Protein Composition of Different Sized Casein Micelles in Milk after the Binding of Lactoferrin or Lysozyme

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ABSTRACT: Casein micelles with bound lactoferrin or lysozyme were fractionated into sizes ranging in radius from ∼50 to 100 nm. The κ-casein content decreased markedly and the α_S-casein/β-casein content increased slightly as micelle size increased. For lactoferrin, higher levels were bound to smaller micelles. The lactoferrin/κ-casein ratio was constant for all micelle sizes, whereas the lactoferrin/α_S-casein and lactoferrin/β-casein ratio decreased with increasing micelle size. This indicates that the lactoferrin was binding to the surface of the casein micelles. For lysozyme, higher levels bound to larger casein micelles. The lysozyme/α_S-casein and lysozyme/β-casein ratios were nearly constant, whereas the lysozyme/κ-casein ratio increased with increasing micelle size, indicating that lysozyme bound to α_S-casein and β-casein in the micelle core. Lactoferrin is a large protein that cannot enter the casein protein mesh; therefore, it binds to the micelle surface. The smaller lysozyme can enter the protein mesh and therefore binds to the more charged α_S-casein and β-casein.

KEYWORDS: *milk, lactoferrin, lysozyme, casein micelles, size, binding, interactions*

INTRODUCTION

Bovine milk naturally contains low levels of lactoferrin (0.02 to 0.35 mg/mL)[1] and very low levels of lysozyme (0.05−0.22 μg/mL).[2] Recently, it has been shown that the lactoferrin naturally found in bovine milk is partitioned between the casein micelles and the serum phase.[1] When high levels of lactoferrin or lysozyme are added to milk, they have been found to bind to the casein micelles.[3] However, the binding of lactoferrin or lysozyme have different effects on the micelles. The binding of lactoferrin caused the casein micelles to initially swell and then dissociate on prolonged holding, with the effect being greater at higher temperatures.[3] This dissociation resulted in the milk becoming progressively more transparent with time after adding the lactoferrin. In contrast, low levels of lysozyme (up to about 0.5%) had no effect on the casein micelle size, whereas higher levels caused the complete destabilization and flocculation of the casein micelles.[3] In addition, the maximum binding of lactoferrin to the casein micelles in skim milk was about 0.15 mM, whereas for lysozyme it was about 3 times higher at more than 0.4 mM.

The reason for the different effects between lactoferrin (M ≈ 78 kDa) and the much smaller lysozyme (M ≈ 14 kDa) when bound to the casein micelles in milk has not been resolved. One hypothesis is that the two basic proteins may be binding to different casein molecules within the casein micelles, with the larger lactoferrin molecules binding preferentially to the κ-casein at the micelle surface, whereas the smaller lysozyme molecules may be able to penetrate the casein micelles more effectively and therefore bind to all of the casein molecules within the micelles.

As κ-casein is preferentially located on the surface of the casein micelles, smaller casein micelles have higher κ-casein content than larger micelles in size fractionated samples.[4−6] Therefore, if lactoferrin or lysozyme binds preferentially to the surface of the casein micelles, a higher level of lactoferrin or lysozyme would be expected to bind to smaller casein micelles than to larger casein micelles. However, if the lactoferrin or lysozyme is bound throughout the casein micelles then similar levels of lactoferrin or lysozyme would be expected to be bound to all size classes of casein micelles.

The current study attempts to elucidate whether lactoferrin or lysozyme is binding preferentially to the surface of the casein micelles or uniformly throughout the casein micelles. Lactoferrin or lysozyme is first bound to the casein micelles, and then the casein micelles are fractionated into different size classes. By examining the composition and in particular the ratio of lactoferrin or lysozyme to the individual caseins in different sized casein micelles, it may be possible to determine whether lactoferrin or lysozyme binds preferentially to individual caseins (or the casein micelle surface).

MATERIALS AND METHODS

Milk Supply. Fresh skim milk was sourced from a local supply. Sodium azide was added (0.02%w/v) as a preservative, and the milk was held at 25 °C. A stock solution of lactoferrin at a concentration of about 200 mg/mL and a pH of 6.67 was prepared, and combinations of milk (41 parts) and water/lactoferrin (9 parts) were added together to give lactoferrin levels of about 0, 1, and 2% in milk. Similarly, a stock solution of lysozyme at a concentration of about 30 mg/mL and pH 6.67 was prepared, and combinations of milk (41 parts) and water/lysozyme (9 parts) were added together to give lysozyme levels of about 0, 0.25, and 0.5% in milk. This procedure diluted the milk to 82% of its initial concentration. The experimental milk samples were at ∼pH 6.67.

The composition of the individual caseins (α_S-casein, β-casein, and κ-casein) in the original skim milk was determined against standard

curves of pure casein mixtures of known composition using the microfluidic chip sodium dodecyl sulfate–polyacrylamide gel electrophoresis (MF-PAGE) method, as has been described previously.[7] The individual casein concentrations as mg/mL of milk was determined, and using reported molecular weights of the caseins,[8] their molar concentrations in the milk were calculated. As fresh milk samples were used, the concentrations varied between individual milk samples, but the average molar concentrations in the fresh skim milk samples were 0.52 ± 0.01 mM, 0.58 ± 0.02 mM, 0.18 ± 0.02 mM, and 1.18 ± 0.01 mM for the α_S-casein, β-casein, κ-casein, and total casein, respectively. The molar masses used were 23.5 kDa, 24 kDa, 19 kDa, and 23 kDa for the α_S-casein, β-casein, κ-casein, and total casein respectively.

