The Effect of the N-Linked Glycans on Structural Features and Physicochemical Functions of Soybean β-Conglycinin Homotrimers

Nobuyuki Maruyama[a], Mohamad Ramlan Mohamed Salleh[a], Koji Takahashi[b], Kazuhiro Yagasaki[c], Hideyuki Goto[d], Naho Hontani[a], Shuko Nakagawa[a], and Shigeru Utsumi[a,*]

[a]Laboratory of Food Quality Design and Development, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan, [b]National Institute of Crop Science, National Agricultural Research Organization, Tsukuba, Ibaraki 305-8518, Japan, [c]Nagano Chushin Agricultural Experiment Station, Shiojiri, Nagano 399-6461, Japan, and [d]Department of Food Science, Ishikawa Agricultural College, Nonoichi, Ishikawa 921-8836, Japan

ABSTRACT: β-Conglycinin is a trimeric protein consisting of three subunits, α, α′, and β, which are N-glycosylated. The α and α′ subunits contain extension regions in addition to core regions common to all subunits. We purified homogeneous trimers consisting of only α, α′, or β from mutant soybean cultivars containing β-conglycinin lacking one or two subunits: α homotrimers from an α′-lacking mutant, α′ homotrimers from an α-lacking mutant, and β homotrimers from an α- and α′-lacking mutant. Structural features and physicochemical functions of the three homotrimers were examined and compared with those of recombinant homotrimers having no N-linked glycans. The native homotrimers have secondary structures very similar to those of the recombinant ones. In analogy with the recombinant homotrimers, the native ones exhibit different thermal stabilities from one another (β > α′ > α), and the native α and α′ homotrimers exhibit better solubility, emulsifying ability, and heat-induced association than the native β homotrimer. Further, the N-linked glycans contribute to solubilities of the three subunits at low ionic strength (μ = 0.08) and to the emulsifying ability of the native β homotrimer. N-Linked glycans also prevent heat-induced associations of the native α and α′ homotrimers but do not contribute to the secondary structure and the thermal stability of β-conglycinin.

Paper no. J10008 in *JAOCS 79*, 139–144 (February 2002).

KEY WORDS: β-Conglycinin, N-linked glycan, physicochemical function, soybean, structural feature.

Soybean proteins play an important role in foods, and their applications are based on their physicochemical functions. Thus, knowledge of the structure–physicochemical function relationships of soybean proteins is important in extending their applications in foods and in developing new functional ingredients (1,2).

β-Conglycinin is one of the major components of soybean protein. It is a trimeric protein composed of three subunits: α (~67 kDa), α′ (~71 kDa), and β (~50 kDa) (2,3). 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) (3). The extension regions of the α and α′ subunits exhibit lower absolute homologies (57.3%) and a highly acidic property (3). The α, α′, and β subunits are glycosylated at specific Asn sites (α, Asn199 and Asn455; α′, Asn215 and Asn471; and β, Asn328) (2). The N-glycosylation site of the β subunit corresponds to the latter site of the α and α′ subunits (2).

Preparation of homogeneous molecular species composed of only one kind of subunit is necessary to investigate structure–physicochemical function relationships of β-conglycinin constituent subunits. However, it is very difficult to obtain a large amount of such species from soybean seeds because many molecular species having different subunit compositions are present in soybean seeds (4,5). In a previous study, we prepared homotrimers of the individual subunits and deletion mutants (α_c and α'_c) lacking the extension regions by means of an *Escherichia coli* expression system (3) and demonstrated that the physicochemical functions of the β-conglycinin constituent subunits are different from one another (6). Further, we elucidated the roles of their core and extension regions in the structural features and physicochemical functions (6). Recently, mutant soybean cultivars containing β-conglycinin lacking the α subunit, the α′ subunit, or the α and α′ subunits were developed (7,8). Therefore, the native homotrimers of the individual subunits can be purified easily using these mutant soybean cultivars. In this study, we purified the native homotrimers from these mutants and revealed the roles of the carbohydrate moieties in the structural features and physicochemical functions by comparing those of the recombinant homotrimers having no carbohydrate moieties.

