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Effects of Salts and Denaturants on Thermocoagulation of Proteins

Kazuko Shimada* and Setsuro Matsushita

Thermocoagulation of proteins containing different amounts of hydrophobic amino acids was investigated with regard to the effects of salts and denaturants. The formation of thermocoagulum of egg albumin was enhanced by the addition of salts, and the effect of salts followed the lyotropic series. For bovine serum albumin, salts of the higher order on the lyotropic series enhanced fromation of coagulum but those of the lower order inhibited it when the salt concentrations were increased. For soybean protein, an increase in turbidity at alkaline pH was observed when salts were added, while formation of thermocoagulum was inhibited. Guanidine hydrochloride enhanced the coagulum formation in a manner similar to that of salts. Sodium dodecyl sulfate and urea suppressed thermocoagulation. From the effects of salts and denaturants on thermocoagulation of these proteins, the mechanism of coagulum formation can be surmised from the standpoint of the hydrophobic amino acid content of proteins.

Thermocoagulation of proteins is widely utilized in food processing. However, there has been very little past research on this subject. Heat–treated solutions of serum albumin have been found to form an opaque coagulum, a clear gel, or an intermediate clot depending on the pH of the medium (Jensen et al., 1950). Seideman et al. (1963) reported that some factors (pH, addition of sucrose, etc.) affected the coagulation temperature of egg white. Differences in coagulation of egg white resulting from application of conventional heat and electronic exposure were compared by Baldwin et al. (1967). It has been observed that the microstructures of soybean protein curds and yeast protein curds which were examined by an optical microscope and a scanning electron microscope varied according to pH (Lee and Rha, 1978; Tsintsadze et al., 1978).

The three-dimensional network of the protein coagulum is believed to be formed by hydrophobic interactions, hydrogen bonds, and ionic attractions, but the mechanism of formation is still not well understood. Our previous paper showed that the amount of hydrophobic amino acids in proteins easily forming thermocoagulum differed from those in proteins forming thermoreversible gel (Shimada and Matsushita, 1980b). In this paper, we report the effects of salts and denaturants on thermocoagulation of proteins containing various amounts of hydrophobic amino acids. Egg albumin which coagulates on heating and bovine serum albumin and soybean protein which gel on heating were chosen as models in this study.

MATERIALS AND METHODS

Materials. Egg albumin was purchased from Nakarai Chemicals Ltd., Kyoto. Bovine serum albumin (BSA) (demineralized) was obtained from Povite Producten N.V. (Amsterdam, Holland). These proteins were defatted by acetone before use. Soybean protein solution, prepared from an aqueous extract of defatted soybean meal, was powdered by acetone treatment. Other chemicals were reagent grade.

Heat Treatment and Turbidimetry. Heat treatment was carried out with 5 mL of protein solution in a glass tube (105 × 15 mm). Protein solutions were heated in a

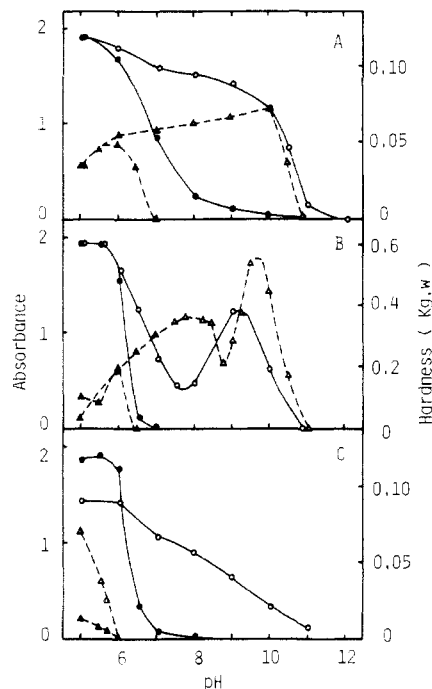Research Institute for Food Science, Kyoto University, Kyoto, Japan.

