JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Role of κ-Casein in the Association of Denatured Whey Proteins with Casein Micelles in Heated Reconstituted Skim Milk

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Reconstituted skim milk at pH from 6.5 to 7.1 was unheated, preheated (68 °C/20 min), or heated at 90 °C for 20−30 min. On preheating, the size of the casein micelles decreased by about 5−20 nm, with a greater effect at higher pH. The casein micelle size of the heated milk at pH 6.5 increased by about 30 nm when compared to that of the unheated or preheated milk. As the pH was increased before heating, the particle size gradually decreased so that, at pH 7.1, the size was markedly smaller than that for the unheated milk and slightly smaller than that for the preheated milk. High levels (about 85%) of denatured whey protein associated with the casein micelles at pH 6.5, and this level decreased as the pH increased so that, at pH 7.1, low levels (about 15%) were associated with the micelles. Low levels of α_S-casein and β-casein were found in the serum regardless of the heat treatment or the pH of the milk. At pH 6.5, low levels (about 10%) of κ-casein were also found in the milk serum. In the unheated milk, the level of serum κ-casein increased slightly with increasing pH; in the heated samples, the level of serum κ-casein increased markedly and linearly with increasing pH so that, at pH 7.1, about 70% of the κ-casein was in the serum phase. The results of this study indicate that the pH dependence of the levels of serum phase κ-casein may be responsible for the change in distribution of the whey proteins between the colloidal and serum phases. This is the first report to demonstrate significant levels of dissociation of κ-casein from the micelles at pH between 6.5 and 6.7, although this dissociation phenomenon is well known on heating milk at high temperatures at pH above 6.7.

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

INTRODUCTION

When milk is heated at temperatures above about 70 °C, the whey proteins denature (*1*, *2*). These denaturation reactions, and the other processes associated with heat treatments, are of great interest as they are used to manipulate the physicochemical and functional properties of many of the current dairy products. However, the denaturation of the whey proteins is only the first step in a complex series of aggregation reactions. These aggregation reactions of the denatured whey proteins are more important determinants of the functional properties of the milk than the denaturation reactions.

Our recent studies have shown that the interaction of the denatured whey proteins with the casein micelles appears to be strongly dependent on the pH of the milk at heat treatment (*3*, *4*). If the milk pH is low (about pH 6.5), most of the denatured whey protein interacts with the casein micelles. Progressively increasing the pH before heat treatment reduces the level of whey protein interacting with the casein micelles so that, at pH 6.7, only about 30% of the denatured whey protein is associated with the casein micelles, and, at higher pH, very low levels of whey protein are associated with the colloidal phase (*3*−*5*). This pH-dependent association of denatured whey proteins with the casein micelles affects the physical properties of the milk such as the colloidal particle size (*3*, *4*, *6*), the viscosity (*3*, *7*), and the turbidity (*8*), as well as some of the functional properties such as acid gelation and aggregation (*5*, *6*, *9*−*11*).

Numerous studies on the heat stability of milk have demonstrated that progressively higher levels of κ-casein dissociate from the casein micelles as the pH of milk is increased above pH 6.7 before heat treatment (*8*, *12*−*17*). This dissociation of κ-casein has been used to explain the unusual pH dependence of the heat stability of milk at high temperatures (above about 120 °C). At 140 °C, the heat stability versus pH profile for skim milk displays a heat stability local maximum and a heat stability local minimum at about pH 6.7 and pH 6.9, respectively, with the heat stability increasing again at higher pH (*16*, *18*). As denatured whey proteins are known to interact with κ-casein via thiol−disulfide exchange reactions (*19*−*22*), this dissociation

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10.1021/jf062734m CCC: $37.00
Published on Web 04/07/2007

of κ-casein-rich protein at pH above 6.7 may explain the low levels of denatured whey proteins interacting with the casein micelles at elevated pH. As studies on the heat stability of milk at its natural concentration report that little or no dissociation of κ-casein occurs at pH 6.7 or lower, this cannot explain the pH-dependent association of denatured whey proteins with the casein micelles at pH below 6.7 (*13*−*16*).

The studies on the heat stability of milk were at extremely high temperatures, usually at 120 °C or above, whereas the studies on the interaction of denatured whey proteins with the casein micelles were at lower temperatures, usually below about 100 °C. Studies have shown that the dissociation behavior of casein from the micelles is dependent on the temperature (*8*, *17*, *23*); however, no detailed studies have been conducted at steps between pH 6.5 and pH 6.7, and no studies have examined the relationship between the interaction of denatured whey proteins with the casein micelles and the dissociation of κ-casein from the casein micelles over a wide pH range. Therefore, this study was initiated to investigate the relationships between the particle size changes, the association of denatured whey proteins with the casein micelles, and the dissociation of casein, particularly κ-casein, from the casein micelles in milk adjusted to pH in the range 6.5−7.1 before heating at 90 °C. Emphasis was placed on samples at pH between 6.5 and 6.7 as this is the region where the greatest change in association of whey proteins with the casein micelles occurs (*4*).

