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Physicochemical Properties of Native and Recombinant Mungbean (*Vigna radiata* L. Wilczek) 8S Globulins and the Effects of the N-Linked Glycans

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We have previously cloned and characterized the cDNAs of three isoforms of the 8S globulin of mungbean, expressed the major 8Sα isoform in *Escherichia coli*, and purified and successfully crystallized it (Bernardo, A. E. N.; Garcia, R. N.; Adachi, M.; Angeles, J. G. C.; Kaga, A; Ishimoto, M.; Utsumi, S.; Tecson-Mendoza, E. M. *J. Agric. Food Chem.* **2004**, *52*, 2552−2560). Herein, we report the physicochemical and emulsifying properties of the native 8S and recombinant 8Sα globulin or vicilin. The circular dichroism spectra analysis of the native 8S and recombinant 8Sα globulins revealed that the recombinant 8Sα formed a secondary structure close to that of the native 8S. Further, gel filtration analysis showed that 8Sα was able to assemble into trimers. The native 8S and recombinant 8Sα globulins were soluble at pH 3.4 and at pH 7.4−9.0 at low ionic strength, $\mu = 0.08$. Interestingly, the native 8S was more soluble at pH 7.0 and pH 7.4 than the recombinant 8Sα at $\mu = 0.08$. Both forms were very soluble at pH 3.4−9.0 at high ionic strength, $\mu = 0.50$. The native form exhibited a higher T_m (69.2, 79.5, and 83.8 °C) than the recombinant form (65.6, 71.6, 77.5 °C) at $\mu = 0.1$, 0.2, and 0.5, respectively. The recombinant form was found to have greater surface hydrophobicity than the native form. There was little difference in the emulsifying ability between the native 8S and 8Sα at pH 3.4 and pH 7.6. The results indicate that the presence of N-linked glycans is not essential in the assembly and stable conformation of the mungbean vicilin. However, the N-linked glycans might have contributed to the higher solubility at low ionic strength, greater thermal stability, and decreased surface hydrophobicity of the native vicilin as compared to the recombinant 8Sα. On the other hand, the N-linked glycans showed little effect on the emulsifying ability of the protein.

KEYWORDS: Functional properties; 8S globulins; mungbean; N-linked glycans; recombinant; physicochemical properties; vicilin; *Vigna radiata*

INTRODUCTION

The usefulness of legume proteins in food and nonfood systems is dependent on their physicochemical and functional properties. Functional properties are derived from the protein's physicochemical properties such as solubility, water binding, emulsification, foaming, gelling, and surface hydrophobicity (*1*). Except for a report on the thermal denaturation (*2*) and basic physicochemical properties of mungbean storage proteins (*3*), there is a dearth of information on the physicochemical and functional properties of mungbean vicilin.

Mungbean is increasingly becoming a more important leguminous crop in Asia and other parts of the world and is a major source of proteins in developing countries. The major storage protein in mungbean seeds is the 8S globulin or vicilin, which comprises 89% of the total globulins (*3*). Three isoforms of the cDNA for this protein were isolated, cloned, and characterized by Bernardo and co-workers (*4*). The 8Sα, 8Sα′, and 8S*β* had molecular weights of 51 973, 51 627 or 51 758, and 51 779, respectively. The different isoforms were highly homologous with each other, from 88% to 92%. A single site of glycosylation was observed about 90 amino acids from the C-terminus. The major isoform 8Sα was expressed in *Escherichia coli* and was successfully crystallized.

Soybean *β*-conglycinin is one of the best-studied vicilin proteins with respect to its physicochemical and functional properties (*5*−*8*). It is a trimeric protein, which is composed of

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three subunits, α (~67 kDa), α′ (~71 kDa), and β (~50 kDa) (*9*). The α and α′ subunits consist of the core domain and extension domain, while the β consists of only the core domain. All types of the subunits are N-glycosylated at specific sites (*9*). Working on recombinant α and α′ homotrimers and deletion mutants of β-conglycinin, the N-linked glycans and extension domain were shown not to be essential for the correct folding and formation of stable trimers of said protein as evaluated by CD, gel filtration, and density-gradient centrifugation (*5*). Similar results were obtained in hazelnut vicilin wherein the native glycosylated form and the unglycosylated recombinant form share similar secondary structures as indicated by CD spectroscopy (*10*). N-Linked glycans usually do not play an essential role in the maintenance of the overall folded structure once the protein has folded (*11*). Their effects on the properties of the polypeptide moiety are also usually small.

