Covalent Polymerization of Acidic Subunits on Heat-Induced Gelation of Soybean Glycinin

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ABSTRACT

Heat-induced gelation of soybean glycinin was studied using polyacrylamide gel electrophoresis and gel filtration. As protein concentration increased, buffer-soluble components in the gel tended to contain more polymerized acidic subunits that were bonded by disulfide bonds. Trypsin digestion of intact glycinin, which is selective and causes limited degradation of acidic subunits, prevented polymerization of acidic subunits and gelation. These results suggest that polymers of acidic subunits of buffer-soluble components in the gel participate in heat-induced gelation of soybean glycinin.

Proteins contribute significantly to the functional behavior and quality of foods (Kinsella 1981). The possession of a range of functional properties considerably extends the potential use of various proteins in food applications. Heating during processing, preserving, and cooking often alters functional properties. Heating causes chemical and physical changes that are involved in protein denaturation and gelation. Little is known about the relationship between chemical and physical changes and functional properties of soybean proteins on heating.

Wolf and Tamura (1969) reported that during heating soybean glycinin was converted into buffer-insoluble aggregates and buffer-soluble dissociates. Catsimpoolas et al (1971) suggested that the antigenicity of glycinin required the native conformation of glycinin. Hashizume and Watanabe (1979) reported that turbidity, an indicator of denaturation, depended on ionic strength during heating. Koshiyama et al (1980-81) studied the dependence of the conformational change of glycinin on ionic strength. By analyzing the heat-induced products on the basis of knowledge of subunit structure of glycinin, Yamagishi and co-workers (1980) confirmed that the mechanism of thermal denaturation of glycinin is based on sulfhydryl/disulfide interchange among subunits. They also indicated that the mechanism was one in which glycinin disappeared with the concomitant appearance of the acidic subunits and was followed by precipitation of the basic subunits polymerized by disulfide bonds. Further, Yamagishi and co-workers (1982b) indicated that all glycinin molecules were converted to the soluble aggregates through the release of some acidic subunits. Release of acidic subunits from soluble aggregates determines the degree of polymerization and oligomerization of aggregates as heat-induced end products. German et al (1982) and Damondaran and Kinsella (1982) suggested that glycinin does not aggregate in the presence of β-conglycinin. Yamagishi et al (1983a) found that heat-induced interactions between glycinin and β-conglycinin result from preferential associations of glycinin basic subunits with β-conglycinin β-subunits, and glycinin acidic subunits with β-conglycinin $\alpha\alpha'$-subunits. Utsumi et al (1984) investigated interactions between β-conglycinin and glycinin during heating and obtained similar results to Yamagishi et al (1983a).

Extensive studies have provided basic knowledge of thermal denaturation of soybean glycinin at low protein concentrations ($<$0.5%), and these are considered as the basis of thermal denaturation (gelation) of glycinin at high protein concentration. However, the molecular basis of the gelation mechanism is yet to be elucidated. Mori et al (1982) investigated the process at initial stages of heating under conditions in which gels are formed and noted the significance of the intermolecular disulfide exchange reaction, which has been discussed by Yamagishi et al (1980). Mori et al (1982) determined that soluble aggregates, a transient intermediate in the course of gel formation, associate resulting in gels at high protein concentrations. Because they did not refer to the end products in the course of gelation, it is not clear whether the soluble aggregates aggregate or disaggregate on further heating. We believe that studies on the subunit structure of gels will contribute to a better understanding of the gelation mechanism and have investigated heat-gelled products using purified glycinin at high concentration based on knowledge of the process at low protein concentration (Yamagishi et al 1980). The present investigation also provides molecular insight into rheological properties of heat-induced glycinin gel.

MATERIALS AND METHODS

Materials

Miyagishirome variety soybeans were used throughout this work. Defatted meal was prepared from soybeans by grinding, defatting with *n*-hexane, and removing the solvent at room temperature.

All the reagents were the highest grade. Sepharose CL-2B and CL-6B were obtained from Pharmacia Fine Chemicals.

Purified glycinin was prepared by the method described by Yamagishi et al (1980).

Electrophoresis

Acetic acid-urea and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed by methods described by Yamagishi et al (1981a, 1983b).

