Heat-induced Transparent Gel Formation of Bovine Serum Albumin

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The formation of transparent gels by 6% bovine serum albumin (BSA), pH 7.5, was examined by one- and two-step heating methods. Heating of the BSA solutions at various NaCl concentrations produced transparent gels at 25—50 mM NaCl and transparent sols at 0—20 mM NaCl (one-step heating method). The transparent sol obtained by heating without NaCl was reheated after mixing with various amounts of NaCl (two-step heating method I). The result was almost identical to that obtained by the one-step heating method. However, when the first heating was done with 10 mM NaCl, transparent gels were obtained over a wide range of NaCl concentrations with a second heating (two-step heating method II). Analyses of gels obtained at various NaCl concentrations by gel permeation chromatography and transmission electron microscopy showed the presence of linear polymers in the heated BSA sol (10 mM NaCl) and gel networks formed by the linear polymers (20 mM NaCl). The mechanism of transparent gel formation in BSA may be similar to that in ovalbumin.

The ability to form a gel is an important property of food proteins. Many studies have been done on protein gelation. However, the mechanism by which heat induces gel formation in globular protein molecules is still not clear.

In previous papers, we showed that heating ovalbumin solutions produces transparent or turbid gels depending upon the medium, pH, ionic strength, and protein concentration. Transparent ovalbumin gel is obtained by heating of ovalbumin solutions at pHs distant from the isoelectric point and at certain salt concentrations (one-step heating method). On the other hand, transparent gels can be obtained over a wide range of salt concentrations by the two-step heating method. In this method, the ovalbumin solution is first heated without salt, which produces a transparent sol. After cooling, salt is added to the solution, and then the mixture is heated. We have previously suggested a model for the formation of transparent gels. Essentially, ovalbumin molecules are believed to aggregate to form linear polymers after being heated at a low ionic strength. Then, after the second heating in the presence of salt, these polymers associate to form gel networks. In this paper, we hope to demonstrate the general applicability of our model to another protein besides ovalbumin: i.e. bovine serum albumin (BSA). The ability of BSA to form gels has been previously reported.

In these experiments, transparent gels of BSA were prepared by the one- and two-step heating methods. The existence of linear polymers in heated BSA solution was examined by transmission electron microscopy (TEM).

Materials and Methods

BSA. BSA (fraction V) was purchased from Sigma Chemical Co. (St. Louis, U.S.A.). The BSA was thoroughly dialyzed against distilled water containing 0.02% (w/v) NaNO₃ and 0.1 mM EDTA at 5°C. The protein concentration was measured using the absorbance at 280 nm, based on $E_{1%}^{1cm} = 6.67$. The pH of the dialyzed solution was adjusted by the addition of 1 N HCl or 1 N NaOH.

Gel preparation. Based on preliminary experiments, the BSA concentration was chosen as 6%, and pH as 7.5 except as otherwise noted. Three heating methods were used: the one-step heating method, and two-step heating methods I and II. In the one-step heating method, 6% BSA solution containing 0—500 mM NaCl was put into glass tubes (5 × 120 mm (Fig. 1) or 10 × 120 mm (Fig. 2)) and heated for 20 min at 85°C. In the two-step heating methods, 6% BSA solution without NaCl (method I) or with 10 mM NaCl (method II) was heated for 20 min at 85°C. After cooling to 20°C, 1—5 mM NaCl solution was added to the solution to make various concentrations, and the mixtures were reheated. The formation of BSA gel was judged by placing a steel ball (φ = 3 mm, 0.25 g) on the top of the sol or gel.

Measurement of gel hardness. BSA gel was prepared in a stainless cup (25 mm i.d. × 6 mm height) which was sealed with a rubber sheet, glass plate, and clippers. The gel hardness was measured with a Rheonner RE-3305 (Yamaden Co., Ltd., Tokyo) by using a plunger 8 mm in diameter. The cup containing the gel was set on the instrument, and moved upward at a rate of 5 mm/s until the gel was compressed by 3 mm.

Measurement of gel turbidity. Gel was prepared in a special test tube (11 mm × 100 mm). The turbidity was measured using the absorbance at 600 nm by applying the test tube directly to the photometer.

Gel permeation chromatography. Gel permeation chromatography was done on a high-pressure liquid chromatograph (Shimadzu LC-5A) with a TSK Gel G 4000SW column (7.5 × 600 mm, Toyo Soda Co.) as described by Koseki et al.

