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Controlled Proteolysis and the Properties of Milk Gels

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Milk gels induced by partial proteolysis of the κ-casein followed by acidification were studied, and their gelation behavior was compared to that of milk gels induced by simultaneous acidification and renneting, using dynamic rheology. There were generally two stages (at pH values below and above 5.0) in the gelation of the milk whose κ-casein had been partially proteolyzed and acidified. The onset of gelation was at higher pH as the degree of κ-casein proteolysis increased. The development of G' immediately after the onset of gelation was faster in the milk gels induced by simultaneous acidification and renneting, because of the continuing κ-casein proteolysis. Preheat treatment caused the onset of gelation to occur at higher pH than for unheated milk. However, the maximum tan δ during gelation always occurred at the same pH (for a given concentration of acidulant), and its value and position were independent of the extent of renneting and whether the milks had been heat treated. The results are discussed in terms of the interactions between casein micelles occurring during gelation.

KEYWORDS: Milk; renneting; acidification; gelation; partial renneting; casein micelles

INTRODUCTION

Many dairy products are based on the gelation of milk by acidification and/or renneting. Both of these processes produce gels, but the mechanisms of gel formation, and the properties of the final gels, are quite different.

At the normal pH of milk, the casein micelles are stabilized by electrostatic and steric repulsion of the hairy layer on the surface of the casein micelles (*1*). This hairy layer is composed of the negatively charged glycomacropeptide (GMP) of the κ-casein fraction of the milk protein. During the rennet coagulation of milk, the κ-casein is specifically cleaved by rennet and the GMP (κ-casein peptide 106−169) is split off. As a result of this reaction, the casein micelles become less stable, but extensive aggregation only occurs after at least 85−90% κ-casein has been cleaved (*2*). In contrast, in the acid coagulation of milk, the casein micelles lose their electrostatic repulsion when the negative charge is titrated as the pH drops; the decrease in charge accompanies the collapse of the hairy layer, and therefore loss of steric stabilization as well (*3*). These effects on the micellar surface decrease the stability of the casein micelles and lead to aggregation as the isoelectric point of casein (pH 4.6) is approached. There have been many studies of milk gels induced by either rennet or acid (*4*−*7*). The structure and rheological properties of milk gels induced by renneting at different pH values have been described (*8*).

There is considerably less information on the gelation behavior of milk induced by simultaneous acidification and renneting (*9*, *10*). The combination of the two effects when both acidification and rennet action occur concurrently gives behavior quite different from that of strictly acid or rennet milk gels. For example, the storage modulus of these mixed gels is considerably larger than that of either of the gels alone. Although these studies showed the effect of high and low concentrations of rennet on the gel properties, there was no quantitative measurement of the amounts of proteolysis of the κ-casein during the reactions where simultaneous acidification and renneting occurred.

In another study (*11*), milk was subjected to controlled proteolysis by rennet, and then an inhibitor was added to stop the enzymatic reaction at different degrees of proteolysis. In these experiments the acidification step followed the partial removal of the hairy layer. This study showed that the pH at which gelation occurred increased with the extent of κ-casein proteolysis, and generally agreed with the study of Lucey et al. (*10*) that the elasticity of the mixed gels was stronger than that of either of the gels alone.

This latter study (*11*) was performed only on unheated milk, so the possible effects of the change in gelation mechanism caused by heating the milk were not considered. Similarly, although Lucey et al. (*10*) did consider the behavior of heated milk, they did not study the effect of controlled proteolysis. The objectives of the research described in this paper were to study the gelation behavior of milk gel induced by partial κ-casein proteolysis and acidification, to study the κ-casein proteolysis during the gelation of milk induced by simultaneous acidification and renneting, and to compare the gelation behavior of these two different processes. In addition, we studied the gelation behavior of both heated and unheated milks under conditions of controlled proteolysis and of simultaneous acidification and renneting. To attempt to distinguish between "rennet coagulation" and "acid coagulation" in the mixed systems, we

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used high and low concentrations of rennet, a range of partially renneted milks, and two levels of addition of the acidulant GDL.

MATERIALS AND METHODS

Materials. Instant low-heat skim milk powder was supplied by Parmalat (London, ON, Canada). Glucono-δ-lactone (GDL) and pepstatin A were obtained from Sigma-Aldrich Co, Inc. (St. Louis, MO). The rennet was double strength Chymostar brand supplied by Rhodia Inc. (Madison, WI), and the coagulation strength was 570 IMCU/mL.

Reconstituted Skim Milk and Heat Treatment. Standard reconstituted skim milk was prepared to a concentration of 10% (w/v) by adding low-heat skim milk powder (SMP) to Milli-Q water; NaN_3 (0.2 g/L) was added to prevent bacterial growth. The reconstituted milks were stirred for 1 h at 25 °C and stored at 4 °C overnight. For heat treatment, samples of the skim milks (100 mL) in beakers were heated in a water bath at 80 °C for 30 min. After the heat treatment, the milks were rapidly immersed in ice and cooled to 30 °C. After resuspension of the SMP or after heat treatment, 1 mM Ca was added to the milks by adding a 0.1 M solution of $CaCl_2$ in Milli-Q water to the milk at a ratio of 1:100 (v/v).

Preparation of Milks with Partial Proteolysis of κ-Casein. The κ-casein proteolysis was carried out at 30 °C by adding rennet to the milks. A volume (2.5 μL) of the stock rennet solution was diluted into water (1 mL). This diluted rennet was then added to milk at dilutions of 175 or 29 μL/25 mL of milk (these are termed high and low rennet concentrations, respectively).

