# α-Lactalbumin Enhances the Gelation Properties of Bovine Serum Albumin

Naotoshi Matsudomi,*,† Toru Oshita,† Kunihiko Kobayashi,† and John E. Kinsella‡

Department of Applied Biochemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753, Japan, and Department of Food Science, University of California, Davis, California 95616

The effects of addition of α-lactalbumin (α-La) to bovine serum albumin (BSA) on gelling properties were investigated. A BSA concentration of 4% (w/v) was required for formation of a self-supporting gel following heating at 80 °C for 30 min in 100 mM sodium phosphate buffer (pH 6.8). Solutions of α-La, even up to a protein concentration of 8% (w/v), did not gel under the same conditions. The addition of 3% α-La to 6% BSA caused a significant increase in gel hardness, and the gels thus obtained were transparent. BSA and α-La interacted to form soluble aggregates through thiol–disulfide interchange reaction during gel formation. The soluble aggregates formed during heating of the mixture of BSA and α-La had lower molecular weights than those formed from BSA alone. The enhancing effect of α-La on gel hardness of BSA was ascribed to the formation of a finer, more uniform gel matrix.

## INTRODUCTION

Whey proteins are useful as functional ingredients in the manufacture of processed foods (Kinsella, 1984; Kinsella and Whitehead, 1989). Gelation is one of the most important functional properties of proteins in food systems. The ability of gels to act as a matrix for holding water, lipids, sugars, flavors, and other ingredients is useful in food applications and in new product development (Kinsella, 1979). The mechanisms of gelation of whey proteins have been studied by many investigators (Schmidt, 1981; Dunkerley and Zadow, 1984; Mulvihill and Kinsella, 1987; Zirbel and Kinsella, 1988; Shimada and Cheftel, 1989; Rector et al., 1991). However, the mechanisms responsible for the formation of the three-dimensional network of whey protein are not fully understood because of the complicated interactions among the different proteins of whey. Whey protein consists of three major components, β-lactoglobulin (β-Lg), α-lactalbumin (α-La), and serum albumin (BSA). These proteins have different structures and molecular properties (Brunner, 1977) and possess different gelling properties (Mulvihill and Kinsella, 1988; Paulsson et al., 1986; Yasuda et al., 1986; Matsudomi et al., 1991b). It is possible that interactions among these proteins may significantly affect the gelling properties of whey protein.

We have demonstrated that α-La and β-Lg interact during heating and that the addition of α-La to β-Lg solutions markedly enhances the strength of the resultant thermal gels (Matsudomi et al., 1992). In this study the mechanism of the interactions between α-La and BSA which enhanced gel properties was determined.

## MATERIALS AND METHODS

**Protein and Chemicals.** Bovine serum albumin (BSA, Product No. A-9647), α-lactalbumin (α-La, Product No. L-6010), *N*-ethylmaleimide (NEM), and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical Co. (St. Louis, MO). Proteins were desalted by dialysis prior to use and freeze-dried. All other chemicals were of reagent grade.

**Method of Gelation and Measurement of Hardness of the Gel.** One-milliliter samples of the protein solutions in 0.1 M sodium phosphate buffer (pH 6.8, heating buffer) were placed into a Sigmacoated tube (6.0 mm in diameter) and heated at 80 °C for 30 min in a water bath. The hardness of the gel sections (5.0 × 6.0 mm) was then measured with a tensile tester (Tensilon UTM-II, Toyo Baldwin Co.) as described previously (Matsudomi et al., 1991a).

**Heat Treatment of Proteins.** Each protein solution (0.2% α-La, 0.2% BSA, and 0.2% α-La plus 0.2% BSA), in the heating buffer, was heated at 80 °C for 30 min in thin tubes as described previously (Matsudomi et al., 1987). NEM (5 mM) was added to protein solutions before heat treatment. Under these conditions, no gel coagulum was formed during heating.

**Gel Filtration by High-Performance Liquid Chromatography (HPLC).** Gel filtration of each heat-treated protein solution was done by HPLC (L-6000, Hitachi) on a TSK Gel G-3000SW column (Tosoh Co., 0.75 × 30 cm). A 20-μL portion of each protein solution was loaded on the column at a flow rate of 0.5 mL/min using the heating buffer as an eluent. A UV detector (L-4000, Hitachi) was used to monitor the effluent at 280 nm. The chromatogram was depicted by using a chromatointegrator (D-2500, Hitachi).

**Gel Electrophoresis.** SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was done in a slab gel made from 5% stacking gel and 20% separating gel according to the method of Laemmli (1970).