Heat Treatment Procedure

Purified glycinin was stored as a solution at 6° C and heat treated as follows. The protein solution was adjusted to protein concentrations of 0.5, 1, 2, 3, 4, 5, and 10% with standard buffer (35 m*M* potassium phosphate buffer containing 0.4*M* NaCl, pH 7.6). Protein solutions were heated at 100° C for 5 min in an oil bath. Test tubes were fitted with loose stoppers (glass marbles) to reduce evaporation. At the end of the heating period, the test tubes were removed from the oil bath, and 0.02*M* *N*-ethylmaleimide (NEM) was added before cooling in ice.

Standard buffer solution was added to each heated sample. The suspension was agitated thoroughly with magnetic stirring for 30 min and centrifuged at 20° C and 4,500 × g for 10 min. The insoluble fraction was washed with standard buffer three times. The buffer-soluble and buffer-insoluble fractions were subjected to SDS-PAGE and gel filtration.

Partial Cleavage of Intact Glycinin by Trypsin Digestion

Glycinin (2 mg/ml in standard buffer) was digested with trypsin (twice crystallized, Worthington Biochemical Corp.) at 30° C for 1, 3, 6, 10, 30, and 60 min. A 0.4% (w/w) ratio of trypsin to protein was used. At each time interval, test tubes were removed, and soybean trypsin inhibitor was added at a twofold weight excess

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over the level of trypsin to stop the reaction. The samples were analyzed by SDS-PAGE, adjusted to 10% protein concentration, and then heat-gelled.

Instrumentation

Difference-second derivative ultraviolet (UV) absorption spectra were measured in a Yanagimoto high-order derivative UV-visible spectrophotometer (Yamagishi et al 1981b, 1982a, 1984). Fluorescence spectra were measured with a Hitachi MPF-4 spectrophotofluorimeter (Yamagishi et al 1981b, 1982a, 1983b). Circular dichroic (CD) spectra were measured by the method of Yamagishi et al (1983c).

RESULTS

Changes in subunit structure were observed following heat gelation at different protein concentrations. The lowest concentration of protein required for gelation was 2–3%. At lower protein concentrations (0.5 or 1%), electrophoresis (Fig. 1) of buffer-soluble components gave monomers and dimers of acidic subunits as major components and trimers as minor components. With further increases in protein concentration, monomer, dimer, and trimer concentrations decreased, whereas higher polymers remaining at the origin of the gel tended to increase (Fig. 1). The dimers, trimers, and polymerized subunits were transformed into monomers of acidic subunits in the presence of 2-mercaptoethanol (2-ME) (Fig. 1). As the self-supporting gel was formed, a band was observed between the positions of the monomers and the dimers, and its mobility was similar to that of intermediary subunits (Fig. 1). On the other hand, electrophoresis of buffer-insoluble components gave monomers, oligomers, and polymerized subunits (remaining in the origin of the gel; Fig. 2A).

Distributions of molecular weights of respective buffer-soluble components were compared using gel filtration on Sepharose CL-2B in the presence of 0.5% SDS. Elution patterns of respective buffer-soluble components are shown in Figure 3A. The elution profile indicated that the major peak (H-1) of the buffer-soluble components at the high protein concentration was a high molecular weight fraction, whereas peak (L-2) at the low protein concentration was a lower molecular weight fraction. On the other hand, the elution profile of buffer-insoluble components at the high protein concentration was similar to that of the low protein concentration (Fig. 3B). As shown in Figure 4, SDS-PAGE indicated that the high molecular weight fraction (H-1) in the buffer-soluble components contained polymers (remaining at the origin of the gel, Fig. 4A) consisting of acidic subunits (Fig. 4B).

The course of tryptic digestion of native glycinin over time as analyzed by SDS-PAGE is shown in Figure 5. SDS-PAGE of the digests in the absence of 2-ME (Fig. 5A) indicated that the time required for cleavage of intermediary subunits to the lower molecular weight fragments was 3–6 min. SDS-PAGE (Fig. 5B) in the presence of 2-ME indicated that acidic subunits decrease in their molecular weights during enzymatic digestion under conditions used here, but basic subunits did not.