The concentrations of lactoferrin and lysozyme in the stock solutions were determined by UV absorbance at 280 nm using known extinction coefficients.[8,9] When lactoferrin or lysozyme were added to milk, the milk samples were diluted to 82% of its original concentration, and therefore, the final casein concentrations in the experimental milk samples were 0.42, 0.40, 0.15, and 0.97 mM for the α_S-casein, β-casein, κ-casein, and total casein, respectively. In milk samples with lactoferrin added, the molar concentration lactoferrin in the experimental milk samples was either 0.105 or 0.21 mM. For milk samples with lysozyme added, the molar concentration of lysozyme was either 0.145 or 0.29 mM.

Centrifugation of Milk Samples to Determine the Binding of Lactoferrin to Casein Micelles. In preliminary studies where the binding of lactoferrin and subsequent dissociation of the casein micelles was monitored, the casein micelles were separated from the milk serum by centrifugation ($\sim$27,000g, 25 °C, 1 h in a bench centrifuge). The level of protein in the original milk samples and supernatants was determined by MF-PAGE.

Differential Centrifugation of Milk Samples. Milk samples (1 mL) were centrifuged at $\sim$20,000g and at 25 °C in an Eppendorf 5417R benchtop centrifuge (Eppendorf AG, Hamburg, Germany). The milk samples were centrifuged for a set time and then the supernatant removed for the next centrifugation run for a longer time. The times used were 2, 5, 10, 15, 30, and 60 min.

MF-PAGE. An Agilent 2100 Bioanalyzer system and the associated Protein 80 kit (Agilent Technologies, Waldbronn, Germany) were used for the electrophoresis of milk samples, as has been described in detail previously.[7]

Particle Size, Scattering Intensity, and Zeta Potential Measurements. Particle sizing using dynamic light scattering and zeta potentials using laser Doppler electrophoresis were performed using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, Worcestershire, U.K.) as has been described previously.[10,11] For the zeta-potential experiments, the milk samples were diluted in a calcium imidazole buffer (20 mM imidazole, 5 mM CaCl$_2$, and 30 mM NaCl, pH 7.0) before injecting into the zeta potential cell. The zeta potential measurements were performed at 20 °C and with an applied voltage of 50 V. For the sizing and scattering intensity measurements, the casein pellets obtained by centrifugation were redispersed in exactly 1 mL of calcium imidazole buffer to bring the volume back to that of the original milk. Milk samples or redispersed casein micelles were accurately diluted with calcium imidazole buffer so that reliable scattering intensities could be determined. The sizing experiments were performed at 20 °C. The sizing experiment provided the z-average radius of the particles and the back scattering intensity of the solution, whereas the zeta potentials were derived from the electrophoretic mobilities determined experimentally.

■ RESULTS AND DISCUSSION

Preliminary experiments showed that it was possible to fractionate reconstituted skim milk into different size classes; however, the variation in κ-casein content between the different sizes was very small (results not shown). This may be due to the heat, evaporation, drying, and reconstitution steps inducing some aggregation of the casein particles, thus changing the relationship between surface area and κ-casein content. When fresh skim milk was centrifuged, casein micelles could be

fractionated into different size classes, and these fractionated casein micelles had κ-casein contents inversely related to the radius of the casein micelles, as has been reported previously.[4−6] Interestingly, it was reported that in milk samples of single cows with varying casein micelle size, there was no correlation between κ-casein content and average casein micelle size.[12]

All further experiments used fresh skim milk. However, as a solution of lactoferrin or lysozyme needed to be added to the milk samples, the milk samples were diluted by these additions. By using concentrated lactoferrin and lysozyme solutions, dilution of all milk samples was controlled to less than 20% in all samples (including controls where water was added). Analysis of particle size and casein contents of supernatants after centrifugation revealed that the colloidal structure was unaffected by this dilution as the particles size and supernatant casein content was indistinguishable between undiluted and diluted milk samples (results not shown).

Fresh skim milk was mixed with lactoferrin/water combinations to give milk samples with lactoferrin levels of about 0, 1, and 2% in the milk samples. As lactoferrin initially binds to the casein micelles, and then the casein micelles subsequently disintegrate,[3,13] preliminary experiments were conducted to determine the optimum reaction time where maximum binding of lactoferrin and minimum disintegration of the casein micelles occurred. Lactoferrin levels of 1 and 2% level were added to the milk samples, and the change in micelle radius, scattering intensity, binding of lactoferrin to the casein micelles, and dissociation of the casein micelles was monitored with time after addition (Figure 1).