To prepare milks with different extents of proteolysis of κ-casein, individual samples of milk were treated with the higher concentration of rennet for different times up to 2 h, and then the rennet activity was stopped by the addition of a 1 mM solution of pepstatin A. Ethanol (100%, 7.3 mL) was used to dissolve 5 mg of the pepstatin, with warming to 60 °C. This was then added to the renneted milks at a ratio of 1:100. It is estimated that this dilution is unlikely to significantly affect the behavior of the milk. Both heated and unheated milks were reacted with rennet in this way; it should be noted that the heating, if used, always preceded the rennet treatment.

To estimate the extent of proteolysis of κ-casein, the milk was precipitated with trichloroacetic acid to a final concentration of 8% (v/v), at which all the whey proteins are precipitated but the GMP still remains in the supernatant (*12*). The degree of κ-casein proteolysis was determined by measuring the GMP content in the TCA supernatants using reversed-phase HPLC (Thermo, Mississauga, ON, Canada) according to the modified methods of Lopez-Fandino et al. (*13*). The measured values of GMP were compared with those from the completely renneted milk (i.e., the values where the production of GMP leveled off for unheated milks (see **Figure 3**). To ensure that the inhibition by pepstatin A was permanent in the range of pH 6.6−4.6, the GMP content of partially renneted milks was measured before and after the acidification process; it was confirmed that there was no increase in the free GMP in the milk after the initial addition of the pepstatin. This showed that there was no evidence that the inhibitory properties of the pepstatin could be reversed during acidification.

Acid and Rennet Gelation of the Milks. Milks and renneted milks were acidified by the addition of GDL at concentrations of 1% or 2% (w/v). The required amount of GDL was added to the milk, and the whole was agitated for 1 min to dissolve the acidulant. A sample was taken for the rheometer (see below), and the remainder was held at 30 °C and used to measure the pH at regular intervals, using an AR15 pH meter (Fisher Scientific, Mississauga, ON, Canada). From these measurements, a pH−time curve was constructed which could then be used to describe the course of the pH change in the rheometer. The concentrations of GDL were selected to give fast and slow acidification; since we were interested in studying the mechanism, we used one concentration of GDL (1%) where the rate of acidification was very slow.

For the measurement of simultaneous acidification and renneting, the two concentrations of rennet defined above were used. In these experiments, the rennet activity was not stopped, and the kinetics of the GMP release during the combined rennet and acidification treatment were measured by taking samples at defined times and precipitating the protein with 8% TCA as described above.

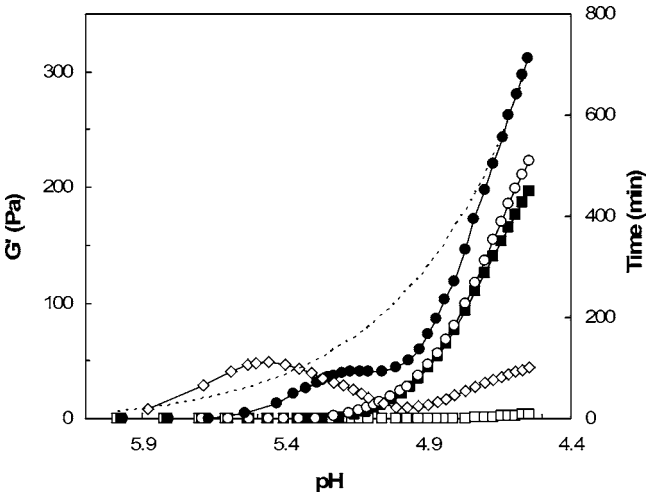

Figure 1. Gelation behavior (elastic modulus of the gel) of unheated reconstituted skim milks previously treated with chymosin to different degrees of κ-casein proteolysis and then acidified with 1% GDL: (□) milk with no rennet treatment; (■) milk with 22% κ-casein proteolysis; (○) milk with 38% κ-casein proteolysis; (●) milk with 62% κ-casein proteolysis; (◇) milk with 94% κ-casein proteolysis. The broken line and right-hand axis show the relation between pH and time for the milk treated with 1% GDL.

Dynamic Rheological Measurements. A controlled-stress rheometer AR 1000 (TA Instruments, New Castle, DE) was used, with a Couette measuring system consisting of two concentric cylinders of diameters 30 and 28 mm. All experiments were run at a temperature of 30 °C. Untreated milks, or milks subjected to controlled proteolysis, were treated with 1% or 2% GDL; for the experiments involving simultaneous acidification and renneting, the required amount of rennet was added at the same time as the GDL. In all cases, the mixtures were stirred for 1 min and an appropriate volume (20 mL) of the mixture was transferred to the rheometer. A cover was placed on the cylinder to prevent evaporation. The oscillation frequency was 0.1 Hz, and the strain applied was set to 1%. Gel points were defined as the points where $G' = G''$. Experiments were continued until an approximate pH value of 4.5 had been attained (approximately 12 h for 1% GDL and 2 h for 2% GDL).

RESULTS

Gelation Behavior of Partially Renneted Skim Milk. The changes in the elastic modulus, G', of reconstituted skim milk samples with different extents of proteolysis of their κ-casein are shown in **Figure 1** for acidification with 1% GDL. Milk which had not been rennet-treated before it was acidified showed gelation only at a low pH (~4.8). The gel development was very slow, and G' was less than 10 Pa even when the pH had decreased to 4.6. This weak gelation process is well-known for milks which are acidified without any prior heat treatment (*14*).

