JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Evaluation of the Solubility and Emulsifying Property of Soybean Proglycinin and Rapeseed Procruciferin in Relation to Structure Modified by Protein Engineering

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The presence or absence of a highly negatively charged extension region in β-conglycinin (*J. Agric. Food Chem.* **1999**, *47*, 5278) and the length of a highly negatively charged variable region IV in glycinin (*J. Agric. Food Chem.* **2004**, *52*, 8197) are important determinants of solubility and emulsifying property. To examine the effects of the variable region IV from proglycinin A1aB1b and A3B4 and of the extension region from β-conglycinin α′ (α′ext) on solubility and emulsifying properties in detail, several mutants of proglycinin, procruciferin, and β-conglycinin were designed and prepared in *Escherichia coli*. Nine out of 10 mutants were expressed at high levels in *E. coli* and shown to be homotrimer similar to the wild types as assessed by gel filtration. The position of the introduced negatively charged region as well as the amino acid composition were demonstrated to affect solubility at $\mu = 0.08$. All of the proglycinin, procruciferin, and β-conglycinin mutants with the α′ext in the C-terminus, especially the proglycinin mutant, exhibited excellent emulsifying ability and emulsion stability. These indicate that improvement of emulsifying properties by insertion of the α′ext in the C-terminus may be generally applicable to seed globulins.

KEYWORDS: Emulsion; rapeseed; procruciferin; proglycinin; protein engineering; solubility; soybean

INTRODUCTION

Solubility is a very important physical property of food proteins because it limits their functional behaviors. Emulsification, a functional property widely utilized in the food industry, is greatly affected by solubility. Proteins with both hydrophilic and hydrophobic regions can exhibit emulsifying ability (*1*). In oil−water emulsion systems, proteins migrate to and interassociate with the oil−water interface to be adsorbed and be unfolded there, depending on their structure (*2*). This emulsion formation is known to be thermodynamically unstable such that the ability of an emulsifier to hold the emulsion as long as possible is very important for food applications. Thermal stability (*3*) has been reported to have some correlation with emulsifying ability, while pH has been shown to significantly influence emulsifying properties (*4*).

Soybean proteins are used extensively as emulsifier in concentrated emulsions such as comminuted meat products. However, their use as surface-active agents in dilute emulsion and foam type products is very limited (*5*). This could be due to the low adsorption of soy protein isolate at the oil−water interface when used at low concentration. Rapeseed is much less popular than soybean. However, rapeseed proteins may also be used in the food industry, provided that their functional properties are improved. An earlier study showed that cruciferin has lower solubility than glycinin at $\mu = 0.5$ but higher at $\mu = 0.08$, and that the emulsifying ability of glycinin was in general better than cruciferin (*6*).

The major soybean proteins are glycinin and β-conglycinin, which account for 40% and 30% of the total seed proteins, respectively (*1*, *7*). The emulsifying activity of soy proteins has already been extensively studied (*3*, *5*, *8*−*10*). Native glycinin was reported to exhibit surface behavior and, consequently, foaming and emulsifying properties, which are limited by its closely packed globular conformation, low surface hydrophobicity, and low molecular flexibility (*11*, *12*). Its solubility and surface-active properties were improved when the oligomeric structure of glycinin was appropriately dissociated with simultaneous unfolding of the acidic and basic polypeptide chains (*13*). It was later confirmed that the acidic polypeptide of soybean glycinin has good emulsifying ability (*5*). On the other hand, analysis of the individual β-conglycinin subunits showed that the extension region of α and α′ subunits conferred better solubility and emulsifying ability while the core regions determined the thermal stability (*3*, *14*). The extension region is rich in negatively charged residues. This is consistent with the report that the negatively charged variable region IV of

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10.1021/jf050871y CCC: $30.25
Published on Web 10/06/2005

glycinin also contributes to its solubility and emulsifying ability, although the extent is lower than that of the extension region (*15*). The presence or absence of the highly negatively charged extension region in β-conglycinin (*3*) and the length of the highly negatively charged variable region IV in glycinin (*15*) are therefore important determinants of solubility and emulsifying property.

Studies on glycinin crystal structures indicated that the two proglycinin molecules combine to form the mature glycinin between IE-faces after processing (*16*, *17*). Based on the structures, the recombinant proprotein instead of the mature protein can be engineered to assess modifications before utilization in genetic crop improvement.

As for rapeseed, the total protein is composed of 60% cruciferin (*18*). Unlike soybean proteins, reports on the emulsifying ability of rapeseed proteins are limited except for a recent study on the emulsifying properties of cruciferin (*6*). Most of the reports are on rapeseed meal and/or rapeseed isolate (*4*, *19*).

This present paper focuses on efforts at producing soybean proglycinin and rapeseed procruciferin with improved emulsifying ability and solubility through protein engineering. We hereby report on the effects of the variable region IV from proglycinin A1aB1b and A3B4 and of the extension region from β-conglycinin α' on solubility and emulsifying properties in detail. We constructed several proglycinin A1aB1b mutants involving insertion or replacement of the variable region IV from A1aB1b or A3B4 subunits, or addition or insertion of the extension region from β-conglycinin α' at the N-terminus, within the variable region IV, or at the C-terminus. We also prepared procruciferin mutants with additional variable region IV or extension region at the C-terminus, and a β-conglycinin α' mutant, which has an extension region at the C-terminus instead of the N-terminus. The emulsifying activity and emulsion stability of the modified proteins as well as their solubility as a function of pH were determined and compared to their recombinant wild type forms. The effects of the mutations on thermal stability were also investigated. It is hoped that structure−function relationships drawn from this study can widen the utilization of soybean and rapeseed crops. Better understanding of the properties of proproteins can also contribute to appropriate strategies for genetic crop improvement.