MATERIALS AND METHODS

Milk Supply. Low heat skim milk powder was obtained from Fonterra Co-operative Group, New Zealand. This powder is manufactured with minimal heat treatment (pasteurization, ∼72 °C, ∼15 s) to maintain high levels of native whey proteins. Reconstituted skim milk samples of 10% total solids (w/w) were prepared by adding the low heat skim milk powder to water (purified through a Milli-Q apparatus (Millipore Corp., Bedford, MA)). A small quantity (0.02%) of sodium azide was added to each of the milk samples as a preservative. The milk samples were stirred for at least 12 h at ambient temperatures (about 20 °C) before further use to ensure the re-equilibration of mineral components.

Adjustment of pH and Heat Treatments. Sub-samples of skim milk were adjusted to pH values between 6.5 and 7.1 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 final pH reading and minor readjustment. For the heated milks, sub-samples of each milk (6 mL) were transferred to glass vials and heated, with continuous rocking, in a thermostatically controlled oil bath at 68 °C for 20 min or at 90 °C for 20, 25, or 30 min. After heat treatment, the milk samples were cooled to room temperature by immersion of the glass vials in cold running water.

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.), as has been described previously (*3*, *4*, *7*). Skim milk samples were dispersed in Ca-imidazole buffer (20 mM imidazole, 5 mM $CaCl_2$, 30 mM NaCl, pH 7.0) before particle size measurement.

Centrifugation. Milk volumes of 1 mL were placed in small plastic tubes of 1.5 mL total volume. Serum whey proteins were defined as those that did not sediment from the milk during centrifugation at 14 000 rev/min (21 000*g* average) for 1 h at 20 °C in an Eppendorf centrifuge Type 5417C (Eppendorf AG, Hamburg, Germany). As has been discussed previously (*5*, *6*, *9*), this method of centrifugation is sufficient to completely sediment the casein micelles while retaining the smaller complexes of denatured whey proteins and κ-casein in the serum phase. After centrifugation, the clear supernatant (serum phase) was carefully poured from the pellets (colloidal phase). The β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) content of these serum samples was determined by gel electrophoresis and laser densitometry.

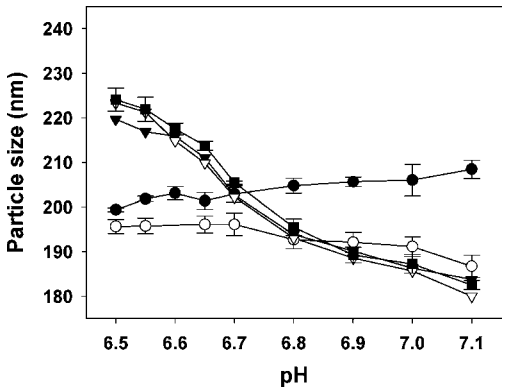

Figure 1. Effect of pH on the particle size of casein micelles in milk: ●, unheated milk; ○, preheated milk (68 °C/20 min); ▼, milk heated at 90 °C for 20 min; ▽, milk heated at 90 °C for 25 min; ■, milk heated at 90 °C for 30 min. Error bars on selected points represent typical standard deviations of repeated measurements.

Native Polyacrylamide Gel Electrophoresis (native-PAGE). The casein and the denatured whey proteins were removed from the milk samples 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, as has been described previously (*2*).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The level of protein in the milk samples and the serum was determined using SDS-PAGE under reducing conditions, as has been described previously (*3*, *4*, *8*). Standard protein samples of known protein concentrations for the milk proteins were included in each gel at various concentrations to generate standard curves.

Laser Densitometry. 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 milk protein bands were determined using the Imagequant software associated with the densitometer. For native-PAGE, the quantity of β-LG and α-LA in the heated samples was calculated as a percentage of that in the unheated milk. For SDS-PAGE, the quantity of each protein in the milk and serum samples was determined as an absolute concentration (in mg protein per g milk or serum sample) that was calculated from the standard curves generated from the protein standards.

All experiments reported were repeated at least twice with the same milk samples and analyzed statistically using an analysis of variance test. In addition, the experiments were repeated with 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 AND DISCUSSION

Milk samples were adjusted to pH values in the range from pH 6.5 to pH 7.1. Control samples were left unheated, and experimental samples were heated at 68 °C for 20 min (preheated samples) or at 90 °C for 20, 25, or 30 min. The size of the particles in each milk sample was monitored (**Figure 1**). For the unheated milk, the particle size increased by about 5 nm as the pH was increased from 6.5 to 7.1. Preheat treatment (68 °C/20 min) decreased the casein micelle size relative to that of the unheated milk at all pH. At pH values between 6.5 and 6.7, the particle size of the preheated milk remained relatively constant, whereas, above pH 6.7, the particle size decreased as the pH was increased. A statistical analysis indicated that, although the changes in size between adjacent points were not always statistically significant, the overall increase in size between samples at low pH (∼pH 6.5) and high pH (∼pH 7.1)

