels prepared from milks in which the denatured whey proteins were predominantly associated with the casein micelles were markedly higher than those of acid gels prepared from milk samples in which the whey proteins were pre-denatured in the absence of casein and, therefore, not associated with the casein micelles. This indicated that the association of denatured whey proteins with the casein micelles is important for the marked increase in the *G′* of acid gels made from heated milk (*24*).

In recent studies, it has been shown that the interaction of denatured whey proteins with the casein micelles is markedly dependent on small shifts in the pH of the milk at heating (*12*, *13*). At pH 6.5, ∼70% of the denatured whey proteins are associated with the casein micelles. The level of association decreases with increasing pH so that only ∼30% are associated at pH 6.7. As the pH of the milk is increased above ∼6.7 before heating, the κ-casein progressively dissociates from the casein micelles, and only low levels of denatured whey proteins associate with the casein micelles at these elevated pH values (*26*, *27*).

By adjusting the pH of the milk to between 6.5 and 7.1 prior to heat treatment, it is possible to produce milk samples with different levels of whey proteins associated with the casein micelles. The current study was conducted to examine the effect of the milk pH at heating, and therefore the level of whey

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proteins associated with the micelles, on the rheological properties of acid gels prepared from these milk samples. The level of whey protein denaturation, the level of denatured whey proteins associated with the casein micelles, and the level of soluble denatured whey proteins were also monitored so that any relationships between the rheological properties of the acid gels and the denaturation of whey proteins or their association with the micelles could be examined.

MATERIALS AND METHODS

Milk Supply. Fresh skim milk samples were obtained from the Bavarian State Dairy, Freising, Germany. Low-heat skim milk powder (whey protein nitrogen index >6; 37% protein) was obtained from Fonterra Co-operative Group, Pahiatua Manufacturing Site, New Zealand. Reconstituted skim milk samples were prepared by adding low-heat skim milk powder to purified (reverse osmosis followed by filtration through a Milli-Q apparatus) water to a final concentration of 10% (w/w) total solids. The reconstituted skim milk samples were allowed to equilibrate at ambient temperature (~20 °C) for at least 10 h before further treatment. A small amount of sodium azide (0.01% w/v) was added to all milk samples as a preservative.

Adjustment of pH and Heat Treatments. The pH of the milk samples was adjusted by the slow addition of 3 M HCl or 3 M NaOH to stirred solutions. The pH was allowed to equilibrate for at least 2 h, and then minor readjustments were made. The milk samples were transferred to glass tubes and heated for the desired times (from 0 to 30 min) in a thermostatically controlled water bath preset to 90 °C. The heating time included the time to reach the experimental temperature, which was ~30 s. After heat treatment, the milk samples were cooled by immersion in cold running water until the temperature was below 30 °C. The samples were stored for 6 h at ambient temperature after heat treatment and before any further analysis.

Gel Formation. The milk samples were carefully readjusted to the natural pH (pH 6.7) with 3 M HCl or 3 M NaOH. They were acidified using glucono-δ-lactone (GDL) at a 2% (w/w) level and at 30 °C. In preliminary experiments, the pH change with time was monitored using a combination glass electrode type InLab 422 (Mettler Toledo, Urdorf, Switzerland) and standard pH-meter. The results showed that the pH change with time was the same for all milk samples regardless of the pH at heat treatment. The pH of all milk samples was ~4.2 after 6 h. Little further change in pH occurred on holding for an additional 4 h.

Rheological Measurements. The rheological properties of the acidified milks were monitored with time using low-amplitude dynamic oscillation, as has been described previously (*23*, *25*). A Carrimed CSL100 rheometer (TA Instruments U.K., Cirencester, Gloucestershire, U.K.) and a cone (4 cm, 4°) and plate arrangement were used for all experiments. GDL was added to the milk, the milk was stirred for 30 s, and then 1.2 mL was transferred to the rheometer plate and the plate raised to give the required gap between the cone and plate. A water trap and cover arrangement was placed over the sample to prevent evaporation. The strain applied was 0.01.

Initial measurements involved monitoring gelation as the milk was acidified. The samples were oscillated at a frequency of 0.1 Hz, and the temperature of the sample was maintained at 30 °C. Measurements were taken every 5 min for 5.5 h. Gelation was defined as the point at which the storage modulus (G') was ≥1 Pa. After 5.5 h, the effect of the time scale of the applied strain on the rheological properties of the set gel was determined by varying the frequency from 0.01 to 10 Hz. After gelation was complete, the temperature of the system was reduced to 5 °C, and the rheological properties of the set gel at 5 °C were monitored for a period of 15 min.

