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Effects of Adding Low Levels of a Disulfide Reducing Agent on the Disulfide Interactions of β-Lactoglobulin and κ-Casein in Skim Milk

Nguyen H. A. Nguyen,[†] Marie Wong,[†] Skelte G. Anema,*[,‡] Palatasa Havea,[‡] and Fanny Guyomarc'h[§,∥]

[†]Institute of Food, Nutrition and Human Health, Massey University, Albany, Private Bag 102 904, North Shore Mail Centre, Auckland, New Zealand

[‡]Fonterra Research Centre, Private Bag 11029, Palmerston North, New Zealand

[§]INRA, and [∥]Agrocampus Ouest, UMR 1253, Science et Technologie du Lait et de l'Œuf, 65 rue de St Brieuc, F-35 042 Rennes Cedex, France

ABSTRACT: Low concentrations of a disulfide reducing agent were added to unheated and heated (80 °C for 30 min) skim milk, with and without added whey protein. The reduction of the β-lactoglobulin and κ-casein disulfide bonds was monitored over time using electrophoresis. The distribution of the proteins between the colloidal and serum phases was also investigated. κ-Casein disulfide bonds were reduced in preference to those of β-lactoglobulin in both unheated and heated skim milk (with or without added whey protein). In addition, in heated skim milk, while the serum κ-casein was reduced more readily than the colloidal κ-casein, the distribution of κ-casein between the two phases was not affected.

KEYWORDS: *κ-Casein, β-lactoglobulin, skim milk, disulfide reducing agent, disulfide bonds*

INTRODUCTION

The heat-induced thiol/disulfide exchange reactions between β-lactoglobulin (β-lg) and κ-casein (κ-cn) have been intensely studied because these reactions are involved in determining the functional properties of heated-milk products.[1] Thiol/disulfide exchange reactions are initiated by a thiol group and its reaction with a disulfide bond. In bovine milk, β-lg is the main source of thiol groups, while disulfide bonds can be found in β-lg, κ-cn, α-lactalbumin (α-la), and α_{s2}-casein.

A few studies have shown that the firmness of the gels made by heating (60 °C for 1 h) milk at pH 5.5 (acid-heat gels) was improved by adding low levels of a disulfide reducing agent to milk.[2,3] It was proposed that the addition of low quantities of disulfide reducing agent increased the number of available thiol groups, which in turn promoted the formation of intermolecular disulfide bonds.[2,3] However, Hashizume and Sato reported that milks treated with low levels of a reducing agent formed weaker acid-heat gels than milks without added reducing agents.[4] The authors suggested that the disulfide reducing agent prevented the formation of disulfide bonds during setting of the acid-heat gel and this resulted in a weaker gel.[4]

There are no studies investigating the effects of adding low levels of reducing agents to milk on the interactions between the proteins in unheated or heated milks. Therefore, the aim of this work is to examine the effect of adding low levels of β-mercaptoethanol (β-ME) to unheated or heated skim milk (with or without added whey protein isolate) on the disulfide bonding of the milk proteins. The level of β-lg and κ-cn involved in disulfide bonding and the distribution of the proteins between the colloidal and serum phases were determined by monitoring their changes with time after adding the reducing agent.

MATERIALS AND METHODS

Preparation of Milk Samples. Skim milk of 10% (w/w) total solids (control milk) was prepared by adding the appropriate quantity of low-heat skim milk powder (Fonterra Co-operative Group, New Zealand) to Milli-Q water. Skim milk of 10% total solids with 1% extra whey protein added (whey-protein-fortified milk) was prepared by adding whey protein isolate (WPI 895, Fonterra Co-operative Group, New Zealand) (1.15 g) to a sub-sample of the control milk (100 g), which raised the protein level in the milk by 1%. A small quantity of sodium azide (~0.01%, w/v) was added to the milk samples as a preservative. The milk samples were allowed to stir for at least 6 h at room temperature and were then kept in the cold room before further use.