**Estimation of Molecular Weight of Soluble Aggregates.** Chromatograms on the TSK column of each heat-treated protein solution were detected by a low-angle laser light scattering photometer (LS-8, Toyo Soda Co.) and a precision differential refractometer (RI-8000, Toyo Soda Co.). The molecular weight of soluble aggregates was estimated from the ratio of the output of the LS photometer, $(\text{output})_{LS}$, to that of the refractometer, $(\text{output})_{RI}$, by eq 1 (Takagi and Hizukuri, 1984), where $K$ is a

$$\text{MW} = K(\text{output})_{LS}/(\text{output})_{RI} \quad (1)$$

constant depending on the instrumental and experimental conditions and is determined by using standard protein. Native BSA [molecular weight (MW), 67 000] was used as a molecular weight standard.

The weight-average molecular weight of the soluble aggregates was determiend by eq 2 (Takagi and Hizukuri, 1984), where

$$\text{MW} = K(\text{area})_{LS}/(\text{area})_{RI} \quad (2)$$

$(\text{area})_{LS}$ and $(\text{area})_{RI}$ are the total areas in the elution peak of the LS photometer and the refractometer, respectively.

**Measurement of Molecular Weight Distribution.** Chromatograms of the soluble aggregates make it possible to estimate the molecular weight of the fraction eluted at a particular

* Author to whom correspondence should be addressed.
† Yamaguchi University.
‡ University of California.

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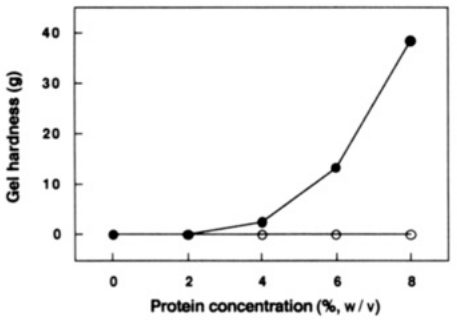


**Figure 1.** Effects of protein concentration on gel hardness. Gels were formed by heating α-La (○) and BSA (●) at 80 °C for 30 min.

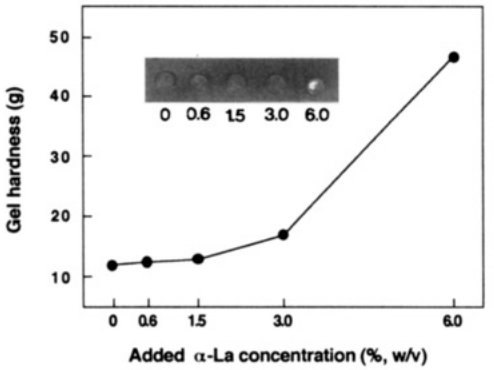


**Figure 2.** Hardness and appearance of gels caused by heating mixtures of BSA and α-La at 80 °C for 30 min. Gels were made from the mixture of 6% BSA and α-La with various concentrations.

retention time from eq 1 and its relative population from the output of the refractometer. The molecular weight distribution curves of soluble aggregates were drawn according to eq 3 (Kato

$$f(\mathrm{M}) = k(\mathrm{output})_{\mathrm{RI}} \tag{3}$$

and Takagi, 1987), where $f(\mathrm{M})$ is a weight fraction of the component with a particular molecular weight, $k$ is a constant, and $(\mathrm{output})_{\mathrm{RI}}$ is the output of refractometer when the component was eluted. The distribution curves were subsequently normalized according to eq 4 (Kato and Takagi, 1987).

$$k\int_0^\infty (\mathrm{output})\, \mathrm{dM} = 1 \tag{4}$$

## RESULTS AND DISCUSSION

**Effect of Protein Concentration on Gel Hardness.** The heat-induced gelation of globular proteins is dependent on the experimental conditions (Hegg, 1982; Rector et al., 1989). The hardnesses of the gels formed by heating BSA and α-La at different protein concentrations were measured (Figure 1). The BSA gels were transparent, had a smooth texture, and exhibited good water-holding capacity with little syneresis. The hardness of BSA gels increased exponentially with increasing protein concentrations, and a BSA concentration of 4% (w/v) was required for the formation of a self-supporting gel. The α-La did not gel under these conditions even up to a protein concentration of 8% (w/v).

**Effect of α-La on Hardness of Gels.** The addition of α-La to BSA enhanced gel hardness. A mixture of 6% BSA with various concentrations of α-La was heated for 30 min at 80 °C, and the hardness and appearance of gels formed were measured (Figure 2). Addition of α-La above 3% caused a remarkable increase of gel hardness of α-La/BSA mixtures, although the α-La itself did not form gels. Gels made from the mixture of BSA and α-La remained transparent (Figure 2). The addition of α-La to BSA caused gel hardness to increase without loss in transparency, reflecting the interaction of these proteins during gelation.

