Formation of New Protein Structures in Heated Mixtures of BSA and α-Lactalbumin

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The heat-induced protein−protein interactions of α-lactalbumin (α-La) and bovine serum albumin (BSA), dispersed in a pH 6.8, 10% whey protein concentrates (WPC) permeate, were followed using alkaline and sodium dodecyl sulfate (SDS) 1D and 2D polyacrylamide gel electrophoresis (PAGE) and size-exclusion high-performance liquid chromatography (SE-HPLC). Heated (75 °C) 5% BSA solution contained large disulfide-bonded BSA aggregates, although some monomer BSA (SDS-monomeric BSA) could be dissociated from the aggregates by SDS. In contrast, similarly heated α-La solutions contained small quantities of several monomeric forms of α-La and dimeric α-La but no large aggregates. When 10% solutions of 1:1 (w/w) mixtures of α-La and BSA were heated, large disulfide-bonded aggregates and SDS-monomeric BSA and α-La were present. However, heated 2% mixtures contained more modified α-La monomers, α-La-dimers, and α-La-trimers, fewer large disulfide-bonded aggregates, and less SDS-monomeric α-La or BSA. These results suggest that BSA forms disulfide-bonded aggregates that contain available thiol groups that can catalyze the formation of differently structured α-La monomers, dimers, higher polymers, and adducts of α-La with BSA.

Keywords: *Homogeneous aggregates; heterogeneous aggregates; disulfide bond interchange; α-lactalbumin−BSA adducts; α-lactalbumin dimers; α-lactalbumin trimers; thermal denaturation; disulfide-bonded aggregates; size-exclusion high-performance liquid chromatography; two-dimensional polyacrylamide gel electrophoresis*

INTRODUCTION

The heat-induced denaturation, aggregation, and gelation of whey protein concentrates (WPCs) and isolates (WPIs) have been studied extensively over the past few years (Aguilera, 1995; Bowland and Foegeding, 1995; Havea et al., 1998). The major protein components of whey include *β*-lactoglobulin (*β*-Lg) (50%), α-lactalbumin (α-La) (20%), and bovine serum albumin (BSA) (5%), and it is often accepted that the characteristics of the major protein, *β*-Lg, dominate the behavior of the WPCs and WPIs. However, closer examination indicates that the other whey proteins play important roles in determining the characteristics of heat-induced gels (Hines and Foegeding, 1993; Matsudomi and Oshita, 1996).

There have been some studies of the heat-induced changes to *β*-Lg, α-La, and BSA (Paulsson and Dejmek, 1989). Although *β*-Lg has been studied extensively in different buffers using a wide range of techniques, neither α-La nor BSA has been studied as extensively from a food science perspective. As part of an exploration of the factors affecting the gelation of WPCs, we have examined heat effects in the model system of α-La and BSA in WPC permeate on the protein aggregation using polyacrylamide gel electrophoresis (PAGE).

Previous work has shown that when α-La is heated in solution, it can repeatedly undergo the same thermal transition without forming a gel (Rüegg et al., 1977; Paulsson et al., 1986; Relkin et al., 1993). Studies of the reversible changes in the spectral properties of the protein (e.g., Hiraoka et al., 1980) implicate protein conformational changes that are pH, calcium, and ionic strength dependent (Bernal and Jelen, 1984, 1985; Relkin et al., 1993). However, more extensive heating in the absence of calcium (Chaplin and Lyster, 1986) gives rise to disulfide-bonded α-La dimers, trimers, etc. as well as some altered monomeric protein.

By contrast, BSA readily forms gels when heated (Boye et al., 1996), but there are relatively few studies (Bernal and Jelen, 1985) of the molecular rearrangements that are the consequences of heat treatment of this protein.

When added to *β*-Lg or BSA solutions, α-La increases the rigidity of the resultant gels and irreversibly forms disulfide-bonded polymers (Calvo et al., 1993; Matsudomi et al., 1993, 1994; Matsudomi and Oshita, 1996). Studies, using PAGE and other techniques, of the heat-induced aggregation of mixtures of α-La and *β*-Lg indicate that both covalent bonding and noncovalent bonding are involved in the aggregation process (Matsudomi and Oshita, 1996; Gezimati et al., 1997; Dalgleish et al., 1997).

Results from the analysis of heat-treated *β*-Lg have suggested that there is more than one mechanism of protein−protein association as a consequence of the heat treatment. Some aggregates that are intact at pH 8.8 dissociate in the presence of sodium dodecyl sulfate (SDS) (McSwiney et al., 1994a,b), whereas others are dissociable only after disulfide-bond reduction. Exten-

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sion of that study to the heat-induced aggregation and gelation of 10% (w/w) solutions of a 1:1 mixture of β-Lg and BSA (Gezimati et al., 1996a) or α-La (Gezimati et al., 1996b, 1997) indicated that similar types of aggregates could occur in these mixtures. More recently, we (Havea et al., 1998) showed that when WPC solutions were heated, particularly at concentrations below 3% (w/v), and then examined by two-dimensional (2D) PAGE, there was evidence for disulfide-bonded dimeric forms of α-La and for noncovalent aggregates involving monomer α-La.

The present investigation extends that study by examining the protein components in heated solutions of a 1:1 (w/w) mixture of BSA and α-La in greater detail.

MATERIALS AND METHODS

Proteins and Chemicals. α-La (product L-5385), BSA (product A-4378), the molecular weight standards, and the gel buffer salts were obtained from Sigma Chemical Co. (St. Louis, MO). The extent of the changes to the proteins in these samples was assessed using alkaline−PAGE, and the most appropriate samples were selected for further examination. In all cases there were small quantities of protein impurities that were usually other whey or blood proteins and polymers of α-La or BSA. A commercial WPC powder, derived from acid whey, was obtained from the New Zealand Dairy Board, Wellington, New Zealand. Most of the reagents used for the preparation of electrophoresis gels were obtained from Bio-Rad Laboratories, Hercules, CA. Water was purified using a MilliQ system (Millipore Corp., Bedford, MA).

