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Characterization and Crystallography of Recombinant 7S Globulins of Adzuki Bean and Structure−Function Relationships with 7S Globulins of Various Crops

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The recombinant proteins Adzuki 7S1, Adzuki 7S2, and Adzuki 7S3 were prepared through the *Escherichia coli* expression systems of three kinds of adzuki bean cDNAs. The recombinant proteins exhibited intrinsic thermal stabilities, surface hydrophobicities, and solubilities, although the homology of their amino acid sequences ranged from 95−98%. To understand why these individual proteins exhibited different properties, their three-dimensional structures were elucidated. The three proteins were successfully crystallized, and the three-dimensional structures of Adzuki 7S1 and Adzuki 7S3 were determined. The properties and structures of these two proteins were comprehensively compared with those of recombinant 7S globulins (soybean β-conglycinins β and α'c and mungbean 8Sα) reported previously. It was likely that cavity sizes, hydrogen bonds, salt bridges, hydrophobic interactions, and lengths of loops determine the thermal stabilities of 7S globulins, and results indicated that cavity sizes strongly contribute to such stability. Surface hydrophobicity was also found to be determined not only by distributions of hydrophobic residues on the molecular surface. Furthermore, solubility at neutral and weak alkaline pH values at $\mu = 0.08$ was found to be dominantly influenced by the electrostatic surface potentials.

KEYWORDS: Adzuki bean; soybean; 7S globulin; β-conglycinin; physicochemical properties; crystal structure

INTRODUCTION

Elucidation of structure−function relationships of seed storage proteins is a prerequisite for developing theoretically new food and/or food materials based on seed storage proteins. Most storage proteins of crop seeds are composed of many kinds of subunits, resulting in molecular heterogeneity; that is, many molecular species with different subunit compositions are present in seeds. As a first step to elucidate structure−function relationships, individual constituent subunits should be characterized and their three-dimensional structures should be determined by using molecular species composed of only one kind of subunits. However, this is very difficult because of their molecular heterogeneity. To prepare homogeneous proteins, we have utilized *Escherichia coli* (*E. coli*) expression systems of cDNAs encoding individual subunits. Furthermore, we have characterized soybean β-conglycinin subunits (α, α', and β) (*1*) and glycinin subunits (A1aB1b, A1bB2, A2B1a, A3B4, and A5A4B3) (*2*) and determined some of their three-dimensional structures (*3*−*6*).

As described in a previous paper (*7*), we cloned three kinds of cDNAs encoding adzuki bean 7S globulins. In this paper, we constructed *E. coli* expression systems of the three cDNAs, characterized three recombinant 7S globulins, crystallized them, and determined the three-dimensional structures of two of them. Moreover, we deduced general structure−function relationships of 7S globulins by comparing the three-dimensional structures and the functional properties of various 7S globulins, which had been earlier reported on recombinant forms.

MATERIALS AND METHODS

Construction of Expression Plasmids Encoding Adzuki Bean 7S Globulins. Three kinds of cDNAs encoding adzuki bean 7S globulins cloned previously (*7*) were amplified using primers for the N- and C-terminals designed on the basis of the nucleotide sequences. The primers for the C-terminals contained a *Bam*HI site next to the stop

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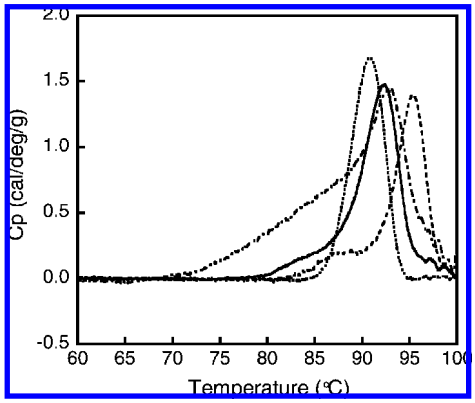

Figure 1. DSC scan of Adzuki 7S1, Adzuki 7S2, Adzuki 7S3, and β-conglycinin β at $\mu = 0.5$. Profiles of Adzuki 7S1, Adzuki 7S2, Adzuki 7S3, and β-conglycinin β are shown by solid line, dashed line, dashed and single-dotted line, and dotted line, respectively. The data of β-conglycinin β were adopted from ref *1*.

Table 1. Elution Times of Each Recombinant Protein on Hydrophobic Columns

	elution times (min)		
column	Adzuki 7S1	Adzuki 7S2	Adzuki 7S3
butyl Sepharose	45.8	49.1	49.0
phenyl Sepharose	65.3	66.5	62.3

codon. PCR products were blunted and then digested with *Bam*HI. The resultant DNA fragments were inserted into the *Nco*I (filled-in) and *Bam*HI sites of pET-21d vector (Novagen) to construct the three expression plasmids, pEAdzuki 7S1, pEAdzuki 7S2, and pEAdzuki 7S3. Sequences were confirmed by the dideoxy sequencing method using ABI Prism 3100 DNA analyzer (Applied Biosystems).

Expression of Recombinant Adzuki Bean 7S Globulins. The expression plasmids were transformed into *E. coli* strains HMS174(DE3), BL21(DE3), AD494(DE3), and Origami(DE3). For small-scale expression, 40 μL of fully grown culture was inoculated in the fresh 4 mL of LB medium containing 0.17 or 0.5 M NaCl and 50 μg/mL carbenicillin and cultured at 37 °C. At $A_{600} = 0.4-0.6$, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. The recombinant proteins were expressed by incubating the culture at 20 °C for 40 h. The cells were harvested by centrifugation at 6700*g* for 20 min at 4 °C and stored at −20 °C until used. Cells were resuspended (15 mg of cells/mL of buffer) in buffer A (35 mM sodium phosphate, pH 7.6, 0.4 M NaCl, 1 mM EDTA, 0.1 mM *p*-amidinophenylmethanesulfonyl fluoride (*p*-APMSF), 1 μg/mL leupeptin, 1 μg/mL pepstatin A) and disrupted by sonication on ice. The insoluble fraction was separated from the soluble fraction by centrifugation at 17800*g* for 10 min. The total, soluble, and insoluble fractions were analyzed by SDS-PAGE (*8*) using 11% acrylamide as described previously (*9*). Expressed recombinant proteins were identified on the basis of their expected sizes.

