Molecular Interaction in Alkali Denatured Soybean Proteins

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ABSTRACT

Water extractable, acid-precipitated soybean proteins were treated with NaOH. Molecular interaction of soybean proteins denatured with alkali occurred above 8% concentration at pH 12.3. Under these conditions, a remarkable increase of solution viscosity or gelation was observed after dialysis against phosphate buffer, pH 7.2. On the other hand, protein treated at less than pH 11.0 and at more than 8% concentration did not increase in viscosity after dialysis. The results suggest that soybean proteins change their conformation above pH 11.0. Gelation was strongly inhibited by addition of urea or mercaptoethanol. When the proteins were treated at pH 13.4, their viscosity did not increase after dialysis, although the structure was more disorganized. Decomposition of cystinyl residue occurred near pH 11.0 and increased gradually at higher pH. Interaction during dialysis after alkali treatment above pH 11.0 apparently results from intermolecular bonds (hydrogen, hydrophobic, disulfide bonds) formed by amino acid residues that become accessible to each other by conformational changes. At pH 7.2 and 10.4, the solutions of alkali-treated proteins showed high viscosities. This seems to be caused by aggregation and increased hydration of proteins not involving changes of the structure.

Today soybean proteins are processed to produce textured, meat-like products based on spun soy fibers. In such processes, soybean proteins are first dissolved in a strong alkaline solution to yield a "dope." The dope is then extruded into an acid-salt coagulation bath and the fibers are formed. Investigations about alkali denaturation of soybean proteins have been reported by Kelley and Pressey (1) and Koshiyama (2). For example, it is known that formation of a good fiber requires 13.5% protein dissolved in 0.95% NaOH (1). Koshiyama (2) showed that the 7S component dissociated into a 2.5S unit at pH 11.0, and a 1.8S entity above pH 12.5. Furthermore, the subunits unfolded by exposure to alkali. However, if the buffer (0.01M glycine-NaOH) contained 0.5M NaCl, 7S component appeared unchanged up to pH 10.5. Although alkali-induced conformational changes, fiber producibility of alkali-denatured soybean proteins, and molecular interaction produced by heat denaturation (3,4) have been studied, molecular interactions of alkali-denatured soybean proteins have not been examined in detail. In this paper, we describe dependence of molecular interaction in alkali-denatured protein on pH and protein concentration before and after dialysis against phosphate buffer (pH 7.2, $\mu = 0.5$).

MATERIALS AND METHODS

Acid-precipitated soybean proteins were prepared as follows: Defatted meal was extracted with water (water:meal ratio of 10:1) for 1 hr. at 40° C. The extract, after removing insolubles by centrifugation ($10{,}000 \times g$), was adjusted to pH 4.5 with 1N HCl to precipitate proteins, which were collected and washed with water several times.

For preparation of alkali-denatured soybean proteins, NaOH solution was added to the protein suspended in water in the case of more than 5% protein

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concentration, and to the protein dissolved in phosphate buffer (pH 7.2, $\mu = 0.5$) in the case of below 1% concentration. The mixture was stirred for 1 hr. at room temperature. The resulting alkali-denatured soybean protein (ADSP) was then analyzed by physicochemical methods. Because the solution showed a decrease of pH with time, the pH of alkali-treated protein solution was determined as follows: With low protein concentration (below 1%), pH immediately after addition of NaOH was regarded as the pH of the solution. At high protein concentration (5 to 10%), the pH of the dope was measured 1 hr. after addition of NaOH. On neutralization of the protein solution, the proteins were dialyzed against the phosphate buffer described above until they reached pH 7.2.

Viscosity of the protein solutions was measured with a single determination at 25°C. by a Brookfield Synchro-Lectric viscometer. However, to avoid the effect of shear rate, the rate of the rotation of the viscometer was operated as slowly as possible. Results are reported as apparent viscosities in centipoises.

Ultraviolet difference spectra were carried out in a Hitachi 124 spectrophotometer at room temperature.

