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Structure−Physicochemical Function Relationships of Soybean β-Conglycinin Heterotrimers

NOBUYUKI MARUYAMA,† MOHAMAD RAMLAN MOHAMED SALLEH,† KOJI TAKAHASHI,‡ KAZUHIRO YAGASAKI,§ HIDEYUKI GOTO,|| NAHO HONTANI,† SHUKO NAKAGAWA,† AND SHIGERU UTSUMI*,†

Laboratory of Food Quality Design and Development, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan; National Institute of Crop Science, National Agricultural Research Organization, Kannondai 2-1-18, Tsukuba, Ibaraki 305-8518, Japan; Nagano Chushin Agricultural Experiment Station, Shiojiri, Nagano 399-6461, Japan; and Department of Food Science, Ishikawa Agricultural College, 1-308 Suematsu, Nonoichi, Ishikawa 921-8836, Japan

We purified four single molecular species of β-conglycinin heterotrimers consisting of the α and β subunits or the α′ and β subunits from mutant soybean cultivars lacking the α or α′ subunit, respectively, and examined their structural features and physicochemical functions. The extent of the hydrophobicities of the heterotrimers was related to the number of the α or α′ subunit. The thermal stabilities of the heterotrimers were mainly conferred by the subunit which had lower thermal stability. Solubilities at low ionic strength ($\mu = 0.08$) of the heterotrimers containing the α or α′ subunit were very similar to those of the α and α′ homotrimers, respectively. Emulsifying abilities and heat-induced associations of the heterotrimers containing one β subunit were similar to those of the α or α′ homotrimer, whereas those of the heterotrimers containing two β subunits were similar to those of the β homotrimer.

KEYWORDS: β-Conglycinin; structure−physicochemical function relationship; solubility; thermal stability; soybean

INTRODUCTION

β-Conglycinin is one of the dominant storage proteins of soybean seeds, and the analysis of its structure−physicochemical function relationships is imperative for extending the applications of soybean proteins in foods. β-Conglycinin is a trimeric protein composed of three subunits: α (∼67 kDa), α′ (∼71 kDa), and β (∼50 kDa) (*1*). The α and α′ subunits are composed of extension regions and core regions, whereas the β subunit consists of only the core region. The core regions of three subunits exhibit high absolute homologies with one another (90.4, 76.2, and 75.5% between α and α′, between α and β, and between α′ and β, respectively) (*1*). The extension regions of the α and α′ subunits exhibit lower absolute homologies (57.3%) and a highly acidic property (*1*). Many molecular species of β-conglycinin containing homo and heterotrimers are present in normal soybean seeds (*2, 3*). In a previous paper (*4*), the structural features and physicochemical functions of the native homotrimers of the individual subunits of β-conglycinin purified from mutant soybean cultivars lacking the α and/or α′ subunits were compared with those of recombinant homotrimers prepared by means of the *Escherichia coli* expression system (*1, 5*). We demonstrated how the extension and core regions and the carbohydrate moieties contribute to the structural stability, solubility, emulsifying ability, and heat-induced association of β-conglycinin (*1, 4, 5*). To elucidate the structure−physicochemical function relationships of β-conglycinin in more detail, it is necessary to study the structural features and physicochemical functions of heterotrimers.

Here, we purified four single molecular species of heterotrimers consisting of the α and β subunits or the α′ and β subunits from the mutant soybean cultivars which contain β-conglycinin lacking the α′ or α subunit, respectively, and investigated their structure−physicochemical function relationships.

MATERIALS AND METHODS

Purification of Native β-Conglycinin Heterotrimers from the Mutant Soybean Cultivars. Heterotrimers were prepared from the mutant soybean cultivars which contain β-conglycinin lacking the α′ or α subunit (*6, 7*). They were purified by the methods identical to those for the native α and α′ homotrimers (*4*). The heterotrimer composed of two α subunits and one β subunit was designated α2β1, and the other homo- and heterotrimers were designated in a manner similar to that of α2β1.

