Reconstitution of Single Molecular Species from Isolated Subunits of Glycinin

Guoyan Zhang, Satoshi Tsunokawa, Yukako Hayashi, Shinya Matsumoto, Yasuki Matsumura, and Tomohiko Mori*

Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

ABSTRACT: Ion-exchange HPLC systems using POROS 20 HQ and Mono Q HR 10/10 columns were applied to isolate glycinin subunits under denaturing conditions. Analyses by SDS-PAGE, N-terminal amino acid sequence, and sucrose density gradient centrifugation showed that the pseudoglycinin from the highly purified homo-subunit, A_3B_4, was reconstituted. The A_3B_4 pseudoglycinin was similar to the native glycinin with respect to molecular size, subunit structure, and secondary structure. The hexameric pseudoglycinin dissociated into trimers after long storage at pH 7.6.

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KEY WORDS: Glycinin, pseudoglycinin, reconstitution, subunit.

Soybean proteins play important roles in food processing systems based on their excellent nutritional qualities and desirable functionalities, such as gelation, emulsification, and foaming (1). 11S globulin (glycinin), a legumin-type protein, is a dominant storage protein in soybean seeds. Glycinin is a hexameric protein composed of five constituent subunits: $A_{1a}B_{1b}$, A_2B_{1a}, $A_{1b}B_2$, A_3B_4, and $A_5A_4B_3$ (2). Each subunit is composed of an acidic polypeptide (acidic pI) and a basic polypeptide (basic pI). The acidic and basic polypeptides are linked together by a disulfide bond.

Among various soybean cultivars, there are differences in the subunit compositions of glycinin (3). In the same soybean cultivar, glycinin exhibits heterogeneity of molecular species (4). Formation of the glycinin molecule from a single subunit is possible. Earlier work by Mori and Utsumi (5), Utsumi *et al.* (6), and Nakamura *et al.* (7) reported the formation of pseudoglycinins from artificial A-B subunits composed of the isolated acidic and basic polypeptides, and from the isolated native A-B subunits. Furthermore, gelation phenomena of the pseudoglycinins were studied, and the contribution of each A-B subunit to the gel hardness was found to be different (7,8).

It has become more and more important to elucidate the protein functionalities at the molecular level, such as the 3-D structure. For X-ray structure analysis, a homogeneous molecular species of protein with a single subunit is required to obtain good crystallization. Glycinin is difficult to crystallize because of molecular heterogeneity. Recently, there have been several studies concerning glycinin species that have consisted of only a single subunit species (9–11). These single homogeneous glycinins, together with the *in vitro* reconstitution of a single molecular species of glycinin from a single subunit species, is expected to be a good approach for structural analysis of glycinin and for functional property studies. The objective of this research was therefore to prepare well-purified A-B subunits from native glycinin by using anion-exchange HPLC systems and to reconstitute the single molecular species of glycinin from the purified A-B subunit.

*To whom correspondence should be addressed.
E-mail: morit@kais.kyoto-u.ac.jp

EXPERIMENTAL PROCEDURES

Materials. Soybean (*Glycine max*, var. Shirotsurunoko) was kindly supplied by the Faculty of Horticulture of Chiba University (Chiba, Japan). All chemicals were reagent grade.

Preparation of glycinin. 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 vol (vol/vol) of 63 mM Tris-HCl buffer (pH 7.8) containing 10 mM 2-mercaptoethanol (2-ME) and allowed to stand for 1 h at 20°C with gentle stirring. The homogenate was filtered through gauze and then centrifuged at $9000 \times g$ for 15 min at 0°C. Chilled acetone was added slowly to the supernatant to 60%. The precipitate was collected by centrifugation, washed once with chilled acetone, washed once with diethyl ether, dried, and stored at 4°C until used. The glycinin fraction was isolated on a DEAE-Toyopearl (Tosoh, Tokyo, Japan) column equilibrated with 35 mM potassium phosphate buffer (pH 7.6) containing 0.15 M NaCl, 10 mM 2-ME, and 0.02% NaN_3 at 4°C, with a linear elution gradient of 0.15–0.4 M NaCl (12).