MATERIALS AND METHODS

Purification of native β-conglycinin homotrimers from the mutant soybean cultivars. The α or α′ homotrimer-rich fraction

*To whom correspondence should be addressed.
E-mail: utsumi@food2.food.kyoto-u.ac.jp

was prepared from mutant soybean [*Glycine max* (L.) Merr.] seeds that contain β-conglycinin lacking the α′ or α subunit, respectively, by the procedure of Nagano *et al.* (9). The proteins in the native α or α′ homotrimer-rich fraction were fractionated using ammonium sulfate. The precipitate of 65 to 85% saturation was dissolved in buffer A [35 mM sodium phosphate, pH 7.6, 0.25 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride (*p*-APMSF), 0.02% NaN_3] and was dialyzed against buffer A. The dialyzates were subsequently applied to a Mono Q HR 10/10 column (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) equilibrated with buffer A. The native α and α′ homotrimers were eluted with a linear gradient from 0.25 to 0.5 M NaCl over a period of 250 min. Fractions of 4.5 mL were collected at a flow rate of 1.5 mL/min. The native β homotrimer was purified by ammonium sulfate fractionation (70 to 85%) from the glycinin-rich fraction (10) of the mutant soybean seeds, which contain β-conglycinin lacking both the α and α′ subunits.

Protein measurement. Protein concentrations of the samples were determined using a Protein Assay Rapid Kit (Wako, Osaka, Japan) with BSA as the standard.

Circular dichroism (CD) studies. Secondary structures of the native homotrimers were evaluated by CD measurement as described previously (3). CD spectra were recorded with a Jasco model J720 spectropolarimeter. Measurement was carried out in buffer B (35 mM sodium phosphate, pH 7.6, 0.4 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM *p*-APMSF, 0.02% NaN_3).

Solubility as a function of pH. Solubilities of the native homotrimers were measured as described previously (6). Protein solutions (0.8 mg/mL) were kept at 4°C for 18 h at various pH values at μ (ionic strength) = 0.5 and 0.08. After centrifugation, 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.

DSC measurement. DSC experiments were carried out on a Microcal MC-2 ultrasensitive microcalorimeter (Micro Cal Inc., Northampton, MA) as described previously (3). All DSC experiments were performed with a protein concentration of 0.5 mg/mL in buffer B.

Analysis of heat-induced association. Heat-induced associations of the native homotrimers were examined as described previously (6). Each homotrimer (1 mg/mL) in buffer B without 10 mM 2-mercaptoethanol was heated at 70, 80, or 90°C for 5 min. After heating, the solutions were passed through a membrane filter (0.22 μm). The filtered samples were fractionated *via* gel filtration chromatography using KW804 and SB806M columns (Showa Denko, Tokyo, Japan) and 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 for calculations of molecular masses of heat-induced aggregates.

Analysis of emulsifying ability. Emulsifying abilities of the native homotrimers were measured as described previously (6). To prepare emulsions, 0.25 mL soybean oil and 1.5 mL of the native homotrimers (0.5 mg/mL) in buffer B were homogenized for 30 s with a high-speed homogenizer (model NS-50; Nichion Irikakikai Ltd., Chiba, Japan) and further sonicated using an ultrasonic homogenizer (model US-150; Nihonseiki Kaisha Ltd., Tokyo, Japan) for 1 min. The particle size distribution of the emulsions was measured using a laser light-scattering instrument (model LA 500; Horiba Seisakusho Ltd., Kyoto, Japan). Each sample was analyzed several times and a representative typical pattern was presented.

RESULTS AND DISCUSSION

CD studies. Figure 1 shows the CD spectra of the native homotrimers. The CD spectrum of the native α homotrimer was very similar to that of the native α′ homotrimer, but the native β homotrimer was somewhat different from those of the native α and α′ homotrimers (Fig. 1A). These results coincide well with our previous demonstration that the difference in the CD spectra between the native α and α′ homotrimers and the native β homotrimer is due to the presence of the extension regions in the native α and α′ homotrimers (6). The CD spectra of the native α, α′, and β homotrimers were almost identical to those of the recombinant α, α′, and β homotrimers, respectively (Fig. 1B–D), indicating that the carbohydrate moieties of the native homotrimers do not affect the secondary structures. Recently, we determined the 3-D structures of the native and recombinant β homotrimers and demonstrated that the carbohydrate moieties do not influence the final structure of the β homotrimer (11). The result obtained here is consistent with X-ray crystallographic data for β homotrimers.

Solubility. The native α, α′, and β homotrimers were soluble at all pH values examined here at μ = 0.5 in analogy with the recombinant α, α′, and β ones (6). At μ = 0.08, the native α and α′ homotrimers were insoluble only in the vicinity of pH 5.0, although the native β homotrimer was insoluble at pH 4.8 to 8.5 (Fig. 2). The difference in the solubilities of the native α and α′ homotrimers and the native β homotrimer is consistent with the results obtained with the recombinant homotrimers (6) and is due to the presence of the extension regions in the native α and α′ homotrimers.