Centrifugation. Soluble whey proteins were defined as those that did not sediment from the milk during centrifugation at 14000 rpm (25000*g* average) for 1 h at 20 °C in an Eppendorf centrifuge type 5417C. The sample volume was 1 mL and was placed in a small plastic tube of 1.5 mL total volume. The clear supernatant was carefully poured from the pellet. The protein content and composition of the supernatants were determined by gel electrophoresis and laser densitometry. When centrifuged milk was compared with the original milk, a correction factor of 0.95 was applied to the supernatant samples to account for steric exclusion effects of the colloidal components (*28*). Preliminary experiments indicated that this centrifugation method provided a separation between colloidal and soluble phases almost identical to that of the ultracentrifugation method that has been used previously (*12*, *13*).

Gel Electrophoresis and Laser Densitometry. The casein and the denatured whey proteins were removed from the milk by adjusting the pH to 4.6 and centrifuging out the precipitate using a bench centrifuge. The resultant supernatant was analyzed for native whey protein content using native polyacrylamide gel electrophoresis (native-PAGE), as has been described previously (*2*). The level of soluble whey proteins in the centrifugal supernatants was determined using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, as has been described previously (*27*).

Native-PAGE and SDS-PAGE gels were scanned using a Molecular Dynamics model PD-SI computing densitometer (Molecular Dynamics Inc., Sunnyvale, CA) and integrated as has been described previously (*12*, *13*). The quantity of each protein in the ultracentrifugal supernatants was determined as a percentage of that in the original milk samples.

All experiments were fully replicated at least twice, including milk preparation, pH adjustments, heat treatments, and acidification. Experiments using different milk sources produced results similar to those reported.

RESULTS AND DISCUSSION

For all gelation experiments, the G' used in the discussion refers to the final G' value measured after a gelation time of 5.5 h, whereas the gelation pH and gelation time refer to the pH and time at which G' became ≥1 Pa. In preliminary experiments, a sample of milk at pH 6.7 was heated at 90 °C for 30 min, cooled, held for 6 h, and subsequently adjusted to pH 6.5 or 7.1 and then back to pH 6.7 with NaOH and HCl ranging in concentration from 0.1 to 3 M. The samples were then acidified with GDL, and the rheological properties were monitored with time. The pH adjustment with the lower concentration base and acid caused a significant reduction in the final G' of the acid gels (up to 40 Pa) when compared with the sample that had received no pH adjustment. As the concentration of the acid was increased, the difference between the pH-adjusted samples and the unadjusted samples became smaller (results not shown). This reduction in G' on pH adjustment was attributed to the dilution of the milk by the addition of acid and base. With 3 M acid or base, the differences between the samples were small, and therefore this was used for all subsequent pH adjustments. To avoid local pH extremes, a 10 μL micropipet was used to slowly add the acid or base to vigorously stirred milk samples. The changes in pH with time after GDL addition were unaffected by the various treatments applied, indicating that the chemical hydrolysis of GDL was unchanged by the pH adjustments and heat treatments (results not shown). These results indicate that the changes in the acid gelation properties are not related to the change in the colloidal calcium phosphate content of the micelles on pH adjustment and heat treatment.

The changes in G' with time after GDL addition for reconstituted and fresh skim milk samples that were pH adjusted to values in the range from pH 6.5 to 7.1 and heated (90 °C/30 min) are shown in **Figure 1**. Key gelation parameters are summarized in **Table 1**. Unheated milk samples are included for comparison. It should be noted that the pH of all samples was readjusted to pH 6.7 prior to the start of the gelation experiments. In agreement with literature reports, the acid gels prepared from unheated skim milk had very low G' values, long gelation times, and low gelation pH values, whereas those from

