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Rennet-Induced Aggregation of Heated pH-Adjusted Skim Milk

Skelte G. Anema,*,† Siew Kim Lee,† and Henning Klostermeyer

Lehrstuhl für Chemie der Biopolymere, Technische Universität München, 85350 Freising, Germany

Supporting Information

ABSTRACT: Heated (20−100 °C/0−30 min) skim milks (pH 6.5−7.1) were diluted in buffer (pH 7.0). Rennet was added, and the particle size with time was measured. For all samples, the size initially decreased (lag phase) and then increased (aggregation phase). Milks heated at ≤60 °C had short lag phases and rapid aggregation phases regardless of pH. Milks heated at >60 °C at pH 6.5 had long lag phases and slow aggregation phases. As the pH increased, the lag phase shortened and the aggregation phase accelerated. The aggregation time was correlated with the level of whey protein associated with the casein micelles and with the level of κ-casein dissociated from the micelles. Heated milks formed weak gels when renneted. It is proposed that the milks heated at low pH have whey proteins associated with the casein micelles and that these denatured whey proteins stabilize the micelles to aggregation by rennet and therefore inhibit gelation. In the milks heated at higher pH, the whey proteins associate with κ-casein in the serum and, on rennet treatment, the κ-casein-depleted micelles and the serum-phase whey protein/κ-casein complexes aggregate; however, the denatured whey proteins stabilize the aggregates so that gelation is still inhibited.

KEYWORDS: milk, denatured whey protein, casein micelle, chymosin, rennet, heating, pH, gelation, aggregation

INTRODUCTION

The heat treatment of milk results in the denaturation of the whey proteins, and these denatured whey proteins can interact with κ-casein to form serum-phase or micelle-bound aggregates depending on the pH of the milk.[1,2] It is now generally agreed that, as the pH of milk is progressively increased from about pH 6.5 to about pH 7.1 before heat treatment, the denatured whey proteins are progressively transferred from being complexes associated with κ-casein at the casein micelle surface to serum-phase complexes with κ-casein that has dissociated from the micelles.[1−3]

The pH-dependent change in the distribution of denatured whey proteins and κ-casein between the serum and colloidal phases affects the functional properties of the milks in acid gelation applications. The final G' of acid gels increases markedly as the pH of the milk at heating increases, which corresponds to an increase in the serum-phase denatured whey protein/κ-casein complexes.[4−6] However, the few studies on the rennet-induced gelation of heated milks indicated that there was little effect of pH on the properties of the gels formed. The rennet-induced gels from heated milks had low G' when compared with unheated milks, regardless of the pH of the milks at heating.[7,8]

It was initially believed that the primary (enzymatic) phase of the renneting reaction is inhibited by heat treatments.[9−11] However, more recent studies have indicated that, under relatively mild heating conditions, this primary phase is only slightly affected by the heat treatment of the milk or the pH of the milk at heating.[7,8,12,13] This indicates that the Phe^{105}−Met^{106} bond of κ-casein is accessible to the enzymes in rennet even when the κ-casein has associated with denatured whey proteins. Therefore, the changes in gel formation for heated milk when compared with unheated milk appear to be due to the inhibition of the secondary (aggregation or gelation) phase of the renneting reaction. It has been proposed that the denatured whey proteins inhibit the aggregation of the casein micelles, thereby slowing gel formation, and that this inhibition occurs regardless of whether the denatured whey proteins are associated with the casein micelles or in the serum phase.[7,9,12] However, the reduced calcium activity in heated milks, as a result of the conversion of soluble calcium and phosphate to an insoluble colloidal phase, may also contribute to the slower gel formation, and this is supported by the observation that added calcium salts or pH cycling of heated milks can restore their rennet gelation characteristics to a certain extent.[9,14,15]

Recently, the inhibition of rennet-induced gelation of heated milks was studied using systematic exchange experiments in which proteins and other components from different phases of unheated and heated milks were interchanged.[8] The denatured whey proteins, whether associated with the micelles or in the serum phase, were found to inhibit gelation. However, changes to the casein micelles and nonprotein components in the heated milks also inhibited gelation. It was concluded that the inhibition of the rennet-induced gelation of heated skim milk is a complex process and may be due to synergistic effects of heat-induced changes to the casein micelles, the denatured whey proteins (both serum and micelle bound), and other nonprotein serum components.

As the heat treatment of milk at different pH values can result in the formation of casein micelles with different κ-casein and whey protein concentrations,[1,2] it would be expected that these milks would have different stabilities toward rennet; however, gelation experiments indicate that this may not be the case.[7,8] Gelation by rennet is not an objective measure of the stability of the casein micelles as it indicates only the formation of a network structure of the casein micelles. It is possible for the casein

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micelles to be destabilized by rennet without the formation of a gel. This study was therefore conducted to examine the very early stages of the rennet-induced destabilization of the casein micelles in heated milks. Particle size analysis was used as an indicator of the aggregation of the casein micelles. The denaturation of the whey proteins, their association with the casein micelles, and the dissociation of κ-casein from the casein micelles were also monitored so that relationships between the stability and the composition of the casein micelles could be evaluated.

MATERIALS AND METHODS

Milk Supply. Reconstituted skim milk was prepared from low-heat skim milk powder (Fonterra Co-operative Group, New Zealand) and water to give a milk concentration of 10% (w/w) total solids. Fresh skim milk samples were obtained from the Bavarian State Dairy, Freising, Germany. 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 values of the milk samples were adjusted to pH 6.5−7.1 by the slow addition of 3 M HCl or 3 M NaOH to stirred samples. The pH was allowed to equilibrate for at least 2 h with minor readjustments during this time. Subsamples of milk (6 mL) were transferred to glass tubes and heated for the desired times (from 0 to 30 min) in a thermostatically controlled water bath preset to the required temperature (from 20 to 100 °C). After heating, the samples were cooled by immersion in cold running water and then stored for 12 h before use.

Gel Electrophoresis and Laser Densitometry. Milk samples (1 mL) were placed in small plastic tubes of 1.5 mL total volume and then centrifuged at 14000 rpm (25000*g* average) for 1 h at 20 °C in an Eppendorf centrifuge type 5417R. The supernatant was poured from the pellet, and the composition of these supernatants was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, as described previously.[16] The SDS-PAGE gels were scanned using a Molecular Dynamics model PD-SI computing densitometer (Molecular Dynamics Inc., Sunnyvale, CA) and integrated, as described previously.[16] The quantity of each protein in the ultracentrifugal supernatants was determined as a percentage of that in the original milk samples.