Glycinin samples digested for various times were concentrated to 10% and heated to convert them into gels. Heating of glycinin digested for more than 6 min caused precipitation as determined by low-speed centrifugation, but samples digested for 3 min did not. When self-supporting gels were subjected to low speed centrifugation, sediments did not occur (Fig. 6). Under the conditions used here, enzymatic digestion for 3 min did not affect gelation, but digestion for 6 min did (Fig. 6). Heat-induced products of the digests were separated into buffer-insoluble and buffer-soluble components by low-speed centrifugation. The components were analyzed by gel filtration and electrophoresis in the presence of SDS. As shown in Figure 7, the elution profiles of

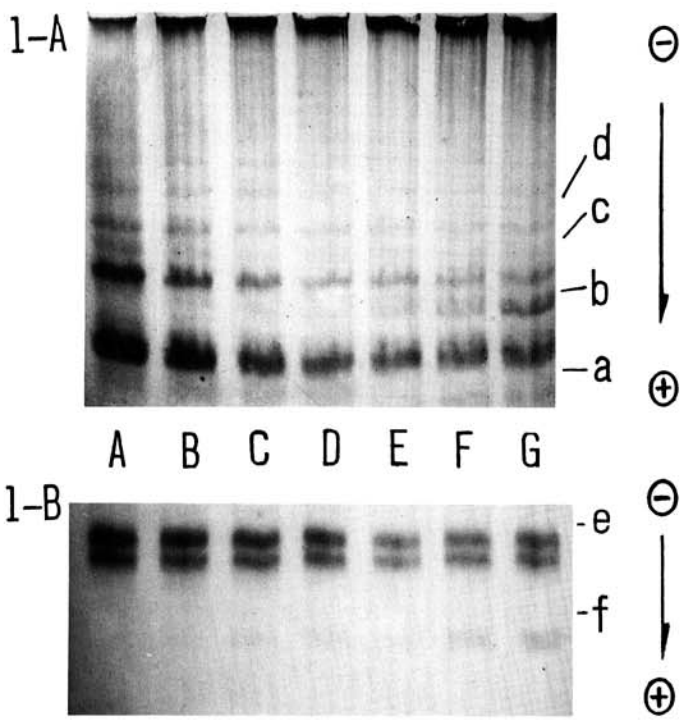

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of buffer-soluble components in the heat-induced products at the various protein concentrations. Concentration of gel was 3.75%. **A,** Heat-induced products without 2-mercaptoethanol; **B,** heat-induced products treated with 2-mercaptoethanol. Protein concentrations were **(A)** 0.5, **(B)** 1, **(C)** 2, **(D)** 3, **(E)** 4, **(F)** 5, and **(G)** 10%. Symbols **(a)**, **(b)**, **(c)**, and **(d)** indicate monomers, dimers, trimers, and tetramers of acidic subunits, respectively. Symbols **(e)** and **(f)** indicate positions of acidic and basic subunits, respectively.