MATERIALS AND METHODS

Construction of Expression Plasmids for Mutants. *A. Proglycinin A1aB1b Mutants.* Schematic representations of proglycinin A1aB1b WT and its seven mutants are shown in **Figure 1A**. To construct expression plasmids for the mutants, the expression plasmids pEA1aB1b for proglycinin A1aB1b (*20*), pECα' for β-conglycinin α' (*14*), and pEA3B4 for proglycinin A3B4 (*21*) were used as templates for PCR. The different primers used in amplifying the desired mutant cDNAs by PCR using KOD plus (TOYOBO) are as follows: **α'A1bB1b**; pECα' as template, 5′-TAATACGACTCACTATAGGG-3′ (20mer), and 5′-TGGTTCTCTTTGAGACTCAGAACCTTC-3′ (27mer), pEA1aB1b as template, 5′-TTCAGTTCCAGAGAGCAGCCTC-3′ (22mer) and 5′-CATGGTATATCTCCTTCTTAAAGTTAAACAAAA-3′ (33mer), **A1aα'B1b**; pECα', 5′-GTGGAGGAAGAAGAAGAATGCGAAGAAGGTC-3′ (31mer) and 5′-TGGTTCTCTTTGAGACTCAGAACCTTC-3′ (27mer), pEA1aB1b, 5′-AAACACTCCCAACGCCCCCGAGG-3′ (23mer) and 5′-GTCTTTACCCTTGGACTGTGGCTTC-3′ (25mer), **A1aB1bα'**; pECα', 5′-GTGGAGGAAGAAGAAGAATGCGAAGAAGGTC-3′ (31mer) and 5′-TGGTTCTCTTTGAGACTCAGAACCTTC-3′ (27mer), pEA1aB1b, 5′-TAGAATTCCGGATCCGAATTCGAGCTC-3′ (27mer) and 5′-AGCCACAGCTCTCTTCTGAGACTCC-3′ (25mer), **A1aB1bII**; pEA1aB1b, 5′-CCCACGGACGAGCAGCAACAAAGAC-3′ (25mer) and 5′-TCTTCTGCTTTTGCTTTGGCTTCCTCG-3′ (27mer), pEA1aB1b, 5′-CAACAAAGAGGACAAAGCAGCAGAC-3′ (25mer) and 5′-AGGTTGTTGAGGCTCTTCAAATGTG-3′ (25mer), **A1aB1bIII**; pEA1aB1b, 5′-CCCACGGACGAGCAGCAACAAAGAC-3′ (25mer) and 5′-TCTTCTGCTTTTGCTTTGGCTTCCTCG-3′ (27mer), pEA1aB1b, 5′-GGAAAGCATCAGCAAGAAGAAGAAAAC-3′ (27mer) and 5′-TTTCTGGCTTTGATGACCTCCTTGC-3′ (25mer), **A1aB1bIV**; pEA3B4, 5′-AAGTGGCAAGAACAAGAAGACGAAGATG-3′ (28mer) and 5′-TCTAGTCTGACATCCTCTTCCACGTGG-3′ (27mer), pEA1aB1b, 5′-AATGGCATTGACGAGACCATATGCA-3′ (25mer) and 5′-TGGTTTTATCACGCTCAGACCTCCTTT-3′ (27mer).

Vectors for α'A1aB1b, A1aα'B1b, and A1aB1bα' were amplified by 30 cycles of denaturation at 95 °C for 20 s, annealing at 50 °C for 30 s, and elongation at 68 °C for 10 min, and the reaction was extended for 8 min at 68 °C. The DNA fragment encoding the α' extension region was amplified using 40 cycles of 95 °C for 20 s, 55 °C for 30 s, and 68 °C for 2 min, and an extension of 8 min at 68 °C. The resulting fragment was phosphorylated and blunted before its ligation with the corresponding vectors to construct the expression plasmids pEα'A1aB1b, pEA1aα'B1b, and pEA1aB1bα'. The plasmids for A1aB1bII, A1aB1bIII, and A1aB1bIV were amplified using 30 cycles of 95 °C for 20 s, 55 °C for 30 s, and 68 °C for 10 min, and an extension of 8 min at 68 °C. DNA fragments for the variable region IV of A1aB1b (A1aIV) and A3B4 (A3IV) were amplified using 45 cycles of 96 °C for 10 s, 55 °C for 10 s, and 68 °C for 10 s. The resulting fragments were phosphorylated and blunted. They were then ligated with corresponding plasmids to construct pEA1aB1bII, pEA1aB1bIII, and pEA1aB1bIV. The plasmid for A1aB1bIV(c) was amplified using 25 cycles of 96 °C for 20 s, 58 °C for 10 s, and 74 °C for 7 min, while the DNA fragment for A1aIV was amplified using 30 cycles of 96 °C for 20 s, 58 °C for 10 s, and 74 °C for 1 min. The vectors for A1aB1bIV(c) and the DNA fragment were both digested with *Xho*I prior to phosphorylation and ligation to construct the expression plasmid pEA1aB1bIV(c).

B. Procruciferin and β-Conglycinin α' Mutants. **Figure 1B** shows the schematic representations of procruciferin, β-conglycinin α', and their mutants. Expression plasmids pETCRU2/3a for cruciferin (*22*), pECα' for β-conglycinin α' (*14*), and pECα'c for β-conglycinin α' core region (*14*) were used in constructing expression plasmids pEcru+A1aIV(c), pEcru+α'(c), pEcore+α'(c) for cru+A1aIV(c), cru+α'(c), and core+α'(c), respectively. The different primers used in PCR are as follows: **cru+A1aIV(c)**; pEA1aB1b as template, 5′-CCCACGGACGAGCAGCAACAAAGAC-3′ (25mer) and 5′-CCGCTCGAGCTATCTTCTGCTTTTGCTTTGGCTTCCTCG-3′ (39mer), pETCRU2/3a as template, 5′GACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCAC-3′(44mer) and 5′-AGCATCAGCCTTCCTTGGCCC CGTAG-3′ (28mer), **cru+α'(c)**; pECα', 5′-GTGGAGGAAGAAGAAGAATGCGAAGAAGGTC-3′ (31mer) and 5′-CCGCTCGAGCTATGGTTCTCTTTGAGACTCAGAACCTTCAC-3′ (41mer), pETCRU2/3a, 5′-GACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCAC-AC-3′(44mer) and 5′-AGCATCAGCCTTCCTTGGCCCTCCGTAG-3′ (28mer), **core+α'**(c); pEα', 5′-GTGGAGGAAGAAGAAGAATGCGAAGAAGGTC-3′ (31mer) and 5′-CCGCTCGAGCTATGGTTCTCTTTGAGACTCAGAACCTTCAC-3′ (41mer), pECα'c, 5′-GACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCAC-3′ (44mer) and 5′-GTAAAAAGCCCTCAAAATTGAAGACAAAG-3′ (29mer).

Vectors for cru+A1aIV(c), cru+α'(c), and core+α'(c) were amplified using 25 cycles of 96 °C for 20 s, 58 °C for 10 s, and 74 °C for 7 min. DNA fragments encoding A1aIV and the α' extension region were amplified using 30 cycles of 96 °C for 20 s, 58 °C for 10 s, and 74 °C for 1 min. After PCR reaction, the vectors and DNA fragments were digested with *Xho*I. The DNA fragments were phosphorylated and then ligated to their corresponding vectors to construct the expression plasmids pEcru+A1aIV(c), pEcru+α'(c), and pEcore+α'(c).

Protein Expression. *A. Proglycinin A1aB1b and Its Mutants.* The expression plasmids were transformed into *E. coli* BL21(DE3), HMS174(DE3), AD494(DE3), and JM109(DE3). After assessment of the expression efficiency and solubility of the recombinant proteins, the most suitable *E. coli* strain for each plasmid was selected to be as follows: AD494(DE3) for A1aB1b, JM109(DE3) for A1aα'B1b, and HMS174(DE3) for A1aB1bα', A1aB1bII, A1aB1bIII, A1aB1bIV, and