Particle Size Analysis during Rennet Treatment. Particle sizes were obtained by photon correlation spectroscopy using the methods described previously.[17] Particle sizing of the diluted milk samples was performed at 25 °C using a Malvern Zetasizer 3 instrument and the associated AZ10 sizing cell (Malvern Instruments, Malvern, Worcestershire, U.K.). A subsample of milk (20 μL) was added to calcium−imidazole buffer (1 mL of a buffer containing 20 mM imidazole, 30 mM NaCl, 5 mM $CaCl_2 \cdot 2H_2O$, pH 7.0) in the glass tube of the sizing cell. The sample was allowed to equilibrate for 5 min, and then five measurements were performed to get the initial size of the particles. Rennet (Chymogen 570, 570IMCU, Chr. Hansen, Hørsholm, Denmark) was diluted with water (1:120 rennet to water), then 10 μL was added to the sample, and the particle size was measured repeatedly until it had increased about 100 nm above the initial particle size. For particle sizing in undiluted milk, a Malvern Zetasizer Nano ZS (Malvern Instruments) was used. The unheated and heated milk samples were carefully readjusted to the natural pH (~6.7) with 3 M HCl or 3 M NaOH and then allowed to equilibrate for 12 h. A subsample of milk (1 mL) was placed in the sizing cell. It was allowed to equilibrate for 5 min, and then five measurements were performed to get the initial size of the particles. Diluted rennet (50 μL) was added to the milk, and the particle size measurements were continued until the size had increased about 100 nm above the initial particle size.

Gel Formation and Rheological Measurements. The rheological properties of the milks during renneting were monitored using a Carrimed CSL100 rheometer (TA Instruments UK, Cirencester, Gloucestershire, U.K.) and a cone (4 cm, 4°) and plate arrangement, as described previously.[7] The applied strain was 0.01, the frequency of oscillation was 0.1 Hz, and the temperature was controlled at 30 °C. The milk samples were carefully readjusted to the natural pH (6.7) with 3 M HCl or 3 M NaOH and then allowed to equilibrate for 12 h. Rennet was diluted with water (1 part rennet with 3 parts water), a subsample of the dilute rennet (40 μL) was added to a subsample of milk (1300 μL), and the mixture was upturned several times to mix. The milk sample was then transferred to the rheometer plate, the plate was raised to the required gap, and the experiment was started. The samples were oscillated at a frequency of 0.1 Hz, a strain of 0.01, and a temperature of 30 °C. Measurements were taken every 5 min for 1 h.

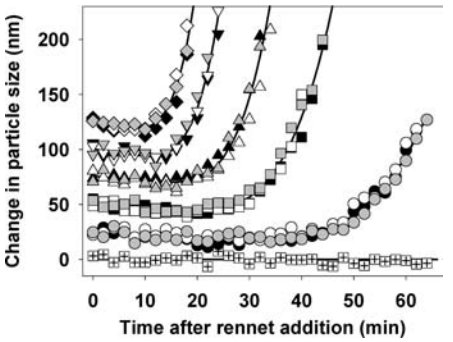

Figure 1. Changes in particle size on the rennet treatment of unheated milks. Milk samples (20 μL) were diluted in calcium−imidazole buffer (1 mL). The level of rennet (diluted 1:120 with water) added to the samples was (⊞) 0 μL, (○) 5 μL, (□) 7.5 μL, (△) 10 μL, (▽) 15 μL, or (◇) 20 μL. The different colored symbols of the same shape represent replicate experiments. For clarity, each curve was displaced from the previous curve by 25 nm.

Statistical Analysis. All experiments were fully replicated at least twice, including milk preparation, pH adjustments, heat treatments, and renneting experiments. Statistical analyses of key parameters were performed using the EZAnalyze program.[18] Any results reported as being significant had $P \leq 0.05$.

RESULTS

Particle Size Changes in Rennet-Treated Unheated Skim Milk Samples. A particle sizing technique was used to study the rennet-induced aggregation of the casein micelles in heated milk, independent of other variables. For each experiment, milk was diluted in buffer and a volume of (diluted) rennet was added to the sample. The changes in particle size of the samples were monitored with time. The changes in particle size for unheated milk samples with added rennet levels from 0 to 20 μL are shown in Figure 1. For the sample with no added rennet, the size did not change over a period of more than 1 h, indicating that the casein micelles were stable in the buffer system. When rennet was added, the size initially decreased slightly and then markedly increased, indicating that the casein micelles were sufficiently destabilized to start aggregating. As expected, the addition of higher levels of rennet resulted in a more rapid destabilization of the casein micelles, with aggregation occurring more rapidly as the rennet level was increased. The size changes in the milk samples with time at each rennet level were analyzed in triplicate and were found to be reproducible (Figure 1). This indicates that it should be possible to use this particle sizing technique to monitor the changes in stability of the casein micelles in milk during rennet treatment.

Two points from the particle size versus time curves, as shown in Figure 1, are used in the discussion. The time taken for the