Figure 1. Relationship between turbidity and formation of coagulum. Protein solutions were heated at 80 °C for 30 min. (A) 2.8% egg albumin solution; (B) 2.8% BSA solution; (C) 4.6% soybean protein solution. (● and ▲) Absence of salt; (O and △) presence of 0.3 M sodium chloride; (—) turbidity; (---) hardness of coagulum.

water bath at 80 °C for 30 min, and after termination of heating, the tubes were immediately cooled in ice–water. Turbidity was measured with a Klett-Summerson Photoelectric colorimeter using a filter, No. 66 (red filter). Protein concentration was determined by drying the sample to constant weight at 110 °C.

Texturometer Measurement. Textural properties of the coagulum were determined by a Texturometer (General Food Co. GXT-2) using a visco-type plunger and a cup of 24-mm diameter. The clearance between plunger and plate was adjusted to 0.3 mm. Hardness was measured from the first chew.

Electrometric Titration. Titration was carried out in a water-jacketed vessel at 25 °C on a Radiometer PHA 943 pH meter using a G2222C glass electrode and a K4112 calomel electrode. The procedure was the same as that described in a previous paper (Shimada and Matsushita, 1980a).

Isoelectric point measurement was performed according to the procedure of Nozaki and Tanford (1967). The h value is defined as the net charge in 100 amino acid residues.

RESULTS

Relationship between Thermocoagulation and Turbidity. Egg albumin solutions were heated, after which changes in turbidity and hardness of coagulums were determined (Figure 1A). Both an increase in turbidity and formation of coagulum were observed at more alkaline pH values in the presence of 0.3 M sodium chloride compared with the absence of salt. Figure 1B shows the change in turbidity and the formation of coagulum after heat treatment of BSA solution. For BSA solution, as with egg albumin, an increase in turbidity and formation of coagulum were observed at more alkaline pH with the addition of salts. Although the results for BSA were somewhat complicated, both egg albumin and BSA revealed a similar relationship between an increase in turbidity and forma-
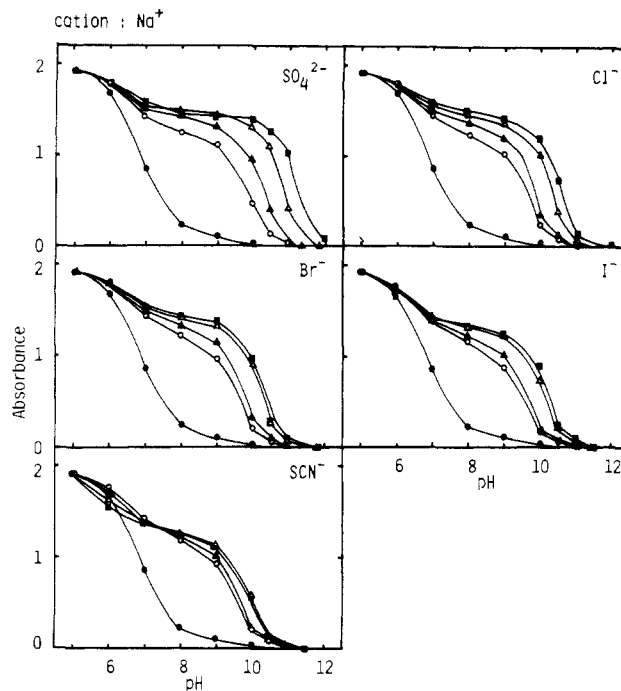

Figure 2. Effects of various anions on thermocoagulation of egg albumin. Protein concentration was 2.8%. Concentration of salts: (●) 0; (O) 0.05; (▲) 0.1; (△) 0.2; (■) 0.3 M.
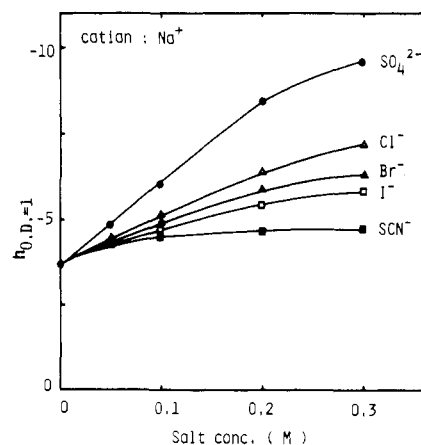

Figure 3. Relationship between concentration of salts and $h_{OD=1}$.

tion of coagulum. In the presence of 0.3 M sodium chloride, soybean protein showed an increase in turbidity at alkaline pH, but formation of coagulum was inhibited (Figure 1C). The coagulum from soybean protein is soft compared with those from egg albumin or BSA, and, therefore, the high protein concentration was used for soybean protein. Gelatin did not become turbid and did not form coagulum even though the protein was heated with 0.3 M sodium chloride at the isoelectric point (not shown here). For investigation of the effects of salts and denaturants on thermocoagulation, the turbidity method was mainly used for egg albumin and BSA.

