Mechanism of Heat-Induced Gelation and Gel Properties of Soybean 7S Globulin

Takashi NAKAMURA, Shigeru UTSUMI* and Tomohiko MORI*

Hohnen Oil Co., Ltd., 1-2-3, Otemachi, Chiyoda-ku, Tokyo 100, Japan

** Research Institute for Food Science, Kyoto University,*

Uji, Kyoto 611, Japan

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The gelation of 7S globulin when heated at 100°C and 0.5 ionic strength was investigated. The minimum protein concentration required for gel formation was 7.5%. The minimum heating times with protein concentrations of 7.5% and above 10% were 20 min and 15 sec, respectively. The hardness of gel formed above 12% protein concentration became maximum after 15 sec heating and then remained constant on subsequent heating. The gels were transparent. The gelation mechanism was presumed to be as follows. Soluble aggregates with molecular weights of around 1 million, the shapes of which are not uniform, are formed at first which then associate with each other randomly to form a cluster. Finally a gel, the structure of which seems to comprise aggregates of clusters, is formed. No SH/S-S exchange reaction participates in this process. These results show that the gel properties and gelation mechanism for 7S globulin are different from those for 11S globulin.

The application of soybean proteins as ingredients of foods depends on their useful functional properties.^{1,2)} The gel forming ability induced on heating of soybean proteins is one of the most important functional properties with respect to their usage in food systems. Elucidation of the mechanism of gelation and the relationship between the physicochemical properties of the gel and the structure of soybean proteins will facilitate the further adoption of soybean proteins in foods. In order to elucidate them, since soybean proteins are composed of four main components, 2S, 7S, 11S and 15S globulins, gelation studies on each component are firstly required and then some on mixtures of them must be performed step by step on the basis of the results for the individual components. Accordingly, we investigated the gelation of 11S globulin, which is one of the most predominant components, and elucidated the fundamental mechanism of the gelation and the contributions of the constituent subunits of 11S globulin to the gelation and gel properties.^{3~10)} Thermal gelation of 11S globulin

is regarded as a three-stage process, *i.e.*, aggregation of 11S globulin molecules to form strands, interaction of strands to form a gel network, and finally an increase in gel hardness involving concurrent stabilization of the gel network structure.^{3,5,6,9,10)}

On the other hand, most gelation studies on 7S globulin have been phenomenastic.^{11,12)} Babajimopoulos *et al.*¹³⁾ and Utsumi and Kinsella¹⁴⁾ demonstrated that hydrogen bonding is the most dominant of the chemical interactions between proteins that lead to the formation of the 7S globulin gel. However, the gelation mechanism and the structural factors contributing to the gelation of 7S globulin are not well known at present.

To elucidate the gelation mechanism of 7S globulin, it is necessary to investigate the dissociation-association reaction which occurs during the course of gel formation as was done in gelation studies on 11S globulin.^{3,6)} In this study, we determined the protein concentration and heating time required for gelation of 7S globulin compared with those for 11S globulin. Also, we presented the presumed

mechanism of heat-induced gelation of 7S globulin on the basis of the results of investigations with respect to the association behavior in the gelation process.

MATERIALS AND METHODS

Materials. Sepharose 6B and DEAE-Sephadex were purchased from Pharmacia Co., Ltd. Sodium dodecyl sulfate (NaDodSO₄), extra pure reagent, was obtained from Wako Pure Chemicals (Japan). 2-Mercaptoethanol (ME), extra pure reagent, was obtained from Nakarai Chemicals (Japan). Other chemicals were of guaranteed reagent grade. Soybeans (*Glycine max*, var. Tsuru-no-ko) were purchased from Mizuno Seed Co., Ltd. (Japan).

Preparation of 7S and 11S globulins. Acetone powder of a crude extract of soybeans was prepared from dry seeds as described previously.¹⁵ Crude 7S and 11S globulin fractions were prepared from the acetone powder according to the method of Thanh *et al.*¹⁶ The 7S globulin fraction was purified further on a Sepharose 6B column (1.6 × 100 cm) equilibrated with 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl, 10 mM ME and 0.02% NaN₃. The 11S globulin fraction was purified further on a DEAE-Sephadex column as described previously.¹⁵ The purities of the 7S and 11S globulins thus obtained were more than 95%.