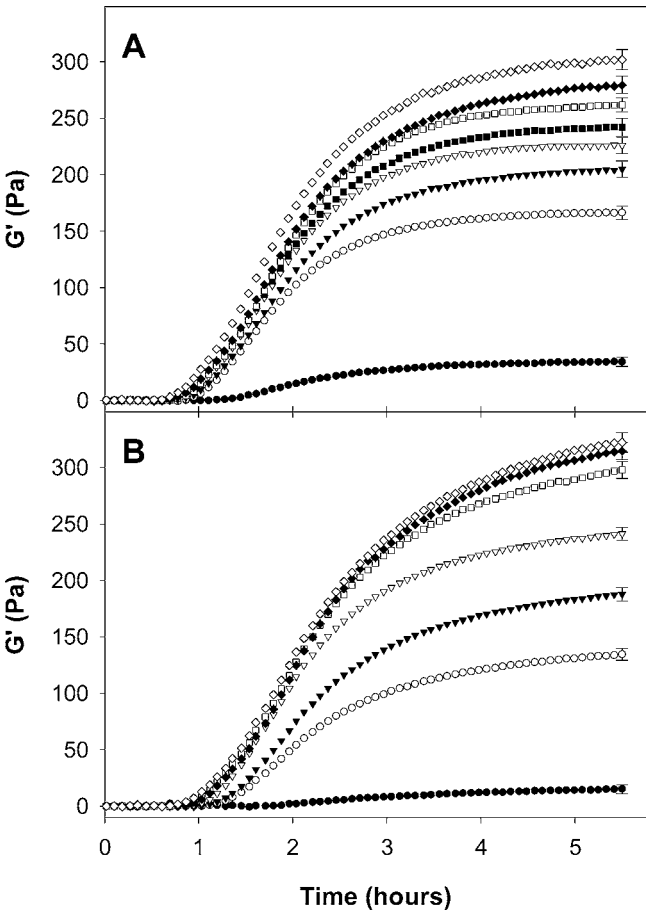

Figure 1. Changes in storage modulus, G', with time after GDL addition for unheated and heated (90 °C/30 min) skim milk samples: (**A**) reconstituted skim milk; (**B**) fresh skim milk: (●) unheated skim milk; (○) heated skim milk, pH 6.5; (▼) heated skim milk, pH 6.55; (▽) heated skim milk, pH 6.6; (■) heated skim milk, pH 6.65; (□) heated skim milk, pH 6.7; (◆) heated skim milk, pH 6.9; (◇) heated skim milk, pH 7.1. Error bars represent the standard deviation of replicate measurements.

the heated milks produced markedly higher G' values and had reduced gelation times and increased gelation pH values (*23*, *25*, *29*).

There was a clear effect of the pH at heating on the final G' of the acid gels (**Figure 1**; **Table 1**). As the pH of the milk was increased from 6.5 to 7.1, the final G' progressively increased so that the G' at pH 7.1 was nearly double and triple that observed at pH 6.5 for the reconstituted and fresh skim milks, respectively. The effect was particularly marked at pH values between 6.5 and 6.7, whereas the effect was smaller at pH values between 6.7 and 7.1. The pH at heating also had an effect on the gelation time and gelation pH, with a decrease in gelation time and an increase in gelation pH as the pH of the milk at heating was increased (**Figure 1**; **Table 1**). In all cases, plots of the logarithm of frequency against the logarithm of the final G' produced straight lines with slopes of ∼0.15 (results not shown), and the final G' values of the acid gels at 5 °C were approximately double those at 30 °C regardless of the pH at heating (**Table 1**). These observations are in accord with literature reports for acid skim milk gels (*23*, *25*, *29*).

In more detailed experiments, samples at pH values between 6.5 and 7.1 were heated for times from 0 to 30 min at 90 °C. The rheological properties during acidification were monitored. In addition, the level of whey protein denaturation and the level of whey proteins associated with the micelles were determined for each sample after heat treatment but before acidification. **Figure 2** shows the change in the final G' of the acid gels with heating time for the milk samples at each pH. The final G' of the acid gels increased markedly with heating time for milk samples heated for short periods (up to ∼10−15 min) and then plateaued for the samples heated for prolonged periods. For the latter samples, the pH of the milk at heating had a marked effect on the final G'. At pH 6.5, the maximum final G' was ∼160 Pa. This final G' increased as the pH of the milk at heating was increased, so that at pH 7.1 the final G' plateaued at values approximately double that observed at pH 6.5 (∼310 Pa), which is consistent with the results observed in **Figure 1**.

Lucey et al. (*24*) heated (80 °C/30 min) milk samples at the natural pH and at pH 7.0 before measuring the rheological properties during acidification. The sample at pH 7.0 had a slightly higher final G' than the sample at the natural pH. However, the effect may have been underestimated as the dilution during pH adjustment was not taken into account and the milk at pH 7.0 was not readjusted to the natural pH before acidification. Our results indicate that larger effects of pH adjustment occur on the acid side of the natural pH of 6.7, with proportionally smaller effects on the alkaline side of the natural pH (**Figures 1** and **2**).