The pH ranges at which the native α and α′ homotrimers were insoluble at μ = 0.08 were narrower compared with those of the recombinant α and α′ ones, respectively (Figs. 2A and 2B). At pH 9.0, the native β homotrimer was soluble, although the recombinant β one was insoluble (Fig. 2C). Therefore, the solubilities of the native α, α′, and β homotrimers at μ = 0.08 are increased by the carbohydrate moieties.

The improvement of the solubility of β-conglycinin is desired for expansion of food usage, because several physicochemical functions, such as emulsifying ability, gelation, and foaming, of proteins are affected by the protein solubility (12). Kitabatake *et al.* (13) indicated that the β-lactoglobulin glycosylated with melibionates or gluconates displayed high solubility even at low ionic strength and isoelectric pH, and that the solubility of the glycosylated β-lactoglobulin in-

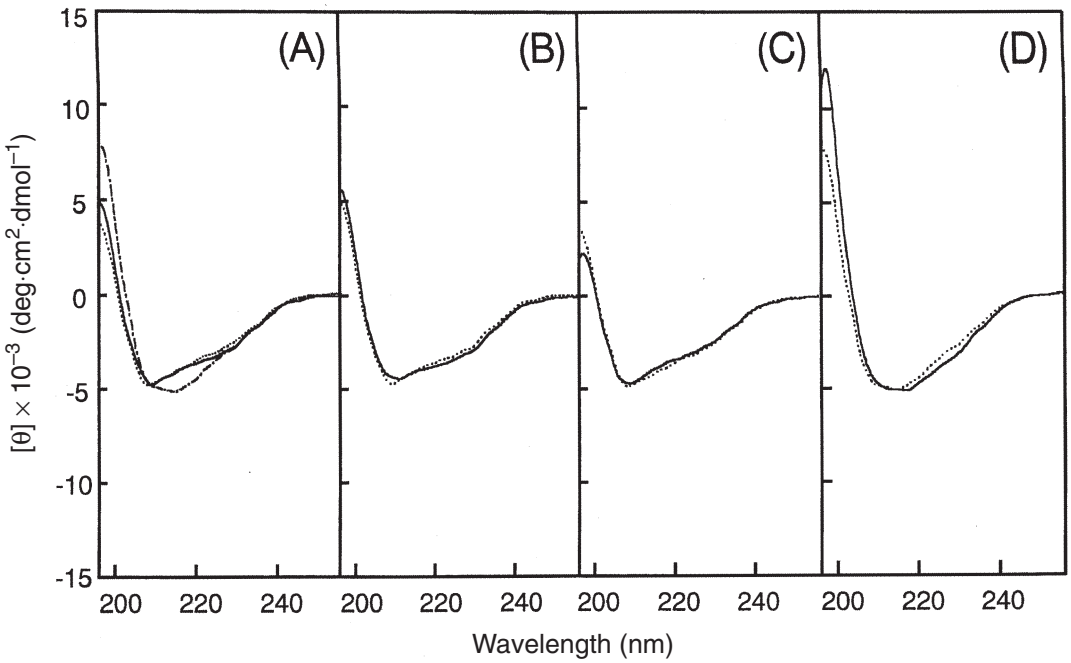

FIG. 1. Circular dichroism (CD) spectra of β-conglycinin homotrimers. CD spectra of (A) native homotrimers α (solid line), α′ (dotted line), and β (dashed and single-dotted line) prepared from soybean mutant cultivars. CD spectra of native homotrimers (dotted line) compared with those of the recombinant homotrimers (3) (solid line): (B) α, (C) α′, (D) β.

creased as the amounts of melibionates or gluconates covalently linked to β-lactoglobulin increased. These, together with our observation, suggest that an increase in the amounts of carbohydrate moieties would improve the solubility of the native β-conglycinin individual homotrimers at μ = 0.08. Therefore, the introduction of N-glycosylation sites by protein engineering is considered a powerful method to improve protein solubility at low ionic strengths.