The amount of heptane bound to hydrophobic regions of alkali-treated

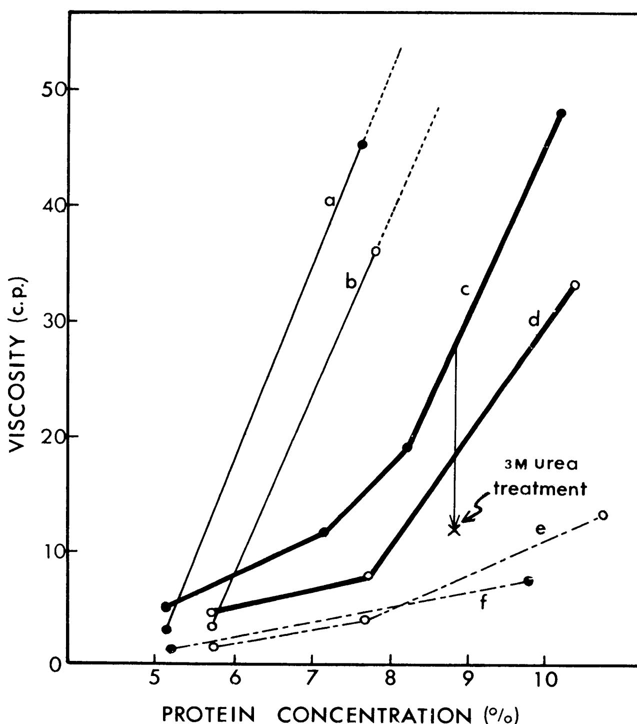


Fig. 1. Dependence of viscosity of ADSP solution on protein concentration. a) and b) ADSP after dialysis against phosphate buffer (pH 7.2, $\mu = 0.5$); c) and d) ADSP; e) and f), ADSP after dialysis against phosphate buffer containing 0.02M 2-mercaptoethanol; closed circles = treatment at pH 12.3; open circles = treatment at pH 12.7; dotted line = gelation.

proteins was measured by gas-liquid chromatography essentially by the procedure of Mohammadzadeh-K et al. (5,6,7). The aqueous protein solution (1.20 ml.) and *n*-heptane (0.9 ml.) were equilibrated for 20 hr. at 25° C. under constant humidity, shaking horizontally at 0.5 revolutions per sec. After equilibration, the sample tube was opened and approximately 0.5 ml. of the aqueous phase was transferred into a small conical centrifuge tube which had been cooled previously in an ice water bath. Three microliters of the equilibrated protein solution was injected directly with a plunger-in-needle-type syringe into a Shimazu GC-4A gas chromatograph equipped with hydrogen flame ionization detectors. The operation was done under the following conditions: Flow rate of nitrogen gas: 20 ml. per min.; column: stainless steel, 150 × 0.3 cm. o.d.; packing: 10% (w./w.) silicon on Chromosorb-W acid-washed, 80 mesh. The temperature parameters were: injection, 150° C., column, 90° C., and detector oven, 170° C. The amount of bound heptane was indicated as the relative increase of the height of heptane peak, assuming the protein concentration to be constant.

In disc electrophoresis, 7.5% polyacrylamide gel in *tris*-glycine buffer, pH 8.3, was employed. The ADSP dialyzed against the phosphate buffer containing 0.02M mercaptoethanol was used as the sample. Protein concentration was kept as low as possible to prevent polymerization of dissociated polypeptide chains during dialysis. No sample gel was used; the sample was layered over the gel with a hypodermic syringe. After electrophoresis of 30 min. at 2 ma. and then 80 min. at 4 ma. per gel, the gel was stained with 1% Amido Black.

Native and denatured proteins were chromatographed on a column (50 × 2.5 cm.) of Sephadex G-200 with the phosphate buffer in the absence of mercaptoethanol. Two-milliliter fractions were collected and the protein content was determined by absorbance at 280 nm.

The destruction of amino acids by alkali treatment was analyzed on a Hitachi KLA-3B amino acid autoanalyzer. The ADSP treated at pH 10.7, 11.4, 12.5, and 13.3 for 1 hr. was dialyzed against phosphate buffer previous to hydrolysis. The sample was hydrolyzed with 6N HCl for 24 hr. at 110° C. in an ampule under high vacuum. The hydrolysate was analyzed according to the ligand method by a single sequential system.