Effects of Salts on Thermocoagulation of Egg Albumin. Effects of anions on thermocoagulation of egg albumin are shown in Figure 2. The range of turbidity shifted to the region of alkaline pH when salt concentrations were increased. (In the absence of salts, proteins generally form coagulum within the narrow range of pH near the isoelectric point.) For a comparison among anions, the pH at which the optical density (OD) was 1 was taken as the critical point. The net charge ($h_{OD=1}$) at the critical pH was derived from the electrometric titration data. Figure 3 shows the relationship between salt con-
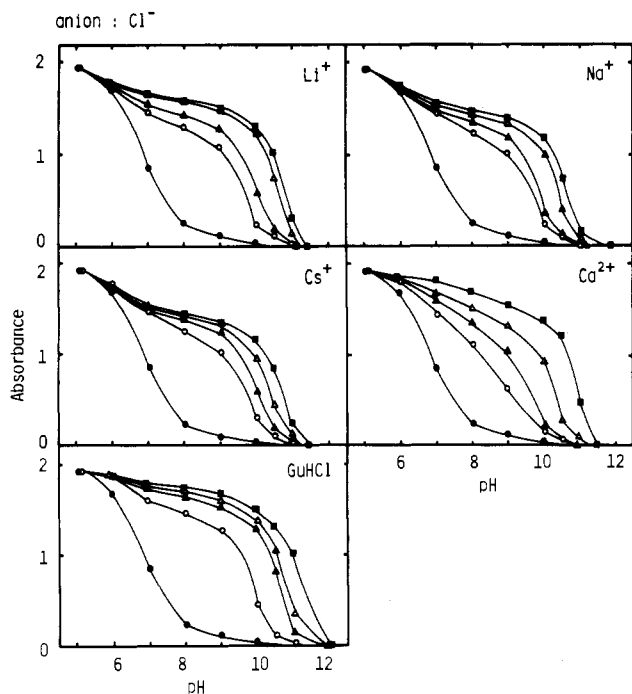
Figure 4. Effects of various cations on thermocoagulation of egg albumin. Protein concentration was 2.8%. Concentration of salts except for calcium chloride: (●) 0; (○) 0.05; (▲) 0.1; (△) 0.2; (■) 0.3 M. Concentration of calcium chloride: (●) 0; (○) 0.1 × 10^{-2}; (▲) 0.25 × 10^{-2}; (△) 0.5 × 10^{-2}; (■) 1 × 10^{-2} M. Concentration of guanidine hydrochloride: (●) 0; (○) 0.05; (▲) 0.1; (△) 0.15; (■) 0.2 M.
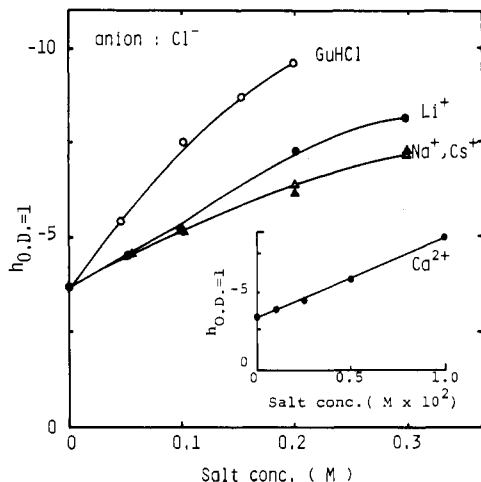

Figure 5. Relationship between concentration of salts and $h_{OD=1}$.

centrations and net charges. Salts affected coagulum formation in the order of SO_4^{2-} > Cl^- > Br^- > I^- > SCN^-.