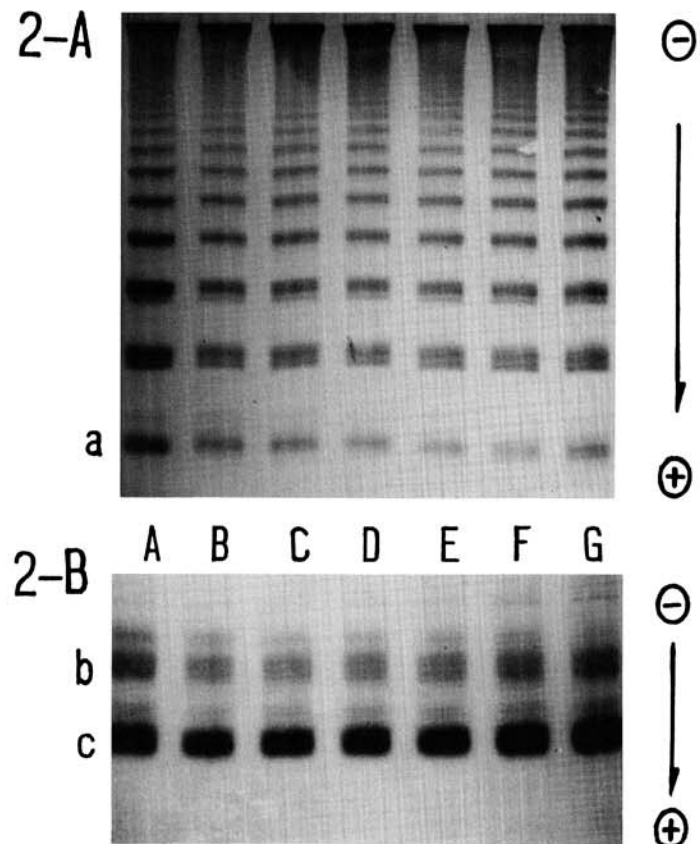

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of buffer-insoluble components in the heat-induced products at various protein concentrations. Concentration of the gel was 5%. **A,** heat-induced products without 2-mercaptoethanol; **B,** heat-induced products treated with 2-mercaptoethanol. Protein concentrations were **(A)** 0.5, **(B)** 1, **(C)** 2, **(D)** 3, **(E)** 4, **(F)** 5, and **(G)** 10%. Symbol **(a)** indicates monomers of basic subunits; various oligomers were detected.

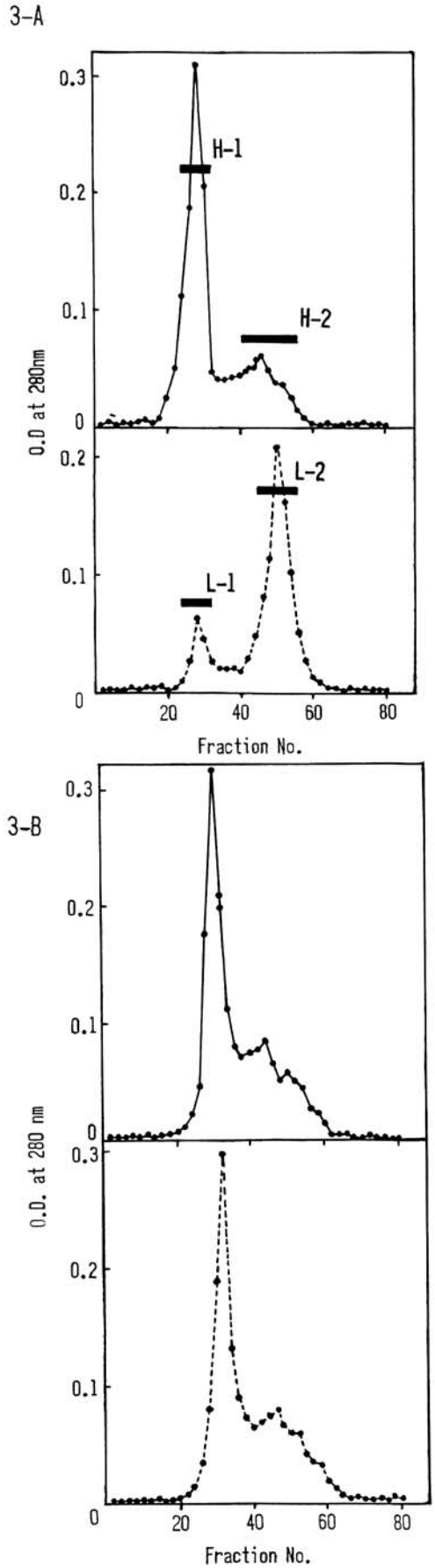

Fig. 3. Gel filtration of heat-induced products of glycinin on Sepharose CL-2B in 0.5% sodium dodecyl sulfate: •——• = 10% protein concentration; •-----• = 0.5% protein concentration. **A,** buffer-soluble components in the heat-induced products; **B,** buffer-insoluble components in the heat-induced products.

buffer-soluble components indicated that the major peak of glycinin digested for 3 min was of higher molecular weight, whereas the sample digested for 6 min was of smaller molecular weight. On the other hand, elution profiles of buffer-insoluble components of glycinin digested for 3 and 6 min were similar (data not shown). When trypsin-digested glycinin was heated, buffer-soluble components of the heat-induced products were analyzed by SDS-PAGE (Fig. 8). SDS-PAGE in the absence of 2-ME (Fig. 8A) indicated that the buffer-soluble components of glycinin digested for less than 3 min contain polymerized subunits at the origin of gel, but glycinin digested longer than 6 min did not. SDS-PAGE in the presence of 2-ME (Fig. 8B) indicated the buffer-soluble components of glycinin digested for less than 3 min consist of intact acidic subunits, but glycinin digested for 6 min did not.

