helping to interpret the NMR spectra.

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Formation of Pseudoglycinins and Their Gel Hardness

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Native subunit proteins of glycinin, the acidic and basic subunits designated as ASI, ASII, ASIII, and ASIV and BS, respectively, were isolated by DEAE-Sephadex column chromatography. Pseudoglycinins were reconstituted from the combinations between BS and each acidic subunits except ASIV. The pseudoglycinins thus formed were similar to native glycinin; they all consisted of reconstituted intermediary subunits that were composed of acidic and basic subunits linked together by disulfide bridges and had molecular weights that were about 6 times that of the intermediary subunit. The hardness of the heat-induced gels from pseudoglycinins was different from those derived from native glycinin, depending on the acidic subunit composition. ASIII appeared to cause a significant increase in the hardness of the gel.

Not only are soybeans used for various kinds of traditional Japanese foods but also their protein products are used commercially as ingredients in foods. The gel-forming ability of soybean protein is of significance with respect to their usage in food systems. It has been reported that the quality of tofu-gel (one of the Japanese traditional foods made from soybeans) differs according to the cultivars used (Smith et al., 1960) and that the proportion of 7S and 11S globulins is responsible for the differences in the physical properties of tofu-gel among soybean cultivars (Saio et al., 1969). The differences in functional properties of 7S and 11S globulins were reviewed extensively by Saio and Watanabe (1978). On the other hand, it has previously been demonstrated that the subunit compositions of 11S globulins of soybeans are different among the cultivars (Mori et al., 1981). Thus, it seems likely that the subunit composition of 11S globulin is related to the physical properties of foods made from soybeans or their isolated proteins. However, very little information is available with respect to the correlation between the physical properties and the protein structure at the subunit level.

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11S globulin (referred to as glycinin), one of the major components of the soybean storage protein, has been shown to be composed of at least three kinds each of acidic and basic subunits (Catsimpoolas, 1969; Kitamura and Shibasaki, 1975). Glycinin has been shown to have intermediary subunits in which the acidic (α) and basic (β) subunits are linked by disulfide bridges in 1:1 ratio and to be composed of $(\alpha\beta)_6$ (Badley et al., 1975; Kitamura et al., 1976; Mori et al., 1979). In a previous paper we investigated the reconstitution of intermediary subunits from native acidic and basic subunits proteins of glycinin and the hybrid intermediary subunits from combinations of native subunit proteins of glycinin and sesame 13S globulin (Mori et al., 1979). Further, preliminary results were obtained for the formation of artificially reconstituted 11S globulins that have different subunit compositions from native 11S globulins, i.e., pseudo and hybrid 11S globulins from combinations of native subunit proteins of glycinin and broad bean legumin (Utsumi et al., 1980a).

In the present study, in order to elucidate how the subunits of glycinin contribute to the physical properties of its gel, we investigated the reconstitution of various pseudoglycinins from its native subunit proteins and the textural properties of the gel made from the pseudoglycinins.

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MATERIALS AND METHODS

Materials. DEAE-Sephadex A-50 and polyacrylamide gradient gel (4–30%) were purchased from Pharmacia Co., Ltd. Urea and 2-mercaptoethanol, extrapure reagent, were obtained from Nakarai Chemicals (Japan). Sodium dodecyl sulfate ($NaDodSO_4$), extrapure reagent, was obtained from Wako Pure Chemicals (Japan). Other chemicals were guaranteed reagent grade.

Preparation of Glycinin and Its Native Subunits. The purified glycinin fraction was prepared from dry soybean seeds (*Glycine max*, var. Tsuru-no-ko) according to the method reported previously (Mori et al., 1979). The purified glycinin fraction was equilibrated with 0.1 M sodium phosphate buffer (pH 6.3) containing 6 M urea and 0.2 M 2-mercaptoethanol and applied to a column of DEAE-Sephadex A-50 equilibrated with the same buffer. Elution was performed by 2000 mL of the buffer containing NaCl in a linear gradient concentration of 0–0.45 M. All operations of the procedure were performed at 5 °C.