Effects of cations on thermocoagulation of egg albumin are shown in Figure 4. The net charges were plotted against salt concentration in Figure 5. The differences among the effects of the cations on the thermocoagulation of egg albumin were less than those observed for the anions. The order of salts affecting coagulum formation was as follows: Ca^{2+} ≫ Li^+ > Na^+ ≃ Cs^+. Guanidine hydrochloride, known as a denaturant of protein, had an effect similar to that of the other salts.

Effects of Salts on Thermocoagulation of BSA. Figures 6 and 7 show the effect of salts on thermocoagulation of BSA. The minimum of the turbidity–pH curve was observed near pH 7.5. For comparison these effects, the pH at which absorbance was 1 on the acidic side of the minimum (shown by an arrow on the Cl^- curve in Figure 6) was taken as the critical pH for coagulum formation.
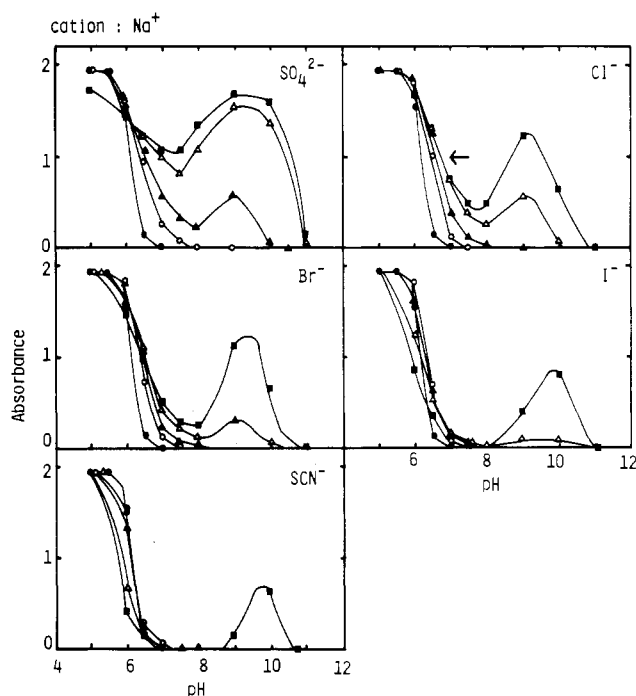

Figure 6. Effects of various anions on thermocoagulation of BSA. Protein concentration was 2.8%. Concentration of salts: (●) 0; (○) 0.05; (▲) 0.1; (△) 0.2; (■) 0.3 M.
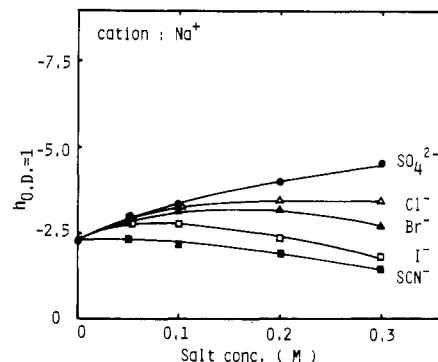

Figure 7. Relationship between concentration of salts and $h_{OD=1}$.

The promotion effect of salts on coagulum formation indicated the following order: SO_4^{2-} > Cl^- > Br^-. The anions of I^- and SCN^- inhibited coagulum formation at high salt concentrations.

Effects of cations on thermocoagulation of BSA are shown in Figures 8 and 9. Coagulum formation was enhanced according to the following order: Ca^{2+} > guanidine hydrochloride > Li^+ > Na^+ ≃ Cs^+. Guanidine hydrochloride influenced coagulum formation more extensively than salts except calcium chloride.

Effects of Salts on Thermocoagulation of Soybean Protein. Changes in turbidity were measured when soybean protein was heated in the presence of salts (Figure 10). Turbidity largely increased at alkaline pH when sodium sulfate was added but did not do so appreciably when sodium thiocyanate was added.