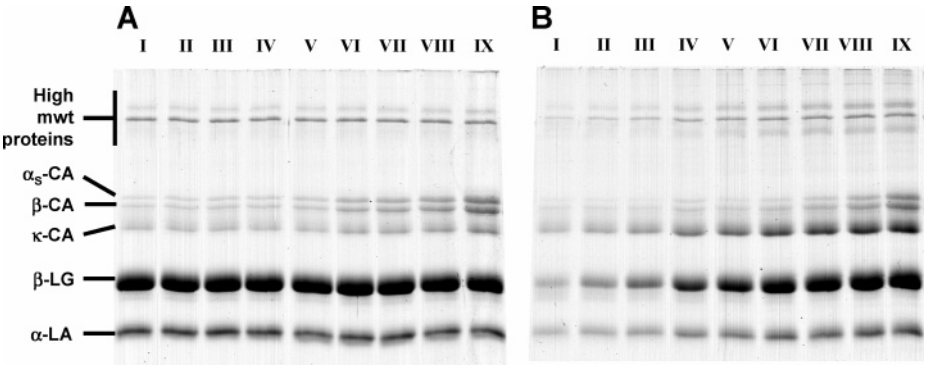

Figure 2. Typical SDS-PAGE patterns for the serum phase proteins in milk: (**A**) unheated milk; (**B**) milk heated at 90 °C for 25 min. Lane I, pH 6.5; lane II, pH 6.55; lane III, pH 6.6; lane IV, pH 6.65; lane V, pH 6.7; lane VI, pH 6.8; lane VII, pH 6.9; lane VIII, pH 7.0; lane IX, pH 7.1.

for the unheated milk, and the overall decrease in size between samples at low pH (~pH 6.5) and high pH (~pH 7.1) for the preheated milks were significant ($p < 0.05$). At each pH, the difference in size between the unheated and preheated milks was statistically significant ($p < 0.05$).

On heat treatment of the milk at 90 °C, and at pH 6.5, the casein micelle size increased markedly, with a total increase of about 25−30 nm when compared to the unheated milk samples, and about 35 nm when compared to the preheated milk samples. As the pH of the milk was increased before heating, the casein micelle size in the heated milk samples gradually decreased so that, at pH 6.7, the size was similar to that of the unheated milk and at higher pH the size was markedly smaller that that of the unheated milk, and slightly smaller than that of the preheated milk. For these heated milks, the decrease in size with pH was statistically significant ($p < 0.05$); however, there was no significant difference between the samples heated at 90 °C for 20, 25, or 30 min.

The changes in particle size on heating, especially between pH 6.5 and pH 6.7, were in good agreement with those reported previously (*3*, *4*, *6*). It is unknown why preheating milk at about 60−70 °C causes a decrease in particle size, although this phenomenon is accompanied by a decrease in the viscosity (*3*, *7*, *24*) and turbidity (*25*) of the milk. This may be related to an increased mineralization of the casein micelles, or rearranged hydrophobic interactions; however, whatever the cause, this decrease in size is irreversible over a reasonable time period (24−48 h after heating).

Analysis by native-PAGE showed that virtually all of the β-LG was denatured after heat treatment at 90 °C for 20−30 min, regardless of the pH at heat treatment (results not shown). For α-LA, native-PAGE showed that the denaturation was somewhat more dependent on both pH and duration of heat treatment. After 20 min of heating, about 90% of the α-LA was denatured at pH 6.5, and this level progressively increased with increasing pH so that, at pH 6.9, virtually all of the α-LA was denatured. Heating for longer holding times progressively denatured more α-LA at each pH; however, the same general trends with increasing pH were observed (results not shown). These denaturation effects were in accord with literature reports on the effect of holding time and pH on the denaturation of whey proteins (*1*, *2*, *26*, *27*).

Representative gels for the protein content of supernatants from the unheated and heated (90 °C/25 min) milk samples at different pH values are shown in **Figure 2**. In the unheated milk (**Figure 2A**), the level of casein (α_S-casein, β-casein, and κ-casein) was low, although this level increased slightly with increasing pH above pH 6.7. The levels of the whey proteins (β-LG, α-LA, and the high molecular weight species) were essentially constant at all pH values. For the heated milk samples, the levels of α_S-casein and β-casein remained low and similar to those in the unheated milk at all pH. In contrast, the levels of κ-casein, β-LG, and α-LA were low at pH 6.5, but increased with increasing pH so that high levels were observed in the supernatants at pH 7.1. The high molecular weight species (which consist of the minor whey proteins such as lactoferrin, bovine serum albumin, and the immunoglobulins) followed trends similar to those for κ-casein, β-LG, and α-LA.