The denaturation of β-lactoglobulin was essentially unaffected by the pH at heating over the narrow pH range used, whereas the denaturation of α-lactalbumin was slightly more rapid at higher pH (results not shown). This is consistent with literature reports (*11*, *13*). The decrease in the individual native proteins was at levels expected from previously published kinetic data (*1*, *2*, *11*). When the β-lactoglobulin and α-lactalbumin were taken to represent the total whey proteins, the differences in the level of denaturation at the different pH values were very small at all heating times (**Figure 3A**).

There was little relationship between the final G' of the acid gels and the level of denaturation when the results at all pH values were considered together (**Figure 3B**). This was particularly apparent at high levels of denaturation (low levels of native protein), where virtually all of the whey protein was denatured yet the G' varied from about 150 to 300 Pa, depending on the pH at heating. There have been reports that show a clear relationship between the level of whey protein denaturation and the firmness of set yogurts (*20*, *22*). In these cases, the milks were heated at their natural pH only. However, when the pH at heating is varied, the final G' of the gels cannot be attributed solely to the denaturation of the whey proteins (**Figure 3B**). Therefore, the final G' must be related to differences in the interactions of the denatured whey proteins with the other proteins in the milk system, including the casein micelles.

The level of total whey protein (α-lactalbumin and β-lactoglobulin combined) associated with the micelles is shown in **Figure 4A**. High levels of whey protein associated with the casein micelles at pH 6.5, and this decreased as the pH at heating increased, so that at pH 7.1 only very low levels (∼10%) were associated with the casein micelles. Similar effects were observed for the individual whey proteins (results not shown). This association behavior is consistent with previous reports (*13*, *27*). There was no relationship between the level of whey protein associated with the casein micelles and the final G' of the acid gels when all pH values were considered together (**Figure 4B**). At pH 6.5, the level of association ranged from 0 to 80% and the G' ranged from 30 to ∼200 Pa, whereas, at pH 7.1, the maximum level of whey protein associated with the micelles was <∼15% for all samples and yet the final G' ranged from 30 to >300 Pa. At pH values between 6.5 and 6.7, an

Table 1. Gelation Properties of Skim Milk Samples Acidified with GDL at 30 °C

	reconstituted skim milk				fresh skim milk			
sample	final G', 30 °C (Pa)	final G', 5 °C (Pa)	gelation time (min)	gelation pH	final G', 30 °C (Pa)	final G', 5 °C (Pa)	gelation time (min)	gelation pH
unheated	34.2 (4.0)[a]	79.3 (4.2)	95 (6)	4.78 (0.04)	15.2 (3.8)	34.8 (4.6)	93 (7)	4.75 (0.05)
heated, pH 6.5	166.4 (6.1)	357.1 (8.3)	52 (4)	5.17 (0.03)	134.5 (5.3)	275.1 (7.7)	72 (3)	5.11 (0.03)
heated, pH 6.55	205.1 (7.1)	431.3 (9.2)	47 (3)	5.21 (0.03)	187.6 (6.1)	430.8 (9.3)	60 (2)	5.12 (0.02)
heated, pH 6.6	225.9 (6.8)	488.3 (9.1)	44 (2)	5.23 (0.02)	241.3 (5.8)	533.1 (12.2)	52 (2)	5.17 (0.02)
heated, pH 6.65	241.9 (8.1)	512.2 (10.7)	40 (3)	5.27 (0.03)	nd[b]	nd	nd	nd
heated, pH 6.7	261.7 (6.4)	555.1 (8.8)	36 (1)	5.33 (0.01)	297.7 (7.4)	615.7 (14.4)	47 (2)	5.21 (0.03)
heated, pH 6.9	279.5 (7.6)	603.6 (9.9)	35 (2)	5.34 (0.02)	314.5 (7.7)	670.9 (12.3)	41 (1)	5.26 (0.01)
heated, pH 7.1	302.0 (8.9)	651.2 (11.3)	34 (2)	5.36 (0.03)	322.0 (8.7)	711.0 (13.7)	39 (2)	5.29 (0.03)

[a] Standard deviations are given in parentheses. [b] Not determined.