Effects of Denaturants on Thermocoagulation. Thermocoagulation of egg albumin was measured in the presence of denaturants. Turbidity shifted down to acidic pH when the sodium dodecyl sulfate (NaDodSO₄) concentration became high, and the formation of coagulum was inhibited (Figure 11A). The relationship between $h_{OD=1}$ and NaDodSO₄ concentration is shown in Figure 11B. The net charge decreased linearly with an increase of NaDodSO₄ concentration. when urea was added to egg albumin, turbidity shifted to acidic pH as the urea con-
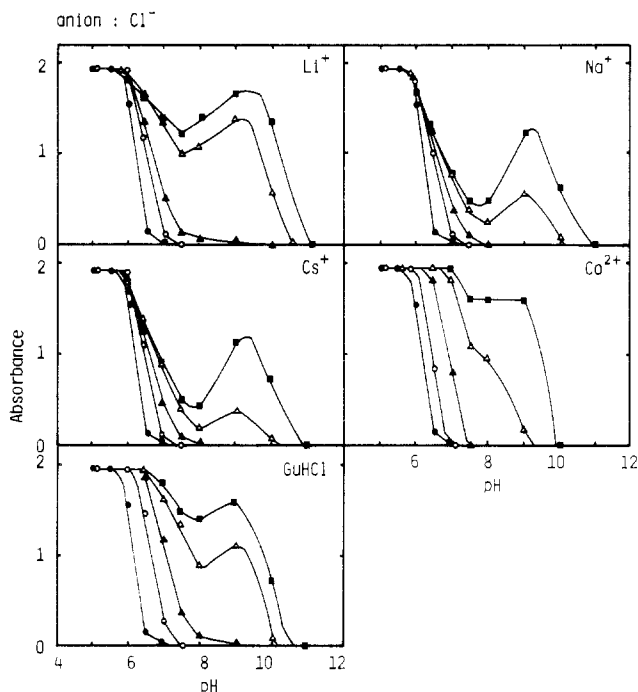
Figure 8. Effects of various cations on thermocoagulation of BSA. Protein concentration was 2.8%. Concentration of salts except for calcium chloride: (●) 0; (○) 0.05; (▲) 0.1; (△) 0.2; (■) 0.3 M. Concentration of calcium chloride: (●) 0; (○) 0.1×10^{-2}; (▲) 0.25×10^{-2}; (△) 0.5×10^{-2}; (■) 0.7×10^{-2} M. Concentration of guanidine hydrochloride: (●) 0; (○) 0.25×10^{-1}; (▲) 0.5×10^{-1}; (△) 0.75×10^{-1}; (■) 1×10^{-1} M.
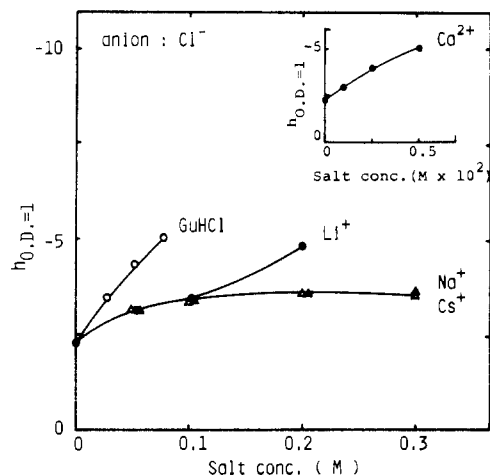

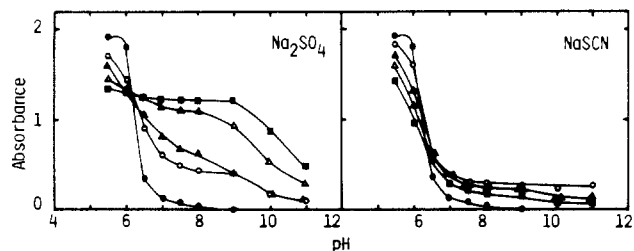**Figure 9.** Relationship between concentration of salts and $h_{OD=1}$.

Figure 10. Effects of salts on thermocoagulation of soybean protein. Protein concentration was 4.6%. Concentration of salts: (●) 0; (○) 0.05; (▲) 0.1; (△) 0.2; (■) 0.3 M.

centration increased (Figure 12A). As with $NaDodSO_4$, the net charge decreased rather linearly as urea concentration increased (Figure 12B).