As the dye binding, and therefore the staining intensity, of the individual proteins is markedly different (*28*), quantitative analysis of the major protein species was performed using standard curves generated from pure protein standards. A total of about 3.5 mg of β-LG/g and about 1.0 mg of α-LA/g was present in the milk serum in native form before heat treatment (**Figure 3A**), which is in the normal range for bovine milk (*29*). On heat treatment at pH 6.5, about 0.5 mg/g (about 14%) of the β-LG and about 0.2 mg/g (about 20%) of the α-LA remained in the serum. As the pH of the milk prior to heating was increased, the levels of β-LG and α-LA remaining in the serum progressively and significantly ($p < 0.05$) increased so that, at pH 7.1, about 3.0 mg of β-LG/g (about 86%) and about 0.75 mg of α-LA/g (about 75%) were in the serum phase (**Figure 3A**). As β-LG and α-LA were virtually completely denatured, these proteins in the serum are described as nonsedimentable denatured whey proteins. There was no significant effect of heating time on the levels of α-LA and β-LG remaining in the serum at each pH (**Figure 3A**).

The level of denatured α-LA and β-LG associating with the casein micelles could be calculated by subtracting the total nonsedimentable α-LA and β-LG (denatured and remaining native protein) in the heated milk samples from the nonsedimentable α-LA and β-LG (native protein) in the unheated milk samples at each pH. For all heating times at 90 °C, the total whey protein associating with the colloidal phase (α-LA and β-LG combined) was strongly and linearly correlated ($r^2 = 0.99$; $p < 0.05$) with the particle volumes (calculated from the sizes shown in **Figure 1**) of the casein micelles in the heated milk samples (**Figure 3B**).

The levels of α_S-casein (about 14.5 mg/g), β-casein (about 11.5 mg/g), and κ-casein (3.0 mg/g) in the milk were in the range reported for bovine milk (*29*). In the supernatants of the unheated and heated milks, the levels of α_S-casein (**Figure 4A**) and β-casein (**Figure 4B**) were low regardless of the heating time or the pH of the milk at heating. For the unheated milk at pH 6.5, about 0.4 mg/g (about 13% of the total) of the κ-casein was found in the serum as nonsedimentable aggregates. There was some effect of pH on the level of nonsedimentable κ-casein in the unheated milk, as the level increased (particularly above

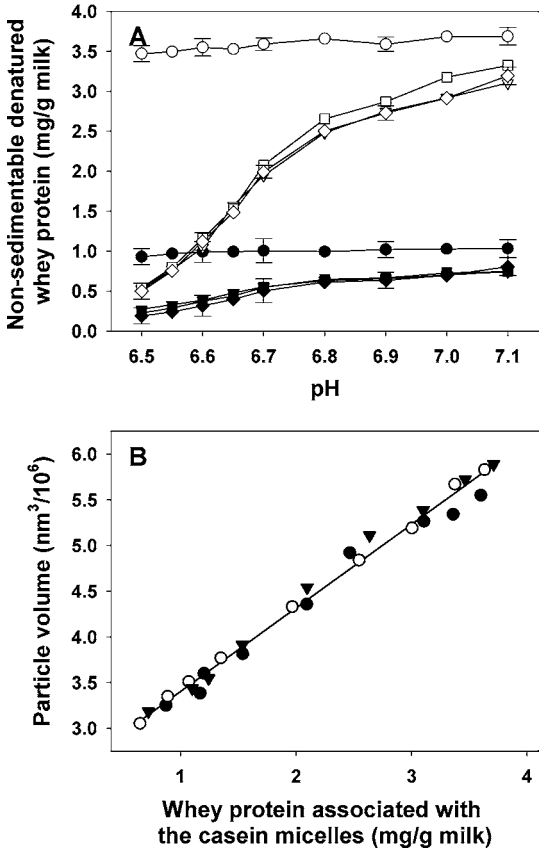

Figure 3. (**A**) Effect of pH on the level of serum phase whey proteins in milk samples. ●,○, Unheated milk; ▼,▽, milk heated at 90 °C for 20 min; ■,□, milk heated at 90 °C for 25 min; ◆,◇, milk heated at 90 °C for 30 min. Open symbols, β-LG; filled symbols, α-LA. (**B**) Relationship between the particle volume for the casein micelles and the level of whey protein associated with the casein micelles for the heated milk samples. ●, Milk heated at 90 °C for 20 min; ○, milk heated at 90 °C for 25 min; ▼, milk heated at 90 °C for 30 min. Error bars on selected points represent typical standard deviations of repeated measurements.