DISCUSSION

When glycinin was subjected to heating at low protein concentration (0.5%), the solutions became turbid and proteins began to precipitate. At higher protein concentrations gelation occurred and their turbidities were slight. As shown in Figure 1, the electrophoretogram of buffer-soluble components in the heat-induced products at 0.5% protein solution consisted of monomers and dimers as major components and oligomers as minor components. These results were in accordance with those previously reported (Yamagishi et al 1980). Increasing protein concentration caused concentrations of monomers and oligomers to decrease, with a concomitant increase in the polymerized subunits (Fig. 1). These results were supported by observations of

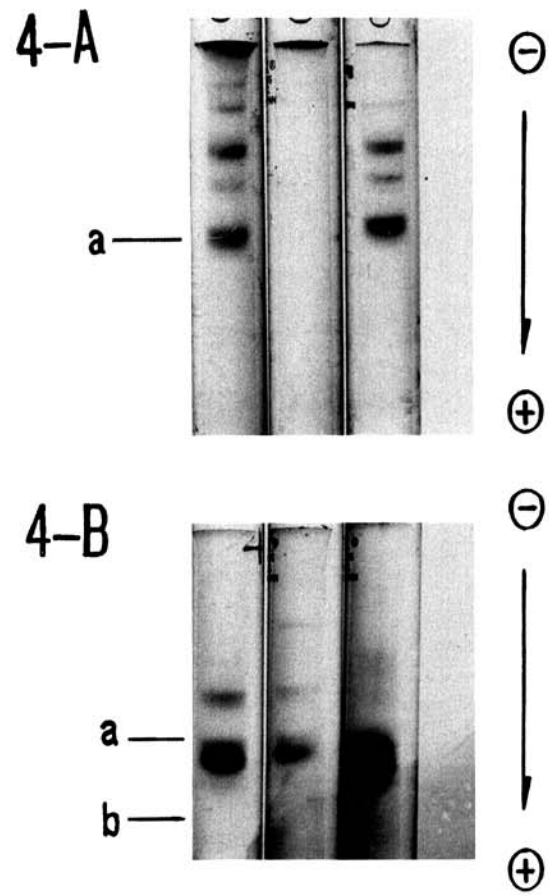

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of buffer-soluble components fractionated by gel filtration. (**A**) unfractionated, (**B**) H-1, (**C**) H-2. Concentration of the gel was 5%. **A,** treated without 2-mercaptoethanol; **B,** treated with 2-mercaptoethanol. H-1, H-2, L-1, and L-2 were depicted in Fig. 3A. Symbols (**a**) and (**b**) indicate the positions of acidic and basic subunits, respectively. Electrophoretic patterns of L-1 and L-2 were similar to those of H-1 and H-2, respectively (data not shown).

gel filtration characteristics in the presence of SDS (Fig. 3). On the other hand, the formation of monomers, oligomers, and polymers in the buffer-insoluble components did not seem to depend on the concentration of protein during heating (Fig. 2). Similarly, these results were supported by those of gel filtration (Fig. 3). Accordingly, gelation or precipitation of glycinin on heating may be attributed to buffer-soluble components. The formation of polymers in the buffer-soluble components distinctly depends on the protein concentration during heating, implying that the polymers play an important role in gel formation of glycinin. The polymers consist of acidic subunits polymerized through disulfide bonds when glycinin is heated at high protein concentration. To investigate the contribution of polymerization of acidic subunits to gelation, we must choose conditions that selectively inhibit polymerization of acidic subunits. One that is considered to selectively and partially degrade acidic subunits of glycinin is trypsin, which partially hydrolyzes protein and makes it possible to selectively degrade acidic subunits of glycinin as shown in Figure 5. Enzymatic degradation of native glycinin causes little conformational change or denaturation before heating. In order to investigate conformational changes in protein caused by enzymatic