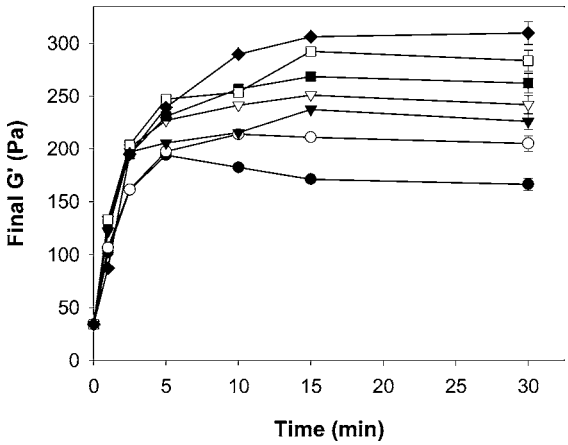

Figure 2. Changes in the final storage modulus (final G') for milk samples heated for various times at 90 °C. The pH values of the milk samples prior to heating were (●) 6.5, (○) 6.55, (▼) 6.6, (▽) 6.65, (■) 6.7, (□) 6.9, and (◆) 7.1. Error bars represent the standard deviation of replicate measurements.

intermediate behavior was observed for both association level and final G'.

From the level of whey protein denaturation and the level of whey protein associated with the casein micelles, it is possible to calculate the level of soluble denatured whey proteins (**Figure 5A**). Low levels (~10%) of soluble denatured whey proteins were observed at pH 6.5. These levels increased markedly as the pH of the milk was increased, so that at pH 7.1 ~80−90% of the whey proteins were soluble but denatured at the longer heating times. When the final G' of the acid gels was plotted against the level of soluble but denatured whey proteins in the milk, the results for all pH values were close to a single curve (**Figure 5B**). Although the denatured whey proteins associated with the micelles have a significant effect on the final G' of the acid gels (**Figure 4B**), those denatured whey proteins that remain in the serum appear to have a more dominant influence over the final G' of the acid gels than those associated with the casein micelles (**Figure 5B**).

After ~15 min of heating, essentially all of the whey proteins were denatured and were distributed between the casein micelle and soluble phases (compare **Figures 3**−**5**). Therefore, a plot of the final G' against the final level of soluble denatured whey protein (i.e., the G' and the level of soluble denatured whey protein observed after 15−30 min of heating) shows the effect of distributing the denatured whey protein between soluble and colloidal phases on the final G' of the acid gels (**Figure 6**). There was a strong positive correlation between the final G' and the level of soluble denatured whey protein ($r = 0.94$, $P < 0.01$). By extrapolation, the final G' of the acid gels would be

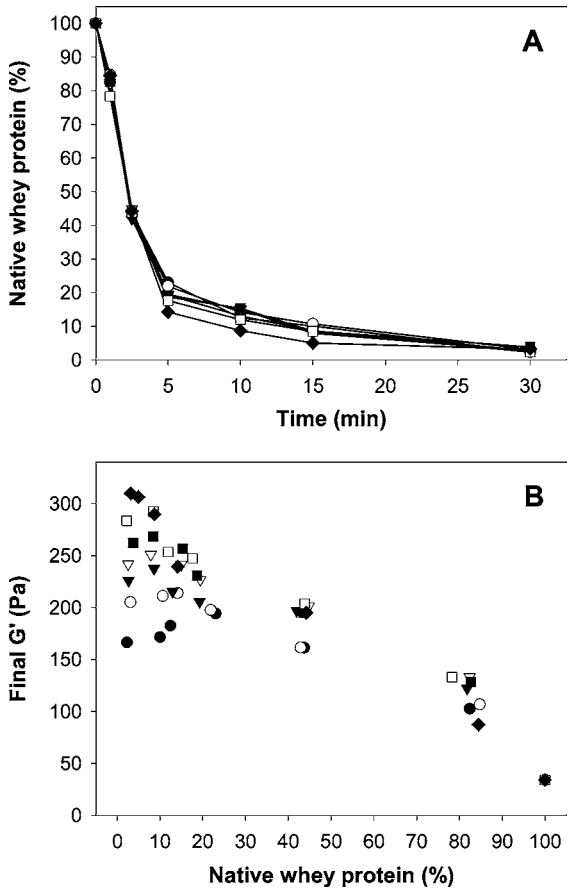

Figure 3. (**A**) Denaturation of whey proteins in skim milk samples that were heated at 90 °C for various times. (**B**) Relationship between the level of total denatured whey protein in heated skim milk and the final storage modulus (final G') for acid gels prepared from the heated milks. The pH values of the milk samples prior to heating were (●) 6.5, (○) 6.55, (▼) 6.6, (▽) 6.65, (■) 6.7, (□) 6.9, and (◆) 7.1.