Protein Measurement. Protein concentrations of the samples were determined using a Protein Assay Rapid Kit (Wako) with bovine serum albumin as the standard.

* To whom correspondence should be addressed. Tel: 81-774-38-3760. Fax: 81-774-38-3761. E-mail: utsumi@soya.food.kyoto-u.ac.jp.
† Kyoto University.
‡ National Agricultural Research Organization.
§ Nagano Chushin Agricultural Experiment Station.
|| Ishikawa Agricultural College.

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Solubility as a Function of pH. Solubilities of the heterotrimers were measured as described previously (*5*). The protein solutions (0.8 mg/mL) were kept at 4 °C for 18 h at various pHs at $\mu = 0.5$ and 0.08. After the solution was centrifuged, protein concentrations in the supernatant were determined using a Protein Assay Rapid Kit (Wako). Solubility was expressed as a percentage of the total protein content in the sample.

Surface Hydrophobicity. Surface hydrophobicities of the homo and heterotrimers were analyzed by hydrophobic chromatography using Phenyl Sepharose 6 Fast Flow and Butyl Sepharose 4 Fast Flow columns (both from Amersham Pharmacia Biotech). Samples were dialyzed against buffer A (35 mM sodium phosphate, pH 7.6, 0.4 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride (*p*-APMSF), and 0.02% NaN_3) containing 2.3 M ammonium sulfate. The dialyzed samples were applied to columns equilibrated with the same buffer. The adsorbed samples were eluted with a linear gradient (2.3−0 M) of ammonium sulfate over a period of 80 min at a flow rate 0.25 mL/min.

DSC Measurement. Differential scanning calorimetry (DSC) experiments were carried out on a Microcal MC-2 ultrasensitive microcalorimeter (Micro Cal Inc.) as described previously (*1*). All DSC experiments were performed with a protein concentration of 0.5 mg/mL in buffer A.

Analysis of Heat-Induced Association. Heat-induced associations of the heterotrimers were estimated as described previously (*5*). Each heterotrimer (1 mg/mL) in buffer A without 10 mM 2-mercaptoethanol was heated at 70, 80, and 90 °C for 5 min. After heating, the solutions were passed through a membrane filter (0.22 μm). The filtered samples were fractionated by gel filtration chromatography using GPC SYSTEM-21 (Showa Denko, Tokyo, Japan) with KW804 and SB806M columns (Showa Denko) and with a refractive index monitor, and were subjected to multiangle laser light scattering experiments. Light scattering was measured by a Dawn DSP-F MALLS (Wyatt Technology, Santa Barbara, CA). Astra software (Wyatt Technology) was used to calculate molecular masses of heat-induced soluble aggregates.

Analysis of Emulsifying Ability. Emulsifying abilities of the heterotrimers were measured as described previously (*5*). To prepare emulsions, 0.25 mL of soybean oil and 1.5 mL of the heterotrimers (0.5 mg/mL) in buffer A were homogenized for 30 s with a high-speed homogenizer (model NS-50, Nichion Irikakikai Ltd) and further sonicated using an ultrasonic homogenizer (model US-150, Nihonseiki Kaisha Ltd). The particle size distribution of the emulsions was measured using a laser diffraction instrument (model LA 500, Horiba Seisakusho Ltd). Each sample was analyzed several times, and a representative typical pattern was presented.

RESULTS AND DISCUSSION

Solubility. In the previous paper (*4*), we demonstrated that at $\mu = 0.5$ all the native homotrimers were soluble at all pHs examined, and that at $\mu = 0.08$ α3 and α′3 were insoluble only in the vicinity of pH 5.0 and β3 was insoluble at pH 4.8−8.5. All heterotrimers (α2β1, α1β2, α′2β1, and α′1β2) were soluble at $\mu = 0.5$ at all pHs examined here in analogy with the native homotrimers. At $\mu = 0.08$, α2β1 and α1β2 were insoluble at pH 4.8−5.4 and pH 4.8−5.7, respectively (**Figure 1A**). These results indicate that at $\mu = 0.08$ the solubility of α2β1 is very close to that of α3 and that of α1β2 is similar to that of α3 rather than that of β3. The heterotrimers composed of the α′ and β subunits also exhibited almost the same phenomena (**Figure 1B**) as did the heterotrimers composed of the α and β subunits.