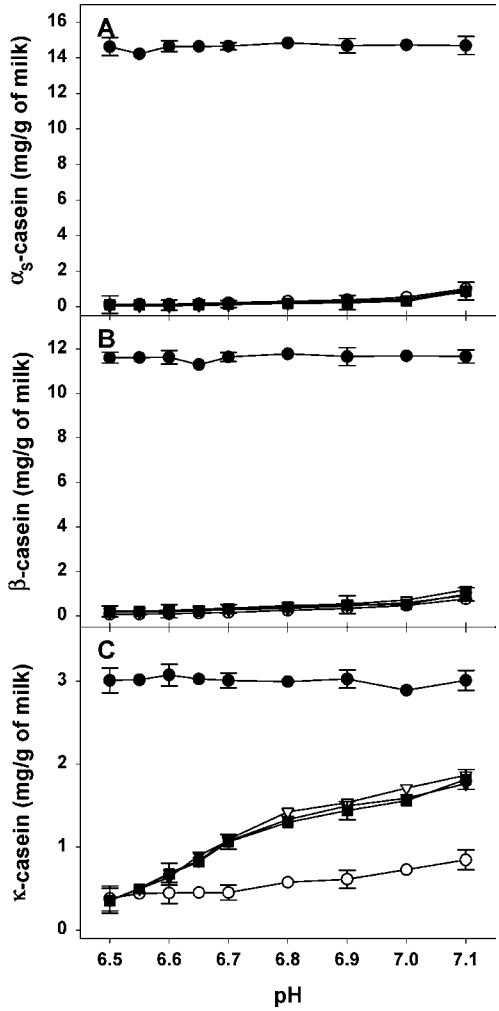

Figure 4. Effect of pH on the level of total casein and serum phase casein in milk samples: (**A**) α_s-casein; (**B**) β-casein; (**C**) κ-casein. ●, Total casein in milk; ○, serum phase casein in unheated milk; ▼, serum phase casein in milk heated at 90 °C for 20 min; ▽, serum phase casein in milk heated at 90 °C for 25 min; ■, serum phase casein in milk heated at 90 °C for 30 min. Error bars on selected points represent typical standard deviations of repeated measurements.

pH 6.7) with increasing pH so that, at pH 7.1, about 0.8 mg/g (about 23%) of the κ-casein was found in the serum. Although the increase in κ-casein was small, it was found to be statistically significant when the changes over the entire pH range were considered ($p < 0.05$). In the heated milk, at pH 6.5, about the same level of κ-casein was found in the serum as in the unheated milk (about 0.4 mg/g, about 13% of the total). As the pH was increased before heating, the level of κ-casein in the serum increased significantly ($p < 0.05$) so that, at the natural pH of the milk (pH $\approx$ 6.7), about 1.1 mg/g (about 37%) of the κ-casein was in the serum, and this increased to 1.9 mg/g (about 63%) at pH 7.1 (**Figure 4C**).

The particle volume of the casein micelles (calculated from the sizes shown in **Figure 1**) in the heated milk samples was linearly correlated with the level of κ-casein remaining with the colloidal phase ($r^2 = 0.98$; $p < 0.05$; **Figure 5A**), and, as this volume was also correlated with the level of whey protein associated with the casein micelles (**Figure 3B**), it was not surprising to find that the levels of nonsedimentable κ-casein and nonsedimentable denatured whey protein were strongly correlated as well ($r^2 = 0.99$; $p < 0.05$; **Figure 5B**).

The relationship between the particle size changes and the level of denatured whey protein associating with the casein micelles (**Figure 3B**) or the level of κ-casein remaining with the colloidal phase (**Figure 5A**) for the milk samples heated at 90 °C indicates that these changes in size may be related to changes in the protein composition of the casein micelles. An increase in size is observed when high levels of denatured whey protein are associated with the colloidal phase, as is observed on heating milk at pH 6.5, and a decrease in size is observed when significant levels of κ-casein are dissociated from the micelles, as is observed on heating milk at pH 7.1. The great difficulty in interpreting these results is determining whether the association of the denatured whey proteins with the casein micelles is directly responsible for the change in size/volume of the casein micelles, by increasing the diameters of the individual particles as the proteins interact, or whether there is some associated phenomenon, such as aggregation of the casein micelles, that is related to the level of whey protein or κ-casein that is in the serum phase or associated with the casein micelles. The strong relationship between the level of whey protein associating with the colloidal phase and the size/volume of the casein micelles (**Figure 3B**; (*3*, *4*)), the observation that the size change plateaus on prolonged heating (*3*, *4*), and the relationships between the protein composition of the micelles and size (**Figures 3B** and **5A**), viscosity (*7*), and turbidity (unpublished results) seem to suggest that the size changes are