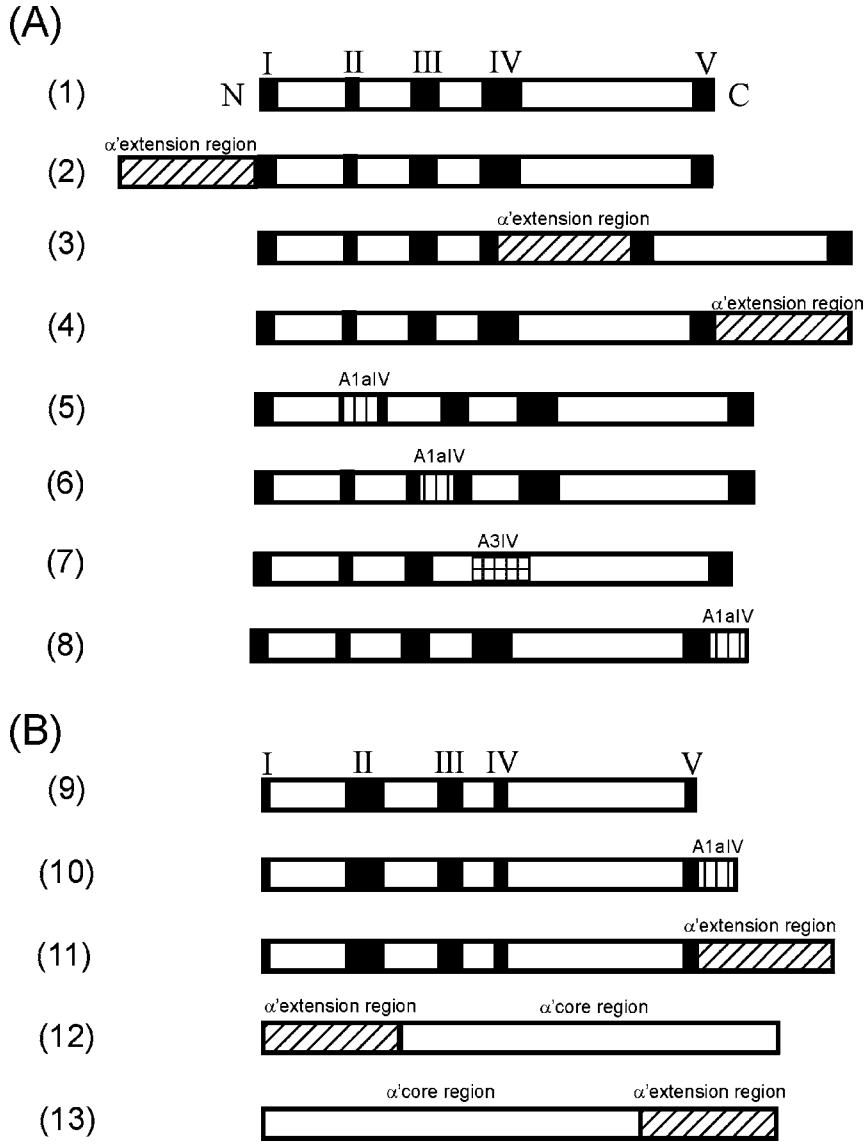

Figure 1. Schematic diagram of (**A**) proglycinin A1aB1b WT and its mutants and (**B**) procruciferin WT, β-conglycinin α′ WT, and their mutants. (1) Proglycinin A1aB1b WT; (2) α′A1aB1b; (3) A1aα′B1b; (4) A1aB1bα′; (5) A1aB1bII; (6) A1aB1bIII; (7) A1aB1bIV; (8) A1aB1bIV(c); (9) procruciferin WT; (10) cru+A1aIV(c); (11) cru+α′(c); (12) β-conglycininα′ WT; and (13) core+α′(c). In proglycinin A1aB1b and procruciferin WTs, open and closed boxes indicate the conserved regions and the five variable regions, respectively, among various 11S globulin sequences. The extension region of α′ is represented by a diagonal lined box; the variable regions IV of A1aB1b (A1aIV) and A3B4 (A3IV) are represented by vertical lined and crisscrossed boxes, respectively.

A1aB1bIV(c). One milliliter of an overnight culture was inoculated into 500 mL of modified LB medium containing 0.37 M NaCl, and incubated at 37 °C until OD_{600} of about 0.60 (8 flasks, 4 L). At this point, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The proteins were expressed by incubating the culture at either 20 °C for 40 h (A1aB1b, A1aB1bα′, A1aB1bII, A1aB1bIII, and A1aB1bIV(c)) or 37 °C for 14 h (A1aα′B1b and A1aB1bIV). The cells were harvested by centrifugation. Mutant α′A1aB1b was not expressed in any *E. coli* and under the conditions tested.

B. Procruciferin, β-Conglycinin α′, and Their Mutants. Procruciferin WT and its mutants cru+A1aIV(c) and cru+α′(c) were expressed in *E. coli* AD494(DE3). IPTG at a final concentration of 1 mM was added to the culture when it reached OD_{600} of about 0.60 (8 flasks, 4 L). The culture was grown in modified LB medium containing 0.5 M NaCl at 20 °C for 20 h. β-Conglycinin α′ was expressed by *E. coli* HMS174-(DE3) containing pECα′ as described previously (*3*), while pECore+α′-(c) was expressed by HMS174(DE3) at 20 °C for 20 h.

Protein Extraction and Purification. *A. Proglycinin A1aB1b and Its Mutants.* Except when NaCl concentration was specified, buffer A used in extraction and purification procedures consisted of 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM (*p*-amidinophenyl)-methanesulfonyl fluoride (*p*-APMSF), 1 μg/mL pepstatin A, and 1 μg/mL leupeptin.

The cells were harvested by centrifugation at 4800*g* using RPR-9-2 (HITACHI) rotor in SCR-60B centrifuge. The *E. coli* cells were sonicated to disrupt the cells and to release the recombinant proteins in buffer A. Thirty-five percent ammonium sulfate was added to the supernatant containing the protein of interest to precipitate out some *E. coli* proteins after centrifugation at 4800*g*. The 35% ammonium sulfate supernatant was applied on a Butyl-Toyopearl hydrophobic column (Tokyo, Japan) and eluted using a gradient of 30% and 0% as initial and final ammonium sulfate saturation in buffer A. Pooled fractions containing the protein of interest were dialyzed against the buffer A containing 0.15 M NaCl, and subsequently applied to a Mono Q column (Amersham Pharmacia Biotech). Proteins were eluted by gradient elution of 0.15 and 0.6 M NaCl, respectively.

B. Procruciferin and β-Conglycinin α′ and Their Mutants. The recombinant proteins of procruciferin and its mutants were obtained from the sonicated *E. coli* cells. After centrifugation at 4800*g*, the supernatant was treated with ammonium sulfate at 35% saturation for procruciferin WT or 45% for cru+A1aIV(c) and cru+α′(c). The precipitate obtained was dialyzed against buffer B containing 0.05 M

NaCl and applied on Q-Sepharose column. A linear gradient elution of 0.05−0.5 M NaCl was done. Buffer B consisted of 50 mM Tris buffer (pH 8.0) containing 1 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM *p*-APMSF, 1 μg/mL pepstatin A, and 1 μg/mL leupeptin. β-Conglycinin α' was purified as described previously (*3*). core+α'(c) was purified according to the same method used with β-conglycinin α'.

Gel Filtration. To assess the molecular assembly of the protein samples, gel filtration chromatography using Hi-Prep 16/60 Sephacryl S-200 HR column was conducted. Each protein was eluted with buffer A at a flow rate of 0.5 mg/mL. The void volume of the column and elution volume of protein standards (*14*) were determined as reference.

Protein Measurement and SDS-PAGE Analysis. The amount of protein was determined using Protein Rapid Assay Kit (Wako, Osaka, Japan), and bovine serum albumin was used as a standard. SDS-PAGE was performed using 11% acrylamide gel according to the method of Laemmli (*23*). The protein bands were analyzed by densitometric scanning using the program Scion Image (Scion Corporation) after staining with Coomassie Brilliant Blue R-250.