Surface Hydrophobicity. Surface hydrophobicity is an important factor for physicochemical functions such as emulsifying and foaming abilities (*8, 9*). We assessed the surface hydrophobicities of the homo- and heterotrimers by measuring their elution times of the hydrophobic chromatography (i.e., the longer the elution time, the higher the surface hydrophobicity). We used two columns containing a phenyl and a butyl group

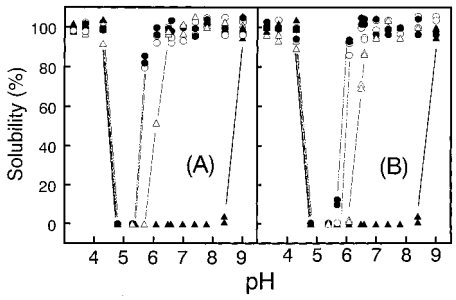

Figure 1. Dependency of the solubility of β-conglycinin heterotrimers composed of the α and β subunits (A) and of the α′ and β subunits (B) on pH at ionic strength 0.08 compared with those of the homotrimers (*4*). Solubilities of α3 and α′3, α2β1 and α′2β1, α1β2 and α′1β2, and β3 are shown by dashed and double-dotted line with closed circles, dashed and single-dotted line with open circles, dashed line with open triangles, and solid line with closed triangles, respectively.

Table 1. Elution Times of the Homo- and Heterotrimers on Hydrophobic Chromatography

	elution time (min)						
column	α3	α′3	α2β1	α′2β1	α1β2	α′1β2	β3
butyl sepharose	48.6	47.9	47.1	45.9	43.8	43.0	38.2
phenyl sepharose	69.2	69.2	67.3	67.3	64.7	64.3	56.7

(**Table 1**). Both columns gave similar results, showing that the hydrophobicities of α3 and α′3 were higher than that of β3, although there were slight differences in the hydrophobicities of α3 and α′3 using the two columns. The hydrophobicity of α3 as evaluated by a phenyl sepharose column was identical to that of α′3, but that of α3 evaluated by a butyl sepharose column was somewhat higher than that of α′3.

In case of the heterotrimers composed of the α and β subunits, both α2β1 and α1β2 were eluted between α3 and β3 and the orders of elution were similar for both columns: α3 > α2β1 > α1β2 > β3. In parallel, the orders of elution of the heterotrimer composed of the α′ and β subunits on both columns were also α′3 > α′2β1 > α′1β2 > β3. Thus, the extent of the hydrophobicities of the heterotrimers is related to the number of the α or α′ subunit.

Thermal Stability. DSC profiles of the heterotrimers are shown in **Figure 2**.

α2β1 gave one peak at 78.5 °C and α1β2 gave a major peak at 82.5 °C with a minor peak at 78.5 °C (**Figure 2A**). Compared with the T_m values of the homotrimers in a previous paper (*4*), the peak of α2β1 (78.5 °C) and the minor peak of α1β2 (78.5 °C) were almost identical to that of α3 (78.2 °C), and the major peak of α1β2 (82.5 °C) was between the peaks of α3 (78.2 °C) and β3 (87.0 °C). On the other hand, the T_m value of α′2β1 (80.3 °C) was slightly lower than that of α′3 (82.6 °C), and that of α′1β2 (82.1 °C) was close to that of α′3 (**Figure 2B**). These results indicate that each subunit in the heterotrimers does not contribute equally to the thermal stabilities of the heterotrimers, and that the thermal stability is significantly conferred by that of the subunit which has lower thermal stability. Once the subunit with lower thermal stability denatures in the heterotrimer, the trimeric structure is probably broken, resulting in the complete denaturation of the counterparts. However, α1β2 and α′2β1 exhibited behaviors different from those of the other heterotrimers and homotrimers. Although we cannot fully explain this, it is possible that the modes of the subunit interactions governing the formation of the heterotrimers are slightly variable depending upon the subunit compositions of

