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Molecular species of glycinin in some soybean cultivars

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Abstract

A_4 polypeptide-containing (Shirotsurunoko and York) and A_4 polypeptide-lacking (Raiden and Suzuyutaka) soybean cultivars were used to investigate the heterogeneity of glycinin molecular species. Purification of glycinin by DEAE-Toyopearl column chromatography afforded molecular species eluting before the glycinin fraction. Analysis of this fraction by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and sucrose density gradient centrifugation indicated that this protein consisted of A_1 and A_2 polypeptides. The A_4-containing soybean cultivars contained less of this protein than the A_4-lacking soybean cultivars, as exhibited by the size of the early peak appearing during column chromatography. Alkaline PAGE and N-terminal amino acid sequence analysis confirmed that the A_1- and A_2-rich molecular species in the A_4 polypeptide-lacking cultivars consisted of the A_{1a} and A_2 polypeptides. Estimation of the molecular mass by gel permeation chromatography and multi-angle laser light scattering (GPC–MALLS) indicated that the A_{1a}- and A_2-rich molecular species were similar to a monomer of glycinin.

Keywords: *Glycine max*; Leguminosae; Glycinin; Molecular species; Soybean cultivars

1. Introduction

Storage globulins in soybean seeds, which comprise most soybean proteins, can be grouped mainly into two types based on their sedimentation coefficients: 7S globulin (β-conglycinin) and 11S globulin (glycinin). Glycinin, which has been studied quite extensively (Staswick et al., 1981; Mori et al., 1984; Nielsen, 1985; Wright, 1987; Nielsen et al., 1989), is a hexamer composed of constituent subunits. Each subunit is composed of an acidic (acidic pI, A) and a basic (basic pI, B) polypeptide, which are linked together by a disulfide bond. Five major subunits have been identified, $A_{1a}B_{1b}$, A_2B_{1a}, $A_{1b}B_2$, A_3B_4 and $A_5A_4B_3$ (Utsumi et al., 1997), which can be divided into two groups based on amino acid sequence homology: group I ($A_{1a}B_{1b}$, A_2B_{1a}, $A_{1b}B_2$) and group II (A_3B_4 and $A_5A_4B_3$) (Nielsen, 1996). The subunit composition of glycinin varies among different soybean cultivars (Mori et al., 1981), and each molecular species of glycinin shows heterogeneity (Utsumi et al., 1981), which probably affects the function of soybean proteins. However, there is little information available on the heterogeneity of molecular species from the standpoint of subunit compositions.

Anion exchange column chromatography is very useful in the purification of glycinin. The fraction eluting later as a single broad peak has been shown to be glycinin, and the fraction eluting earlier is thought to be mainly β-conglycinin. However, during the preparation of glycinins from various soybean cultivars, we repeatedly observed that the fraction eluting before glycinin varied considerably among different soybean cultivars with regard to both subunit content and composition. In this study, we analyzed this earlier-eluting fraction to clarify the presence of a new molecular species of glycinin.

2. Results and discussion

2.1. DEAE-Toyopearl column chromatography

Crude extracts of storage globulins were obtained from various soybean cultivars (Shirotsurunoko, York, Raiden and Suzuyutaka) by cryoprecipitation and were analyzed by SDS-PAGE in the presence of 2-mercaptoethanol (2-ME) (Fig. 1A and B). SDS–PAGE revealed that the cryoprecipitated fraction contained glycinin as

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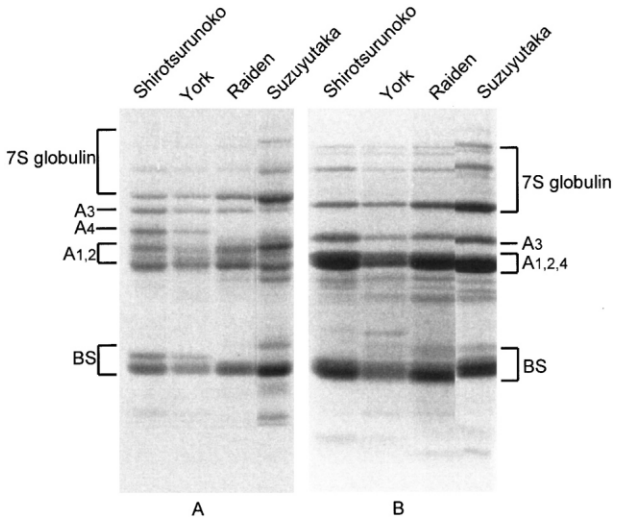