Table 2. Overview of the Statistics of Data Collection and Refinement

	Adzuki 7S1	Adzuki 7S3
data statistics		
space group	$P2_1$	$P2_12_12_1$
observed reflections	424225	378726
unique reflections	104746	69062
R_{merge} (%)	5.4	5.6
completeness (%)	97.8	100.0
unit-cell parameters (Å)	$a = 101.6$, $b = 48.6$, $c = 119.8$ $\beta = 97.1°$	$a = 66.9$, $b = 99.3$, $c = 216.9$
resolution (Å)	50−1.8	50−2.25
refinement statistics		
final model	1156 amino acid residues, 755 water molecules, 4 calcium ions, 3 acetate ions	1144 amino acid residues, 357 water molecules, 3 calcium ions, 3 acetate ions, 3 citrate ions
resolution range (Å)	15.0−1.8	15.0−2.25
reflections used	104561	68683
average *B* factor (Å^2)		
protein atoms	33.3	39.2
water molecules	42.2	41.8
R factor (%)	19.3	19.4
R_{free} (%)	22.8	23.7

For large-scale expression, 1 mL of each overnight culture was inoculated in 500 mL of LB medium containing 0.17 M (for pEAdzuki 7S1) and 0.5 M (for pEAdzuki 7S2 and pEAdzuki 7S3) NaCl and 50 μg/mL carbenicillin. The cells were incubated at 37 °C until A_{600} reached 0.4−0.6. At this point, IPTG was added to a final concentration of 1 mM. The recombinant proteins were expressed by incubating the culture at 20 °C for 40 h. The cells were harvested by centrifugation at 6700*g* for 20 min at 4 °C and stored at −20 °C until used.

Protein Extraction and Purification. The recombinant proteins were extracted from *E. coli* cells by sonication in buffer A. Extracts were subjected to ammonium sulfate fractionation. The fractions of 30−50% saturation containing recombinant proteins were dissolved in buffer A and dialyzed against buffer B [35 mM sodium phosphate, pH 7.6, 0.1 M NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM *p*-APMSF, 1.2 μM leupeptin, 0.2 μM pepstatin A, 0.02% (w/v) NaN_3] and subsequently applied on a Q Sepharose HR column (GE Healthcare Bioscience). Elution was performed with a linear gradient of 0.1−0.35

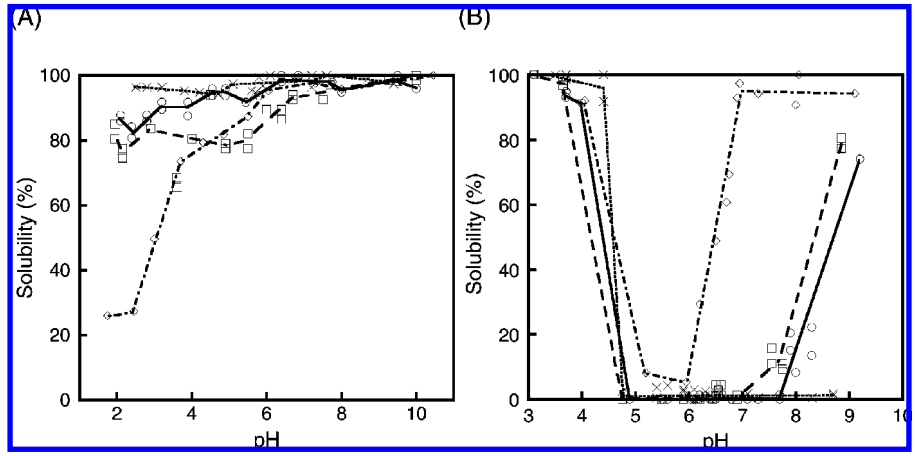

Figure 2. pH dependence of solubilities of Adzuki 7S1, Adzuki 7S2, Adzuki 7S3, and β-conglycinin β at $\mu = 0.5$ (**A**) and 0.08 (**B**). Adzuki 7S1, Adzuki 7S2, Adzuki 7S3, and β-conglycinin β are shown by solid line with circles, dashed line with squares, dashed and single-dotted line with diamonds, and dotted line with crosses, respectively. The data of β-conglycinin β were adopted from ref *20*.