It was found possible to partially rennet milk to about 94% of the maximum and still give a product that was stable over the time scale of the experiment. The partially renneted milks with different extents of κ-casein breakdown gave gelation behavior as a function of pH that was quite different from that of untreated milk (**Figure 1**). In all cases, the onset of gelation was at higher pH, and stronger gels were formed. As the extent of κ-casein breakdown was progressively increased, the gelation started at higher pH (*11*). At lower extents of breakdown of κ-casein (22% and 38%), the values of G' kept steadily increasing after the onset of gelation, which occurred at a pH of about 5.2. However, at medium (62%) or high (94%) extents

of κ-casein breakdown, the behavior of G' showed two stages. In the first stage, gelation started at relatively high pH (5.55 and 5.95, respectively), after which the values of G' increased to maximum values, and then leveled off to a plateau (at 62% breakdown) or decreased to a minimum value (at 94% proteolysis). In the second stage, the values of G' began to increase again at a pH of about 5.0. In the milk with 62% κ-casein breakdown, the development of G' was fast and the final values of G' were larger than the maximum value of G' in the first stage; in fact, they were the largest values measured in all of the experiments. In contrast, the most highly renneted milk (94%) gave a development of G' during the second stage which was very slow, so that even when the pH had reached 4.6, the value of G' was still lower than its maximum value in the first stage. This slow increase in G' could be caused by weak gel development and/or extensive syneresis and shrinkage of the gel. We did not observe detachment of the gel from the wall during the experiment, although whey separation was obvious when the measurement cell was dismantled and the gel was fractured. This slow development of G' at the second stage had previously been observed also when a high rennet concentration was used in simultaneous acidification and renneting of milk (*10*). In fact, our overall gelation behavior with 94% renneted milk is similar to that found when acidification and rapid renneting take place simultaneously (see below and ref *10*). The comparison of the gelation behavior of milk under these two different processes will be considered later in this paper.

What is especially remarkable in these results is the very large increase in the elastic modulus of the acid gel when only a relatively small amount (22%) of the κ-casein has been removed by the rennet. That is, the elastic modulus of the gel is not linearly dependent on the extent of change of the surface of the casein micelles. This was also shown by previous results (*11*). Successive increases in proteolysis increase the modulus, but not to the same extent. Therefore, for unheated milk, the behavior of the acid gel is very much influenced by the exact quantity of intact κ-casein that remains on the casein micelles.

Gelation Behavior of Partially Renneted Heated Skim Milk. The gelation behavior of heated milk acidified with 1% GDL was in some respects similar to that of the unheated milk (**Figure 2**). As is well established, we found that the gelation started at higher pH (~5.2) in unrenneted heat-treated milk, and the gel development was much faster, compared to that of unheated milk. The value of G' increased to about 160 Pa at pH 4.6, which is more than 10 times stronger than that of the gel made from unheated milk. This result is in agreement with many other studies of the acidification of milk after heating (*15*−*17*). As was found for unheated milks, the pH values of the onset of gelation in heated milks with partial κ-casein proteolysis increased with the extent of breakdown of κ-casein, and were also higher than those of unheated milks with similar extents of κ-casein proteolysis. However, the differences in the pH of gelation were smaller at larger extents of proteolysis. This could arise from changes in the interactions between the denatured whey proteins and casein micelles as a result of rennet action on the whey protein/casein micelle and whey protein/κ-casein complexes formed in the heated milk (*10*). In contrast to the behavior of unheated milks, the shapes of the G' developments at different extents of κ-casein breakdown were qualitatively similar to each other during acidification, although quantitatively different. In all of the partly proteolyzed samples there appeared to be two stages in the increase in G', which increased to a maximum value and then leveled off to a plateau or decreased slightly before a major increase started at about pH 5.0. However, the values of G' at any pH, and the relative importance of the first stage, increased with the degree of breakdown of κ-casein. Again, these behavior patterns qualitatively follow those observed previously for simultaneous acidification and renneting (*10*). At the natural pH (6.6) of milk, the κ-casein proteolysis in heated milk was a little lower than in unheated milk (*18*−*20*).

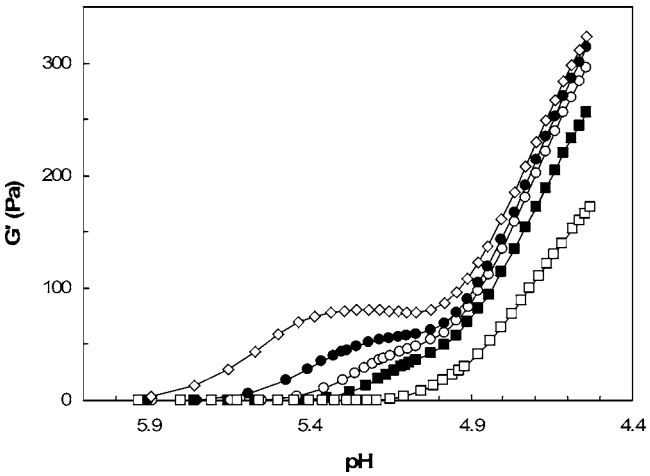

Figure 2. Gelation behavior (elastic modulus of the gel) of reconstituted skim milks heated at 80 °C for 30 min before being treated with chymosin to different degrees of κ-casein proteolysis and then acidified with 1% GDL: (□) heated milk with no rennet treatment; (■) heated milk with 20% κ-casein proteolysis; (○) heated milk with 26% κ-casein proteolysis; (●) heated milk with 46% κ-casein proteolysis; (◇) heated milk with 81% κ-casein proteolysis.

In contrast to the behavior of the unheated milk, where even low extents of renneting had a very large effect on the gel strength at low pH, the G' values of the gels from heated milks show a significant, but proportionately smaller, dependence on the extent of renneting. However, even here the largest effect was found at the lowest extent of renneting. It should be noted that the partially renneted unheated milks all show values of G' higher than those of the untreated heated milk at pH < 5.0. This showed how important the effect of even small extents of renneting is. At the other end of the scale, it was noted that the heated and extensively renneted milks did not show the much reduced G' that was typical of corresponding samples from highly renneted unheated milks.