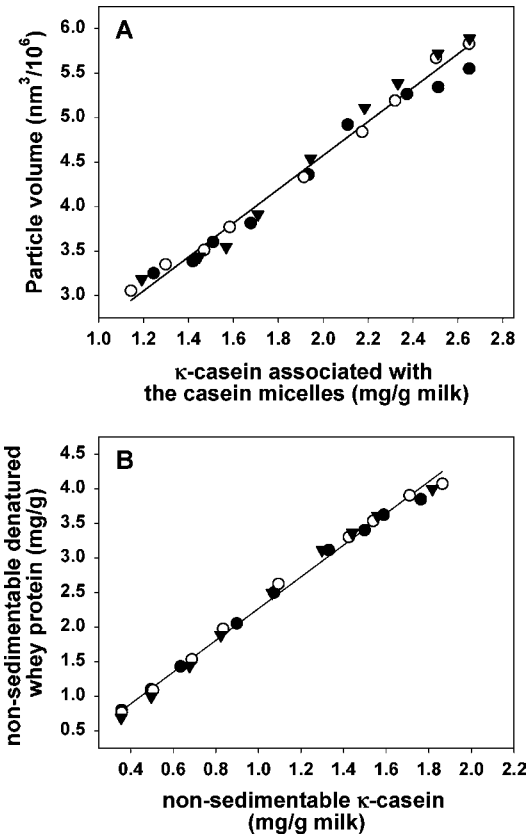

Figure 5. (**A**) Relationship between the particle volume for the casein micelles and the level of κ-casein associated with the casein micelles for the heated milk samples. ●, Milk heated at 90 °C for 20 min; ○, milk heated at 90 °C for 25 min; ▼, milk heated at 90 °C for 30 min. (**B**) Relationship between the serum phase denatured whey protein and the level of serum phase κ-casein for the heated milk samples. ●, Milk heated at 90 °C for 20 min; ○, milk heated at 90 °C for 25 min; ▼, milk heated at 90 °C for 30 min.

a direct consequence of the distribution of protein between the colloidal and serum phases, rather than an associated aggregation reaction.

It is extremely difficult to determine the direct interactions between the various protein species in heated milk, and within the colloidal and serum phases. In particular, it is difficult to conclusively determine the direct interactions between the denatured whey protein and κ-casein, and the predominant bonding involved in the interactions. It is generally accepted that the denatured whey proteins interact with κ-casein via thiol−disulfide exchange reactions forming new intermolecular disulfide bonds between the various milk protein species. However, other interactions, such as hydrophobic interactions or hydrogen bonding, are likely to be involved in forming and maintaining the aggregate structures. In a recent study, our group conclusively identified the specific disulfide bonds between κ-casein and denatured β-LG in model systems and in heated milk at the natural pH (*22*), thus confirming that intermolecular disulfide bonds were involved in the aggregation between of these proteins. A similar analysis of colloidal and serum phase materials from heated pH-adjusted milks showed that disulfide bonds between κ-casein and the denatured whey proteins β-LG existed in the protein aggregates from both phases (unpublished results). This confirmed that intermolecular disulfide bonds were formed between denatured β-LG and κ-casein regardless of whether the κ-casein was present in the serum or colloidal phase of the milk.

Literature reports have shown that κ-casein is the predominant casein dissociating from the micelles at elevated pH (above pH 6.7) and temperatures (*8*, *12*−*17*, *23*). Anema and Klostermeyer (*8*) showed that, when milk at pH above 6.7 was heated, the level of α_S-casein and β-casein dissociating from the micelles increased with temperature up to about 70−80 °C and then decreased again at higher temperatures. In a subsequent study, it was shown that the decrease in dissociation of α_S-casein and β-casein at these higher temperatures was related to the denaturation of the whey proteins and their interactions with κ-casein (*23*). It was postulated that all of the caseins dissociated from the micelles on heating. On subsequent cooling, the dissociated κ-casein stabilized the dissociated α_S-casein and β-casein as small serum phase aggregates if the heating temperature was below about 70 °C. However, above about 70 °C, κ-casein was associated with denatured whey proteins, and this interaction prevented κ-casein from stabilizing the other caseins and they reassociated with the casein micelles or formed larger aggregates on subsequent cooling (*23*).

The observation of the dissociation of κ-casein throughout the pH range from 6.5 to 7.1, but particularly at pH below 6.7, on the heating of milk (**Figure 4C**) is of great interest. The unusual heat stability of milk at high temperatures (about 120−140 °C), particularly the occurrence of the maximum and the minimum in the heat stability at about pH 6.7 and pH 6.9, respectively, has been attributed to the pH-dependent dissociation of κ-casein from the micelles (*13*−*16*). In these studies on the heat stability of milk, very little κ-casein was dissociated from the casein micelles at pH 6.7 or below, whereas, at higher pH, progressively more κ-casein dissociated as the pH was increased. In another study on the dissociation of κ-casein on heating milk at temperatures below 100 °C (*8*), there was a small increase in the level of dissociated κ-casein in milk heated at pH 6.7 and 90 °C so that about 25% was in the serum phase at pH 6.7, as compared to about 15% at pH 6.5.

Most of the earlier studies used very high centrifugal forces, above 50 000*g* and often as high as 100 000*g*, to sediment the casein micelles. In this study, and in other studies (*3*, *4*, *7*, *9*), it has been shown that a centrifugal force of 25 000*g* is sufficient to deposit the casein micelles in unheated milk. These milder centrifugation conditions may be more selective in separating serum phase aggregates from the casein micelles when compared to the markedly higher forces used in earlier studies. However, it must also be noted that no detailed studies on the dissociation behavior of casein micelles in heated milk have been conducted at pH steps between 6.5 and 6.7.