Preparation of Pseudoglycinins. Each isolated acidic subunit was mixed with the basic subunits and allowed to stand for 1 h at room temperature. The mixtures were dialyzed against 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl, 40% (v/v) glycerol, and 0.02% NaN_3. After dialysis, the sample was centrifuged on a linear sucrose density gradient (10–30% w/v). After centrifugation, the gradient was divided into 0.4-mL fractions and measured at 280 nm simultaneously with an ISCO density gradient fractionator. The details of the procedure have been described in the previous paper (Utsumi et al., 1980a).

Electrophoreses. $NaDodSO_4$ gel electrophoresis was performed according to the method of Laemmli (1970) at room temperature with 10% polyacrylamide gels in the presence or absence of 2-mercaptoethanol as described previously (Mori and Utsumi, 1979). Polyacrylamide gradient gel electrophoresis was performed according to the procedure described previously (Utsumi et al., 1981). Gel electrofocusing in the presence of urea and 2-mercaptoethanol was performed according to the method of Wrigley (1971) with a slight modification as described previously (Utsumi et al., 1980b).

Method of Gelation and Determination of Hardness of Gel. Twenty-microliter aliquots of the protein solutions in 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl were taken in disposable micropipets (1.68 mm in the inside diameter), sealed carefully at one end with poly(vinylidene chloride) film. The micropipets containing the protein solutions were then heated at 100 °C in a water bath for 20 min, followed by rapid cooling to room temperature by immersing in tap water. The gels thus formed in the micropipets were taken out and cut into 5-mm lengths.

Gel hardness was then measured with a texturometer (General Foods Corp., GXT-2) using a Lucite plunger of 18-mm diameter. The clearance between plunger and plate was adjusted to 0.1 mm. The validity and detailed application of the above-mentioned micromethod will be published elsewhere.

RESULTS AND DISCUSSION

Fractionation of Native Subunit Proteins of Glycinin. Native subunit proteins of glycinin were fractionated by chromatography on a DEAE-Sephadex column in the presence of urea and 2-mercaptoethanol as described under Materials and Methods. As shown in Figure 1, glycinin was separated into five fractions. The first peak that flowed through the column and the following four peaks eluted with a gradient of NaCl were basic subunits and acidic subunits, respectively (Mori et al., 1979). The basic subunit fraction and each acidic subunit fraction (the underlined part of each peak) were termed BS (M_r 18300 and 19000) and ASI (M_r 34800), ASII (M_r 34800), ASIII (M_r 38000), and ASIV (M_r 34800), respectively, in the order of elution from the column as shown in Figure 1. The molecular weight (M_r) of each subunit was determined by $NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown).

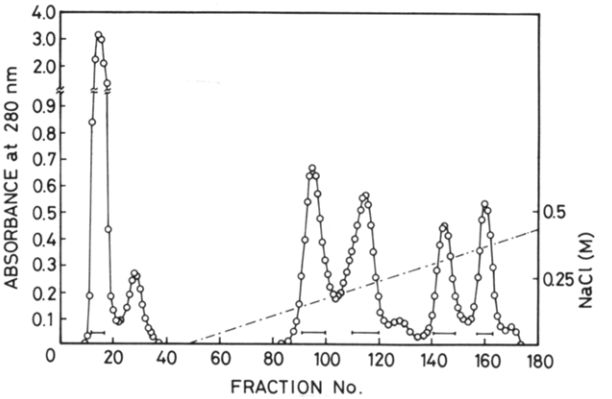

Figure 1. Fractionation of the native subunits of glycinin using a DEAE-Sephadex A-50 column. The purified glycinin preparation (650 A_{280} units) was fractionated on a 5 × 20 cm column at 5 °C as described under Materials and Methods. The flow rate was 40 mL/h, and 16 mL was collected in each fraction. (-o-) Absorbance at 280 nm; (- - -) concentration of NaCl.

