Formation of Pseudoglycinins from Intermediary Subunits of Glycinin and Their Gel Properties and Network Structure

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Intermediary subunits of soybean 11S globulin (glycinin) designated as IS I, IS II and IS III were isolated by DEAE-Sephadex column chromatography. Pseudoglycinins composed of one of the intermediary subunits alone were reconstituted. The pseudoglycinins were similar to the native glycinin as to molecular size, subunit structure and secondary structure. The turbidity and hardness of the heat-induced gels formed from pseudoglycinins were different from those derived from the native glycinin, depending on the constituent intermediary subunits. The results indicate that IS II is closely related to the generation of the gel turbidity and IS III plays an important role in increasing the gel hardness. The hardness of the gel seems to be determined by both the length and extent of branching of the constituent strands of the gel network structure.

The gel forming ability of soybean proteins is one of the most important and desirable functional properties for their usage for conventional foods and as meat extenders. It is very important to elucidate how the constituents of soybean proteins contribute to the gel forming ability and the physical properties of the gels for their further adoption in soybean protein foods and for the breeding of useful soybean cultivars. From such a standpoint, we have investigated the gelation mechanism of 11S globulin (referred to as glycinin) which is one of the most predominant storage proteins of soybean.1,2

Glycinin is composed of six kinds of both acidic and basic subunits which constitute intermediary subunits linked to each other by disulfide bridges in a 1:1 ratio.3~5) Employing various pseudoglycinins reconstituted from the isolated constituent subunits and various cultivars of glycinins which have different subunit compositions, we found that the constituent acidic subunits contribute differently to the formation and hardness of the gels: ASIV (the most acidic subunit) and ASIII (the highest molecular weight subunit) play important roles in facilitating the gel formation and increasing the gel hardness, respectively.6,7)

Recent investigations on the biosynthesis of 11S globulin have demonstrated that 11S globulin is synthesized as a single polypeptide precursor consisting of covalently linked acidic and basic subunits, and then it is processed post-translationally to form both the acidic and basic subunits.8~12) This indicates that the specific acidic-basic pairing is primarily established at the step of translation. Therefore, it may be better to regard the intermediary subunits as constituent units of glycinin.

In the present study, in order to elucidate how the intermediary subunits of glycinin contribute to the physical properties of its gel, we compared the gelation and the physical properties of gels of pseudoglycinins formed from the isolated intermediary subunits with those of the native glycinin.

MATERIALS AND METHODS

Materials. Urea and 2-mercaptoethanol (ME), ex-
trapure reagents, were obtained from Nakarai Chemicals. Sodium dodecyl sulfate (NaDodSO4), electrophoretic grade, was purchased from Wako Pure Chemical Industries. DEAE-Sephadex A-50 and molecular weight marker proteins (phosphorylase b, MW 94,000; bovine serum albumin, MW 67,000; ovalbumin, MW 43,000; carbonic anhydrase, MW 30,000; trypsin inhibitor, MW 20,100; a-lactalbumin, MW 14,400) were obtained from Pharmacia Co., Ltd. Other chemicals were of guaranteed reagent grade.

Preparation of glycinin and its intermediary subunits. The purified glycinin was prepared from dry soybean seeds (Glycine max, var. Tsuru-no-ko) according to the method reported previously.5) The purified glycinin was dialyzed against 0.035 m potassium phosphate buffer (pH 7.6) containing 0.4 m NaCl. After exclusion of ME, the glycinin solution was equilibrated with 0.09 m sodium phosphate buffer (pH 6.6) containing 6m urea and 0.001 m EDTA degassed with nitrogen, and then applied to a column of DEAE-Sephadex A-50 equilibrated with the same buffer. Elution was performed with 1600 ml of the buffer containing a linear concentration gradient of 0 to 0.3 m NaCl. All operations were performed at 5 °C and all the buffers were degassed with nitrogen.

