JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of pH on the Association of Denatured Whey Proteins with Casein Micelles in Heated Reconstituted Skim Milk

SKELTE G. ANEMA* AND YUMING LI

Food Science Section, Fonterra Research Centre, Private Bag 11029, Palmerston North, New Zealand

Skim milk was adjusted to pH values between 6.5 and 6.7 and heated (80, 90, and 100 °C) for up to 60 min. Changes in casein micelle size, level of whey protein denaturation, and level of whey protein association with the micelles were monitored for each milk sample. Changes in casein micelle size were markedly affected by the pH at heating. At low pH (6.5−6.55), the casein micelle size increased markedly during the early stages of heating, and the size plateaued on prolonged heating. The maximum increase in size was ∼30−35 nm. In contrast, at high pH (6.7), much smaller changes in size were observed on heating and the maximum increase in size was only ∼10 nm. An intermediate behavior was observed at pH values between these two extremes. The rate of denaturation of the major whey proteins, α-lactalbumin and β-lactoglobulin, was essentially unaffected by the pH at heating for the small pH changes involved in this study, and the changes in casein micelle size were poorly related to the level of whey protein denaturation. In contrast, the level of denatured whey proteins associating with the micelles was markedly dependent on the pH at heating, with high levels of association at pH 6.5−6.55 and low levels of association at pH 6.7. Changes in casein micelle size were related to the levels of denatured whey proteins that were associated with the casein micelles, although there was a small deviation from linearity at low levels of association (<15%). Further studies on reconstituted and fresh milk samples at smaller pH steps confirmed that the association of whey proteins with the casein micelles was markedly affected by the pH at heating. These results indicate that the changes in casein micelle size induced by the heat treatment of skim milk were a consequence of the whey proteins associating with the casein micelles and that the level of association was markedly influenced by small pH changes of the milk. It was not possible to determine whether the association itself influenced the casein micelle size or whether parallel reactions involving micellar aggregation caused the increase in micelle size as whey protein association progressed.

KEYWORDS: Milk; denatured whey proteins; casein micelles; α-lactalbumin; β-lactoglobulin; κ-casein

INTRODUCTION

Heating milk, or solutions containing β-lactoglobulin and κ-casein, results in the formation of a heterogeneous complex between these protein species (*1*−*6*). The major interaction appears to involve thiol−disulfide exchange reactions between the denatured β-lactoglobulin and κ-casein at the micelle surface (*2*, *3*, *6*). However, some studies suggest that ionic and/or hydrophobic interactions may play a significant role in the aggregation of the denatured whey protein with the casein micelles, particularly in the early stages of the reaction (*4*, *7*).

The conditions under which milk is heated appear to influence the level of association of whey proteins with the casein micelles. About 80% of the denatured β-lactoglobulin associated with the casein micelles when the temperature of the milk was increased slowly (*3*, *8*). In contrast, only about half of the denatured β-lactoglobulin and α-lactalbumin was found to be associated with the casein micelles when the temperature of the milk was increased rapidly. The rest of the denatured whey protein remained in the milk serum as disulfide-bonded and/or hydrophobically associated aggregates (*9*−*11*). Corredig and Dalgleish (*8*, *12*) suggested that, on heating of milk, α-lactalbumin and β-lactoglobulin initially aggregate in the serum phase at a ratio dependent on the initial individual whey protein concentrations. These complexes subsequently associate with the casein micelles on prolonged heating.

The association of denatured whey proteins with the micelles is dependent on the pH at which the milk is heated. Smits and van Brouwershaven (*3*) showed that, in heated model systems containing redispersed casein micelles with added β-lactoglobulin and α-lactalbumin, the association of β-lactoglobulin with the micelles decreased as the pH of the milk was increased from pH 6.3 to 7.3. Anema and Klostermeyer (*13*), Corredig and Dalgleish (*8*), Oldfield et al. (*14*), and Anema and Li (*15*) also reported a pH dependence of whey protein association with the micelles; however, the absolute association levels varied among

* Corresponding author (telephone +64 (6) 350 4649; fax +64 (6) 356 1476; e-mailskelte.anema@fonterraresearch.com).

10.1021/jf025673a CCC: $25.00
Published on Web 02/13/2003

the independent studies. No detailed studies have been performed over the narrow pH range between pH 6.5 and 6.7, which encompasses the natural pH range for milk.

In a recent study, it was shown that the micelle size increased when milk at pH 6.55 was heated and that this size change could be related to the association of whey proteins with the casein micelles (*15*). If the association of denatured whey proteins with the casein micelles is pH dependent, especially over the natural pH range for milk (pH 6.5−6.7), then some variation in the casein micelle size of heated milks may be expected. This could also explain why large differences in size changes are observed in heated milks obtained from individual cows (*16*). This study examined the effect of milk pH on the changes in casein micelle size and related these changes to the denaturation of the major whey proteins and their association behavior with the casein micelles.

MATERIALS AND METHODS

Milk Supply. Fresh milk samples were obtained from a local farm. The milk samples were skimmed at 40 °C, and the resultant skim milk was used. Low-heat skim milk powder was obtained from Kiwi Cooperative Dairies, Pahiatua, New Zealand. This milk powder had a whey protein nitrogen index of >6 (*17*) and contained ∼37% protein on a dry basis. Experimental skim milk samples were prepared by reconstituting low-heat skim milk powder to 10% (w/w) total solids in purified (reverse osmosis followed by filtration through Milli-Q apparatus) water. The reconstituted skim milk samples were allowed to equilibrate at ambient temperature (∼20 °C) with gentle stirring for at least 4 h before further treatment. A small amount of sodium azide (0.01% w/v) was added to all milk samples as a preservative.