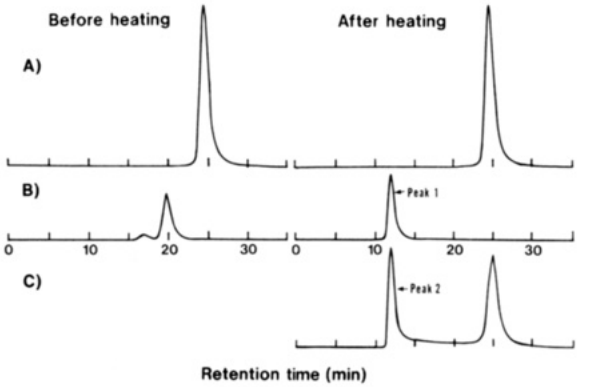


**Figure 3.** HPLC elution patterns of products formed by heating α-La (A), BSA (B), and a mixture of α-La and BSA (C) at 80 °C for 30 min. Each heat-denatured protein solution (0.2% α-La, 0.2% BSA, and 0.2% α-La plus 0.2% BSA) was analyzed by HPLC on TSK Gel G-3000SW (0.75 × 30 cm), at a flow rate of 0.5 mL/min of the elution buffer of 0.1 M sodium phosphate buffer, pH 6.8. Elution from the column was monitored at 280 nm.

**Heat-Induced Interaction of BSA with α-La.** Soluble aggregates of polymerized molecules were formed during the early stages of heat-induced gelation of proteins, and subsequent polymerization results in the formation of a rigid gel network (Nakamura et al., 1986; Kitabatake et al., 1989). Therefore, such gelling properties observed in the mixture of BSA and α-La may have been due to the formation of a specific aggregate by the interaction between BSA and α-La. The formation of soluble aggregates in heated solutions of individual and mixed BSA and α-La was analyzed by gel filtration on HPLC (Figure 3). Heating (30 min at 80 °C) of the α-La solution (0.2%) did not show any change in elution patterns (Figure 3A). The BSA solution (0.2%) formed soluble aggregates having a retention time of 12 min with the disappearance of the monomer (Figure 3B). In the case of the mixture of BSA (0.2%) and α-La (0.2%), the peak area of soluble aggregate increased in proportion to the decrease in the peak area of α-La monomer (Figure 3C). This suggested that BSA and α-La interact upon heating to form soluble aggregates. The soluble aggregates obtained by heating BSA alone (peak 1) and the mixture of BSA and α-La (peak 2) were collected separately and analyzed by SDS–PAGE (Figure 4). The protein in peak 1 did not enter the gel and, in the absence of 2-mercaptoethanol (ME), remained on top of the stacking gel, while this protein was dissociated almost completely into the BSA monomer in the presence of ME (Figure 4A), suggesting that the soluble aggregate had been polymerized by disulfide bonds. Most of protein in peak 2 remained on top of the separating gel in the absence of ME and was dissociated into the monomers of BSA and α-La after reduction with ME (Figure 4B), indicating that BSA and α-La interact to form soluble aggregates through thiol–disulfide interchange reaction during heating. On the other hand, when the BSA solution (0.2%) and the mixture of BSA (0.2%) and α-La (0.2%) were heated independently in the presence of NEM (5 mM), which blocks thiol groups, soluble aggregates having a retention time of 12 min were formed, respectively, as well as the aggregates without NEM (data not shown). These soluble aggregates were analyzed by SDS–PAGE with and/or without ME (Figure 5). In the SDS–PAGE, without ME, the soluble aggregate formed from BSA solution in the

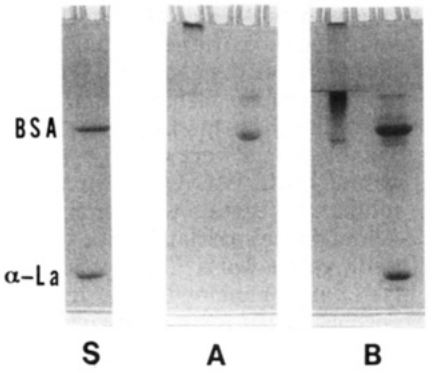


**Figure 4.** SDS–PAGE of soluble aggregates formed from a BSA solution and a mixture of α-La and BSA following heating at 80 °C for 30 min. S, standard solution containing α-La and BSA; A, aggregate from BSA solution (peak 1); B, aggregate from mixture of α-La and BSA (peak 2). The right lane of each slab gel was treated with ME.