DSC Analysis. DSC analysis was conducted using 1 mg/mL protein solution in 35 mM sodium phosphate buffer (pH 7.6) containing 0.4 M NaCl, 1 mM EDTA, 0.02% NaN_3, 0.1 mM *p*-APMSF, 1 mg/L pepstatin A, and 1 mg/L leupeptin ($\mu = 0.5$). Scanning was recorded using Microcal MC-2 Ultra Sensitive Microcalorimeter (Micro Cal Inc., Northampton, MA) at a rate of 1°/min.

Solubility Analysis as a Function of pH. Solubility as a function of pH was determined using the method described previously (*14*). Protein solutions (0.8 mg/mL) whose ionic strength was adjusted to attain $\mu = 0.08$ and $\mu = 0.5$ at various pH were incubated at 4 °C for 18 h. The sample was then centrifuged, and the amount of protein that remained in the supernatant was determined. Solubility is expressed as percent of the remaining soluble protein.

Emulsifying Ability As Assessed by Particle Size and Stability. Oil-in-water emulsions were prepared using the method described previously (*13*). One-and-one-half milliliter of sample containing either 0.5 or 1 mg/mL protein sample plus 0.25 mL of soybean oil was homogenized for 30 s using a high-speed homogenizer (Nichion Irikakikai Ltd.) set at 2.2×10^4 rpm. The homogenate was then sonicated to further disperse the particles using an ultrasonic homogenizer (Nihonseiki Kaisha Ltd.) for 1 min. The emulsifying activity of the protein samples was analyzed by measuring the particle size distribution and mean particle diameter using a laser light scattering instrument (model LA 500, Horiba Seisakusho Ltd.). Analysis of each sample was conducted several times. Emulsions were kept at room temperature without agitation and visually observed after 20 h to assess stability. Emulsions were prepared at both high ($\mu = 0.5$) and low ($\mu = 0.08$) ionic strength conditions. At $\mu = 0.5$, the sample was in 35 mM sodium phosphate buffer (pH 7.6) containing 0.4 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.02% NaN_3, 0.1 mM *p*-APMSF, 0.14 μg/mL pepstatin A, and 0.5 μg/mL leupeptin. Except for sodium phosphate buffer (pH 7.6), which is at 10 mM, and NaCl, which is at 0.05 M, all of the rest of the chemicals in $\mu = 0.08$ were the same as that of the high ionic strength buffer.

RESULTS AND DISCUSSION

Expression and Assembly. Our group has previously proposed the criteria for judging the formation of proper conformation of modified proglycinins as follows: [1] solubility should be comparable to that of globulins, that is, the expressed proteins should be highly soluble in saline buffer just like globulins, [2] there must be self-assembly into trimers, and [3] high level expression (above 10% of total *E. coli* proteins) (*24*−*26*). Based on these criteria, it was therefore possible to determine whether the modified proteins form almost the same core structure as that of wild-type proteins in this study.

Proglycinin A1aB1b WT expressed in AD494(DE3) as soluble protein was estimated at about 15% of total cell proteins (data not shown). However, the expression level of its mutants in AD494(DE3) was about 1−6% of the total cell proteins. Yet

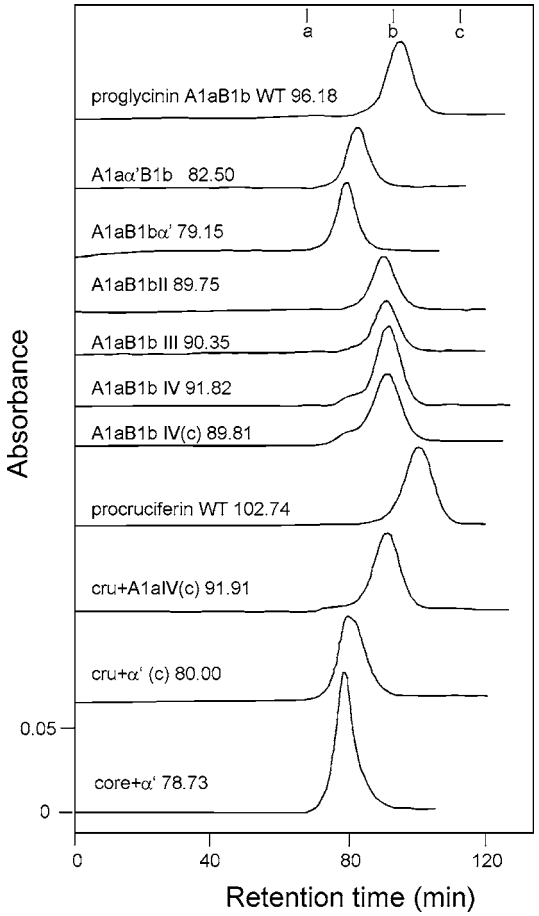

Figure 2. Gel filtration elution profiles of expressed and purified proteins using Sephacryl S-200 HR column 16/60. Flow rate was 0.5 mL/min. The positions of the void volume (**a**) and the elution positions of catalase (240 kDa) (**b**) and bovine serum albumin (67 kDa) (**c**) are indicated.

when other *E. coli* strains specifically JM109(DE3) and HMS174(DE3) were used, the mutant proteins were expressed at 10−15% of the total cell proteins, except for α'A1aB1b, which was not expressed by any of the strains used, suggesting that the presence of the extension region at the N-terminus of proglycinin inhibits its correct folding. The expression level of procruciferin WT as well as its mutants in AD494(DE3) was more than 15% of the total cell proteins (data not shown). The recombinant modified proteins just like the WTs were soluble in high salt buffer ($\mu = 0.5$) as described below.

To test if the expressed proteins form proper quaternary structure as homotrimers and to investigate the effects of modifications on their molecular dimensions, their retention times in a gel filtration column were measured (**Figure 2**). Because the mutations introduced in this study resulted in a dramatic increase in molecular size due to the insertion or addition of a segment with 42 (A1aIV) or 141 (α' extension region) amino acid residues, or replacement of a variable region composed of 42 amino acids (A1aIV) with a peptide of 70 amino acids (A3IV), all of the purified mutants exhibited faster retention times than their respective WTs. Basically, the retention times were inversely proportional to the molecular masses of the inserted or added regions. For instance, the mutants containing the extension region eluted faster than those that contain variable region IV. Results shown in **Figure 2** suggest that the mutant proteins self-assembled into trimers. Except for mutant α'A1aB1b, all of the modified proglycinin A1aB1b and procruciferin as well as their WTs satisfy the three

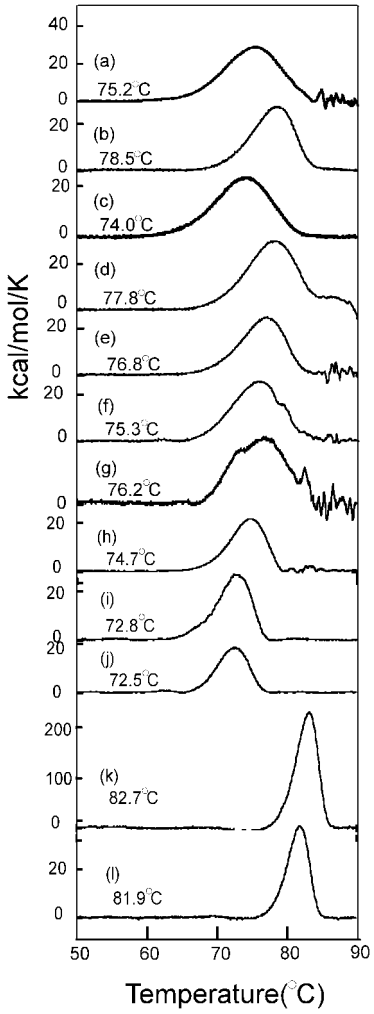

Figure 3. DSC profiles of proglycinin A1aB1b WT, procruciferin WT, and β-conglycinin WT, and their mutants. (**a**) Proglycinin A1aB1b WT; (**b**) A1aα'B1b; (**c**) A1aB1bα'; (**d**) A1aB1bII; (**e**) A1aB1bIII; (**f**) A1aB1bIV; (**g**) A1aB1bIV(c); (**h**) procruciferin WT; (**i**) cru+A1aIV(c); (**j**) cru+α'(c); (**k**) β-conglycininα' WT; and (**l**) core+α'(c).

criteria stated above, indicating that the modified proteins formed the proper conformation like their WT forms.

