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Physicochemical and Structural Properties of 8S and/or 11S Globulins from Mungbean [*Vigna radiata* (L.) Wilczek] with Various Polypeptide Constituents

Chuan-He Tang*,†,‡ and Xin Sun†

†Department of Food Science and Technology, and ‡State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou 510640, P. R. China

Two kinds of globulins, 8S and 11S globulins, with various polypeptide constituents, were well fractionated from acid- and salt-extracted mungbean globulins using DEAE-Sepharose fast flow column chromatography. The physicochemical and conformational properties, including amino acid composition, surface charge and hydrophobicity, free sulfhydryl group (SH) and disulfide bond (SS) contents, protein solubility, thermal and emulsifying properties, as well as secondary and tertiary conformations, were evaluated. Remarkable differences in polypeptide composition, surface charge and hydrophobicity, SS contents, protein solubility, thermal and emulsifying properties, and secondary and tertiary conformations were observed between 8S and 11S globulins. The physicochemical and conformational properties of the vicilins also varied with the heterogeneity of their polypeptides, but to a relatively limited extent. The emulsifying ability of these globulins was distinctly dependent on their protein solubility (or net charge), surface hydrophobicity and polypeptide heterogeneity. The thermal properties were similar among various vicilins, but distinctly different between the vicilins and 11S globulins. The circular dichrosim spectral analyses revealed that there were no marked differences in secondary and tertiary conformations between various vicilins, but the secondary, tertiary and quaternary conformations of 11S globulins were much more unordered and flexible than the vicilins. These results suggested good relationships between the physicochemical properties and conformational features of these globulins from mungbean, which could be useful for the utilization of these proteins in the food industry, and providing a working direction of mungbean breeding or protein engineering to improve its physicochemical properties.

KEYWORDS: Mungbean; vicilin; 8S globulins; 11S globulins; physicochemical property; conformation; characterization; DEAE-Sepharose column chromatography; *Vigna radiata* (L.) Wilczek

INTRODUCTION

During recent decades, there has been gradually increasing interest in the exploitation of plant storage proteins from legume sources, as a kind of food ingredient with good nutrition and functionality. The utilization of these proteins requires the investigation of their physicochemical and functional properties, and even structural properties in detail. In many cases, it has been well recognized that there are close relationships between the physicochemical functions and conformations of plant storage globulins, like soy β-conglycinin (*1*−*4*). Thus, the investigation and elucidation of structure−function relationships of legume storage proteins would be of vital importance for the development of legume proteins with excellent functionalities, e.g. emulsifying or foaming properties.

Generally, legume storage proteins include the globulins and albumins, and the globulins are mainly composed of 7S or 8S (vicilins) and 11S (legumin) globulins (*5*). The protein composition of legumes remarkably varies with the variety of the legumes, and even in the same variety, various isoforms of globulins (and vicilins in particular) with different polypeptide constituents may be present. The differences in polypeptide composition increase the difficulty to understand and manipulate the properties of these proteins, and thus limit their utilization in many food formulations. To overcome this difficulty and further elucidate the structure−function relationships of legume storage globulins, there is a dearth of information on the physicochemical (and/or functional) and conformational properties of purified individual globulins, e.g. vicilin (7−8S) and legumin (11S), with different polypeptide constituents.

Mungbean [*Vigna radiata* (L.) Wilczek] is increasingly becoming a more important leguminous crop in Asia and other parts of the world, and even is a major source of proteins in some developing countries. It contains 17−26% protein, and the major storage protein in mungbean seeds is the 8S globulin or vicilin, which comprises 89% of the total globulins (*6*). Other globulins, e.g., 11S and basic 7S globulins, are minor, with contents of about 7.6 and 3.4%, respectively (relative to total globulins). The mungbean vicilin is mainly composed of four polypeptides with molecular weights (MW) of about 60, 48, 32, and 26 kDa,

*Corresponding author. Tel: (086)20-87111707. Fax: (086)20-87114263. E-mail chtang@scut.edu.cn.

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respectively, and the 11S globulins are composed of acidic and basic subunits with MW of 40 and 24 kDa, respectively (*6*). Three isoforms of the cDNA of the major mungbean vicilin (8S globulin), 8Sα, 8Sα′, 8S*β*, have been isolated, cloned, and characterized by Bernardo and co-workers (*7*). The different isoforms were highly homologous with each other, from 88 to 92%. The major isoform 8Sα was expressed in *Escherichia coli*, and successfully purified and crystallized.

Although the physicochemical and functional properties of soybean storage globulins and *β*-conglycinin (vicilin) in particular have been well studied (*1*−*4*), the information on the properties of mungbean globulins is very limited. Based on the work of successful cloning and crystallization for recombinant 8Sα globulin, Garcia and co-workers reported the physicochemical and emulsifying properties of native 8S and recombinant 8Sα globulin or vicilin (*8*). The secondary structure, protein solubility (as a function of pH and ionic strength), thermal stability, surface hydrophobicity and emulsifying ability were evaluated, and it was shown that the presence of N-linked glycans is not essential in the assembly and stable conformation of mungbean vicilin, but it might contribute to the higher protein solubility at low ionic strength, greater thermal stability, and decreased surface hydrophobicty of the native vicilin as compared to the recombinant 8Sα (*8*). Factually, the native vicilin (8S globulin) in this work is still a combination of various vicilin isoforms with different polypeptide constituents. Therefore, the comparison of the properties of this native vicilin with recombinant 8Sα globulin cannot well reveal the structure−function relationships of these vicilins. The fractionation and purification of various vicilin isoforms with different polypeptide constituents, and then comparison of their properties, would be a good choice to elucidate their structure−function relationships. On the other hand, to the best of our knowledge, there is no report about the properties of 11S globulins from mungbean.