Adjustment of pH and Heat Treatments. Subsamples of skim milk samples were adjusted to pH values between 6.5 and 6.7 by the slow addition of 1 M HCl or 1 M NaOH to well-stirred solutions. The milk samples were allowed to equilibrate for 2 h before the final pH reading and minor readjustment. The milk samples were preheated at 68 °C for 10 min before further use. Subsamples of milk (6 mL) were transferred to glass vials and heated, with continuous rocking, in a thermostatically controlled oil bath preset to temperatures in the range from 75 to 100 °C for the desired time. After heat treatment, the milk samples were cooled to room temperature by immersion of the glass vials in cold running water. The reported heating times include the come-up time, which was ∼30 s at all temperatures.

Particle Size Analysis. Particle size measurements were made by photon correlation spectroscopy using a Malvern Zetasizer 4 instrument and the associated ZET5110 particle sizing cell (Malvern Instruments Ltd., Malvern, Worcs., U.K.). The temperature of the cell was maintained at 20 ± 0.5 °C for the duration of the experiments. Measurements of the dynamics of the scattered light were collected at a scattering angle of 90° only. Average diffusion coefficients were determined by using the method of cumulants and were translated into average particle diameters using the Stokes−Einstein relationship for spheres. Skim milk samples were dispersed in calcium imidazole buffer (20 mM imidazole, 5 mM $CaCl_2$, and 30 mM NaCl, pH 7.0), as has been described previously (*18*). Preliminary experiments showed that similar particle size changes were obtained when skim milk ultrafiltrate or calcium imidazole buffer was used as the dispersant. Calcium imidazole buffer was used in preference due to its ease of preparation and storage stability.

Ultracentrifugation. Soluble whey proteins were defined as those that did not sediment from the milk during ultracentrifugation at 30000 rev/min (63000*g* average) for 1 h at 20 °C in a Beckman L8-80M ultracentrifuge and the associated Beckman Ti-80 rotor (Beckman Instruments Inc., Palo Alto, CA). The clear supernatant was carefully removed from the pellets. The protein content and composition of the supernatants was determined by gel electrophoresis and laser densitometry. When centrifuged milk was compared with the original milk, a correction factor of 0.95 was applied to the supernatant samples to account for steric exclusion effects of the colloidal components.

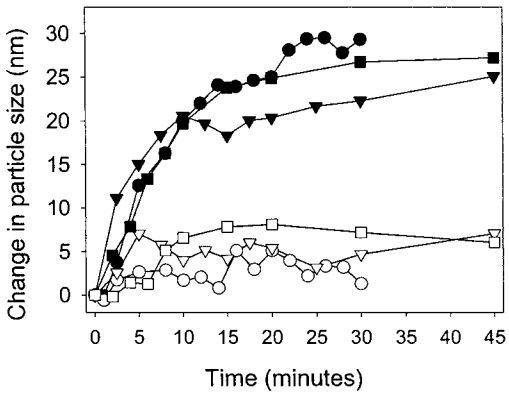

Figure 1. Changes in casein micelle size for skim milk samples at pH 6.55 (black symbols) and 6.70 (white symbols) that were heated at 90 °C for various times: (○, ●) milk 1; (□, ■) milk 2; (▽, ▼) milk 3.

Gel Electrophoresis and Laser Densitometry. The level of native whey protein in the unheated and heat-treated milk samples was determined using native polyacrylamide gel electrophoresis (native-PAGE), as has been described previously (*19*). The casein and the denatured whey proteins were removed from the milk by adjusting the pH to 4.6 and centrifuging out the precipitate using a bench centrifuge. The resultant supernatant was analyzed for native whey protein content using native-PAGE. The level of soluble whey proteins in the ultracentrifugal supernatants was determined using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, as has been described previously (*13*).

Native-PAGE and SDS-PAGE gels were scanned using a Molecular Dynamics model PD-SI computing densitometer (Molecular Dynamics Inc., Sunnyvale, CA). The integrated intensities of the major whey protein (α-lactalbumin and *β*-lactoglobulin) and casein (α_{S1}-, α_{S2}-, *β*-, and *κ*-casein) bands were determined using Imagequant software associated with the densitometer. The quantity of each protein in the ultracentrifugal supernatants was determined as a percentage of that in the original milk samples. The dye-binding properties of α-lactalbumin and *β*-lactoglobulin were determined from standard curves of the purified proteins and found to be very similar for the two proteins under the SDS-PAGE system used here. As a consequence, it was valid to determine the level of total whey protein associated with the casein micelles by comparing the combined intensities of the major whey protein bands (*β*-lactoglobulin and α-lactalbumin) in the heated samples with the corresponding bands in the unheated samples.

All experiments reported have been repeated on several different milk samples. Although some variations existed between individual milks, the same trends and relationships as reported here have been found for all samples examined to date.