The molecular shape of the modified proteins could also affect retention time. The retention time of A1aB1bα' (79.2 min) was found to be faster than that of the A1aα'B1b (82.5 min). This is consistent with our expectation that the extension region in A1aB1bα' is more protruding into the solvent than within A1aα'B1b. The extension region in the former is attached at the C-terminus, which is located at the outer side of the molecule (*14*, *16*), while the extension region in A1aα'B1b is inside of a polypeptide. Moreover, the retention time of A1aB1bII (89.8 min) was faster than that of A1aB1bIII (90.4 min) and was almost the same as that of A1aB1bIV(c) (89.8 min). The position of variable region III is nearer to the center of the molecule than that of variable region II (*16*). Thus, the inserted variable region in A1aB1bII would be more protruding to the solvent. It seems that the added variable region in A1aB1bIV(c) is not as protruding such as in the case of the extension region in A1aB1bα'.

DSC Analysis. Proglycinin A1aB1b WT and its six mutants were purified, and DSC analysis was conducted to compare their thermal stabilities (**Figure 3**). Comparing the T_m values of A1aB1b mutants (**Figure 3b**−**g**) to that of the WT (**Figure 3a**) (75.2 °C), A1aα'B1b (**Figure 3b**) exhibited the most different T_m value (78.5 °C), followed by A1aB1bII (77.8 °C). Only A1aB1bα' exhibited a lower T_m value than that of the WT, although the difference was only 1.2 °C. The other three mutants exhibited slightly higher T_m values. The DSC results for procruciferin, β-conglycinin α', and their mutants are also shown in **Figure 3**. As compared to the T_m value of procruciferin WT, the T_m values of both cru+A1aIV(c) and cru+α'(c) were about 2 °C lower. In the case of core+α'(c), the T_m value was about 1 °C lower than that of β-conglycinin α' WT. These results demonstrate that the addition of the variable region IV or extension region does not greatly affect the thermal denaturation points of these globulins. Modification in the variable region seems acceptable despite the drastic insertions. These results indicate that the tertiary structure of all of the modified proteins constructed here can be assumed to fold like WT.

In general, insertion or addition of a flexible segment into or at terminals of a protein contributes to increased conformational entropy at the unfolded state, and increased heat capacity change between the folded and unfolded states of proteins (*27*). Here, the inserted or added variable region IV and extension region would have flexible structures to some extent, and we suggest that they could change their structure depending on the environment like ionic strength. Also, they would have lower heat capacity than the core structure, which includes β-barrels and helices (*16*). Therefore, the modifications we made should lead the protein toward the more unstable state based on increased entropy at the unfolded state.

However, some A1aB1b mutants had higher T_m values than the WT. In particular, the increased T_m value of A1aα'B1b by 3.3 °C can be noted especially since the T_m values of A1aB1bα', cru+α'(c), and core+α'(c) were lower than those of A1aB1bWT, procruciferin WT, and β-conglycinin α', respectively. The C-terminal end is located on the side of A1aB1b molecule (*16*) and the α' molecule (*28*), whereas the variable region IV is located on the IE-face, the face of the molecule containing the inter-disulfide bond (*16*). We attribute the heat stabilization of A1aα'B1b to the possible electrostatic charge−charge interaction of the α' extension region inserted in the variable region IV with the IE-face. The IE-face is slightly more positively charged than the IA-face, or the face of the molecule containing the intra-disulfide bond, and the side of molecule (*16*). It is possible that the positively charged IE-face interacts with the highly negatively charged α' extension region in the case of A1aα'B1b. Moreover, the higher T_m values of A1aB1bII and A1aB1bIII can also be noted. Variable region III is located on the IA-face, while variable region II is located on the IE-face (*16*). The T_m value of A1aB1bII is higher than that of A1aB1bIII, and this is consistent with our assumption on the effect of charge−charge interaction. These results are of interest in understanding the reasons why and where the variable regions in glycinin are inserted.

Previously, we reported that the insertion of a charged region into a site in procruciferin corresponding to the variable region IV of glycinin did not stabilize the protein structure at high ionic condition, although the protein structure was stabilized at low ionic condition (*29*). Probably, the inserted charged region interacts differently in proglycinin and in procruciferin due to their charge differences. For instance, a charged variable region exists between the acidic and basic chains in proglycinin, but not in procruciferin. The position of the inserted variable region might not depend on the folding motif of jelly roll barrel but on its interaction with the core region.

Solubility. Solubility is a basic physicochemical property of food proteins. Here, we investigated the solubility of engineered