Thermal stability. DSC profiles of the native α, α′, and β homotrimers are shown in Figure 3. The native β homotrimer exhibited the highest thermal denaturation midpoint temperature (T_m) (87.0°C), followed by the native α′ homotrimer (82.6°C) and the native α homotrimer (78.2°C). The order of the T_m values among the native α, α′, and β homotrimers was identical to that of the recombinant ones. The T_m values of the native α and α′ homotrimers were similar to those of the recombinant ones, respectively (Figs. 3A and 3B). This clearly indicates that the carbohydrate moieties of the native α and α′ homotrimers do not influence their thermal stability. On the other hand, the T_m value of the native β homotrimer was 3.8°C

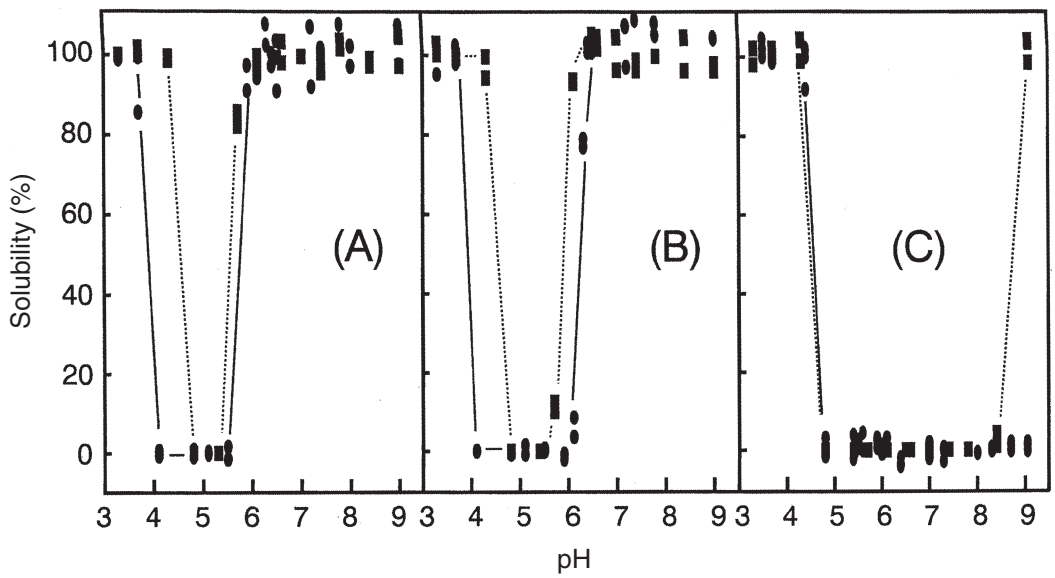

FIG. 2. Dependency of the solubilities of β-conglycinin native homotrimers (dotted line with squares) on pH at ionic strength 0.08 compared with those of the recombinant homotrimers (solid line with circles) (6): (A) α, (B) α′, (C) β.

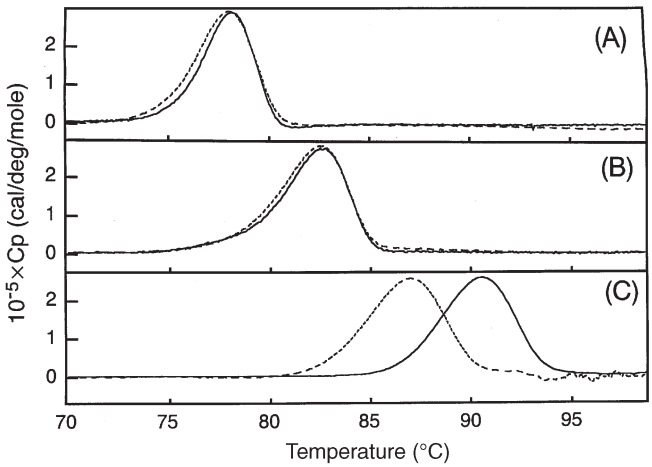

FIG. 3. DSC scans of β-conglycinin native homotrimers (dotted line) compared with those of the recombinant homotrimers (solid line) (6): (A) α, (B) α′, (C) β. Cp, [specific heat capacity.