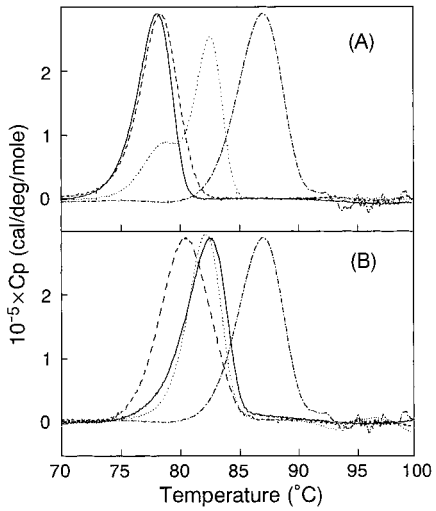

Figure 2. DSC scans of β-conglycinin heterotrimers composed of the α and β subunits (A) and of the α′ and β subunits (B) compared with those of the homotrimers (*4*). Profiles of α3 and α′3, α2β1 and α′2β1, α1β2 and α′1β2, and β3 are shown by solid line, dashed line, dotted line, and dashed and single-dotted line, respectively.

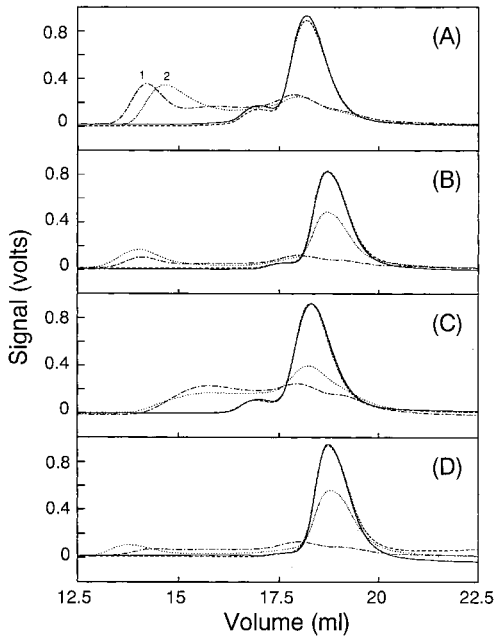

Figure 3. Elution patterns of heat-treated β-conglycinin heterotrimers: α2β1 (A), α1β2 (B), α′2β1 (C), and α′1β2 (D), which were heated at 70 °C (dashed line), 80 °C (dotted line), and 90 °C (dashed and single-dotted line). Nonheated samples are shown by solid line. Numbers indicate the peaks.

the heterotrimers. Recently, we succeeded in X-ray crystallography of β3 (*10*). Further X-ray crystallography of the other subunits and heterotrimers will shed light on the reason for this behavior.

Heat-Induced Association. Heat-induced associations of the heterotrimers at pH 7.6 and $\mu = 0.5$ were determined by gel filtration chromatography and multiangle laser light scattering (**Figure 3**). The amounts of the intact species of all the heterotrimers decreased by heating, depending on their T_m values. α2β1 formed soluble aggregates by heating at ≥80 °C, similar to results obtained with α3 and α′3 (*4*). The α2β1 soluble aggregates (panel A, peaks 1 and 2) had molecular masses of approximately 2−8 million Da, although the molecular masses

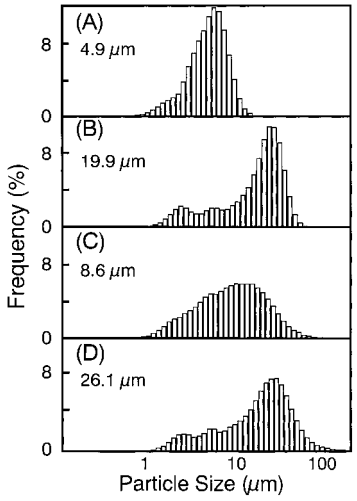

Figure 4. Particle size distributions of emulsions from β-conglycinin heterotrimers: (A) α2β1; (B) α1β2; (C) α′2β1; (D) α′1β2.