Fig. 1. SDS–PAGE of crude glycinins from various soybean cultivars (Shirotsurunoko, York, Raiden and Suzuyutaka) in the presence of 2-ME. A, in the presence of urea; B, in the absence of urea. $A_{1,\,2,\,3,\,4}$ and BS denote the acidic subunits A_1, A_2, A_3, and A_4 and basic subunits, respectively.

a major component and β-conglycinin as a minor component. Furthermore, the glycinins of these soybean cultivars had different subunit compositions. That is, while all four cultivars contained A_3 polypeptide and A major polypeptides (A_{1a}, A_{1b}, A2) (Fig. 1B) glycinins from Shirotsurunoko and York contained A_4 polypeptide, whereas those from Raiden and Suzuyutaka did not (Fig. 1A). This is consistent with previous findings (Mori et al., 1981).

Crude cryoprecipitated glycinins from each cultivar were fractionated by DEAE-Toyopearl chromatography as shown in Fig. 2. In the case of Shirotsurunoko and York, the ratio of peak I, eluting in the earlier region of the chromatogram, to peak II, eluting in the latter region, were small, whereas those for Raiden and Suzuyutaka were large.

Each fraction eluting from the column was characterized by SDS–PAGE (Fig. 3). Compared with the earlier-eluting single peak (peak I) of Shirotsurunoko and York, which showed almost no glycinin (Fig. 3A: lane I, Fig. 3B: lane I), the first peak (peak Ia) from Raiden and Suzuyutaka contained a mixture of β-conglycinin and glycinin polypeptides (Fig. 3C: lane 16 and Fig. 3D: lane 16), but not A_3 polypeptide. Peak Ib (from Raiden and Suzuyutaka) contained glycinin polypeptides including low levels of A_3 (Fig. 3C: lanes 23, 25, 29 and Fig. 3D: lanes 21, 28, 36). These results suggest that A_4 polypeptide-lacking cultivars (Raiden and Suzuyutaka) may contain new A major polypeptides.

Since it is well known that native glycinin (an 11S hexamer) dissociates into trimers under different environmental conditions of ionic strength, pH, denaturant and high temperatures (Utsumi et al., 1997), it is possible that the new molecular species found in peak Ib may or may not be derived from native glycinin. To clarify this point, 11S glycinin was purified from each cultivar by cryoprecipitation and sucrose density gradient centrifugation (Fig. 4). Similar sedimentation profiles were obtained for each cultivar (Fig. 4) revealing typical 11S and 7S peaks. Analysis of these profiles by SDS–PAGE