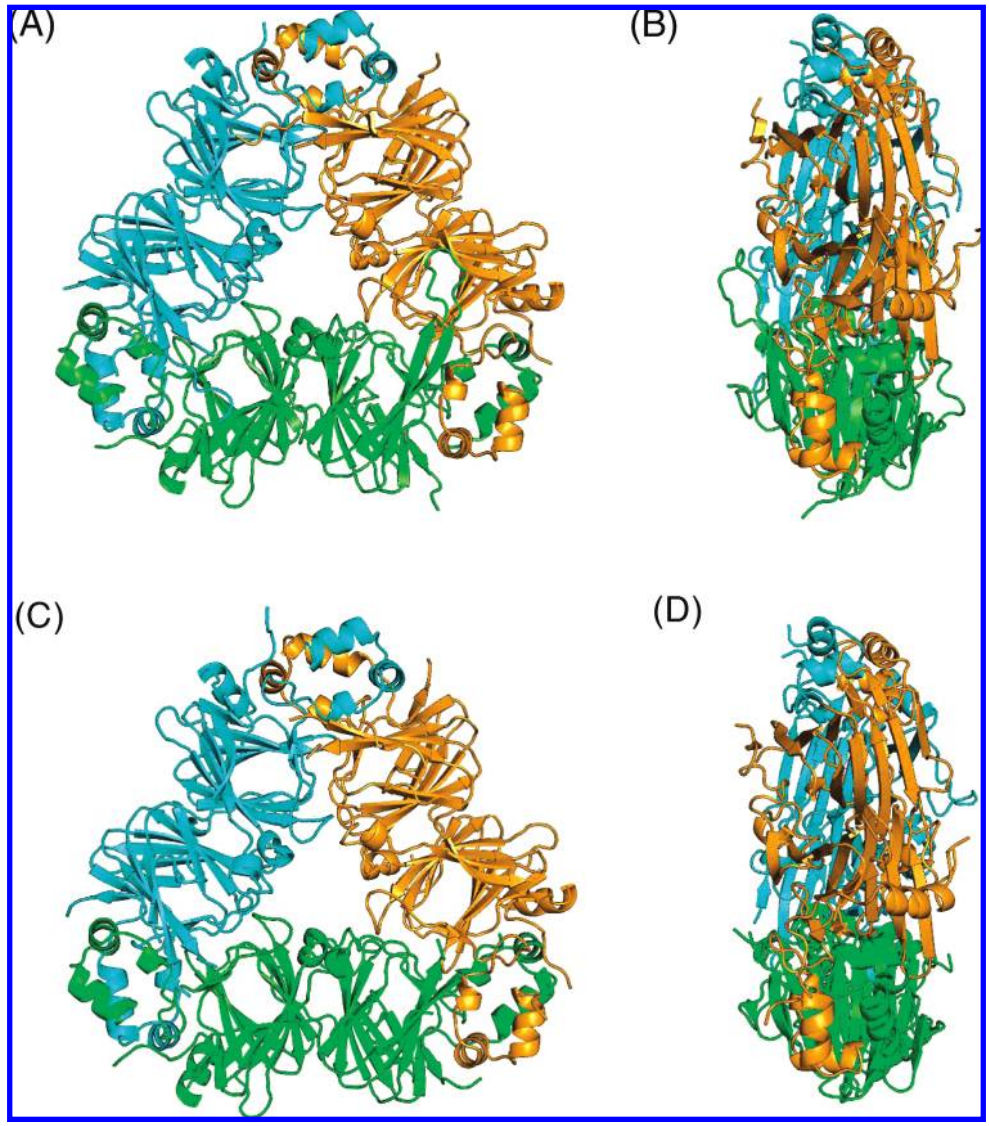

Figure 3. Overall structures of Adzuki 7S1 (**A**, **B**) and Adzuki 7S3 (**C**, **D**) trimers. The three subunits are shown in green, cyan, and orange. (**A**, **C**) Trimers are seen along 3-fold axes. (**B**, **D**) Views after 90° rotation around the vertical axes of (**A**) and (**C**), respectively.

M NaCl in buffer B over a period of 200 min at 2 mL/min.

Analysis of Self-Assembly into Trimers. Self-assembly of individual recombinant 7S samples was analyzed using a HiPrep 16/60 Sephacryl S-200 HR column (GE Healthcare Bioscience) at pH 7.6 and $\mu = 0.5$ at a flow rate of 0.5 mL/min as described previously (*1*).

DSC Measurement. Recombinant 7S samples were dialyzed against buffer A ($\mu = 0.5$). After dialysis, the samples were subjected to the DSC measurement that was carried out on a Microcal MC-2 ultrasensitive microcalorimeter (Micro Cal) as described previously (*1*). All DSC experiments were performed with a protein concentration of 0.5 mg/mL. A DSC scan rate of 1 °C/min was used for all experiments.

Surface Hydrophobicity. Surface hydrophobicities of recombinant 7S samples were analyzed as described previously (*7*) except for the starting concentration of ammonium sulfate [35% saturation instead of 2.3 M (48.75%)]. The chromatography was performed with a linear gradient of ammonium sulfate 35−0% over a period of 55 min at a flow rate of 0.25 mL/min. A flow of 0% ammonium sulfate buffer was continued until 100 min to elute the protein.

Emulsifying Ability. The emulsifying abilities of recombinant 7S samples were measured as described previously (*10*). Protein samples were dialyzed against buffer A for $\mu = 0.5$ and buffer C [10 mM sodium phosphate, pH 7.6, 0.05 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM *p*-APMSF, 1.2 μM leupeptin, 0.2 μM pepstatin A, 0.02% (w/v) NaN_3] for $\mu = 0.08$. Dialyzed samples (1.5 mL at 1.0 mg/mL, $\mu = 0.5$ or 0.08) and 0.25 mL of soybean oil were homogenized and sonicated. Each sample was analyzed three times, and the average particle sizes of the emulsions were evaluated.

Solubility as a Function of pH. The solubilities of recombinant 7S samples as a function of pH were measured as described previously (*10*). The protein solutions (0.8 mg/mL) were kept at 4 °C for 18 h at various pH values at $\mu = 0.5$ and 0.08. After centrifugation, protein concentrations in the supernatant were determined using a Protein Assay Rapid Kit (Wako Pure Chemical Industries). Solubility was expressed as percentage of the total protein content in the sample.

Crystallization. The recombinant adzuki bean 7S globulins were subjected to crystallization using a hanging drop vapor diffusion method.

The purified proteins were dialyzed against buffer D [10 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM EDTA, 0.1 mM *p*-APMSF, 0.5 μg/mL leupeptin, 0.2 μM pepstatin A, 0.02% (w/v) NaN_3] and concentrated to 10 mg/mL using a Vivaspin 20 10000 MWCO PES (Vivascience). Crystallization screening was performed using Crystal Screen and Crystal Screen 2 (Hampton Research) and Wizard I and Wizard II (Emerald BioStructures). On the basis of the screening results, 5 μL of Adzuki 7S1 and 7S3 solutions was mixed with 5 μL of bottom solutions [34% (v/v) 2-methyl-2,4-pentanediol, 0.1 M acetate, pH 4.8, for Adzuki 7S1, 30% (v/v) 2-methyl-2,4-pentanediol, 0.2 M ammonium acetate, 0.1 M sodium citrate, pH 5.6, 2.0 M ammonium sulfate for Adzuki 7S3] and equilibrated against 1 mL reservoir solutions at 20 °C to get bigger crystals.