Sample Preparation. WPC solutions (10%, w/w) were prepared by dissolving WPC powder in water, the mixtures were then stirred for 2 h at room temperature using a magnetic stirrer, and the pH was adjusted to pH 6.8 using 0.1 M NaOH or HCl. WPC permeate was obtained using an ultrafiltration unit equipped with an SO-HF-131-VIV filter and was then used to dissolve α-La and BSA to give 10% (w/w) solutions of the proteins. The pH was readjusted to 6.8. The permeate monovalent cation—mostly potassium—concentration was ~400 mM and calcium concentration was ~35 mM. The protein solutions were mixed in a ratio of 1:1, and some aliquots of this were diluted 1:4 with permeate. Small (0.4 mL) samples of the mixtures were transferred into 2 mL round-bottomed Beckman polyallomer centrifuge tubes (0.35 mm wall thickness, 11 mm internal diameter, 34 mm height) that were closed with appropriately fitted lids and placed in a water bath thermostatically controlled at 75 °C. Tubes and samples were removed after heating for up to 48 min for the 2% solutions and up to 10 min for the 10% solutions. (It took 25 s for the samples to attain 74.9 °C.) The tubes and heated WPC solutions were immediately placed in ice water for 5 min and then held at room temperature (~20 °C) for 2 h. The heated solutions (plus unheated controls) were then analyzed using one-dimensional (1D) PAGE. The samples of the 2% solutions that had been heated for 36 min and samples of the 10% solutions that had been heated for 6 min were analyzed by 2D PAGE.

PAGE. The protein samples were analyzed using the Mini-Protean II dual slab cell system (Bio-Rad Laboratories, Hercules, CA). The discontinuous PAGE systems for both the 1D and 2D separations have been described by Manderson et al. (1998) and Havea et al. (1998), respectively.

Size-Exclusion High-Performance Liquid Chromatography (SE-HPLC). The HPLC equipment was supplied by GBC Scientific Equipment Ltd., Victoria, Australia, and included a model LC 1200 UV/vis detector and an LC 1150 pump. The samples were prefiltered using 0.45 μm filters and separated on a Superdex 75 HR column using a flow rate of 0.5 mL/min of a 20 mM imidazole, 50 mM NaCl solution adjusted to pH 6.9.

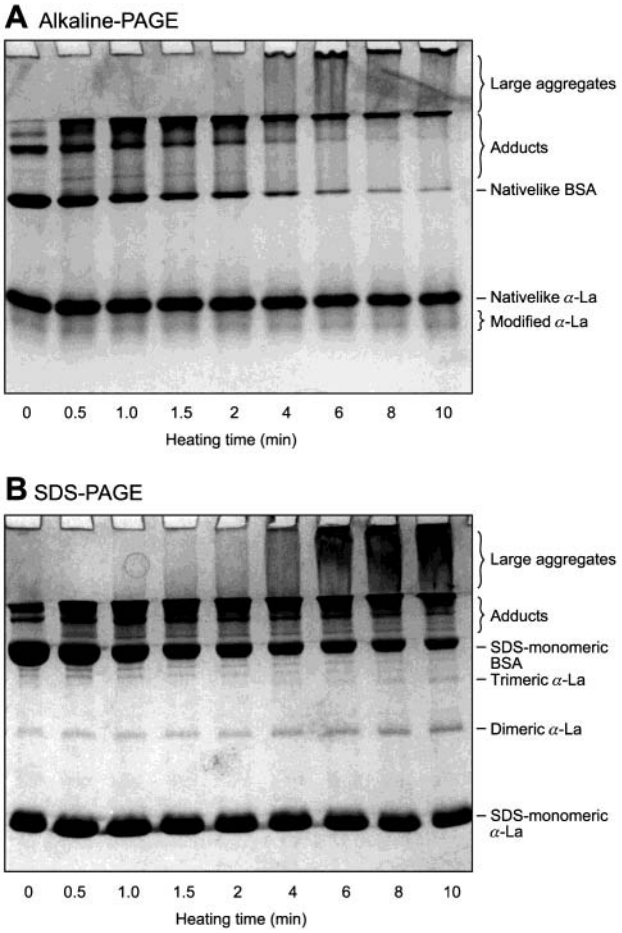

Figure 1. Alkaline-PAGE (A) and SDS-PAGE (B) patterns showing the effect of heat treatment on a 1:1 mixture of α-La and BSA in WPC permeate at a total protein concentration of 10% (w/w). The water bath temperature was 75 °C, and samples were taken at 0, 0.5, 1.0, 1.5, 2, 4, 6, 8, and 10 min after the samples reached 74.9 °C (25 s). Further experimental details are given in Materials and Methods.

RESULTS

Effect of Heating Solutions of BSA and α-La. *(i) Unheated Protein Mixtures.* The alkaline- and SDS-PAGE patterns of the unheated protein mixtures (5% BSA and 5% α-La for Figure 1, and 1% BSA and 1% α-La for Figure 2) showed intense monomer bands and fainter bands corresponding to dimers and other impurities (lane 1, Figures 1A, 1B, 2A, and 2B).

(ii) 10% Protein Mixtures. Heating the 10% mixtures at 75 °C gave patterns (Figure 1A) in which the intensities of the nativelike BSA band and the various low-mobility bands decreased with heating time. The intensity of the α-La band (Figure 1A) also decreased with heating time. There were concurrent increases in the concentrations of material in the region ahead of the nativelike α-La band (Figure 1A) corresponding to the modified α-La of Chaplin and Lyster (1986). There were also increases and then decreases in the amounts of material in the region between the BSA band and the beginning of the stacking gel, which could have been various reaction products of BSA. As the various band intensities within the gel decreased with increased heating time, there was an increase in the amount of material caught at the top of the resolving gel and caught within or above the stacking gel. This is labeled as "large aggregates" in Figure 1A.