Transmission electron microscopy (TEM). The heated 6% BSA solution containing 0—20 mM NaCl was diluted 1500-fold with 4 mM sodium phosphate buffer, pH 7.5, and 1% BSA containing 500 mM NaCl was diluted 200-fold with the same buffer containing 500 mM NaCl. These solutions were negatively stained with 2% potassium phosphotungstate on a carbon-coated grid, and observed with a Hitachi H-700H transmission electron microscope operating at 100 kV.

Results

Effects of pH on gel formation

The effects of pH on gel formation in 6% BSA solution in the absence of salt were examined by the one-step heating method (Fig. 1). Gels formed between pH 4.0 and 6.5. The gels were transparent (pH 4.0 and 6.5), translucent (pH 4.5

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Fig. 1. Effects of pH on Heat-induced Gel Formation in 6% BSA Solution in the Absence of NaCl by the One-step Heating Method.
BSA solution was adjusted to various pHs by the addition of 1 M NaOH or 1 M HCl and heated at 85°C for 20 min. A steel ball in each tube indicates whether BSA gelled or not.

Fig. 2. Effects of NaCl Concentration on Heat-induced Gel Formation of 6% BSA Solution, pH 7.5, by One-step Heating Method.
BSA solutions containing various concentrations of NaCl were heated at 85°C for 20 min. A steel ball in each tube indicates whether BSA gelled or not.

and 6.0), and turbid (pH 5.0 and 5.5). Turbid gels were obtained at a pH near the isoelectric point (pI) of BSA (5.2), and transparent gels were obtained at acidic or basic pH somewhat distant from the pI. The transparent solutions were obtained at pH quite distant from the pI. These results are compatible with those of other researchers.4,6,9,13,17

Effects of NaCl concentration
Gel formation by heat-denatured proteins is controlled by the balance between hydrophobic interactions and electrostatic repulsive forces. The effects of salt concentration on gel formation by BSA were examined by the one-step heating method (Fig. 2). No gel was formed at NaCl concentrations of 20 mM or below. Gels were obtained at NaCl concentrations of 25 mM or above: transparent gels (25–50 mM NaCl), translucent gels (100 mM NaCl), and turbid gels (200, 500 mM NaCl). These results were then compared with results obtained by two-step heating methods I and II.

Comparison of one-step heating method and two-step heating methods I and II
Transparent and viscous sol was obtained by the heating of 6% BSA solution containing 0–20 mM NaCl. These sols were examined for the two-step heating method. The sols containing 15 and 20 mM NaCl during first heating were difficult to mix concentrated NaCl solution due to their high viscosity. The sol containing 0 or 10 mM NaCl was reheated after mixing with various concentrations of NaCl (two-step heating methods I or II). The effects of NaCl concentration on the turbidity and hardness of BSA gels prepared by the one-step heating method (Fig. 3a) and those prepared by the two-step heating method I (Fig. 3b) were very similar. By both methods, transparent gels were obtained at low concentrations of NaCl (25–50 mM NaCl). Gel hardness

Fig. 3. Turbidity and Hardness of Gels Prepared by the One-step Heating Method and Two-step Heating Methods I and II.
(a) one-step heating method; (b) two-step heating method I; (c) two-step heating method II.

Fig. 4. Gel Permeation Chromatography of BSA Solution Heated without and with 10 mM NaCl.
TSK G4000 column was eluted with 20 mM Na-phosphate buffer, pH 7.0, at 20°C. (a) native solution; (b) heated solution in the absence of NaCl; (c) heated solution in the presence of 10 mM NaCl.
increased with increasing NaCl concentration and attained almost constant values with NaCl concentrations greater than 100 mM NaCl. Gel turbidity also increased with increasing NaCl concentration. Turbid gels were obtained at NaCl concentrations above 200 mM. However, the increase of turbidity was slightly less with two-step heating method I than with the one-step heating method.

The effects of NaCl concentration on the hardness of gels prepared by two-step heating method II (Fig. 3c) were almost the same as those observed by the other two methods. However, the increase of turbidity with increasing NaCl concentration was less than those observed with the other two methods. Transparent or translucent gels were obtained over a wide range of NaCl concentration by two-step heating method II.