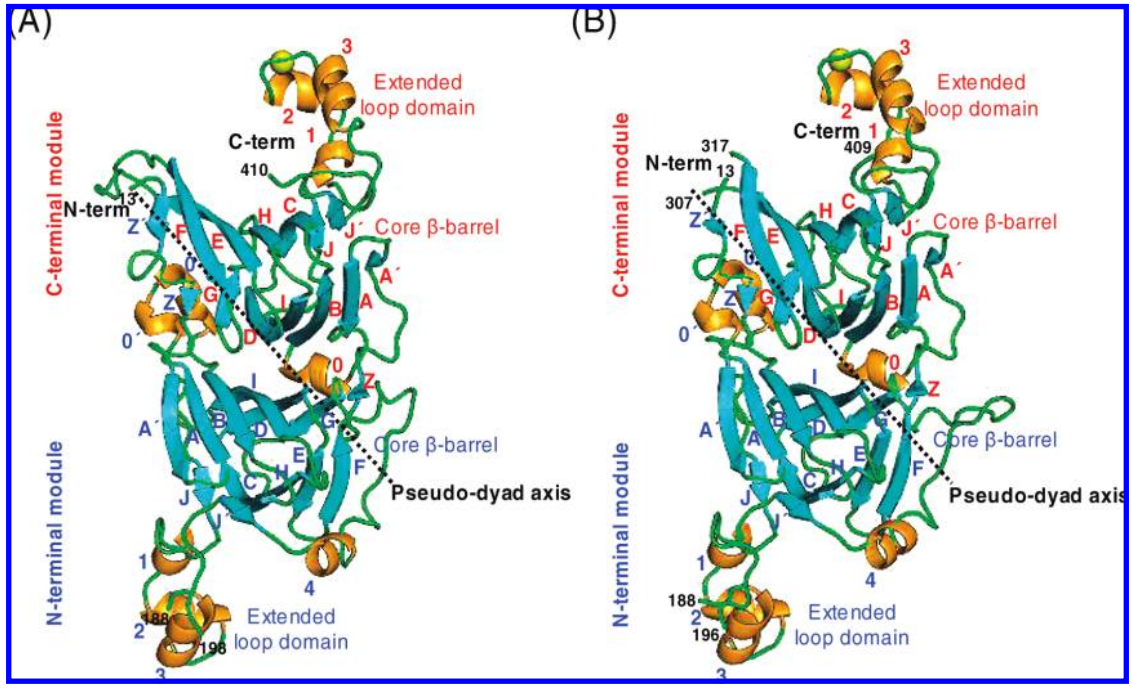

Figure 4. Monomers of Adzuki 7S1 (**A**) and Adzuki 7S3 (**B**). The β-strands, the α- and 3_{10}-helices, and loops are shown in cyan, orange, and green, respectively. The broken line in the figure is the pseudodyad axis of the N- and C- terminal modules. Calcium ions are shown in yellow as a sphere model.

Data Collection and Refinement. Crystals were frozen and stored in liquid nitrogen. The diffraction data were collected by CCD and imaging-plate detectors for Adzuki 7S1 and Adzuki 7S3, respectively, in SPring-8 (Japan) using beam line BL38B1. The collected images were processed by HKL2000 (*11*). The crystal structures of Adzuki 7S1 and Adzuki 7S3 were determined by the molecular replacement method as implemented in *CNS* v. 1.1 (*12*). The refined crystal structure of the recombinant β-conglycinin β homotrimer (*3*) and a resultant Adzuki 7S1 structure were used as the probe structure for Adzuki 7S1 and Adzuki 7S3, respectively. Model building and refinement were performed using *TURBO-FRODO* (AFMB-CNRS) and *CNS*, respectively. $|F_o| - |F_c|$ and $2|F_o| - |F_c|$ maps were used to locate the correct models.

Structure Comparison. Crystal structures of Adzuki 7S1 and Adzuki 7S3 were compared with those of various legume 7S globulins. The coordinates of soybean β-conglycinin β (1ipk), β-conglycinin α'c (1uik), mungbean 8Sα (2cv6), French bean phaseolin (2phl), and jackbean canavalin (1dgw) were taken from the PDB (*13*). These models were superimposed by a fitting program, *TOP* (*14*), part of the *CCP*4 suite (*15*), and were calculated by the *RIGID* program implemented in *TURBO-FRODO*. Ribbon plots were prepared using *PyMOL* (http://www.pymol.org). The cavity sizes were estimated by *CASTp* (*16*). Hydrogen bonds were assigned using *CONTACT*, part of the *CCP*4 suite (*15*), in which the definition of the cutoff distance between the acceptor and donor atoms was 2.5−3.2 Å, the minimum calculated angle O−H−N was 120°, and the minimum angle donor atom −O−C was 90°. Surface hydrophobicity and electrostatic surface potential were calculated using *GRASP* (*17*). The file "full.crg" was used for charge assignment, wherein the histidine residue has no charge. The protein secondary structures were calculated using *JOY* (*18*).

RESULTS AND DISCUSSION

Expression and Purification. The expression plasmids were transformed into *E. coli* strains HMS174(DE3), BL21(DE3), AD494(DE3), and Origami(DE3). On the basis of preliminary small-scale expression, we decided to use HMS174(DE3) as a host for all expression plasmids, because it gave the highest expression level of soluble proteins in all cases (data not shown).

The expressed recombinant proteins were purified to near homogeneity by ammonium sulfate fractionation and anion-exchange column chromatography using Q Sepharose HR (data not shown).

Self-Assembly of Recombinant Proteins. To assess self-assembly into trimers of individual recombinant adzuki bean 7S globulins, recombinant and native protein samples were subjected to gel filtration column Sephacryl S-200 HR. The elution times were 96.2, 96.5, and 95.9 min for Adzuki 7S1, Adzuki 7S2, and Adzuki 7S3 (data not shown). Under the same condition, the native adzuki bean 7S globulins eluted at 94.0 min (*7*). The results indicate that the native globulins eluted more rapidly than the recombinant proteins. This could be due to the fact that the native adzuki bean 7S globulins have one or two carbohydrate moieties. Therefore, the molecular dimensions of the native globulins are larger than those of recombinant forms. The results also demonstrate that Adzuki 7S1, Adzuki 7S2, and Adzuki 7S3 were trimers like the native 7S globulins of adzuki bean.

Thermal Stability. The DSC profiles of Adzuki 7S1, Adzuki 7S2, and Adzuki 7S3 at $\mu = 0.5$ are shown in **Figure 1**. Previously, we reported that recombinant soybean β gives a peak with a thermal denaturation midpoint temperature (T_m) of 90.8 °C (*19*). Adzuki 7S1 and Adzuki 7S3 exhibited peaks with the T_m values of 92.4 and 92.5 °C, respectively. However, Adzuki 7S3 had a broad denaturation profile starting at around 70 °C. On the other hand, Adzuki 7S2 gave a peak with the T_m value of 95.0 °C. Therefore, the three recombinant proteins showed characteristic patterns of thermal stabilities. When compared with the DSC patterns of the native adzuki bean 7S globulins, which exhibited two peaks with T_m values of around 85 and 92 °C (*7*), the lower peak of the two peaks can be attributed to the broad denaturation profile of Adzuki 7S3, which started at around 70 °C. In addition, previously we demonstrated that thermal stabilities of heterotrimers of β-conglycinin depend on subunit compositions (*19*).