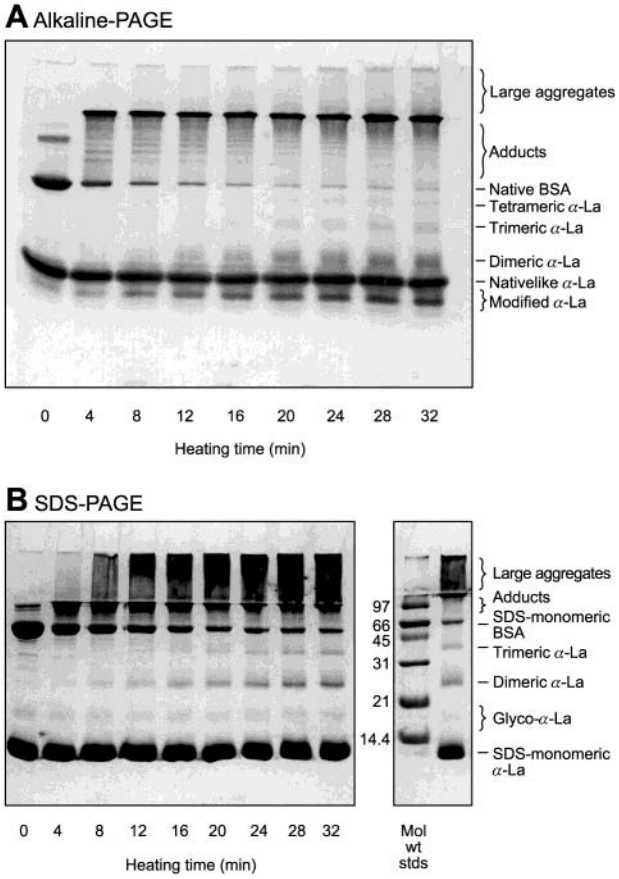

Figure 2. Alkaline-PAGE (A) and SDS-PAGE (B) patterns showing the effect of heat treatment on a 1:1 mixture of α-La and BSA in WPC permeate at a total protein concentration of 2% (w/w). The water bath temperature was 75 °C, and samples were taken at 0, 4, 8, 12, 16, 20, 24, 28, and 32 min after the samples reached 74.9 °C (25 s). Further experimental details are given in Materials and Methods.

Analysis of the same samples on SDS-PAGE (Figure 1B) indicated that the concentrations of both SDS-monomeric α-La and BSA decreased steadily with increased heating time, although the rate of decrease was less than that for the nativelike proteins (Figure 1A). There was an increase in material in bands that had mobilities corresponding to dimeric and trimeric α-La (Figure 1B). There were two fine bands that ran slightly more slowly than BSA, which could have corresponded to adducts of BSA and one or two molecules of α-La, respectively. The quantity of material in the large aggregate region of Figure 1B increased steadily for most of the heating period.

It was also noted that the decrease in the concentration of monomer BSA was markedly faster when a 10% solution of BSA was heated at 75°C (not shown) than when BSA was heated in the 5% BSA, 5% α-La mixtures (Figure 1), although no BSA was observable after heating for 4 min using either gel system. This result complements the results of Gezimati et al. (1996a) who found that heating a 5% solution of BSA resulted in a slower loss of BSA than from a mixture containing 5% β-Lg and 5% BSA. (Overall, the rate of loss of nativelike BSA is greatest for 10% BSA, intermediate for 5% BSA plus 5% β-Lg, and slowest for 5% BSA.) Gezimati et al. (1997) considered that there was a generalized protein effect rather than specific interactions between the two proteins.

(iii) 2% Protein Mixtures. Examination of the heated 2% mixtures by both alkaline- and SDS-PAGE gave slightly different results (Figure 2) from those obtained using 10% solutions (Figure 1). The rate of loss of both nativelike and SDS-monomeric protein was slower than from the 10% solutions, confirming the earlier reports (Gezimati et al., 1996a; Havea et al., 1998). The increase in the quantity of material that did not enter the resolving gel was slower, and there was more material with intermediate mobilities in the resolving gels (Figure 2) with well-resolved banding patterns.

(a) Alkaline-PAGE. In the alkaline gel (Figure 2A), there were at least five bands or regions with greater mobility than BSA, and these have been labeled as modified monomeric α-La, nativelike α-La, dimeric α-La, trimeric α-La, and tetrameric α-La. The bands with mobilities greater than BSA were similar to those reported by Chaplin and Lyster (1986) in α-La solutions heated for 20 min at 100 °C. Although the bands just ahead of the nativelike α-La were apparent in the unheated mixture, the band intensity increased with heating time. The other bands between the nativelike α-La and BSA also increased in intensity with the extent of heating, but they were not apparent in the unheated mixture. Similarly, the series of bands with mobilities lower than that of BSA, which were not present in the unheated sample (Figure 2A, lane 1), initially increased in intensity and then diminished in intensity with heating time. The distribution of stain intensity among the bands in this region moved to the lower mobility bands with increased heating time. The band that was present in the unheated mixture, and could have been dimeric BSA (lane 1, Figure 2A), was no longer present after only 4 min of heating time. The quantity of large aggregates that did not enter the stacking or the resolving gels also increased with heating time.

(b) SDS-PAGE. Examination of the same samples by SDS-PAGE (Figure 2B) also showed bands with mobilities comparable with those of BSA, α-La, glyco-α-La, dimeric and trimeric α-La, and large aggregates and labeled as such, in accord with Figure 1B. Glyco-α-La (Baumy and Fauquant, 1989; Slangen and Visser, 1999) was identified by comparative PAGE analysis (Kinghorn et al., 1995). The material in the large aggregates that entered the stacking gel may have corresponded to adducts of α-La and BSA. Reduction of the heated protein samples with mercaptoethanol prior to PAGE analysis gave essentially only α-La and BSA (not shown), indicating that most of the heat-induced modifications and aggregates were the result of heat-induced disulfide bond-interchange reactions.

2D PAGE Analysis of Heated Protein Solutions. The protein aggregate bands observed in alkaline and SDS gels (Figures 1 and 2) were further characterized using two kinds of 2D PAGE as outlined by Havea et al. (1998). In the first kind (alkaline- then SDS-PAGE) a heated protein sample was separated by alkaline-PAGE and a gel lane was excised, immersed in SDS buffer, and used as the sample for the SDS second dimension (e.g., Figure 3A). In the second kind (SDS-PAGE, reduction, then SDS-PAGE) a heated protein sample was separated by SDS-PAGE and a gel lane excised, immersed in a warm solution of mercaptoethanol in SDS buffer, and used as the sample for the second SDS dimension (e.g., Figure 3B). The identifica-