Thus, this study aimed (1) to fractionate and purify the 8S and 11S globulins from mungbean, including various vicilin isoforms with different polypeptide constituents; (2) to characterize and compare the physicochemical and structural properties of these globulins; and (3) to establish possible structure−physicochemical function relationships of mungbean vicilins. The amino acid composition, surface charge and hydrophobicity, free sulfhydryl group (SH) and disulfide bond (SS) contents, protein solubility, thermal and emulsifying properties, as well as secondary and tertiary conformations, were evaluated.

MATERIALS AND METHODS

Materials. Mungbean [*Vigna radiata* (L.) Wilczek] seeds, cultivated in the North-East area of China, were purchased from three local supermarkets (Guangzhou, China), and their composite was applied. The seeds were soaked in deionized water for 12 h at 4 °C and dehulled manually. The dehulled seeds were freeze-dried, ground and defatted by Soxhlet extraction with hexane to produce the defatted flour. 5,5′-Dithio-bis 2-nitrobenzoic acid (DTNB), 2,4,6-trinitrophene sulfonic acid (TNBS) and 1,8-anilinonaphthalenesulfonate (ANS) reagents were purchased from Sigma (St. Louis, MO). Bovine serum albumin (BSA) was obtained from Fitzgerald Industries International Inc. (Concord, MA). All other chemicals used were of analytical or better grade.

Preparation of Acid- and Salt-Extracted Mungbean Globulins (Control). The acid- and salt-extracted mungbean globulins (control) were prepared according to the process described by Hall et al. (*9*), with slight modifications. This process was applied mainly to isolate vicilin-type globulins from legume seeds. The defatted flour (100 g) was dispersed in 2 L of 0.5 M NaC1 solution containing 0.025 M HCl (pH 3.5). The resultant dispersion was gently stirred at room temperature for 2 h. The slurry was centrifuged (30000*g*, 20 min) at 4 °C in a CR22G centrifuge (Hitachi Co., Japan), and the supernatant was diluted with 5-fold volumes of deionized water (0−4 °C). Then, the obtained precipitate was collected by centrifugation at 12000*g* for 20 min at 4 °C. The pellet was dissolved in 0.5 M NaCl solution, and reprecipitated twice as above. The last obtained precipitate was finally dissolved in 0.5 M NaCl solutions and dialyzed against deionized water at 0−4 °C for 48 h, and then lyophilized to produce the crude globulins (control; denoted as G).

Fractionation of the Globulins by DEAE-Sepharose Chromatography. The crude mungbean globulins (G; about 10 g) were further fractionated using DEAE-Sepharose fast flow column chromatography with AKTA Purifier (GE Co. Ltd., USA), according to the process described by Gueguen et al. (*10*). Elution was performed using 50 mM phosphate buffer (PBS; pH 7.0) with a gradient of 0 to 0.5 M NaCl, at a flow rate of 2.5 mL per minute. The eluent was collected at 10 mL per tube. The collected fractions, pooled according to the requirement, were resolved by sodium dodycyl sulfate−polyacryamide gel electrophoresis (SDS−PAGE), and dialyzed against deionized water at 4 °C and further freeze-dried to produce various fractionated globulins (denoted as G1−G4).

SDS−PAGE. SDS−PAGE was performed on a discontinuous buffered system according to the method of Laemmli (*11*) using 12% separating gel and 4% stacking gel. The gel was stained with 0.25% Coomassie brilliant blue (R-250) in 50% trichloroacetic acid, and destained in methanol−water solution containing 7% (v/v) acetic acid and 40% (v/v) methanol. The tested protein samples for SDS−PAGE under reducing conditions were prepared by directly mixing the collected fractions with electrophoretic sample buffer (2×), namely, 0.25 M Tris-HCl buffer (pH 8.0) containing 2.0% (w/v) SDS, 0.1% (w/v) bromophenol blue, 50% (v/v) glycerol and 10% (v/v) *β*-mercaptoethanol (2-ME). The protein samples for SDS−PAGE under nonreducing conditions were prepared by the same process, just without the presence of 2-ME.

The relative percentages of individual polypeptides of vicilins (7S globulins) were estimated by scanning the SDS−PAGE gels, using the gel analysis software TotalLab v2005 (Nonlinear Dynamics, NE1 2ET, U.K.). The purity of vicilin (7S globulin) or 11S globulin was calculated by combined percentages of all their polypeptides (relative to total dyed proteins).

Amino Acid Analysis. The protein samples were hydrolyzed with 6 N HCl for 24 h at 110 °C in a sealed tube. Amino acid composition was determined using automatic amino acid analyzer (Waters M510, USA), with PICO.TAG column. The determination was carried out at 38 °C, detection wavelength 254 nm and flow rate 1.0 mL per minute. Amino acid composition was reported as g/100 g protein.