Isolation of glycinin subunits. The purified glycinin was equilibrated with 90 mM sodium phosphate buffer (pH 6.6) containing 6 M urea and 1 mM EDTA according to the method of Nakamura *et al*. (7), and then applied to a column of POROS 20 HQ from PerSeptive Biosystems, Inc. (Framingham, MA) connected to the HPLC system. Elution was performed with the same equilibrated buffer containing a linear gradient of NaCl from 0 to 0.3 M. The isolated fraction was rechromatographed on a Mono Q HR 10/10 column from Pharmacia Biotech (Uppsala, Sweden) connected to the HPLC system. The fraction was eluted as above with a linear gradient of NaCl from 0.15 to 0.21 M. All operations were carried out at 5°C, and all buffers were degassed with nitrogen.

Reconstitution of pseudoglycinin from isolated subunits. The isolated subunit at 0.5% concentration was dialyzed

against 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl, 40% (vol/vol) glycerol, and 0.02% NaN_3 without agitation at 20°C for 48 h with one change of buffer, and then equilibrated with the buffer as above without glycerol at 5°C. The sample was subsequently centrifuged at 154,000 × *g* on a 10 to 30% (wt/vol) linear sucrose gradient in 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl at 20°C for 20 h in a Hitachi RPS 40T rotor. After centrifugation, the gradient was examined at 280 nm with an ISCO density gradient fractionator.

Gel electrophoresis. SDS-PAGE was performed as described by Laemmli (13) in a 12.5% slab gel at room temperature. The gel was stained with Coomassie Brilliant Blue R-250.

N-Terminal amino acid sequence analysis. After electrophoresis, the sample fragments on the gel were blotted onto polyvinylidene difluoride (PVDF) membrane at 25°C at constant 1 mA per cm^2 membrane for 90 min. The Ponceau S-stained bands in the PVDF membrane were excised with a razor blade for amino acid sequence analyses. Subsequently, N-terminal amino acid sequences were determined by a protein sequencer, Model 492 Procise (Applied Biosystems, Framingham, MA), with detection of phenylthiohydantoin (PTH)-amino acids.

Circular dichroism (CD) spectra. CD spectra measurements were made with a JASCO model J-720 spectropolarimeter with a cell path length of 0.2 cm over the range of 190–250 nm (far UV). The sample was measured at 25°C at 1 mg/mL of protein concentration.

Protein determination. Protein was determined by using the method of Lowry *et al*. (14). The Bradford method (15) was used where precise concentration of protein was not strictly required, such as SDS-PAGE.

RESULTS AND DISCUSSION

Isolation of A-B subunits of glycinin. Glycinin prepared from acetone powder on a DEAE-Toyopearl column (data not shown) was applied to the HPLC system using a POROS HQ column in the presence of 6 M urea as has been described. Three distinct peaks were fractionated as shown in Figure 1A (peaks 1, 2, and 3). The fractionated peaks were subsequently characterized by SDS-PAGE in the absence and presence of 2-ME (Fig. 1B). Compared with native glycinin (Fig. 1B, lane N), isolated peaks 1 and 2 were composed of acidic and basic polypeptides that were linked *via* disulfide bonds. Peak 1 contained acidic polypeptides A_1 (A_{1a}, A_{1b}) and A_2 and basic polypeptides. Peak 2 contained acidic polypeptides A_3 and basic polypeptides B_4. As Fontes *et al.* (16) demonstrated, peak 3 contained acidic polypeptide A_4, shown as a broad band in SDS-PAGE (Fig. 2B, lane 3), but no basic polypeptides. This is consistent with previous work reported by Nakamura *et al.* (7) and Mori *et al.* (3), where the A_4 polypeptide was thought