The mungbean 8Sα and the β-subunit of soybean β-conglycinin share some similarities in structure. The mungbean isoform exhibits about 61% homology with the β-subunit of soybean β-conglycinin (*4*), and both do not have the extension domain found in the α and α′ subunits of β-conglycinin (*5*). Further, both the mungbean 8Sα and the β subunit have a single glycosylation site at an equivalent position, whereas the α- and α′ subunits of β-conglycinin have two (*4*, *9*).

This paper reports on the comparative analysis of native mungbean 8S globulin and the recombinant 8Sα in terms of circular dichroism spectra, solubility, thermal stability, surface hydrophobicity, and emulsifying ability and discusses the effects of N-linked glycans on said properties.

EXPERIMENTAL PROCEDURES

Materials. Mungbean var Pag-asa 7 was obtained from the Institute of Plant Breeding, College of Agriculture, University of the Philippines Los Baños. *E. coli* HMS174(DE3) harboring the pET-21d (Novagen Inc.) containing the cDNA encoding the mature 8Sα globulin (*4*) was used for the production of the recombinant 8Sα globulin.

All of the chemicals used were of highest purity and were purchased from various suppliers.

Purification of the Recombinant 8Sα and the Native 8S Globulins. The recombinant 8Sα globulin was expressed in and purified from *E. coli* as described previously (*4*). *E. coli* HMS174(DE3) carrying the expression plasmid for mature 8Sα globulin was grown on LB medium containing 50 μg/mL carbenicillin at 37 °C. At OD_{600} of 0.8, isopropyl-thio-β-D-galactopyranoside (IPTG) was added to induce production of the protein. After 72 h cultivation at 20 °C, the bacterial cells were harvested by centrifugation at 5000*g* for 20 min at 4 °C. The cells were resuspended in buffer A [35 mM potassium phosphate (KPi) buffer, pH 7.6, 0.4 M NaCl, 1 mM EDTA, 0.1 M *p*-amidinophenylmethlysulfonyl fluoride (APMSF), 1 μg/mL leupeptin, 1 μg/mL pepstatin A] and sonicated. The resultant crude protein solution was subjected to a series of purification steps as follows: ammonium sulfate fractionation (35% cut), hydrophobic interaction chromatography using Butyl toyopearl eluted with ammonium sulfate gradient of 30−0% in buffer A and anion exchange chromatography (Mono Q HR 10/10, Pharmacia Biotech) developed at a flow rate of 1.5 mL/min with a gradient of 0.1 to 0.4 M NaCl. The 8Sα globulin fractions were resolved by SDS-PAGE and dialyzed against 5 mM KPi buffer, pH 7.6, 0.30 M NaCl, 1 mM EDTA, 0.10 mM APMSF, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A.

Purification of the native 8S globulin was as described previously (*3*). Mungbean variety Pag-asa 7 seed meal was homogenized in 0.4 M NaCl in buffer A for 1 h on ice and centrifuged at 13 500*g* for 15 min at 4 °C. The supernatant was collected and dialyzed against 35 mM KPi buffer, pH 7.6, containing 0.10 M NaCl, 1 mM EDTA, 0.10 mM APMSF, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A for 72 h to precipitate the total seed globulins. The globulins were collected by centrifugation and then resuspended in buffer A. The suspension was brought to 60% ammonium sulfate saturation, dialyzed against buffer A for 72 h, and developed on Sephacryl S200 column at a flow rate of 1 mL/min using buffer A with 0.5 M NaCl to isolate the native 8S (MW 48 000).

Protein Measurement. The protein content of the samples was determined using the Bradford method (*12*) with bovine serum albumin as the standard.