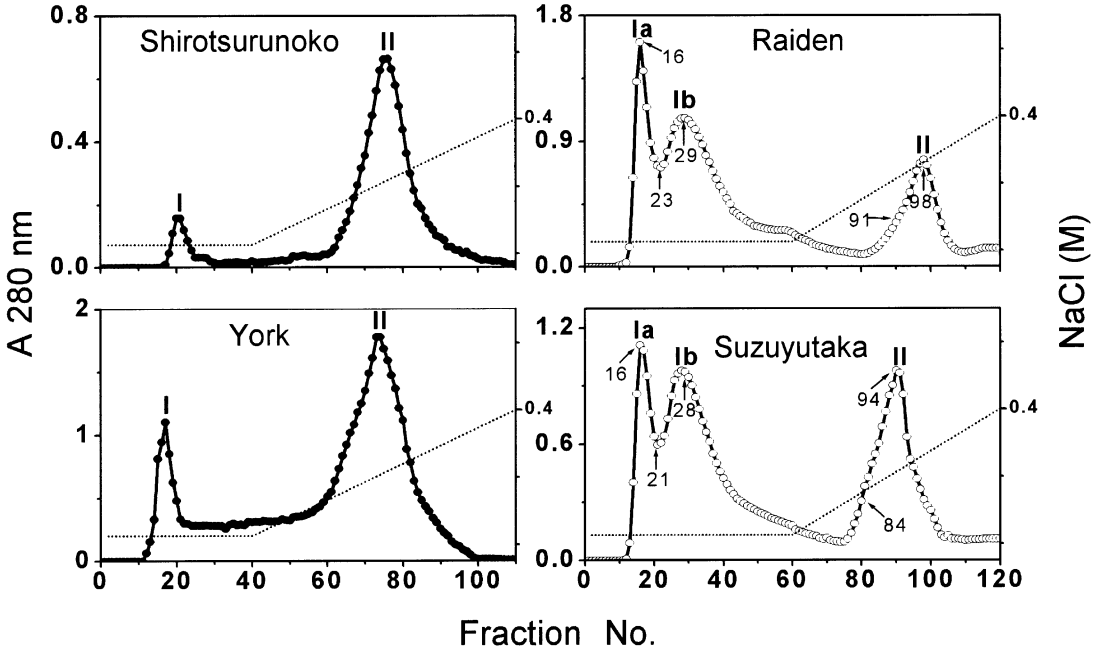

Fig. 2. DEAE-Toyopearl column chromatography of crude glycinin fractions prepared by cryoprecipitation from the four soybean cultivars. ●: absorbance at 280 nm for Shirotsurunoko and York, ○: absorbance at 280 nm Raiden and Suzuyutaka, ····: concentration of NaCl. I, Ia, Ib and II denote the peaks and the Arabic numerals on the chromatogram are the fraction numbers.

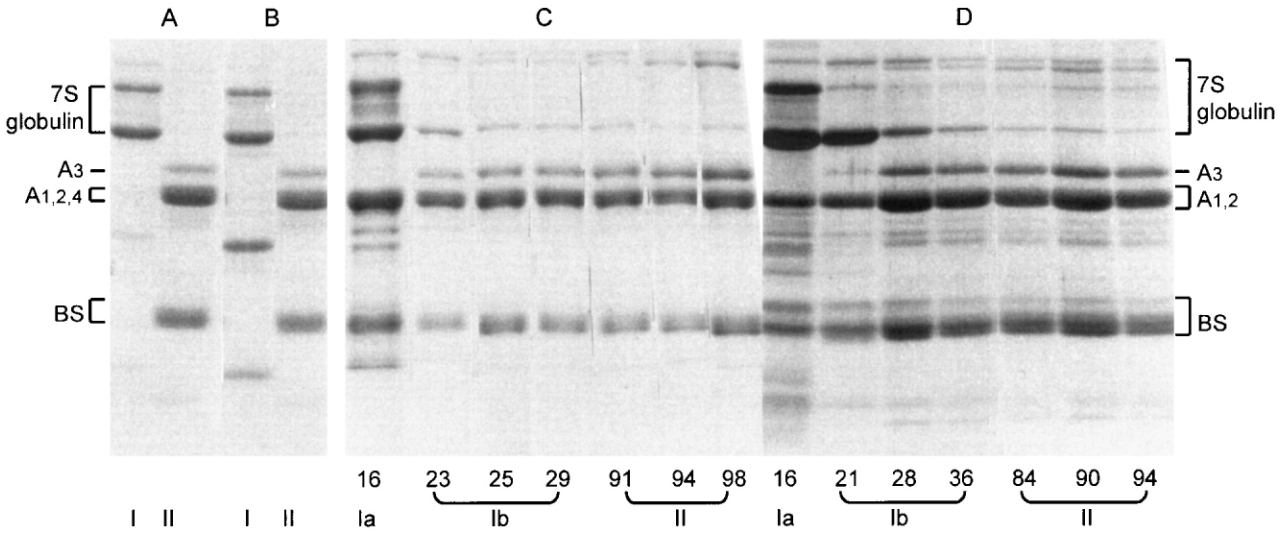

Fig. 3. SDS–PAGE of selected fractions obtained from DEAE-Toyopearl column chromatography of cryoprecipitated glycinins (Fig. 2) in the presence of 2-ME and without urea. Migration was from top to bottom. $A_{1,2,3,4}$ and BS denote the acidic subunits A_1, A_2, A_3, and A_4 and basic subunits, respectively. A, B, C and D represent the soybean cultivars Shirotsurunoko, York, Raiden and Suzuyutaka.

(data not shown) confirmed the presence of β-conglycinin and glycinin in each respective peak.