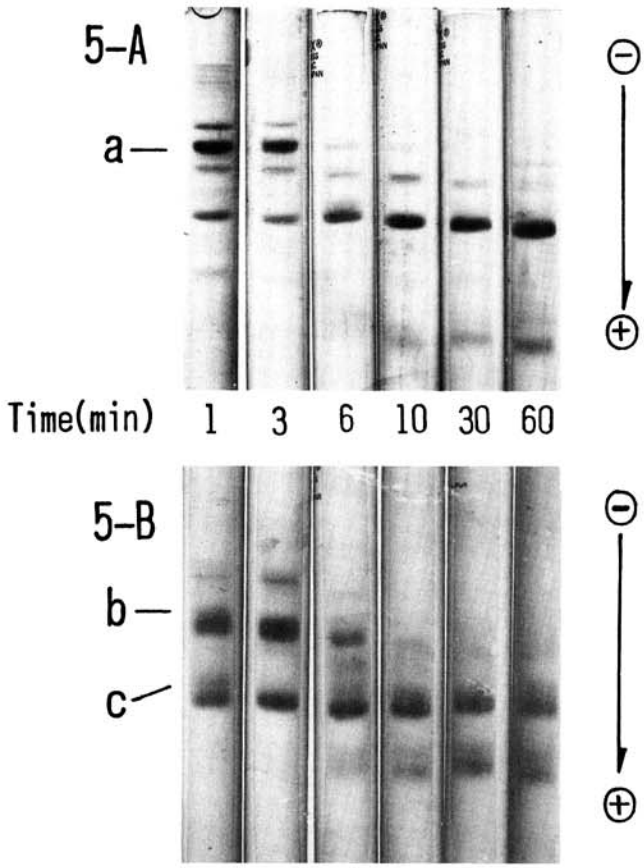

Fig. 5. Time of the selective degradation of acidic subunit of glycinin (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). **A,** the digests without 2-mercaptoethanol; **B,** the digests treated with 2-mercaptoethanol. Symbols (**a**), (**b**), and (**c**) indicate the positions of intermediary, acidic, and basic subunits, respectively.

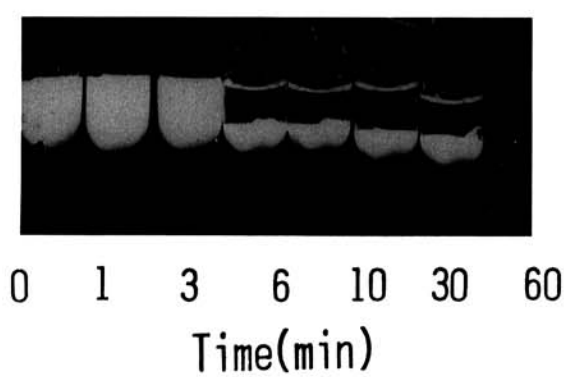

Fig. 6. Effect of enzymatic digestion of glycinin on gelation.