RESULTS

Protein Concentration Dependence of Viscosity

Viscosity of undialyzed ADSP solution increased remarkably above approximately 8% protein concentration at pH 12.3 as illustrated in Fig. 1. Although the viscosity decreased a little when the protein solution was adjusted to pH 12.7, changes in viscosity depending on the protein concentration were almost similar to the former treatment. When ADSP was dialyzed against phosphate buffer, viscosity increased even more rapidly and gelation occurred above 8% protein concentration. It is apparent that a pronounced molecular interaction of ADSP occurred above 8% protein concentration as a result of alkali treatment. However, addition of 0.02M mercaptoethanol or 3M urea to the dialysis buffer inhibited gel formation strongly. This suggests that hydrogen, hydrophobic, and disulfide bonds contribute to the formation of a network structure. Dialysis of 10% ADSP produced a very tough, insoluble gel, which exhibited no beany flavor. Similar alkali treatment of proteins have been shown to produce such insoluble gels (8), and to reduce beany flavor in soybeans (9,10).

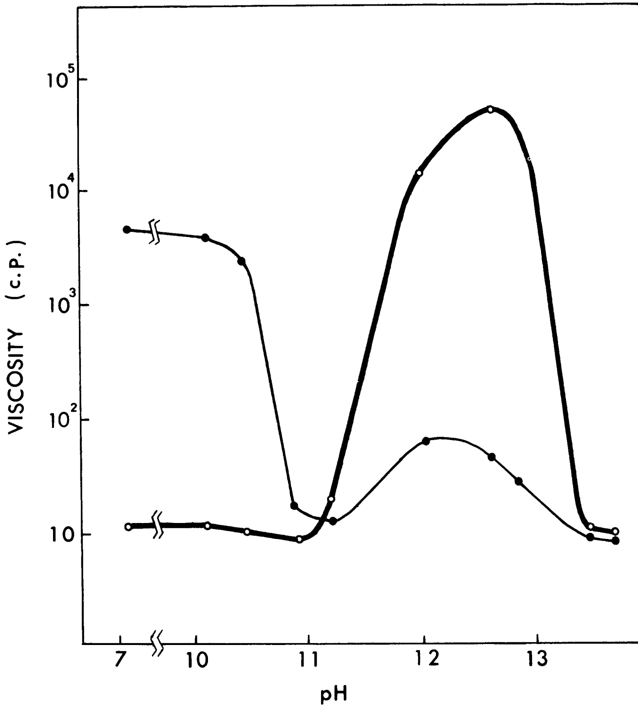


Fig. 2. Dependence of viscosity on pH for ADSP (10.4% protein concentration). Closed circles = before dialysis; open circles = after dialysis against phosphate buffer.

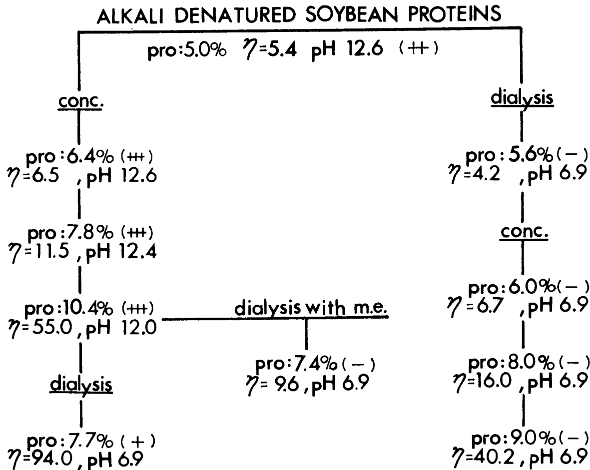


Fig. 3. Changes in viscosity of ADSP on concentration with Carbowax 6000. Dialysis = dialysis of ADSP against phosphate buffer; conc. = concentration of protein solution by Carbowax 6000; pro = protein concentration; m.e. = 2-mercaptoethanol. Spinability: +++ = excellent; ++ = good; + = slight; − = none.