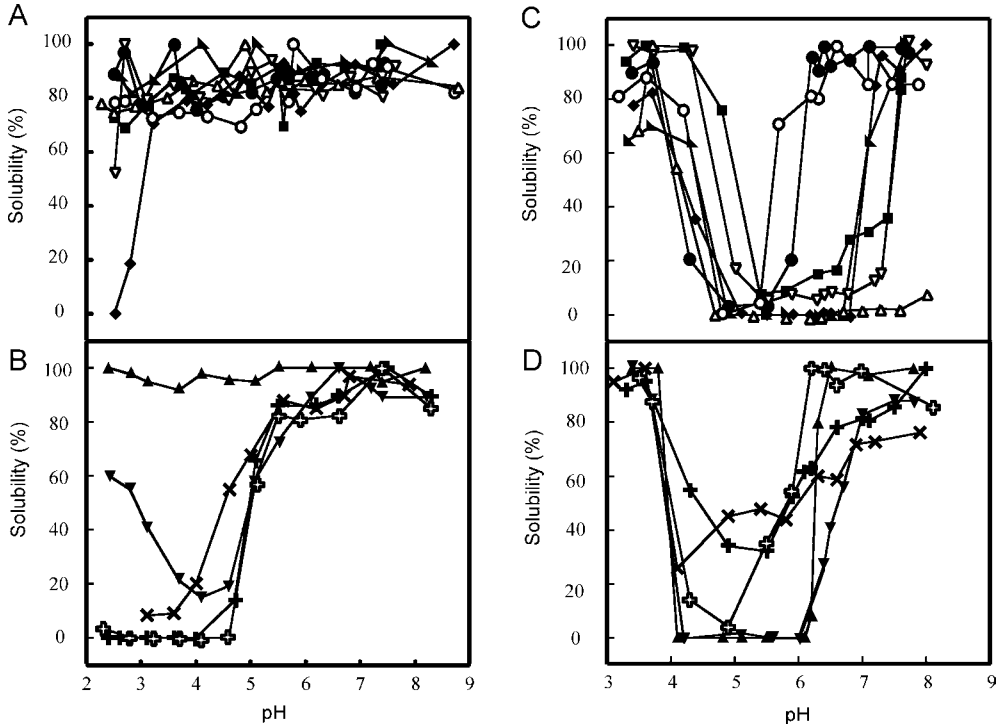

Figure 4. Solubility as a function of pH. (**A**) Proglycinin A1aB1b WT and its mutants at $\mu = 0.5$; (**B**) procruciferin WT, β-conglycinin α' WT, and their mutants at $\mu = 0.5$; (**C**) proglycinin A1aB1b WT and its mutants at $\mu = 0.08$; and (**D**) procruciferin WT, β-conglycinin α' WT, and their mutants at $\mu = 0.08$. ■ is proglycinin A1aB1b WT; ○ is A1aα'B1b; ● is A1aB1bα'; △ is A1aB1bII; ▽ is A1aB1bIII; ◆ is A1ab1bIV; right ▲ is A1aB1bIV(c); × is procruciferin WT; + is cru+A1aIV(c); cross is cru+α'(c); ▲ is β-conglycinin α' WT; ▼ is core+α'(c).

proteins at both high ($\mu = 0.5$, **Figure 4A** and **B**) and low ($\mu = 0.08$, **Figure 4C** and **D**) ionic strength conditions. At $\mu = 0.5$ and 0.08, the solubility of the proteins was measured at pH 2.0−9.0 (**Figure 4A** and **B**) and 3.0−8.0 (**Figure 4C** and **D**), respectively. In **Figure 4A**, proglycinin A1aB1b WT and all of its mutants except A1aB1bIV were soluble at all pH's measured. The solubility of A1aB1bIV decreased dramatically at pH 2−3. We cannot explain why A1aB1bIV exhibits a profile different from those of the others. On the other hand, at $\mu = 0.08$ (**Figure 4C**), proglycinin A1aB1b WT showed precipitation between pH 5.0 and 7.5. The solubility of engineered proteins differed from that of the WT. A1aB1bα' and A1aα'B1b showed significantly increased solubility at neutral pH. This is probably due to the increase in acidic amino acid composition imparted by the inserted or added extension region. The solubility curves of A1aB1bα' and A1aα'B1b shifted by 0.5−1.0 pH unit toward the acidic side on the acidic side of the curve, and by 1.5−2.0 pH units toward the acidic side on the basic side of the curve, respectively, as compared to WT. As a result, A1aα'B1b was insoluble within a narrower acidic pH range (pH 4.8−5.3) than A1aB1bα' (pH 4.2−5.8) despite their having the same amino acid composition. Moreover, the pH range where A1aB1bIV and A1aB1bIV(c) precipitated shifted by 1.0 pH unit to the acidic side on the acidic side of the curve, and by 0.5 pH unit to the acidic side on the basic side of the curve relative to the WT. The difference in solubility between WT and its modified versions A1aB1bIV and A1aB1bIV(c) was observed basically because of their different amino acid composition. In these cases, the introduction of acidic residues made the curve shift to the acidic side. However, A1aB1bII and A1aB1bIII exhibited different pH shift profiles despite their having the same amino acid composition as A1aB1bIV(c). A1aB1bII was insoluble at pH 5−8, while A1aB1bIII had a curve pattern similar to that of WT. These results indicate that the positioning of the introduced negatively charged regions as well as the amino acid composition are both important factors affecting solubility.

Procruciferin mutants slightly differed in solubility from their WT at both $\mu = 0.5$ and 0.08. At $\mu = 0.5$ (**Figure 4B**), the solubility of both mutants was lower at pH between 4 and 5 than that of procruciferin WT. These profiles were very different from those of proglycinin and its mutants. This is probably caused by the difference in amino acid compositions and amino acid sequences. At $\mu = 0.08$ (**Figure 4D**), cru+α'(c) was more soluble at pH near neutral and more insoluble at pH between 4.2 and 5.5 than procruciferin WT. Likewise, cru+A1aIV(c) exhibited a similar tendency to a limited extent. Previously, we demonstrated that exchanging the variable region IV of procruciferin with that of proglycinin A3B4 resulted in a phenomenon similar to cru+α'(c) (*29*). These results indicate that the number of acidic amino acids is an important factor determining pH dependence of solubility.

In **Figure 4B**, considerable differences in solubility between β-conglycinin α' WT and core+α'(c) were observed at $\mu = 0.5$. While β-conglycinin α' WT was soluble at all measured pH's, the solubility of core+α'(c) decreased dramatically at pH below 6 with the minimum at pH 4.0. In contrast to the result at $\mu = 0.5$, the solubility profiles of β-conglycinin α' WT and core+α'(c) were similar at $\mu = 0.08$ (**Figure 4D**). Because the amino acid composition is the same between the two proteins, the difference in the position of the extension region (N- and C-terminals or attachment sides) must have affected the solubility. Based on the crystal structure of β-conglycinin α' core (*28*), the terminal residues Asn6 and Ala393 are located on the surface and the side of the plane trimer structure of the built model, respectively. Thus, it is likely that the direction of the extension region against the core region plays a key role in their solubility profile.

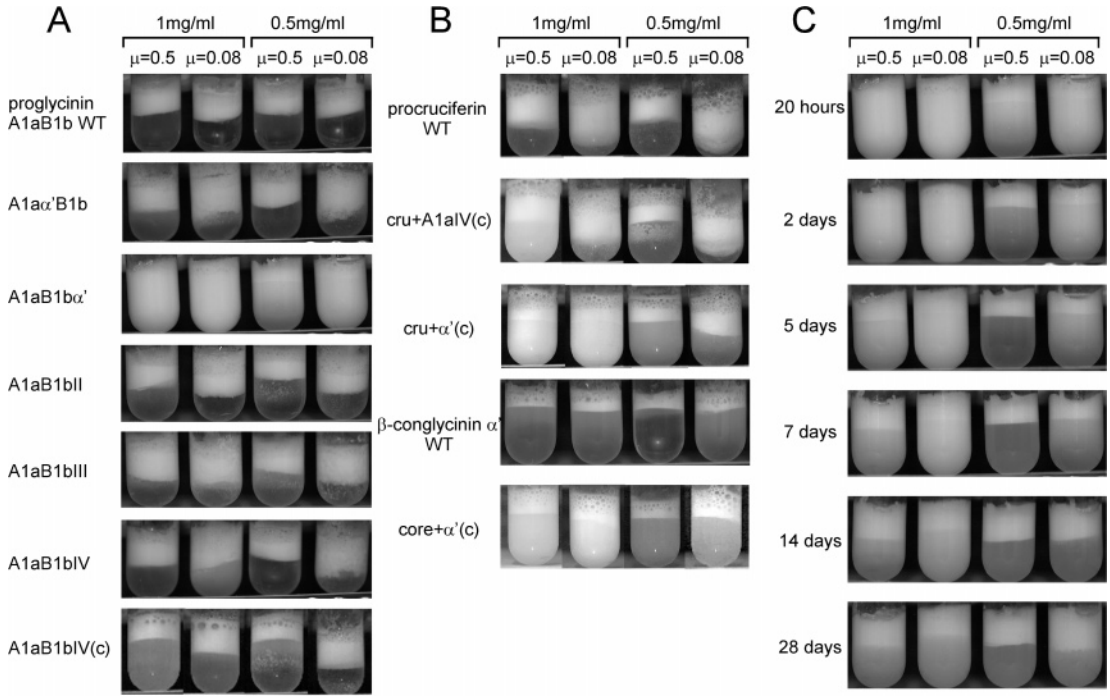

Figure 5. Emulsion stability. (**A**) Emulsions formed using proglycinin A1aB1b WT and its mutants as emulsifier after 20 h. (**B**) Emulsions formed using procruciferin WT, β-conglycinin α' WT, and their mutants as emulsifier after 20 h. (**C**) Emulsion stability of A1aB1bα' until 28 days at room temperature.