Surface Hydrophobicity. We assessed the surface hydrophobicities of Adzuki 7S samples using phenyl and butyl

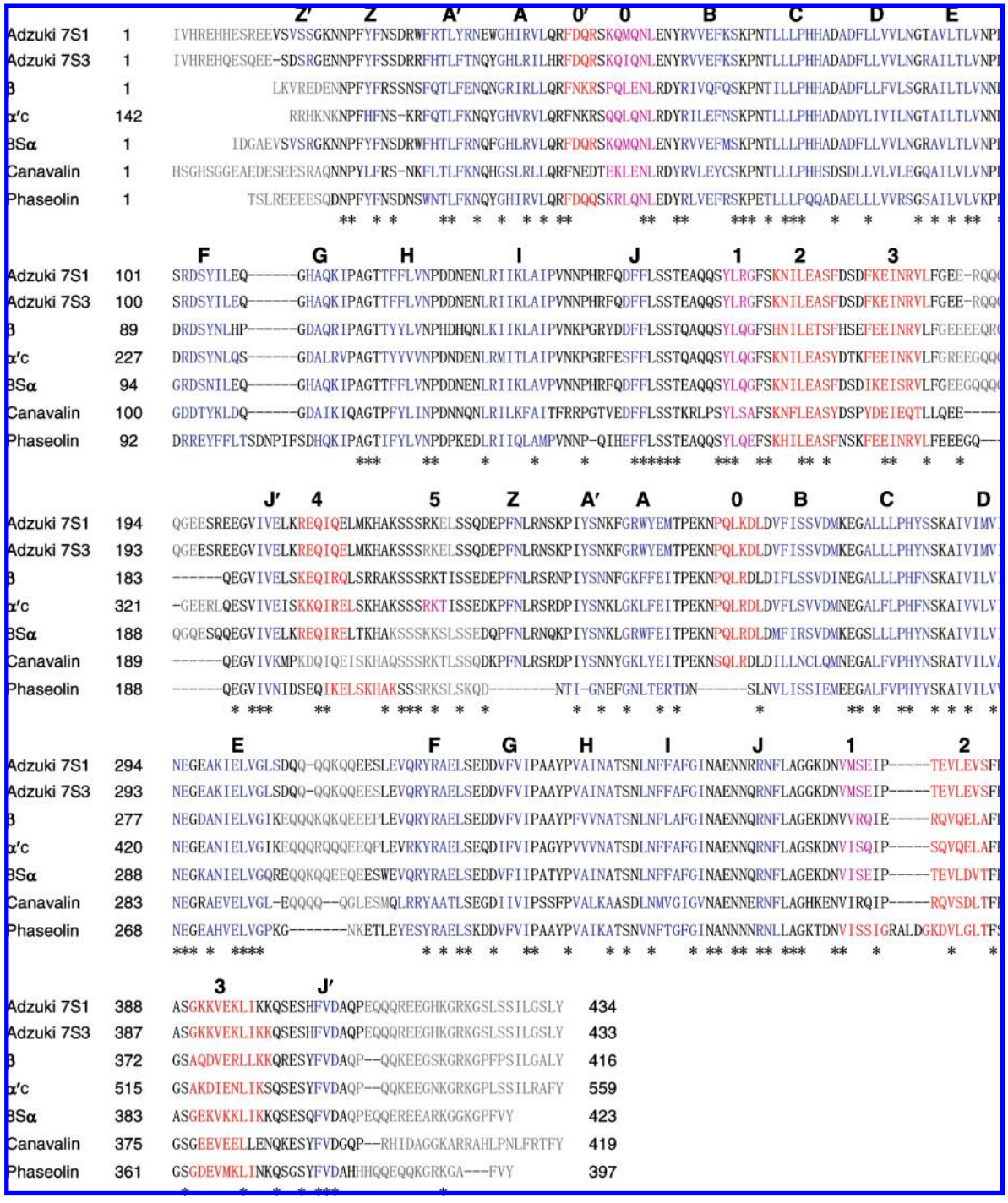

Figure 5. Structure-based sequence alignment of Adzuki 7S1 and Adzuki 7S3 with other 7S globulins for which crystal structures have been reported. Purple, red, and pink represent β-strands, α-helices, and 3_{10}-helices, respectively. The symbols '-' and '*' represent gaps and conserved residues, respectively. Residues omitted from the final models are shown in gray. β, β-conglycinin β; α'c, β-conglycinin α'c; 8Sα, mungbean 8Sα; canavalin, jackbean canavalin; phaseolin, French bean phaseolin.

Sepharose columns (**Table 1**). Adzuki 7S1 had lower hydrophobicity than Adzuki 7S2 and Adzuki 7S3 as evaluated by the butyl Sepharose column. On the other hand, Adzuki 7S3 had lower hydrophobicity than Adzuki 7S1 and Adzuki 7S2 as evaluated by the phenyl Sepharose column. These mean that Adzuki 7S1 had fewer aliphatic amino acids on the surface than did Adzuki 7S2 and Adzuki 7S3, and Adzuki 7S3 had fewer aromatic amino acids on the surface than the others.

Solubility. We examined the solubilities of Adzuki 7S1, Adzuki 7S2, and Adzuki 7S3 at different pH values at $\mu = 0.5$ and 0.08 as described previously (*10*).

At both $\mu = 0.5$ and 0.08, Adzuki 7S1 and Adzuki 7S2 exhibited patterns similar to one another (**Figure 2**). However, Adzuki 7S3 exhibited a lower solubility at lower pH at $\mu = 0.5$ and a high solubility at pH 6−9 at $\mu = 0.08$. This could be due to Adzuki 7S3's containing one more acidic amino acid and two fewer basic amino acid than Adzuki 7S1 and Adzuki 7S2 (*7*).

Emulsifying Ability. We assessed the emulsifying abilities of Adzuki 7S1, Adzuki 7S2, and Adzuki 7S3 by measuring the sizes of the emulsions formed at $\mu = 0.5$ and 0.08 as described previously (*10*).

Table 3. Thermal Stabilities and Structural Features

	Adzuki 7S1	Adzuki 7S3	β-conglycinin β	β-conglycinin α′c	mungbean 8Sα
T_m values (°C)	92.4	92.5	90.8	83.3	77.5
size of cavities (Å^3)	3632	3539	4754	5464	6441
no. of intermonomer hydrogen bonds (no. of salt bridges)	13 (0)	12 (0)	14 (1)	18 (0)	16 (1)
no. of intramonomer hydrogen bonds (no. of salt bridges)	312 (5)	292 (5)	244 (3)	294 (6)	332 (12)
hydrophobic residue areas on molecular surface (Å^2)	5887	5152	5679	5761	5353
length of loops (% of loops to total residues)	219 (50.5%)	213 (49.2%)	203 (48.8%)	208 (49.8%)	214 (50.6%)
no. of Pro residues (% of Pro to total residues)	18 (4.1%)	18 (4.2%)	21 (5.0%)	19 (4.5%)	19 (4.5%)

At pH 7.6 and $\mu = 0.5$, the three samples formed emulsions with average particle sizes of around 50 μm. This value is close to that of soybean β-conglycinin β (52.9 μm) (*20*). At pH 7.6 and $\mu = 0.08$, the emulsion particles of Adzuki 7S1 and Adzuki 7S2 were not measurable because of their insolubilities. Although Adzuki 7S3 was soluble under this condition, the emulsifying ability was worse than that at $\mu = 0.5$. In conclusion, none of the recombinant adzuki bean proteins exhibited good emulsifying abilities similar to that of β-conglycinin β (*20*). This is probably because Adzuki 7S globulins have high thermal stabilities.