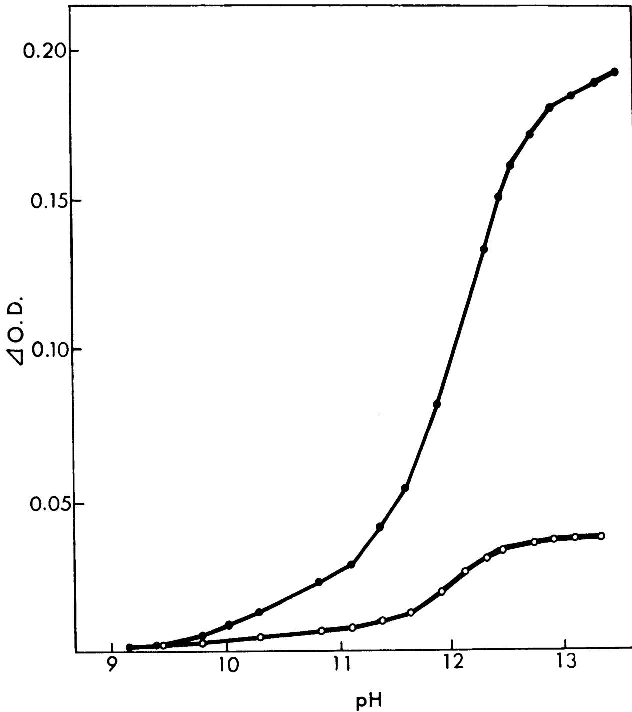


Fig. 4. Dependence of ultraviolet difference spectra on pH for ADSP. Closed circles = 245 nm.; open circles = 295 nm.

pH Dependence of Viscosity

The effect of pH variation on viscosity of ADSP is shown in Fig. 2. This experiment was performed at 10.4% protein concentration which was sufficiently high to cause molecular interactions (Fig. 1). With nondialyzed ADSP below pH 10.4, the viscosity was very high, but decreased rapidly between pH 10.4 and 10.9. At the same time, the solution changed from milky white to transparent brown. Between pH 11.2 and 12.2, the viscosity increased a little and then decreased again.

In contrast, the dialyzed solution was low in viscosity between pH 7.2 and 11.0, but the viscosity of the solution increased rapidly at higher pH. Between pH 12.0 and 12.7, the dialyzed solution gelled. Then above pH 12.8, the viscosity of the dialyzed solution decreased greatly and at pH 13.4 the viscosity was similar to that of ADSP.

Changes in Viscosity on Concentration with Carbowax® 6000

A 5% solution of ADSP at pH 12.5 was prepared as a starting material. The solution was dehydrated with Carbowax 6000 that was packed in a cellophane tube. The viscosity increased in proportion to concentration (Fig. 3). Spinability also improved. During dialysis of the concentrated ADSP against phosphate buffer, the viscosity increased significantly. Gel producibility deteriorated after a

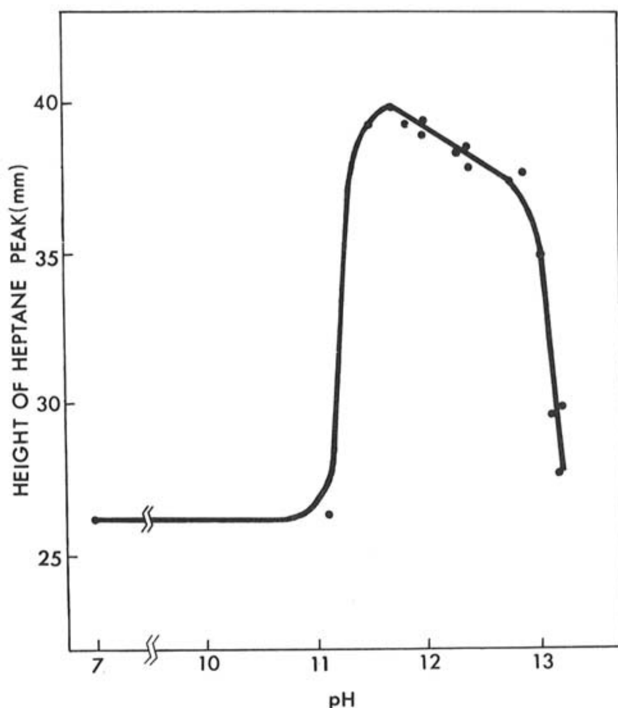


Fig. 5. Heptane bound to ADSP as a function of pH. The amount of heptane bound to proteins was measured as the height of heptane peak on chromatogram.