In a very recent study, Donato and Dalgleish (*30*) examined the effect of the pH on the distribution of protein components between serum and colloidal phases in heated milk. Although there was no quantitative determination of the individual casein proteins and no detailed examination at pH between 6.5 and pH 6.7, this study did show that the level of serum protein in milk heated at pH 6.7 was markedly higher than that in milk heated at pH 6.5. However, this study also showed that there was relatively small changes in serum protein concentrations at pH above 6.7, especially for the proteins involved in heat-induced disulfide aggregation (primarily β-LG and κ-casein), which seems to contrast the results of this study (**Figures 2**−**4**). In addition, the serums appeared to have higher levels of α_S-casein and β-casein than observed in the present study (**Figures 2** and **4**), which suggests that the centrifugation conditions may not have effectively deposited all of the casein micelles.

Figure 6**A** shows the ratios of whey protein to κ-casein in the serum phase protein material and the colloidal phase protein

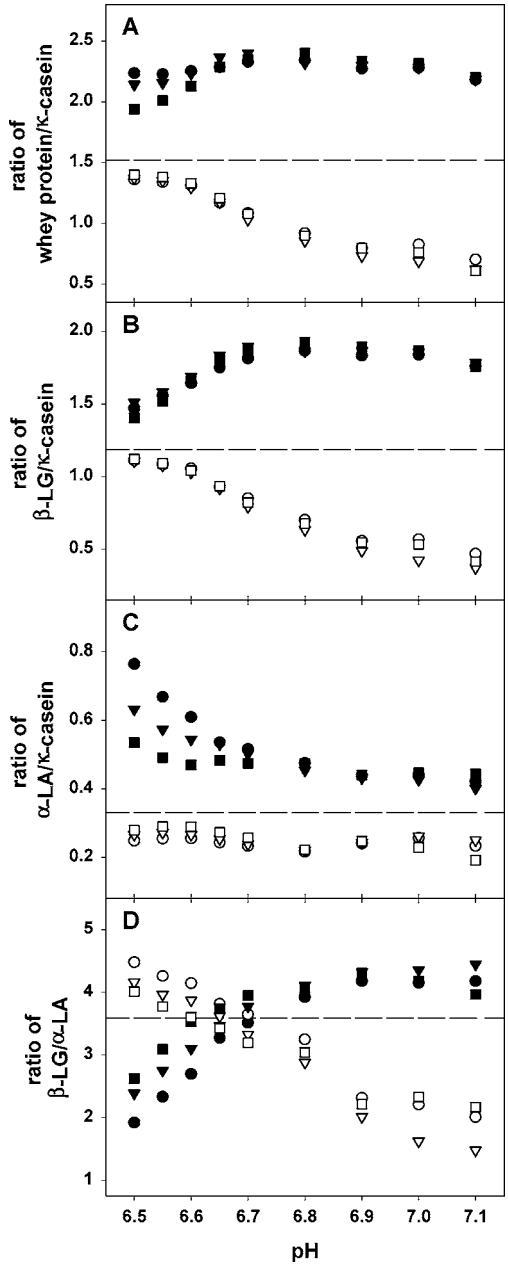

Figure 6. Effect of pH on the ratio of protein components in unheated milk (dashed line), and for the serum phase protein (filled symbols) and colloidal phase protein (open symbols) in the heated milk samples. (**A**) Ratio of whey protein (α-LA + β-LG) to κ-casein; (**B**) ratio of β-LG to κ-casein; (**C**) ratio of α-LA to κ-casein; (**D**) ratio of β-LG to α-LA. ●,○, Milk heated at 90 °C for 20 min; ▼,▽, milk heated at 90 °C for 25 min; ■,□, milk heated at 90 °C for 30 min.

material. At pH 6.5, the ratio of whey protein to κ-casein in the colloidal phase protein material was about 1.4 and was slightly lower than that observed in the original milk. This ratio was found to decrease with increasing pH from about 1.4 at pH 6.5 to about 0.61 at pH 7.1. For the serum phase protein material, the ratio of whey protein to κ-casein was higher than that observed in the colloidal phase protein material at all pH values. The ratio was found to vary only a small amount with pH, increasing from about 2.2 to about 2.4 with an increase in pH between 6.5 and 6.7, and then decreasing again at higher pH to a ratio of about 2.2. There was some effect of heating time at pH below 6.7 with a higher ratio at shorter heating times.

In the colloidal phase protein material, the ratio of β-LG to κ-casein at pH 6.5 was only slightly lower than that observed in the original milk, and this ratio progressively decreased from about 1.1 to about 0.4 as the pH was increased. In contrast, in the serum phase protein material, the ratio of β-LG to κ-casein was markedly higher than that observed in the original milk and was found to increase from about 1.4 to 1.9 as the pH at heating was increased from pH 6.5 to 6.7, and then to decrease slightly with increasing pH to about 1.7 at pH 7.1 (**Figure 6B**). The ratio of α-LA to κ-casein in the colloidal phase protein material was relatively constant at about 0.3, and slightly lower than that observed in the original milk (0.35). The ratio of α-LA to κ-casein in the serum phase protein material was relatively constant at about 0.45 at pH 6.7 or above; however, at pH below 6.7, the ratio was dependent on the heating time, with the ratio decreasing as the heating time increased (**Figure 6C**). This may be a reflection of the small differences in denaturation level for this protein at lower pH. As a consequence, the β-LG/α-LA ratio in the colloidal phase protein material progressively decreased from about 4 to about 2 as the pH was increased from 6.5 to 7.1. In contrast, the β-LG/α-LA ratio in the serum phase protein material increased from about 2.5 to 4 as the pH was increased from 6.5 to 6.7 and then remained relatively constant at about 4 as the pH was increased further (**Figure 6D**).