RESULTS

Particle Size Changes in Heated Milk. Although there have been a few studies examining the association of whey proteins with the casein micelles, no detailed studies have been performed over the narrow range between pH 6.5 and 6.7. In addition, no studies have examined the changes in casein micelle size when milks at various pH values have been heated. **Figure 1** shows the changes in casein micelle size when three skim milk samples at pH 6.55 and 6.7 were heated at 90 °C for up to 30 min. At pH 6.55, the casein micelle size increased markedly, with a total increase of ∼25−30 nm. In contrast, at pH 6.7, the casein micelle size increased by only ∼5−10 nm over the heating period. A similar behavior was observed for both the fresh and the reconstituted skim milk samples, and the results were reproducible as long as the milk pH and the heating conditions were carefully controlled. In some milk samples, a decrease in casein micelle size of ∼3−5 nm was observed at very short heating times, and this preceded the gradual increase

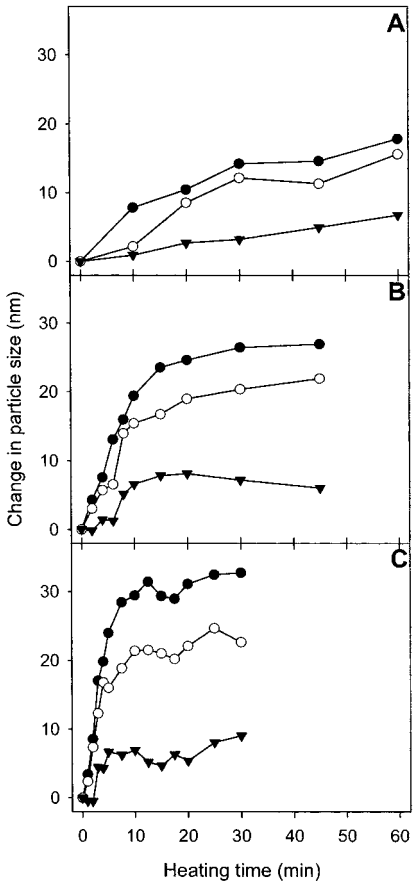

Figure 2. Changes in casein micelle size for skim milk samples at pH 6.55 (●), pH 6.60 (○), and pH 6.70 (▼) that were heated at 80–100 °C for various times: (A) 80 °C; (B) 90 °C; (C) 100 °C.

in size. This effect could be minimized, but not eliminated, by preheating the milk to 68 °C for a few minutes. No change in casein micelle size was observed when whey protein depleted milk samples were heated, whereas the addition of β-lactoglobulin to the whey protein depleted milk reproduced the behavior shown in **Figure 1** (results not shown). This indicates that the changes in casein micelle size were due to the presence of the whey proteins.

In more detailed experiments, reconstituted skim milk samples were adjusted to pH 6.55, 6.6, and 6.7 and then were heated at 80, 90, or 100 °C for times up to 60 min. The heated milk samples were analyzed for casein micelle size, the level of whey protein denaturation, and the level of whey proteins associated with the casein micelles. **Figure 2** shows the changes in casein micelle size induced when the milk was heated. The heat treatment of the milk caused an increase in casein micelle size at all heating temperatures. At 80 °C, the size increased relatively slowly throughout the heating time, whereas at 90 or 100 °C, the size increased rapidly during the initial period of heating and tended to plateau on prolonged heating. At all three heating temperatures, there was a strong dependence of the change in size on pH, with the largest increases (up to 30−35 nm) observed at pH 6.55, intermediate increases (up to 20−25 nm) at pH 6.6, and only small increases in size (5−10 nm) at pH 6.7. No measurable changes in polydispersity were observed, which indicates that, within instrument limits, the size distribution was not broadening (results not shown).

Whey Protein Denaturation. The observation that the casein micelle size increases when milk is heated, but no size increase is observed when whey protein depleted milk is heated, indicates

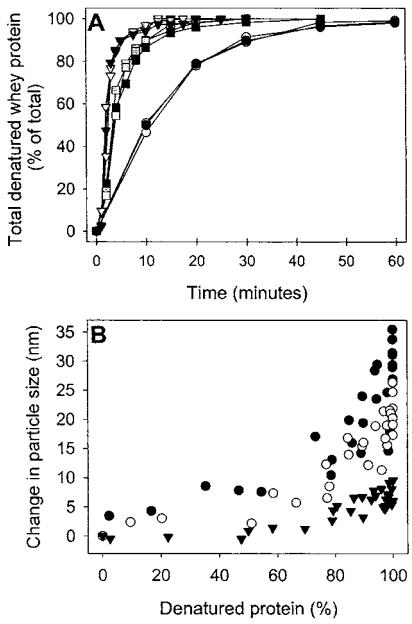

Figure 3. (A) Denaturation of whey proteins in skim milk samples at pH 6.55 (white symbols), pH 6.60 (gray symbols), and pH 6.70 (black symbols) that were heated at 80–100 °C for various times: (circles) 80 °C; (squares) 90 °C; (triangles) 100 °C. (B) Relationship between the level of total denatured whey protein and the particle size for skim milk samples at pH 6.55 (●), pH 6.60 (○), and pH 6.70 (▼) that were heated at 75–100 °C for various times.

that the size change is related to the association of the denatured whey proteins with the casein micelles. The results of **Figures 1** and **2** suggest that the reactions involved in increasing the casein micelle size are dependent on small shifts in pH. This indicates that the denaturation of the whey proteins or the level of association of the whey proteins with the casein micelles is pH dependent. **Figure 3A** shows the changes in the levels of total denatured whey protein (defined as the denatured α-lactalbumin and β-lactoglobulin combined) during heating of the milk samples at the various pH values. Similar trends were observed for the denaturation of α-lactalbumin and β-lactoglobulin (results not shown).