When BSA solutions were heated in the presence of $NaDodSO_4$ or urea, turbidity shifted slightly to acidic pH as the denaturant concentrations increased (Figure 13).
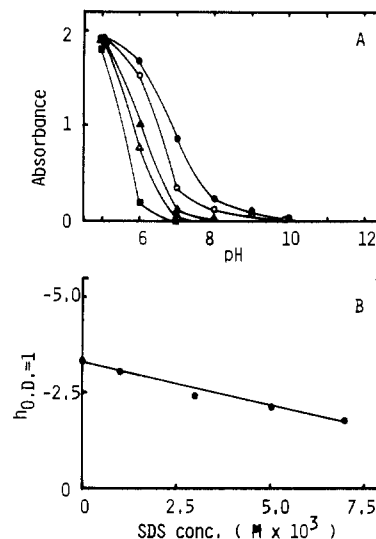

Figure 11. Effects of $NaDodSO_4$ on thermocoagulation of egg albumin. Protein concentration was 2.8%. Concentration of $NaDodSO_4$: (●) 0; (○) 1×10^{-3}; (▲) 3×10^{-3}; (△) 5×10^{-3}; (■) 7×10^{-3} M.
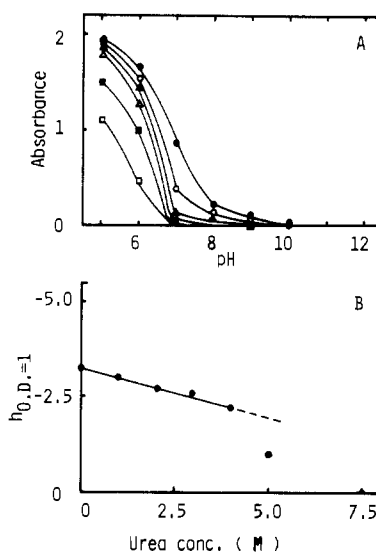

Figure 12. Effects of urea on thermocoagulation of egg albumin. Protein concentration was 2.8%. Concentration of urea: (●) 0; (○) 1; (▲) 2; (△) 3; (■) 4; (□) 5 M.
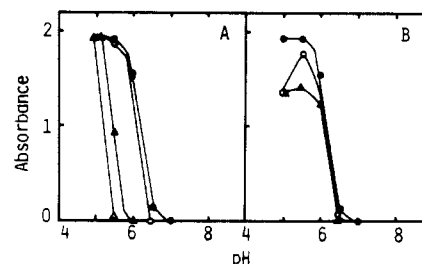
Figure 13. Effects of denaturants on thermocoagulation of BSA. Protein concentration was 2.8%. (A) Concentration of $NaDodSO_4$: (●) 0; (○) 3×10^{-3}; (▲) 5×10^{-3}; (△) 7×10^{-3} M. (B) Concentration of urea: (●) 0; (○) 3; (▲) 5 M.

DISCUSSION

The network structures of proteins formed by heating differ from thermoirreversible coagulum to thermoreversible gel depending on certain conditions such as the variety of protein, pH, heating temperature, presence or absence of denaturant, and others. In a previous paper, we reported that proteins forming thermoirreversible coagulum (coagulation-type protein) and those forming

thermoreversible gel (gelation-type protein) could be distinguished by the mole percent of hydrophobic amino acids in each protein (Shimada and Matsushita, 1980b).

The effect of salts on proteins is rather complicated. It has been reported that salts rupture ionic attractions on proteins, affect hydrogen bonds, and indirectly enhance hydrophobic interactions (Nandi and Robinson, 1972). Our investigation of the effect of salts on thermocoagulation of egg albumin shows that the critical pH for coagulum formation shifted to more alkaline pH as salt concentrations increased (Figures 2 and 4).

Electrostatic forces of the protein do not participate in some intra- and interactions when under electrometric titration; that is, proteins are fully denatured in 6 M guanidine hydrochloride. Consequently, it seems likely that the actual net charge at each pH varies under different conditions. For comparison of the effect of salts on coagulum formation, the net charge of the protein at the critical pH was used (Figures 3 and 5). The higher the absolute value of $h_{OD=1}$, the more alkaline was the pH at which coagulum formed. The effect of ions on coagulum formation indicated an order of $SO_4{}^{2-} > Cl^- > Br^- > I^- > SCN^-$ for anions and $Ca^{2+} \gg Li^+ > Na^+ \simeq Cs^+$ for cations. These orders follow the lyotropic series of ions.