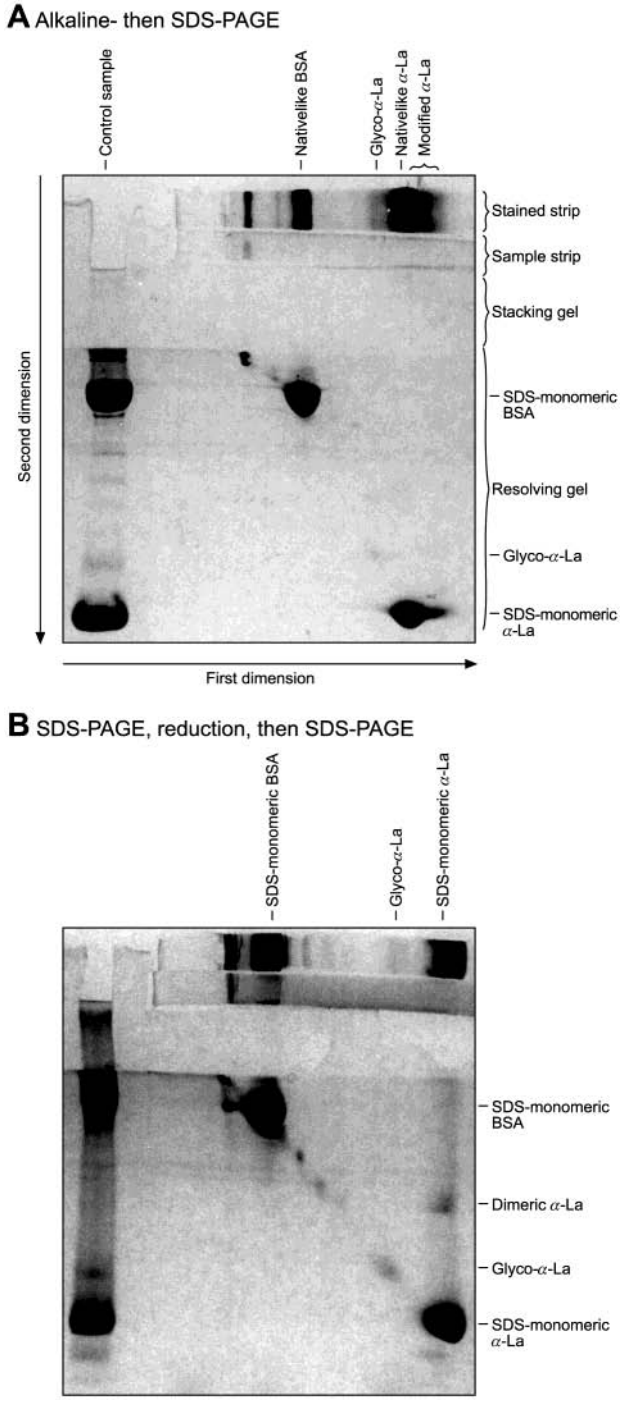

Figure 3. 2D (alkaline-, then SDS-) PAGE (A) and 2D (SDS-, then SDS- after sample reduction) PAGE (B) patterns of a sample of an unheated 1:1 mixture of α-La and BSA in WPC permeate at a total protein concentration of 2% (w/w). Further experimental details are given in Materials and Methods.

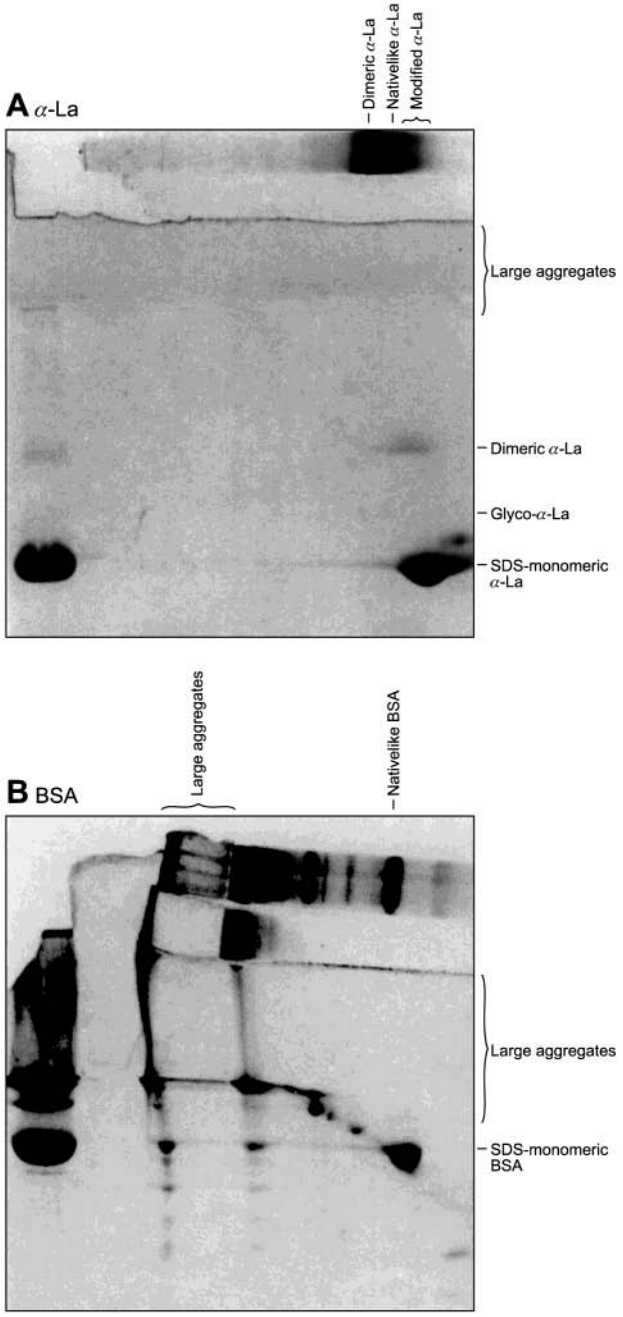

Figure 4. 2D (alkaline-, then SDS-) PAGE patterns of (A) a 5% (w/v) sample of α-La heated for 6 min at 75 °C and (B) of a 5% (w/v) sample of BSA heated for 6 min at 75 °C. Further experimental details are given in Materials and Methods.

tions made for the bands in Figures 1 and 2 have been transferred to the figures containing the 2D gel patterns.

(i) Unheated Mixture. Figure 3A shows the 2D alkaline- and then SDS-PAGE pattern for the 1:1 mixture of BSA and α-La. Clearly, the most mobile major band in both gel systems was α-La, and this was a single symmetrical spot in the 2D PAGE patterns (Figure 3). Similarly, the major low-mobility bands and spot in each system was BSA. The remaining bands, streaks, and spots were either minor contaminants of the commercial products used in this study or artifacts formed during the analysis.

(ii) Heated Individual Proteins. The 2D alkaline- and then SDS-PAGE patterns that arose from heating 5% α-La and 5% BSA separately in permeate are shown in parts A and B of Figure 4, respectively.