Effects of Simultaneous Acidification and Renneting. The extents of κ-casein breakdown as a function of pH in simultaneously acidified and renneted milks are shown in **Figure 3**. The extent of κ-casein breakdown increased after the rennet and acid action started, and continued throughout the acidification. Rennet action is dependent on both time and pH (*21*). With 1% GDL and the higher concentration of rennet, the breakdown of κ-casein was more than 80% complete by the time the pH of 5.7 was reached. At the lower concentration of rennet, this level of breakdown was reached only at pH 5.4. After the milk was preheated, the renneting followed a similar trend, but it appeared to be a little slower than that of the unheated milk. The optimal pH for rennet activity in milk is in the region of 6.0−5.5 (*21*), so during acidification the pH of the milk may have passed the optimal pH and further decreases in pH may have retarded the renneting reaction.

Comparison of Gelation with Partial or Continuous Renneting. Comparisons of the gelation of unheated milk by simultaneous acidification and renneting are shown in **Figure 4** for milks acidified using 1% GDL. Results from high and

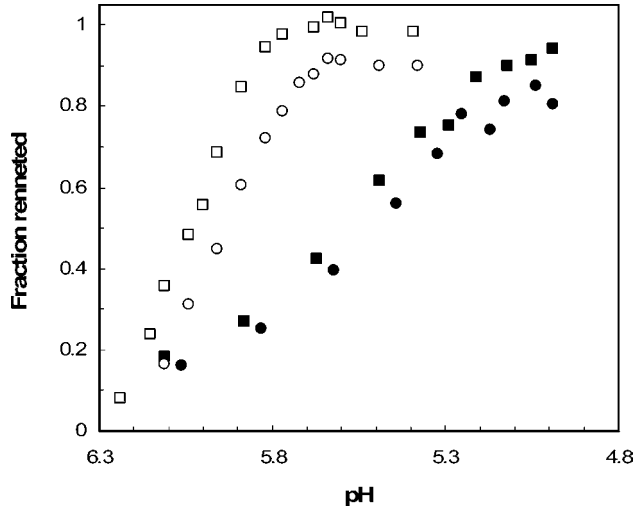

Figure 3. Kinetics of breakdown of κ-casein in milks simultaneously acidified with 1% GDL and renneted, measured by the analysis of CMP as described in the text. The kinetics are described as the extent of renneting as a function of the pH attained. Results are shown for two different concentrations of rennet. Key: (□) unheated milk treated with the higher concentration of chymosin (see the text for details); (■) unheated milk treated with the lower concentration of chymosin; (○) heated (80 °C, 30 min) milk treated with the higher concentration of chymosin; (●) heated milk treated with the lower concentration of chymosin.

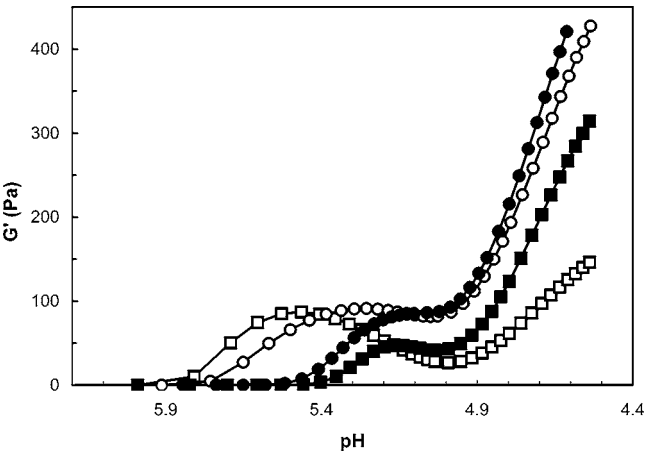

Figure 4. Gelation behavior of heated (○, ●) and unheated (□, ■) milks that are being continuously renneted by high (○, □) and low (●, ■) levels of chymosin during acidification by 1% GDL. The kinetics of chymosin action are shown in **Figure 3**.

low rennet concentrations are shown, and correspond to the renneting behavior shown in **Figure 3**. For the gels induced by simultaneous acidification and renneting, and with the higher concentration of rennet, the onset of gelation was at a pH of 5.8, at which point the extent of κ-casein breakdown was about 75%. At the lower rennet concentration, the onset of gelation was at pH 5.4, at which the extent of κ-casein breakdown was about 55%. In comparison, the gelation in milks with partial renneting of 94% and 62% (**Figure 1**) started earlier than in the milks with high and low concentrations of rennet, respectively. However, the initial rates of increase of G' in the gels made by simultaneous acidification and renneting were higher than in the gels made from partially renneted milks, and this presumably arises from the continuing breakdown of κ-casein during gelation when rennet is present. In both of the continuously renneted systems, G' increased to a maximum and then decreased to a minimum value or leveled off before increasing

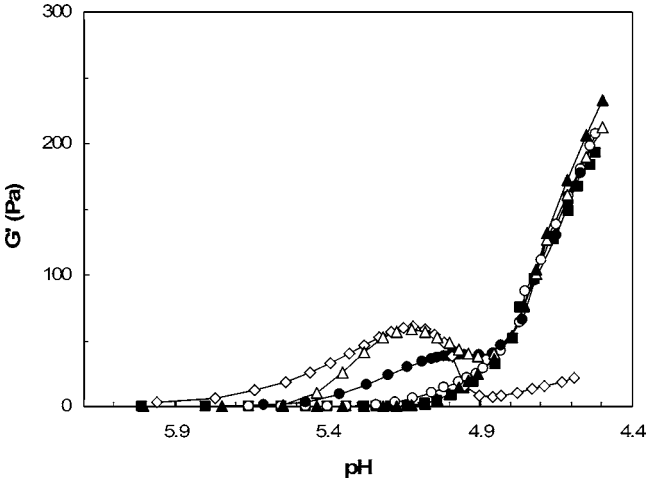

Figure 5. Gelation behavior (elastic modulus of the gel) of unheated reconstituted skim milks previously treated with chymosin to different degrees of κ-casein proteolysis and then acidified with 2% GDL: (■) milk with 22% κ-casein proteolysis; (○) milk with 38% κ-casein proteolysis; (●) milk with 62% κ-casein proteolysis; (◇) milk with 94% κ-casein proteolysis. Also shown are the results for unheated milks subjected to continuous proteolysis by high (△) and low (▲) concentrations of chymosin during acidification with GDL.