The apparent casein micelle radius increased after the addition of lactoferrin, and this reached a maximum after about 2 to 3 h. For the milk samples with 1% added lactoferrin, the radius did not change further on holding for about 2 to 7 h, but for the milk samples with 2% added lactoferrin, the radius decreased with holding time beyond 3 h (Figure 1A). The scattering intensity increased initially on adding lactoferrin and then decreased progressively with holding time (Figure 1B). The binding of lactoferrin with the casein micelles and the dissociation of casein from the micelles are shown in Figure 1C. The time shown is when centrifugation was started, and as this step took 1 h, the actual elapsed time is therefore 1 h longer. It was evident that maximum association of lactoferrin with the micelles occurred after 1 h and that by 2 h the casein micelles had started to dissociate, with dissociation increasing with longer holding times (Figure 1C).
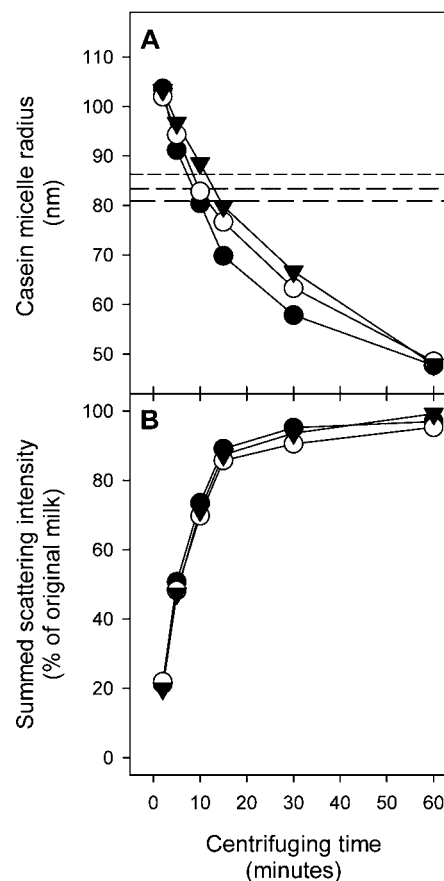
These effects were similar to what we observed previously, although maximum association occurred faster, and dissociation occurred sooner than in our previous experiments.[3,13] This may be because we used slightly higher temperatures (25 °C in this study compared with 20 °C with most experiments in our previous study), and higher temperatures are known to accelerate the dissociation phenomenon.[3] Alternatively, the dilution of milk may reduce ionic strength and promote electrostatic repulsion and the subsequent dissociation reactions. The data in Figure 1 indicate an increased size, while scattering intensity decreases steadily from the start. This is consistent with a swelling of the micelles, which results (at finite scattering angles) in a decreased scattering as the form factor falls off at a smaller angle.

On the basis of the results in Figure 1, it was decided to start fractionation of the casein micelles 1 h after lactoferrin addition as this would encompass the maximum association, while
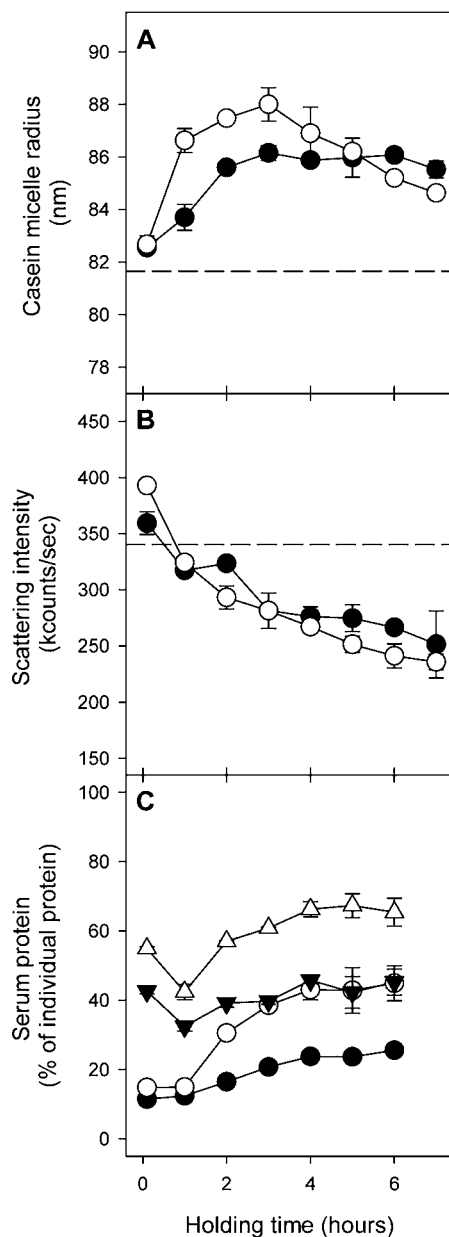
Figure 1. Change in (A) casein micelle size, (B) scattering intensity, and (C) serum casein (●,○) and lactoferrin (▼,△) levels with time after adding lactoferrin. Filled symbols, milk with 1% added lactoferrin; open symbol, milk with 2% added lactoferrin. Dashed lines represent the size and scattering intensities of milk without added lactoferrin.

Figure 2. (A) Casein micelle size of different fractions and (B) summed scattering intensities of fractions as centrifuging time was increased. Dashed lines represent the radius of micelles in the original milk samples. Long dashed line and ●, milk without added lactoferrin; medium dashed line and ○, milk with 1% added lactoferrin; short dashed line and ▼, milk with 2% added lactoferrin.

keeping casein micelle dissociation to a minimum. Milk samples with 0, 1, and 2% lactoferrin added were allowed to interact for about 1 h, and then several tubes with 1 mL aliquots were subjected to differential centrifugation. One aliquot of pelleted micelles from each centrifugation step was mixed with 1 mL of calcium imidazole buffer, and the casein micelles were redispersed for size and scattering intensity measurements (Figure 2). It is recognized that there may be a small bias as the lactoferrin in the milk samples with the shortest centrifuging time has a shorter contact time with the casein micelles than the milk samples with the longest centrifuging time. However, based on the results in Figure 1 this effect was considered to be small. In addition, over 80% of the casein micelles were deposited in the first four centrifuging steps, which correspond to a total of only 32 min centrifuging time (Figure 2B).

The casein micelles in the original milk and in each redispersed fraction were accurately diluted for particle sizing measurements. The casein micelles pelleted at the shortest centrifugation times were the largest with radii of about 100 nm, and the size progressively decreased after each centrifuging step so that those collected after the longest centrifuging run were about 50 nm in radius (Figure 2A). The same general trends were observed for the milk samples with 0, 1, or 2% added lactoferrin, although at each centrifugation step, the micelles were generally larger as more lactoferrin was added. This may be a consequence of the swelling of the micelles when the lactoferrin was bound.