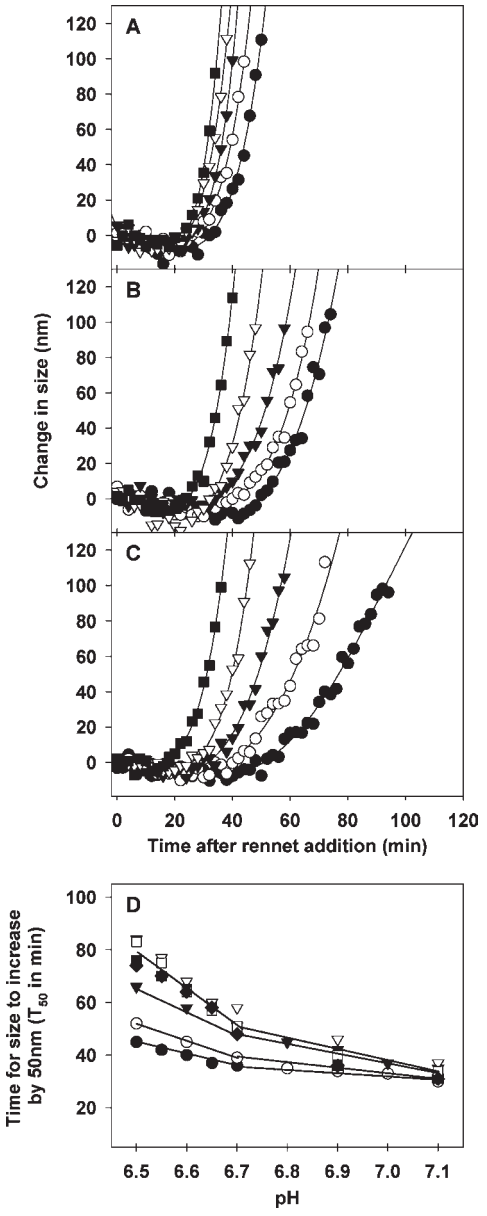

Figure 2. Changes in particle size on the rennet treatment of (A) unheated skim milk, (B) skim milk heated at 90 °C for 2.5 min, and (C) skim milk heated at 90 °C for 30 min. Milk samples (20 μL) were diluted in calcium−imidazole buffer (1 mL) before the addition of rennet (10 μL of 1:120 diluted rennet). The pH values of the milk samples were (●) 6.5, (○) 6.6, (▼) 6.7, (▽) 6.9, and (■) 7.1. (D) Effect of pH on the time taken for the particle size to increase by 50 nm from the initial size (T_{50}) after the addition of rennet to the milk samples. The milk samples were unheated (●) or heated at 90 °C for (○) 1 min, (▼) 2.5 min, (▽) 5 min, (■) 10 min, (□) 15 min, or (◆) 30 min. Each point represents the average of replicate measurements.

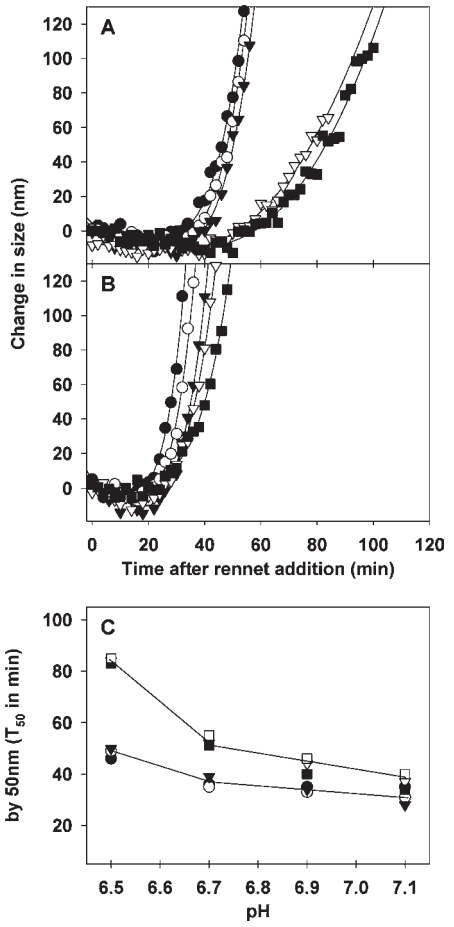

Figure 3. Effect of pH on the changes in particle size on the rennet treatment of skim milk heated at different temperatures for 30 min. The milk samples were at (A) pH 6.5 or (B) pH 7.1 before heat treatment. Milk samples (20 μL) were diluted in calcium−imidazole buffer (1 mL) before the addition of rennet (10 μL of 1:120 diluted rennet). The heating temperatures of the milks were (●) 20 °C, (○) 40 °C, (▼) 60 °C, (▽) 80 °C, and (■) 100 °C. (C) Effect of pH on the time taken for the particle size to increase by 50 nm from the initial size (T_{50}) after the addition of rennet to the milk samples. The milk samples were heated for 30 min at (●) 20 °C, (○) 40 °C, (▼) 60 °C, (▽) 80 °C, (■) 90 °C, or (□) 100 °C. Each point represents the average of replicate measurements.

milks to start increasing in size is referred to as T_{agg}. As T_{agg} was relatively difficult to obtain accurately, for comparative purposes, the time for the size to increase by 50 nm from the initial size is used in the detailed discussion and is referred to as T_{50}.

Particle Size Changes on the Rennet Treatment of pH-Adjusted Skim Milk Samples Heated at 90 °C for Different Times. Milk samples were adjusted to pH 6.5−7.1 and then heated at 90 °C for up to 30 min. The changes in particle size on the addition of rennet to unheated milks and heated milks were monitored after the samples had been dispersed in buffer. The changes in size for the unheated milks and the milks heated for 2.5 and 30 min are shown in Figure 2, panels A−C, respectively. T_{50} plotted against pH for the unheated and heated milks is shown in Figure 2D. For the unheated milks, T_{agg} (Figure 2A) and T_{50} (Figure 2D and Supporting Information) decreased slightly as the pH of the milks increased. This decrease in T_{agg} and T_{50} with an increase in the pH of the milk was small but was consistently observed. It is not known why unheated milks adjusted to slightly higher pH would aggregate more quickly than those at lower pH. One possibility is the change in mineral balance on pH adjustment of the milks. Milks at higher pH will have a higher colloidal calcium phosphate level than milks at lower pH,[19] and this should be maintained when the milks are dispersed in the buffer used for particle sizing. As the casein micelles are more mineralized at higher pH, this may make them aggregate somewhat

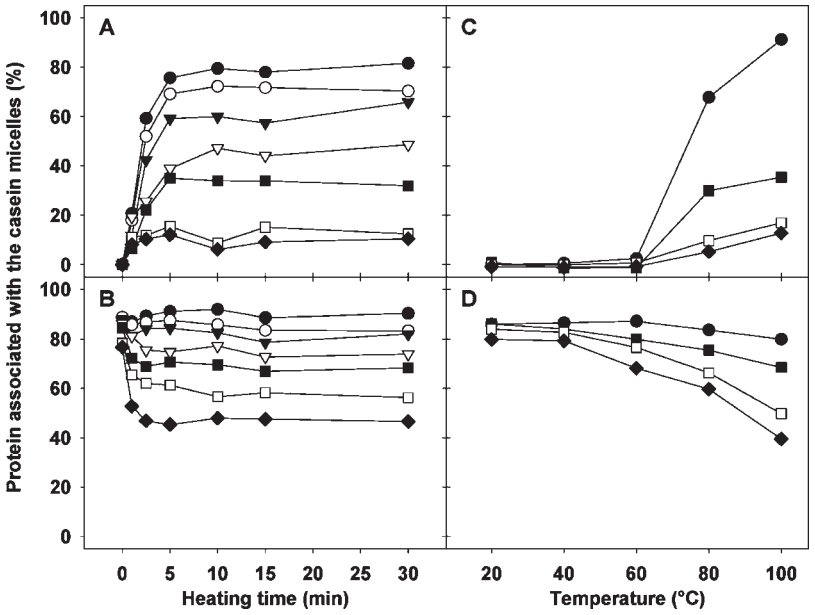

Figure 4. Percentage of (A, C) β-lactoglobulin and (B, D) κ-casein associated with the casein micelles in skim milk samples that were heated at (A, B) 90 °C for various times or (C, D) different temperatures for 30 min. The pH values of the milk samples were (●) 6.5, (○) 6.55, (▼) 6.6, (▽) 6.65, (■) 6.7, (□) 6.9, and (◆) 7.1.

earlier on subsequent rennet treatment. However, it is also possible that there are small increases in the soluble casein levels as the milk pH is increased, as has been reported previously.[3,16] The dissociation of casein may make the casein micelles more susceptible to aggregation by rennet and, therefore, account for the slightly faster aggregation rate as the pH of the unheated milk is increased (Figure 2).