Zeta Potential. The zeta potential profiles of various mungbean globulins as a function of pH were measured using a Zetasizer Nano ZS (Malvern Instrument Ltd., Malvern, Worcestershire, U.K.) in combination with a multipurpose autotitrator (model MPT-2, Malvern Instruments, Worcestershire, U.K.). Freshly prepared protein dispersions were diluted to 2 mg/mL with deionized water and filtered through a 0.45 *μ*m HA Millipore membrane prior to analysis. Titration was performed from pH 10.0 to pH 2.0 with 0.25 and/or 0.025 N NaOH or HCl under constant stirring.

Surface Hydrophobicity (H_0). The H_0 was determined with the fluorescence probe ANS according to the method of Haskard and Li-Chan (*12*). Serial dilutions in 0.01 M PBS (pH 7.0) were prepared with the protein samples (stock solutions; 1.5%, w/v) to a final concentration of 0.004−0.02% (w/v). ANS solution (8.0 mM) was also prepared in the same buffer. Twenty microliters of ANS solution was added to 4 mL of each dilution, and fluorescence intensity (FI) of the mixture was measured at 390 nm (excitation) and 470 nm (emission) using an F4500 fluorescence spectrophotometer (Hitachi Co., Japan). The initial slope of the FI versus protein concentration (mg/mL) plot (calculated by linear regression analysis) was used as an index of H_0.

Free Sulfhydryl Group (SH) and Disulfide Bond (SS) Contents. The free SH and SS contents of protein samples were determined by the method of Beveridge et al. (*13*). Protein samples (75 mg) were dissolved in 10 mL of Tris-Gly buffer (0.086 M Tris, 0.09 M glycine, 0.04 M EDTA, pH 8.0) containing 8 M urea. The solution was gently stirred overnight until a homogeneous dispersion was achieved. For SH content determination, 4 mL of the Tris-Gly buffer was added to 1 mL of protein solution. Then 0.05 mL of Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoic acid in Tris-Gly buffer, 4 mg/mL) was added, and absorbance was measured at 412 nm after 5 min. For total SH content [SH + reduced SS] analysis, 0.05 mL of

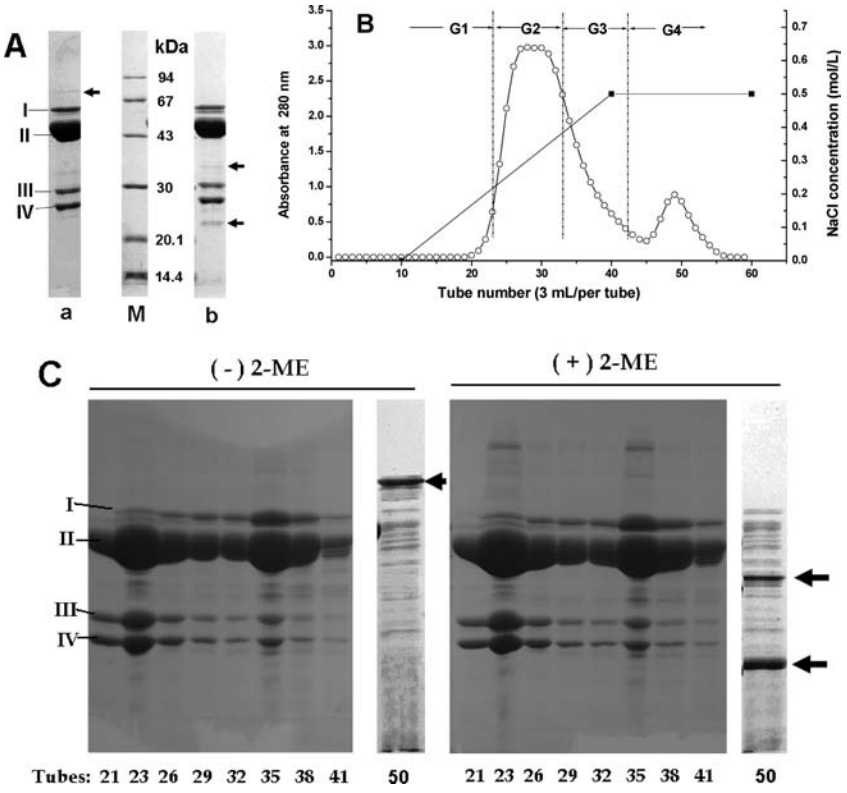

Figure 1. (**A**) SDS−PAGE analysis of total mungbean globulins (control; G). Lanes a and b: with and without 2-ME, respectively. M: protein markers. (**B**) Ion-exchange chromatography of total mungbean globulins on a DEAE-Sepharose column by gradient elution with NaCl. Fractions no. 20−23, no. 24−34, no. 35−44 and no. 45−55 were collected as G1, G2, G3 and G4, respectively. (**C**) SDS−PAGE profiles of the globulins in collected tubes in the absence and presence of 2-ME. The numbers (I−IV) indicated the main four polypeptides or subunits of 8S globulins (vicilin). The arrows show the polypeptides or its associated form (disulfide bond bridged) of 11S globulins.