At a given heating time, the level of denatured α-lactalbumin, denatured β-lactoglobulin, and the total denatured whey protein increased with an increase in heating temperature and, at a given heating temperature, the level of denaturation increased with the duration of the heat treatment. The pH of the milk at heating had very little effect on the level of denatured protein over the narrow pH range used in this study. Oldfield et al. (*14*) also reported that the rates of denaturation of both α-lactalbumin and β-lactoglobulin were unaffected by pH over the pH range of 6.48−6.83 and at temperatures of 80−90 °C, although some small differences were observed at lower temperatures. As a consequence, there was no relationship between the changes in casein micelle size and the denaturation of the total whey protein (**Figure 3B**). Most of the change in casein micelle size occurred after more than ∼80% of the whey protein had denatured.

Association of Denatured Whey Proteins with Casein Micelles. The association of the major whey proteins (α-lactalbumin, β-lactoglobulin, and total whey protein) with the casein micelles was monitored by an ultracentrifugation/SDS-PAGE technique. At all temperatures, the level of total denatured whey protein that associated with the micelles increased rapidly during the initial period of heating and tended to plateau on prolonged heating (**Figure 4**). It was evident that, at all three

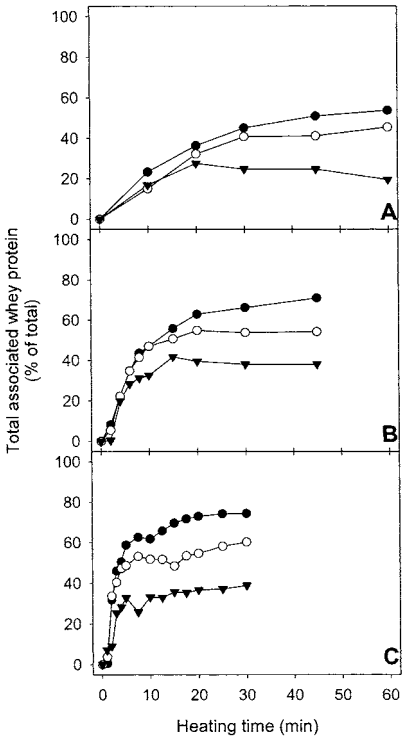

Figure 4. Level of association of whey proteins with casein micelles in skim milk samples at pH 6.55 (●), pH 6.60 (○), and pH 6.70 (▼) that were heated at 80–100 °C for various times: (A) 80 °C; (B) 90 °C; (C) 100 °C.

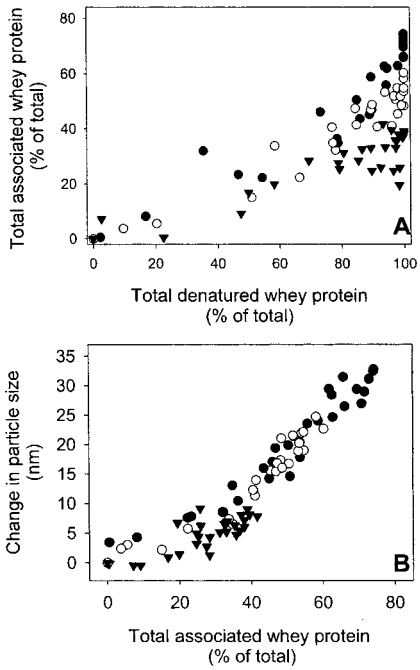

Figure 5. Relationships between (A) the level of denatured whey protein and the level of whey protein associated with casein micelles and (B) the level of associated total whey protein and the particle size for skim milk samples at pH 6.55 (●), pH 6.60 (○), and pH 6.70 (▼) that were heated at 75–100 °C for various times.

heating temperatures, the association behavior was strongly dependent on pH. Up to 70% of the total denatured whey protein associated with the casein micelles at pH 6.55, and this decreased to about 50 and 30% as the pH was increased to pH 6.6 and 6.7, respectively. A similar behavior was observed for α-lactalbumin and β-lactoglobulin (results not shown).

The relationship between the level of total denatured whey protein and the level of total whey protein associated with the micelles is shown in **Figure 5A**. When samples at all pH values were considered, there was little relationship between the level of denatured whey protein and the level that associated with the micelles. For example, at a denaturation level of ~95%, up to 70% of the denatured whey protein was associated with the micelles if the milk pH was 6.55, but only ~30% associated at pH 6.7. Clearly, not all denatured whey protein associated with the micelles, and the level of association decreased markedly with increasing pH.

Figure 5B shows the relationship between the level of total whey protein associated with the casein micelles and the changes in particle size for the milk samples at the three pH values. There was a strong, almost linear, relationship between the amount of whey protein associated with the casein micelles and the casein micelle size at all temperatures and at all pH values investigated. This indicates that the strong pH dependence of the particle size change (**Figures 1** and **2**) was a consequence of the pH dependence of the interaction of denatured whey proteins with the micelles. However, it was not possible to determine whether the size changes observed were due explicitly to the association of denatured whey proteins with the casein micelle surface or were due to the partial aggregation of the casein micelles that occurs at the same time and is proportional to the levels of whey proteins that have associated with the casein micelles.