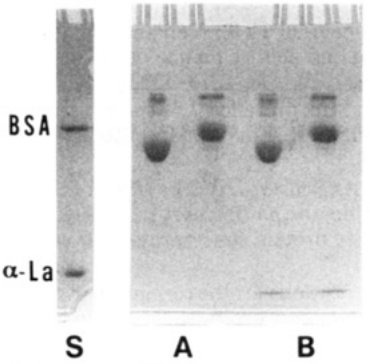


**Figure 5.** SDS–PAGE of soluble aggregates formed from a BSA solution and mixture of BSA and α-La in the presence of NEM following heating at 80 °C for 30 min. S, standard solution containing α-La and BSA; A, aggregate from BSA with NEM; B, aggregate from mixture of α-La and BSA with NEM. The right lane of each slab gel was treated with ME.

presence of NEM was dissociated to the monomer by SDS alone. The soluble aggregates formed from the mixed system were also dissociated in the BSA monomer by SDS alone, and in the presence of ME, no α-La was detected on the slab gel. This result indicates that α-La had not been incorporated into the soluble aggregates formed from the mixture of BSA and α-La in the presence of NEM. The intensity of protein bands of these soluble aggregates on SDS–PAGE was identical with that obtained before and after reduction with ME treatment, although the electrophoretic mobility of BSA decreased because of an increase of the hydrodynamic radius of the protein by cleavage of intramolecular disulfide bonds (Wang and Damodaran, 1990). The results indicated that in the presence of NEM these soluble aggregates formed from the BSA solution and the mixture of BSA and α-La represent products formed by noncovalent interactions between the BSA molecules. The results (Figures 4 and 5) indicate that the interaction between BSA and α-La, induced by heating, is mainly caused by disulfide cross-links. BSA has 1 thiol group and 17 disulfide bonds per monomer (Spencer and King, 1971); α-La has no thiol group and 4 disulfide bonds per monomer (Brew et al., 1970). The thiol group in the BSA molecule would easily participate in reduction of the disulfide bond in the α-La molecule following heating (30 min at 80 °C) which is above the thermal transition point for BSA (65 °C) and the heat denaturation temperature of α-La (77 °C) at pH 6.65 (Larson and Rolleri, 1955). Thus, it is suggested that such a thiol–disulfide interchange reaction between BSA and

**Table I. Apparent Molecular Weight of Soluble Aggregates Formed by Heating**

| protein system | MW (×10⁶) | protein system | MW (×10⁶) |
|---|---|---|---|
| BSA | 2.5 | NEM-modified[a] | |
| BSA + α-La | 1.6 | BSA | 4.6 |
| | | BSA + α-La | 4.8 |

[a] The BSA solution and the mixture of BSA and α-La were heated for 30 min at 80 °C in the presence of *N*-ethylmaleimide (5 mM).

α-La enhances the formation of soluble progel aggregates. On the other hand, to determine whether hydrophobic interactions contribute to the formation of soluble aggregates formed from the mixture of BSA and α-La, the soluble aggregate fraction eluted by gel filtration on HPLC was treated with 0.5% SDS and then analyzed using the elution buffer (0.1 M sodium phosphate, pH 6.8) containing 0.1% SDS. The HPLC elution patterns of the soluble aggregate in the presence of SDS were measured by light scattering (LS), refractive index (RI), and absorption at 280 nm, respectively. The peak area of the soluble aggregate detected with those detectors did not show any change in the presence of 0.1% SDS, and a significant change in the apparent molecular weight average of the soluble aggregate was not observed (data not shown), suggesting that hydrophobic interactions were not involved in the formation of soluble aggregates formed from the mixture of BSA and α-La. Thus, it was concluded that the soluble aggregates had been formed only by disulfide cross-links between BSA and α-La.