of the α3 soluble aggregates by heating at ≥80 °C are approximately 1−2 million Da as described in the previous paper (*4*). Furthermore, the amount of the α3 large soluble aggregates was less than that of α2β1. Similar tendencies were observed in case of α′2β1 (panel C). Therefore, heterotrimers which contain one β subunit form more and bigger soluble aggregates than do α3 and α′3.

On the other hand, α1β2 and α′1β2 became turbid when heated at ≥80 °C. On gel filtration, after removal of the turbid materials by membrane, their heat-induced soluble aggregates were barely observed despite the decreases in the amount of their intact species (panels B and D), indicating formation of insoluble aggregates which were removed before gel filtration. Previously, we observed that β3 forms insoluble aggregates, but not soluble aggregates at all, by heating at a temperature higher than its T_m value (*4*). Thus, the behaviors of heat-induced associations of heterotrimers which contain two β subunits resemble that of β3 rather than those of α3 and α′3.

The results obtained here indicate that the inclusion of β subunit in the heterotrimers enhances the increases in size and amount of aggregates. In the previous studies (*4*, *5*), we showed that N-linked glycans prevent heat-induced associations, and that the extension regions prevent heat-induced precipitation due to their highly acidic properties. Possibly, N-linked glycans and the extension regions hinder the close contact of molecules denatured by heating. Because the β subunit has only one N-linked glycan and no extension region, unlike the α and α′ subunits, the β subunit may tend to associate upon heating despite its lower surface hydrophobicity. The presence of β subunits in heterotrimers may therefore enhance the size and amount of aggregates.

Emulsifying Ability. We assessed the emulsifying abilities of the heterotrimers by measuring the sizes of the emulsions (**Figure 4**). Compared with the average particle sizes of emulsions of the homotrimers reported in the previous paper (*4*), the average particle sizes of α2β1 (4.9 μm) and α′2β1 (8.6 μm) were very close to those of α3 (5.2 μm) and α′3 (9.8 μm), respectively. On the other hand, the average particle sizes of α1β2 (19.9 μm) and α′1β2 (26.1 μm) were closer to that of β3 (28.5 μm) than to those of α3 (5.2 μm) and α′3 (9.8 μm), respectively. In other words, the emulsifying abilities of the heterotrimers containing one β subunit are similar to that of α3 or α′3, and those containing two β subunits are similar to that of β3.

Surface hydrophobicity is an important factor for physicochemical functions such as the emulsifying ability, because it is related to the binding ability of a protein to oil (*8*, *9*). Previously, we measured the surface hydrophobicity of the recombinant homotrimers by using the hydrophobic probe 8-anilino-1-naphthalene sulfonic acid (ANS), and indicated that the order of the hydrophobicity is $\alpha'3 \gg \alpha3 \gg \beta3$ (*5*). However, this order was completely different from that of the emulsifying abilities of the recombinant and native homotrimers ($\alpha3 > \alpha'3 > \beta3$) (*4*, *5*), being consistent with the report that there is no convincing reason that the fluorescence enhancement by ANS is due to the hydrophobic interactions (*11*). Nevertheless, the order of the emulsifying ability was similar to that of the hydrophobicity evaluated by the hydrophobic columns, especially a butyl sepharose column ($\alpha3 > \alpha'3 > \beta3$) described above, indicating that hydrophobic column chromatography is suitable for evaluation of hydrophobicity of a protein in relation to its emulsifying ability.