2-ME and 4 mL of Tris-Gly buffer were added to 1 mL of the protein solution. The mixture was incubated for 1 h at room temperature. After an additional hour of incubation with 10 mL of 12% TCA, the mixtures were centrifuged at 5000*g* for 10 min. The precipitate was twice resuspended in 5 mL of 12% TCA and centrifuged to remove 2-ME. The precipitate was dissolved in 10 mL of Tris-Gly buffer. Then 0.04 mL of Ellman's reagent was added to 4 mL of this protein solution, and the absorbance was measured at 412 nm after 5 min. The calculation was as follows: μM SH/g = $73.53 \times A_{412} \times D/C$; where A_{412} is the absorbance at 412 nm, C is the sample concentration (mg/mL), D is the dilution factor (5 and 10 are used for SH and total SH (SH + reduced SS) content analysis, respectively), and 73.53 is derived from $10^6/(1.36 \times 10^4)$ (1.36×10^4 is the molar absorptivity (*14*), and 10^6 is for the conversion from molar basis to μM/mL basis and from mg solids to g solids). Half of the value after subtracting the SH value from the total SH value was defined as the SS content.

Protein Solubility (PS). An aqueous solution (1.0%, w/v) of protein samples was stirred magnetically for 30 min, and then with either 0.5 N HCl or 0.5 N NaOH, the pH of the solutions was adjusted to the desired values. After 30 min of stirring, the pH was readjusted if necessary. Then it was centrifuged at 8000*g* for 20 min at 20 °C in a CR22G centrifuge (Hitachi Co., Japan). After appropriate dilution, the protein content of the supernatant was determined by the Lowry method (*15*) using BSA as the standard. The PS was expressed as grams of soluble protein per 100 g of total protein. All determinations were conducted three times.

Differential Scanning Calorimetry (DSC). The thermal transition of various protein samples was examined using a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, DE 19720), according to the procedure of Meng and Ma (*16*) with some modifications. Approximately 2.0 mg of the samples were weighed into aluminum liquid pans (Dupont), and 10 μL of 0.05 M PBS (pH 7.0) was added. The pans were hermetically sealed and heated from 20 to 110 °C at a rate of 5 °C/min. A sealed empty pan was used as a reference. Onset temperature (T_m), peak transition or denaturation temperature (T_d), enthalpy change of the endotherm (ΔH) and cooperativity, represented by the width at half-peak height ($\Delta T_{1/2}$), were computed from the thermograms by the Universal Analysis 2000, Version 4.1D (TA Instruments-Waters LLC). All experiments were conducted in triplicate. The sealed pans containing protein samples and buffers were equilibrated at 25 °C for more than 6 h.

Emulsifying Activity Index (EAI). EAI was determined according to the method of Pearce and Kinsella (*17*), with minor modifications. For emulsion formation, 15 mL of 0.05−1.0% (w/v) protein solutions in 50 mM PBS (pH 7.0) and 5 mL of corn oil were homogenized in ULTRA-TURRAX T25 digital homogenizer (IKA Co., Germany) at 24,000 turn/min for 1 min. Fifty microliters of emulsion was taken from the bottom of the homogenized emulsion, immediately after homogenization, and diluted (1:100, v/v) in 0.1% (w/v) SDS solution. After shaking in a vortex mixer for 5 s, the absorbance of dilute emulsions was read at 500 nm using a Spectrumlab 22PC spectrophotometer (Shanghai Lengguang Technology Co. Ltd., Shanghai, China). EAI values were calculated using the following equation:

$$\text{EAI } (\text{m}^2/\text{g}) = \frac{2 \times 2.303 \times A_0 \times \text{DF}}{c \times \phi \times (1-\theta) \times 10000}$$

where DF is the dilution factor (100), c the initial concentration of protein (g/mL), ϕ the optical path (0.01 m), θ the fraction of oil used to form the emulsion (0.25), and A_0 the absorbance of diluted emulsions respectively. Measurements were performed at least in quadruplicate.

Far-UV and Near-UV Circular Dichroism (CD) Spectroscopy. Far-UV and near-UV CD spectra were obtained using a MOS-450 spectropolarimeter (BioLogic Science Instrument, France). The far-UV CD spectroscopic measurements were performed in a quartz cuvette of 2 mm with a protein concentration around 0.1 mg/mL in 10 mM PBS (pH 7.0). The sample was scanned from 190 to 250 nm. The near-UV CD spectroscopy measurements were performed in a 1 cm quartz cuvette with a protein concentration around 1.0 mg/mL. The sample was scanned over a wavelength range from 250 to 320 nm. For both measurements, the spectra were an average of eight scans. The following parameters

Table 1. Relative Percents of Individual Major Polypeptides and/or Subunits, and Protein Purity of Various 8S and 11S Globulins as Estimated by Densitometry Analysis[a]

	rel percent of individual polypeptides and/or subunits[b] (%)						
	8S globulins				11S globulins		
sample	I	II	III	IV	acidic	basic	protein purity[c] (%)
G (control)	12.8 (1.1) b	60.2 (1.3) b	10.2(0.5) b	12.8(0.1) b	−	−	96.1(0.8) a
G1	− d	55.2(1.0) c	18.3(0.0) a	22.1(0.5) a	−	−	95.6(0.6) a
G2	6.3(0.2) c	68.4(0.2) a	9.7(0.1) b	12.5(0.1) b	−	−	96.9(0.1) a
G3	20.2(0.6) a	60.5(1.1) b	4.7(0.2) c	6.6(0.0) c	−	−	92.0(0.9) b
G4	−	−	−	−	23.6	27.7	45.6(6.7)