By comparison with the 2D pattern for the unheated mixture (Figure 3A), there was a new small spot and an indistinct band that corresponded to the modified α-La and α-La dimers, respectively, of Chaplin and Lyster (1986) and are labeled as such in Figure 4A.

The 2D pattern for the heated BSA (Figure 4B) showed a number of spots. The largest spot corresponded to monomer in both the alkaline and SDS dimensions. There was a small spot that probably corresponds to dimer in both dimensions (cf. Figures 1A

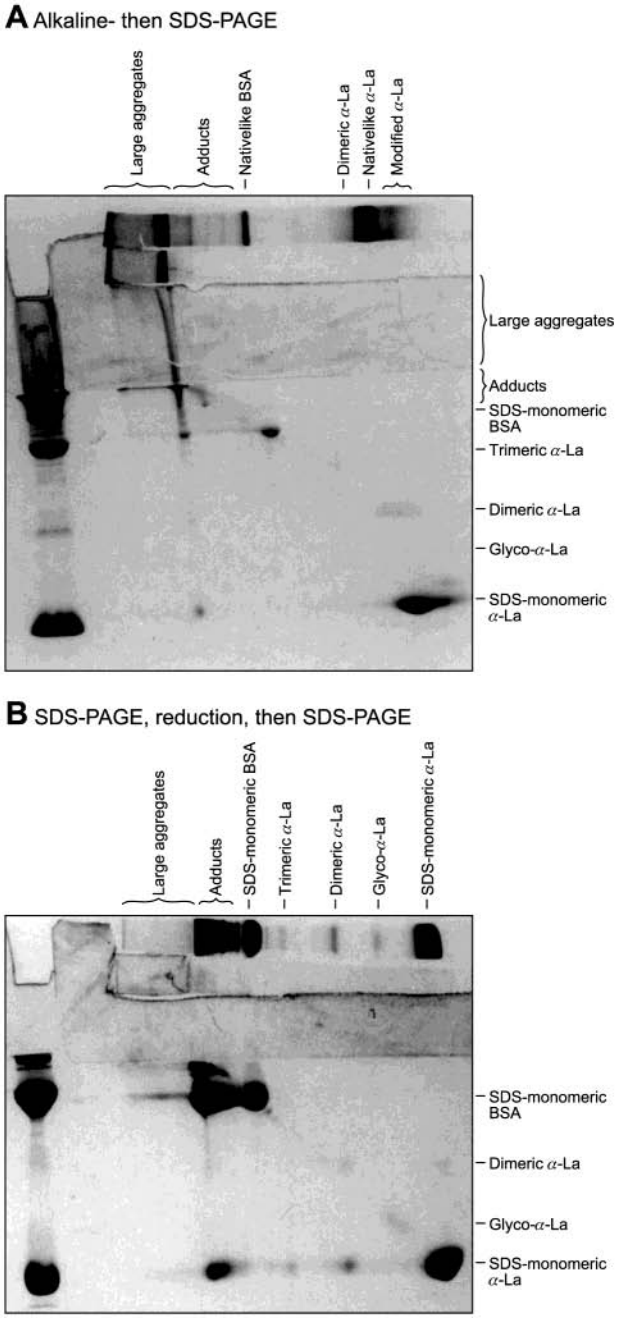

Figure 5. 2D (alkaline-, then SDS-) PAGE (A) and 2D (SDS-, then SDS- after sample reduction) PAGE (B) patterns of a sample of a 10% (w/w) mixture of 1:1 BSA/α-La heated for 6 min at 75 °C. Further experimental details are given in Materials and Methods.

and 2A). In the horizontal line corresponding to monomeric BSA, there were two spots that corresponded to monomer in the SDS dimension and to large aggregates (top of the resolving gel and top of the stacking gel, respectively) in the alkaline dimension. Clearly, this was SDS-monomeric BSA that had been dissociated from the heat-induced large aggregates by SDS. There were also spots/bands corresponding to various large aggregates in both dimensions. These were found to be dissociated into monomeric BSA after mercaptoethanol reduction (Havea, 1998) using 2D PAGE.

(iii) Heated 10% Mixture. The 2D PAGE patterns (Figure 5) show more spots than one would expect from a combination of those of the separately heated proteins, i.e., Figure 4. Comparison of Figure 5A with Figure 3A (unheated mixture) showed several notable differences. There were the modified monomeric α-La and dimeric α-La seen when α-La was heated (Figure 4A) and the small spot that might be dimeric BSA. There were also spots corresponding to SDS-monomeric BSA and α-La that had dissociated from the large aggregates.

When another portion of the same sample was run on SDS-PAGE before and after mercaptoethanol treatment (Figure 5B), there were the expected spots of monomeric α-La, glyco-α-La, and BSA along the diagonal. There was a spot that could have been dimeric α-La, which may have indicated that a small quantity of nonreducible dimer was present. In addition, there was a horizontal line of spots of α-La that was monomer after reduction and had molecular weights corresponding to monomer, dimer, trimer, tetramer, and much greater molecular aggregations before mercaptoethanol treatment, indicating that α-La had probably formed disulfide-bonded aggregates with itself and with BSA.

Similarly, there was a horizontal smear of material that was monomer BSA after mercaptoethanol treatment that apparently originated from the bands that migrated a little more slowly than BSA and appeared to be adducts of one or more α-La molecules per BSA molecule (Figures 1 and 2 and in the SDS dimension of Figure 5A). It is significant that the obvious bands in the stained sample slice in Figure 5B did NOT give rise to many spots on the diagonal; i.e., they were aggregates that were nearly all reducible to monomer BSA and α-La.

(iv) Heated 2% Mixture. The 2D PAGE patterns of a sample of a 2% mixture of α-La and BSA heated in permeate for 36 min at 75 °C are shown in Figure 6. In the 2D alkaline- and then SDS-PAGE pattern the region near the α-La monomer was well resolved and several spots were discernible. The α-La dimer and trimer band areas were also clear, and a tetramer was apparent in the alkaline dimension, but it appeared to have the same mobility as BSA in the SDS dimension. All of these polymers gave rather diffuse spots, suggesting that there may have been several molecular species of very similar molecular weight but with varying sizes, shapes, and charges as suggested by Manderson et al. (1998) for comparable 2D PAGE patterns of heat-treated β-Lg. There also appeared to be some protein in the region between the monomers and the dimer spot. The quite sharp bands that were observed between the BSA bands and the top of the resolving gel in some of the lanes in Figure 2 were not especially obvious in either the 1D or 2D PAGE patterns (Figure 6A).