again. From **Figure 4**, at high concentration of rennet, when the κ-casein breakdown was rapid, G' increased to a relatively high value at the maximum (pH $\approx$ 5.5) and then decreased to a minimum at pH 5.0 before increasing again. For low rennet concentration, G' had a maximum at pH $\approx$ 5.5 and then a minimum at pH 5.0 before increasing rapidly. Interestingly, the progress of the gelation at low rennet concentration resembled very closely that of the 62% renneted milk, even though the former ended up with a greater extent of breakdown by the end of the reaction.

As described above, when the partial κ-casein breakdown was over 80%, G' in the second stage increased slowly. In the milk gel induced by simultaneous acidification and a high concentration of rennet, a similar phenomenon was observed. Thus, when 80% κ-casein proteolysis occurred before the acidification or before G' reached the maximum value during the first stage, the development of G' in the second stage was slow, while when 80% κ-casein breakdown occurred after the maximum G' in the first stage, such as when low rennet was used, there was little impact on the second-stage development of G'.

In heated milks that were simultaneously renneted and acidified the gelation also started at higher pH when partial κ-casein breakdown was higher or the rennet concentration was higher. After preheat treatment, the onset of gelation of the milk occurred at a higher pH value than that of the unheated milk at the same degree of partial κ-casein proteolysis or the same rennet concentration. At the second stage of the gelation, G' of the milk gels increased rapidly and followed a similar trend even though the extent of κ-casein breakdown was different in the two cases and they had reached the final gelation stage in different ways.

Acidification of Milks with 2% GDL. When milk was acidified with 2% GDL, the gelation started at higher pH when the partial κ-casein breakdown was higher or the rennet concentration was higher (**Figure 5**). For the partially renneted micelles, the onsets of gelation in 2% GDL were almost at the same pH as those of milks acidified with 1% GDL. However, for the simultaneous acidification and renneting, the onset of gelation in milk acidified with 2% GDL occurred at lower pH

than milk acidified with 1% GDL. This is caused by the different relative rates of κ-casein breakdown and acidification at different GDL concentrations. The κ-casein proteolysis in milk acidified with 1% GDL was 80% when the pH was decreased to 5.8, while in milk acidified with 2% GDL κ-casein breakdown was only 30% at pH 5.8. During the first stage, the milk with high rennet and the milk with 94% renneting behaved very similarly; this was also true for the milk with low rennet and the 38% renneted milk.

The second stage of the gelation also started at the same pH for all the milks, even though the first stage started at different pH values when different extents of κ-casein were hydrolyzed. However, at 2% GDL, it started at pH $\approx$ 4.9 instead of pH 5.0. In our results, the extent of κ-casein breakdown had no impact on the development of the gel in the second stage, except for the milk with very high proteolysis (94%), which showed an exaggerated version of what was seen at 1% GDL. However, even the milk treated with high rennet did not show the decrease typical of the highly renneted milks. This may suggest that breakdown of the κ-casein is not complete in this milk, and measurements of the liberation of CMP (not shown) confirmed this. The results on the behavior of the partially renneted milks acidified by 2% GDL differ somewhat from those given previously (*11*); these authors suggested that the extent of κ-casein breakdown had a more significant effect on the gel at the second stage than at the first stage. Also, in their results, the second stage of the gel development seems to start at different times (i.e., different pH values) depending on the extent of κ-casein breakdown.

Development of the Gels in the Different Systems. In gels, the tan δ function is the ratio of the viscous and elastic moduli G'' and G'. The change in tan δ indicates that different types of bonds are forming in the gel. When gelation occurs, the elastic modulus of the bonds increases faster than the viscous modulus so that tan δ decreases. In the acid gelation of nonrenneted unheated milk, tan δ has been shown to decrease continuously after the onset of gelation as the pH continues to decrease (*10*, *22*). However, during the acid gelation of partially proteolyzed or continuously renneted milks (**Figure 6**), tan δ first decreased as the gel started to form, increased to a maximum at about pH 5.0, and then decreased again as the pH continued to drop. This observation is in agreement with others (*9*, *10*). The increase in tan δ in the gelation indicated that the type of bonds in the gel kept changing and for a time the viscous modulus of the gel increased faster (or rather decreased more slowly) than the elastic modulus, although both G'' and G' show the same overall pattern of development. The maximum in tan δ in fact occurs when both G' and G'' are at their minimum values.

If the other conditions (temperature, GDL) remained the same, the maxima in tan δ always occurred at the same pH and the maximum values were also the same, no matter how the milk gels were produced. Preheat treatment caused no change in the position of the maximum in tan δ, but the value at the maximum was somewhat lower (**Figure 6A,B**). Increasing the concentration of GDL (**Figure 6C**) caused a shift of the pH at the maximum to a lower value (because the acidification speed was faster). In the milk with 94% partial proteolysis, the tan δ curve had a shape different from those of the others in both 1% and 2% GDL. After the onset of gelation at around pH 6.0, the value of tan δ increased similarly to those of the other milks, but it had a second minimum at around pH 5, where the other milks gave a maximum value, before decreasing again (**Figure 6A,C**). It was noted also that the heated milk with the highest degree of proteolysis had a lower value of tan δ than the other heated milks (**Figure 6B**). This suggests that a more complicated gelation process occurs at the highest levels of partial κ-casein breakdown. However, no similar behavior was observed in the milk subjected to a high level of rennet (**Figure 6A**).