The summed scattering intensity of each fraction of casein micelles was determined as a percentage of the scattering intensity of the original milk (Figure 2B). Each micelle fraction was dispersed in a volume of buffer equivalent to that of the original milk, and then each fraction was diluted the same as the original milk for sizing. The scattering intensity of samples at each progressive centrifuging time was summed, and this summed scattering intensity should be similar to that of the original milk if all casein micelle units are accounted for in each centrifuged fraction and if the micelles are well dispersed. The summed scattering intensities do sum up close to 100% (Figure 2B), which indicates that, within error, most of the original casein micelles are accounted for in the combined fractions. As casein micelles ranging in radius from 50 to 100 nm were

separated, this provides casein micelles with surface areas increasing by a factor of about 4.

Separate aliquots of casein micelle pellets were recentrifuged to squeeze out the excess serum phase, and then the remaining pellets were analyzed by MF-PAGE to determine the protein composition of the different sized casein micelles. The scattering intensities obtained from the sizing measurements were used to estimate the dilutions required for the electrophoresis. Using the dilution factors and the intensities of the casein bands, it was possible to estimate the proportion of the caseins accounted for in the fractions compared with the unfractionated milk samples. When the individual caseins were summed across all fractions, it was found that virtually all of the α_S-casein and β-casein and approximately 90% of the κ-casein was accounted for (Figure 3). This indicates that almost all of the casein micelles were accounted for in the fractions and that little if any casein remained in the final supernatant.

From the dilution factors and by comparing the intensities of each individual casein against that in the original milk (for which the concentrations had been determined), it was possible to calculate the concentration (in mg/mL) of the individual caseins in each redispersed pellet. From this, the total mass of casein and therefore the proportion on a mass basis of each individual casein could be calculated. As the casein micelle radius (R) increased from about 50 to 100 nm, the proportion of α_S-casein and β-casein in the casein micelles increased, whereas the proportion of κ-casein decreased (Figure 4). This is similar to that observed by Donnelly et al.[6] but markedly contrasts with the results of Dalgleish et al.[5] who reported constant α_S-casein levels across all casein micelle sizes, whereas the level of κ-casein and β-casein was inversely correlated so that large micelles had low levels of κ-casein and high levels of β-casein, whereas the opposite was observed for small micelles.

κ-Casein is found predominantly on the surface of the casein micelles; therefore, κ-casein content would scale with R^2 and total casein with R^3, and therefore, the relative κ-casein over total casein as R^{-1}. On the basis of this, increasing the casein micelle radius by a factor of 2 would decrease κ-casein content by a factor of 2, as is observed (Figure 4C). If α_S-casein and β-casein were predominantly in the interior of the casein micelles, then the proportions of α_S-casein and β-casein would be expected to scale with, R^3 and therefore, the relative proportion should be constant with micelle size. However, the proportions of α_S-casein and β-casein increase with increasing micelle size. The increase in α_S-casein and β-casein may be explained as follows: The total α_S-casein and β-casein will be proportional to $(c_1 \cdot R^3)$, whereas the total κ-casein will be proportional to $(c_2 \cdot R^2)$, where c_1 and c_2 are constants. Therefore, the total casein will be proportional to $(c_1 \cdot R^3 + c_2 \cdot R^2)$. For casein micelles with a radius of 75 nm, the ratio of κ-casein to total casein was found to be ~0.15 (Figure 4C). Therefore, $c_2 \cdot R^2 / (c_1 \cdot R^3 + c_2 \cdot R^2) = 0.15$, which gives $c_2 = c_1 \cdot (0.176 \times 75) = c_1 13.2$.

On the basis of these calculations, the proportion of α_S-casein and β-casein would scale as follows:

$$c_1 \cdot R^3 / (c_1 \cdot R^3 + c_2 \cdot R^2)$$

$$= c_1 \cdot R^3 / (c_1 \cdot R^3 + c_1 13.2 R^2)$$

$$= R^3 / (R^3 + 13.2 E^2) = 1 / (1 + 13.2 / R)$$

Therefore, for casein micelles of $R = 75$ nm, the proportion of α_S-casein and β-casein combined would be 0.85, which is in agreement with the experimental results (Figure 4A and B). If
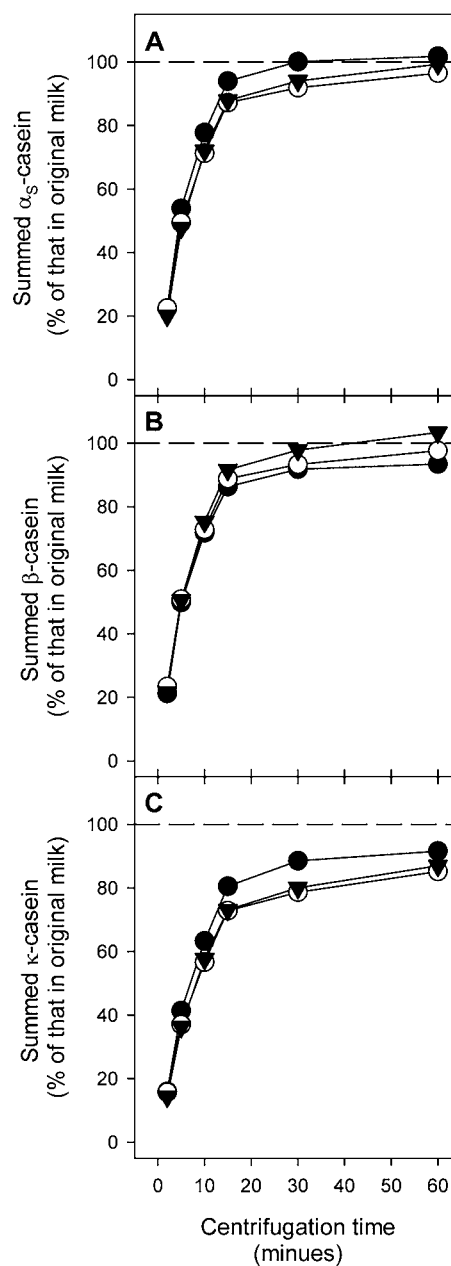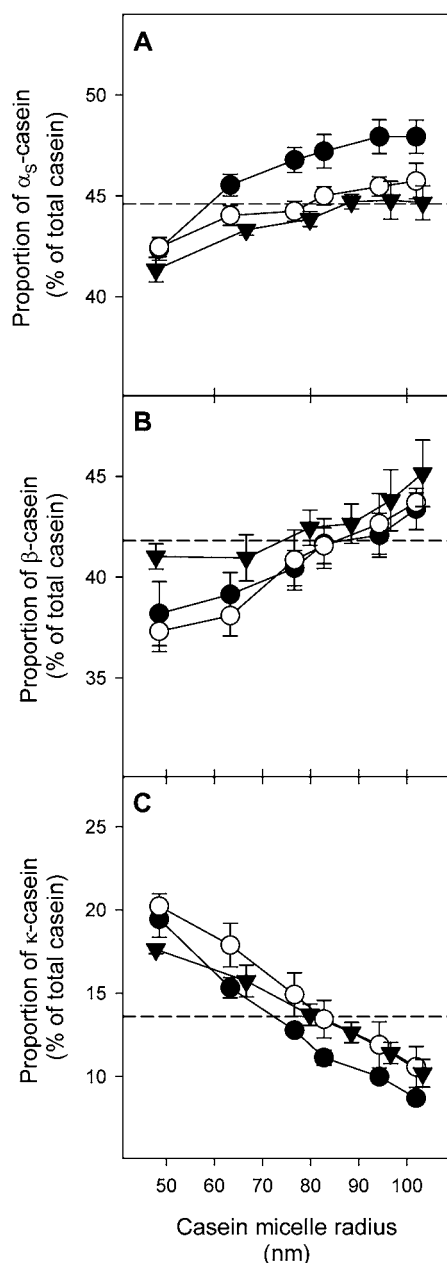

Figure 3. Summed concentrations of (A) α_s-casein, (B) β-casein, and (C) κ-casein of fractions as centrifuging time was increased. The concentrations are in mg/mL and are reported as a percentage of each protein in the original milk. ●, milk without added lactoferrin; ○, milk with 1% added lactoferrin; ▼, milk with 2% added lactoferrin.

the radius is increased from 50 to 100 nm, the proportion of α_S-casein and β-casein combined is calculated to increase from 0.79 to 0.88 (a total increase of about 10%), which appears to be in agreement with the experimental results (Figure 4A and B).

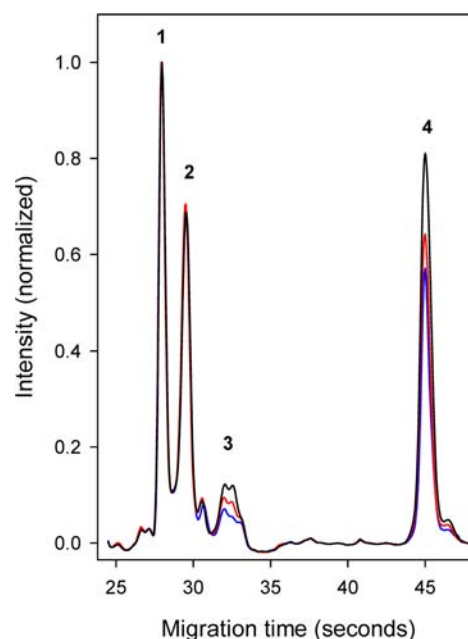Using the same reasoning, the proportion of κ-casein to total casein is:

$$c_2 \cdot R^2 / (c_1 \cdot R^3 + c_2 \cdot R^2)$$

$$= 13.2 c_1 \cdot R^2 / (c_1 \cdot R^3 + 13.2 c_1 \cdot R^2)$$
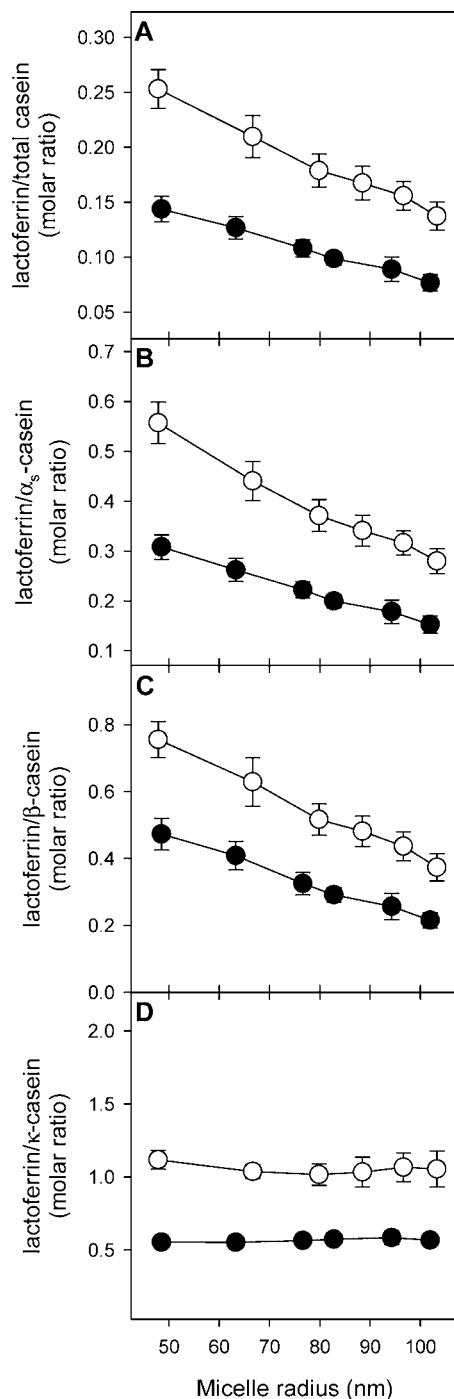
$$= 13.2 / (R + 13.2)$$