For the heated milks, a similar general behavior was observed at all heating times (Figure 2B−D). T_{agg} and T_{50} were considerably longer for the milks heated at pH 6.5 than for the unheated milks. As the pH of the milks was increased from pH 6.5 to 7.1, T_{agg} and T_{50} decreased markedly so that, at pH 7.1, they were similar to those for the unheated milks (Figure 2B,C). A greater overall effect of pH was observed for the milk samples heated for 30 min than for those heated for 2.5 min, mainly because of the marked increases in T_{agg} and T_{50} for the milk samples heated at low pH when compared with those heated at higher pH. This can be clearly seen in Figure 2D, in which T_{50} is plotted against the pH of the milk at heat treatment. For the heated milks at pH 6.5 and for heating times of 5 min or longer, T_{50} was almost twice that of the unheated milks. However, as the pH of the milks was increased, the difference in T_{50} between the heated milks and the unheated milks became progressively smaller, so that the T_{50} values were indistinguishable at pH 7.1 regardless of the heating conditions. It is also evident that, at any given pH, the T_{50} values for the milks heated for 5 min or longer were indistinguishable from each other, whereas those for the milks heated for 1 or 2.5 min had a behavior that was between those of the unheated milks and those of the milks heated for ≥5 min (Figure 2D).

Effect of Heating Temperature on the Changes in Particle Size on Rennet Treatment of Skim Milk Samples. The changes in particle size on rennet treatment of milk samples at pH 6.5 and 7.1 that were heated at 20−100 °C for 30 min are shown in Figure 3, panels A and B, respectively. Rennet treatment of the samples heated at 20−60 °C at pH 6.5 produced similar curves, with the size initially decreasing slightly and then increasing markedly, with T_{agg} at about 30 min after rennet treatment. In contrast, on rennet treatment of the samples heated at 80 or 100 °C at pH 6.5, T_{agg} was considerably longer, at about 60 min, although the samples heated at 80 and 100 °C were not significantly different from each other. On rennet treatment of the samples heated at pH 7.1, T_{agg} increased slightly as the heating temperature increased from 20 to 100 °C. However, as T_{agg} was <30 min for all samples heated at pH 7.1, aggregation occurred in a much shorter time than for the corresponding samples at pH 6.5. This difference was relatively small for the samples heated at 20−60 °C but was quite marked for the samples heated at 80 and 100 °C (compare panels A and B of Figure 3).

The effect of heating temperature and pH can be seen more clearly when the T_{50} values for all samples at all pH values and heating temperatures are compared (Figure 3C and Supporting Information). At each temperature, T_{50} decreased as the pH increased. The decrease was small for the samples heated at 20−60 °C, with T_{50} decreasing from about 45−50 min at pH 6.5 to about 30−35 min at pH 7.1. In contrast, the decrease was quite marked for the samples heated at 80 and 100 °C, with T_{50} decreasing from over 80 min at pH 6.5 to about 40 min at pH 7.1.

Whey Protein Denaturation in Heated Skim Milks. On heating the milks at 90 °C, β-lactoglobulin progressively denatured as the heating time was increased so that all of the β-lactoglobulin was denatured at heating times of 5 min or longer regardless of the milk pH (results not shown). However, not all of the α-lactalbumin was denatured on heating, and the denaturation was somewhat dependent on the pH, with generally higher levels denatured at pH 7.1 than at pH 6.5, although the differences were significant only at the extreme pH values and for heating times between 5 and 15 min (results not shown). For milk at pH 6.5, the percentage of denatured α-lactalbumin increased from about 40% after 5 min of heating to >90% after 30 min of heating. At pH 7.1, denaturation increased from about 65% to >90% for the same heating times.

In milks heated for 30 min, no denaturation of β-lactoglobulin or α-lactalbumin was observed for the samples heated at temperatures up to 60 °C, as expected, because this is below the denaturation

temperature of these whey proteins. However, on heating at 80 °C, about 85% of the α-lactalbumin and β-lactoglobulin was denatured at pH 6.5, and this increased to nearly 100% at pH 7.1; at 100 °C, virtually all of the α-lactalbumin and β-lactoglobulin was denatured at all pH values (results not shown). The denaturation percentages for β-lactoglobulin and α-lactalbumin and the effects of temperature, heating time, and pH on this denaturation were in accord with those predicted from kinetic experiments.[20−22]

Association of Whey Proteins and κ-Casein with the Casein Micelles in Heated Skim Milk. When milks were heated at 90 °C for different times, the percentage of denatured β-lactoglobulin associating with the casein micelles was markedly dependent on the pH of the milk at heating (Figure 4A and Supporting Information). About 80% of the denatured β-lactoglobulin associated with the casein micelles when the milk pH was 6.5 and the heating time was 5 min or longer. As the pH of the milk increased, the percentage of β-lactoglobulin associating with the casein micelles progressively decreased so that only about 10% was associated with the casein micelles when the milk pH was 7.1. Similar behavior was observed for α-lactalbumin, although it took about 10 min of heating for maximum association to be achieved at each milk pH (results not shown).

As the pH of the milk was increased prior to heating at 90 °C, the percentage of κ-casein remaining associated with the casein micelles decreased (Figure 4B and Supporting Information). Similar behavior was observed at all heating times, with about 90% of the κ-casein being associated with the micelles for the milks heated at pH 6.5, and this progressively decreased with increasing pH so that about 45% was associated with the casein micelles when the milk pH was 7.1.

For the milks heated at temperatures from 20 to 100 °C for 30 min, the percentage of denatured β-lactoglobulin associated with the micelles was also dependent on the temperature and the pH of the milks at heating (Figure 4C and Supporting Information). At temperatures up to 60 °C, no β-lactoglobulin was associated with the micelles, as expected, because this protein does not denature and interact at temperatures below about 70 °C. However, at 80 and 100 °C, β-lactoglobulin associated with the casein micelles. At each temperature, higher percentages were associated at lower pH and, at each pH, higher percentages were associated at 100 °C than at 80 °C (Figure 4C). Similar behavior was observed for α-lactalbumin (results not shown).