Association Behavior with Small pH Changes. To confirm the strong pH dependence of whey protein association with the casein micelles, and the effect on casein micelle size, the experiment was repeated using a different milk sample adjusted to pH values from 6.5 to 6.7, with steps of only 0.05 pH unit. The milk samples were heated for up to 30 min at 90 °C only. The particle size change, the level of association of whey proteins with the micelles, and the relationship between these two results were monitored (**Figure 6**). Even at pH steps as small as 0.05 pH unit, it was possible to measure the progressive increase in the size of the casein micelles as the pH before heating was decreased (**Figure 6A**). As observed in the earlier experiments, the change in casein micelle size was accompanied by an increase in the level of association of the whey proteins with the micelles (**Figure 6B**), so that a relationship between size changes and association levels existed at all pH values examined (**Figure 6C**). Similar results were obtained when a fresh milk sample was used in place of the reconstituted skim milk, indicating that the association of denatured whey proteins with the casein micelles was strongly dependent on very small changes in pH in both fresh and reconstituted milk samples. The use of a single heating temperature reduced the noise in the plots (**Figure 6C**).

At low levels of association, the size increase of the casein micelles was not as great as observed when higher levels of whey protein associated with the micelles. This produced plots with a marked curvature at low association levels (**Figures 5B** and **6C**). This effect may have been due to some shrinkage of the micelle core during the early stages of heating, possibly through the increased mineralization of the micelle or the increase in hydrophobic interactions at the higher temperatures. Some reduction in casein micelle size during the early stages of heating has been previously observed during turbidity (*20*), viscosity (*21*), and size (*15*) measurements. A slight reduction in the core micelle size will negate the increase in size as a consequence of the whey protein association at low levels of association and hence the size increase will not be as large as expected from the association level. Alternatively, this could

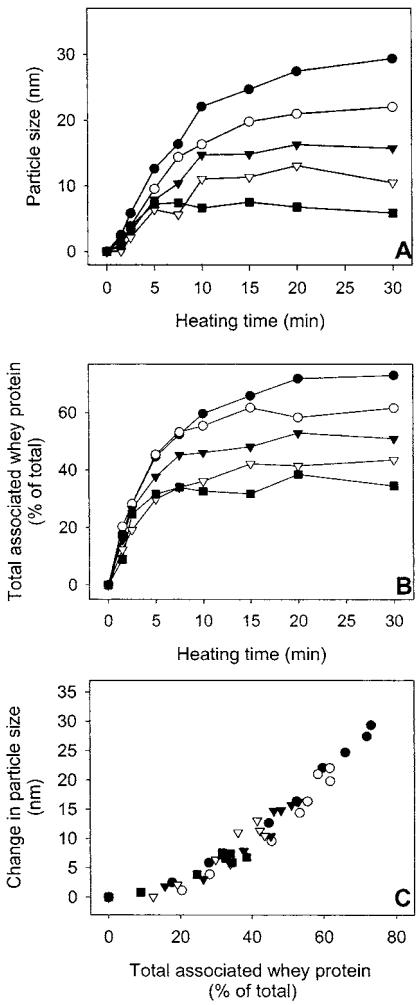

Figure 6. (A) Changes in casein micelle size for skim milk samples at pH values between 6.5 and 6.7 that were heated at 90 °C for various times. (B) Level of association of whey proteins with casein micelles in skim milk samples at pH values between 6.5 and 6.7 that were heated at 90 °C for various times. (C) Relationship between the level of associated total whey protein and particle size for skim milk samples at pH values between 6.5 and 6.7 that were heated at 90 °C for various times. (●) pH 6.50; (○) pH 6.55; (▼) pH 6.60; (▽) pH 6.65; (■) pH 6.70.

be due to the diffuse nature of the casein micelle surface. Initially, the denatured whey proteins will need to penetrate the hairy micelle surface to interact with the disulfide bonds of κ-casein, and therefore the size may not increase proportionally with the level of association. Subsequent association may occur with those whey proteins already attached to the micelle surface and, as the associating proteins no longer need to penetrate the hairy surface, the micelle volume will increase proportionally with the level of association.

DISCUSSION

There have been numerous studies on the association of β-lactoglobulin with κ-casein; however, many have been in model systems, and no studies have examined the effect of small changes in pH (0.05 unit) over the narrow range used in the present study. Smits and van Brouwershaven (*3*) showed that when model milk systems were heated at 90 °C, a maximum of ~83% of the β-lactoglobulin associated with the micelles at pH 5.8, and this decreased to ~76% at pH 6.3, ~44% at pH 6.8, and ~24% at pH 7.3. Corredig and Dalgleish (*8*) examined the association of whey proteins with the casein micelles for milk heated at pH 5.8, 6.2, and 6.8. They reported that, in general, an increased amount of whey protein complexed with the micelles at lower pH. Similarly, Oldfield et al. (*14*) examined the association behavior of whey proteins with the micelles for milk samples at pH 6.48, 6.60, and 6.83. On heating at 90 °C, ~90% of the whey protein associated with the micelles at pH 6.48, and this decreased to 80% at pH 6.60 and 60% at pH 6.83. Oldfield et al. (*14*) suggested that the decreased association of whey proteins with the casein micelles at elevated pH was due to the partial dissociation of κ-casein from the micelles, which is known to occur when milk is at a pH >6.7 (*13*, *22*−*24*).