Heat Treatment of Milk Samples. Sub-samples of milk (6 mL) were transferred to small sealable glass vials and then heated at 80 °C for 30 min in a thermostatically controlled oil bath. After heating, the samples were rapidly cooled using cold tap water.

Addition of Disulfide Reducing Agent to Milk Samples. β-ME (Aldrich Chemistry, St. Louis, MO) was initially diluted (1:9 β-ME/water) and then added to unheated or heated milk to give β-ME levels of 4.3, 7.1, 17, and 43 mM. The milk with added β-ME was shaken on a vortex mixer for 10 s and then left to react at 20 °C in a thermostatically controlled water bath for different time periods. The milk container was sealed to minimize exchange of oxygen with the environment.

Centrifugation of Milk Samples. Milk samples were centrifuged at 21000*g* and 25 °C for 60 min using a bench centrifuge (Centrifuge 5417R, Eppendorf AG, Hamburg, Germany) to separate the colloidal (pellet) and the serum (supernatant) proteins. The supernatant was carefully separated from the pellets, and the protein composition of the original milks and their supernatants was determined by electrophoresis.

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Previous studies have used this method and demonstrated that it can efficiently separate the colloidal and serum phases.[5−7]

Gel Electrophoresis and Laser Densitometry. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed using a Bio-Rad mini-gel slab electrophoresis unit (Bio-Rad Laboratories, Richmond, CA) as described elsewhere.[8] The milk and supernatant samples were analyzed under nonreducing and reducing conditions. Nonreducing conditions meant no further β-ME was added to samples, whereas reducing conditions involved the addition of an excess of β-ME (~20 μL) to 1 mL diluted sample, which was then heated (100 °C for 10 min). Comparisons between the reducing supernatant and reducing milk gave information on the distribution of the proteins between the colloidal and serum phases. Comparing the nonreducing supernatant and milk to the reducing milk gave information on the levels of proteins participating in disulfide bonds. After electrophoresis, the gels were processed and integrated using the method by Anema and Klostermeyer.[8]

Microfluidic Chip SDS−PAGE (MF−Electrophoresis). An Agilent 2100 bioanalyzer system and the associated Protein 80 kit (Agilent Technologies, Waldbronn, Germany) were used for MF−electrophoresis. The MF−electrophoresis works on the same principle as SDS−PAGE and has been explained in details previously.[9] The milk samples were prepared and analyzed in the same way as using gel electrophoresis described above.

RESULTS

Effect of Adding Reducing Agent into Unheated Milk Systems. The MF−electropherograms of the nonreduced and fully reduced skim milk are shown in panels A and B of Figure 1, respectively. In the nonreduced milk, the majority of κ-cn was disulfide-linked to form oligomers;[10−12] thus, the level of monomeric κ-cn was low (peak VI in Figure 1A). The low level would correspond to monomeric κ-cn with an intramolecular disulfide bond.[12] In fully reduced control milk, the disulfide bonds were broken; thus, the level of monomeric κ-cn was at a maximum (Figure 1B). Similarly, α_{s2}-casein naturally occurs as a mixture of monomers (with one intramolecular disulfide bond) or as disulfide-linked dimers.[13] Hence, in nonreduced control milk electrophoregrams, both monomeric and dimeric α_{s2}-casein peaks were observed (peaks V and VII in Figure 1A, respectively), whereas in fully reduced control milk, only monomeric α_{s2}-casein was detected (peak V in Figure 1B). A small peak for VII was still detected in fully reduced control milk. This peak is probably bovine serum albumin (BSA) because this runs at a similar position to dimeric α_{s2}-casein.

In the nonreduced control milk, native β-lg and α-la still contain intramolecular disulfide bonds,[14,15] whereas these bonds are disrupted upon fully reducing the milk. While the reduction of disulfide bonds did not affect the mobility of α-la (peak I in panels A and B of Figure 1), the nonreduced and reduced states of β-lg had different electrophoretic mobilities (peak II in panels A and B of Figure 1), as also observed previously.[16] One of the two disulfide bonds in β-lg was reported to be more reactive to thiol groups than the other.[17] Hence, β-lg can be either fully reduced (i.e., both disulfide bonds are broken) or partially reduced (i.e., only one bond is broken). The current method was not able to distinguish partially reduced β-lg from nonreduced or fully reduced β-lg. Therefore, in this study, the reduced peak was assumed to represent a fully reduced β-lg. On the basis of this analysis, the reduction of disulfide bonds of β-lg and κ-cn can be examined by monitoring the increases in intensity of the reduced β-lg peak and the monomeric κ-cn peak.