High percentages of κ-casein were associated with the micelles for the sample heated at 20 °C. At each pH, the percentage of κ-casein associated with the micelles decreased as the temperature was increased. However, at any heating temperature above 20 °C, the percentage of κ-casein remaining associated with the micelles decreased as the pH at heating increased (Figure 4D and Supporting Information). The overall effect can be seen clearly for the sample at pH 7.1; the percentage of κ-casein associated with the micelles decreased from about 85% to about 40% as the temperature was increased from 20 to 100 °C. The levels of α_s-casein and β-casein associated with the micelles were high regardless of the pH of the milks and the heating conditions employed. Some effects of temperature and pH were observed, with slight decreases in colloidal α_s-casein and β-casein with increasing pH, especially when the milks were heated at temperatures of 60 and 80 °C (results not shown). Overall, the results on the effects of temperature, heating time, and pH on the association state of proteins with the casein micelles were in accord with previous studies on the heat-induced, pH-dependent interaction between denatured whey proteins and the casein micelles and the dissociation of κ-casein from the casein micelles.[3,16,23,24]

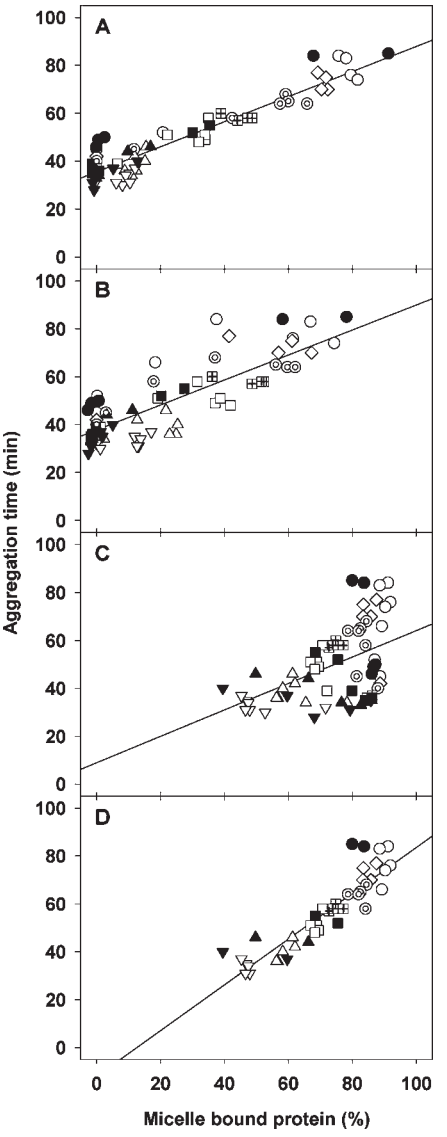

Figure 5. Relationship between the time taken for the particle size to increase by 50 nm (T_{50}) and (A) the percentage of micelle-bound β-lactoglobulin, (B) the percentage of micelle-bound α-lactalbumin, (C) the percentage of micelle-bound κ-casein (all data), and (D) the percentage of micelle-bound κ-casein (selected data omitted, see text). The milk samples were heated either at 90 °C for various times (open symbols) or at different temperatures for 30 min (solid symbols). The pH values of the samples were (○, ●) 6.5, (◇) 6.55, (◎) 6.6, (⊞) 6.65, (□, ■) 6.7, (△, ▲) 6.9, and (▽, ▼) 7.1.

Relationships between Protein Associations in Heated Skim Milk Samples and Their Renneting Properties. When the changes in the denaturation of the whey proteins were compared with the changes in particle size on rennet treatment, there was no relationship between T_{50} and the percentage of whey protein denaturation (results not shown). This was expected because there was only a small effect of the pH at heating on the denaturation of the whey proteins but a large effect of the pH at heating on the particle size changes in the milks during rennet treatment. However, when the denatured whey proteins associated with the casein micelles (Figure 4A,C) were compared with the changes in particle size on rennet treatment (Figures 2 and 3), there was a significant positive correlation ($r^2 = 0.89$; $P < 0.01$) between

the percentage of denatured β-lactoglobulin associated with the casein micelles and T_{50} (Figure 5A) regardless of the temperature, pH, or heating time of the milk samples. There was a similarly significant but weaker correlation (r^2 = 0.7; P < 0.01) between denatured α-lactalbumin associated with the casein micelles and T_{50} (Figure 5B).

There appeared to be a poor relationship between micelle-bound κ-casein and T_{50} (Figure 5C); however, when the results for the samples heated at 60 °C or below and heated at 90 °C for 1 min were excluded, there was a significant positive correlation (Figure 5D; r^2 = 0.82; P < 0.01). On heating at ≤60 °C or at 90 °C for 1 min, significant percentages of κ-casein had dissociated from the casein micelles at pH 7.1 (Figure 4B,D) but only small percentages of β-lactoglobulin and α-lactalbumin denatured under these conditions. The T_{50} values for the milks heated at ≤60 °C (Figure 3C) or at 90 °C for 1 min (Figure 2D) were only slightly longer than those for the unheated milks. These results suggest that the dissociation of κ-casein from the casein micelles alone has a limited effect on the rennet-induced aggregation of the casein micelles. However, the significant correlations between the percentages of denatured β-lactoglobulin and α-lactalbumin associated with the casein micelles and the T_{50} values for the acid gels suggest that the casein micelles coated in denatured whey proteins (as observed on heating at low pH) are more resistant to rennet-induced aggregation than those with low percentages of denatured whey proteins associated with the casein micelles (as observed on heating at high pH).

Investigation of Fresh Skim Milk Samples. Fresh skim milk samples were adjusted to pH 6.5−7.1 and then either left unheated or heated at 90 °C for 15 min. The particle size change on rennet treatment when the samples were dispersed in buffer was examined. This produced results virtually identical to those for the reconstituted skim milks, with T_{agg} and T_{50} decreasing slightly with increasing pH for unheated fresh milk (Figure 6A) and decreasing markedly with increasing pH for heated milks (Figure 6B).

A recent advance in particle sizing technology allows photon correlation spectroscopy to be performed on higher concentration samples. This technique, called noninvasive backscatter, measures the particle size from backscattered laser light and shifts the measuring position closer to the sample cell wall for higher concentration samples, thus minimizing multiple scattering events. This technique was used on one fresh skim milk sample that was adjusted to pH 6.5, 6.7, or 6.9 and then either left unheated or heated at 90 °C for 15 min. After heat treatment, the heated and unheated samples were held at room temperature overnight, and then all were readjusted back to the initial pH of the milk (~6.7 ± 0.01). Rennet was added to the milks, and the changes in particle size were monitored without dilution.