In a previous study (*5*), we demonstrated that the extension region and the structural stability are important factors for the emulsifying ability of homotrimers. This is supported by the report that the balance of hydrophilicity and hydrophobicity in a protein and its suitable conformational change at the interface between oil and water are important for emulsifying ability (*12*). Therefore, the extension region, the structural stability, and surface hydrophobicity are important factors for emulsifying abilities of homotrimers. However, for example, $\alpha2\beta1$ showed emulsifying ability similar to that of $\alpha3$, although the number of extension regions and the hydrophobicity of $\alpha2\beta1$ are less and lower than those of $\alpha3$, respectively. This indicates that factors other than those cited above may influence the emulsifying abilities of heterotrimers.

ABBREVIATIONS USED

DSC, differential scanning calorimetry; *p*-APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride; ANS, 8-anilino-1-naphthalene sulfonic acid.

LITERATURE CITED

(1) Maruyama, N.; Katsube, T.; Wada, Y.; Oh, M. H.; Barba de la Rosa, A. P.; Okuda, E.; Nakagawa, S.; Utsumi, S. The roles of the *N*-linked glycans and extension regions of soybean β-conglycinin in folding, assembly and structural features. *Eur. J. Biochem.* **1998**, *258*, 854−862.

(2) Thanh, V. H.; Shibasaki, K. Heterogeneity of beta-conglycinin. *Biochim. Biophys. Acta* **1976**, *439*, 326−338.

(3) Thanh, V. H.; Shibasaki, K. Major proteins of soybean seeds. Subunit structure of β-conglycinin. *J. Agric. Food Chem.* **1978**, *26*, 692−695.

(4) Maruyama, N.; Mohamad Ramlan, M. S.; Takahashi, K.; Yagasaki, K.; Goto, H.; Hontani, N.; Nakagawa, S.; Utsumi, S. The effect of the N-linked glycans on structural features and physicochemical functions of soybean β-conglycinin homotrimers. *J. Am. Oil Chem. Soc.* **2002**, *79*, 139−144.

(5) Maruyama, N.; Sato, R.; Wada, Y.; Matsumura, Y.; Goto, H.; Okuda, E.; Nakagawa, S.; Utsumi, S. Structure−physicochemical function relationships of soybean β-conglycinin constituent subunits. *J. Agric. Food Chem.* **1999**, *47*, 5278−5284.

(6) Takahashi, K.; Banba, H.; Kikuchi, A.; Ito, M.; Nakamura, S. An induced mutant line lacking the α subunit of β-conglycinin in soybean (*Glycine max* (L.) Merril). *Breed. Sci.* **1994**, *44*, 65−66.

(7) Takahashi, K.; Mizuno, Y.; Yumoto, S.; Kitamura, K.; Nakamura, S. Inheritance of the α-subunit deficiency of β-conglycinin in soybean (*Glycine max* (L.) Merril) line induced by γ-ray irradiation. *Breed. Sci.* **1996**, *46*, 251−255.

(8) Nakai, S.; Li-Chan, E. Hydrophobicity-functionality relationship of food proteins. In *Hydrophobic Interactions in Food Systems*; CRC Press: Boca Raton, FL, 1988; pp 23−41.

(9) Nakai, S. Structure−function relationships of food proteins with an emphasis on the importance of protein hydrophobicity. *J. Agric. Food Chem.* **1983**, *31*, 676−683.

(10) Maruyama, N.; Adachi, M.; Takahashi, K.; Yagasaki, K.; Kohno, M.; Takenaka, Y.; Okuda, E.; Nakagawa, S.; Mikami, B.; Utsumi, S. Crystal structures of recombinant and native soybean β-conglycinin β homotrimers. *Eur. J. Biochem.* **2001**, *268*, 3595−3604.

(11) Penzer, G. R. The solution conformation and some spectroscopic properties of 1, N 6-ethenoadenosine monophosphate, a fluorescent analogue of AMP. *Eur. J. Biochem.* **1973**, *34*, 297−305.

(12) Damodaran, S. Protein-stabilized forms and emulsions. In *Food Proteins and Their Applications*; Damodaran, S., Paraf, A., Eds.; Marcel Dekker: New York, 1997; pp 57−110.

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