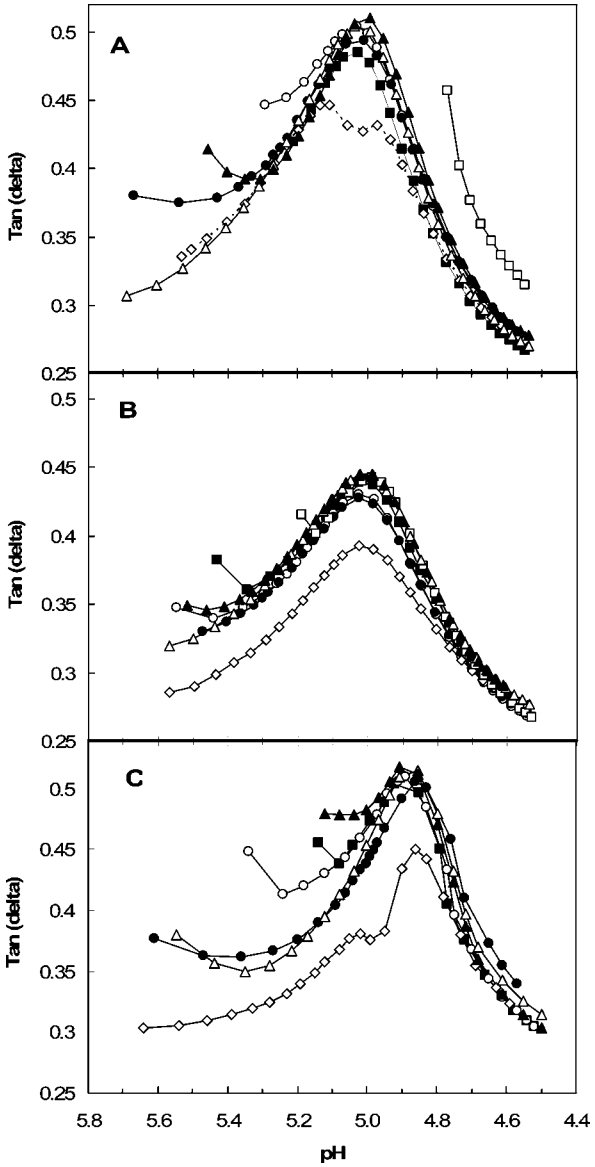

Figure 6. Variation in tan δ of the milks studied by partial κ-casein proteolysis and by simultaneous acidification and renneting. **(A)** Unheated milks treated with 1% GDL: (□) milk with no chymosin treatment; (■) milk with 22% κ-casein proteolysis; (○) milk with 38% κ-casein proteolysis; (●) milk with 62% κ-casein proteolysis; (◇) milk with 94% κ-casein proteolysis; continuous proteolysis by high (△) and low (▲) concentrations of chymosin. **(B)** Heated milks treated with 1% GDL: (□) milk with no chymosin treatment; (■) milk with 20% κ-casein proteolysis; (○) milk with 26% κ-casein proteolysis; (●) milk with 46% κ-casein proteolysis; (◇) milk with 81% κ-casein proteolysis; continuous proteolysis by high (△) and low (▲) concentrations of chymosin. **(C)** Unheated milks treated with 2% GDL: (■) milk with 22% κ-casein proteolysis; (○) milk with 38% κ-casein proteolysis; (●) milk with 62% κ-casein proteolysis; (◇) milk with 94% κ-casein proteolysis; continuous proteolysis by high (△) and low (▲) concentrations of chymosin.

The onset of gelation of unheated milk without κ-casein breakdown or continuous renneting occurred at lower pH (4.8 at 1% GDL) than where the maximum tan δ occurred (**Figure 6A**). All of the other renneted and partially renneted milks

started to gel at pH values above 5.0. Heat-treated milks gave an onset of gelation at higher pH (pH 5.2 at 1% GDL). In all of these, the onset of gelation is at a higher pH than the pH of the maximum in tan δ, so the maximum in tan δ could be detected. This is in general agreement with the suggestion that a maximum in tan δ appears in any acidified milk system that forms gels at pH $\geq$ 5.3 (*10*). However, the results demonstrated that the extent of renneting had virtually no effect on the relation between viscous and elastic moduli of the forming gels.

Mixtures of Renneted and Nonrenneted Milks. As a comparison with the behavior of the partly renneted milks, some studies were made on 1:1 mixtures of partly renneted milks with untreated milks (**Figure 7**). For these, the renneting was performed on one batch of milk, and was stopped at defined values by adding pepstatin. This milk was then mixed with an equal volume of the same milk that had not been renneted but had the same pepstatin concentration added. After mixing, the mixtures were acidified with 1% GDL. Results for unheated milk treated with 1% GDL are shown in **Figure 7**. The results confirm the remarkable effect of the partial rennet treatment on the gelation capability of the casein micelles at low pH. It was anticipated that the dilution of the renneted milk would decrease the strength of the acid gel by about 50%; however, this was not the case. For the mixtures containing milks renneted to 22% and 38%, the elastic moduli of the gels at pH 4.6 were found to be about 80% of their values in the unmixed milks. Evidently, the partly renneted milks are capable of disproportionately strengthening an acid gel. It is difficult to understand how this phenomenon can arise. The effect of similar mixing of partially renneted heated milks with unrenneted heated milks was considerably less; for the mixture with milk partially renneted to 32%, the resulting G' at pH 4.6 was 65% of the difference between the values for unrenneted heated milk and the unmixed 38% renneted milk. This is much closer to the expected average.