For the unheated milks, the particle size increased rapidly after the addition of rennet. T_{agg} and T_{50} were about 7.5 and 15 min, respectively, and there was little discernible difference between the samples at different pH values (Figure 6C). For the heated milks, the particle size did not increase as rapidly, with T_{agg} for each milk being about 15 min. Once the milks started to aggregate, it was clear that the aggregation rate was more rapid for the milk at pH 6.9 (T_{50} = 37 min) than for the milk at pH 6.5 (T_{50} = 55 min), with the milk at pH 6.7 being intermediate (T_{50} = 43 min).

Although the overall renneting behavior of the undiluted milk was similar to that of the milks diluted in buffer, there were distinct differences. For the unheated milks, there was limited effect of pH on T_{agg} and T_{50} in the undiluted samples, but a significant effect on these parameters in the diluted samples (compare panels C and A of Figure 6). Similarly, for the heated milks, there was a much smaller effect of pH on T_{agg} and T_{50} in the undiluted milks than in the diluted milks (compare panels D and B of Figure 6).

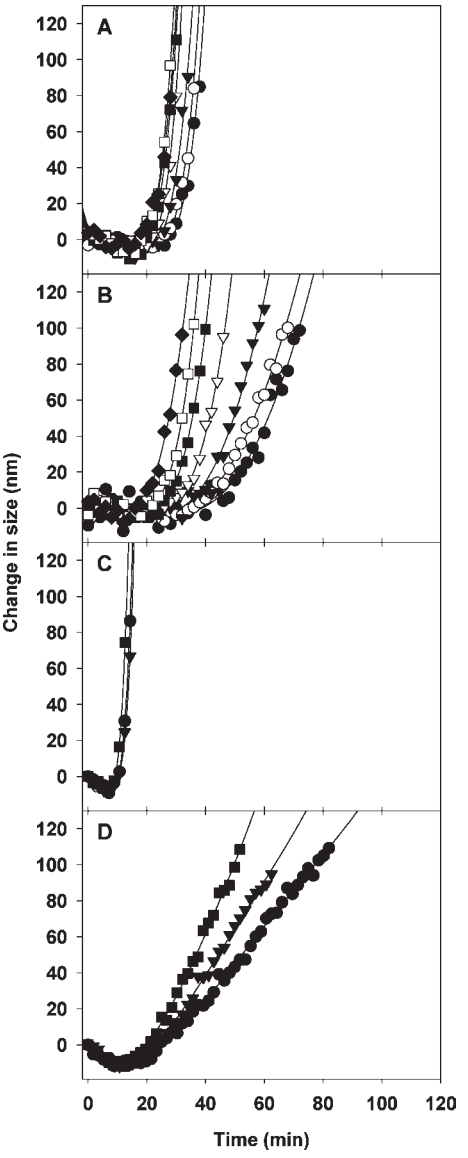

Figure 6. Changes in particle size on the rennet treatment of (A, C) unheated fresh skim milk and (B, D) fresh skim milk heated at 90 °C for 15 min: (A, B) milk samples (20 μL) diluted in calcium−imidazole buffer (1 mL) before the addition of rennet (10 μL of 1:120 diluted rennet); (C, D) milk samples readjusted to pH 6.7 before the addition of rennet (10 μL of 1:80 diluted rennet per mL of milk). The pH values of the milk samples were (●) 6.5, (○) 6.6, (▼) 6.7, (▽) 6.8, (■) 6.9, (□) 7.0, and (◆) 7.1.

Recently, the effect of serum and colloidal components on the rennet-induced gelation of heated and unheated milks was studied by exchanging serum and colloidal components from milks treated under different conditions.[8] It was shown that the denatured whey proteins inhibited the gelation of milks regardless of whether they were in the serum phase or associated with the micelles. However, heat-induced changes to the casein micelles and to the nonprotein serum-phase components also inhibited gelation. It was suggested that inhibition of the gelation of heated milks was complex and probably due to synergistic

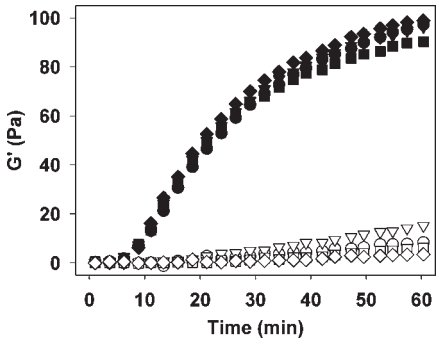

Figure 7. Changes in storage modulus (G') with time after the addition of rennet to unheated milks (solid symbols) and skim milk samples heated at 90 °C for 30 min (open symbols). The pH values of the milk samples at heating were (●,○) 6.5, (▼, ▽) 6.7, (■, □) 6.9, and (◆, ◇) 7.1. All samples were readjusted back to the natural pH (6.67) before the addition of rennet (40 μL of 1:3 diluted rennet per 1.3 mL of milk).

effects of heat on the casein micelles, the whey proteins, and nonprotein serum-phase components.

In the diluted milks of the current study, the effects of nonprotein serum-phase components were essentially eliminated by diluting the milk into a buffer. Hence, any effects on the renneting properties were due to the changes to the protein components as a consequence of the pH adjustments and the heat treatments. In contrast, for the undiluted milks, as all milk components were still present, the effects on the renneting properties would have been due to changes to both the protein components and the nonprotein components. In addition, in the undiluted milks, the volume fraction of the casein micelles is considerably higher, the calcium activity would be lower, and the concentration of rennet to protein was lower than in the diluted milks. Some of these effects may account for the differences in the aggregation behavior between the undiluted milks and the diluted milks as both the protein components and the nonprotein components in pH-adjusted and heated milks have been shown to inhibit their rennet gelation of the milks.[8] The combined effects will be observed in the undiluted milks, whereas only the effects of the protein components will be observed in the diluted milks.