Method of gelation and determination of turbidity and hardness of gels. Twenty-microliter aliquots of a protein solution in 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl (heating buffer) were taken up into micropipettes (Drummond Scientific Co., 200 μ l), followed by heating at 100°C for various times in a water bath. The turbidity of the gels formed in the micropipettes was measured by scanning as such with a Shimadzu dual-wavelength chromatoscanner, Model CS-910. The hardness of the gels was measured with a texturometer (General Food Corp., GXT-2). The details of the procedures were given in previous papers.^{4,5}

Heating of 7S globulin. 7S globulin solutions in the heating buffer were heated at 100°C in thin test tubes (50 μ l and 100 μ l per tube for 7.5% and 0.5% protein solutions, respectively) as described previously.³

Sucrose density gradient centrifugation. The heat-treated 7S globulin solutions were centrifuged at 20°C in a 12 ml 15~40% (w/v) linear sucrose gradient in the heating buffer at 152,000 × *g* for 60 min in a Hitachi RPS 40T rotor. After centrifugation, the gradient was examined for absorbance at 280 nm with an ISCO density gradient fractionator. For sucrose gradient centrifugation, 2.5 mg of protein was used per experiment.

Electrophoreses. Polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Davis¹⁷ as described previously.¹⁸ NaDodSO₄-PAGE was performed according to the method of Laemmli¹⁹ in the absence of ME. For PAGE and NaDodSO₄-PAGE, 100 μ g and 50 μ g of protein were used, respectively.

Electron microscopy.

i) **Negative staining.** A small drop of a protein solution was applied to a carbon-coated electron microscope grid, stained with potassium phosphotungstate and then examined under a Hitachi H-700H electron microscope.

ii) **Thin sectioning.** Small gel pieces were fixed, dehydrated, embedded and sectioned. The thin sections were stained and then viewed under a Hitachi H-700H electron microscope.

The details of the procedures were given in a previous paper.⁶

Protein determination. Protein was determined by the method of Lowry *et al.*²⁰

RESULTS AND DISCUSSION

Gelation and gel properties

7S and 11S globulins at various concentrations were heated for appropriate times, and the minimum times required for the formation of self-supporting gels were determined, as shown in Fig. 1. The degree of gelation, *i.e.*, sol, gel-like but not self-supporting, and self-supporting gels, could be judged macroscopically. The lowest concentrations for the formation of self-supporting gels of the 7S and 11S globulins within 20 min heating were 7.5% and 2.5%, respec-

FIG. 1. Minimum Heating Time for Gelation as a Function of Protein Concentration.

Δ, 7S globulin; ○, 11S globulin.

FIG. 2. Effects of Heating Time and Protein Concentration on Gel Hardness of 7S and 11S Globulins. Triangles and circles denote the 7S and 11S globulins, respectively.

tively. In the case of 7S globulin, the times required for gel formation were 20 min and 15 sec for protein concentrations of 7.5% and more than 10%, respectively. On the other hand, in the case of 11S globulin, the higher the protein concentration, the shorter the time required for gel formation.

The effects of the heating time and the protein concentration on the gel hardness of the 7S and 11S globulins are shown in Fig. 2. The gel hardness of the 7S and 11S globulins increased with the protein concentration. Although the gel hardness of 11S globulin increased with the heating time and then reached a plateau, that of 7S globulin at a concentration of above 12% was the highest after 15 sec heating, which is the minimum heating time required for gel formation, and at 10% concentration it increased up to 1 min heating. In other words, the gel hardness of 7S globulin did not change with the heating time, indicating that the dependency of the gel hardness of 7S globulin on the heating time is low. The gel hardness of both globulins showed a dependency on the protein concentration.

7S and 11S globulins were heated to obtain gels, and then the turbidity of the gels was determined (Fig. 3). 11S globulin formed turbid gels and the turbidity increased with decreasing protein concentration.⁵⁾ On the other hand, 7S globulin formed a transparent gel at any protein concentration.

FIG. 3. Turbidity of Gel as a Function of Protein Concentration.

The symbols are the same as in Fig. 2. The closed triangles (dotted line) show that the 7S globulin solution does not form a gel, being in a sol state.

The results obtained here (Figs. 1~3) indicate that 7S globulin is different in gelation and gel properties from 11S globulin.