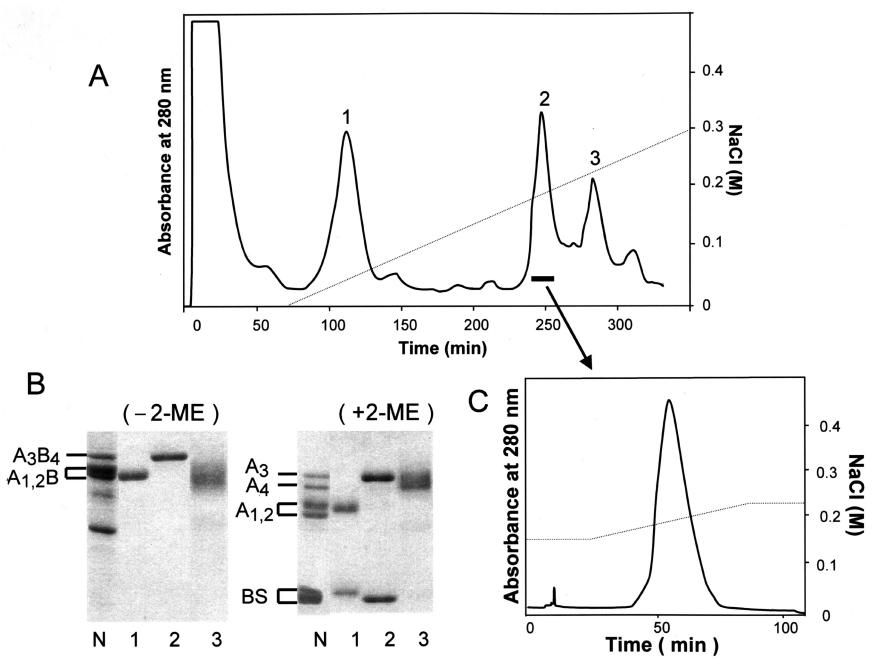

FIG. 1. (A) HPLC patterns of subunits from glycinin on a POROS 20 HQ column (PerSeptive Biosystems, Inc., Framingham, MA) as described in the Experimental Procedures section. The numbers on the chromatogram indicate each eluting peak. (B) SDS-PAGE of each peak fractionated by POROS 20 HQ column chromatography in the presence of urea. N indicates native glycinin; lanes 1, 2, and 3 represent the peak numbers on the chromatograph. $A_{1,2}$, A_3, A_4, and BS represent acidic and basic polypeptides of glycinin, respectively. (C) HPLC pattern of fraction isolated from POROS 20 HQ column chromatography (shown as peak 2 in part A) on Mono Q HR 10/10 column (Pharmacia Biotech, Uppsala, Sweden) as described in the Experimental Procedures section.

to be linked to the A_5 polypeptide and basic polypeptide to form an A-B subunit (17), not through a disulfide bridge but through a noncovalent bond that could be disrupted by denaturants such as urea and SDS. Therefore, as the counterpart of the A_4 polypeptide, the B_3 polypeptide was presumed to elute earlier, in 50 min, along with the A_5 polypeptide.

The fraction containing single subunit species of A_3B_4 (Fig. 1A, peak 2) was collected and rechromatographed by using a Mono Q HR column in the presence of 6 M urea (Fig. 1C). One major peak was obtained. SDS-PAGE analysis of the fraction in the presence and absence of 2-ME showed the same results as in Figure 1B (lane 2) (data not shown), suggesting that a highly purified A_3B_4 fraction was obtained by using the Mono Q HR column.

Reconstitution and characterization of pseudoglycinin. The highly purified A_3B_4 subunit from the chromatography was reconstituted by dialyzing against the potassium phosphate buffer without agitation to remove urea. The reconstituted products were examined by sucrose density gradient centrifugation (Fig. 2A) to check the extent of reconstitution. The reconstituted product contained more than 80% of an 11S-size component (peak II), and less than 20% of an 7S-size component (peak I) (18). Unreacted monomers or larger aggregates of the subunit were hardly ever observed in any replicates. Each fraction was characterized by SDS-PAGE in the absence and presence of 2-ME (Fig. 2B).

To further identify each polypeptide, the bands observed in the presence of 2-ME were then transferred to a PVDF membrane by electroblotting and analyzed for N-terminal amino acid sequences. Since the full amino acid sequences of the glycinin subunits in the Shirotsurunoko cultivar used in this study have not been determined, known sequence data of the soybean cultivar CX635-1-1 were used for comparison. Sequences (NH_2-Ile Thr Ser Ser Lys Phe Asn) corresponding to the known A_3 polypeptide were detected clearly in the purified fraction. Since the specific pairing of acidic and basic subunits exists in glycinin A-B subunits (17), the bands on SDS-PAGE (–2-ME) were identified as A_3 and B_4 polypeptides. It has been demonstrated that the amino acid sequence of each subunit of glycinin is somewhat different among the cultivars (19). Considering the full sequences of the A_3B_4 subunit, we were unsure with this method whether there was a micro heterogeneity between soybean cultivars of Shirotsurunoko and CX635-1-1. However, the basic structures and protein chemical properties are considered to be substantially similar even though the sequence homogeneity of the A_3B_4 subunit among different cultivars is not so strict (20).