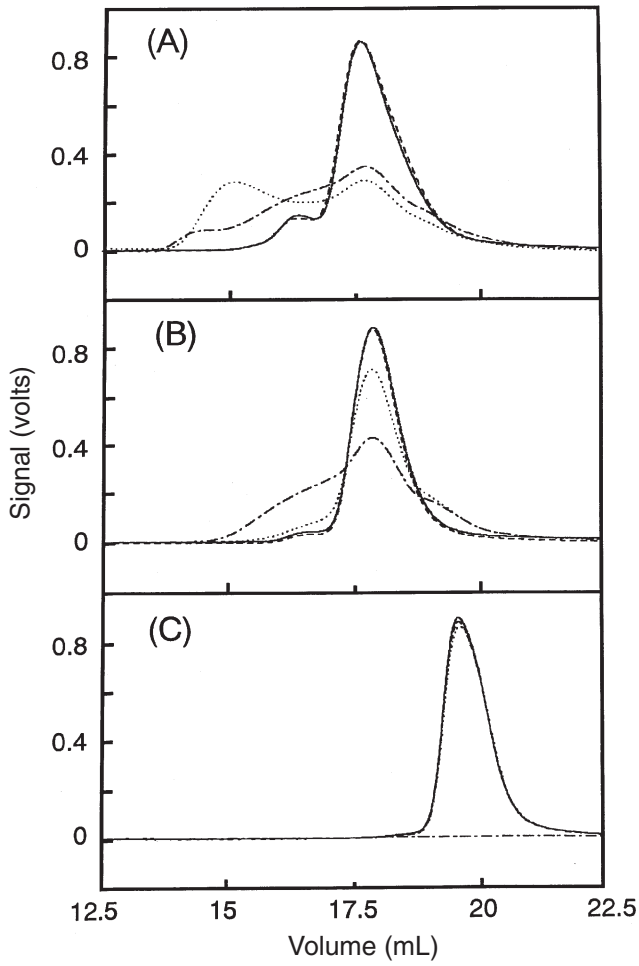

FIG. 4. Elution patterns of heat-treated β-conglycinin native homotrimers. The native α (A), α′ (B), and β (C) homotrimers were heated at 70°C (-----), 80°C (·····), or 90°C (-·-·-). Nonheated samples are shown by a (——) line.

lower than that of the recombinant β one (Fig. 3C). The number of the carbohydrate moieties of the native β homotrimer is half of those of the native α and α′ homotrimers (2). In taking into account this fact and the results obtained for the results of the native α and α′ homotrimers, one can assume that the carbohydrate moiety of the native β homotrimer does not affect its thermal stability. A sequencing study revealed that Phe13 and Phe174 in the recombinant β homotrimer were replaced by Leu in the native β one from the mutant soybean (11). Our recent X-ray analysis indicated that the conformation of the polypeptide backbone of the regions including the replaced sites of the recombinant β homotrimer was identical to those of the native β homotrimer, but small cavities were made by the replacement of the two residues in the native β homotrimer (11). Eriksson *et al.* (14) found that the decrease in the stability of the mutant T4 lysozymes substituted in the core, Leu → Ala, Phe → Ala and Leu → Ala/Phe → Ala, depends on the increase in the size of the cavity. Therefore, the difference in the T_m values between the native and recombinant β homotrimers may be due to the small cavities in the mutation sites.

Wang *et al.* (15) examined the role of the carbohydrate moiety in the thermal stability of glycoproteins and demonstrated that the destabilization effect of deglycosylation seems to depend on the carbohydrate content. They indicated that the deglycosylation of ovotransferrin and avidin, which contain 2.2 and 10% carbohydrate per protein, respectively, did not show measurable variations in T_m values. β-Conglycinin contains approximately 5% carbohydrate per protein as glucose (16). Thus, the contents of the carbohydrate moieties of the β-conglycinin homotrimers are probably too small to influence the thermal stability.

Heat-induced association. To examine heat-induced association, heated samples were fractionated by using gel filtration chromatography (Fig. 4). The native α and α′ homotrimers formed soluble aggregates with a concomitant decrease of the intact species by heating at >80°C, which was close to the T_m values of the native α and α′ homotrimers (Figs. 4A and 4B). The efficiency of the conversion of the intact species of the native α homotrimer to soluble aggregates at 80°C was higher than that of the native α′ homotrimer. This is probably due to the difference in the T_m values (α, 78.2°C; α′, 82.7°C). In contrast, the native β homotrimer did not form soluble aggregates at all at temperatures higher than T_m, but instead formed insoluble aggregates depending on its T_m value. The difference in the solubilities of heat-induced aggregates between the native α and α′ homotrimers and the native β homotrimer was consistent with the results from our study on recombinant homotrimers, indicating that the difference is due to the presence of the extension regions in the native α and α′ homotrimers (6).