Crystallization. Although the sequence identities among Adzuki 7S1, Adzuki 7S2, and Adzuki 7S3 are quite high, 95−98% (*7*), thermal stabilities, surface hydrophobicities, and solubilities are different among three samples as described above. To understand why individual samples exhibit different properties, their three-dimensional structures were determined. We crystallized the three samples, Adzuki 7S1, Adzuki 7S2, and Adzuki 7S3, under various conditions and succeeded in crystallization of all samples. However, only the crystals of Adzuki 7S1 and Adzuki 7S3 gave sufficient diffraction.

Final Model Qualities. The crystal structures of Adzuki 7S1 and Adzuki 7S3 could be determined at 1.8 and 2.25 Å resolution, respectively. The data collection and refinement statistics are summarized in **Table 2**. A high level of 99.7% of the amino acid residues in Adzuki 7S1 and Adzuki 7S3 were within the allowed region of the Ramachandran plot (*21*) as calculated using *PROCHECK* (*22*). We designated the three monomers as A, B, and C. The final models of Adzuki 7S1 monomers A, B, and C contained 390, 382, and 379 residues, respectively, and those of Adzuki 7S3 contained 375, 380, and 377 residues, respectively.

In all of the monomers of Adzuki 7S1 and Adzuki 7S3, the N termini (residues 1−12, 1−10, and 1−12 and residues 1−13, 1−12, and 1−12 of Adzuki 7S1 A, B, C and Adzuki 7S3 A, B, C monomers, respectively) and the C termini (residues 412−434, 410−434, and 411−434 and residues 410−433 of Adzuki 7S1 A, B, C and all Adzuki 7S3 monomers, respectively) were not included in the final models. Furthermore, three regions were not included in most monomers. One of them was the region comprising residues 188−197. In the case of Adzuki 7S1, the regions of residues 188−196, 189−197, and 188−197 were not included in the models of the monomers A, B, and C, respectively. In the case of Adzuki 7S3, the regions of residues 188−195, 188−194, and 189−195 were not included in the models of the monomers A, B, and C, respectively. The second region was 222−227. In the case of Adzuki 7S1, residues 226−227 were not included in the model of monomers B and C, although the model of monomer A contained this region completely. In the case of Adzuki 7S3, residues 222−226 and 223−226 were not included in the models of monomers A and C, respectively, although the model of monomer B contained this region completely. The other region was 307−316. In the case of Adzuki 7S1, residues 308−313 and 308−314 were not included in the models of monomers B and C, respectively, although monomer A contained this region completely. In the case of Adzuki 7S3, residues 307−314, 307−316, and 307−313 and 315−316 were not included in the model of monomers A, B, and C, respectively. The $2|F_o| - |F_c|$ maps in these regions were too thin to trace the correct sequences. Most residues located in these regions are hydrophilic and probably located on the molecular surface. In the soybean β-conglycinin β trimer, all of the corresponding residues also gave diffuse densities, with the exception of residues 205−209 (corresponding to 222−226 and 221−225 in Adzuki 7S1 and Adzuki 7S3, respectively). Moreover, all monomers of mungbean 8Sα, canavalin, and phaseolin gave diffuse densities in these regions. These findings, together with the fact that the resolution of Adzuki 7S1 was the highest among these 7S globulins, indicate that these regions were disordered.

All monomers contained a single residue, His404 in Adzuki 7S1 and His403 in Adzuki 7S3, in the disallowed region. The corresponding residues in all of the models of 7S globulins of known crystal structure also had $\varphi-\psi$ angles similar to those of His404 in Adzuki 7S1 and His403 in Adzuki 7S3 (*3, 4, 23−25*).

Overall Structures. The asymmetric unit of the Adzuki 7S1 and Adzuki 7S3 crystals contains a trimer. The overall trimeric structures of Adzuki 7S1 and Adzuki 7S3 are shown as ribbon

Figure 6. Structural comparison of overall structures. Adzuki 7S3 (green) and the other 7S globulins (orange, soybean β-conglycinin β; light green, soybean β-conglycinin α′c; blue, mungbean 8Sα; pink, jackbean canavalin; yellow, French bean phaseolin) are superimposed onto Adzuki 7S1 (red).