Gelation mechanism

To elucidate the gelation process in the case of 7S globulin, a protein concentration of 7.5% is preferable, because the gelling times at and above 10% are very short (15 sec), as shown in Fig. 1. The association-dissociation behavior of 7S globulin (7.5%) in the gel formation process was investigated by PAGE in comparison with the case of a protein concentration of 0.5%, at which 7S globulin does not form a gel (Fig. 4). In the case of 0.5% protein concentration (Fig. 4A), on heating for 15 sec and 1 min the 7S globulin solution gave only complexes which could not enter the separation gel, and on heating for 10 and 20 min it gave complexes which could not enter the stacking gel in addition to the complexes formed on heating for a shorter time. In the case of 7.5% protein concentration, the 7S globulin solution on heating gave both complexes which could not enter the separation and the stacking gels at all heating times, and the amount of the larger complexes was much greater than

FIG. 4. PAGE of 7S Globulin Solutions Heated for Various Times.

Protein concentrations: A, 0.5%; B, 7.5%. C denotes the native 7S globulin.

FIG. 5. NaDodSO₄-PAGE of 7S Globulin Solutions Heated for Various Times.

The symbols are the same as in Fig. 4.

that of the smaller complexes (Fig. 4B). The PAGE patterns were almost the same with any heating time. Since turbidity was not observed in the heated 7S globulin solutions (Fig. 3), the complexes which could not enter the stacking gel represent soluble aggregates formed on heat treatment at 100°C. The minimum molecular weights of the soluble aggregates which could not enter the separation and stacking gels are more than 1 million and a few millions, respectively, as judged from the concentrations of acrylamide used. With both protein concentrations, unchanged 7S globulin and dissociated subunits were scarcely observed in the separation gel regardless of the heating time. In other words, 7S globulin has a tendency to associate to form

FIG. 6. Sucrose Density Gradient Centrifugation of 7S Globulin Solutions Heated for Various Times.

Protein concentrations: A, 7.5%; B, 0.5%. The gradient was divided into regions I to III. Regions I and II correspond to the soluble aggregates with molecular weights of around 1 million and 4 millions, respectively. The arrows (a, b and c) show the sedimentation positions of the soluble aggregates with molecular weights of 1.8×10^6 , 4.0×10^6 and 8.0×10^6 , respectively, which were formed in the case of 11S globulin. Heating times: —, 0 min; ···, 15 sec; ---, 1 min; —·—, 10 min; — — —, 15 min (A) and 20 min (B).

soluble aggregates under the heating conditions employed here.

To examine the molecular forces contributing to the formation of soluble aggregates, the heated 7S globulin solutions were analyzed by NaDodSO₄-PAGE in the absence of ME (Fig. 5). Regardless of the protein concentration and the heating time, the heated 7S globulin gave only bands corresponding to the constituent subunits, α , α' and β , similar to in the case of the native 7S globulin. This clearly demonstrates that an SH/S-S exchange reaction is not involved in the formation of the soluble aggregates. Moreover, 7S globulin can form a gel even in the presence of N-ethylmaleimide which prevents the disulfide exchange reaction or ME which cleaves disulfide bonds (data not shown) in analogy with the case of heating at 80°C.¹³⁾ These available data suggest that noncovalent interactions are important in the formation

of a gel of 7S globulin.

Sucrose density gradient centrifugations were carried out to investigate in detail the association behavior of 7S globulin. As shown in Fig. 6, heating for 15 sec resulted in the formation of soluble aggregates (region I) with molecular weights of around 1 million, and no peak corresponding to the native 7S globulin or the dissociated subunits at both 0.5% and 7.5% protein concentrations was seen. However, the molecular weights of the soluble aggregates at 7.5% protein concentration are slightly higher than those at 0.5%. Further aggregation (regions II and III) is more remarkable at 7.5% than at 0.5%, as can be seen by the broadness of the peak of the soluble aggregates at 7.5%. Heating for more than 10 min caused the formation of soluble aggregates with molecular weights of around 4 millions (region II) with the concomitant disappearance of the aggregates with molecular weights smaller than 4 millions at both protein concentrations. This could be because of the continued increase in the size of soluble aggregates. However, though the formation of soluble macroaggregates distributed in region III was observed in the case of 7.5% protein concentration, such macroaggregate formation was not observed in the case of 0.5%. This may be why a gel is not formed at 0.5% protein concentration. The average molecular weight of the soluble macroaggregates may be more than 80 millions, as judged on comparison of their sedimentation positions with that of the molecular weight marker, TMV (data not shown). Hashizume *et al.*²¹⁾ demonstrated that soybean 7S protein (7S globulin) is converted to aggregates with sizes of 28S and 43S on heating at 0.1 ionic strength and pH 7.6. The soluble aggregates with molecular weights of 1×10^6 and 4×10^6 which were formed at 0.5 ionic strength, as described above, seem to be similar to these aggregates reported by Hashizume *et al.*²¹⁾