The association behavior with pH for the present study and from the studies reported by Smits and van Brouwershaven (*3*), Corredig and Dalgleish (*8*), and Oldfield et al. (*14*) shows the same general trends, with a reduced association level at higher pH. However, there was some variation in association levels at comparable pH. This may be related to the separation (centrifugation) techniques and/or the analysis techniques. For example, the present study used a centrifugal force of ~63000*g*, which was found to be sufficient to deposit the casein micelles. Higher *g* forces increased the level of whey protein deposited with the pellet without increasing the levels of casein deposited, indicating that larger whey protein aggregates were being deposited (*8*, *15*). The study of Oldfield et al. (*14*) used a substantially higher centrifugal force of 175000*g*; this may have caused the deposition of some of the larger whey protein aggregates, and this would have increased the apparent level of association with the micelles. It is also possible that the difference in association levels between these different studies is related to the method used to heat the milk. Corredig and Dalgleish (*10*) have shown that the higher levels of whey proteins associated with the casein micelles when skim milk was heated using indirect heating systems than when a direct steam injection system was used.

None of the previous studies examined the effect of pH on the association behavior of denatured whey proteins with the casein micelles when the milk pH was adjusted to values over the narrow range between 6.5 and 6.7. This study has clearly shown that this association behavior has a strong pH dependence over this narrow pH range. However, it is unknown why small shifts in pH cause such major changes in the level of association of denatured whey proteins with the casein micelles. The denaturation of α-lactalbumin and β-lactoglobulin was not markedly affected by pH over this narrow range (**Figure 3**). Therefore, the increased interaction is not due to higher levels of denatured whey proteins available for association at the lower pH. Similarly, the reactivity of sulfhydryl groups and the rate of the sulfhydryl−disulfide exchange reaction increase with increasing pH (*25*); therefore, an increased association may be expected at higher pH, not at lower pH as is observed. It is possible that the change in reactivity or availability of sulfhydryl and disulfide groups with changes in pH may shift the preferred interaction pathway between the serum and colloidal phases.

Differences in levels of association of denatured whey proteins with the casein micelles may be influenced by the dissociation of κ-casein from the micelles at higher pH. However, the literature indicates that there are only low levels of dissociation of κ-casein in unconcentrated milk unless the pH is increased above ~6.7 (*13*, *22*−*24*). Under the heating and centrifugation conditions used in this study, there was a only a very small difference in the level of soluble κ-casein in all samples. In the unheated milk, the level of soluble κ-casein

was ~17% of the total, which is similar to that reported previously (*13*, *23*). Heated milk at pH 6.5 had a level of soluble κ-casein similar to that in the unheated milk, and this level increased slightly as the pH was increased so that ~23% of the total κ-casein was soluble at pH 6.7 (results not shown). It is uncertain whether this level of dissociation is sufficient to account for the changes in association of denatured whey proteins with the casein micelles, as this would require a high ratio of denatured whey protein to κ-casein (at least 3:1 or 4:1) in the soluble complexes, when compared with those associated with the micelles (ratio of about 1.5:1). A ratio of about 1:1 for β-lactoglobulin to κ-casein in the micelles from heated milk has been reported, although this was affected by the conditions at heating (*8*, *10*, *12*, *26*). The ratio of α-lactalbumin to κ-casein in the micelles was dependent on the initial concentration of α-lactalbumin and the presence of β-lactoglobulin (*26*). In pure protein systems, the ratio of β-lactoglobulin to κ-casein in aggregated species is dependent on the ratio of the individual components before heating. However, a maximum ratio of 3:1 for β-lactoglobulin to κ-casein was observed when a large excess of β-lactoglobulin was used (*27*, *28*).

It is assumed that the major interaction between whey proteins and the casein micelles involves the sulfhydryl−disulfide exchange reactions between the free sulfhydryl groups of denatured β-lactoglobulin (or a whey protein aggregate with a free sulfhydryl group) and the disulfide bonds of the κ-casein polymer. As the disulfide bonds of κ-casein are found near the boundary between the *para*-κ-casein region (associated with the micelle core) and the glycomacropeptide region (the flexible hair), the denatured whey protein complex is required to penetrate through the entire hairy layer in order to interact with the disulfide bond of κ-casein (*11*, *29*). As the pH is increased, the micelle surface charge and, in particular, the charge on the macropeptide hairs will increase. This will cause the hairs to extend further from the micelle surface and therefore may, through charge and possibly steric repulsions, reduce the propensity of interactions between denatured whey proteins and casein micelles. This may, at higher pH, increase the tendency for serum phase aggregation reactions to occur, either among denatured whey proteins or even between denatured whey proteins and the low levels of serum phase κ-casein.