Sodium Dodecyl Sulfate-Polyacryamide Gel Electrophoresis (SDS-PAGE). All electrophoresis experiments were done on 11% gels. The gels were run at 150 V for 55 min. Electrophoresis was carried out using Tris-Glycine buffer pH 8.3 (*13*). The gels were stained with 0.05% Coomassie Brilliant Blue R250 in methanol:acetic acid:water (50:40:10) and then destained with the acidified methanol solution without the dye to visualize the protein bands.

Circular Dichroism. The secondary structures of the native and recombinant forms of mungbean 8S globulins were determined using a Jasco model J720 spectropolarimeter. Far UV spectra were recorded between 197 and 250 nm using a quartz demountable cell of 0.1 mm path length and protein concentration of 2 mg/mL. Data were expressed in terms of mean residue ellipticity (deg cm^2 $dmol^{-1}$).

Gel Filtration. To assess the self-assembly of the recombinant 8Sα, gel filtration chromatography using FPLC-HiLoad 26/60 Superdex 200 (Amersham Pharmacia) was conducted. The protein was eluted with buffer A at a flow rate of 1.0 mL per min.

Solubility as a Function of pH. Samples of 100−200 μL of the native and recombinant mungbean 8S globulins (1 mg/mL) were dialyzed against various buffers differing in pH and ionic strength as previously described (*6*). After 18 h of dialysis, the samples were recovered and centrifuged for 5 min at 23 500*g* at 4 °C. The supernatant was subjected to SDS-PAGE analysis, and the amount of protein in each 8S band was estimated using Scion Image.

Thermal Stability. Native 8S and recombinant 8Sα globulins (2.0 mL of 2.0 mg protein/mL) were dialyzed overnight against 0, 0.1, and 0.4 M NaCl ($\mu = 0.1$, 0.2, and 0.5, respectively) in 35 mM KPi buffer, pH 7.6, 1 mM EDTA, 0.1 mM APMSF, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A. Differential scanning calorimetry (DSC) experiments were carried out using a Microcal MC-2 ultrasensitive microcalorimeter (Microcal Inc.).

Surface Hydrophobicity. Surface hydrophobicity was measured using two hydrophobic interaction chromatography columns, phenyl sepharose 6 Fast Flow and butyl sepharose 4 Fast Flow, at a flow rate of 0.25 mL/min using a gradient of 2.3−0 M ammonium sulfate in buffer A. The samples (0.25 mg/mL) were dialyzed twice against buffer A before injection. Elution times of the native 8S and recombinant 8Sα were noted.

Emulsifying Ability. Protein solutions (2 mg/mL) were dialyzed against 35 mM potassium phosphate buffer, pH 7.6 at $\mu = 0.1$, 0.2, and 0.5. For the emulsification test, 1.5 mL of the protein solution and 0.25 mL soybean oil were homogenized for 30 s at maximum speed followed by 1 min sonication at 100 μA. Immediately after sonication, the particle size of the emulsion was determined using a Horiba LA-500 Particle Size Analyzer.

RESULTS AND DISCUSSION

CD Spectra. Circular dichroism spectra of the native 8S and recombinant 8Sα globulins showed only slight differences (**Figure 1**). The CD spectra indicated that the recombinant 8Sα was able to fold correctly to the secondary and presumably to tertiary structure similar to that of the native form. Earlier, we reported that the recombinant 8Sα could form crystals (*4*). The purified 8Sα showed a molecular size of about 160 000 when applied to a HiLoad 26/60 Superdex 200 column, indicating that the recombinant form could assemble into trimers (**Figure 2**). These results strongly suggest that the recombinant 8Sα has a correct structure and thus can be used for studying its physicochemical and functional properties. The results also

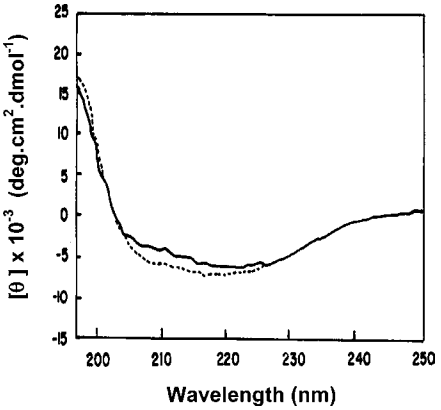

Figure 1. Circular dichroism spectra of native (solid line) and recombinant (dotted line) mungbean 8S globulins.