The sucrose gradient purified glycinin fractions (11S) from each cultivar were collected and further fractionated by DEAE-Toyopearl chromatography (Fig. 5). Only the elution patterns for the Shirotsurunoko (Fig. 5A) and Raiden extract (Fig. 5B) are shown, since those for the York and Suzuyutaka were identical to those found in Shirotsurunoko and Raiden, respectively. Elution patterns consistent with the results in Fig. 2 were obtained. The A_4-lacking cultivar (Fig. 5B) produced two major peaks (Ia and Ib) that eluted before initiation of the salt gradient, while the A_4-containing cultivar (Fig. 5A) only produced a single minor peak (peak I) in this region of the chromatogram.

SDS–PAGE analysis of major peaks depicted in Fig. 5 is shown in Fig. 6. Interestingly, the earlier-eluting peak in DEAE-Toyopearl column chromatography of Shirotsurunoko (lane I, Fig. 6A) contained A major polypeptides and basic polypeptides. This is in contrast to the absence of glycinin subunits in peak I, when the 7S globulin-containing cryoprecipitation fraction was directly applied to the DEAE-Toyopearl column (Figs. 2 and 3). This is probably due to the extremely high content of conglycinin contaminants in the cryoprecipitation fraction, which resulted in a relatively lower content of glycinin subunits, which could not be detected by electrophoresis.

Fig. 6B shows the electrophoresis patterns of an A_4-lacking cultivar (Raiden) in the presence of 2-ME. Results similar to those shown in Fig. 3 were obtained, suggesting that a large amount of A_3-, A_4-lacking polypeptides of glycinin was present in the earlier-eluting peak shown as lane Ia. In addition to this, a lane corresponding to Raiden in Fig. 1A, in which glycinin from Raiden was separated by SDS–PAGE in the presence of urea and 2-ME, shows the absence of A_4 subunit. Together, the results obtained for peak I (Shirotsurunoko and York) and peak Ia (Raiden and Suzuyutaka) in Figs. 2 and 5 suggest the existence of an A_3-, A_4-lacking molecular species (A major polypeptide-rich molecular species) of 11S in native glycinin from both types of cultivars. The earlier-eluting fraction in DEAE-Toyopearl column chromatography of A_4-containing cultivars contained a micro-level of this molecular species, whereas A_4-lacking cultivars contained a much higher level.

To further characterize this new molecular species, we attempted to purify large amounts of it from A_4-lacking cultivars. The fraction of peak Ia from Raiden (Fig. 2) was collected, concentrated, and further purified by sucrose density gradient centrifugation to remove the 7S globulin contaminant (Fig. 7).

2.2. Amino acid sequence analysis

Since the molecular size of A_{1a}, A_2 and A_{1b} polypeptides are similar, they appear as a single band on SDS–PAGE. To clarify whether the subunit compositions of the new molecular species were the same as those in glycinin, which contains all of the A major polypeptides (Staswick and Nielsen, 1983), urea alkaline gel electrophoresis was carried out. Acidic polypeptides were observed in the regions of A_1 and A_2 polypeptides, as shown in Fig. 8. The observed bands of the new molecular species were then transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting, and analyzed for N-terminal amino acid sequences by Edman degradation. Sequences corresponding to known A_{1a} and A_2 were detected,