In a recent study by Guyomarc'h et al. (*31*), the ratio of whey proteins (β-LG + α-LA) to κ-casein in the serum phase for skim milk heated at the natural pH was found to be between 3 and 4, which is slightly higher than that observed in this study (about 2.4 at the natural pH, **Figure 6A**). Interestingly, Guyomarc'h et al. (*31*) found that the ratio of whey proteins to κ-casein in the colloidal phase fraction was about 2 to 3, which is also markedly higher than that observed in this study (about 1.1 at the natural pH, **Figure 6A**). The ratio of whey proteins to κ-casein found in the milk in our study was about 1.5 (**Figure 6A**), which is in the normal range reported for bovine milk (*29*). However, in the study by Guyomarc'h et al. (*31*), both the serum phase fraction and the colloidal phase fraction had ratios markedly higher than normal, which suggests either that the milk used had an unusual protein composition or that the analytical method employed overestimated the whey protein level or underestimated the κ-casein level. Guyomarc'h et al. (*31*) found a ratio of β-LG to α-LA of about 2−4 in both the serum phase fraction and the colloidal phase fraction for milk at the natural pH, which is in reasonable agreement with the observations in this study (about 3.5 in both fractions, **Figure 6D**).

When milk is heated, there is a dissociation of casein, particularly κ-casein, from the micelles, and, at sufficiently high temperatures, the whey proteins denature. The dissociation of the κ-casein is strongly dependent on the pH at heating and is a rapid phenomenon as compared to the denaturation reactions (*17*, *23*). Therefore, at temperatures above about 70 °C, the interaction between the denatured whey protein and κ-casein can involve either the serum phase κ-casein or the colloidal phase κ-casein. The results in **Figure 5B** suggest that the distribution of denatured whey protein between the colloidal and serum phases is determined by the dissociation of κ-casein. At pH 6.5, most of the κ-casein is in the colloidal phase (**Figure 4C**), and therefore the predominant interaction of the denatured whey proteins is with the colloidal phase κ-casein; therefore, the ratio of the whey proteins to κ-casein in the colloidal phase is similar to the ratio found in milk (**Figure 6**). As the pH of the milk is increased, progressively more κ-casein is found in the serum phase. There appears to be a preferential reaction

between the denatured whey protein and the serum phase κ-casein as the ratio of denatured whey protein to colloidal phase κ-casein decreases as more κ-casein is found in the serum. This may be a consequence of the easier access of the disulfide bonds of the serum phase κ-casein than the colloidal phase κ-casein for thiol−disulfide interchange reactions with the denatured whey proteins. However, the interactions will also be diffusion limited, and the interactions between denatured whey proteins and the serum phase κ-casein will probably be more rapid than the interactions between denatured whey proteins and the colloidal phase κ-casein.

As a consequence, when there is a segregation of κ-casein between the colloidal and serum phases, the ratio of whey protein to κ-casein is higher in the serum phase than in the colloidal phase. However, when the level of κ-casein in the serum phase becomes very high, the ratio of whey protein to serum phase κ-casein will decrease as there is insufficient whey protein in the milk to maintain the high ratio. This may be the cause of the small decrease in the ratios of whey protein to κ-casein at pH above 6.7 (**Figure 6**), as higher levels of κ-casein are dissociated from the micelles at these pH values. Ultimately, when (or if) all of the κ-casein is in the serum phase, all of the whey protein will also be in the serum phase, and the ratio of denatured whey protein to κ-casein will be the same as that observed in the original milk.

In conclusion, this study has confirmed that there is a pH-dependent segregation of denatured whey protein between the serum and colloidal phases, with high levels of whey proteins associating with the casein micelles at pH 6.5, and the level decreasing with increasing pH so that most of the denatured whey protein is found in the serum at pH 7.1. A pH-dependent dissociation of κ-casein was also observed, and, interestingly, significant dissociation of κ-casein occurred at pH below 6.7, which has not previously been reported. The strong relationship between the serum phase denatured whey proteins and the serum phase κ-casein indicates that the distribution of the denatured whey protein between the colloidal and serum phases appears to be controlled by the distribution of κ-casein. The reason why a pH-dependent dissociation of κ-casein occurs over such a narrow pH range, and particularly at below pH 6.7, is unknown and warrants further investigation.

ACKNOWLEDGMENT

We would like to thank Mike Boland and Lawrie Creamer for useful discussions, and Claire Woodhall for proofreading this manuscript.

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Received for review September 24, 2006. Revised manuscript received January 15, 2007. Accepted February 21, 2007. This work was supported by the New Zealand Foundation for Research, Science, and Technology, contract number DRIX0201.

JF062734M