Preparation of pseudoglycinins from isolated intermediary subunits. Each isolated intermediary subunit was dialyzed against 0.035 m potassium phosphate buffer (pH 7.6) containing 0.4 m NaCl, 40% (v/v) glycerol and 0.02% NaN3. After dialysis, the sample was centrifuged on a linear sucrose density gradient (10~30%, w/v). After centrifugation, the gradient was divided into 0.4ml fractions, with simultaneous measurement of the absorbance at 280nm, with an ISCO density gradient fractionator. The procedure followed the method for the reconstitution from isolated subunits described previously.13)

Electrophoreses. NaDodSO4 gel electrophoresis was performed according to the method of Laemmli14) at room temperature with 10% polyacrylamide gels in the presence or absence of ME as described previously.15) Alkaline urea gel electrophoresis was carried out in 7m urea according to the method of Davis16) in 7 m urea as described previously.15)

Measurement of circular dichroism (CD) spectra. CD measurements were performed with a Durrum-JASCO Model J-500 at room temperature. The measurements were made in 1 mm cells and the protein concentration was 0.25 mg/ml. The mean residue ellipticity, [θ], was expressed as the dimension of degrees-square centimeter-decimoles.−1

Methods of gelation and determination of turbidity and hardness of gels. Twenty-microliter aliquots of the protein solution in 0.035 m potassium phosphate buffer (pH 7.6) containing 0.4 m NaCl (heating buffer) were taken into micropipettes (Drummond Scientific Co., 200 μl) and heated at 100 °C in a water bath for 20 min. The turbidity of the gels formed in the micropipettes was measured by scanning as such with a Shimadzu dual-wavelength chromatocaner, 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.6,17)

Transmission electron microscopy. A small piece of the gel was fixed, dehydrated, embedded and sectioned. The thin sections were stained and viewed under a Hitachi H-700H electron microscope. The details of the procedures were given in a previous paper.21

Protein determination. Protein was determined by the method of Lowry et al.18)

RESULTS AND DISCUSSION

Fractionation and characterization of intermediary subunits of glycinin

The intermediary subunits of glycinin were fractionated by chromatography on a DEAE-Sephadex column in the presence of 6 m urea as described in MATERIALS AND METHODS. As shown in Fig. 1, glycinin was separated into five fractions. These fractions (the underlined parts) were termed peaks 0, I, II, III and IV in order of elution from the column, as indicated in Fig. 1.

Each fraction was characterized by gel electrophoresis under different conditions as shown in Figs. 2 and 3. Peak 0 contained a basic subunit with a molecular weight (MW) of 19,000 and a presumed contaminant (MW 24,000), but no acidic subunit (Fig. 2B). Peak I contained intermediary subunit IS I (MW 58,000), which is composed of ASI (MW 37,000) and BS (MW 21,000), peak II intermediary subunit IS II (MW 58,000), which is composed of ASH (MW 38,000) and BS (MW 20,000), peak III intermediary subunit IS III (MW 62,000), which is composed of ASIII (MW 42,000) and BS (MW 20,000), and peak IV had no intermediary subunit but ASIV (MW 38,000) (Figs. 2 and 3). Identification of each acidic subunit (ASI, ASH, ASIII and ASIV) was carried out on the basis of its mobility of alkaline urea gel electrophoresis, as
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One gram of the purified glycinin preparation was fractionated on a 2.3 x 70 cm column at 5°C as described in MATERIALS AND METHODS. The flow rate was 20 ml/hr, and 20 ml fractions were collected. —○—, absorbance at 280 nm; ———, concentration of NaCl.

Fig. 1. Fractionation of the Intermediary Subunits of Glycinin by DEAE-Sephadex Column Chromatography.

Fig. 2. NaDodSO₄ Gel Electrophoresis of Each Peak Fractionated by DEAE-Sephadex Column Chromatography.