The difference between Figures 5A and 6A (in which the protein concentrations were 10% and 2%, respectively) was the relatively greater concentration of the α-La species that appeared to be dimer, trimer, and nonnativelike monomers at the lower concentration. The absence of SDS-monomeric BSA or α-La in the patterns indicated that much less of these species was associated with the large aggregates when the protein concentrations were lower.

The 2D SDS-PAGE patterns for the heated mixture before and after mercaptoethanol treatment are shown in Figure 6B. The pattern was quite similar to that shown in Figure 5B, although a number of the bands and spots were more distinct and more intense. In particular, the protein species that dissociated from the large aggregates caught at the top of the resolving gel prior to mercaptoethanol treatment clearly released not

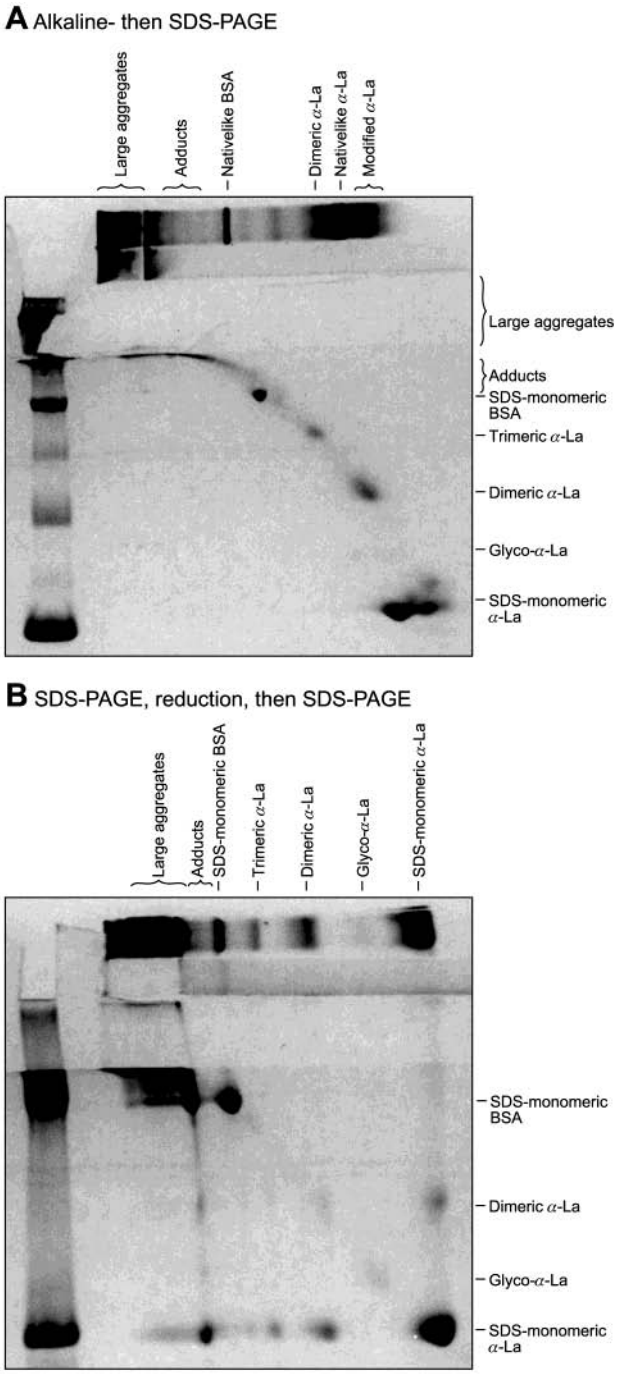

Figure 6. 2D (alkaline-, then SDS-) PAGE (A) and 2D (SDS-, then SDS- after sample reduction) PAGE (B) patterns of a sample of a 2% (w/w) mixture of 1:1 BSA/α-La heated for 36 min at 75 °C. Further experimental details are given in Materials and Methods.

only α-La but also glyco-α-La and dimeric α-La.

The spot that ran as monomer in alkaline-PAGE but dimer in SDS-PAGE (cf. Figures 4A, 5B, and 6B) was also quite distinct and may have been an artifact of the analytical procedure as discussed by Manderson et al. (1998). The dimeric α-La spot on the left-hand side of the 2D pattern may well have had the same origin.

SE-HPLC Separation of Protein Products. Many of the heated samples were analyzed using SE-HPLC, and some typical results are shown in Figure 7. The peaks corresponding to monomer BSA (ca. 17 min) and α-La (ca. 22.5 min) were well separated, and the material between them of intermediate size increased

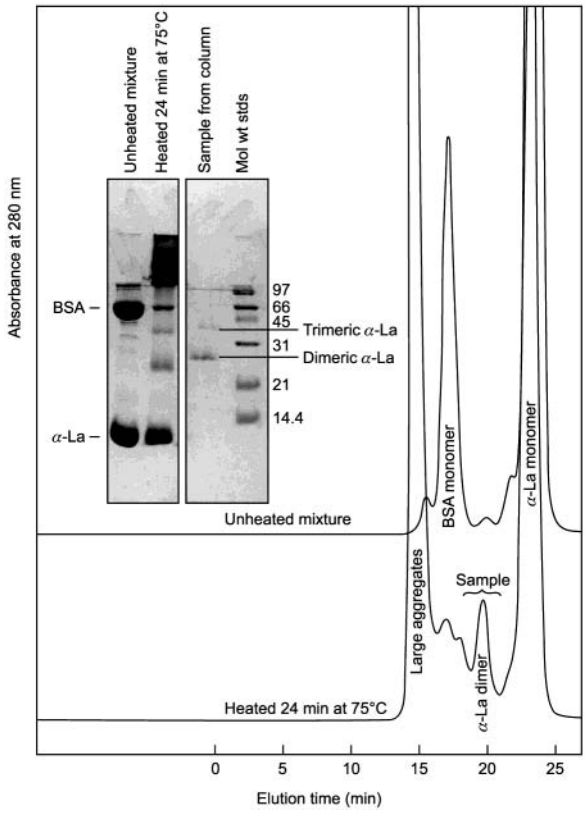

Figure 7. SE-HPLC chromatograms showing the change in the HPLC pattern with heat treatment. Heat treatment was at 75 °C on a 1:1 mixture of α-La and BSA in WPC permeate at a total protein concentration of 2% (w/w). Inset: SDS-PAGE pattern of the SE-HPLC column fractions. Further experimental details are given in Materials and Methods.

in intensity with heating time as the monomer peaks decreased. There was also material of higher molecular weight that eluted close to the void volume (ca. 14.5 min). Some of the material from the peaks was collected and electrophoresed in the 1D SDS-PAGE system, and the gel pattern is shown as an inset in Figure 7. It is clear that the eluate collected from near the 19 min peak on the chromatogram contained a mixture of dimeric and trimeric α-La, demonstrating that these polymers can be isolated from the heat-treated mixture.