Figure 1C illustrates how the monomeric κ-cn and reduced β-lg peaks changed upon adding β-ME to unheated control milk. After 1 h, the peak for reduced β-lg remained low,

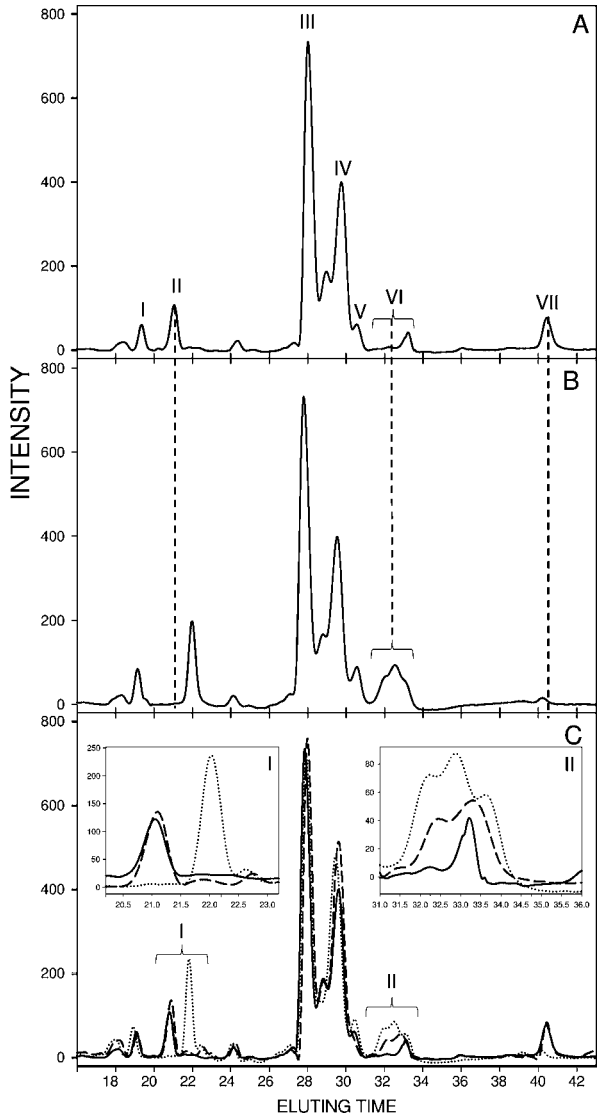

Figure 1. Electropherograms, obtained from MF−electrophoresis of (A) nonreduced and (B) fully reduced skim milk: I, α-la; II, β-lg; III, β-casein; IV, α_{s1}-casein; V, monomeric α_{s2}-casein; VI, κ-cn; and VII, dimeric α_{s2}-casein. (C) Overlapped electropherograms of (···) fully reduced control milk, (—) nonreduced control milk, and (- - -) control milk that had been reacted with 7.1 mM β-ME for 1 h. (Top left, inset I) β-lg peak and (top right, inset II) κ-cn peak.

indicating that a low level of β-lg was reduced (inset I of Figure 1C). In contrast, κ-cn was reduced more readily, because within the first hour, the level of monomeric κ-cn increased to ~50% of the total κ-cn present in the control milk (inset II of Figure 1C).

When β-ME was added to unheated control milk, the reduction of disulfide bonds of κ-cn and β-lg occurred over 2−3 h, after which no further reduction occurred (panels A and B of Figure 2). At time ≥1 h, the level of monomeric κ-cn and reduced β-lg increased as the concentration of β-ME increased from 4.3 to 43 mM (panels A and B of Figure 2). The level of κ-cn being reduced was always higher than that of β-lg, regardless of the β-ME concentration and time.