Fifty micrograms of protein was electrophoresed in the absence (A) and presence (B) of ME as described in MATERIALS AND METHODS. Migration is from top to bottom. N indicates the native glycinin. The numbers under the gels are the peak numbers on DEAE-Sephadex column chromatography. S indicates the molecular weight marker proteins. IS, AS and BS represent intermediary, acidic and basic subunits of glycinin, respectively.

shown in Fig. 3. ASIV forms an intermediary subunit with the counterpart basic subunit not through a disulfide bond but a noncovalent bond,⁵,¹⁹ therefore the basic subunit in peak 0 could be the counterpart of ASIV. These results with respect to the intermediary subunit structure seem to coincide fundamentally with those reported by Staswick et al.²⁰,²¹

Formation of pseudoglycinins from isolated intermediary subunits

The reconstituted products from each isolated intermediary subunit prepared as described in MATERIALS AND METHODS were fractionated by sucrose density gradient centrifugation (Fig. 4). In the absorbance patterns, the sedimentation positions of the 7S size (half-molecule of the 11S component) and the 11S size are indicated by arrows. As shown in Fig. 4, the extent of the formation of the 11S component from any intermediary subunits was more than 80%. Unreacted intermediary subunits and larger aggregates than an 11S component were not detected at all in any case.
Electrophoresis was performed in the absence (A) and presence (B) of ME as described in Materials and Methods. For the native glycinin and the fractionated samples, 100 \( \mu g \) and 50 \( \mu g \) of protein were used, respectively. Migration is from top to bottom. The symbols are the same as in Fig. 2.

Thus, the intermediary subunits have a tendency to associate to form 11S components composed of homologous intermediary subunits. In other words, the association is not random, whereas the specificity of interactions among intermediary subunits is not strict in the formation of the 11S component. This may be one of the reasons for the occurrence of heterogeneity of glycinin molecules. For the reconstitution from the intermediary subunits, we employed no agitation during dialysis against the buffer containing 40\% glycerol. In the case of agitation, the extent of the formation of an 11S component was extremely low and larger complexes were formed (data not shown). Thus, urea should be excluded slowly during the dialysis. Although there is no clear explanation for this phenomenon, the intermediary subunits may refold slowly and thereby satisfy the size specificity for the reconstitution of an 11S component. Otherwise the association of the intermediary subunit proceeds randomly resulting in little reconstitution.

In the case of the combination of ASIV and peak 0, neither the formation of a 7S nor that of an 11S component was observed. No clear explanation for this phenomenon can be offered at the present time.

The reconstituted 11S components (pseudoglycinins) from each intermediary subunit fractionated by sucrose density gradient centrifugation were analyzed by NaDodSO4 gel electrophoresis in the absence (Fig. 5, A) or presence (Fig. 5, B) of ME. In either the absence or presence of ME, the pseudoglycinins showed the same patterns as those of the isolated intermediary subunits shown in Fig. 2. This indicates that the reconstituted pseudoglycinins have a similar subunit structure to that of the native glycinin.

In order to compare the secondary structure of each pseudoglycinin with that of the native glycinin, the CD spectra were measured as shown in Fig. 6. The native glycinin and all the pseudoglycinins had similar spectra. This indicates that the pseudoglycinins have similar secondary structures to that of the native glycinin.
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Fig. 5. NaDodSO₄ Gel Electrophoresis of Pseudoglycinins.
Fifty micrograms of each pseudoglycinin obtained on sucrose density gradient centrifugation was electrophoresed in the absence (A) and presence (B) of ME as described in Fig. 2. Migration is from top to bottom. S, molecular weight marker proteins; N, native glycinin; I, pseudoglycinin from IS I; II, pseudoglycinin from IS II; III, pseudoglycinin from IS III. IS, AS and BS are the same as in Fig. 2.

Fig. 6. CD Spectra of Pseudoglycinins.
---, native glycinin; ----, pseudoglycinin from IS I; ------, pseudoglycinin from IS II; ---, pseudoglycinin from IS III.