[a] Different letters (a−d) indicate a significant difference at the $p < 0.05$ level in the same column between G, G1, G2 and G3. The character "−" within the table indicates the absence of the polypetides and/or subunits (or very low levels). For the legends G, G1, G2, G3 and G4, refer to **Figure 1**. [b] Means and standard deviations of the estimated data in the presence and absence of 2-ME (except G4, where only the data in the presence of 2-ME was provided). [c] Protein purity (%) represents the relative percents of combined individual polypeptides and/or subunits of 8S or 11S gloublins (relative to total stained bands).

were used: step resolution, 1 nm; acquisition duration, 1 s; bandwidth, 0.5 nm; sensitivity, 100 mdeg. The cell was thermostated with a Peltier element at 25 °C, unless specified otherwise. The concentration of the proteins was determined by the Lowry method (*15*) using BSA as the standard. Recorded spectra were corrected by subtraction of the spectrum of a protein-free buffer. A mean value of 112 for the amino acid residue was assumed in all calculations, and CD measurements expressed as mean residue ellipticity (θ) in $deg \cdot cm^{-2} \cdot dmol^{-1}$. The secondary structure compositions of the samples were estimated from the far-UV CD spectra using the CONTIN/LL program in CDPro software, using 43 kinds of soluble proteins as the reference set (*18*). Each data was the means of duplicate measurements.

Statistical Analysis. An analysis of variance (ANOVA) of the data was performed, and a least significant difference (LSD) test with a confidence interval of 95% was used to compare the means.

RESULTS AND DISCUSSION

Fractionation by DEAE Ion Exchange Chromatography and SDS−PAGE Analyses. Under reducing condition, the total globulin sample (control; G) was mainly composed of four bands (I−IV) with MW of 58, 50, 30, and 26 kDa, respectively (**Figure 1A**). The four bands were unaffected by the presence of 2-ME, and thus clearly attributed to the main polypeptides of 8S mungbean globulins. Similar SDS−PAGE profiles for 8S globulins (vicilin) have been observed in previous literature (*19*, *20*). Besides the major polypeptides of 8S globulins, individual acidic and basic subunits (with 2-ME) or disulfide bond-associated subunits (without 2-ME) of 11S globulins were observed, but the amount is minor (**Figure 1A**). Based on the densitometry analysis, the purity of vicilin in the G was about 96.1%, and the relative contents of individual polypeptides I−IV were 12.8, 60.2, 10.2 and 12.8%, respectively (**Table 1**). In the control, the polypeptide II was the major polypeptide of the vicilin.

The control (G) was fractionated on a DEAE-Sepharose column using a linear gradient elution with NaCl (0−0.5 M) (**Figure 1B**). No peak appeared peak when the sample was introduced to the column and then eluted with the buffer without NaCl. A major peak was eluted in the range of 0−0.5 NaCl, and upon further elution with 0.5 M NaCl, a minor peak appeared (**Figure 1B**). The protein compositions for the fractions eluted at various tubes were analyzed by SDS−PAGE under reducing and nonreducing conditions (**Figure 1C**).

As expected, the major peak was attributed to the 8S globulins, composed of the polypeptides I−IV (**Figure 1B**). However, the relative ratios of different polypeptides (I−IV) to a great extent varied with the elution time (or NaCl concentration in the elution buffer). The first eluted vicilin component (tubes no. < 23) was mainly composed of the polypeptides II, III and IV, while in the last eluted components (e.g., at tubes no. 35 or 38), the relative content of the polypeptides III and IV remarkably decreased, and the polypeptide I increased, with eluting NaCl concentration increasing (**Figure 1C**).

By contrast, the minor peak (centered at around 49−50 tubes) mainly consisted of the 11S globulins, with relative purity of about 45.6% (relative to total stained bands; **Table 1**). The purity of 11S globulins was much higher than that for 11S-enriched globulin fraction of mungbean proteins, fractionated using combined ammonium sulfate precipitation and chromatography on Mono Q HR column (*20*). This suggested that, in the present case, combined acid/salt-extraction technique and DEAE ion exchange chromatography would be more effective to fractionate 8S and 11S mungbean globulins.

To elucidate the differences in physicochemical and structural properties of vicilin-type (8S) and 11S globulins, with different polypeptide constituents, the protein fractions eluted at tubes no. 20−23, no. 24−34, no. 35−44 and no. 45−55, respectively, were pooled and dialyzed to remove the salts, and further freeze-dried to produce various 8S and/or 11S globulins (denoted as G1, G2, G3 and G4, respectively). The protein compositions of G1, G2 and G3 were similar to that of control (G), and mainly composed of 8S globulins, while G4 was enriched with 11S globulins (**Figure 1C**). The approximate protein yields of G1, G2, G3 and G4 were about 10.0, 57.6, 27.2 an 5.2%, respectively. The relative ratios of individual polypeptides (especially polypeptides I−IV for 8S globulins), estimated by the densitometry analysis, are summarized in **Table 1**. There were significant differences in percents of individual polypeptides among various fractionated samples (G1−G4). In G1, the polypeptide I was almost absent, and the polypeptides III and IV (18.3 and 22.1%) were significantly higher than the control (G). The relative contents of the polypeptides III and IV considerably decreased in the order G1 > G2 > G3, while that of the polypeptide I contrarily increased (**Table 1**). Furthermore, the relative content of the polypeptide II was highest in G2, and least in G1. These differences in polypeptide constituents would warrant the investigation of the physicochemical and conformational properties of 8S and 11S mungbean globulins, as affected by the polypeptide heterogeneity.