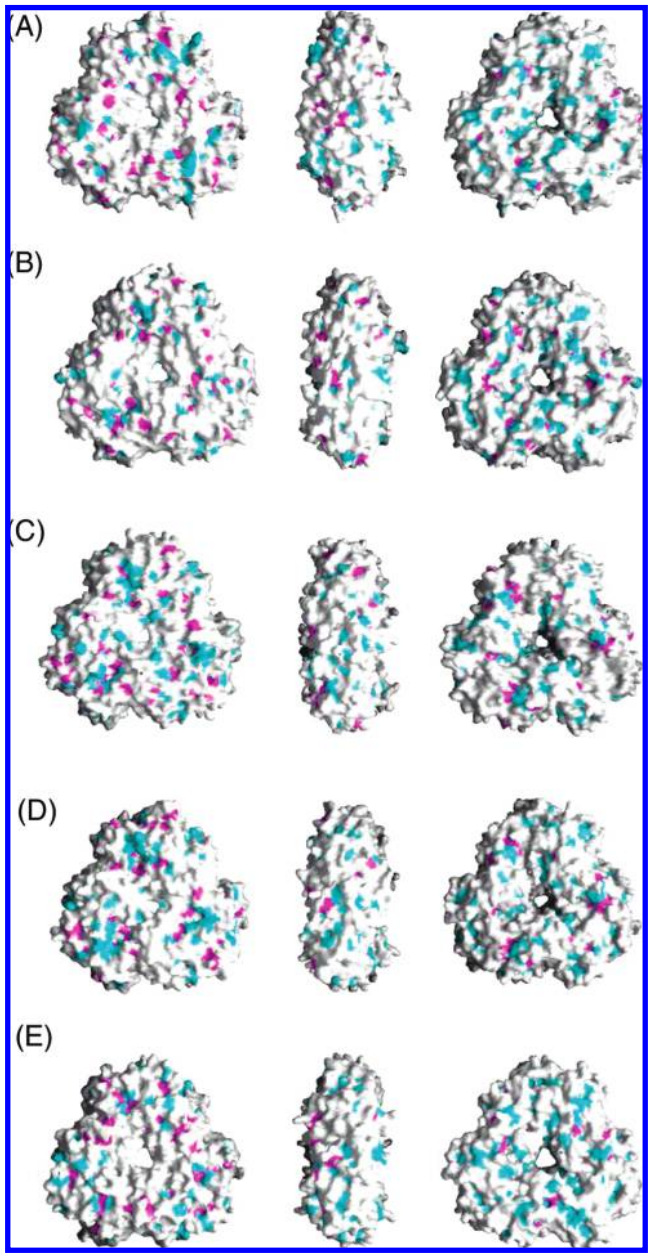

Figure 7. Distributions of hydrophobic residues on molecular surfaces. Structures are represented as white molecular surface models: (**A**) Adzuki 7S1; (**B**) Adzuki 7S3; (**C**) β-conglycinin β; (**D**) β-conglycinin α'c; (**E**) mungbean 8Sα. Aromatic and aliphatic hydrophobic residues are shown in pink and cyan, respectively. The center and rightmost diagrams represent a view after 90° rotation of the leftmost diagram around the vertical axis.

Table 4. Proportion of Hydrophobic Residues to the Surface Area

	Adzuki 7S1	Adzuki 7S3	β-conglycinin β	β-conglycinin α'c	mungbean 8Sα
aromatic hydrophobic residues on molecular surface (%)	3.3	3.1	3.3	3.2	3.1
aliphatic hydrophobic residues on molecular surface (%)	10.3	9.0	10.2	10.6	10.0

models in **Figure 3**. Although there was a little difference, their basic structures were close to those of β-conglycinin β, β-conglycinin α'c, mungbean 8Sα, canavalin, and phaseolin (*3, 4, 23*−*26*).

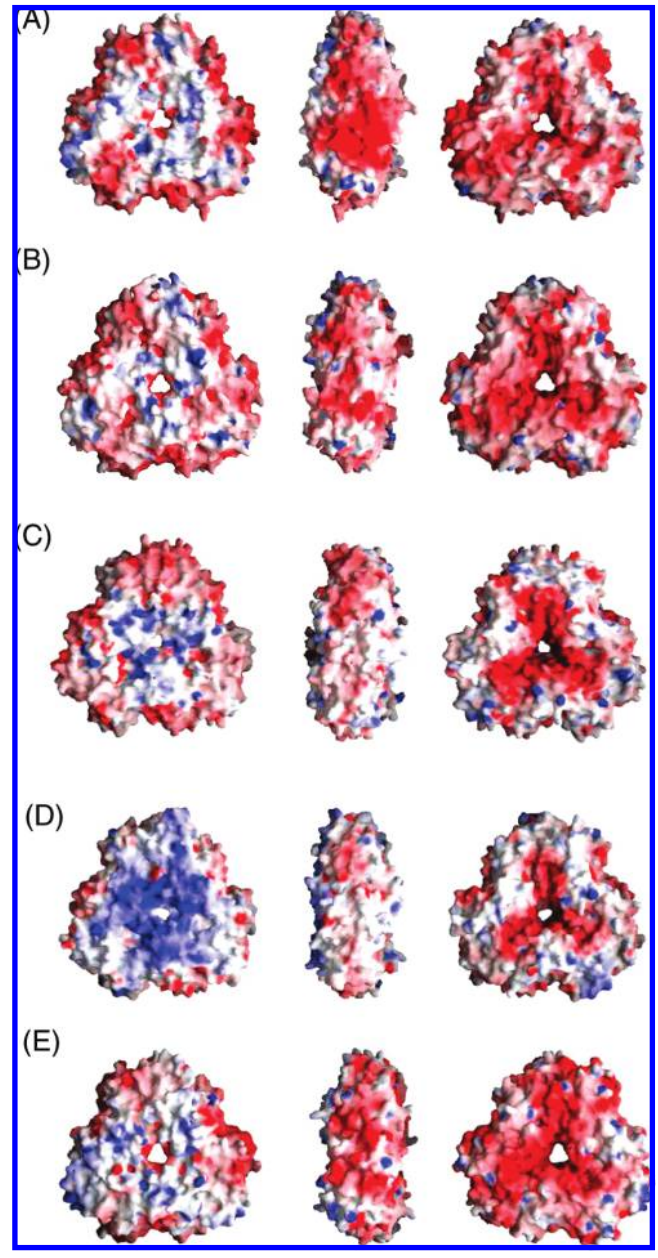

Figure 8. Electrostatic surface potentials. Structures are represented as white molecular surface models: (**A**) Adzuki 7S1; (**B**) Adzuki 7S3; (**C**) β-conglycinin β; (**D**) β-conglycinin α'c; (**E**) mungbean 8Sα. The electrostatic potential surfaces are drawn in the range from $-20k_BT$ (red) to $+20k_BT$ (blue), where k_B is the Bolzmann constant and T is the absolute temperature (K). The diagrams are in the same directions as those of **Figure 7**.

Both of the adzuki bean 7S globulin trimers had approximate dimensions of 93 × 93 × 45 Å. **Figure 4** shows monomeric models of Adzuki 7S1 and Adzuki 7S3. Monomers A and B were adopted as Adzuki 7S1 and Adzuki 7S3 monomers, respectively, because their $2|F_o| - |F_c|$ maps were most visible among those of three monomers. Each monomer can be divided into two very similar modules, the N- and C-terminal modules, related by a pseudodyad axis. Each module contained a core β-barrel (jelly roll) domain and an extended loop domain containing several helices. The secondary-structure elements were named according to the strands and helices in soybean β (*3*). Each β-barrel consisted of two β-sheets, A′ABIDG and J′JCHEF. Moreover, in both monomers, calcium ions were octahedrally coordinated with oxygen atoms of helix 2 and the

loop between helix 2 and helix 3 of the C-terminal module and two water molecules with a distance of 2.42 ± 0.31 Å. The helix−loop−helix motif coordinated by calcium ions is called an E−F hand (*27*). Soybean β-conglycinin β did not have this calcium ion site. The structure around the corresponding site of β-conglycinin β was different from those of the calcium binding sites of Adzuki 7S1 and Adzuki 7S3, because the interactions between subunits were slightly different. In the case of Adzuki 7S1, Ala388 of monomer A and Val184 of monomer C are hydrogen-bonded. However, in the case of β-conglycinin β, the corresponding residues, Gly372 of monomer A and Val172 of monomer C, have no interactions. These seem to result in the differences of orientations of Pro387 (the adjacent residue of Ala388) of monomer A in Adzuki 7S1 and Pro371 (the adjacent residue of Gly372) of monomer A in β-conglycinin β. This is likely to result in a difference in the loop conformation between helix 2 and helix 3 of the C-terminal module between Adzuki 7S1 and β-conglycinin β. It is assumed that the slight difference in the conformation determines the binding or unbinding of calcium ions.