~160 Pa if all of the denatured whey proteins were associated with the casein micelles, whereas the final G' would be ~320 Pa if all of the denatured whey proteins remained in the serum. Intermediate values for G' were observed when the level of denatured whey protein associated with the casein micelles was between these two extremes.

Lucey et al. (*24*) showed that acid gels prepared from milk samples with the whey proteins bound to the casein micelles had markedly higher final G' values than those from unheated milk, which is in broad agreement with the results presented here (**Figures 4** and **6**). However, Lucey et al. (*24*) found that milk samples with soluble denatured whey protein aggregates produced acid gels with markedly lower final G' values than

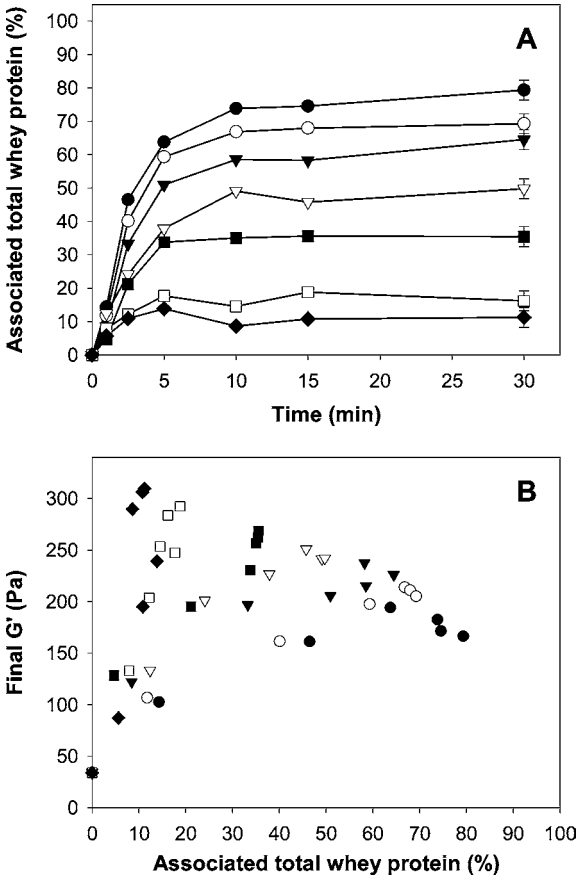

Figure 4. (**A**) Level of whey proteins associated with the casein micelles in skim milk samples that were heated at 90 °C for various times. (**B**) Relationship between the level of total whey protein associated with the casein micelles in the heated skim milk samples and the final storage modulus (final *G′*) for acid gels prepared from the heated milks. The pH values of the milk samples prior to heating were (●) 6.5, (○) 6.55, (▼) 6.6, (▽) 6.65, (■) 6.7, (□) 6.9, and (◆) 7.1. Error bars represent the standard deviation of replicate measurements.