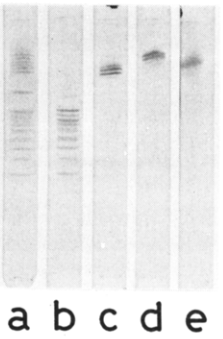

Figure 2. Polyacrylamide gel electrofocusing of isolated native subunits of glycinin. 0.03–0.1 A_{280} unit of protein was electrophoresed as described under Materials and Methods. Gel a, native glycinin; gel b, BS; gel c, ASI; gel d, ASII; gel e, ASIII.

Each peak was analyzed by polyacrylamide gel electrofocusing as shown in Figure 2. The basic subunit fraction gave all the bands of the constituent basic subunits of native glycinin. Each acidic subunit fraction gave multiple bands. Thus, the acidic subunit fractions are mixtures of some constituent acidic subunits of native glycinin, although each of them was eluted as a single peak on DEAE-Sephadex column chromatography.

Formation of Pseudoglycinin. The reconstituted products from the combinations of each acidic subunit fraction and basic subunit fraction were fractionated by sucrose gradient centrifugation (Figure 3). In the absorbance patterns, peaks I and II correspond to 7S (half-molecule of the 11S component) and 11S components, respectively. As shown in Figure 3A, the formation of the 11S component from the combination of ASI and BS was about 90%. On the other hand, in the cases of ASII and BS and ASIII and BS (parts B and C of Figure 3), the formation of 11S components was lower and the areas of 7S components were larger than that of the other combination. Unreacted acidic subunits were scarcely detected

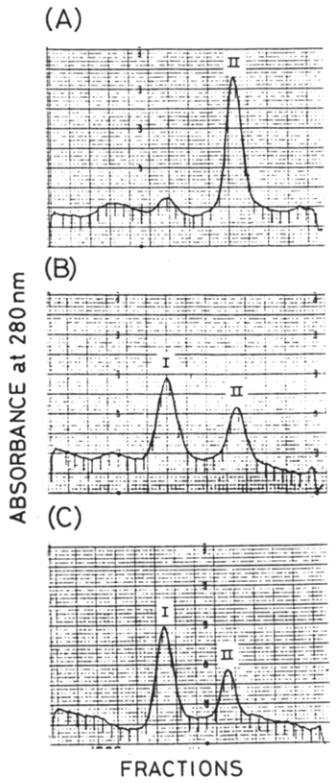

Figure 3. Sucrose density gradient centrifugation of the reconstituted products. The isolated acidic and basic subunits were combined and reconstituted at a weight ratio of approximately 1:2.5 in combinations of BS and ASI (A), ASII (B), and ASIII (C), and then the reconstituted products were fractionated by sucrose gradient centrifugation as described under Materials and Methods. Sedimentation is from left to right. Absorbance range is 0–0.2.

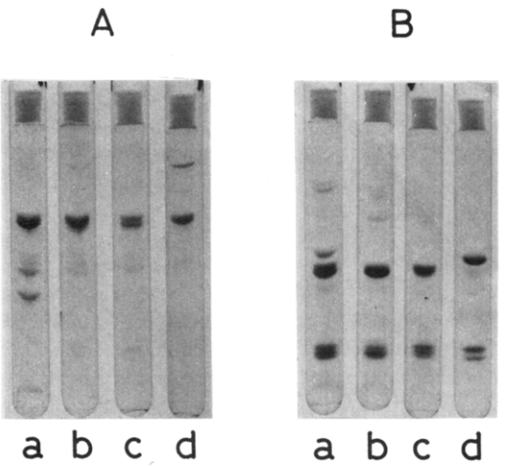

Figure 4. NaDodSO$_4$–polyacrylamide gel electrophoresis of pseudoglycinins. 0.03 A_{280} unit of each pseudoglycinin obtained from the sucrose gradient centrifugation was electrophoresed in the absence (A) and presence (B) of 2-mercaptoethanol as described under Materials and Methods. Migration is from top to bottom. Gel a, native glycinin; gel b, ASI plus BS; gel c, ASII plus BS; gel d, ASIII plus BS.