For all further experiments, a concentration of 7.1 mM β-ME was used to examine the effect of introducing thiol groups into milk systems. This concentration was chosen because 7.1 mM β-ME did not reduce all available disulfide bonds and,

therefore, allowed for the investigation of introducing a controlled number of new thiol groups into the system, which was the primary purpose of the study.

When 7.1 mM β-ME was added to the unheated whey-protein-fortified milk, the level of monomeric κ-cn was higher than the level of reduced β-lg, as also observed in control milk (Figure 3A). However, the percentage of monomeric κ-cn was

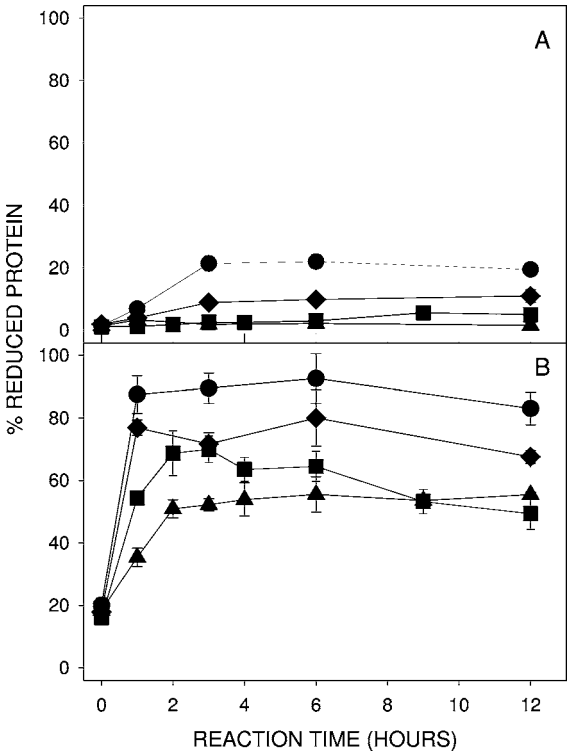

Figure 2. Impact of the reaction time on the percentage of (A) reduced β-lg and (B) monomeric κ-cn over the total of that protein in unheated control milk with concentrations of β-ME: (▲) 4.3 mM, (■) 7.1 mM, (♦) 17 mM, and (●) 43 mM of the disulfide reducing agent. Analysis used MF−electrophoresis, and each data point is the mean value of 2−4 replicates. The error bar is the standard deviation of the replicates.

lower in whey-protein-fortified milk compared to that in control milk, while the percentage of reduced β-lg was the same in both milks. An estimation of the total number of disulfide bonds in the system and the number of bonds being reduced indicated that, after 6 h, the total number of disulfide bonds of κ-cn and β-lg being reduced was similar in both control and whey-protein-fortified milks ($\sim 5.8 \times 10^{-5}$ mmol mL^{-1}), although that level in control milk appeared slightly higher than that level in whey-protein-fortified milk during the early stages of the reaction (Figure 3B).

Effect of Adding Reducing Agent into Heated Milk Systems. Figure 4 shows the electropherograms of fully reduced control milk, nonreduced heated control milk, and heated control milk treated with 7.1 mM β-ME for 6 h. Because whey proteins and κ-cn interact mainly via disulfide bonds in heated control milk,[18] the nonreduced heated skim milk electropherogram showed very low levels of β-lg (either reduced or nonreduced) and low levels of monomeric κ-cn. The addition of β-ME into the heated control milk can reduce the intermolecular disulfide bonds; hence, the level of reduced β-lg and monomeric κ-cn would be expected to increase as a function