Amino Acid Composition. The amino acid compositions of various mungbean globulin samples are shown in **Table 2**. The relative percents of amino acids with different characters (acidic, basic, nonpolar and polar) are also included. The data for G1 was almost the same as that for G2, and thus not provided. As expected, these globulins contained low levels of surfur-containing amino acids (Met and Cys), but were rich in aspartic acid/asparagines and glutamic acid/glutamine which impart acidic characteristic to the proteins (*21*). The relative ratio of acidic (Asx + Glx) and basic (His + Arg + Lys) amino acids was highest in G2 (1.74), followed by G3 (1.58), and least in G4 (1.52) (**Table 2**),

suggesting that the acidic characteristic of 11S globulins was less than that of 8S globulins, and 8S globulins with higher content of polypeptide I (and lower polypeptides III and IV) exhibited higher acidic characteristics than those with lower content of polypeptide I. As for uncharged amino acids, the relative content of nonpolar amino acids (Ala + Pro + Val + Met + Ile + Leu + Phe) for G2 was distinctly higher than that of G3 and G4. Much higher polar amino acids (Ser + Gly + Thr + Tyr + Cys) were observed in G4 than in other globulins. These differences in amino acid composition may be related to the differences in physicochemical properties of these globulins, e.g., surface net charge and hydrophobicity.

Zeta Potential. Figure 2 shows the zeta potential (ζ) profiles of various mungbean globulin samples as a function of pH. Except G4, all the globulins exhibited similar trends of the pH-dependence of zeta potential, namely, the ζ progressively increased from a negative value to a positive value (e.g., from −38.5 mW to 38.2 mW for G1) with pH decreasing from 9.8 to 3.5. This reflects that the electrostatic repulsion pattern was gradually changed from between negatively charged proteins to between positive charged proteins, as a result of gradual protonation of carboxyl groups and deprotonation of amino groups of the proteins. Upon further decrease in pH, the ζ on the contrary slightly decreased (**Figure 2**). The decrease may be due to the hydrolysis of glutamine and/or asparagine into glutamic acid and/or glutamic acid at high acidic pH.

Although the ζ of various fractionated 8S globulins was similar at pH above 7.0, evident differences in isoelectric point (pH at ζ = 0; I_p) and zeta potential values at acidic pH conditions were observed (**Figure 2**). The I_p decreased in the order G1 (5.04) > G2 (4.81) > G3 (4.55) > G4 (4.39). Furthermore, higher positive potential values were observed at pH below I_p (e.g., of G3) for the 8S globulins eluted earlier on ion chromatography, while higher negative potential values were found at acidic pH above I_p (e.g., of G1) for those eluted later. This is consistent with the acidic/basic amino acid ratio of these globulin samples (**Table 2**), indicating a close relationship between surface charge (or zeta potential) and the amino acid composition.

Unexpectedly, the ζ of G4 progressively decreased from 33.8 to 0 mV as the pH gradually decreased from 10 to about 2.0 (**Figure 2**). This suggests that the surface charged groups of 11S globulins are completely different from those of vicilin-type or 8S globulins. Although the underlying mechanism for this phenomenon is not yet fully known, it might be closely related to high uncharged polar amino acid contents in this protein (**Table 2**).

Surface Hydrophobicity (H_0). Surface hydrophobicity (H_0), measured using ANS^- as fluorescence probe, is an important parameter indicative of hydrophobic clusters on the surface of the proteins. The H_0 of various mungbean globulin samples are shown in **Table 3**. The H_0 of all the fractionated samples was significantly ($p < 0.05$) higher than that of control (G). This might be due to the influence of additional solubilization, dialyzation

Table 2. Amino Acid Compositions of Various Mungbean Globulins[a]

	mungbean globulin samples (% protein)			
amino acids	G	G2	G3	G4
Asx[b]	12.3	11.9	11.4	10.1
Glx[b]	20.5	21.9	21.1	20.4
Ser	5.5	5.8	5.8	6.2
Gly	2.8	3.1	3.4	4.1
His	4.1	3.7	4.0	4.1
Arg	7.7	7.6	8.0	8.2
Thr	2.4	2.6	3.1	3.3
Ala	3.6	3.2	3.1	2.9
Pro	4.9	4.5	4.8	5.8
Tyr	2.7	2.8	3.4	4.0
Val	4.8	4.2	4.3	4.5
Met	0.3	0.3	0.5	0.9
Cys	0.04	0.05	0.05	0.07
Ile	4.4	4.2	4.2	4.1
Leu	9.1	9.0	8.2	7.6
Phe	6.8	7.1	5.9	5.0
Lys	8.1	8.2	8.6	8.8
charged[c]				
acidic	32.8	33.8	32.5	30.6
basic	19.8	19.4	20.6	20.1
acidic/basic	1.66	1.74	1.58	1.52
uncharged[d]				
nonpolar	34.0	32.4	30.1	30.8
polar	13.5	14.3	15.8	17.5

[a] Each data is the mean of duplicate measurements. For the legends G, G2, G3 and G4, refer to **Figure 1**. [b] Asx: Asp + Asn. Glx: Glu + Gln. [c] Acidic amino acids: Asx + Glx. Basic amino acids: His + Arg + Lys. [d] Nonpolar amino acids: Ala + Pro + Val + Met + Ile + Leu + Phe. Polar amino acids: Ser + Gly + Thr + Tyr + Cys.