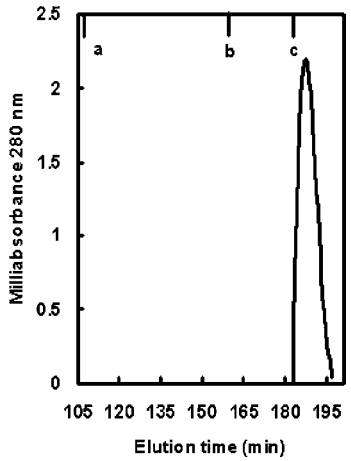

Figure 2. Gel filtration profile of recombinant 8Sα globulin on a HiLoad 26/60 Superdex 200 column. The positions of the void volume (**a**) and the elutions of mature glycinin (334 kDa) (**b**) and proglycinin (167 kDa) (**c**) are indicated.

indicate that the presence of N-linked glycan is not essential in the correct folding and assembly of the mungbean vicilin similar to the findings on soybean β-conglycinin (*5*) and hazelnut vicilin (*10*). Recently, this was confirmed by X-ray crystallography of the recombinant mungbean 8Sα (will be described elsewhere).

Solubility. Solubility is an important property that dictates the functional behavior of proteins and their potential applications to food processing. Good solubility is required in functional properties such as gelation, foaming, and emulsification. The solubility of the native 8S and the recombinant 8Sα globulin was measured at high ($\mu = 0.5$) and low ($\mu = 0.08$) ionic strengths. Both the native 8S and the recombinant 8Sα globulins were soluble at pH 3.4 and at pH 7.4−9.0 at low ionic strength ($\mu = 0.08$, **Figure 3A**). Notably, the native 8S was 50% and 20% more soluble than the recombinant 8Sα at pH 7.0 and 7.4, respectively, at low ionic strength. Solubility also declined for both after reaching the maximum solubility at pH 8.2. The higher solubility of the native vicilin even at low ionic strength could be due to the presence of carbohydrate moieties in the native 8S. Similarly, the N-linked glycans contributed to the solubilities of the three subunits, α, α′, and β, of β-conglycinin at low ionic strength ($\mu = 0.08$) (*8*). These results indicate the important role of N-linked glycans in preventing the aggregation of the protein molecules.

On the other hand, both native 8S and recombinant 8Sα were soluble at pH 3.4−9.0 at higher ionic strength $\mu = 0.5$ (**Figure 3B**). Recombinant soybean β-conglycinin proteins were also

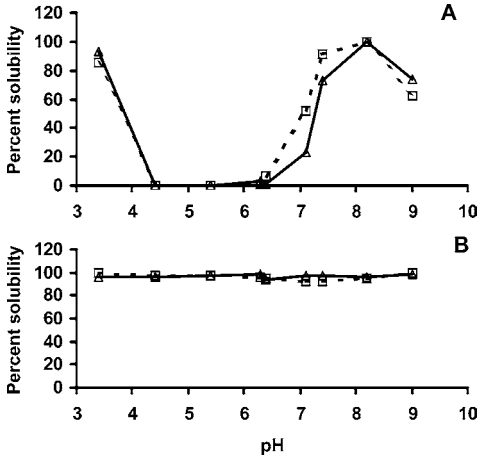

Figure 3. Solubility of native and recombinant mungbean 8S globulins at various pH conditions at (**A**) $\mu = 0.08$ and (**B**) $\mu = 0.50$ (dashed line with □, native; solid line with △, recombinant).