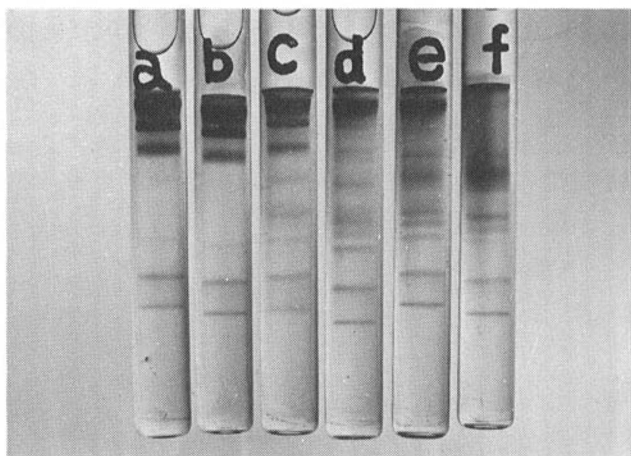


Fig. 6. Disc electrophoretic patterns of ADSP adjusted to the indicated pH for 1 hr. and dialyzed against phosphate buffer containing 0.02M 2-mercaptoethanol. a) native; b) pH 11.0; c) pH 11.5; d) pH 12.0; e) pH 12.5; and f) pH 13.0.

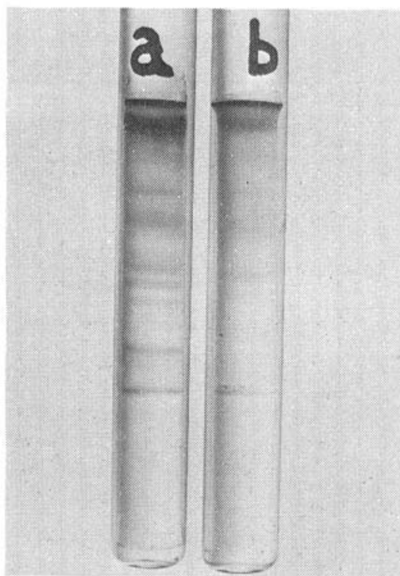


Fig. 7. Disc electrophoretic patterns of ADSP treated at pH 12.5 and dialyzed a) against phosphate buffer containing 0.02M 2-mercaptoethanol; and b) against phosphate buffer.

few days even if ADSP was prepared under a suitable condition to make a gel by dialysis.

As reported by Donovan and White (11), loss of cystine and conformational change by exposure of protein to alkali increased at longer times. For this reason, the concentrated ADSP would show a higher viscosity if dehydration had been performed more rapidly. It took 7 days to get a final protein concentration of 10.4% by this method. The addition of mercaptoethanol to the concentrated solution produced a decrease in viscosity after dialysis.

On the other hand, the solution which was dialyzed before concentration to 9.0% protein had a lower viscosity than the 7.7% protein solution of the previous experiment where the reverse procedure was used. This means that the unfolded protein may reassume its globular state on neutralization; thus intermolecular interaction must be rather weak. Spinability likewise disappeared as a result of dialysis. We then did the following experiments to find some relations between conformational changes and the interactions.

Ultraviolet Difference Spectra

Tyrosine has a characteristic absorption at 245 and 295 nm. on ionization above pH 8.5. As shown in Fig. 4, difference spectra were recognized from pH 9.6 and increased remarkably above pH 11.0. This result is similar to that obtained on 7S component by Koshiyama (2). Taking into account the unchanged viscosity of dialyzed protein between pH 9.6 and 11.0 (Fig. 2), ionization of tyrosine in this pH region suggests loosening of phenolic bonds, but not involving the destruction of the fundamental structure.