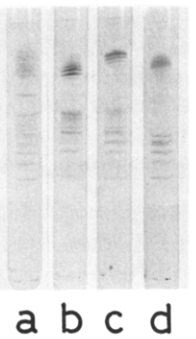

Figure 5. Polyacrylamide gel electrofocusing of pseudoglycinins. 0.1 A_{280} unit of each pseudoglycinin obtained from the sucrose gradient centrifugation was electrophoresed as described under Materials and Methods. Gel a, native glycinin; gel b, ASI plus BS; gel c, ASII plus BS; gel d, ASIII plus BS.

in any of the cases. In the case of the combination of ASIV and BS, neither the formation of 7S nor that of 11S components was observed. No clear explanation for this can be offered at the present time.

The reconstituted 11S components (pseudoglycinins) of each combination obtained from the sucrose gradient centrifugation were analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis. The results of the electrophoresis in the absence and presence of 2-mercaptoethanol are shown in parts A and B of Figure 4, respectively. In the absence of 2-mercaptoethanol (Figure 4A), all the pseudoglycinins gave bands with molecular weights of around 50 000 that seem to correspond to the intermediary subunits. On the other hand, in the presence of 2-mercaptoethanol (Figure 4B), all the pseudoglycinins gave bands corresponding to the acidic and basic subunits used but no bands corresponding to those observed in the absence of 2-mercaptoethanol. The molar ratio of the acidic and basic subunits given by each pseudoglycinin was approximately 1:1. These results indicate that the band with a molecular weight of around 50 000, observed in the absence of 2-mercaptoethanol, may be regarded as the intermediary subunit composed of acidic and basic subunits that are linked by disulfide bridges in the ratio of 1:1.

In the formation of an intermediary subunit ASI randomly selected both of the basic subunits with molecular weights of 19 000 (BSI) and 18 300 (BSII) as a counterpart (Figure 4B, gel b), while ASII and ASIII exhibited some affinity for BSI, as is evident from its higher presence in contrast to BSII in the pseudoglycinins in comparison with the situation that exists in the native glycinin (Figure 4B, gels c and d). So that more could be known about such difference of the basic subunit composition, the pseudoglycinins obtained from the sucrose gradient centrifugation were analyzed by polyacrylamide gel electrofocusing in the presence of 2-mercaptoethanol (Figure 5). A comparison of the electrofocusing patterns of the pseudoglycinins indicates that some slower migrating basic subunits were lacking in the case of the combination of ASIII and BS, while all the constituent basic subunits were present in the case of other combinations.

For examination of the size of each pseudoglycinin, 11S component fractions obtained from the sucrose gradient centrifugation were analyzed by polyacrylamide gradient gel electrophoresis. As shown in Figure 6, the pseudoglycinin from the combination of ASI and BS gave a main band with a molecular weight of approximately 340 000 and a minor band with a molecular weight of about 70 000 (lane 3). The pseudoglycinin from the combination of ASII and BS gave a band with a molecular weight of about 60 000, and that from the combination of ASIII and BS gave a main band with a molecular weight of approximately 200 000 and a minor band with a molecular weight of about 370 000 (Figure 6, lanes 4 and 5). These may be due to dissociation of the pseudoglycinins to substructural components during the electrophoresis. The 7S components formed from these two combinations also gave bands with molecular weights of approximately 60 000 and 200 000, respectively (data not shown). The molecular weight of

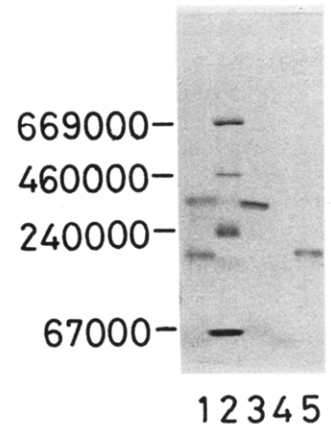

Figure 6. Polyacrylamide gradient gel electrophoresis of pseudoglycinis. 0.02 A_{280} unit of each pseudoglycinin obtained from the sucrose gradient centrifugation was electrophoresed as described under Materials and Methods. Migration is from top to bottom. Lane 1, native glycinin; lane 2, standard proteins (thyroglobulin, M_r 669 000; ferritin, M_r 460 000; catalase, M_r 240 000; bovine serum albumin, M_r 67 000); lane 3, ASI plus BS; lane 4, ASII plus BS; lane 5, ASIII plus BS.