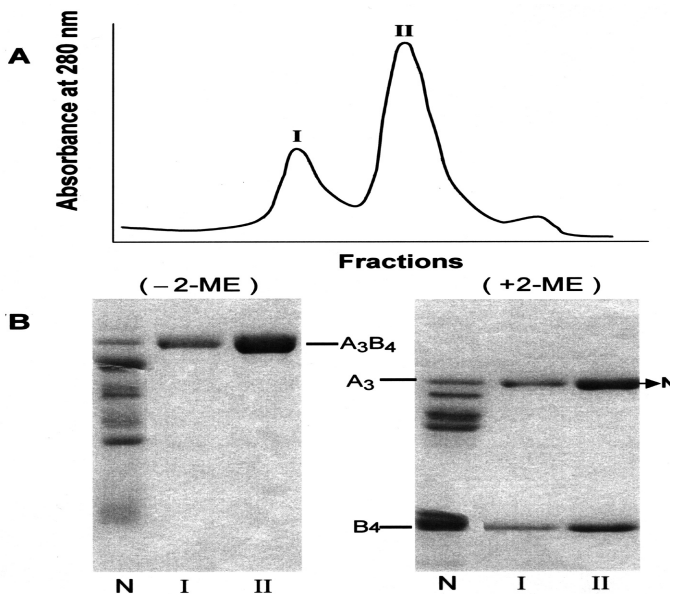

FIG. 2. (A) Sucrose density gradient centrifugation of the reconstituted products. Sedimentation is from left to right. (B) SDS-PAGE of each fraction in the presence of urea. N represents native glycinin; I and II represent 7S-size and 11S-size components; A_3 and B_4 represent acidic and basic polypeptides, respectively.

In the case of both the 7S- and 11S-size components, a single band corresponding to the A_3B_4 subunit (–2-ME) and a single band corresponding to the A_3 or B_4 polypeptide (+2-ME) were detected, respectively. The electrophoretic patterns obtained here were the same as those of the isolated subunit shown in Figure 1B. This indicated that the 11S-size component, the reconstituted pseudoglycinin, has a subunit structure similar to that of the native glycinin. The 7S-size component was a half-molecule of the pseudoglycinin. In previous work, the half-molecule of glycinin was referred to as 7S to distinguish it from the 7S globulin, conglycinin (20).

On reconstituting, the isolated A_3B_4 subunit had a tendency to associate with the 11S component composed of a homologous subunit. To obtain higher recovery of reconstituted products, urea should be excluded slowly without agitation during dialysis. In the case of agitation, we found an extremely low proportion of pseudoglycinin formed, and the reconstituted products were very complex in size distribution (data not shown). Although there is no clear explanation for this phenomenon, the subunit was presumed to assemble slowly and thereby satisfy the size specificity for the reconstitution of an 11S component. Otherwise, the association of the subunit proceeded too randomly, resulting in a low extent of reconstituted glycinin of 11S size.

To compare the secondary structure of the reconstituted 11S component with that of the native glycinin, CD spectra were measured as shown in Figure 3. There was only a slight difference in the spectra between the examined samples. This indicates that there was no significant difference in secondary structure between the reconstituted 11S component, A_3B_4-pseudoglycinin, and the native glycinin. This result, together with the same sedimentation position on sucrose density gradient centrifugation of the pseudoglycinin and native glycinin, suggests that A_3B_4-pseudoglycinin may have a molecular structure similar to that of the native glycinin.