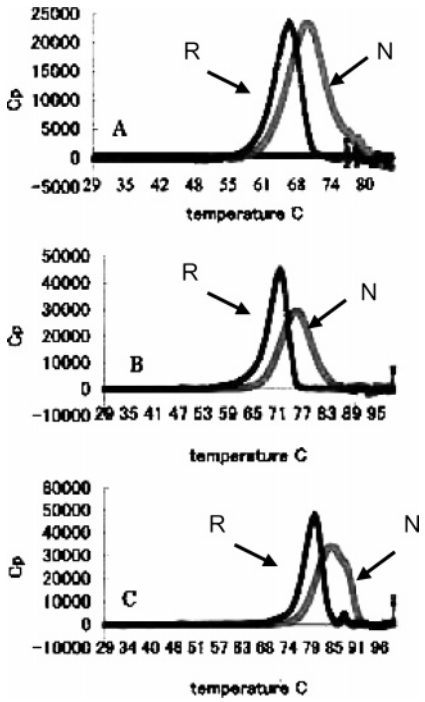

Figure 4. DSC scans of native 8S and recombinant mungbean 8Sα globulin in 35 mM KPi, pH 7.6 containing 1 mM EDTA, 10 mM APMSF, and 1.0 μg/mL pepstatin A and leupeptin. (**A**) $\mu = 0.1$, (**B**) $\mu = 0.2$, (**C**) $\mu = 0.5$. N, native; R, recombinant.

found to be soluble at any pH examined at ionic strength of 0.5 similar to native β-conglycinin (*8*). At low ionic strength of $\mu = 0.08$, the recombinant β homotrimer of β-conglycinin was insoluble at pH > 4.8 (*8*), while 8Sα was insoluble only at pH 4.4−6.4. This could perhaps be explained by the presence of different charge potentials on the molecular surface of the 8Sα as compared to that of the β homotrimer revealed by X-ray crystallography, resulting in the aggregation of the latter at the pH > 4.8 (will be described elsewhere).

Thermal Stability. Structural stability is one of the important factors related to heat-induced gel forming and emulsifying abilities. The thermal stability of the recombinant 8Sα and native 8S globulins measured at pH 7.6, $\mu = 0.1$, 0.2, and 0.5, by differential scanning calorimetry was 65.6−77.5 °C and 69.2−87.9 °C, respectively (**Figure 4** and **Table 1**). The native form exhibited a higher T_m than the recombinant form at all ionic

Table 1. Melting [Midpoint] Temperature of Native 8S and Recombinant 8Sα Globulins of Mungbean at Different Sodium Chloride Concentrations Obtained via Differential Scanning Calorimetry

	T_m (°C)	
[NaCl]	native	recombinant
0 M	69.2	65.6
0.10 M	75.9	71.6
0.40 M	83.8, 87.9	77.5

Table 2. Elution Times of the Native 8S and Recombinant Mungbean 8Sα Globulins Globulin on Hydrophobic Chromatography

	elution time (min)	
column	native	recombinant
butyl sepharose	40	52
phenyl sepharose	66	78

strengths studied by a 7.5−12% increase in T_m observed in the native form over those of the recombinant form. At $\mu = 0.5$, the native form had a T_m peak at 83.8 °C and a shoulder at 87.9 °C. The results could be explained by the fact that the native form consists of a mixture of at least three mungbean 8S isoforms, α, α′, and β, all of which are N-glycosylated (*4*), while the recombinant form would be a homotrimer of the major isoform 8Sα, which is not N-glycosylated. These results suggest that for mungbean vicilin, N-linked glycans influence the thermal stability of the protein. However, in the case of soybean β-conglycinin, N-linked glycans do not exert influence on the thermal stability (*5*). Alternatively, therefore, the subunits other than α could contribute to the higher thermal stability. The observation of a shoulder in the native 8S supports this interpretation, indicating the presence of molecular species having a different subunit composition. In fact, native β-conglycinin was observed to exhibit two peaks of T_m of 79 and 83.1 °C, which were close to the T_m values of the recombinant α and α′ homotrimers.