Figure 5. Electrophoretic traces of differently sized casein micelles from milk with 2% added lactoferrin. The casein micelles were obtained from milk centrifuged for 2 min (blue trace), 10 min (red trace), or 30 min (black trace). The peaks identified are (1) β-casein; (2) α_s-casein; (3) κ-casein; and (4) lactoferrin.

Figure 4. Proportion of (A) α_s-casein, (B) β-casein, and (C) κ-casein in differently sized casein micelles. The casein micelles were obtained from ●, milk without added lactoferrin; ○, milk with 1% added lactoferrin; and ▼, milk with 2% added lactoferrin. Dashed lines represent the proportion of the protein in the original milk samples.

The proportion of κ-casein is ~0.15 for casein micelles with a radius of 75 nm (Figure 4C), and if the radius is decreased to 50 nm or increased to 100 nm, the proportion of κ-casein is calculated to increase to 0.21 or decrease to 0.12, respectively, which agrees closely with that observed for the control milk (Figure 4C). The observation that the proportion of α_s-casein seems to level off a bit while β-casein seems to increase a bit at larger micelle sizes would be consistent with the proposal that about 10% of the surface hairs is β-casein.[14]

Selected electropherograms of the different sized casein micelles from the milk samples with 2% added lactoferrin are shown in Figure 5. In all casein micelle samples, it was possible to dilute the milk samples so that the α_s-casein and β-casein peaks were of nearly equivalent intensity; however, to ease comparisons, the electropherograms were normalized to the β-casein peak. As the casein micelle size decreased, the proportion of κ-casein increased, and concomitantly, the proportion of lactoferrin also increased. These results indicate that higher levels of lactoferrin bound to smaller casein micelles (Figure 5). It should be noted that the peak intensities are not proportional to molar concentrations as each protein produced different intensities at a given concentration; however, it was possible to convert these to molar concentrations based on the known protein concentrations in the original milk samples.

The molar ratio of lactoferrin to total casein and to each of the individual caseins was determined (Figure 6). The lactoferrin to total casein ratio decreased markedly as the micelle size increased. In fact, the ratio decreased to approximately half as the micelle radius increased from 50 to 100 nm at both 1% and 2% addition levels of lactoferrin (Figure 6A). Therefore, significantly less lactoferrin bound to large micelles than small micelles regardless of the level added to the milk. Similar decreases in lactoferrin to α_s-casein (Figure 6B) and lactoferrin to β-casein (Figure 6C) ratios with increasing casein micelle size was observed. However, the lactoferrin to κ-casein ratio was essentially constant at all micelle sizes (Figure 6D). This suggests that lactoferrin is preferentially binding to the κ-casein at the surface of the casein micelles. Further indications that lactoferrin binds to the casein micelle surface come from zeta potential measurements. The addition of lactoferrin to skim milk caused a progressive decrease in zeta potential and an increase in the casein micelle radius due to swelling (Table 1).[3] The decrease in surface charge was correlated with the level of lactoferrin bound to the casein micelles, as was the increase in casein micelle size.

When 2% of lactoferrin was added to milk, the saturation binding of about 1.1% lactoferrin to the casein micelles was achieved, which is similar to that obtained in our previous study.[3] On the basis of this maximum binding, the lactoferrin to

Figure 6. Molar ratios of lactoferrin to (A) total casein; (B) α_s-casein, (C) β-casein; and (D) κ-casein in differently sized casein micelles. The casein micelles were obtained from ●, milk with 1% added lactoferrin; and ○, milk with 2% added lactoferrin.

κ-casein molar ratio is very close to unity. Lactoferrin may form oligomers (dimers or tetramers) at the calcium level found in milk,[15,16] so the molecular binding ratio of lactoferrin oligomers to κ-casein may be lower than unity.