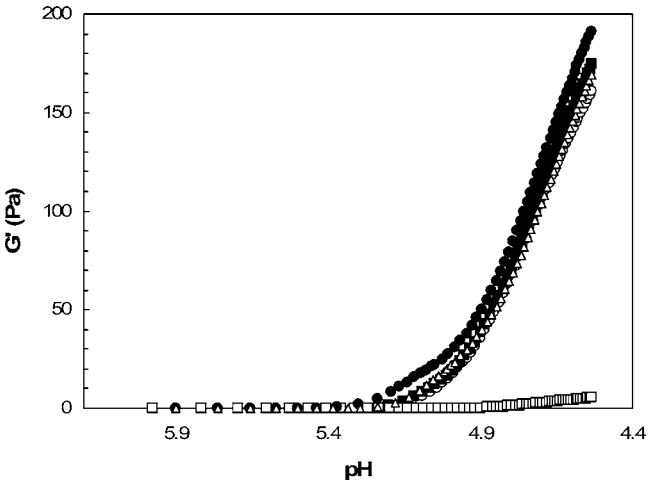

Figure 7. Gelation behavior (elastic modulus of the gel) of reconstituted skim milks previously treated with chymosin to different degrees of κ-casein proteolysis and then mixed with equal volumes of unproteolyzed milks before being acidified with 1% GDL: (□) unheated milk proteolyzed to 15% and then mixed with unheated milk; (○) unheated milk proteolyzed to 22% and then mixed with unheated milk; (■) unheated milk proteolyzed to 32% and then mixed with unheated milk; (●) heated milk proteolyzed to 32% and mixed with heated milk; (△) heated milk proteolyzed to 20% and then mixed with heated milk.

DISCUSSION

The results presented here are in general agreement with those that have been published previously (*9*−*11*). It is clear that partial or continuous renneting has a large effect on the acid-induced gelation of milk and the properties of the acid gel. However, relative to the gelation induced by either acid or rennet alone, where the mechanisms are at least qualitatively understood, it is not completely clear what factors control the mixed coagulation. It is especially important to try to explain the large increase in the value of G' in the acid gel formed from milk where only a rather small amount of renneting has occurred. At pH values below 5.0, all of the different milks studied show a major increase in the strength of the gel. However, there are significant differences in the behavior at higher pH values that need to be discussed.

In understanding the gel properties, it is necessary to distinguish between (i) the interactions that define whether aggregation and subsequent gelation can occur and (ii) the interactions that determine the properties of the gel. These need not be the same. As the casein micelles approach one another, they are subject to long-range forces which determine whether the particle surfaces can approach one another closely. On the other hand, once close approach has been achieved, the interactions that hold the casein micelles together are short-range and may depend critically on local compositions and conformations at the points where the micelles touch. The internal structures and properties of the casein micelles may also become important. Castillo et al. (*23*) have suggested that there are two reactions that they term the aggregation rate and the curd-firming rate. However, these do not distinguish the rennet and the acid contributions to the two processes.

The rate of micellar aggregation (i.e., whether a gel can be formed) is defined by the breakdown of the stability of the casein micelles. These particles are stable because their surface layer of κ-casein macropeptide (GMP) carries a net negative charge at neutral pH and also provides a highly hydrated layer around the micelle (*24*−*28*). This layer provides both electrostatic and (especially) steric stabilization to the casein micelles, and its state controls their aggregation. Rennet removes the hairy layer, reduces the charge of the particle, as assessed by the ζ-potential (*11*, *29*), and eventually completely destroys the steric stabilization (*25*, *30*). However, the stabilizing power of the GMP is so large that it is necessary to remove a great part of the hairy layer, between 80% and 95% at the natural pH of milk, to allow coagulation (*2*, *21*, *31*). Once aggregation has started, hydrophobic interactions between regions of para-κ-casein and calcium bridges between other caseins have been suggested as being important in forming the gel (*28*, *32*).

The extent of breakdown of κ-casein needed to cause aggregation becomes smaller as the pH is decreased (*21*). This may be explained partly because the charge on the micelle is less at lower pH, but more importantly the thickness of the stabilizing layer of the κ-casein, and hence its capacity for steric stabilization, decreases as the pH drops (*30*, *33*, *34*). Thus, according to the geometrical model of renneting (*35*), the amount of κ-casein needing to be broken to produce a reactive "hot spot" will be smaller. A gel can therefore be formed more readily than at higher pH values; conversely, micelles renneted to greater extents will form gels at higher pH, as is seen in our results.

At the same time as the pH is changing the surfaces of the casein micelles, their internal structures are modified, because the micellar calcium phosphate is progressively dissolved between pH 6.5 and pH 5.0 (*36*−*39*); this does not cause dissociation of the proteins as long as the temperature is maintained above 25 °C (*37*). The loss of steric stabilization

seems to be complete by about pH 5.6, as estimated by the changes in micellar radius (*33*). As the surface charge is further reduced by titration, repulsive forces are insufficient to prevent coagulation.