Structure Comparison. Figure 5 shows a structure-based sequence alignment of Adzuki 7S1 and Adzuki 7S3 with other 7S globulins, β-conglycinin β, β-conglycinin α′c, mungbean 8Sα, jackbean canavalin, and French bean phaseolin, the structures of which have already been determined. Common among these proteins were α-helices 2 and 3 in the N- and C-terminal modules, 3_{10}-helices 0 and 1 in the N-terminal module, and all β-strands except for strand Z′ in the N-terminal module and strand Z in the C-terminal module.

The structure of Adzuki 7S1 was compared with those of the other 7S globulins described above including Adzuki 7S3 by superimposing their structures on that of Adzuki 7S1 (**Figure 6**). The rms deviations were 0.47 Å for the superimposition of 363 common C^{α} atoms of Adzuki 7S3, 0.64 Å for 357 C^{α} atoms of β-conglycinin β, 0.70 Å for 353 C^{α} atoms of β-conglycinin α′c, 0.55 Å for 359 C^{α} atoms of mungbean 8Sα, 0.84 Å for 331 C^{α} atoms of canavalin, and 0.99 Å for 325 C^{α} atoms of phaseolin calculated by the *TOP* and *RIGID* programs. The order of these rms deviations corresponded to the dendrogram shown in a previous paper (*7*). These legume 7S globulin structures have similar topologies with one another.

Relationships of Thermal Stabilities and Structures. The thermal stabilities and structures of Adzuki 7S1, Adzuki 7S3, β-conglycinin β, β-conglycinin α′c, and mungbean 8Sα were compared comprehensively. The T_m values, the sizes of cavities, the numbers of intermonomer and intramonomer hydrogen bonds and salt bridges, hydrophobic residue areas on the molecular surface, the lengths of loops, and the number of proline residues are summarized in **Table 3**. It is known that cavities are observed inside the molecule and that they decrease the thermal stabilities (*28*). Hydration of nonpolar groups apparently destabilizes proteins (*29*). Shorter loops are a stabilizing factor (*30, 31*). Proline residues also stabilize the protein structures.

The order of T_m values was Adzuki 7S3 ≥ Adzuki 7S1 > β-conglycinin β > β-conglycinin α′c > mungbean 8Sα. The order of cavity sizes was Adzuki 7S3 ≤ Adzuki 7S1 < β-conglycinin β < β-conglycinin α′c < mungbean 8Sα. These orders inversely correspond to each other; thus, larger cavity sizes are likely to lead to lower thermal stabilities. On the other hand, no correlation was observed between the other factors and thermal stabilities. Thus, thermal stabilities of proteins are probably determined by these factors collectively, although the contribution of cavity sizes is significant. In addition, it is known that the binding of calcium ion stabilizes the protein structures (*32*). Considering that calcium ions coordinate in the structure of Adzuki 7S1 and Adzuki 7S3, it is possible that the calcium ions contribute to the high thermal stabilities of Adzuki 7S1 and Adzuki 7S3.

Surface Hydrophobicities and Distributions of Hydrophobic Residues. Surface hydrophobicities and distributions of hydrophobic residues on the molecular surfaces of the 7S globulins were compared. Hydrophobicity evaluations using butyl and phenyl Sepharose columns (*17, 33*) showed the order of aromatic hydrophobicity to be β-conglycinin α′c > Adzuki 7S1 > β-conglycinin β ≥ Adzuki 7S3 > mungbean 8Sα. On the other hand, the order of aliphatic hydrophobicity was β-conglycinin α′c > β-conglycinin β ≥ Adzuki 7S3 > Adzuki 7S1 > mungbean 8Sα. Distributions of aromatic and aliphatic hydrophobic residues based on individual 7S globulin structures and the proportions of the areas occupied by their residues to the surface area are shown in **Figure 7** and **Table 4**, respectively. The hydrophobicities described above did not correlate with the values in **Table 4**. For example, β-conglycinin α′c and mungbean 8Sα had the strongest and weakest hydrophobicities, respectively, among all of the 7S globulins, but they gave similar percentages of both hydrophobic residues. Other 7S globulins had a similar pattern. Therefore, it may be concluded that surface hydrophobicities are determined not only by distributions of hydrophobic residues on the molecular surfaces.

Solubilities and Electrostatic Surface Potentials. Solubilities of various 7S globulins at neutral and alkaline pH values at $\mu = 0.08$ (**Figure 2B**) were compared with their electrostatic surface potentials (**Figure 8**). As electrostatic interactions between polypeptides are suppressed by the presence of salt at $\mu = 0.5$, the differences of solubilities at $\mu = 0.08$ were examined. Adzuki 7S1, Adzuki 7S3, and mungbean 8Sα are soluble at around pH 8 and $\mu = 0.08$, whereas β-conglycinin β and β-conglycinin α′c are insoluble under these conditions even at pH 9 (*18, 33*). In the case of β-conglycinin β, the center and the periphery of one surface had positive and negative potentials, respectively, and those of the other surface had negative and positive potentials, respectively (**Figure 8C**). In the case of β-conglycinin α′c, positive or negative potentials were closely distributed at the center of each of two surfaces, respectively. These potential distributions of β-conglycinin β and α′c lead to charge−charge interactions forming long aggregates of β-conglycinin β, resulting in precipitation. On the other hand, the potential distributions of Adzuki 7S1, Adzuki 7S3, and mungbean 8Sα are not like those of β-conglycinin β and α′c, resulting in no charge−charge interaction and high solubilities. Thus, electrostatic surface potentials are likely to influence largely the solubilities of 7S globulins at neutral and weakly alkaline pH at $\mu = 0.08$.

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