Table 1. Emulsifying Activity of the Protein Samples at High ($\mu = 0.5$) and Low ($\mu = 0.08$) Ionic Strength Conditions As Indicated by Mean Droplet Diameter

	mean droplet diameter (mm)			
	$\mu = 0.5$		$\mu = 0.08$	
sample	1 mg/mL	0.5 mg/mL	1 mg/mL	0.5 mg/mL
A1aB1b WT[a]	2.8/0.2[b]	3.0/0.4	3.7/0.2	6.2/0.1
A1aα′B1b	14.0/0.2	4.3/1.0	2.5/0.2	7.5/1.0
A1aB1bα′	1.6/0.05	2.0/0.09	1.6/0.03	1.2/0.04
A1aB1b II	5.0/1.0	4.4/0.3	30.2/2.0	42.2/3.4
A1aB1b III	33.9/2.0	11.6/1.0	64.9/3.8	69.4/4.2
A1aB1b IV	4.2/0.2	3.7/0.2	2.8/0.1	68.4/4.0
A1aB1b IV (c)	2.7/0.1	3.9/0.3	3.4/0.2	12.1/0.6
procruciferin WT	6.5/3.0	10.1/2.0	33.1/2.7	52.3/3.2
cru+A1aIV (c)	14.5/0.4	10.3/0.3	44.2/1.0	58.9/2.0
cru+α′(c)	4.4/0.1	5.3/0.1	4.3/0.2	8.7/0.2
β-conglycinin α′ WT	3.0[c]	4.1	3.5	3.6
core+α′ (c)	2.6/0.1	4.7/0.1	2.4/0.2	3.5/0.06

[a] Proglycinin A1aB1b WT. [b] Standard error. [c] Reference *3*.

Emulsification. The emulsifying properties of WT proteins and mutants were studied at two protein concentrations (0.5 and 1.0 mg/mL) and two ionic strength conditions ($\mu = 0.5$ and 0.08) and were investigated based on two criteria: emulsifying ability and emulsion stability. The emulsifying ability of the proteins was analyzed by measuring the average particle size of the emulsion droplets using laser light scattering instrument (**Table 1**). The mutants A1aα′B1b, A1aB1bII, A1aB1bIII, A1aB1bIV, and A1aB1bIV(c) gave similar or worse particle sizes as compared to proglycinin A1aB1b and β-conglycinin α′ WT. The particle sizes at $\mu = 0.08$ were generally bigger than those at $\mu = 0.5$ for these mutants. In particular, A1aB1bII and A1aB1bIII showed significantly large particle sizes. This suggests a possibility that variable regions II and III should be removed rather than elongated to improve emulsifying properties at $\mu = 0.08$. In contrast to the other five mutants, only A1aB1bα′ exhibited significantly smaller particle sizes than those of proglycinin A1aB1b and β-conglycinin α′ WTs, and it exhibited a sharp peak particle distribution (data not shown). Moreover, it is notable that the particle size of A1aB1bα′ is remarkably smaller than that of A1aB1bIV(c). Both A1aB1bα′ and A1aB1bIV(c) involved modification at the C-terminal end, but the added sequences are different. In addition, A1aα′B1b did not exhibit a small particle size like A1aB1bα′. Therefore, these results indicate that the combined effect of the inherent property of the α′ extension region and its positioning at the C-terminus improves the emulsifying ability of proglycinin A1aB1b.

To confirm the effect of the addition of the extension region at the C-terminus of a protein on its emulsifying ability, the emulsifying properties of more mutants were analyzed and compared to those of their respective WTs. We constructed mutants cru+A1aIV(c) and cru+α′(c) to test the effect of highly negatively charged regions at the C-terminus of procruciferin. The remarkable effect of the extension region at the C-terminus of procruciferin was observed. The effect was not as great in the case when A1aIV was used because the emulsion particle size was not as small as that of proglycinin A1aB1b and β-conglycinin α′. Significant improvement was observed though in cru+α′(c). For example, the emulsion mean droplet diameter of cru+α′(c) was 4.4 and 4.3 μm, while those of WT procruciferin was 6.5 and 33.1 μm at $\mu = 0.5$ and 0.08, 1 mg/mL protein concentration, respectively.

β-Conglycinin α′ subunit has an extension region at its N-terminus. We changed the position of the extension region from the N-terminus to the C-terminus of the core domain of β-conglycinin α′ to investigate its positional effect on emulsifying ability. As shown in **Table 1**, slightly improved particle size was observed at 1.0 mg/mL protein concentration at both ionic strength conditions. This suggests that the extension region at the C-terminal side is more effective than that at the N-terminal for emulsifying properties.

Furthermore, we examined the emulsion stability of the WT and mutants. We sealed and kept the test tubes containing the emulsion without agitation at room temperature, and the stability of the emulsions was observed visually. The emulsion of proglycinin A1aB1b WT completely separated into water and

cream phase after 20 h (**Figure 5A**). Those of A1aα′B1b, A1aB1bII, A1aB1bIII, A1aB1bIV, and A1aB1bIV(c) were also mostly separated. However, no separation in the A1aB1bα′ emulsion was observed, indicating that A1aB1bα′ has a good property for forming stable emulsion. A slight separation was observed though in the case of emulsion at condition having 0.5 mg/mL protein concentration and $\mu = 0.5$. **Figure 5B** shows the emulsion of procruciferin WT, β-conglycinin α′ WT, and their mutants at 20 h after emulsion formation. The emulsion of cru+A1aIV(c) was significantly more stable than that of procruciferin WT and A1aB1bIV(c) at $\mu = 0.5$ and 1 mg/mL condition, although the particle size of cru+A1aIV(c) was not improved as described above. As another noteworthy point, it appears that both procruciferin WT and cru+A1aIV(c) have greater foaming ability than the other proteins at $\mu = 0.08$. On the other hand, emulsion of cru+α′(c) was significantly much more stable than those of procruciferin WT and cru+A1aIV-(c). Like A1aB1bα′, the emulsion stability of cru+α′(c) was best at $\mu = 0.08$ and 1 mg/mL. Similarly, the emulsion stability of core+α′(c) was significantly improved, although the mean droplet diameter was similar to that of its WT. In conclusion, the attachment of the extension region at the C-termini of proglycinin A1aB1b, procruciferin, and β-conglycinin α′ core remarkably improved emulsion stability, and this modification may be generally applicable to any seed globulins.