In a previous paper, we demonstrated that almost all the intact species of the recombinant α homotrimer were converted to the soluble aggregates having molecular masses of 2 to 3 million daltons by heating at >80°C (6). On the other hand, approximately 70% of the intact species of the native α homotrimer were converted to the soluble aggregates having molecular masses of approximately 1 to 2 million daltons by heating at 80°C (Fig. 4A). Similarly, the size and amount of the soluble aggregates of the native α homotrimer were

smaller and fewer than those of the recombinant α one by heating at 90°C. Similar tendencies in the case of the recombinant and native α′ homotrimers were observed. These results indicate that the carbohydrate moieties of the native α and α′ homotrimers inhibit the heat-induced associations, being consistent with the observation that the N-linked glycan of the glycosylated proglycinin by protein engineering prevents protein–protein interactions induced by heating (17).

Emulsifying ability. The emulsifying abilities of the native α, α′, and β homotrimers were assessed by measuring the particle sizes of the emulsions (the smaller the size, the better the emulsion). The native α homotrimer exhibited the best value (5.2 μm) among the three homotrimers, followed by the native α′ (9.8 μm) and β (28.5 μm) homotrimers (Fig. 5). This order is consistent with that among the recombinant α, α′, and β homotrimers (6). The average particle sizes of the emulsions of the native α and α′ homotrimers were close to those of the recombinant α and α′ ones, respectively. On the other hand, the average particle size of emulsions of the native β homotrimer was smaller than that of the recombinant one. The presence of the carbohydrate moiety and/or the difference in thermal stability may influence the difference in emulsifying ability between the native and recombinant β homotrimers.

Since the recombinant β homotrimer was constructed by using the cDNA prepared from the developing seeds of soybean var. Wasesuzunari, the β homotrimer was purified from the seeds of soybean var. Wasesuzunari and its emulsifying ability was examined. The emulsifying ability of the native β homotrimer from var. Wasesuzunari was almost identical to that of the native β homotrimer from the mutant soybean cultivar, indicating that the carbohydrate moieties influence the emulsifying ability of the native β homotrimer, although they do not affect those of the native α and α′ homotrimers.

Why is the emulsifying ability of only the native β homotrimer improved by the carbohydrate moiety? Conformational changes of protein occur at the oil and water interface (18). As a result, hydrophobic segments lie on the interface and hydrophilic ones protrude into the aqueous phase. Therefore, the balance of these two types of segments is important in providing excellent emulsifying ability. The extension regions of the α and α′ homotrimers are considered to be hydrophilic segments and are presumably large enough to exhibit good emulsifying abilities. On the other hand, the carbohydrate moiety of the β homotrimer, which does not have the extension region, is probably valuable as a hydrophilic segment.

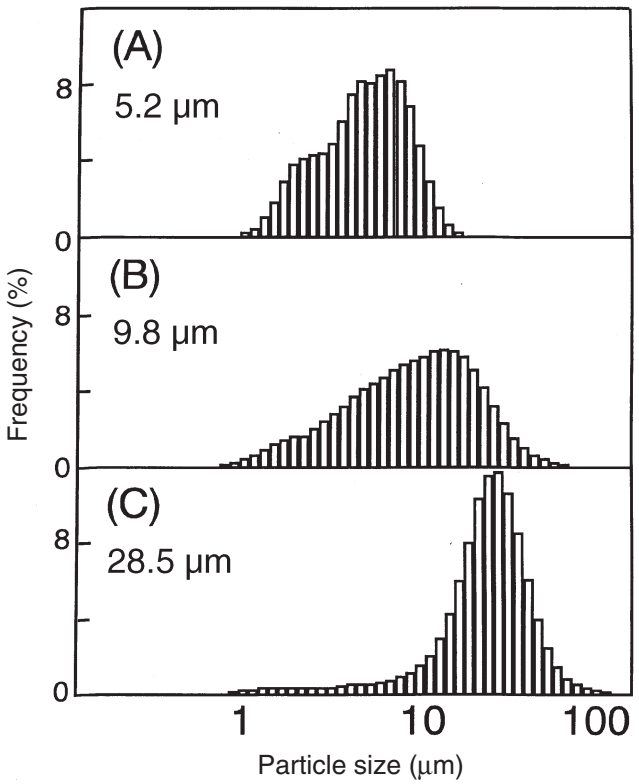

FIG. 5. Particle size distributions of emulsions from β-conglycinin native homotrimers: (A) α, (B) α′, (C) β. Numbers indicate the average particle sizes of the emulsions.

ACKNOWLEDGMENTS

This work was supported in part by grants to S.U. and N.M. from the Ministry of Education, Science and Culture of Japan, and to S.U. from Fuji Foundation for Protein Research, The San-Ei Gen Foundation for Food Chemical Research, and The Salt Science Research Foundation.

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[Received June 15, 2001; accepted December 12, 2001]