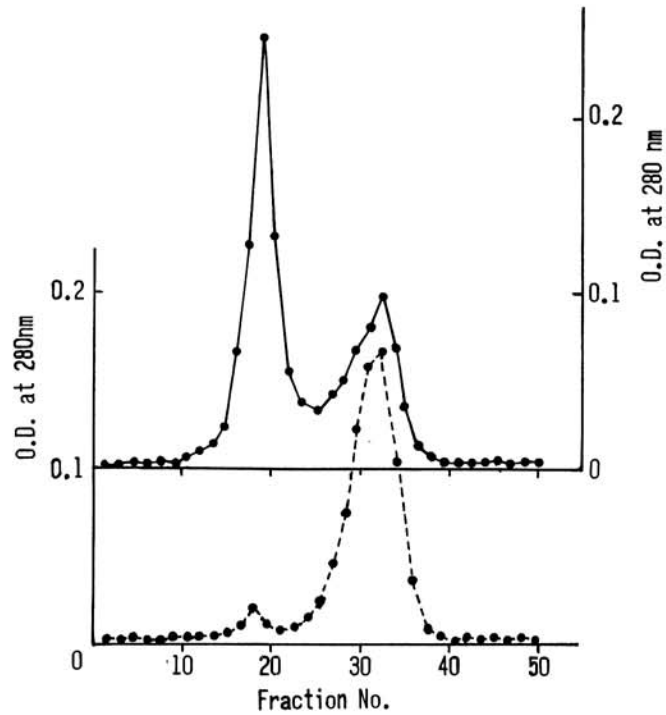

Fig. 7. Gel filtration of buffer-soluble components in the heat-induced products of trypsin-digested glycinin on Sepharose CL-6B in 0.5% sodium dodecyl sulfate: •-----• = glycinin digested for 6 min; •——• = glycinin digested for 3 min.

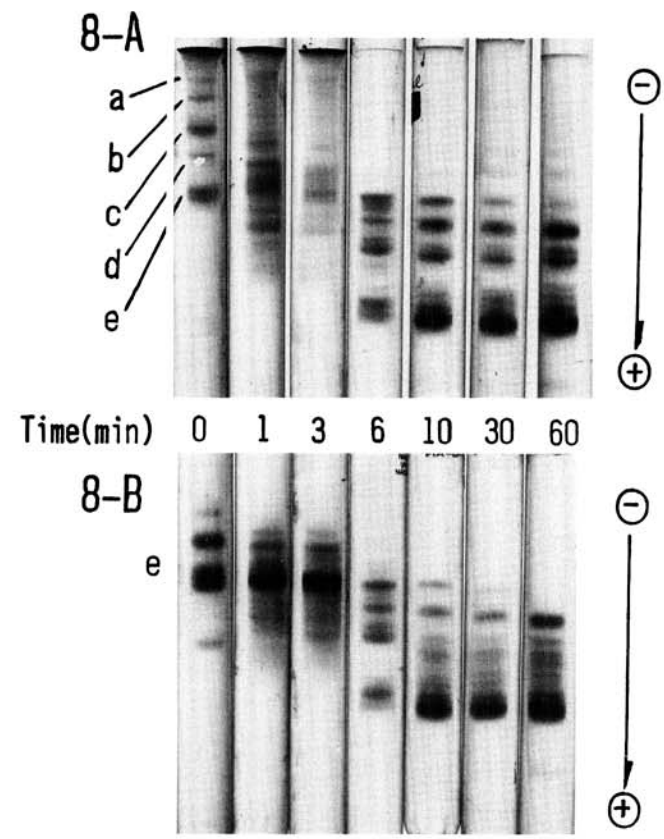

Fig. 8. Sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of heat-induced products of trypsin-digested glycinin. Symbols (**a**), (**b**), (**c**), (**d**), and (**e**) indicate the positions of acidic tetramers, trimers, dimers, and intermediary and acidic subunits, respectively.

digestion of glycinin, CD, difference-second derivative UV absorption spectra, and fluorescence spectra were measured (data not shown). These measurements did not show the evidence of conformational changes. Accordingly, partial degradation of an acidic subunit did not change the conformation of native glycinin. Enzyme-digested glycinin before heating retains the conformation of native glycinin. Nevertheless, the digests were no longer gelled on heating following the selective degradation of acidic subunit (Fig. 5B). SDS-PAGE and gel filtration indicated the digest that did not gel did not contain the disulfide-bonded polymer of acidic subunits. Thus, selective treatment of acidic subunits in the glycinin molecule interferes with polymerization of the acidic subunits on heating. The reasons for this interference by pretreatment with enzyme are as follows: 1) Disulfide interchange among acidic subunits is not caused by heating of the digests, because the pathway for heat denaturation of the digests is different from native glycinin; 2) a segment with sulfhydryl or disulfide residue, or both, which substantially participates in the sulfhydryl/disulfide exchange reaction, is cleaved upon enzymatic digestion and as a result looses the possibility for sulfhydryl and disulfide exchange among their residues. Even if heating causes the exchange in a manner similar to that for heat denaturation of native glycinin, there is little polymerization of acidic subunits. On the other hand, buffer-insoluble components in heat-induced products of the digests are similar to those of native glycinin. When the 6-min digests did not gel upon heating, the heat-treated products could be separated clearly into supernatant and precipitate (Fig. 6). This phenomenon is similar to that observed in low concentration solutions of native protein. On the other hand, electrophoresis indicated that buffer-insoluble components in the heat-induced products of the digests are similar to those of native glycinin at the low protein concentration. Accordingly, the polymerization of acidic subunits was found to play an important role in heat-induced gelation of soybean glycinin.