T_m correlates positively with increased ionic strength. The T_m of both the native 8S and the recombinant 8Sα increased as a response to increase in ionic strength. For the native 8S, the observed T_m values were 69.2, 75.9, and 83.9 and 87.9 °C at $\mu = 0.1$, 0.2, and 0.5, respectively, whereas 8Sα showed T_m values of 65.6, 71.6, and 77.5 °C at $\mu = 0.1$, 0.2, and 0.5, respectively. Similarly, the thermal denaturation of pea vicilins increased from ∼70 to ∼84 °C in response to an increase in ionic strength from 0.03 to 0.5 (*14*). Salt inhibits aggregation and denaturation of proteins and thus stabilizes the quaternary structure (*15*). This is based on the electrostatic screening effect at the higher ionic strength and is a common property of vicilins as well as legumins.

Surface Hydrophobicity. Hydrophobicity affects solubility, foaming, and emulsifying properties (*16*). The surface hydrophobicities of the native 8S and recombinant 8Sα were analyzed using butyl sepharose and phenyl sepharose columns. From both columns, 8Sα eluted 12 min later than the native 8S, indicating that the recombinant form had greater surface hydrophobicity than the native form (**Table 2**). The elution time was longer in the phenyl sepharose column (66 and 78 min, for the native and recombinant protein, respectively) than in the butyl sepharose column (40 and 52 min, for the native and recombinant protein, respectively). Because the recombinant protein

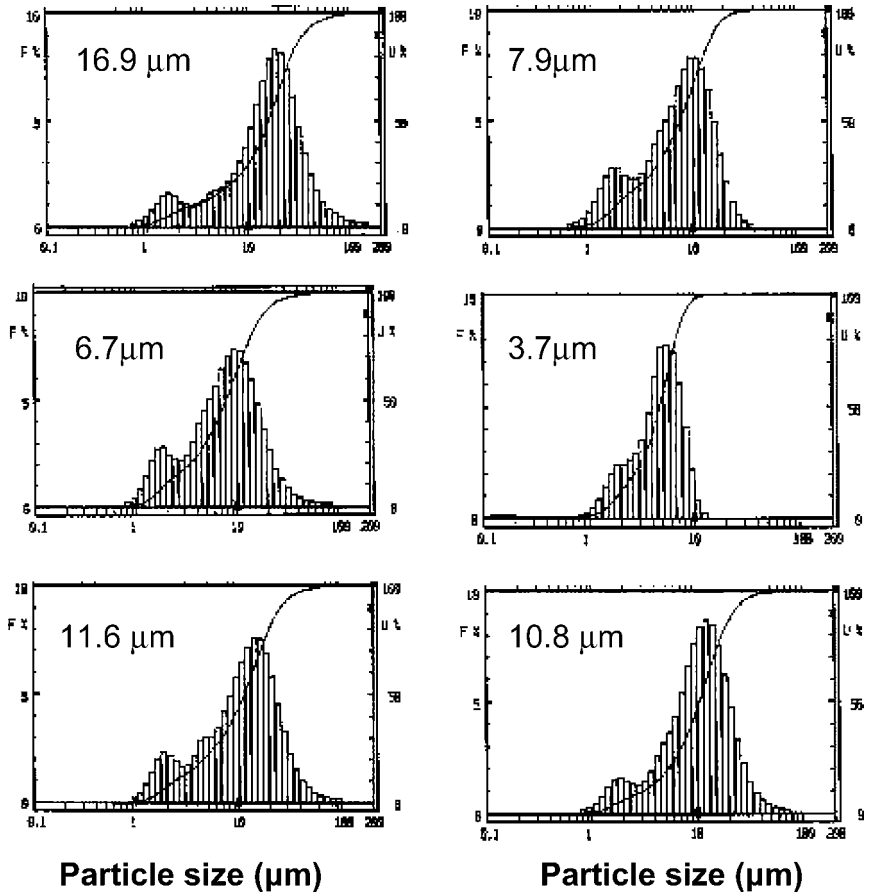

Figure 5. Particle size distribution of emulsions from native and recombinant 8S globulins at pH 7.6. Left column, native; right column, recombinant. Row 1, $\mu = 0.1$; row 2, $\mu = 0.2$; row 3, $\mu = 0.5$. Numbers indicate average particle sizes of the emulsions.