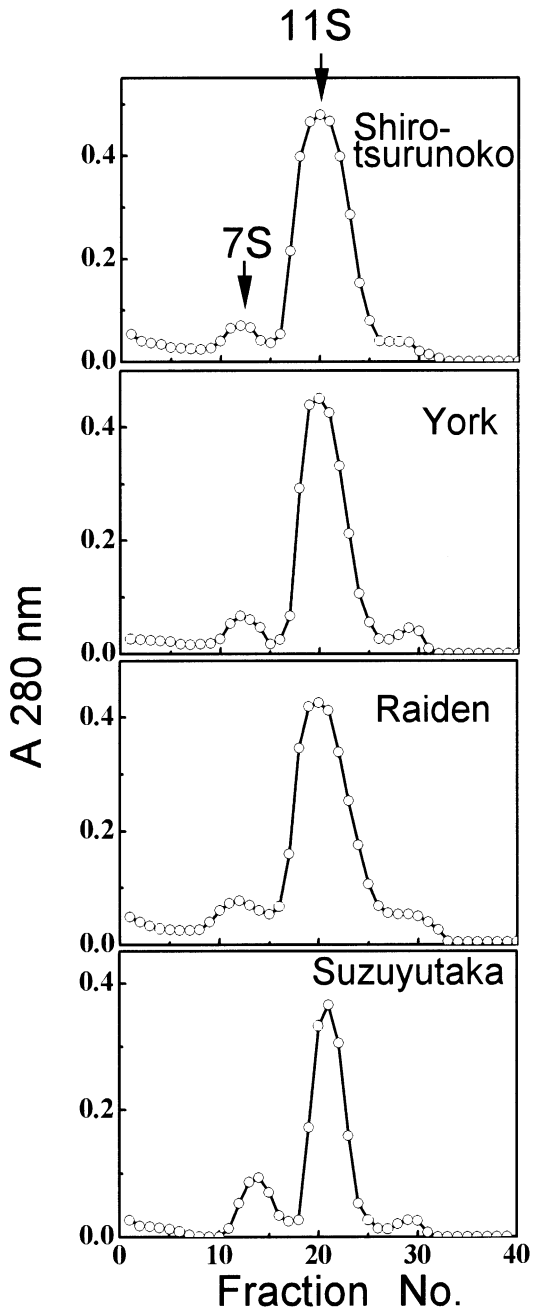

Fig. 4. Sucrose density gradient centrifugation of the crude glycinins obtained by cryoprecipitation. Sedimentation is from left to right.

while A_{1b} was, somewhat surprisingly, not found. We were unable to determine whether A_{1b} polypeptide was entirely absent from this new molecular species or merely undetectable by this method. Nevertheless, our results indicate that the new molecular species is at least enriched in A_{1a} and A_2 polypeptides. Furthermore, since the acidic and basic components of each subunit are synthesized from the same messenger RNA (Staswick and Nielsen, 1983), pairs of acidic and basic subunits are presumably present such that the new A_{1a}-, A_2-rich molecular species contained $A_{1a}B_{1b}$ and A_2B_{1a} subunits as the major components.

2.3. Molecular mass analysis

The molecular masses of the A_{1a}-, A_2-rich molecular species and glycinin from Raiden cultivar were determined by GPC–MALLS. The results showed that the A_{1a}-, A_2-rich molecular species had a molecular mass of $3.760 \pm 0.089 \times 10^5$ g/mol, and glycinin had a molecular mass of $3.865 \pm 0.251 \times 10^5$ g/mol. There was almost no difference in molecular mass between the new molecular species and glycinin, suggesting that the A_{1a}-, A_2-rich molecular species is structurally similar to a molecule of glycinin.

3. Concluding remarks

Although the molecular diversity and assembly of glycinin have been investigated using soybean isogenic lines (Yagasaki et al., 1997), the contribution of each subunit to the formation of the hexameric structure, and how the subunits interact with each other are still unknown. Our results are somewhat interesting from the standpoint of the assembly mechanism. Assembly of the glycinin complex begins in the endoplasmic reticulum (ER) when the subunits are in a precursor form called proglycinin. Proglycinin subunits are transported from the ER to the vacuoles as trimers (Dickinson et al., 1989), the assembly of which into hexamers in vivo requires posttranslational cleavage to form the acidic and basic polypeptides of each subunit. Since the two polypeptides remain covalently linked by a disulfide bond, mature glycinin subunits are isolated from seeds as a hexamer (Nielsen et al., 1995). Based on our results, processing of the new molecular species, which was found to have a similar molecular size to glycinin, very likely occurs during the complex assembly of glycinin hexamers from trimers.

In the case of A_4-lacking soybean cultivars, an A_{1a}-, A_2-rich molecular species with weak acidic charges was found in a large amount in the fraction that eluted first during preparative anion exchange chromatography. While the fraction eluting second ahead of glycinin contained all of the constituent subunits, the level of the A_3 subunit was lower than that in the fraction with glycinin. However, in the case of A_4-containing soybean cultivars, only micro-levels of the A major-rich molecular species were found in the fraction eluting first, and we could not determine whether or not it mainly contained A_{1a}, A_2 subunits like that in A_4-lacking cultivars. The fraction containing glycinin contained large amounts of all of the constituent subunits. These findings suggest that the A_3 and A_4 subunits may play an important coordinating role in the organization of subunits during the formation of glycinin hexamers.