Because the emulsion of A1aB1bα′ was surprisingly stable, we monitored its stability until 28 days. At $\mu = 0.08$ and 1 mg/mL protein, the emulsion was still good even after 7 days as shown in **Figure 5C**. Furthermore, no complete separation between cream and water was observed even after 28 days, and A1aB1bα′ was still much better than proglycinin A1aB1b WT after 20 h. A1aB1bα′ exhibited excellent emulsion stability as well as remarkable emulsifying ability.

ABBREVIATIONS USED

WT, wild type; IPTG, isopropyl-β-D-thiogalactopyranoside; *p*-APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DSC, differential scanning calorimetry.

LITERATURE CITED

(1) Utsumi, S. Plant food protein engineering. *Adv. Food Nutr. Res.* **1992**, *36*, 89−208.

(2) Kinsella, J. E.; Damodaran, S.; German, B. Physicochemical and functional properties of oilseed proteins with emphasis on soy proteins. In *New Protein Foods. Vol. 5: Seed Storage Proteins*; Altschul, A. M., Wilcke, H. L., Eds.; Academic Press: Orlando, FL, 1985; pp 107−179.

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

(4) Dev, D. K.; Mukherjee, K. D. Functional properties of rapeseed protein products with varying phytic acid contents. *J. Agric. Food Chem*. **1986**, *34*, 775−780.

(5) Liu, M.; Lee, D.-S.; Damodaran, S. Emulsifying properties of acidic subunits of soy 11S globulin. *J. Agric. Food Chem.* **1999**, *47*, 4970−4975.

(6) Mohamad Ramlan, M. S.; Maruyama, N.; Adachi, A.; Hontani, N.; Saka, S.; Kato, N.; Ohkawa, Y.; Utsumi, S. Comparison of protein chemical and physicochemical properties of rapeseed cruciferin with those of soybean glycinin. *J. Agric. Food Chem*. **2002**, *50*, 7380−7385.

(7) Utsumi, S.; Matsumura, Y.; Mori, T. Structure−function relationships of soy proteins. In *Food Proteins and Their Applications*; Damodarans, S., Paraf, A., Eds.; Marcel Dekker: New York, 1997; pp 257−291.

(8) Wagner, J. R.; Guéguen, J. Surface functional properties of native, acid-treated, and reduced soy glycinin. 2. Emulsifying properties. *J. Agric. Food Chem*. **1999**, *47*, 2181−2187.

(9) Maruyama, N.; Mohamad Ramlan, M. S.; Takahashi, K.; Yagasaki, K.; Goto, H.; Hontani, N.; Nakagawa, S.; Utsumi, S. Structure-physicochemical function relationships of soybean β-conglycinin heterotrimers. *J. Agric. Food Chem*. **2002**, *50*, 4323−4326.

(10) Kinsella, J. E. In *Food Proteins*; Fox, P., Condon, J. J., Eds.; Elsevier Applied Sci. Pub.: London, 1982; p 51.

(11) Kinsella, J. E. Functional properties of soy proteins. *J. Am. Oil Chem. Soc*. **1979**, *56*, 242−258.

(12) Wagner, J. R.; Guéguen, J. Effects of dissociation, deamidation, and reducing treatment on structural and surface active properties of soy glycinin. *J. Agric. Food Chem.* **1995**, *43*, 1993−2000.

(13) Kim, S. H.; Kinsella, J. E. Surface active properties of food proteins: Effects of reduction of disulfide bonds on film properties and foam stability of glycinin. *J. Food Sci*. **1987**, *52*, 128−131.

(14) 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.

(15) Maruyama, N.; Park, K.; Motoyama, S.; Choi, S. K.; Yagasaki, K.; Ishimoto, M.; Utsumi, S. Structure-physicochemical function relationships of soybean glycinin of subunit level assessed by using mutant lines. *J. Agric. Food Chem.* **2004**, *52*, 8197−8201.

(16) Adachi, M.; Takenaka, Y.; Gidamis, A. B.; Mikami, B.; Utsumi, S. Crystal structure of soybean proglycinin A1aB1b homotrimer. *J. Mol. Biol.* **2001**, *305*, 291−305.

(17) Adachi, M.; Kanamori, J.; Masuda, T.; Yagasaki, K.; Kitamura, K.; Mikami, B.; Utsumi, S.; Crystal structure of soybean 11S globulin: Glycinin A3B4 homohexamer. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 7395−7400.

(18) Crouch, M. L.; Sussex, I. M. Development and storage-protein synthesis in *Brassica napus* L. embryos in vivo and in vitro. *Planta* **1981**, *153*, 64−74.

(19) Mahajan, A.; Dua, S.; Bhardwaj, S. Simple physical treatment as an effective tool to improve the functional properties of rapeseed (*Brassica campestris* var. toria) and sesame seed (*Sesamum* indicum) meals. *Int. J. Food Sci. Nutr.* **2002**, *53*, 455−63.

(20) Katsube, T.; Kurisaka, N.; Ogawa, M.; Maruyama, N.; Ohtsuka, R.; Utsumi, S.; Takaiwa, F. Accumulation of soybean glycinin and its assembly with the glutelins in rice. *Plant Physiol.* **1999**, *120*, 1063−74.

(21) Choi, S. K.; Adachi, M.; Ustumi, S. Identification of the bile acid-binding region in the soy glycinin A1aB1b subunit. *Biosci. Biotechol. Biochem.* **2002**, *66*, 2395−2401.

(22) Tandang, M. R. G.; Adachi, M.; Utsumi, S. Cloning and expression of rapeseed procruciferin in *Escherichia coli* and crystallization of the purified recombinant protein. *Biotechnol. Lett*. **2004**, *26*, 385−391.

(23) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(24) Kim, C.-S.; Kamiya, S.; Sato, T.; Utsumi, S.; Kito, M. Improvement of nutritional value and functional properties of soybean glycinin by protein engineering. *Protein Eng*. **1990**, *3*, 725−731.

(25) Katsube, T.; Gidamis, A. B.; Kanamori, J.; Kang, J., II; Utsumi, S.; Kito, M. Modification tolerability of the hypervariable region of soybean proglycinin. *J. Agric. Food Chem*. **1994**, *42*, 2639−2645.

(26) Utsumi, S.; Gidamis, A. B.; Kanamori, J.; Kang, I. J.; Kito, M. Effects of deletion of disulfide bonds by protein engineering on

the conformation and functional properties of soybean proglycinin. *J. Agric. Food Chem.* **1993**, *41*, 687−691.
(27) Kumar, S.; Nussinov, R. How do thermophilic proteins deal with heat? *Cell. Mol. Life Sci.* **2001**, *58*, 1216−1233.
(28) Maruyama, Y.; Maruyama, N.; Mikami, B.; Utsumi, S. Structure of the core region of the soybean beta-conglycinin alpha′ subunit. *Acta Crystallogr., Sect. D* **2004**, *60*, 289−297.
(29) Tandang, M. R. G.; Adachi, M.; Inui, N.; Maruyama, N.; Utsumi, S. Effects of protein engineering of canola procruciferin on its physicochemical and functional properties. *J. Agric. Food Chem.* **2004**, *52*, 6810−6817.

Received for review April 15, 2005. Revised manuscript received August 15, 2005. Accepted September 6, 2005. This work was supported in part by a grant to S.U. from the Ministry of Education, Science and Culture of Japan, to S.U. from The Salt Science Research Foundations, and to M.A. from Mishima Kaiun Memorial Foundation.

JF050871Y