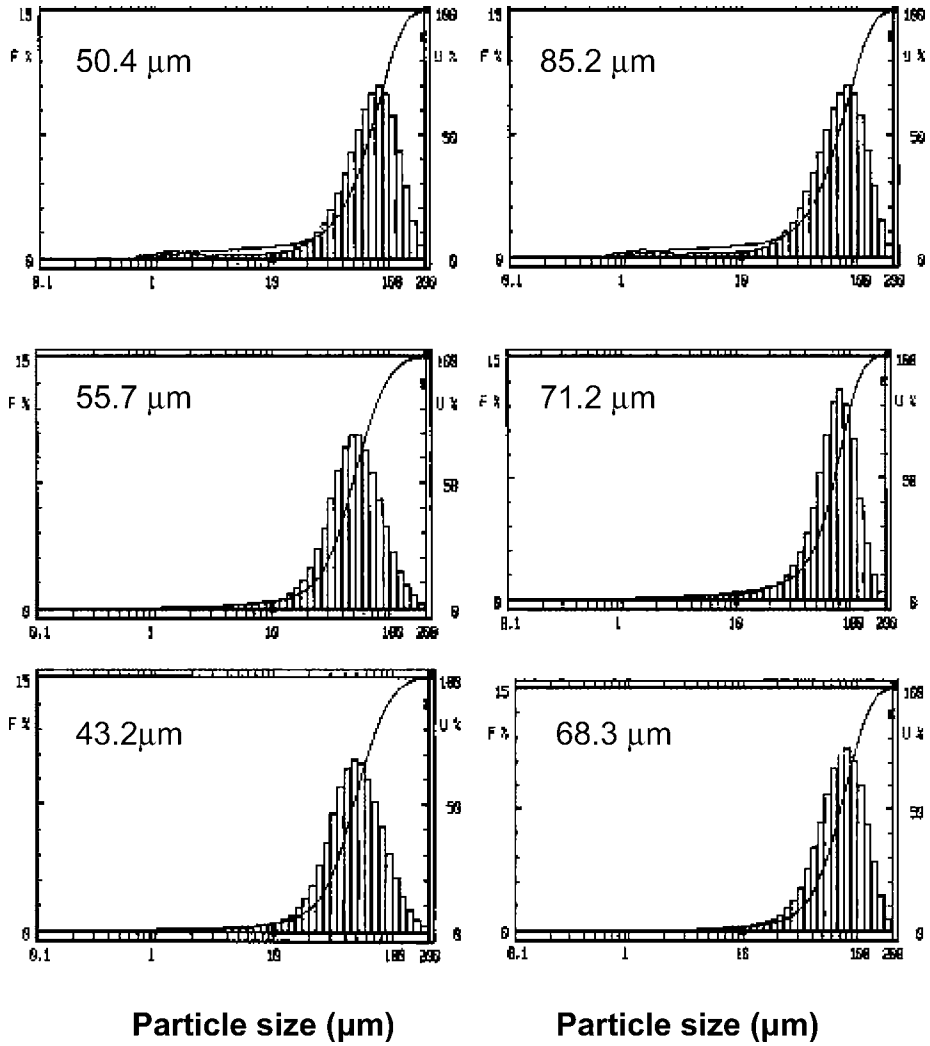

Figure 6. Particle size distribution of emulsions from native and recombinant 8S globulins at pH 3.4. Left column, native; right column, recombinant. Row 1, $\mu = 0.1$; row 2, $\mu = 0.2$; row 3, $\mu = 0.5$. Numbers indicate average particle sizes of the emulsions.

does not contain N-linked glycans, it could be inferred that the hydrophobic surface in the recombinant protein was more exposed than those in the native form, which could be covered by the carbohydrate molecules. The branches of N-linked glycans could reach out more than 3 nm from the protein surface as bulky mobile carbohydrate clusters that are themselves branched (*11*). Hence, the recombinant form had a larger hydrophobic surface available for binding and required a longer elution time. The results indicate that N-linked glycans influence the surface hydrophobicity of the mungbean vicilin, decreasing the hydrophobicity with its presence.