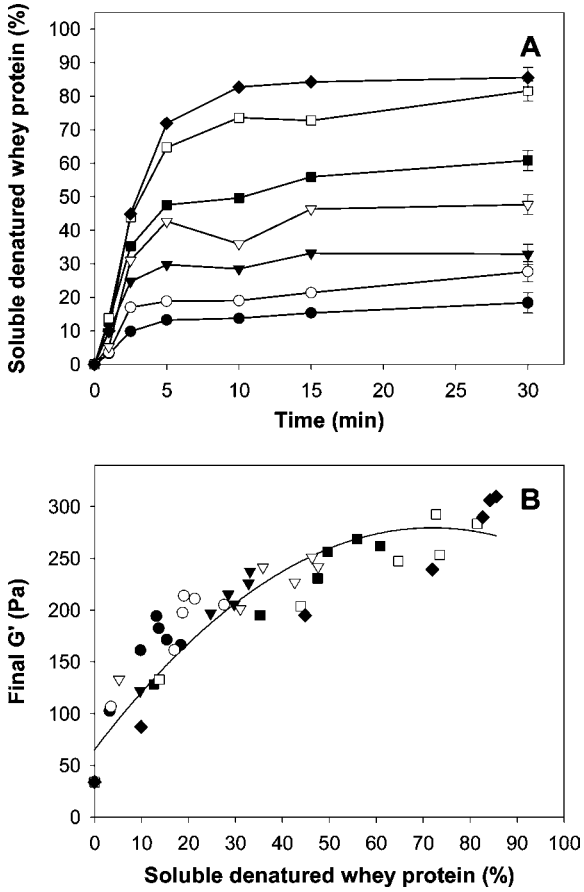

Figure 5. (**A**) Level of soluble denatured whey proteins in skim milk samples that were heated at 90 °C for various times. (**B**) Relationship between the level of soluble denatured whey proteins in the heated skim milk samples and the final storage modulus (final *G′*) for acid gels prepared from the heated milks. The pH values of the milk samples prior to heating were (●) 6.5, (○) 6.55, (▼) 6.6, (▽) 6.65, (■) 6.7, (□) 6.9, and (◆) 7.1. Error bars represent the standard deviation of replicate measurements.

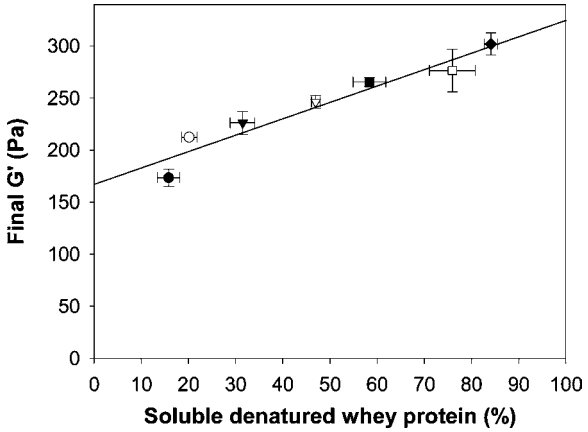

Figure 6. Comparison between the final storage modulus, *G′*, at 30 °C and the maximum level of soluble denatured whey proteins in the heated skim milk samples. The pH values of the milk samples prior to heating were (●) 6.5, (○) 6.55, (▼) 6.6, (▽) 6.65, (■) 6.7, (□) 6.9, and (◆) 7.1. Error bars represent the standard deviation of replicate measurements.

those with micelle-bound whey protein aggregates. In some cases, the final *G′* values were comparable with those from acid gels prepared from unheated milk. This is in marked contrast to the results of this study, which showed that acid gels prepared from milk with predominantly soluble whey protein aggregates had markedly higher final *G′* than those with predominantly micelle-bound whey proteins (**Figures 4**−**6**).

Schorsch et al. (*30*) examined the effect of heating whey proteins in the presence or absence of casein micelles on the subsequent acid gelation properties. The acid-induced gelation occurred at a higher pH and in a shorter time when the whey proteins were denatured separately from the casein micelles than when the whey proteins were heated in the presence of casein micelles. However, the gels formed were weaker and more heterogeneous because of the particulate nature of the denatured whey proteins. It was suggested that the large denatured whey protein aggregates hinder the formation of a casein gel network and that a weak acid gel structure results.

Lucey et al. (*24*) prepared the denatured whey protein solutions by heating the ultracentrifugal supernatant from unheated skim milk, and Schorsch et al. (*30*) dispersed whey protein concentrate in simulated milk ultrafiltrate. These methods of heating whey protein solutions in the absence of casein micelles but in the presence of the soluble milk salts will cause the precipitation of calcium phosphate and a marked reduction in pH when compared with heated milk. As a consequence, large whey protein aggregates may be formed. This is supported by the observation that a large proportion of the soluble aggregates actually cosedimented with the casein micelles during centrifugation, indicating that they were of considerable size (*24*). Transmission electron microscopy showed that large whey

protein aggregates were formed when whey protein solutions were heated in the absence of casein micelles but that these aggregates were not observed when the casein micelles were present during heating (*30*). These large whey protein aggregates may not participate strongly in structural formation with the casein micelles during acidification and, therefore, may act like an inert filler or even weaken the final gel structure.