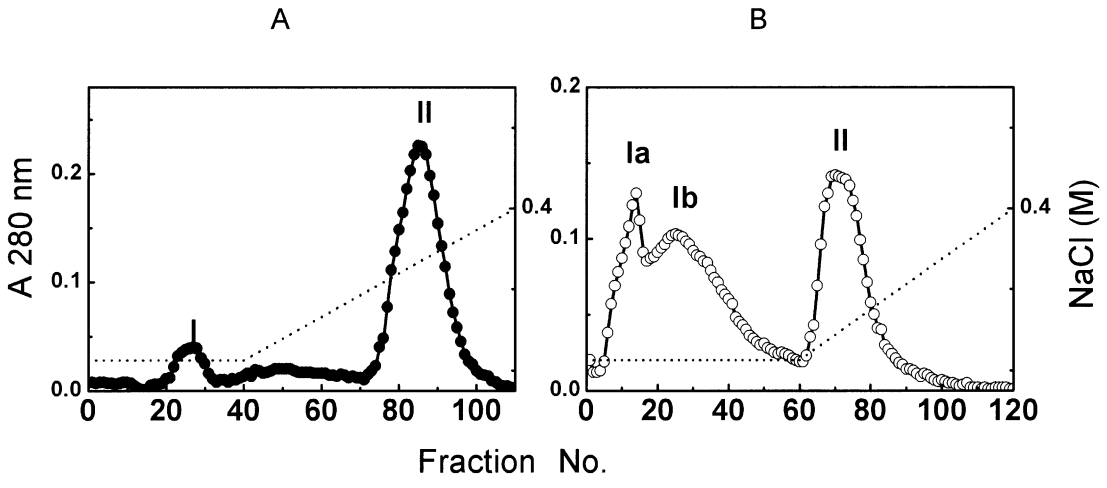

Fig. 5. DEAE-Toyopearl column chromatography of purified 11S glycinin fractions obtained by sucrose density gradient centrifugation in Fig. 4. ● and ○: absorbance at 280 nm for Shirotsurunoko and Raiden, respectively, ····: concentration of NaCl. I, Ia, Ib and II denote the peaks. A and B show the results in the soybean cultivars Shirotsurunoko and Raiden, respectively.