Similar experiments were conducted with lysozyme added to skim milk. As lysozyme caused the casein micelles in the milk to aggregate at levels above 0.5% (Table 1),[3,17] addition levels of 0.25 and 0.5% (w/w) were added to the milk. The casein micelles do not disintegrate when lysozyme is added;[3] therefore, the lysozyme/milk mixture was allowed to react for

**Table 1. Zeta Potentials and Size of Casein Micelles after Adding Different Levels of Lactoferrin or Lysozyme to Milk[a]**

	Milk + Lactoferrin	
lactoferrin added (%)	zeta potential (mV)	size (radius in nm)
0.0	−13.2 (0.41) a	80.9 (0.9) a
0.5	−8.57 (0.95) b	83.8 (0.4) b
1.0	−6.60 (0.61) c	85.6 (0.3) c
1.5	−5.06 (0.52) d	86.7 (0.5) d
2.0	−3.73 (0.53) e	87.5 (0.6) d
	Milk + Lysozyme	
lysozyme added (%)	zeta potential (mV)	size (radius in nm)
0.000	−13.50 (0.44) a	80.5 (0.8) a[a]
0.125	−14.50 (0.41) a	80.1 (0.7) a
0.250	−14.70 (0.45) a	79.3 (0.7) a
0.375	−13.90 (0.30) a	79.4 (0.9) a
0.500	−14.50 (0.42) a	79.8 (0.5) a
0.625	−14.20 (0.61) a	392 (34) e

[a]Standard deviations are given in parentheses. Values with the same letter are not significantly different. Measurements were made after 2 h for lactoferrin and after 6 h for lysozyme.

about 6 h before fractionating the casein micelles by centrifugation.

The same general effects of centrifugation time on micelle size and composition were observed for lysozyme as were observed for lactoferrin (results not shown). Casein micelle sizes ranging in radius from about 50 nm to 100 nm were obtained, and α_s-casein and β-casein content increased (by about 10%), and κ-casein content decreased (by about 50%) as the micelle size increased.

Representative electropherograms of the different sized casein micelles from the milk samples with 0.5% added lysozyme are shown in Figure 7, and the molar ratio of
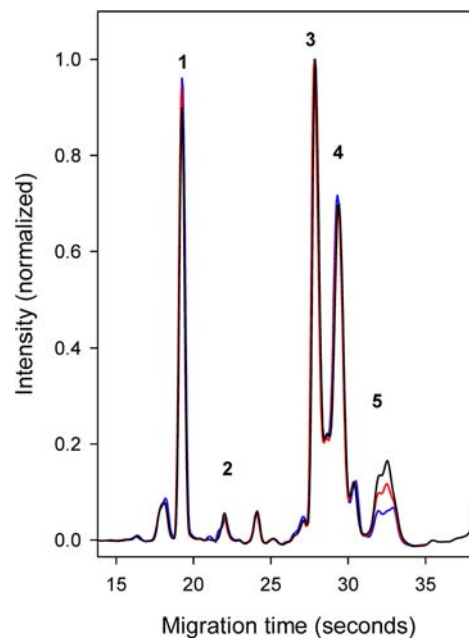
Figure 7. Electrophoretic traces of differently sized casein micelles from milk with 0.5% added lysozyme. The casein micelles were obtained from milk centrifuged for 2 min (blue trace), 10 min (red trace), or 15 min (black trace). The peaks identified are (1) lysozyme; (2) β-lactoglobulin; (3) β-casein; (4) α_s-casein; and (5) κ-casein.
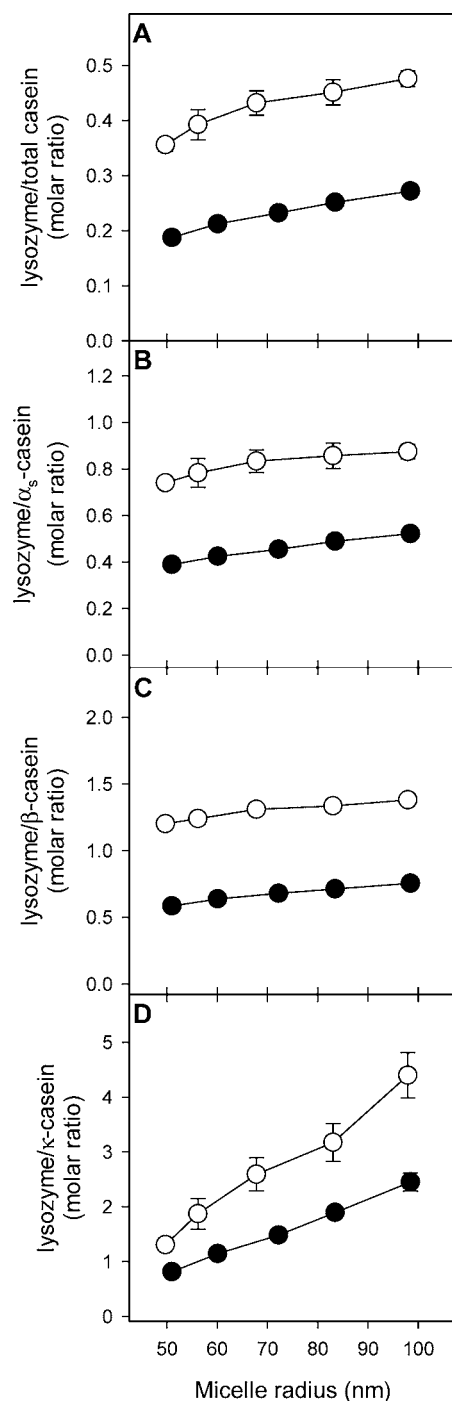
Figure 8. Molar ratios of lysozyme to (A) total casein; (B) α_s-casein; (C) β-casein; and (D) κ-casein in differently sized casein micelles. The casein micelles were obtained from ●, milk with 0.25% added lysozyme; and ○, milk with 0.5% added lysozyme.

lysozyme to the casein components is given in Figure 8. As for the experiments with lactoferrin, when the casein micelle size decreased, the proportion of κ-casein increased. However, unlike the lactoferrin experiments where more bound to smaller casein micelles, for lysozyme more were bound to the larger casein micelles (Figures 7 and 8A). The lysozyme to α_S-casein (Figure 8B) and lysozyme to β-casein (Figure 8C) ratios were nearly constant, with only a very small increase at larger casein micelle sizes. In contrast, the lysozyme to κ-casein ratio increased very markedly as the micelle size increased for both

addition levels (Figure 8D). The same general trends were observed for both 0.25% and 0.5% lysozyme addition levels, although the lysozyme/casein ratios were higher for the samples with the higher lysozyme addition level.

