Gelation Phenomena Induced by Alkali-alcohol Treatment of 7S and 11S Components in Soybean Globulins

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Transparent gels containing about 2% protein were obtained by mixing alkaline dope solution of 7S or 11S soybean proteins with alcohol. The 7S component showed the ability to form a stronger gel than the 11S. This phenomenon depended on pH and alcohol concentration. In 66% ethanol, the viscosity of the 7S and 11S reached maxima at pH 11.4 and 11.2, respectively. Above these pH levels where further unfolding and dissociation into subunits of the protein molecules occur, the viscosity decreased rather. The effectiveness of alcohol to increase viscosity increased in the order; *n*-butanol < *tert*-butanol < *n*-propanol < *iso*-propanol < ethanol < methanol. Alcohols having minor hydrophobicity were more effective for increasing viscosity, but ethylene glycol was ineffective. The addition of NaCl or 2-mercaptoethanol to ethanol-mixed alkaline dope solutions resulted in the remarkable increment of the viscosity, especially for the 7S.

The authors found that the addition of alcohols to an alkaline dope solution of acid precipitated soybean proteins caused the formation of a transparent, heat-reversible gel containing about 2% protein. In alcohol denaturation, a hydrophobic interaction between aliphatic groups of alcohol molecules and hydrophobic sites of proteins has been discussed.^{1~5)} Alcohols which are non-polar and water misible solvents are stable even at high alkaline pH at room temperature. Accordingly, the investigation on the interaction between alkali-denatured proteins and alcohols would reveal the state of hydrophobic core-structure during alkali denaturation. In the previous paper,⁶⁾ it has been shown that, owing to the intermolecular interaction at protein concentration higher than 8%, an alkaline dope solution (pH > 11) of acid precipitated soybean proteins gelled by dialysis against phosphate buffer (pH 7.2, $\mu=0.5$). Considering the parallel relationship between changes in viscosity and solubility of *n*-heptane into the dope solution, the exposure of hydrophobic regions seemed to contribute largely for keeping a gel network structure as well as disulfide and hydrogen bonds. On an alkaline

treatment, soybean 7S and 11S proteins are unfolded irreversibly^{7~9)} followed by partial destruction of a hydrophobic core-structure constituted by the β -structure in the interior of the molecules.^{10~12)}

The present paper describes the changes in viscosity of alkali-alcohol treated 7S and 11S components at various concentrations of alcohols and at various pH. Several kinds of alcohols, ethylene glycol and acetone were employed. The mechanism of the gel formation was speculated by comparing with that in the case of only alkali treatment.

MATERIALS AND METHODS

Preparation of crude 7S and 11S components. The crude 7S and 11S components were prepared from the defatted soybean meal according to the method of the method of Thanh *et al.*¹³⁾ Each fractionated component was precipitated at pH 4.9 and 4.7, respectively, followed by washing with excess of distilled water to remove the residual buffer constituents. After washing, the suspension of each component was almost free from salts. In all experiments, freshly prepared dispersions were used.

Alkali-alcohol treatment. The pH adjustment of each dispersion was performed by adding 1 N NaOH.

However, due to deamination,^{6,14} the pH of the solution decreased gradually with time, so the pH was readjusted 30 min after the first adjustment. The prepared alkaline dope solution was allowed to stand for 1 hr. Then a constant volume of water-alcohol solution (% v/v) was mixed with an aliquot of an alkaline dope solution in a test tube. The mixture was shaken immediately and covered with parafilm to prevent evaporation. After standing for 24 hr, the viscosity was measured. All experiments were carried out at $30 \pm 0.1^\circ\text{C}$. The protein concentration was determined on a weight percentage basis, but this method could not be applied directly because the gravity of alcohol is different from that of water. Accordingly, it was calculated theoretically, assuming water was added instead of alcohol.

Viscosity measurement.

Apparent viscosity was measured with B-type Synchro-Lectric viscometer Model BH using a small sample adaptor Model HH (Toki Sangyo, Co., Ltd.) at $30 \pm 0.1^\circ\text{C}$. In all experiments, the same rotor and rotation velocity were employed.

RESULTS AND DISCUSSION

Alkali-ethanol treated 7S and 11S components showed remarkable changes in viscosity depending on pH and ethanol concentration as illustrated in Fig. 1. The conformational changes of the proteins by alkali are induced electrostatically, while the addition of alcohol converts a solvent to be more hydrophobic environment. Therefore, the increment of viscosity and gel formation induced by mixing ethanol and an alkaline dope solution, is considered to be arisen from a hydrophobic interaction between aliphatic groups of alcohol molecules and hydrophobic regions of proteins. The ethanol-alkaline dope mixture having above 150 cp. (centipoise) did not show fluidity. Under suitable conditions, the gel was transparent being yellow-brownish in color and became a sol phase by heating it. The conversion between the gel and the sol phase was reversible. The 7S component formed a stronger gel than the 11S. The 7S component contains less cystine residues,^{12,15,16} which would mean that the binding force other than disulfide bonds, such as hydrophobic bonds, might contribute to the stabilization of the network structure of the 7S gel. This confor-

FIG. 1. Effect of pH under Various Concentrations of Ethanol on the Apparent Viscosity of the 7S and 11S Dope Solutions Prepared by Alkali-alcohol Treatment. Protein concentrations of the 7S and 11S solutions were 2.28% and 2.34%, respectively.