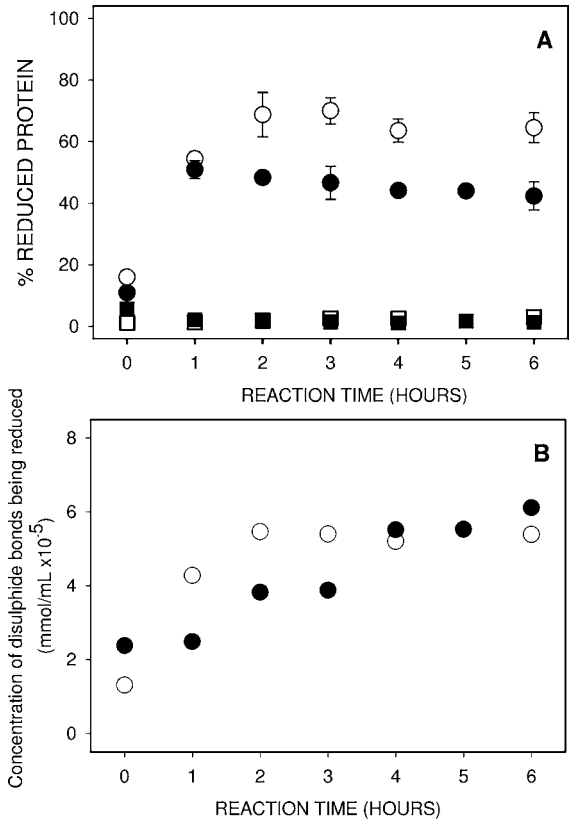

Figure 3. Impact of the reaction time on the (A) percentage of (□ and ■) reduced β-lg and (○ and ●) monomeric κ-cn over the total of that protein in milk and (B) calculated concentration of disulfide bonds being reduced in milk when 7.1 mM β-ME was added to (○) control and (●) whey-protein-fortified milks. Each data point is the mean value of 2−4 replicates. Error bars in panel A represent the standard deviation of the replicates.

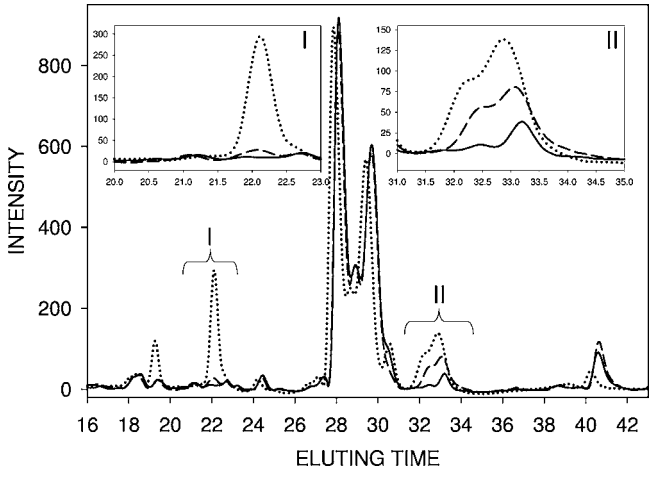

Figure 4. Electropherograms of (···) fully reduced control milk, (—) nonreduced heated control milk, and (- - -) heated control milk that had been reacted with 7.1 mM β-ME for 6 h. (Top left, inset I) β-lg peak and (top right, inset II) κ-cn peak.

of time. After 6 h, the level of reduced β-lg increased slightly (inset I of Figure 4), whereas the level of monomeric κ-cn increased markedly (inset II of Figure 4).

Upon adding 7.1 mM β-ME to heated control milk and whey-protein-fortified milk, the level of monomeric κ-cn was

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always higher than the level of reduced β-lg (Figure 5). However, the level of monomeric κ-cn in heated whey-protein-fortified milk was lower compared to the level in heated control milk. The level of reduced β-lg as a percentage of that present in the milk was similar for control milk and whey-protein-fortified milk (Figure 5A). Consequently, the estimated total number of reduced disulfide bonds was only slightly higher in heated control milk compared to heated whey-protein-fortified milk (Figure 5B).