It also demonstrates that SE-HPLC can be used for quantitation of the mixture at the pH of the elution buffer. In this case the pH was 6.9, and thus, the dimers and trimers seen by SDS-PAGE analysis of the eluted fraction (Figure 7, inset) exist as such at pH 6.9. Consequently, it is likely that the various species observed in the alkaline-PAGE patterns existed in the same state of aggregation at pH 6.9 and at about pH 8.9. However, in the heat-treatment medium, viz. WPC permeate, the state of aggregation could have been greater because of the presence of calcium.

DISCUSSION

In this study, we used electrophoresis to characterize the protein products formed during heating of a 1:1 (w/w) mixture of α-La and BSA in a WPC permeate buffer system. The advantages of this system are that the α-La is in the neutral pH, calcium-loaded form that would be expected to exist in milk and milk products and that the two proteins are well separated electrophoretically so that the α-La dimers and trimers, with molecular

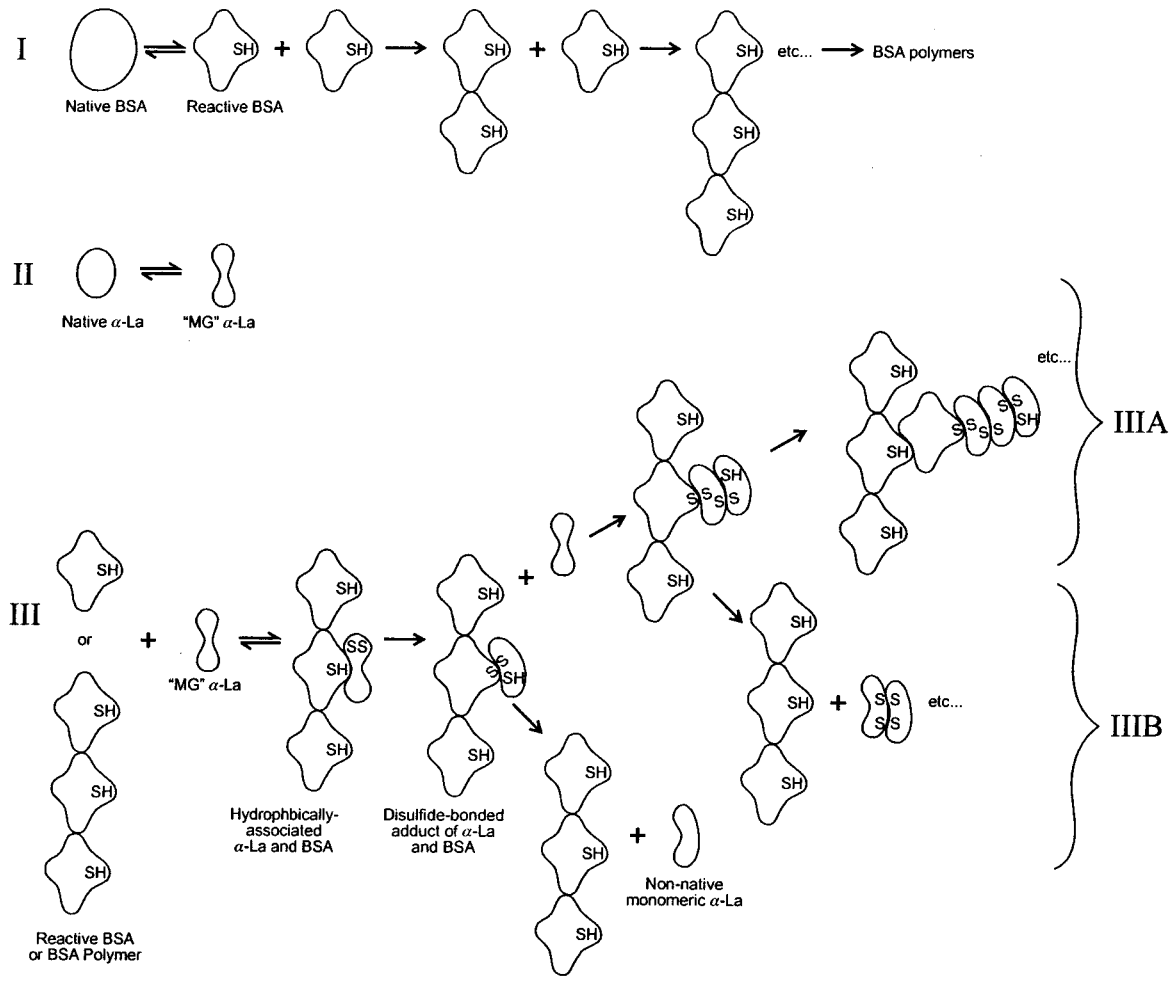

Figure 8. Schematic presentation of a possible mechanism for the formation of α-La dimers, trimers, etc. in a heated solution containing a mixture of α-La and BSA.