Binding of *n*-Heptane to ADSP

This procedure is very valuable for detecting exposed hydrophobic regions of a protein. It is believed that heptane molecules bind to hydrophobic regions of the protein (12,13,14). Changes in solubility of heptane as a function of pH represented in Fig. 5 revealed that a hydrophobic region buried in the molecule began to be exposed near pH 11.0, but above pH 12.9 it was almost destroyed. Unfolding occurs at the former pH and the latter coincides with that at which gel formation decreases. Between pH 11.5 and 12.8, the exposed hydrophobic region seems to keep its structure.

Disc Electrophoresis

Figure 6 shows representative results of dialyzed soybean proteins after alkali treatment investigated by polyacrylamide gel electrophoresis. Between treatment at pH 7.5 and 11.0, no differences of electrophoretic patterns could be seen (Fig. 6, a and b). However, with treatment at pH 11.5, several bands were observed between the slowest and fastest bands that seemed to correspond to subunits of 7S or 11S component (15). Appearance of these bands corresponds with disappearance of the slow-moving bands. More dissociation occurred in the pH range 12.0 to 12.5 (Fig. 6, d and e) whereas at pH 13.0, the slow-moving bands disappeared completely (Fig. 6f). At this pH, there were at least 12 bands including undissociated bands. Although these patterns were reproducible, it is not sure whether the bands represent single-chain subunits or subunit association products (14). When mercaptoethanol was omitted from the buffer system used for dialysis, the electrophoretic pattern and the staining density were different

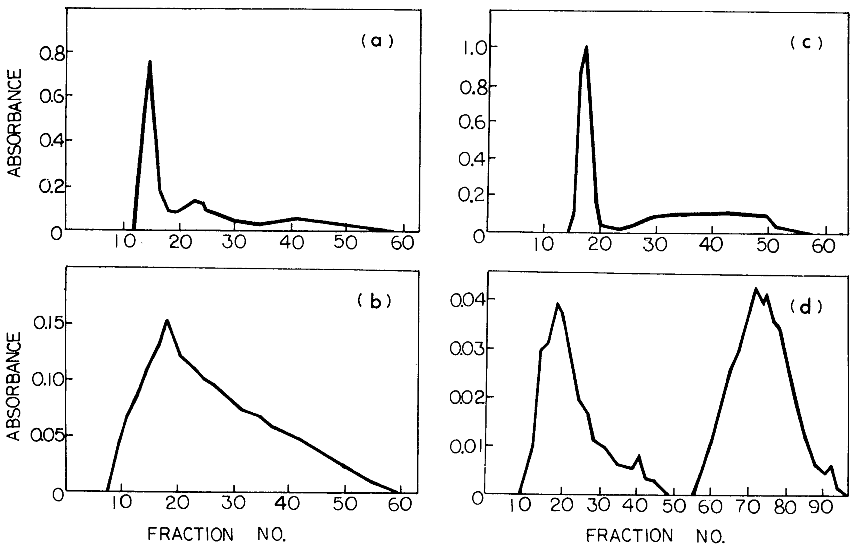


Fig. 8. Sephadex G-200 gel filtration: a) native soybean proteins; b) alkali denatured proteins eluted in phosphate buffer adjusted to pH 12.5 by NaOH; c) dialyzed proteins against phosphate buffer at 6.1% protein concentration; and d) dialyzed protein against phosphate buffer at 0.03% protein concentration. Samples c and d were treated at pH 12.5 before dialysis.

from those in the former experiment (Fig. 7). This is evidence of reforming of disulfide bonds among dissociated subunits during dialysis against the buffer. The range where dissociation begins, pH 11.0 to 11.5, was identical with the pH at which unfolding started as measured by the other methods. That some protein fractions remained at the origins of all gels suggests the formation of macromolecules by polymerization during dialysis or aggregation by acid precipitation (16).

Gel Filtration

Dissociation and polymerization of proteins in the presence of alkali and after dialysis against the buffer were investigated by gel filtration. Elution patterns are shown in Fig. 8. Separation by chromatography on Sephadex G-200 of native acid-precipitated proteins coincided with that obtained by Saio and Watanabe (17). When gel filtration of ADSP was performed in phosphate buffer adjusted to pH 12.5, tailing was observed (Fig. 8b). This tailing suggests a spectrum of dissociation products of the 7S and 11S proteins which in their native state elute in the first peak (fraction 12-18, Fig. 8a). The elution diagram of dialyzed protein at high concentration (in this case, 6.1%) is shown in Fig. 8c and revealed disappearance of the tailing noted when alkali was present (Fig. 8b). This might be attributed to reassociation. At lower protein concentration (approx. 0.03%), the subunits did not reassociate because of insufficient molecular interaction. As a result, a large peak was eluted at the end of fractions, which was expected to be the dissociated subunits (Fig. 8d). This peak seemed to be heterogeneous. A large peak which eluted at first was observed in all experiments.