Emulsifying Ability. The emulsifying ability of a protein is one of the most important functional properties for food applications (*17*). The emulsifying ability of the native 8S and recombinant 8Sα was measured at two pH's of 7.6 (**Figure 5**) and 3.4 (**Figure 6**) and three levels of ionic strength ($\mu = 0.1$, 0.2, and 0.5). The smaller is the size of the particles formed, the better is the emulsion. At pH 7.6, the average sizes of the 8S emulsion particles were 16.9, 6.7, and 11.6 μm at $\mu = 0.1$, 0.2, and 0.5, respectively, while those of 8Sα were 7.9, 3.7, and 10.8 μm at $\mu = 0.1$, 0.2, and 0.5, respectively (**Table 3**, **Figure 5**). At pH 3.4, 8S emulsion particles were 50.4, 55.7, and 43.2 μm at $\mu = 0.1$, 0.2, and 0.5, respectively, while those of 8Sα were 85.2, 71.2, and 68.3 μm at $\mu = 0.1$, 0.2, and 0.5, respectively (**Table 3**, **Figure 6**). At pH 3.4, protein aggregation might have caused the collapse of the interfacial bilayer, resulting in poor emulsion ability. At pH 7.6, protein solubility and surface hydrophobicity were favored. These two properties might have enhanced the adsorption of the protein at the interface, producing smaller emulsions as compared to those observed at pH 3.4. Also, the carboxyl groups of the proteins might have lost their protons at pH 7.6, creating greater electrostatic repulsive interactions, and thus preventing protein aggregation. Proteins at $\mu = 0.2$ and pH 7.6 showed the best emulsifying ability. However, the observed differences in particle sizes between 8S and 8Sα were not so big, and hence the contribution of the carbohydrate moiety to emulsifying ability was low.

Table 3. Average Particle Size of the Emulsions of Native Mungbean 8S and Recombinant 8Sα Globulins with Soybean Oil at pH 7.6 and pH 3.4

	pH 7.6		pH 3.4	
ionic strength (μ)	native μm	recombinant μm	native μm	recombinant μm
0.1	16.9	7.9	50.4	85.2
0.2	6.7	3.7	55.7	71.2
0.5	11.6	10.8	43.2	68.3
	F-test	*a*		*b*
	$LSD_{0.01}$	3.0	$LSD_{0.05}$	24.8

[a] Significant at 1% level. [b] Significant at 5% level.

Results with β-conglycinin were different (*8*). The average sizes of the emulsion particles of native α and α′ homotrimers were close to those of the recombinant α and α′. However, the native β homotrimer gave smaller particle size than the recombinant β homotrimer, which would suggest that the carbohydrate moiety is functioning as a hydrophilic segment in the formation of the emulsions. Therefore, it seems that the contribution of carbohydrate moiety to emulsifying ability is variable.

In contrast, the three subunits of β-conglycinin exhibited the following particle sizes at pH 7.6 and $\mu = 0.5$: α (4.2 μm), α′ (16.4 μm), and β (52.9 μm) (*6*). This showed that the mungbean 8S and recombinant 8Sα had better emulsifying abilities than did the β subunit of β-conglycinin.

Concluding Remarks. The results indicate that the presence of N-linked glycans is not essential in the assembly and stable conformation of the mungbean vicilin. However, the N-linked glycans might have contributed to (1) the higher solubility even at low ionic strength, and (2) decreased surface hydrophobicity of the native vicilin as compared to the recombinant 8Sα. The effect of N-linked glycans on the emulsifying ability was low.

The usefulness of mungbean 8S globulin in food and nonfood systems will be dictated by its physicochemical and functional properties. Protein engineering offers a powerful tool in harnessing the full potential of mungbean 8S globulin as an ingredient in food and nonfood systems. We are now conducting protein engineering studies on the recombinant 8Sα globulin to attempt to further improve its functional properties.

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