●—●, 72% alcohol concentration; ○—○, 66%;
▲—▲, 61%; △—△, 56%; ■—■, 51%; □—□, 45%.

mational feature may be related to the fact that the alkali-alcohol treated solution of the 7S had higher viscosity than the 11S. The maximum viscosity of the 7S alkali-ethanol treated solution shifted from pH 10.4 to 11.4 with increasing alcohol concentration. Whereas all ethanol treatments of the 11S solution showed the maximum at pH 11.2 except in 72% ethanol. In 66% ethanol, both components demonstrated the highest viscosity, and both 7S and 11S solutions increased the viscosity from pH 10.4 and reached maximum at pH 11.4 and 11.2, respectively. Above these pH levels where both components began to unfold and dissociate into their subunits,^{8,9,17} the viscosity decreased rather. Therefore, it was expected that the amount of the exposed hydrophobic regions which could interact with solvent reached maximum at the above described

FIG. 2. Effect of Ethanol Concentration at Various pH on the Apparent Viscosity of the 7S and 11S Dope Solutions Prepared by Alkali-alcohol Treatment.

Protein concentration were 2.28% and 2.34%, respectively.

●—●, pH 8.7; ○—○, pH 10.2; ▲—▲, pH 10.6; △—△, pH 11.2; ■—■, pH 11.7; □—□, pH 12.2; ×—×, pH 12.8.

FIG. 3. Diagram Represented Schematically the Visible Phase of Alkali-alcohol Treated 7S and 11S Dope Solutions.

■, precipitation phase; ▨, turbid sol phase; ▩, turbid gel phase; ○, transparent gel phase; ▤, transparent liquid phase.

pH values, then it decreased through further dissociation process. Conversely, even if the total exposed areas increase under such condi-

FIG. 4. Effect of Alcohols Having Various Kinds of Alkyl Groups and Acetone on the Apparent Viscosity of the 7S Dope Solution Prepared at pH 11.2.

Protein concentration was 2.44% ●—●, methanol; ○—○, ethanol; ▲—▲, *n*-propanol; △—△, *iso*-propanol; ■—■, *n*-butanol; □—□, *tert*-butanol; ×—×, acetone.

tion, the hydrophobic interaction may become weaker because of the split into several small areas by the dissociation. Figure 2 shows the effect of alcohol concentration on the viscosity of the 7S and 11S treated at various pH. With lowering a treatment pH, alcohol concentration giving the maximum viscosity moved to the lower concentration. Figure 3 represents schematically the visible states of alkali-ethanol dope solutions examined in Fig. 1. Gel formation was observed between precipitation and liquid phase. At lower or higher pH region, the protein tended to precipitate or to become turbid.

To investigate the relationship between aliphatic groups of alcohols and the increments of viscosity, several kinds of alcohol, ethylene glycol and acetone were examined. The ability of alcohol to increase viscosity is in the following order; *n*-butanol < *tert*-butanol < *n*-propanol < *iso*-propanol < ethanol < methanol (Fig. 4). The viscosity was influenced extremely by the length and branching of the hydrocarbon chain. It has been revealed that the effectiveness of alcohol denaturation increased with the hydro-

FIG. 5. Effect of Alcohols Having Various Kinds of Alkyl Groups and Acetone on the Apparent Viscosity of the 11S Dope Solution Prepared at pH 11.2.

Protein concentration was 2.62%. ●—●, methanol; ○—○, ethanol; ▲—▲, *n*-propanol; △—△, *iso*-propanol; ■—■, *n*-butanol; □—□, *tert*-butanol; ×—×, acetone.

carbon chain length and decreased with the number of branches of hydrocarbon part.^{1-5, 18-21} Interestingly, the order of gel formation ability and of denaturation ability were completely reversed except below about 46% alcohol concentration. For example, methanol which is the weakest denaturant caused the most dynamic transition in viscosity. In addition, the alcohol concentration which gave the maximum viscosity increased from 46% for *n*-butanol to 72% for methanol. Furthermore, with increasing the chain length, alcohols, even at lower concentration, tended to precipitate proteins. That is to say, the 72% methanol-mixed alkaline dope solution exhibited almost a transparent gel, but the ethanol mixture showed a turbid gel and the proteins were precipitated with *n*-propanol. *tert*- And *n*-butanol led to precipitate even at 67% and 51% concentration, respectively, without gelation. The 11S component demonstrated the same tendency in a function of the chain length and aliphatic groups, although the viscosity was smaller than that of the 7S (Fig. 5).