The casein micelles are markedly changed during the acidification of unheated milk to the pH required to form acid gels. The colloidal calcium phosphate (CCP) is progressively solubilized as the pH is reduced so that essentially all of the calcium and inorganic phosphate are found in the soluble phase at pH 4.6 (*31*). The level of casein transferred to the soluble phase on acidification of milk is strongly dependent on temperature, with high levels transferred to the soluble phase at 4 °C and low levels solubilized at 30 °C (*32*). The solubility of casein diminishes as the milk pH approaches the isoelectric point of the casein (pH ~4.6), and the proteins aggregate to form a gel. In unheated milk, the native whey proteins remain soluble and play no part in the acid gel structure.

Unlike the native whey proteins, the denatured whey proteins are insoluble at their isoelectric points. Therefore, as the pH of heated milk approaches the isoelectric point of the whey proteins (pH ~5.3 for β-lactoglobulin), the whey proteins can aggregate. As a consequence, the gelation of heated milks occurs at a pH considerably higher than in unheated milk (**Table 1**; **Figure 1**). However, it is evident from the results in **Table 1** and **Figure 1** that the pH of gelation increased and the gelation time decreased as the pH of the milk prior to heating was increased. The increased pH prior to heating results in decreased levels of denatured whey proteins associating with the casein micelles and increased levels of soluble denatured whey proteins (**Figures 4** and **5**). These results suggest that the aggregation of the soluble denatured whey proteins occurs at a higher pH than the aggregation of the denatured whey proteins that are associated with the casein micelles. Schorsch et al. (*30*) reported that, when denatured whey proteins were added to casein micelles, the pH of gelation was considerably higher than when the whey proteins were denatured in the presence of the casein micelles. This supports our supposition that soluble denatured whey proteins aggregate at a higher pH than the whey protein/casein micelle complexes.

The observation that the heated skim milk samples with soluble denatured whey proteins produce acid gels with higher final G' than those with the denatured whey proteins associated with the casein micelles may be related to the aggregation process as the milk approaches the isoelectric pH. The rigidity of gels and their resistance to deformation are related to the number and strength of contact points between aggregated particles within a defined area of the structure (*33*). For milk in which the denatured whey proteins are soluble, there will be numerous aggregating particles involving the casein micelles and the soluble denatured whey proteins. Aggregation can occur between the soluble denatured whey proteins, between the denatured whey proteins and the casein micelles, and between casein micelles. Therefore, there is potential for a complex gel network involving a large number of aggregating particles. In contrast, in milk in which the denatured whey proteins are associated with the micelles, there are fewer aggregating particles and the aggregation process will probably involve only entire whey protein/casein micelle complexes. Therefore, there may be fewer contact points in the acid gels formed from milk with the denatured whey proteins associated with the micelles than in those formed from milk with soluble denatured whey proteins.

The pH dependence of the interaction of denatured whey proteins with the casein micelles at pH values >6.7 may be related to the pH dependence of the dissociation of κ-casein from the casein micelles when milk is heated (*26*, *27*, *34*). Low levels of κ-casein are observed in the milk serum at pH values below ~6.7, whereas increasing levels of κ-casein are observed in the milk serum as the pH is increased above pH 6.7. Heat treatment of milk above ~70 °C results in the denaturation of the whey proteins. This denaturation reaction results in increased reactivity of the free thiol groups of β-lactoglobulin, which can be involved in thiol−disulfide exchange reactions with other denatured whey proteins and with κ-casein. As increasing levels of κ-casein are in the serum at pH values >6.7, there may be a preferential interaction of the denatured whey proteins with the serum phase κ-casein and, hence, high levels of soluble denatured whey proteins.

However, there are only low levels of dissociated κ-casein at pH values below 6.7 (*26*, *27*, *34*), yet there is still a marked pH dependence of the association of denatured whey proteins with the casein micelles at pH values between 6.5 and 6.7 (**Figure 4**; *13*). The interaction between denatured whey proteins and the casein micelles probably involves sulfhydryl−disulfide exchange reactions between the free sulfhydryl groups of denatured β-lactoglobulin/whey protein aggregates and the disulfide bonds of κ-casein. Because of the location of the disulfide bonds on the para-κ-casein region of κ-casein, the denatured whey proteins would need to penetrate through the surface glycomacropeptide (hairy) layer of κ-casein in order to interact with the disulfide bonds (*10*, *35*). As the pH is increased, the micelle surface charge and, in particular, the charge on the glycomacropeptide hairs will increase, which will cause the hairs to extend further from the micelle surface. This may reduce the association of denatured whey proteins with the casein micelles when the pH is increased and therefore increase the tendency for serum phase aggregation reactions of the denatured whey proteins.

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