The question arises as to whether the particle size changes shown in **Figures 1**, **2**, and **6** were due to the specific association of denatured whey proteins with the casein micelles or a partial aggregation of the casein micelles that was proportional to the level of whey protein that had associated with the micelles. If the size changes were due solely to the association of the whey proteins with the micelles, then this indicates that the associated whey proteins would need to be highly hydrated or arranged at the micelle surface in such a way that a considerable quantity of serum was entrapped at the micelle surface. This would cause a measurable increase in the viscosity of the milk. Anema and Li (*15*), in a preliminary study, demonstrated that the viscosity of the milk did increase on heating and that the changes in viscosity and volume fraction (calculated from the viscosity) were entirely consistent with the observed changes in casein micelle size.

Jeurnink and De Kruif (*21*) showed that, when milk at pH 6.7 was heated at 90 °C, its viscosity increased. However, they did not measure the levels of whey protein that had associated with the micelles. The viscosity changes reported by Jeurnink and De Kruif (*21*) are consistent with the size changes obtained in this study at a similar pH and with the size and viscosity results of Anema and Li (*15*). Jeurnink and De Kruif (*21*) concluded that the increase in viscosity was due primarily to an increase in volume fraction of the micelles and not due to the permanent clustering of the micelles in these heated samples. This suggests that the particle size, viscosity, and volume fraction changes did not increase due to permanent micellar aggregation. Similarly, the turbidity changes of heated milk at pH 6.7 were considered to be a consequence of the increase in volume fraction through the association of denatured whey proteins with the casein micelles and not due to a change in the interaction between casein micelles (*20*).

Although the studies on viscosity (*15*, *21*) and turbidity (*20*) provide evidence to support the association theory, there is still the possibility that an aggregation process could account for the change in particle size and also the increases in the viscosity, turbidity, and volume fraction. However, the aggregate structures would need to be of sufficient size, structure, and number to occlude the amount of water required to increase the viscosity and volume fraction by the level observed. Without further study, it is not possible to conclude whether such voluminous aggregates could be formed in heated milk, particularly when no change in polydispersity is observed and the size increases by a maximum of only ~30−35 nm.

Similarly, it is difficult to establish whether the association of whey proteins with the casein micelles could increase the micelle size by the level observed without knowledge of the structure of the denatured whey proteins, the composition and structure of the casein micelle surface, and the specific interactions involved in the association reactions. The denatured whey proteins would need to form extended linear polymers that attach to the casein micelle in such a way as to extend the hairy layer and thus the hydration of the micelles. The initial aggregation of β-lactoglobulin in buffer systems does form linear rodlike particles (*30*), but it is unknown whether these findings can be extrapolated to the aggregation in heated milk. In milk, there are approximately two whey proteins available to interact with each κ-casein. This is unlikely to be sufficient to form the polymers required to account for the observed size changes. However, if some of the κ-casein were unavailable for interaction, either due to orientation at the surface or by being located in the micelle interior, then larger whey protein polymers could be formed. Holt and Dalgleish (*31*) indicated that as little as 10% of the total κ-casein was required to form the surface hairy layer. In this case, there may be sufficient whey protein to form linear polymers on the micelle surface that could account for the observed size changes.

The results of this study demonstrated that the changes in casein micelle size on heating milk were markedly dependent on small changes in milk pH values between 6.5 and 6.7, with smaller increases in size at higher pH. The changes in size were related to the interaction of denatured whey proteins with the casein micelles, which was also markedly dependent on pH. About 75−80% of the denatured whey proteins associated with the micelles at pH 6.5, and this decreased to an association level of only ~30% at pH 6.7. These differences in association behavior may explain the variable levels of association between milks heated under different conditions (*8*, *11*, *12*) and also the variations in size changes for heated milks (*16*). Further studies are required to determine why small changes in milk pH cause such a variation in the level of association of denatured whey proteins with the casein micelles. These differences may be due to charge repulsion effects at higher pH, low levels of dissociation of κ-casein, or a preferential aggregation of denatured whey proteins with serum phase components at higher pH.

ACKNOWLEDGMENT

We thank Nick Timmins for assistance with some of the analyses, Mike Boland and Lawrie Creamer for useful discussions, and Claire Woodhall for proofreading the manuscript.

LITERATURE CITED

(1) Zittle, C. A.; Thompson, M. P.; Custer, J. H.; Cerbulis, J. κ-Casein-β-lactoglobulin interaction in solution when heated. *J. Dairy Sci.* **1962**, *45*, 807−810.

(2) Sawyer, W. H. Complex between β-lactoglobulin and κ-casein. A review. *J. Dairy Sci.* **1969**, *52*, 1347−1355.

(3) Smits, P.; van Brouwershaven, J. H. Heat-induced association of β-lactoglobulin and casein micelles. *J. Dairy Res.* **1980**, *47*, 313−325.

(4) Hill, A. R. The β-lactoglobulin-κ-casein complex. *Can. Inst. Food Sci. Technol. J.* **1989**, *22*, 120−123.

(5) Noh, B. S.; Richardson, T. Incorporation of radiolabelled whey proteins into casein micelles by heat processing. *J. Dairy Sci.* **1989**, *72*, 1724−1731.

(6) Jang, H. D.; Swaisgood, H. E. Disulphide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. *J. Dairy Sci.* **1990**, *73*, 900−904.

(7) Mulvihill, D. M.; Donovan, M. Whey proteins and their thermal denaturation−a review. *Ir. J. Food Sci. Technol.* **1987**, *11*, 43−75.

(8) Corredig, M.; Dalgleish, D. G. Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. *Food Res. Int.* **1996**, *29*, 49−55.