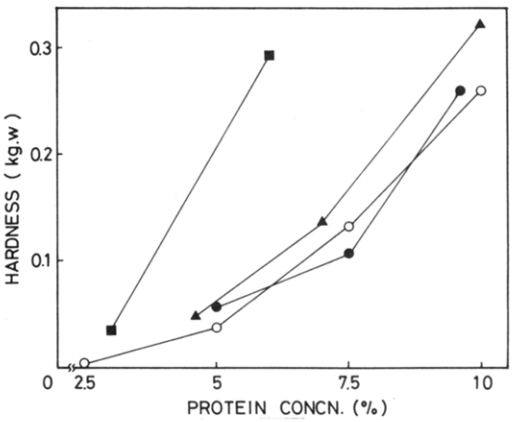

Figure 7. Hardness of heat-induced gels of pseudoglycinins. Native glycinin and the pseudoglycinins were heated to make gel as described under Materials and Methods. The protein concentrations of the pseudoglycinins used were as follows: combination of ASI and BS, 5.0, 7.5, and 9.6; ASII and BS, 4.6, 7.0, and 10%; ASIII and BS, 3.0 and 6.0% (w/v). (○) Native glycinin; (●) ASI plus BS; (▲) ASII plus BS; (■) ASIII plus BS.

each pseudoglycinin was estimated to be about 6 times that of the intermediary subunit.

The native glycinin gave bands corresponding to the 11S-size, the 7S-size, and the intermediary subunits (Figure 6, lane 1). The pseudoglycinins from the combinations of ASII and BS and ASIII and BS dissociated to the substructural components on the electrophoresis (Figure 6, lanes 4 and 5). However, the pseudoglycinin from the combination of ASI and BS hardly dissociated (Figure 6, lane 3). These results suggest that ASII and ASIII are responsible for the fact that glycinin undergoes a dissociation reaction under lower ionic strength in the normal pH range (Wolf and Briggs, 1958). Thus, the dissociation behaviors during the electrophoresis of the pseudoglycinins were different from that of the native glycinin.

These results indicate that the pseudoglycinins can be formed from the isolated subunits of glycinin except in the case of the combination of ASIV and BS and that the pseudoglycinins are similar to the native glycinin with respect to the $(\alpha\beta)_6$ structure.

Gelation of Pseudoglycinins. The pseudoglycinins obtained from the sucrose gradient centrifugation as described above and native glycinin were heated to make a gel, and the hardness of the gels was determined (Figure 7). The lowest gelation concentration for native glycinin was 2.5%, and the gel hardness increased with increasing concentrations of protein and time of heating. The gels formed cannot be reversed to the sol state by reheating unlike the case reported by Catsimpoolas and Meyer (1970), indicating its heat irreversibility.

The gel from the pseudoglycinin from the combination of ASI and BS exhibited similar hardness to that from native glycinin, while that from the combination of ASII and BS was slightly harder. On the other hand, the gel from the pseudoglycinin from the combination of ASIII and BS was significantly harder than that from native glycinin. It is apparent that each gel from the pseudoglycinins exhibits different hardness, depending on the acidic subunit composition. The result suggests that the acidic subunits contribute differently to the hardness of gel and that ASIII, having a larger molecular weight than the other acidic subunits, plays an important role for increasing the hardness of the gel. However, since some kinds of the constituent basic subunits were lacking in the pseudoglycinin from the combination of ASIII and BS (Figure 5, gel d), the possibility still remains that such basic subunits have a different contribution to the hardness of gel than the other basic subunits.

We have previously demonstrated that the subunit compositions of glycinins isolated from the seeds of various cultivars of soybean are different among the cultivars and could be classified into five groups according to differing molecular charges of the subunits (Mori et al., 1981). Therefore, in view of the results obtained here, it is probable that the hardness of gels from glycinins of different cultivars of soybeans could be different among the cultivars, depending on the subunit composition. Studies are currently under way in this respect.

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