These two mechanisms explain why the stability of milk is destroyed in the different processes, but they tell us little about the properties of the gels that are formed (*22*). Even though the gels may be considered as particle gels (*40*, *41*), the rennet gel made at pH > 6.0 is much stronger than an acid gel made from unheated milk (*10*), and for acid gels there is a very large difference in the rheology of gels made from unheated and preheated milks, as shown above and by many other studies (*15*, *42*). The elastic moduli, G', of the gels depend predominantly on the concentration of gelling particles, the number and strengths of the links between them, and the internal strengths of the particles themselves (*43*). In rennet gels, the interparticle forces are thought to be from hydrophobic (via para-κ-casein) and possibly Ca^{2+}-bridging interactions. In the acid gel prepared from unheated milk, it is less clear what local forces (apart from, ultimately, van der Waals forces) are operational. However, the weakness of an acid gel formed from unheated milk may arise at least partly because the interparticle contact is still dominated by the presence of the GMP, which has collapsed but is still present. The GMP is rich in hydroxylated amino acids, some of which are glycosylated, and also acidic and basic residues, including the single phosphoserine residue of the protein (*44*). Thus, the interface between the aggregating particles will tend to be hydrated, and attractive interactions will be partly offset by the hydrophilic tendency of the GMP. It has also been suggested that, at some pH values at least, true coagulation does not occur, but rather that an equilibrium between gelled and nongelled micelles may exist (*3*); this also would result in a weak or flexible gel.

The gels formed during partial renneting and acidification will share some of both of these properties. Gels that are produced with extensive renneting (the 94% prerenneted milk and the high rennet in simultaneous renneting and acidification) show a rapid increase in gel strength at relatively high pH, and are almost certainly held together by the same forces as pure rennet gels. The G' values of acid/rennet gels at about pH 5.5 and pure rennet gels are similar when high levels of rennet are used (that is, when renneting is rapid relative to acidification) (*10*). Our observations for the 94% renneted milk and for the high level of rennet are in agreement with this. On the other hand, in gels formed from milk with less proteolysis, the interaction between the particles will be more mediated by the interparticle layers of GMP. The onset of gelation will therefore be at a lower pH in such systems, to overcome any charge stabilization and also to allow breakdown of steric stabilization. However, the collapse of the GMP layer at lower pH means that even a fairly small gap in the layer will allow interaction between some of the "inner" surfaces of pairs of casein micelles. The large increase in the elastic modulus of the gels below pH 5.0 with even small extents of renneting can be explained in this way. Increasing the extent of the proteolysis will also increase the strength of the gels, because of the increase in the size of the gaps produced in the hairy layer that would lead to more extensive (or possibly more numerous) contacts, either of which will of course increase the gel strength.

In the highly renneted acid gels, our results show, as in ref *10*, that there is a very large decrease in the value of G' as the pH drops between 5.5 and 5.0. This effect can probably be explained because the gels first form at a high pH where there is little dissociation of the micellar calcium phosphate (MCP). As the pH further decreases, the MCP is dissolved from the gel, and this will loosen the bonds within the micelles in the already-formed gel (*10*, *22*, *23*) and may also decrease the role of calcium in the intermicellar bonding. The breakdown of the internal micellar structure and the large surface free of GMP may also promote fusion of the casein micelles and cause extensive changes of the gel structure as seen in ref *45*. Essentially, the gel becomes less of a particulate structure and becomes more continuous, but it can only do this when the micelles are very highly renneted. The lack of a similar effect in highly renneted heated milk may possibly be attributed to the rigidity of the whey proteins attached to the casein micellar surfaces that prevent the fusion of the casein micelles. The mainly rennet gel is initially formed at a pH where there is a large amount of the micellar calcium phosphate left intact. In gels with lower extents of renneting, the gelation starts at a pH where more of the micellar calcium phosphate is already dissolved (*38*, *39*, *46*). Therefore, although milk renneted to 62% starts to coagulate at pH $\approx$ 5.3, it has likely lost most of its MCP and will therefore be less susceptible than the highly renneted sample to loss of structure as the pH drops further. It has been stated that all gels made at pH 5.3 or above will show the decrease in G' (or increase in tan δ) as they are further acidified to about pH 5.0, because of the progressive loss of calcium phosphate in partially gelled micelles (*10*), although it should be pointed out that many of the results on which this statement is based were performed at higher temperatures (40 °C) or at very rapid rates of acidification (GDL concentrations above 3%).

Heated milks broadly follow the same pattern as unheated milks, insofar as rennet action increases the pH of the onset of gelation. The action of heat causes the partial loss of micellar κ-casein, which is removed from the micelle and forms complexes with denatured serum proteins. At the same time some of the whey proteins bind to the casein micelles (*47*−*49*). The casein micelle is still covered by a hairy GMP layer, although it must have reduced density, and partial renneting will induce larger gaps in it. Acidification has been seen to cause collapse of this layer, as in unheated milk (*33*), but the surfaces of the micelles which contact one another in extensively renneted heated milk will be different from those formed in unheated milk. The areas of contact may be larger (because removal of one κ-casein will affect a greater area), but the contact area will almost certainly contain some denatured whey proteins. Thus, in addition to the interactions between the inner surfaces of casein micelles, there will also be interactions between the bound whey proteins. As the pH further decreases, the contacts between the micelles will become stronger as a result of (i) the strong interactions between them as their isoelectric point is approached (*10*, *15*) and (ii) the aggregation and binding of the denatured whey protein/κ-casein complexes from the serum, so the interparticle bonds will become stronger (*14*, *15*, *41*, *50*). These additional effects will offset the effect of the loosening of the gel as the casein micelles lose their micellar calcium phosphate (which seems to be the same in heated and unheated milks) on G'. Therefore, even as the micelles themselves weaken the structure, the intermicellar bonds (mediated by whey protein complexes) become stronger, so the values of G' remain constant. At pH below 5, the removal of some GMP still has a marked effect, although not as large as that found in unheated milk. This may be expected because the contact between the micelles is only partly influenced by the remaining GMP (and there is less of it), but is more determined by the denatured whey proteins. Nevertheless, the substantial increase in G' at

low extents of renneting shows that the removal of GMP greatly increases the interparticle interaction in the gel. This perhaps suggests that the bonds between particles in acidified heated milk are not completely made by whey proteins, and that there is a function also for interactions between caseins as well.

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