(9) Singh, H.; Creamer, L. K. Denaturation, aggregation and heat stability of milk protein during the manufacture of skim milk powder. *J. Dairy Res.* **1991**, *58*, 269−283.

(10) Corredig, M.; Dalgleish, D. G. The binding of α-lactalbumin and β-lactoglobulin to casein micelles in milk treated by different heating systems. *Milchwissenschaft* **1996**, *51*, 123−127.

(11) Oldfield, D. J.; Singh, H.; Taylor, M. W. Association of β-lactoglobulin and α-lactalbumin with the casein micelles in skim milk heated in an ultra-high temperature plant. *Int. Dairy J.* **1998**, *8*, 765−770.

(12) Corredig, M.; Dalgleish, D. G. The mechanisms of the heat-induced interaction of whey proteins with casein micelles in milk. *Int. Dairy J.* **1999**, *9*, 233−236.

(13) Anema, S. G.; Klostermeyer, H. Heat-induced, pH-dependent dissociation of casein micelles on heating reconstituted skim milk at temperatures below 100 °C. *J. Agric. Food Chem.* **1997**, *45*, 1108−1115.

(14) Oldfield, D. J.; Singh, H.; Taylor, M. W.; Pearce, K. N. Heat-induced interactions of β-lactoglobulin and α-lactalbumin with the casein micelle in pH-adjusted skim milk. *Int. Dairy J.* **2000**, *10*, 509−518.

(15) Anema, S. G.; Li, Y. The association of denatured whey proteins with the casein micelles in heated reconstituted skim milk and its effect on casein micelle size. *J. Dairy Res.* **2002**, in press.

(16) Devold, T. G.; Brovold, M. J.; Langsrud, T.; Vegarud, G. E. Size of native and heated casein micelles, content of proteins and minerals in milk from Norwegian red cattle−effect of milk protein polymorphism and different feeding regimes. *Int. Dairy J.* **2000**, *10*, 313−323.

(17) Sanderson, W. B. Determination of undenatured whey protein nitrogen in skim milk powder by dye binding. *N. Z. J. Dairy Sci. Technol.* **1970**, *5*, 46−48.

(18) Anema, S. G. The effect of chymosin on κ-casein-coated polystyrene latex particles and bovine casein micelles. *Int. Dairy J.* **1997**, *7*, 553−558.

(19) Anema, S. G.; McKenna, A. B. Reaction kinetics of thermal denaturation of whey proteins in heated reconstituted whole milk. *J. Agric. Food Chem.* **1996**, *44*, 422−428.

(20) Jeurnink, T. J. M. Changes in milk on mild heating. Turbidity measurements. *Neth. Milk Dairy J.* **1992**, *46*, 183−196.

(21) Jeurnink, T. J. M.; De Kruif, K. G. Changes in milk on heating: viscosity measurements. *J. Dairy Res.* **1993**, *60*, 139−150.

(22) Kudo, S. The heat stability of milk: formation of soluble proteins and protein-depleted micelles at elevated temperatures. *N. Z. J. Dairy Sci. Technol.* **1980**, *15*, 255−263.

(23) Singh, H.; Fox, P. F. Heat stability of milk: pH-dependent dissociation of micellar κ-casein on heating milk at ultra-high temperatures. *J. Dairy Res.* **1985**, *52*, 529−538.

(24) Anema, S. G.; Li, Y. Further studies on the heat-induced, pH-dependent dissociation of casein from the micelles in reconstituted skim milk. *Lebensm.-Wiss. Technol.* **2000**, *33*, 335−343.

(25) Torchinsky, Y. M. *Sulfur in Proteins*; Pergamon Press: Oxford, U.K., 1981.

(26) Dalgleish, D. G.; van Mourik, L.; Corredig, M. Heat-induced interactions of whey proteins and casein micelles with different concentrations of α-lactalbumin and β-lactoglobulin. *J. Agric. Food Chem.* **1997**, *45*, 4806−4813.

(27) Tessier, H.; Yaguchi, M.; Rose, D. Zonal ultracentrifugation of β-lactoglobulin and κ-casein complexes induced by heat. *J. Dairy Sci.* **1969**, *52*, 139−145.

(28) Haque, Z.; Kristjansson, M. M.; Kinsella, J. E. Interaction between κ-casein and β-lactoglobulin: possible mechanisms. *J. Agric. Food Chem.* **1987**, *35*, 644−649.

(29) Holt, C.; Horne, D. S. The hairy casein micelle: evolution of the concept and its implications for dairy technology. *Neth. Milk Dairy J.* **1996**, *50*, 85−111.

(30) Griffin, W. C.; Griffin, M. C. A.; Martin, S. R.; Price, J. Molecular-basis of thermal aggregation of bovine β-lactoglobulin A. *J. Chem. Soc., Faraday Trans.* **1993**, *89*, 3395−3406.

(31) Holt, C.; Dalgleish, D. G. Electrophoretic and hydrodynamic properties of bovine casein micelles interpreted in terms of particles with an outer hairy layer. *J. Colloid Interface Sci.* **1986**, *114*, 513−524.

Received for review May 14, 2002. Revised manuscript received November 11, 2002. Accepted December 16, 2002. This work was supported by the New Zealand Foundation for Research, Science and Technology, contract numbers DRI0001 and DRIX0201.

JF025673A