It is reported that the lyotropic series indicates the strength of bonds between the ion and water molecules and is closely related to the hydration of ions (Luck, 1978; Luck and Schioberg, 1979). One of the functions of salt may be to decrease the amount of water bound to proteins. Salts inhibit interactions between water molecules and hydrophilic groups in proteins, so that protein molecules become difficult to dissolve. Bull and Breese (1970) studied the effects of some salts on water bound to egg albumin. All of the salts studied decreased the amount of water bound to egg albumin, and the effect conformed to the lyotropic series. The major protein fraction of sesame seed, α-globulin, contains subunits which are associated or dissociated in different electrolyte solutions. Prakash and Nandi (1977) showed that the ability of anions in dissociating sesame α-globulin followed the order of $SO_4{}^{2-} < Cl^- < Br^- < ClO_4{}^- < SCN^- \le I^- < CCl_3COO^-$. The first two members were association-inducing ions. The ions of the higher order on the lyotropic series are capable of dehydrating protein so that interactions between protein molecules are enhanced and the thermocoagulation of proteins may occur even at alkaline pH. The above results show that the effective net charge may be lower than the intrinsic net charge ($h_{OD=1}$) obtained from electrometric titration when salts were added.

The effect of anions on coagulum formation of BSA followed the order of $SO_4{}^{2-} > Cl^- > Br^- > I^- > SCN^-$. The first three ions enhanced coagulum formation but the remainder inhibited it as the salt concentrations increased (Figures 6 and 7). The effect of cations on coagulation of BSA was in the order of $Ca^{2+} \gg Li^+ > Na^+ \simeq Cs^+$ (Figures 8 and 9). These orders followed the lyotropic series as in the case of egg albumin. It seems likely that a balance between salting in of polar groups and salting out of nonpolar groups would determine the appropriate condition for coagulum formation.

When salts were added, the turbidity–pH curve for BSA revealed a minimum near pH 7.5. It is thought that this phenomenon is related to the tertiary structure of the BSA molecule and is especially dependent on disulfide bonds. The reason is that the BSA molecule contains three distinct domains, and each domain contains three disulfide loop regions (Brown, 1976). A separate paper will describe in detail this phenomenon.

Guanidine hydrochloride weakens hydrophobic interaction in proteins and inhibits hydrogen bonds and ionic attractions in the same manner as salts. Guanidine hydrochloride actually had a very favorable effect on coagulum formation (Figures 4, 5, 8, and 9). On the other hand, the other denaturants, $NaDodSO_4$ and urea, inhibited coagulum formation (Figures 11–13). In the case of egg albumin, we observed a dependence on protein concentration (Shimada and Matsushita, 1980a,b); hence, the critical pH for coagulum formation decreased near the isoelectric point as the denaturant concentration increased. Because BSA showed very little dependence on protein concentration and thermocoagulation occurred only near the isoelectric pH, the critical pH for coagulation hardly shifted when denaturants were added.

Egg albumin contains many hydrophobic amino acids and easily forms coagulum (coagulation type) and was prompted to form turbidity and coagulum even at alkaline pH by the addition of salts. BSA (thermoreversible gel type), containing a medium number of hydrophobic amino acids, was quicker to form turbidity and coagulum with salts of the higher order on the lyotropic series but was inhibited by salts of the lower order. Soybean protein (thermoreversible gel type), containing fewer hydrophobic amino acids than BSA, was also faster to form turbidity with salts of the higher order on the lyotropic series but was suppressed by salts of the lower order. Furthermore, coagulum formation was inhibited by the addition of any salts. These phenomena may be attributed to the small amount of hydrophobic amino acids and the intricate structures which have subunits (7S and 11S globulins). Gelatin (thermoreversible gel type), which contains a small number of hydrophobic amino acids, did not produce any turbidity and did not form coagulum even at the isoelectric pH when salts were added.

The thermal coagulation is formed after sufficient denaturation of proteins. Hermansson (1978) showed that heat treatment at 80 °C was not enough to denature the 11S soybean protein but was enough to denature the 7S protein. Shen (1980a) indicated that BSA at pH 7 in water denatured above 56 °C. Consequently, it seems likely that it is difficult for the soybean protein to form coagulum, while the coagulation of BSA occurs easily.