From their mobility on SDS–PAGE without ME (Figure 4A,B), the molecular size of the soluble aggregate obtained from the mixture of BSA and α-La was different from those obtained by heating BSA alone. The apparent molecular weight average of these soluble aggregates was determined from the ratio of the peak area of light scattering (LS) to that of the refractive index (RI), as described by Takagi and Hizukuri (1984). The apparent molecular weights of soluble aggregates formed from the BSA alone and the mixture of BSA and α-La in the presence and absence of NEM on heating are given in Table I. The apparent molecular weights of heat-induced aggregates formed from the BSA alone were about 1.5 times those obtained from the mixture of BSA and α-La, which was consistent with the difference in the mobilities of these aggregates on SDS–PAGE, without ME (Figure 4A,B). Apparently BSA and α-La interact to form soluble aggregates through disulfide bond formation during heating, and these are smaller than those formed by heating BSA alone. The apparent molecular weights of soluble aggregates obtained from the BSA alone and the mixture of BSA and α-La, in the presence of NEM, were much greater than those of the aggregates formed without NEM, indicating that the apparent molecular weights of disulfide-induced aggregates were smaller than those of aggregates formed by noncovalent bondings. These results suggest that polymerization of BSA itself in the presence of α-La might be limited by disulfide bond formation between BSA and α-La. The addition of NEM greatly decreased the hardness of BSA gels (Matsudomi et al., 1991b), reflecting the importance of disulfide bonds. Kato et al. (1990) have reported a correlation between decreases in the molecular weight size of soluble aggregate and increases in the gel hardness of dry-heated dried egg white. On the basis of these studies, a decrease in the size of aggregates following the addition of α-La to BSA solutions may play an important role in the formation of stronger, more stable gels by providing a finer gel network.

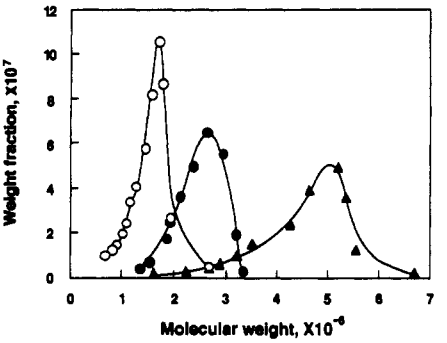


**Figure 6.** Molecular weight distribution curves of soluble aggregates formed by heating BSA (●), mixture of BSA and α-La (○), and BSA with NEM (▲) at 80 °C for 30 min.

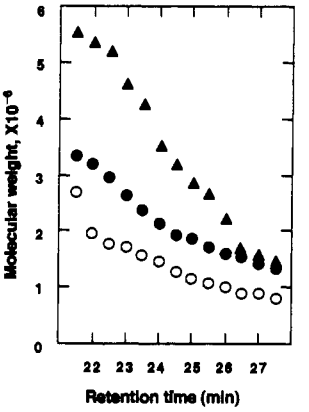


**Figure 7.** Relationship between retention time and molecular weight of soluble aggregates formed by heating at 80 °C for 30 min. Symbols are as in Figure 6.

The molecular weight distribution curves of heat-induced aggregates were derived from the elution patterns of the aggregates monitored by LS and RI (Kato and Takagi, 1987). The molecular weight distribution curves thus obtained are shown in Figure 6. The curve of aggregate from BSA alone showed a wide distribution of molecular weights. In the mixture of BSA and α-La, the curve of the aggregate indicated a narrow molecular weight distribution which shifted toward lower molecular weights as compared to the pattern of aggregate from BSA alone, suggesting a restricted progress of aggregation and the formation of more uniform strands. The curve of aggregate formed from BSA alone in the presence of NEM gave a wide distribution of molecular weights and shifted toward high molecular weights, compared with the pattern obtained without NEM.

The nature of soluble aggregates was estimated by plotting the relationship between molecular weight and retention time derived from the elution patterns of heat-induced aggregates monitored by LS and RI, because the retention time on the gel filtration column reflects the size and mode of aggregate fraction eluted at a given retention time (Figure 7). There was a remarkable shift to shorter retention times for the soluble aggregates formed from the mixture of BSA and α-La. At a given retention time, the molecular weights of heat-induced aggregate formed from the mixture of BSA and α-La shifted to smaller molecular weight as compared with those formed from BSA alone. This suggests that heating a mixture of α-La and BSA caused the formation of aggregate having a less compact structure, as compared to the soluble aggregate formed from BSA alone. The BSA solution heated in the presence of NEM formed more compact soluble aggregates compared to those formed without NEM. Kato et al. (1990) have reported a correlation between decreases in molecular weight size of the soluble aggregate and increases in the gel hardness of dry-heated dried egg white and that the expanded structure of heat-induced soluble aggregate formed from the dry-heated dried egg white might be responsible for the formation of a strong and stable gel network which holds an adequate amount of water. In this study, the expanded structure of uniform soluble aggregate formed from the mixture of BSA and α-La might be correlated with the formation of a firm and stable gel network. Thus, it is concluded that the effect of α-La in enhancing the hardness of BSA gels reflects the formation of a finer, more uniform gel matrix made from BSA and α-La molecules.

## ACKNOWLEDGMENT

Supported in part by a grant from the National Dairy Board.

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Received for review May 18, 1992. Revised manuscript received October 26, 1992. Accepted April 5, 1993.