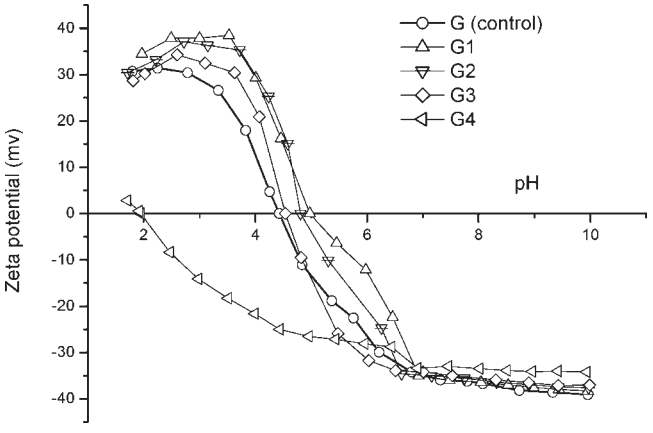

Figure 2. Zeta potential (ζ) profiles of various mungbean globulin samples as a function of pH at 25 °C. I_p within the figure represents the location of isoelectric point at ζ = 0. For the legends G, G1, G2, G3 and G4, refer to **Figure 1**.

Table 3. Surface Hydrophobicity (H_0), Free SH and SS Contents, as Well as DSC Characteristics of Various Mungbean Globulins[a]

		free SH contents (μmol/g protein)			DSC characteristics			
protein samples	H_0	total	exposed	SS contents (μmol/g protein)	T_m (°C)	T_d (°C)	ΔH (J/g)	$\Delta T_{1/2}$ (°C)
G (control)	210.5 d	3.45 ± 0.06 b	3.09 ± 0.22 c	8.13 ± 0.24 c	72.5 ± 0.2 d	80.2 ± 0.2 b	11.5 ± 0.5 a	8.2 ± 0.1 a
G1	256.3 c	3.48 ± 0.14 b	3.31 ± 0.21 b	7.27 ± 0.07 d	73.6 ± 0.6 c	81.2 ± 0.2 b	12.6 ± 0.9 a	8.7 ± 0.4 a
G2	259.3 c	3.26 ± 0.08 c	2.73 ± 0.07 d	7.19 ± 0.09 d	74.2 ± 0.1 c	80.8 ± 0.5 b	11.6 ± 0.3 a	7.7 ± 0.3 ab
G3	278.3 b	3.44 ± 0.08 b	2.98 ± 0.02 c	10.46 ± 0.19 b	74.9 ± 0.2 b	81.6 ± 0.5 b	9.4 ± 0.2 b	7.2 ± 0.1 b
G4	301.3 a	4.55 ± 0.25 a	3.78 ± 0.26 a	16.42 ± 0.27 a	76.2 ± 0.4 a	83.0 ± 0.2 a	6.8 ± 0.1 c	7.1 ± 0.1 b

[a] Different letters (a−d) indicate a significant difference at the $p < 0.05$ level in the same column. T_m and T_d: onset temperature and the denaturation temperature of the prominent endotherm (of vicilin), respectively; ΔH and $\Delta T_{1/2}$: combined enthalpy change of the prominent endotherm, and the width at half-peak height of the major endotherm, respectively. For the legends G, G1, G2, G3 and G4, refer to **Figure 1**.

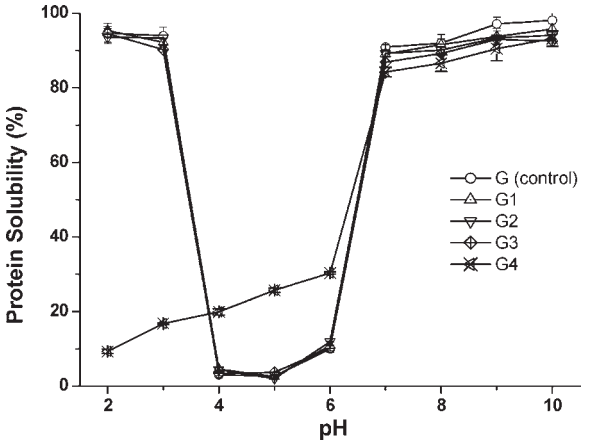

Figure 3. PS profiles of various mungbean globulins as a function of pH. Each data was the means and standard deviation of duplicate measurements. For the legends G, G1, G2, G3 and G4, refer to **Figure 1**.