Two-step heating method II was superior to two-step heating method I as a method preparing transparent gels. The two procedures differed only in the NaCl concentration during the first heating. We believed that this difference in the salt concentration affected the degree of polymerization of heat-denatured BSA molecules. Therefore, we examined the effects of salt concentration on polymerization using gel permeation chromatography and electron microscopy.

**Fig. 5. Transmission Electron Micrographs of Heated BSA Solution.**
The heated 6% BSA solutions containing 0 mM NaCl (a), 10 mM NaCl (b), and 20 mM NaCl (c) were diluted 1500-fold and treated as described in Materials and Methods. The heated 1% BSA solution containing 500 mM NaCl (d) was diluted 200-fold and treated similarly.
Gel permeation of heat treated BSA

Figure 4 shows the elution profiles of native BSA (a), and heat-treated BSA without salts (b), and with 10 mm NaCl (c). Native BSA solution showed two peaks corresponding to the monomer (large peak) and dimer (small peak) of BSA. No such peaks were observed in the curves of Figs. 3b and c. All proteins were present as polymers. The molecular dimensions of polymers were higher in the solution heated with 10 mm NaCl (c) than in the solution heated without NaCl (b). A similar result was obtained by TEM analysis.

Electron microscopy of heated BSA

Figure 5 shows the photographs of heat-treated BSA solutions at different NaCl concentrations. In the absence of NaCl (Fig. 5a), short, rod-like polymers were observed. With 10 mm NaCl (Fig. 5b), long, linear polymers were observed. With 20 mm NaCl (Fig. 5c), an association of linear polymers was observed which, indicated the formation of a gel network. The results show that heat-denatured BSA molecules connected to each other to form linear polymers at low NaCl concentrations. With increasing salt concentration, the length of the linear polymers increased and interactions between linear polymers also increased. At high salt concentrations (> 200 mm), it is believed that the formation of ordered fibrous polymers was interrupted and massive aggregates were formed (Fig. 5d).

Discussion

The heating of 6% BSA solution produced turbid gels at pHs near the pI of BSA, transparent gels at acidic or alkaline pHs slightly distant from the pI and transparent sols at pH quite distant from the pI (Fig. 1). This general tendency is very similar to that obtained by heat-induced gel formation of ovalbumin. 8)

The effects of salt concentration on the turbidity and hardness of BSA gels prepared by the one-step heating method were also similar to those observed in ovalbumin gels. However, one apparent difference between BSA and ovalbumin gels is the degree of hardness they attain. The maximum hardness of 6% BSA at pH 7.5 was about 600 g/cm², while that of 5% ovalbumin at pH 7.5 was 40 g/cm², which had been measured under the same conditions as in this experiment. This difference is thought to be due to the dissimilar molecular properties of the two proteins. However, further experimentation is required to elucidate the actual cause of this difference.

The two-step heating method produced slightly different results for the gel turbidity of BSA and ovalbumin. For ovalbumin, after an initial heating without NaCl, a second heating produced transparent gels over a wide range of NaCl concentrations. However, for BSA, initial heating had to be done with 10 mm NaCl to obtain results after the second heating similar to those observed with ovalbumin (Figs. 3b and c). The difference of conditions suitable for the two-step heating method between the two proteins might be due to the difference of surface hydrophobicity and charge distribution at their denatured states. Gel permeation chromatography (Fig. 4) and electron microscopy (Fig. 5) were used to explain why 10 mm NaCl was required for the first heating of BSA. It is supposed that linear polymers of heat-denatured proteins were formed during the first heating in the absence of salt. Three-dimensional gel networks resulting from the association of these linear polymers were formed during the second heating in the presence of salt. However, the linear polymers that were formed in BSA in the absence of salt were not long enough to produce gel networks (Figs. 4b and 5a). The sol containing long, linear polymers was obtained by the first heating with 10 mm NaCl (method II). This sol produced transparent or translucent gels after a second heating over a wide range of NaCl concentrations. Thus, the molecular model of the formation of transparent gels as a network of linear polymers can be used for BSA gels as well as ovalbumin gels. 8,20) The same model has been used for the formation of hen egg lysozyme gels. 21)

The presence of linear polymers in BSA gels has been reported by Barbui and Joly 22) and Clark et al. 17) However, our results (Figs. 5b and 5c) provide a clearer image of linear polymers than the previous reports and show the role of these polymers in transparent gel formation.

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