Recently, it was reported that the neutral salts affected the electrostatic and the hydrophobic interactions in the salting out of proteins (Melander and Horvath, 1977a,b; Shen, 1980b). The relationship between the amount of hydrophobic amino acids in a protein and the effect of salts and the inhibition of coagulum formation by denaturants indicate that the mechanism of thermocoagulation is largely dependent on hydrophobic interactions among proteins.

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Differences in Subunit Composition of Glycinin among Soybean Cultivars

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The subunit composition of glycinins isolated from the seeds of 18 cultivars of soybean (Japanese, U.S., Korean, and Chinese soybeans) were analyzed by electrophoreses on polyacrylamide gels under various conditions. From the analyses by either sodium dodecyl sulfate or alkaline urea gel electrophoresis, the glycinins of various cultivars could be classified into two groups, one of which contained an extraneous subunit protein, the most acidic one. When glycinins were analyzed by gel electrofocusing in the presence of urea and 2-mercaptoethanol, they could be classified into five groups according to the differing molecular charges of the subunits: group I contained seven acidics and eight basics; group II, seven acidics and seven basics; group III, six acidics and seven basics; group IV, six acidics and five basics; and group V, six acidics and three basics.

Grain legumes are potential sources of edible vegetable proteins for supplementing dietary needs. It is generally known that the major components of the seed storage proteins are responsible for contributing to the quality of foods made from these seeds, their flour and protein products, particularly the physical and nutritional properties. Not only are soybeans used for various kinds of traditional Japanese foods but also their protein products as well are used commercially as ingredients in foods. However, the lack of basic information about soybean protein components has hindered the full and effective utilization of the soybean and its protein products for food. The seed storage proteins of legumes contain legumin (referred to as the 11S component) which occurs in large amounts and appears to have a structure of $\alpha_6\beta_6$, in which α and β are acidic and basic subunit proteins, respectively (Derbyshire et al., 1976). Glycinin, one of the major components of the soybean storage protein, has been shown to be composed of three kinds each of acidic and basic subunits (Catsimpoolas, 1969). Kitamura et al. (1976) have reported the isolation of four kinds each of acidic and basic subunits of glycinin and shown that the acidic and basic subunits are linked together in specific combinations through disulfide bridges with the resulting formation of intermediary subunits. We have been studying the subunit structures of the major components of legume seed storage proteins. Previously, we found that the subunit composition of glycinin was different in Japanese soybean cultivars of Tsuru-no-ko and Raiden (Mori et al., 1979) and also that the subunit composition of legumin differed in broad bean cultivars as to the number and proportion of the subunit proteins (Utsumi et al., 1980). In this paper we report our investigation of the subunit composition of glycinin from a wide range of cultivars of soybean seeds

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Table I. List of Seed Samples Examined with Country of Origin

country	cultivar	sample no.
Japan	Tokachi Nagaha	1
	Shiro Tsuru-no-ko	2
	Rikuu No. 20	3
	Raiden	4
	Goyo Daizu	5
	Sakagami No. 2	6
	Iyo Daizu	7
	Matsuura	8
United States	Hill	9
	Hark	10
	Corsoy	11
	York	12
	Dare	13
	Ford	14
China	Bai-hua-zuo-zi	15
	Tianjin-dachingdou	16
Korea	Kinzu	17
	Huk-tae	18

applying gel electrophoresis under various conditions.

MATERIALS AND METHODS

Materials. The samples of soybean seeds (*Glycine max*) examined and their country of origin are listed in Table I. The cultivars were grown at some places in Japan in 1977 where the seeds of each cultivar normally developed and matured: sample no. 1 and 2 were grown at Hokkaido Central Agricultural Experimental Station; no. 3–5 and 15–18, Iwate University Agronomy Farm; no. 6, The University of Tokyo Agrobiology Farm; no. 7 and 8, Kyushu National Agricultural Experimental Station; no. 9–14, Tohoku National Agricultural Experimental Station. Urea purified for biochemical research was obtained from Nakarai Chemicals (Japan). Ampholine was purchased from LKB Co.

Preparation of Acetone Powder. The seeds of soybean cultivars were soaked overnight in distilled water at 4 °C. Cotyledons from which the germ had been removed were homogenized with 15 volumes (w/v) of 63 mM Tris-HCl buffer (pH 7.8) containing 10 mM 2-mercaptoethanol and allowed to stand for 1 h at 20 °C with gentle