Wolf and Tamura (1969) conducted studies on heat denaturation of glycinin and reported that soluble aggregates are important intermediates in thermal denaturation, and that soluble aggregates formed in the presence of NEM remain unchanged on subsequent heating. They postulated that these intermediates, termed NEM-soluble, aggregate prior to the formation of the soluble aggregate that is formed in the absence of NEM. As sulfhydryl-disulfide exchange was found to play an important role in the formation of heat-induced end products (Yamagishi et al 1980), Yamagishi and co-workers (1981a,b, 1983c) focused on soluble aggregates and dissociates formed in the presence of NEM, which largely affects the sulfhydryl-disulfide exchange reaction. Spectrometric and electrophoretical evidence indicates that glycinin (or its intermediary subunits) prefers to associate through hydrophobic interaction at initial stages of heating, and on subsequent heating, some acidic subunits are released during formation of matured soluble aggregates (1981a). Furthermore, Yamagishi et al (1982b) demonstrated the pathway from the soluble aggregates to end products (precipitates) in the course of thermal denaturation and the role of the acidic subunit which is released during heating. Mori et al (1982) used ultracentrifugation and electrophoresis patterns to study heat denaturation at high protein concentration. The mechanism of heat denaturation of protein solutions at high concentration was substantially the same as those of low protein concentration solutions, i.e., sulfhydryl-disulfide exchange reaction (Wolf and Tamura 1969; Yamagishi et al 1980, 1982b, 1983a, 1984), NEM-soluble aggregates formation (Wolf and Tamura 1969; Yamagishi et al 1981a,b, 1983c), and release of acidic subunits during formation of soluble aggregates (Yamagishi et al 1980, 1981a, 1982b). Mori et al (1982) reported that the soluble aggregates are formed when glycinin is heated both at high and low protein concentrations. On continued heating, soluble aggregates disaggregated into acidic and basic subunits at low protein concentrations, whereas at high concentration they become highly associated, resulting in gel formation. In the end products of the gelling process, we found the polymer of acidic subunits as buffer-soluble aggregates and the polymer consisting of basic subunits as buffer-insoluble aggregates. Our results indicate that glycinin dissociates into its subunits at high protein concentration (gelation) as much as it does at low protein concentration (precipitation). Thus, we are confident that studies at low protein concentrations (Yamagishi et al 1980) are useful in understanding the mechanism of gelation.

Yamagishi et al (1980, 1982b) reported that the formation of precipitation is concomitant with the appearance of monomers of acidic subunits in a buffer-soluble form. Also, Nakamura et al (1985) reported that the formation of soluble aggregates is concomitant with the appearance of acidic subunits when glycinin is heated at high concentrations. Thus, the appearance of acidic subunits is strongly related to various phenomena at heating, irrespective of protein concentration. We speculate that whether a gel results or not is related to the release and polymerization of acidic subunits from the soluble aggregates.

Utsumi and Kinsella (1985), and Mori et al (1986) studied the effects of various reagents on solubility of heat-induced gels of glycinin and so predicted forces involved in gelation. They reported that all of the various molecular forces (covalent and noncovalent bonds) contributed to the formation and maintenance of gel structure. When the effects of various reagents on the hardness and solubility of matured gel were investigated, the results obtained did not seem to reflect significant interactions among subunits and related forces in the course of gel formation. Gelation involves the formation of a three-dimensional matrix through various protein-protein and protein-water binding and immobilizes water within the gel structure. We believe that protein-water interaction plays an important role in gel formation. Acidic subunit monomers and even their polymers have high affinities and water-holding capacities. Further studies on the relationship between release of acidic subunits and their polymerization, and interaction between acidic subunit polymers and water will be reported elsewhere.

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