Changes of Amino Acids

Figure 9 shows that the total amount of acidic and neutral amino acids (except cystine) did not change with alkali treatments while the total amount of basic amino acids decreased a little (7% of that of the native protein). Decreases of

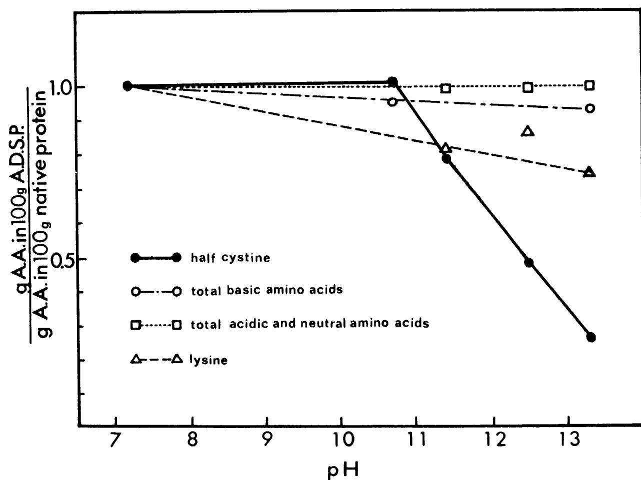
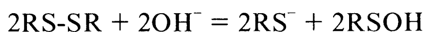


Fig. 9. Changes of amino acids of ADSP with alkali treatment.

basic amino acids might be associated with the odor of ammonia noted during alkali treatment. In general, the amino acids of soybean proteins are not decomposed much with treatment below pH 12, at room temperature and within a few hours (9,18). The amount of cystine was the same as that of the native protein up to pH 10.7, but above this pH it decomposed gradually with increased pH (10,18) and finally at pH 13.3, only 27% of cystine contained in the native protein remained.

DISCUSSION

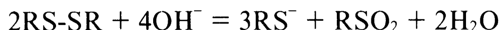
As known already, the native 7S and 11S components have rigid, compact random and β-structures (19,20). In our studies, it has been shown that molecular interaction by alkali treatment of soybean proteins was connected with two factors, chemical and conformational. These factors influence each other closely. For example, the rigid structure unfolds by cleavage of bonds and dissociates into constituent subunits. Consequently, some amino acid residues activated by alkali become accessible. Remarkable conformational changes were observed near pH 11.0, and also decomposition of cystinyl residue correlated with the development of unfolding (Fig. 9). These findings suggest that disulfide bonds may be changed to sulfhydryl and sulfenic acid groups by alkali above this pH and the resulting sulfhydryl residues exist as mercaptide ions (21,22). The cleavage reaction of disulfide bond by hydroxide ion is diagrammed as follows (23):



$$2RS\text{-}SR + 2OH^- = 2RS^- + 2RSOH$$

$$2RSOH = RSH + RSO_2H$$

$$2H^+ + 2OH^- = 2H_2O$$



$$2RS\text{-}SR + 4OH^- = 3RS^- + RSO_2 + 2H_2O$$

Excessively strong alkali treatment leads to destruction of cystine by β-elimination (Fig. 9) (22). Also, deGroot and Slump (18) and Woodard and Short (24) reported that alkali treatment of soybean proteins induced the formation of lysinoalanine which was produced through the addition of the ϵ-amino group of a lysyl residue to the double bond of a dehydroalanyl residue derived from cystine by β-elimination (25,26). In alkali-treated wool (27), the formation of lysinoalanine can result in new intra- and intercross links which are very stable in alkaline solution. There is a possibility that this bond formed in ADSP to a certain extent since lysyl residues decreased significantly (Fig. 9) as also noted for cystine. Upon dialysis, neighboring mercaptide ions may form new disulfide bonds that cause the increase of viscosity or gelation. The decreasing viscosity of ADSP between pH 10.4 and 10.9, and above pH 12.2 might be attributed to an increase in electrostatic repulsive force among molecules, because ϵ-amino group of lysine (pK = 10.53) and guanidyl group of arginine (pK = 12.48) change to a nonionized state in this region, and as a result the relative number of negative charges increases. On the other hand, the high viscosity below pH 10.4 is based on aggregation and an increased hydration of proteins rather than conformational changes, since difference spectra analysis and disc