Considering the sedimentation positions on sucrose density gradient centrifugation (Fig. 4), the intermediary subunit structure (Fig. 5) and the secondary structure (Fig. 6), a similar structure to that of the native glycinin may be renatured in the reconstituted pseudoglycinins.

Gel properties of pseudoglycinins
The pseudoglycinins fractionated by sucrose density gradient centrifugation as described above were heated for 20 min at various protein concentrations, and the turbidity of the gels obtained was compared with that of the native glycinin as shown in Fig. 7. The gel turbidity of all protein samples decreased with increasing protein concentration. The gels from IS I and IS III exhibited lower turbidity than the native glycinin, while that of the gel from IS II was much higher. These results suggest that IS II is closely related to the generation of the turbidity of glycinin gels.

We reported previously that the turbidity of a gel of glycinin has a tendency to increase with increasing sulfhydryl group content. On the other hand, IS II isolated here seems to correspond to the intermediary subunit of A₂-
B_{1a} which was isolated by Staswick et al.,\textsuperscript{20) judging the elution profile on DEAE-Sephadex column chromatography in the presence of 6 M urea. Staswick et al.\textsuperscript{21) reported that A_{2}B_{1a} has the largest number of cysteine and cystine residues among the intermediary subunits. These available data indicate that the reason why IS II is related to the turbidity of the gel is

Fig. 9. Transmission Electron Micrographs of Pseudoglycinin Gels.
A, native glycinin; B, pseudoglycinin from IS I; C, pseudoglycinin from IS II; D, pseudoglycinin from IS III.
its high content of sulfhydryl group.

The hardness of the gels formed from the pseudoglycinins was compared with that of the native glycinin as shown in Fig. 8. The gel hardness of all protein samples increased with increasing protein concentration. The hardness of the gel from IS I was similar to that of the native glycinin gel. The gels from IS II and IS III were significantly softer and harder than the native glycinin gel, respectively. These results suggest that IS III plays an important role in increasing the hardness of the glycinin gel, which coincides with the previous findings on the contribution of acidic subunits to gel hardness in experiments employing glycinins of various soybean cultivars and pseudoglycinins reconstituted from isolated constituent subunits.

Network structure of gels

In order to elucidate why the hardness of the gels is different depending on the intermediary subunit composition, the network structures of the gels from each pseudoglycinin and native glycinin were investigated by transmission electron microscopy (Fig. 9). The gel from IS I, the hardness of which was similar to that of the native glycinin, showed the same network picture as that of the native glycinin (Fig. 9, A and B), indicating that the length and extent of branching of the constituent strands may be almost the same as those of the native glycinin. The gel from IS II, the hardness of which was lower, exhibited a rough network structure (Fig. 9, C). This may indicate that the length and extent of branching of the constituent strands are longer and fewer, respectively, and that the constituent strands are dispersed randomly. On the other hand, the gel from IS III, the hardness of which was higher, exhibited an elaborate network structure (Fig. 9, D), indicating that the length and extent of branching of the constituent strands may be shorter and much greater in number, respectively. These results may demonstrate that the gel hardness of glycinin is decided by the length and extent of branching of the constituent strands of the network structure of the gel, i.e., the elaborateness of the gel network.

Previously we reported that ASIV is closely related to the difference in the time required for gel formation of glycinins with different subunit compositions from various soybean cultivars. On the other hand, as demonstrated in this study, IS III, the constituent acidic subunit of which is ASIII, and IS II, the constituent acidic subunit of which is ASII, play important roles in increasing the gel hardness and gel turbidity, respectively. Furthermore, it has been shown that 7S and 11S globulins are different from each other in gelation and gel properties, and that the contents of 7S and 11S globulins are different among soybean cultivars. These facts clearly indicate that we may be able to control the gelation and the gel properties of soybean proteins through the selection and/or breeding of soybean cultivars by taking into account the protein compositions.

REFERENCES