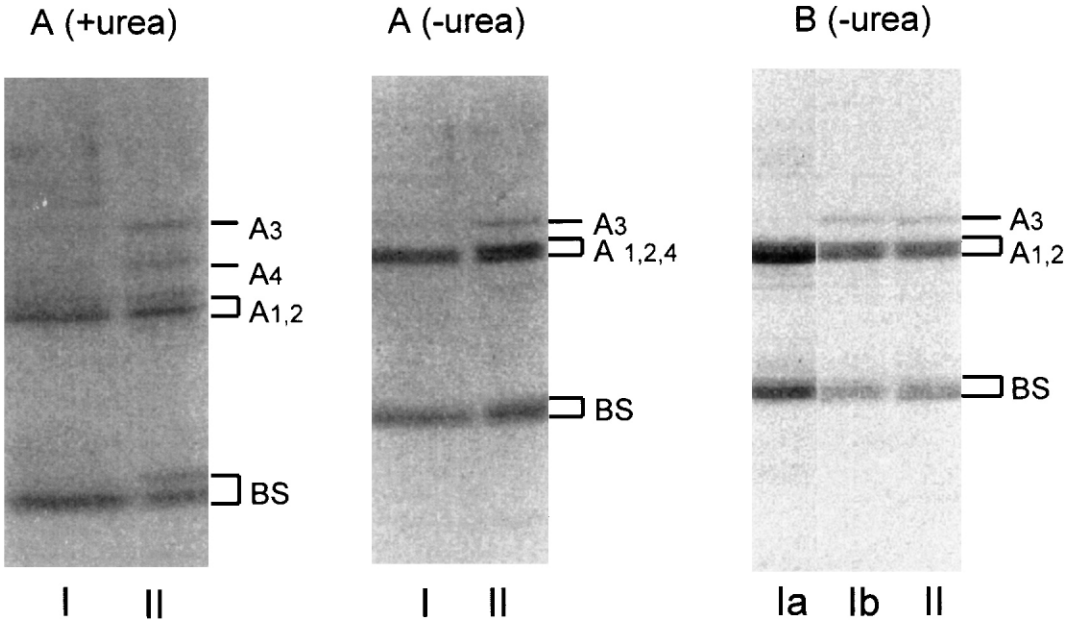

Fig. 6. SDS–PAGE of each peak from DEAE-Toyopearl column chromatography of 11S glycinins (Fig. 5) in the presence of 2-ME. Migration was from top to bottom. The Roman numbers under the gels denote the peaks. $A_{1,\,2,\,3,\,4}$ and BS denote the acidic subunits A_1, A_2, A_3, and A_4 and basic subunits, respectively. A and B show the results in the soybean cultivars Shirotsurunoko and Raiden, respectively.

The coexistence of an A_{1a}-, A_2-rich hexameric molecular species with weak electric charges and other kinds of glycinin molecular species in native glycinin indicates the polymorphism of hexamers in native glycinin and suggests that the combinations of glycinin subunits are not so strict, and indeed may be random. From this point of view, the reconstitution of homohexameric glycinin using each subunit of glycinin is possible and this should provide more information on the structure of glycinin. The present results may lead to new studies on the heterogeneity of glycinin molecular species involving control of the proportion of each subunit of glycinin during the synthesis, assembly and deposition of soybean seed storage proteins.

4. Experimental

4.1. Materials

Four cultivars of soybean seeds (*Glycine max* L. Merr.), Shirotsurunoko, York, Raiden and Suzuyutaka,

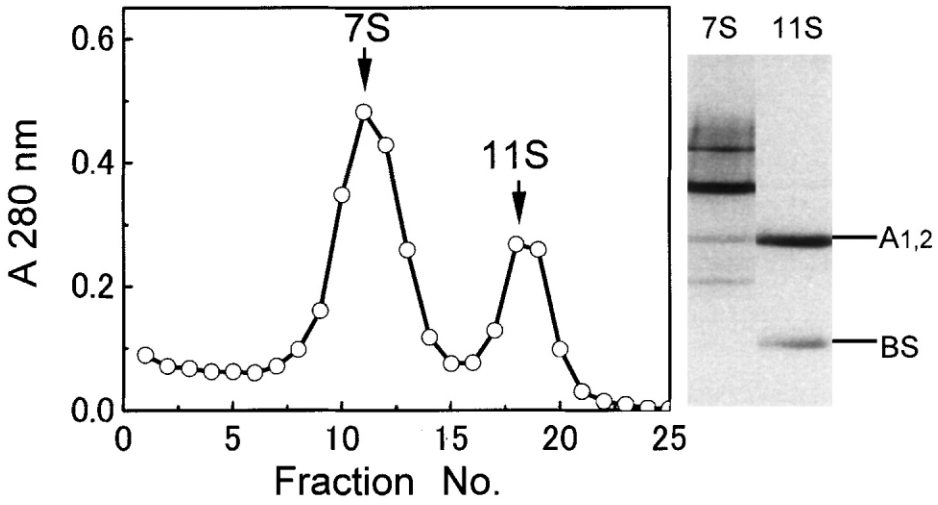

Fig. 7. Chromatogram of sucrose density gradient centrifugation. The sample was obtained from the earlier-eluted fraction in the Raiden cultivar (Fig. 2, peak Ia). Fractions for each peak were analyzed by SDS–PAGE in the presence of 2-ME. $A_{1,2}$ and BS denote the acidic subunits A_1 and A_2 and basic subunits, respectively.