Rheological Properties during the Rennet Treatment of pH-Adjusted Skim Milk Samples Heated at 90 °C for 30 min. Skim milk samples at pH 6.5−7.1 were either left unheated or heated at 90 °C for 30 min. After heating, the samples were held overnight at ambient temperature and then readjusted to the natural pH (~6.7). Rennet was added to the milk samples, and the changes in the rheological properties with time were monitored (Figure 7). At all pH values, heat treatment of the milk increased the time for the milks to gel and reduced the G' of the gels after 60 min of rennet treatment. No significant effect of the pH of the milk at heating on the rheological properties during rennet treatment was observed. This is in agreement with previous results.[7,8]

DISCUSSION

In heated milks, casein micelles and serum phases with markedly different compositions can be generated depending on the pH of the milks at heating (Figure 4).[4,25] It would be expected that this change in distribution of the proteins as a consequence of heating would alter the functional properties of the milks, and this has indeed been established for the heat stability of milks[26,27] and for the acid gelation of milks.[4,5] There have also been some indications that the distribution of proteins may affect the properties of milk powders.[28]

However, in stark contrast, the renneting properties of heated milks, as measured by gelation using rheological techniques, have been reported to be almost totally unaffected by the pH of the milks prior to heating and, as such, the distribution of the proteins between the serum and colloidal phases.[7,8] In all cases, it took a longer time for gels to be formed from the rennet treatment of heated milks, and the gels formed were markedly weaker than those from unheated milks regardless of whether the whey proteins were associated with the micelles or in the serum phase during heating. This was confirmed in the present study (Figure 7). Interestingly, the primary phase of the renneting reaction, when the enzymes in rennet cleave the glycomacropeptide moiety from the κ-casein, is only slightly affected by the heat treatment of the milk,[7,8,12,29] which indicates that any effects of heating are due to changes to the secondary or coagulation phase of the rennet reaction. The longer gelation times and the weaker gels from the rennet treatment of heated milks have been used as evidence that these milks have impaired rennet clotting or coagulation properties.[8,12]

The measurement of the rheological properties of milk during renneting and the commercial application of rennet-treated milks (e.g., in cheese or rennet casein manufacture) require the formation of a gel. This gelation involves the destabilized micelles aggregating to form a three-dimensional network structure that extends through the entire volume and entraps and immobilizes the aqueous phase. The strength of the gel formed will be dependent on the interparticle cross-links in the network structure. However, the system could be destabilized in such a fashion that it does not initially form a gel, but rather a discontinuous precipitate or coagulum. Once a gel is formed, it may be structurally different with a weak network containing few and/or weak interparticle cross-links. This does not necessarily imply that the destabilization of the micelles is impaired, but rather that the structures formed from the destabilized micelles are different. In fact, Vasbinder et al.,[12] using diffusing wave spectroscopy to study the early stages of the renneting reaction, showed that the heat treatment of milk, and in particular the denaturation of the whey proteins, affected the flocculation behavior of the casein micelles by rennet.

By using a particle sizing method, and by dispersing the milks in a buffer solution, it was evident that rennet-induced destabilization of the particles in heated milks was dependent on the pH of the milks at heating (Figure 2); in addition, the correlations between micelle-bound proteins indicated that the effect of the pH at heating was related to the distribution of both denatured whey proteins and κ-casein between the serum and colloidal phases (Figure 5). However, the effects of the pH at heating, although still present, were markedly diminished when the milk was not diluted compared with the milks diluted in buffer (Figure 6), indicating that other heat-induced changes to the milk were influencing the renneting properties, in agreement with Kethireddipalli et al.[8]

Singh et al.[14] examined the effect of the pH at heating on the visual rennet coagulation time and observed a faster coagulation when the pH at heating was decreased from 7.1 to 6.5. Vasbinder and de Kruif used diffusing wave spectroscopy to study the early stage of the rennet destabilization of milk.[30] They observed that heated milks had much lower rates of flocculation than unheated milks and that, for heated milks, a minimum rate of flocculation was observed for samples heated at pH 6.55, with slightly faster