Ethylene glycol did not induce any increase of viscosity (not shown in the figure), which suggests that the introduction of hydroxyl residue inhibits gelation. Acetone also increased the viscosity.

Figure 6 shows the changes in viscosity with time after mixing ethanol. Under favorable conditions, the viscosity increased rapidly within several hours, then increased gradually. The time dependence of viscosity indicates the gradual conformational changes of the protein molecules after adding alcohol. It has been recognized generally that the hydrophobic amino acids tend to be exposed into an alcoholic medium from internal hydrophobic region in the course of unfolding process. However, it is still a question whether a three-dimensional structure (especially, hydrophobic regions) is loosen further with alcohol in the alkaline dope solution. Because, as described before, alcohol having a minor hydrophobicity which has a minor denaturation effect, is more effective for gelation. Furthermore, the alkaline dope solution composed of dissociated subunits (for example, above pH 12) showed low viscosity even by adding alcohol, although

FIG. 6. Effect of Alcohol Treatment Time on the Apparent Viscosity of Alkali-alcohol Treated 7S Dope Solution.

Prepared condition of each sample was as followed: ●—●, at pH 11.8 in 56% ethanol; ○—○, at pH 11.8 in 66% ethanol; ▲—▲, at pH 10.7 in 46% ethanol. Protein concentration was 2.29%.

alcohols rather promote dissociation.²²⁻²⁴⁾ These discrepancies support that the protein-solvent interaction (adsorption of solvent) rather than the structural interaction (formation of three-dimensional network) between protein molecules denatured by alkali and alcohol, is essential to increase viscosity. In the case of a gelation which seems to be based on a protein-protein interaction, for example, by heat-^{25, 26)} or only alkali treatment,⁶⁾ approximately 8% protein concentration is necessary for gelation. So in the about 2% soybean protein solution, direct protein-protein interaction would be weaker.

The viscosity of an alkali-alcohol dope mixture depended greatly on NaCl concentration (Fig. 7). Such finding supports that the protein-solvent interaction plays a very important role to increase viscosity. Excessive addition of NaCl resulted in precipitation of proteins just like salting-out. With raising of the preparation pH, a greater amount of NaCl was required and the increasing rate of viscosity was reduced. Surprisingly, 2-mercaptoethanol showed a more powerful effect for increasing viscosity than NaCl (Fig. 8). Mercaptoethanol is a disulfide-cleavage reagent and inhibited strongly a gel formation in the case of heat- or alkali-treatment. Since the present result was

Fig. 7. Effect of NaCl on the Apparent Viscosity of the 7S and 11S Dope Solutions Prepared by Alkali-alcohol Treatment.

NaCl was added just before alcohol treatment (56% alcohol concentration).

●—●, pH 10.7; ×—×, pH 11.7; ○—○, pH 12.5.

Fig. 8. Effect of 2-Mercaptoethanol on the Apparent Viscosity of the 7S and 11S Dope Solutions Prepared by Alkali-alcohol Treatment.

2-Mercaptoethanol was added just before alcohol treatment (56% alcohol concentration).

●—●, pH 10.6; ×—×, pH 11.7; ○—○, pH 12.5.

completely reversed, mercaptoethanol might behave to change dielectric constant like salt, not to change a conformation through reducing disulfide bonds in the alkali-alcohol dope solution. Shibasaki *et al.*²⁷⁾ reported that mercaptoethanol accelerated the polymerization of proteins in alcohol solution.

The properties of the gel in this study would depend on a nice balance force²⁸⁾; the protein molecules would attract one another at some spots but would be separated by solvent molecules that are attracted at others. If the attraction of the protein molecules for one another were too strong, dispersion in the solvent would not occur and then a precipitate will be formed. The attraction between the proteins and solvent molecules are electrostatic and hydrophobic forces depending on pH, alcohol and protein concentration, temperature and salt concentration. In general, a pK value of carboxyl group rises under a hydrophobic environment,²⁹⁾ and consequently the water structure around the residue is largely changed. Also, exposed hydrophobic region may change a water structure around the protein molecules through the iceberg formation. The dielectric constant of the solvent is lowered by addition of alcohols and glycols, and in consequence,

some destabilization of the protein structure would occur ascribing to increased electrostatic repulsive force of like charges on the protein surface. This effect may be significant in alkaline pH region. At the same time, however, as mentioned by Tanford,³⁰⁾ and Klotz and Franzen,³¹⁾ internal hydrogen bonds become stronger and ion pair formation between charged groups should become favored attributing to lowering of the bulk dielectric constant of the solvent. These phenomena are preferable at high concentration of organic solvent. Considering the difference in protein concentration, optimum pH, the effects of NaCl and mercaptoethanol, the mechanism of the gel formation in the present work is different from those observed by heat- and alkali-treatment.

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