The structure of the soluble aggregates formed on 15 sec heating and the network structure of the gel at 7.5% protein concen-

FIG. 7. Transmission Electron Micrographs of Negatively Stained and Thin Sectioned Materials.

a, Region I in Fig. 6A, 15 sec; b, region II in Fig. 6A, 15 sec; c, region III in Fig. 6A, 15 sec; d, 7S globulin gel; e, region II in Fig. 6B, 20 min. a, b, c and e are of negatively stained materials. d is of thin sectioned material.

tration were visualized on electron microscopy as shown in Fig. 7. In the case of the soluble aggregates with molecular weights of around 1 million in region I, globular species and short strands were seen (Fig. 7a). These aggregates may be composed of five to seven molecules of 7S globulin, as judged from the molecular weights of 7S globulin and the soluble aggregates. The soluble aggregates in region II appeared to be the result of the gathering together into clusters of the soluble aggregates seen in region I (Fig. 7b), and those in region III appeared to be clusters of the soluble aggregates seen in region II (Fig. 7c). Thus, the orientation of the aggregation of 7S globulin induced by heating seems to be random in contrast to the case of 11S globulin, in which the formation of regular strands occurs.⁶⁾ Fig. 7d is a micrograph of ultrathin sections of a 7S globulin gel formed on heating for 20 min. The network structure of the gel can be seen to be a randomly aggregated one like an assembly of the clusters seen in region III, which is in marked contrast to the 11S globulin gel, which is built up of regular strands with a thickness of 10~12 nm.⁶⁾ These

observations suggest that the formation of a network structure and its development are based on the assembly of 7S globulin molecules into clusters. The soluble aggregates with molecular weights of around 4 millions formed at 0.5% protein concentration were in the form of strands (Fig. 7e). These strands seemed to be composed of the soluble aggregates with molecular weights of 1 million, as constituent

units, because the strands of both types of soluble aggregates were of similar thickness. Why the shape of the soluble aggregates with molecular weights of around 4 millions differs with the protein concentration is not clear at present.

The overall scheme proposed for the thermal change of 7S globulin is illustrated below:

The proposed scheme can be summarized as follows: (1) Soluble aggregates with molecular weights of around 1 million are formed when a 7S globulin solution is heated both at low and high protein concentrations. (2) On subsequent heating, the soluble aggregates assemble into strands with a molecular weight of 4 millions in the case of a low protein concentration (process I), while in the case of a high protein concentration the associate randomly with each other to form soluble macroaggregates which results in gel formation (process II). In this process an SH/S-S exchange reaction is not involved, contrary to in the case of 11S globulin.³⁾

On the other hand, we have reported that the constituent subunits of 11S globulin contribute differently to its gelation and gel properties.^{4,7,8,22)} This prompted us to investigate the possibility that the constituent subunits of 7S globulin also contribute differently to the formation of soluble aggregates. Therefore, the distribution of each constituent subunit (α , α' and β) in the fractions obtained on sucrose density gradient centrifugation of 7S globulin heated for 15 sec was examined by NaDodSO₄-PAGE (Fig. 8). The relative proportions of the constituent subunits were calculated by densitometric scanning of the gels and are expressed as percentages of the total subunits. The percentages of the α , α' and β subunits in the native 7S globulin were

FIG. 8. Amount Ratio of Constituent Subunits in Sucrose Density Gradient Fractions.

Solid and dotted lines show the sucrose density gradient centrifugation pattern and the ratio of each subunit, respectively. $\circ$, α subunit; $\square$, α' subunit; $\triangle$, β subunit. Other conditions are given in the text.

37.5%, 25% and 37.5%, respectively, which are shown on the ordinate in Fig. 8. In the fractions near to the top of the gradient where the extent of aggregation is low, the proportions of the α and α' subunits were higher than those of native 7S globulin, while the proportion of the β subunit was lower than that in the case of native 7S globulin. On the other hand, the higher the extent of aggregation, the lower the proportions of the α and α' subunits and the higher the proportion of the β subunit. It has

been demonstrated that 7S globulin consists of seven isomers with different subunit compositions.^{23,24)} Considering this and the above results, it seems likely that the isomers containing the β subunit will have a tendency to form larger aggregates on short time heating. Furthermore, the β subunit may be responsible for such aggregate formation due to its higher content of hydrophobic amino acids, as has been pointed out by Thanh and Shibasaki,²⁵⁾ in their work on self-association at 25°C of 7S globulin subunits.

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