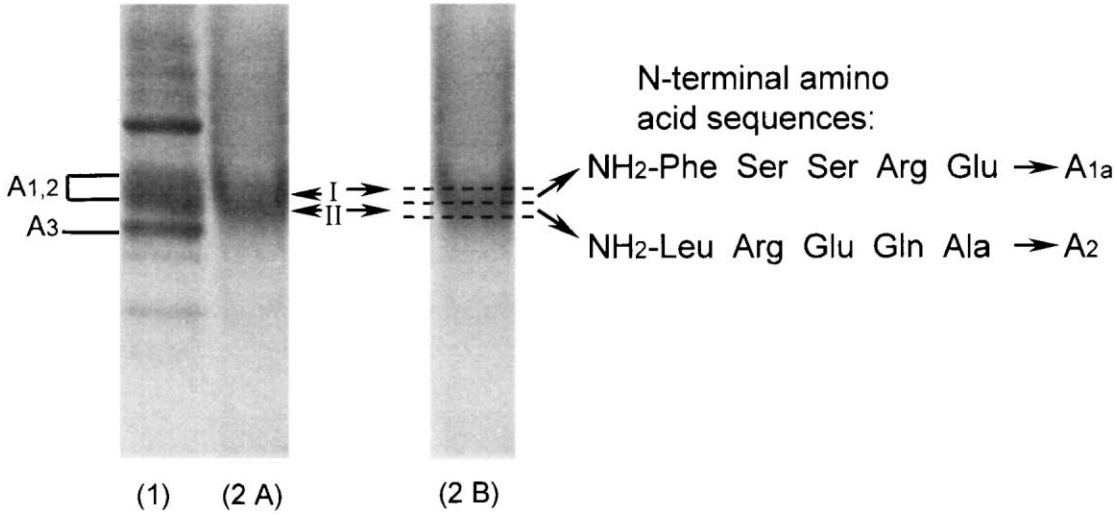

Fig. 8. Alkaline gel electrophoresis patterns: lane (1), glycinin from the Raiden cultivar (Fig. 2, peak II) purified by DEAE-Toyopearl column chromatography; lane (2A), new molecular species purified by sucrose density gradient centrifugation (11S fraction in Fig. 7), lane (2B); polypeptides in lane (2A) electroblotted onto a PVDF membrane for amino acid sequence analysis. I and II represent the regions on the gel excised for sequence analyses.

were kindly supplied by the Faculty of Horticulture, Chiba University, Japan. All chemicals used were reagent grade.

4.2. Acetone powder preparation and cryoprecipitation

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-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 *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.

Cryoprecipitation was used to extract crude glycinin from acetone powder (Mori et al., 1981) according to the method of Thanh et al. (1975).

4.3. Sucrose density gradient centrifugation

Ultracentrifugal analyses were carried out on a 10–30% (w/v) linear sucrose gradient in 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl, 10 mM 2-ME and 0.02% NaN_3 at 35,000 *g* and 20 °C for

20 h in a Hitachi RPS 40T rotor, as described by Mori and Utsumi (1979). After centrifugation, the gradient was examined at 280 nm with an ISCO density gradient fractionator.

4.4. DEAE-Toyopearl column chromatography

The glycinin fraction was isolated on a DEAE-Toyopearl 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 (Zheng et al., 1991).

4.5. SDS–PAGE

Electrophoresis was performed at 20 °C with 12.5% polyacrylamide slab gels according to Laemmli (1970) in the presence of 2-ME with or without 5 M urea.

4.6. Alkaline gel electrophoresis and electroblotting

Electrophoresis was performed by a modification of the method of Davis (1964). In brief, 0.0375 mg/ml potassium hexacyanoferrate and 7 M urea was included in 7.5% polyacrylamide gel and the pH of the gel was adjusted to 9.7 with 0.375 M Tris buffer. The sample buffer contained 7 M urea and 0.2 M 2-ME. Electrophoresis was carried out for 3 h at 4 °C in Tris–glycine buffer (0.01 M Tris and 0.077 M glycine) at a constant voltage of 150 V. After electrophoresis, the polypeptides in the gel were transferred to a PVDF membrane at 25 °C at a constant 1 mA per cm^2 membrane for 90 min (Hirano and Watanabe, 1990). The Ponceau S-stained bands in the PVDF membrane were excised for amino acid sequence analyses.

4.7. N-terminal sequence analysis

N-terminal amino acid sequences were determined by a protein sequencer Model 492 Procise (Applied Biosystems).

4.8. Molecular mass

The molecular mass of the protein was estimated by gel permeation chromatography using KW-804 and SB-806 HQ columns (Showa Denko Co., Tokyo, Japan) combined with a multi-angle laser light scattering (GPC-MALLS) system (DAWN DSP, Wyatt Technology, Santa Barbara, USA). The elution buffer contained 0.4 M NaCl, 35 mM potassium phosphate buffer (pH 7.6) and 0.02% NaN_3.

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