Stability of pseudoglycinin. The reconstituted 11S component in about 0.5% solution was recentrifuged in a sucrose density gradient after storage at 5°C for 1 and 7 d at pH 7.6 in 35 mM potassium phosphate buffer containing 0.4 M NaCl and 0.02% NaN_3 to determine its stability. Not only an

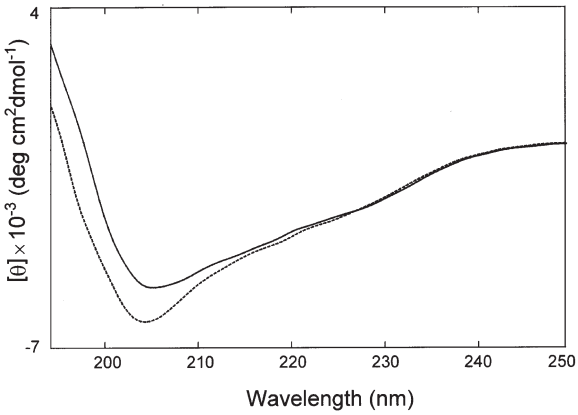

FIG. 3. Circular dichroism spectra of the reconstituted glycinin component in 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl. ——, native glycinin; ------, reconstituted glycinin.

11S-size component (pseudoglycinin) but also a 7S-size component was observed (Fig. 4). It seemed that a decrease in the 11S component took place with an accompanying increase in the 7S component. With 1 d of storage, only about 5% of the 7S component appeared (Fig. 4A), but after 7 d of storage, the 7S component increased to more than 60% (Fig. 4B). This indicates that the reconstituted hexameric A_3B_4-pseudoglycinin dissociated into its trimeric half-molecules.

In glycinin reconstitution experiments using rabbit reticulocyte lysate, synthesized subunits assembled to the half-size 11S (trimer), but not to the full-size 11S (hexamer) (21). In addition to this result, Utsumi *et al.* (11) showed that in *Escherichia coli* expressing glycinin subunits, the trimer formed, but not the hexamer; presumably, the hexamer formed after the processing of the pro-type took place. Because the matured subunits prepared from glycinin were used as the glycinin-reconstituting component, the reassembly conditions and results could not be applied directly to those of the *in vitro* or *in vivo* conditions (11,21). Perhaps some structural factors or processes other than the those of pro-type

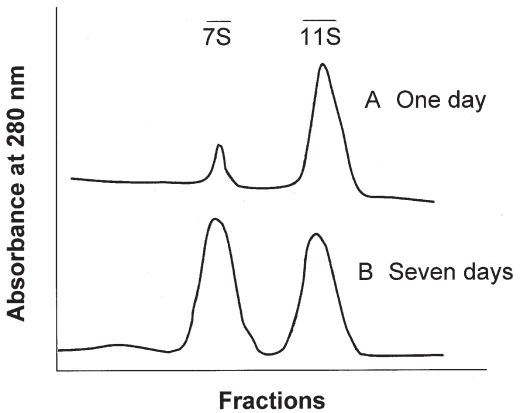

FIG. 4. Recentrifugation of pseudoglycinin in sucrose density gradient. (A) Pseudoglycinin was stored for 1 d at 5°C at pH 7.6 and then centrifuged. (B) Pseudoglycinin was stored for 7 d at 5°C and pH 7.6 and then centrifuged. $\overline{7S}$ and $\overline{11S}$ represent the 7S-size and the 11S-size components, respectively.

subunit assembling to the hexamer may exist and contribute to the instability of the reconstituted glycinin as observed in this study.

Early studies on the stability of native glycinin indicated that glycinin (11S component) partially dissociates into a 7S component when the ionic strength is lowered to 0.01 at pH 7.6 (22), whereas at high ionic strength ($\mu = 0.5$) at pH 7.6, native glycinin is stable at the 11S size (23). The pseudoglycinin we obtained, however, showed dissociation properties even in the presence of high ionic strength ($\mu = 0.5$) over a period of storage. The difference in stability between native glycinin and pseudoglycinin at high ionic strength may be derived from the differences in molecular force in the subunit interactions and/or assembly. The interactions among homosubunits of A_3B_4 are presumed to be weaker than those among hetero-subunits in native glycinin. This is consistent with the result herein of CD spectra in which the conformation of pseudoglycinin species exhibits slight flexibility, and therefore leads to the unstable property compared with native glycinin. It may be desirable to find a better condition for storing A_3B_4-pseudoglycinin for a relatively long time in order to study the functionalities of the pseudoglycinin.

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