These results suggest that the lysozyme is binding preferentially to α_S-casein and β-casein, and therefore is binding in the interior of the casein micelles rather than with κ-casein at the casein micelle surface. When lysozyme was added to skim milk, no change in zeta potential was observed (Table 1), which further supports the suggestion that lysozyme is binding to the α_S-casein and β-casein at the interior of the casein micelles. In a study on the addition of lysozyme to individual caseins, Roos et al.[18] reported that the lysozyme bound to α_S-casein and β-casein but not to κ-casein, which supports the findings of our study.

Therefore, it seems that lactoferrin preferentially binds to the surface of the casein micelles, and lysozyme preferentially binds to the interior of the casein micelles. The differences in binding induce different effects in the casein micelles. Lactoferrin initially caused the micelles to swell and then to disintegrate (Figure 1 and Table 1), whereas lysozyme, when added at sufficiently high levels, caused the micelles to flocculate (Table 1).

It is tempting, at first sight, to suppose that lactoferrin binds directly to the κ-casein at the surface of the casein micelles. However, there are reasons to suggest another mechanism. When lactoferrin is bound to pure κ-casein at pH 6.5, the ratio κ-casein/lactoferrin is about 4.[19] This is consistent with neutralizing the surface charges of lactoferrin. As the mass distribution within a casein micelle is homogeneous,[20] it is possible to calculate the average mesh size of micelles from $\xi = (3/\nu \cdot L)^{0.5}$, where ν is the number density of caseins, and L is the contour length, which is 20 nm. From the data in De Kruif et al.[20] the mesh size (ξ) is calculated to be 4.5 nm.

There is a remarkable difference in the values of the zeta potential (calculated from electrophoretic mobility) of the casein micelles depending on whether the lysozyme of lactoferrin is added to the milk. For lysozyme, the zeta potential does not change (Table 1). Since lysozyme is a very small protein with a radius of about 2.1 nm, it may diffuse into the casein micelle effectively replacing counterions inside the micelle. As a result, the electrophoretic mobility does not change as this is determined mainly by the surface charges.

However, when lactoferrin is added to milk the zeta potential of the casein micelles decreased (Table 1). Lactoferrin consists of two lobes of about 2.5 nm diameter and about 5 nm apart. The radius of gyration is 3.2 nm, which would correspond to an equivalent sphere diameter of 8.3 nm, which is larger than the calculated mesh size. In addition, it has been reported that lactoferrin may form oligomers in the presence of calcium,[16] which will make it even less likely that lactoferrin can permeate into the casein micelles. From these calculations, it is expected that the surface of the casein micelle is composed of a layer permeable to small molecules/proteins such as monomeric β-casein or β-lactoglobulin but not larger molecules/proteins such as lactoferrin.

On the basis of these observations, it appears that the lactoferrin molecules initially adsorb at the "roots" of the κ-casein at the casein micelle surface, and this would scale with surface area as well. Over time, the lactoferrin becomes covered with the strong binding phosphoserine groups of α_s-casein and β-casein, and that requires a (slow) detachment of these proteins from the interior of the casein micelle, causing the

observed disruption of the casein micelle structure (Figure 1).[3,13] It has been shown that pure β-casein and α$_S$-casein bind to lactoferrin; it was also shown that β-casein binds very little lactoferrin at the ionic strength of milk,[19] although the binding of α$_S$-casein or κ-casein with lactoferrin at this ionic strength has not been investigated.

The dissociation and reorganization of the casein micelle after binding lactoferrin disrupts the casein micelle structure. Much of the integrity of casein micelles derives from a cooperative interaction between the caseins. These interactions encompass ion bridges, in particular calcium mediated ones (as sequestration of calcium leads to dissociation of the micelles), hydrophobic/van der Waals and other noncovalent interactions (as adding chaotropes or warm ethanol can dissociate the micelles). Electrostatic interactions are also involved but are probably less important due to the high ionic strength of milk. Adding moderate levels of salt to milk hardly changes the casein micelles.

It is also known that lactoferrin does bind some calcium in a nonspecific manner (unlike iron binding). The highest addition level of lactoferrin to the milk was 2%, and this equates to only about 0.25 mM; therefore, the sequestration of calcium by the added lactoferrin will be negligible relative to the calcium present in milk (about 30 mM). In recent papers, it was suggested that casein micelles have internal hydrated channels;[21,22] however, to be consistent with the calculated mesh size and the surface adsorption of lactoferrin, these channels must be narrow (a few nanometers across).

Addition of lysozyme to milk at a certain critical level causes the milk to flocculate. The effect is quite sudden as below the critical level, no change in size is observed, and above this level, complete and irreversible flocculation is observed.[3] The effect is quite similar to that observed on the acidification of milk where little change is observed when the pH is lowered to a certain critical pH, whereas below this pH, flocculation of the casein micelles occurs.[23,24] This pH-induced flocculation is the result of the collapse of the stabilizing κ-casein hairs on the surface of the casein micelles thus destabilizing the casein micelles. We think that the lysozyme adsorbs preferentially internally (Figure 8), which is supported by the observation that the zeta potential is unchanged (Table 1). However, after saturation internally, it may bind to the κ-casein at the surface, causing the brush to collapse.

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Notes

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