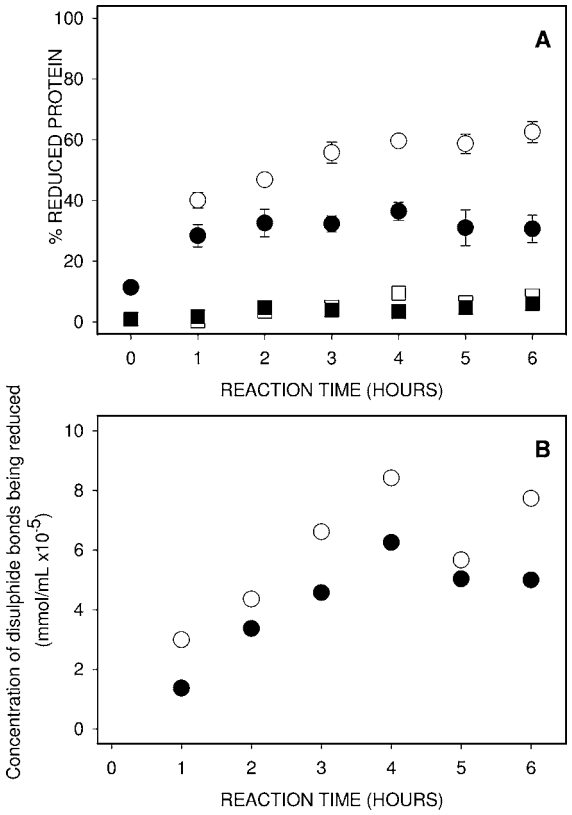

Figure 5. (A) Percentage of (○ and ●) monomeric κ-cn and (□ and ■) reduced β-lg changed as a function time when 7.1 mM β-ME was added to heated milk systems, as analyzed by MF−electrophoresis. (B) Calculated concentration of disulfide bonds being reduced in heated milks: (○) control and (●) whey-protein-fortified milks. Each data point is the mean value of 2−4 replicates. Error bars in panel A represent the standard deviation of the replicates.

Electrophoretic analyses of the serum and milk samples of heated control milk showed that higher levels of serum β-lg and κ-cn were reduced in comparison to the colloidal β-lg and κ-cn (panels A and B of Figure 6). The level of serum β-lg increased slightly over the first 3 h and then remained constant at longer reaction times (Figure 6A). The level of serum κ-cn remained relatively constant, regardless of the reaction time (Figure 6B). Electrophoretic results also showed that the distribution and the interaction of α-la was not affected by β-ME (results not shown).

In heated whey-protein-fortified milk, the disulfide bonds of serum β-lg and serum κ-cn were reduced in preference to those of the colloidal proteins, as also observed in heated control milk. In addition, as the reaction time was prolonged, the percentage of colloidal β-lg having disulfide bonds remained constant, whereas the percentage of κ-cn with disulfide bonds decreased slightly (panels C and D of Figure 6). Regardless of the change in levels of protein participating in disulfide bonds, the distribution of the proteins between the serum and colloidal phases remained constant at any reaction time. As in the heated control milk, α-la in the heated whey-protein-fortified milk did not appear to react with the disulfide reducing agent during the period studied (result not shown).

DISCUSSION

In unheated control milk and whey-protein-fortified milk, the disulfide bonds of κ-cn were reduced in preference to those of β-lg, regardless the level of whey protein in the milk (Figures 2 and 3). In unheated skim milk, κ-cn is found as disulfide-linked oligomers of various sizes because the two cysteine residues (Cys^{11} and Cys^{88}) can form intermolecular disulfide bonds in a random manner.[11] κ-cn has an amphiphilic nature with hydrophobic and hydrophilic regions.[19] The cysteine residues are located in the hydrophobic region, and this region is associated with the interior of the casein micelle. The hydrophilic regions project into the serum and stabilize the micelles.[20,21] However, this layer is porous; therefore, denatured β-lg and small molecules (such as β-ME) can diffuse through to interact with the disulfide bonds of κ-cn.[22]

Nonreduced β-lg has two intramolecular disulfide bonds, Cys^{106}−Cys^{119} and Cys^{60}−Cys^{160}.[14] The Cys^{160}−Cys^{66} disulfide bond is located near the C-terminal and, hence, is probably more accessible than the Cys^{106}−Cys^{119} disulfide bond. However, because lower levels of β-lg were reduced than κ-cn at any β-ME concentration and time, both disulfide bonds of β-lg were considered to be less accessible to β-ME than the intermolecular disulfide bonds of κ-cn.