weights of 29 and 43 kDa, respectively, are reasonably well separated from BSA (molecular weight 66 kDa). Although the intermediate protein aggregates obtained after heating BSA solutions have not been studied in detail, a number of PAGE studies have been made on β-Lg, which is also a protein with a single cysteine residue that is inaccessible to low molecular weight reagents in the native protein. Manderson et al. (1998) used 2D PAGE to show that when β-Lg A at a concentration of ~3 mg/mL was heated at pH 6.7 at temperatures near 80 °C, a portion of the protein material that was monomeric under SDS-PAGE conditions did not behave as a nativelike monomer under alkaline-PAGE conditions. There appeared to be two additional species: unfolded monomeric β-Lg and noncovalently associated aggregates. (The latter was previously reported by McSwiney et al. (1994b) and Gezimati et al. (1996a,b, 1997) as existing in heated 10% β-Lg solution.) In one clear instance (Manderson et al., 1998), aggregates that appeared to be β-Lg trimers in alkaline-PAGE became a mixture of dimers and monomers in SDS buffers. Similarly, tetramers were dispersed into a mixture of covalently bonded dimers and tetramers. However, neither dimers dissociated into monomers to any great extent nor tetramers dissociated into monomers and trimers. It is tempting to suggest that BSA behaves in a fashion similar to that of β-Lg, giving rise to various dimers, trimers, etc. However, we did not identify such aggregates (Figure 4B) partly because the resolving gel pore size in the SDS-PAGE system we used was too small for good resolution of these large molecular aggregates and partly because the original protein preparation contained a number of contaminants. In addition to the disulfide-bonded aggregates in the heat-treated sample, a quantity of SDS-monomeric BSA was dissociated from the aggregates by SDS (Figures 4A, 5A, and 6A). This was, however, a small proportion of the quantity that was released from the aggregates by the combined action of SDS and mercaptoethanol (Figure 5B). When the protein concentration was lowered (Figure 6), the proportion of SDS-monomeric BSA formed was lower. These effects were also found for β-Lg, with a higher proportion of SDS-monomeric β-Lg found in heat-treated 10% WPC than in 2% WPC and a higher proportion of unfolded monomers and dimers found in the lower concentration sample (Havea, 1998; Havea et al., 1998).

Chaplin and Lyster (1986) showed that when a 1.4% solution of α-La was heated at 100 °C for 10 min at pH 7.0 in 0.1 M phosphate buffer, a series of oligomers was formed and there was some loss of ammonia from the system possibly as a result of protein deamidation. Under our lower temperature conditions (pH ~6.8 in WPC permeate (which contained calcium, which affects the thermal transition temperature (Bernal and Jelen, 1984)) and 5% protein concentration at 75 °C for 6 min only a small loss of nativelike monomer was observed (Figure 4A).

Gezimati et al. (1997) listed many of the reactions that could occur in heated whey protein solutions

without distinguishing between BSA, β-Lg, or α-La. Gezimati et al. (1996b) also suggested that BSA formed polymers prior to the unfolding of either α-La or β-Lg at moderate (75 °C) heat-treatment temperatures. In a mixed BSA−α-La system they also suggested that α-La then probably unfolded and formed some sort of adduct with the BSA polymers. We can now speculate a little further than this on the basis of the present results and a greater understanding of the details of the heat-induced polymerization of β-Lg (Havea, 1998; Manderson et al., 1998), and a possible scheme is presented in Figure 8.

Reaction I gives a BSA−thiol-catalyzed polymerization reaction comparable with those outlined for β-Lg (e.g., McKenzie, 1971; Qi et al., 1997; Morgan et al., 1999) without the initial dimer to monomer dissociation but with the transformation of the native monomer to a conformationally different monomer with an accessible thiol. Two of these molecules can then react by way of a thiol-catalyzed disulfide bond interchange to form a dimer, then a trimer, etc. while retaining one accessible thiol per BSA monomer unit.

Reaction II is the reversible conformational changes induced by the heat treatment of α-La, seen using differential scanning calorimetry (Rüegg et al., 1977; Bernal and Jelen, 1985). The product of this reaction can be considered to be somewhat similar to the well-characterized molten globule state of α-La (Doi, 1993; Hirose, 1993) and can be called "molten-globulelike".

Reaction III is the reaction of the molten-globulelike α-La with a reactive BSA molecule or with BSA molecule(s) within larger aggregates. Initially, an adduct that can be dissociated by SDS is likely to form, particularly at high protein concentrations. Once this has undergone a disulfide bond interchange reaction, a free thiol is likely to become available from within the α-La molecule. (Cys^6 and Cys^{120} seem likely candidates (Legowo et al., 1996) because the Cys^6−Cys^{120} disulfide bond is under strain in the native protein (Gohda et al., 1995) and is preferentially reduced (Ikeguchi et al., 1998).) Once the reactive adduct is formed, there are at least two possible reactions that can occur, and these are depicted as reactions IIIA and IIIB in Figure 8. In reaction IIIA a second molten-globulelike α-La molecule adds to the first and continuation of this sequence will lead to a chain of disulfide-bonded α-La molecules. By contrast, reaction IIIB indicates that disulfide bond rearrangement within the reactive adduct could give rise to a non-native form of α-La. This suggests that the unfolded monomer species (Figure 2A) may not be intermediates between native and dimer α-La. In the case of β-Lg it is not clear whether unfolded monomers form or not, although the formation of unfolded monomer proteins is faster than the formation of the dimer (Schokker et al., 1999).

It should be noted that 1:1 BSA to α-La by weight is almost 1:5 in molar terms. (This weight ratio of 1:1 is less than that found in acid whey (∼1:3 to 1:5, depending on the analytical method) and acid WPC (∼1:5) (Elgar et al., 2000). It is likely that if 1:1 molar ratios (1:0.2 by weight) of the proteins had been used, the PAGE patterns would have been different but the overall conclusions would not have changed. However, this 1:0.2 ratio would have been even more distant from the "natural" molar ratio of 1:>15.

The mechanism shown in Figure 8 is also consistent with the finding that the formation of SDS-monomeric α-La was greater in the more concentrated solution (cf. Figures 5 and 6). Pathways IIIA and IIIB are alternatives, and the concentration of the first intermediate along the pathway, the hydrophobic adduct, is determined by its rate of formation, which is determined by the concentrations of thiol-accessible BSA and molten-globulelike α-La. Both of these are proportional to the concentrations of the precursors α-La and BSA. The loss of the hydrophobic adduct could be more complex than just the concentration and the rate of conversion to the "reactive adduct", which involves a thiol−disulfide bond interchange.

However, regardless of the mechanisms involved, it is clear that the presence of BSA in the mixture accelerated the formation of α-La polymers (Figures 5A and 6A) and hydrophobically associated α-La (Figures 5B and 6B).

ABBREVIATIONS USED

SDS, sodium dodecyl sulfate; 1D, one-dimensional; 2D, two-dimensional; PAGE, polyacrylamide gel electrophoresis; SE-HPLC, size-exclusion high-performance liquid chromatography; α-La, bovine α-lactalbumin; BSA, bovine serum albumin; β-Lg, bovine β-lactoglobulin; nativelike, protein that migrated indistinguishably from native protein in alkaline-PAGE; SDS-monomeric, protein that migrated indistinguishably from native protein in SDS-PAGE.

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