electrophoresis revealed no conformational changes until near pH 11.0. The increase in viscosity between pH 11.2 and 12.2 may be caused by unfolding.

We obtained information about an interaction and denaturation by dialyzing alkali-denatured proteins against phosphate buffer pH 7.2, $\mu = 0.5$. Perhaps when denatured proteins are brought back to neutral pH very slowly, their dissociated polypeptide chains are reorganized if the environment permits them to reassume their native conformation. Since up to pH 11.0 the conformational changes were not seen, naturally the viscosity of the dialyzed protein solution was constant even at high concentration. Kelley and Pressey (1) mentioned that the dialyzed (against phosphate buffer pH 7.5 $\mu = 0.5$) soybean proteins after alkali treatment below pH 11 kept almost the same ultracentrifugal patterns as the native proteins. The greatly increased viscosity above pH 11.0 indicated that the proteins began to interact. If dialyzed under conditions where there is no interaction, the stretched protein molecules might reassume the globular state reforming intramolecular disulfide bonds or other corresponding bonds, as recognized by the decreased viscosity and the disappearance of spinability (Fig. 3). The results of gel filtration and changes in viscosity with protein concentration agree with this concept. There are significant differences between two patterns dialyzed under high and low protein concentration (Fig. 8, c and d). We have no explanation why the viscosity of the protein solution adjusted at pH 7.2 decreased greatly by dialysis against the buffer (pH 7.2).

As demonstrated in Fig. 5, an exposed hydrophobic region seems to be stable from pH 11.5 to pH 12.8. Above pH 12.9, however, the region which may exist as a core in the structure is disrupted. Accompanying destruction of this region, the viscosity of the dialyzed protein solution decreased extensively, and also at pH 12.6 where the viscosity of the dialyzed protein solution was highest (Fig. 2), almost half of cystinyl residues contained in the native proteins was broken.

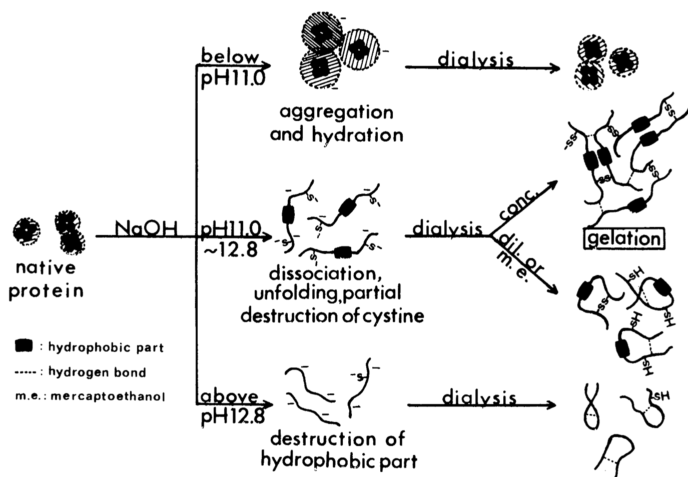


Fig. 10. Schematic representation of molecular interactions of alkali denatured soybean protein before and after dialysis against phosphate buffer as a function of pH and protein concentration.

Considering these findings, hydrophobic bonds may play a large part in making or keeping the three-dimensional network, like the disulfide bonds. Schematic representation of molecular interactions of ADSP based on the foregoing data, is shown in Fig. 10.

It is known that the 11S component has more cystine than the 7S protein (28,29,30). Therefore, it is expected that reactions related to disulfide groups must be mostly due to the 11S component.

Acknowledgment

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