The percentage of reduced β-lg was low in both unheated control milk and whey-protein-fortified milk (Figure 3). However, because whey-protein-fortified milk had ∼3 times the level of β-lg than control milk, the whey-protein-fortified milk had a significantly higher absolute concentration of reduced β-lg molecules. The result in Figure 3B indicates that a higher absolute level of β-lg was reduced when the β-lg content of the milk was raised. Statistically, there was a greater chance for β-ME to interact with β-lg with a higher whey protein concentration in milk. The difference in the total number of disulfide bonds being reduced between control and whey-protein-fortified milk could be due to the levels of reduced α-la and BSA that could not be detected. The addition of whey protein to the milks not only increases the level of β-lg but also the levels of α-la and BSA. As a consequence, with a higher level of β-lg being reduced, the levels of reduced α-la and BSA would also be expected to be higher. In addition, it is possible that a certain amount of β-lg was reduced partially and, thus, may not be detected as reduced β-lg. Even though a higher level of β-lg was reduced in whey-protein-fortified milk and the level of disulfide bonds being reduced was similar in both control and whey-protein-fortified milks, it was clear that κ-cn was reduced preferentially compared to β-lg, regardless of the level of whey protein in the milk.

For the unheated control and whey-protein-fortified milks, the level of κ-cn in the serum and colloidal phases did not change upon adding β-ME (results not shown). This indicates that the monomeric κ-cn was still associated with the casein micelles. Considering the processes involved in the biosynthesis of casein micelles, it has been proposed that monomeric κ-cn (with free thiol groups) stabilizes the aggregates of α- and β-caseins. Then, the thiol groups of κ-cn formed (predominantly intermolecular) disulfide bonds when oxidizing conditions were encountered.[10,23]

In the heated control milk and the heated whey-protein-fortified milk, the disulfide bonds of κ-cn were reduced