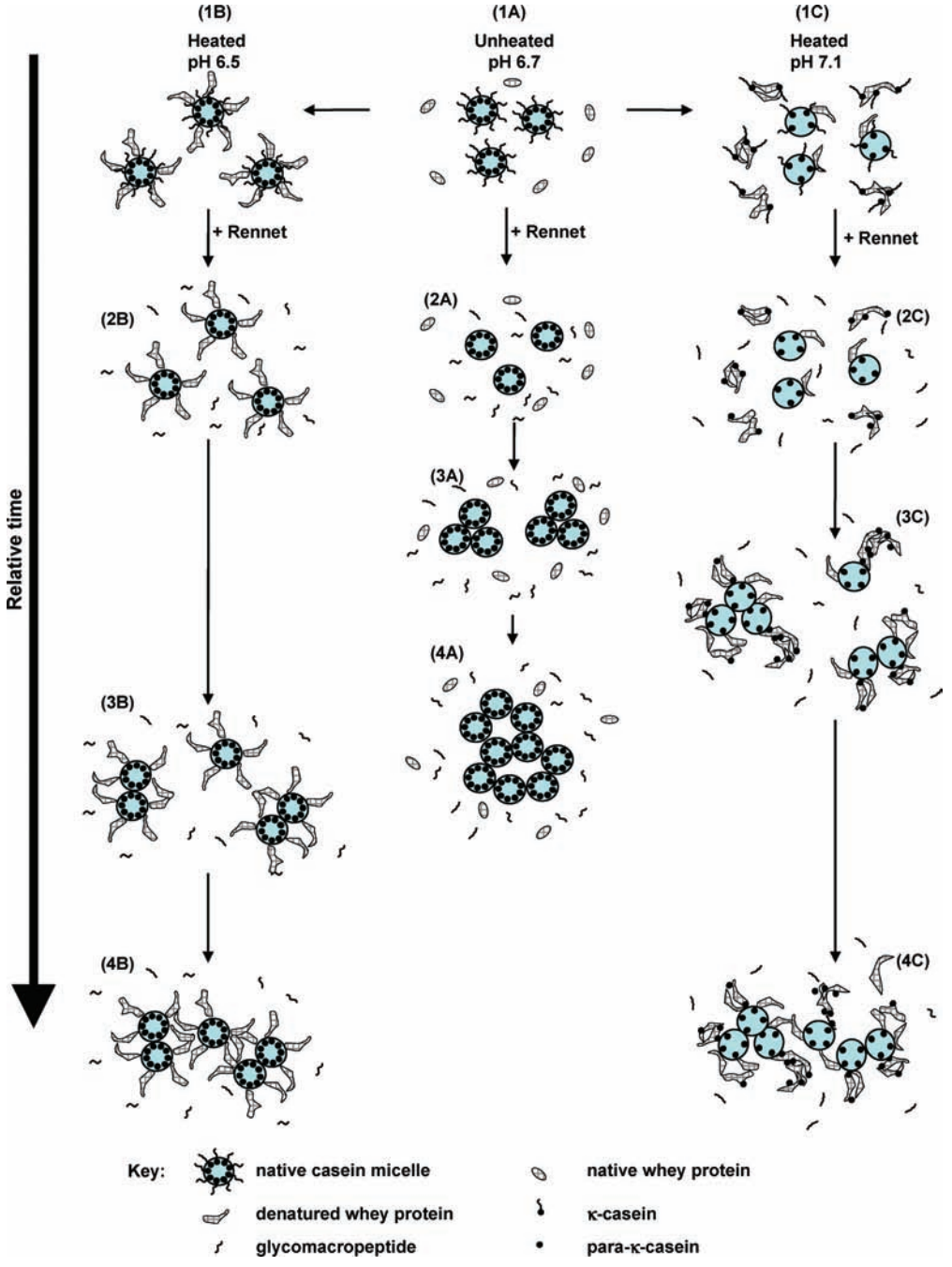

Figure 8. Schematic reaction pathway for the rennet-induced aggregation/gelation of unheated and heated milks.

rates at lower and higher pH values. These observations contrast with those observed here for both diluted milks (Figures 2, 3, and 6B) and undiluted milks (Figure 6D). This indicates that the methods of analyzing the effects of rennet treatment (visual coagulation versus diffusing wave spectroscopy versus particle size analysis) and the conditions of rennet treatment (pH of samples after heat treatment, equilibration times, temperature of rennet treatment, etc.) are important.

Several studies suggest that the association of denatured whey proteins with κ-casein, whether in the serum phase or on the casein micelles, inhibits the aggregation of the casein micelles.[7,9,12] A recent study by Kethireddipalli et al.[29] has shown that the serum-phase complexes between denatured whey proteins and κ-casein do not aggregate on rennet treatment when these complexes are separated from the casein micelle. However, when the κ-casein/whey protein complexes are in the presence of casein micelles (whether from unheated milks or from heated milks), they complex with the casein micelles when rennet treated. This observation helps to explain the effects of the pH at heating on the particle size changes when the heated milks are subsequently rennet treated.

From the results of this study, and those reported in the literature, it is possible to provide a schematic reaction pathway with time for the rennet-induced aggregation/gelation of unheated milks and for milks heated at high and low pH (Figure 8). The unheated milk at the natural pH (~6.7) contains casein micelles and whey proteins (Figure 8(1A)). On heating at

low pH, the whey proteins denature and interact with κ-casein on the micelle surface (Figure 8(1B)) whereas, on heating at pH 7.1, the whey proteins denature and interact with κ-casein in the serum phase (Figure 8(1C)). On the addition of rennet, the enzymatic reaction occurs at a similar rate for the unheated and heated milk samples, releasing the glycomacropeptide to the serum. For the unheated milk, the para-κ-casein is still associated with the casein micelles (Figure 8(2A)). For the milk heated at pH 6.5, the para-κ-casein remains associated with the micelles, as does the denatured whey protein that is disulfide bonded to the para-κ-casein (Figure 8(2B)), whereas, for the heated milks at the initial stages of renneting, the para-κ-casein is predominantly in the serum associated with the denatured whey proteins (Figure 8(2C)).

For the unheated milks, once sufficient κ-casein has been hydrolyzed, the casein micelles aggregate (Figure 8(3A)) and rapidly form a firm gel (Figure 8(4A)). In contrast, for the milks heated at pH 6.5, the denatured whey proteins associated with the micelles partially stabilize the micelles so that the aggregation process is much slower than for the unheated milks (Figure 8(3B)), and these aggregates associate only slowly to form a weak gel (Figure 8(4B)). However, for the milks heated at pH 7.1, the casein micelles (which are depleted in κ-casein and therefore denatured whey proteins) are unstable once renneted and aggregate with other casein micelles and with the serum-phase κ-casein/whey protein complexes (Figure 8(3C)). These aggregated particles will become partially stabilized by the denatured whey proteins from the κ-casein/whey protein aggregates, so that the aggregates associate only slowly to form a gel (Figure 8(4C)), and this gel is considerably weaker than that from the unheated milks. On rennet treatment, both the casein micelles in the milks heated at pH 6.5 and the initial aggregates from the milks heated at pH 7.1 are stabilized by denatured whey proteins; therefore, both systems take a similarly long time to form a gel and the gels are weak, even though the samples at pH 7.1 initially aggregate at a more rapid rate than those at pH 6.5.

The scheme shown in Figure 8 is qualitatively consistent with the experimental results that show that the rates of hydrolysis of κ-casein are similar in unheated and heated milks regardless of the pH of the milks at heating,[7,8,12] that milks heated at pH 7.1 aggregate more rapidly on rennet treatment than those heated at pH 6.5 (Figures 2 and 6) because the renneted κ-casein/whey protein complexes aggregate with the renneted casein micelles,[29] and that heated milks at all pH values take a similarly long time to form (weak) gels compared with unheated milks (Figure 7).[7,8]

Overall, this study has demonstrated that the distribution of denatured whey proteins and κ-casein between the colloidal and serum phases on heating milk at different pH values does influence the stability of the casein micelles to treatment by rennet. Milks in which the denatured whey proteins and κ-casein are predominantly associated with the casein micelles, as observed when the milk is heated at low pH (~6.5), take considerably longer to aggregate than milks in which the casein micelles are depleted in κ-casein and most of the denatured whey proteins and κ-casein are in the serum phase. Clearly, other factors, such as mineral components, also play a role as the effect of pH was greater when the milks were diluted in buffer than in undiluted milks. Although the casein micelles could have been more rapidly destabilized when they were depleted in κ-casein and denatured whey proteins, the milks still took a long time to form gels and the gels were weak compared with those from unheated milks. It is proposed that the denatured whey proteins play a role in stabilizing the aggregation of renneted milk. In milks in which most of the whey proteins and κ-casein are associated with the micelles, the aggregation is slow because of the stabilization of the micelles by the denatured whey proteins. However, in milks in which the casein micelles are depleted in κ-casein and the denatured whey proteins are in the serum phase, the milks initially aggregate rapidly, but the incorporation of the serum-phase denatured whey proteins into the aggregates slows aggregation and, in particular, the gelation process.

■ ASSOCIATED CONTENT

Supporting Information. Additional tables. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Current postal address: Fonterra Research Centre, Private Bag 11029, Palmerston North, New Zealand. Phone: +64 (6) 350 4649. Fax: +64 (6) 356 1476. E-mail: skelte.anema@fonterra.com.

Present Addresses

†Fonterra Research Centre, Private Bag 11029, Palmerston North, New Zealand.

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