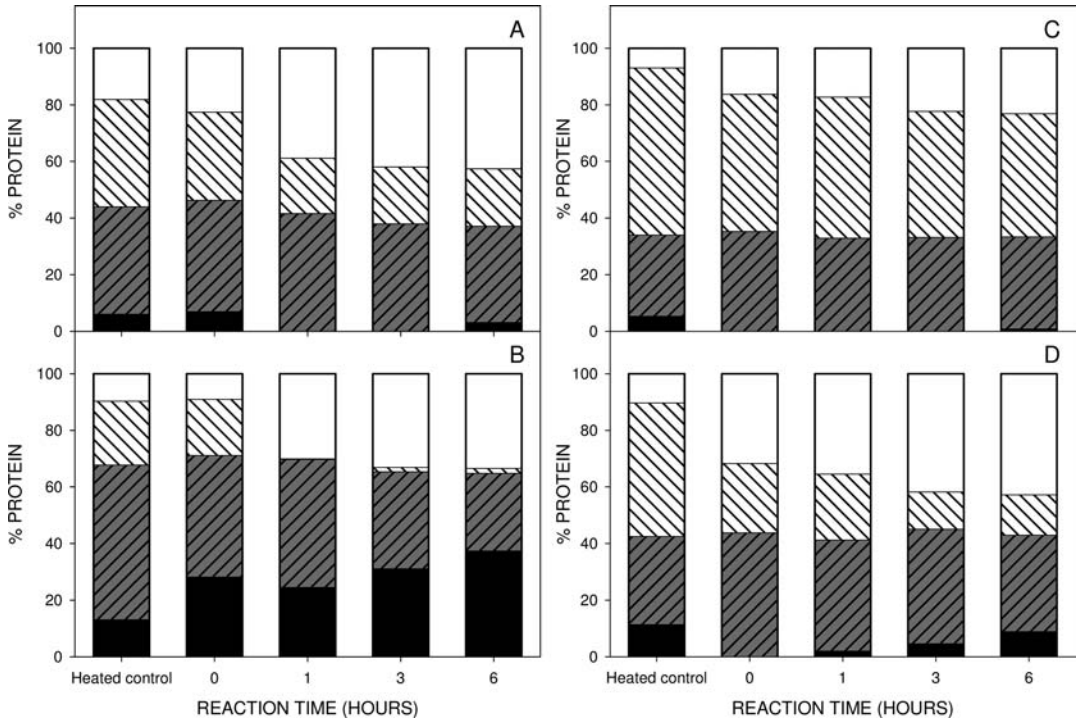

Figure 6. Level of protein being distributed between (white and hatched white bars) serum and (black and hatched gray bars) colloidal phases when 7.1 mM β-ME was added to skim milk: (A) β-lg in control milk, (B) κ-cn in control milk, (C) β-lg in whey-protein-fortified milk, and (D) κ-cn in whey-protein-fortified milk. The level of protein participating in disulfide interactions is demonstrated by hatched bars. The data was obtained from traditional SDS−PAGE. Each data is the mean value of 2−4 replicates.

preferentially to those of β-lg, regardless of the level of whey protein in the milk (Figures 4−6). Because the size of the serum aggregates is reported to decrease with increasing concentrations of κ-cn in the milk system, κ-cn was postulated to be on the surface to control the growth of the aggregates.[24] Although this surface location may account for the preferential reduction of κ-cn disulfide bonds, it seems unlikely that the small reducing agent molecules would be sterically hindered from accessing all of the disulfide bonds in the aggregates. A more plausible explanation is simply that the number of intermolecular disulfide bonds that need to be reduced to form a monomer is higher for denatured β-lg than for κ-cn. In the heated milk system, there are potentially five cysteines that could be involved in intermolecular disulfide bonds for denatured β-lg, whereas for κ-cn, there are only two. Therefore, there is a greater probability of forming κ-cn monomers than β-lg monomers when adding low levels of reducing agents to the heat-induced aggregates, regardless of whether they are in the colloidal or serum phase. Other factors, such as differences in the microenvironment of the cysteine residues, may influence the rate of the reduction, so that those involving κ-cn are reduced preferentially to those involving β-lg. For example, positively charged neighboring amino acid groups may promote the approach of the negatively charged thiolate ion on the reducing agent, whereas negatively charged neighboring groups may inhibit the approach of this thiolate ion.

Interestingly, even though the disulfide bonds of colloidal κ-cn were reduced, the percentage of total colloidal β-lg (with and without disulfide bonds) remained almost constant (panels A and C of Figure 6). This suggests that the intermolecular disulfide bonds between κ-cn and β-lg that connect the heat-induced aggregates to the casein micelle may be less susceptible to reduction by the β-ME than the intermolecular disulfide bonds in polymeric κ-cn. Alternatively, it has been proposed that denatured β-lg may first interact with the casein micelles through hydrophobic interactions and that disulfide bonds are formed subsequently.[25−28] If this is the case, then despite the substantial reduction of κ-cn disulfide bonds, the denatured whey proteins may stay associated with the casein micelles and, therefore, the distribution of β-lg between serum and colloidal phases is not significantly affected.

In conclusion, this study has shown that κ-cn preferentially reacted with the disulfide bond reducing agent compared to β-lg in unheated and heated milks, regardless of the whey protein concentration. In unheated milk, the intermolecular disulfide bonds of κ-cn on the surface of the casein micelles were considered to be more accessible than those of native β-lg, which are intramolecular. In heated milk, the preferential reduction of κ-cn disulfide bonds may be due to fewer of these bonds linking κ-cn to the aggregates than for β-lg or due to a preferential reduction of the disulfide bonds involving κ-cn.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +64-6-350-4649. Fax: +64-6-356-1476. E-mail: skelte.anema@fonterra.com.

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Notes

The authors declare no competing financial interest.

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