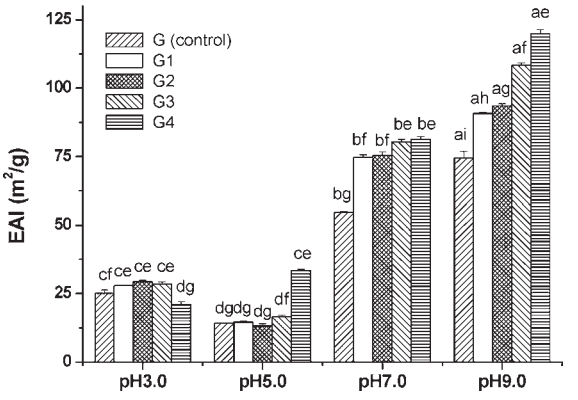

Figure 4. Emulsifying ability index (EAI) of various mungbean globulins at pHs of 3.0, 5.0, 7.0 and 9.0, respectively. Different letters (a−d) on the column top indicate a significant difference at the $p < 0.05$ level, due to the pH difference (in the same sample). Different letters (e−i) on the column top indicate a significant difference at the $p < 0.05$ level (at a given pH value), among various globulin samples. For the legends G, G1, G2, G3 and G4, refer to **Figure 1**.

and freeze-drying treatment (applied for the preparation of these fractionated globulins) on the surface property and conformation of the globulins. The H_0 values of all the globulins increased in the order G1 ≈ G2 < G3 < G4 (**Table 3**). This is well in accordance with the reverse order of their charged amino acid (combined acidic and basic) contents (**Table 2**), suggesting that the globulins with higher contents of charged amino acids might exhibit more compact conformation. The relatively marked difference in H_0 between G1 (or G2) and G3 might reflect that the vicilin with higher content of the polypeptide I exhibited more exposure of hydrophobic clusters on its molecular surface.

Free SH and SS Contents. The total and exposed free SH contents as well as SS contents of various mungbean globulin samples were determined, as also included in **Table 3**. All the tested samples contained much higher SS contents than total free SH contents. Taking together the fact that there are no disulfide bonds maintaining individual polypeptides in 8S globulins, it could be concluded that the disulfide bonds might be present in the intrapolypeptide form. Most of the free sulfhydryl groups were in the exposed form (**Table 3**). The total and exposed free SH contents of G (control) are similar to those for mungbean protein isolate reported in our previous work (*22*). Total free SH contents of various 8S globulins were similar (3.3−3.5 μmol/g protein), but significantly lower than that of G4 (**Table 3**).

The SS content of G1 was almost the same as that of G2, but much less than that of G3. The considerably higher SS content clearly resulted from the contribution of the polypeptide I in 8S globulins. By contrast, the SS content of G4 was even significantly ($p < 0.05$) higher than that of G3 (**Table 3**). This is consistent with the fact that there are disulfide bond bridges between individual acidic and basic polypeptides in 11S globulins (as evidenced by SDS−PAGE analysis; **Figure 1C**).

Protein Solubility (PS) Profiles as a Function of pH. Protein solubility (PS) is an important property for one protein that is related to a number of functional properties of this protein, and thus may affect its application in food formulations. **Figure 3** shows the PS profiles of various mungbean globulin samples as a function of pH in the range pH 2.0−10.0. All the 8S globulins (G1−G3) and the control exhibited similar PS profiles. In each of these samples, a similar minimum PS (about 5%) was observed at pH 4.0−5.0, which is consistent with the I_p data of zeta potential analysis (**Figure 2**). At more than pH 7.0, or less than pH 3.0, the PS was more than 90%, indicating that the electrostatic repulsion interactions between the proteins were of vital importance for the PS of this kind of globulins. Similar PS profiles as a function of pH have been observed in native and recombinant mungbean 8S globulins, or other legume 7S globulins (*8*, *23*, *24*).

The PS profile for G4 was similar to that of other samples at pH 7.0 or above, but completely different at pH 6.0 or below (**Figure 3**). Throughout the I_p range (pH 4.0−6.0), G4 still kept relatively high PS, i.e., 20−26% at pH 4.0−5.0. With the pH decreasing from 6.0 to 2.0, the PS of G4 progressively decreased from 30.8 to 8.9%. This phenomenon is also consistent with the zeta potential analysis of this protein (**Figure 2**).

Thermal Property. The thermal property for a globular protein is also an important property, since it may be related to its heat-induced aggregation and gelation behaviors. The thermal transition of various mungbean globulin samples was determined by DSC technique, and the resultant DSC characteristics including onset temperature (T_m), denaturation peak temperature (T_d), enthalpy change (ΔH) and the peak width at half-height ($\Delta T_{1/2}$) of the major endothermic peak are also included in **Table 3**. All the tested samples exhibited similar DSC thermograms with a prominent endothermic peak (data not shown). The major endothermic peak was clearly attributed to the thermal denaturation of vicilin-type (8S) or 11S globulins.

There were no significant differences in T_d values (80.8−81.6 °C) among various 8S globulins (**Table 3**). The T_d is a measure of thermal stability, and higher T_d value is usually associated with higher thermal stability for a globular protein. Thus, the data suggested that the thermal stability of 8S globulins was unaffected by the differences in polypeptide compositions (especially the polypeptides I and III or IV) and SS contents (within the polypeptides). However, the ΔH values gradually decreased in the order G1 > G2 > G3 (**Table 3**), indicating gradual decrease in the proportion of undenatured protein, or extent of ordered structure (*25*).

The G4 exhibited significantly higher T_m and T_d values (76.2 and 83.0 °C), and much lower ΔH value than various 8S globulins (**Table 3**). The data indicated that the globulins in G4 were more thermally stable, but the extent of ordered structure much less. The higher thermal stability might be attributed to the presence of intrapolypeptide disulfide bonds, or high SS contents. This also agrees with the general viewpoint that the thermal stability of 11S globulins is usually higher than that of 7S or 8S globulins. The much lower ΔH value of the globulins in G4 could be attributed to higher extent of conformational flexibility, as evidenced by the phenomenon that the ΔH order of various

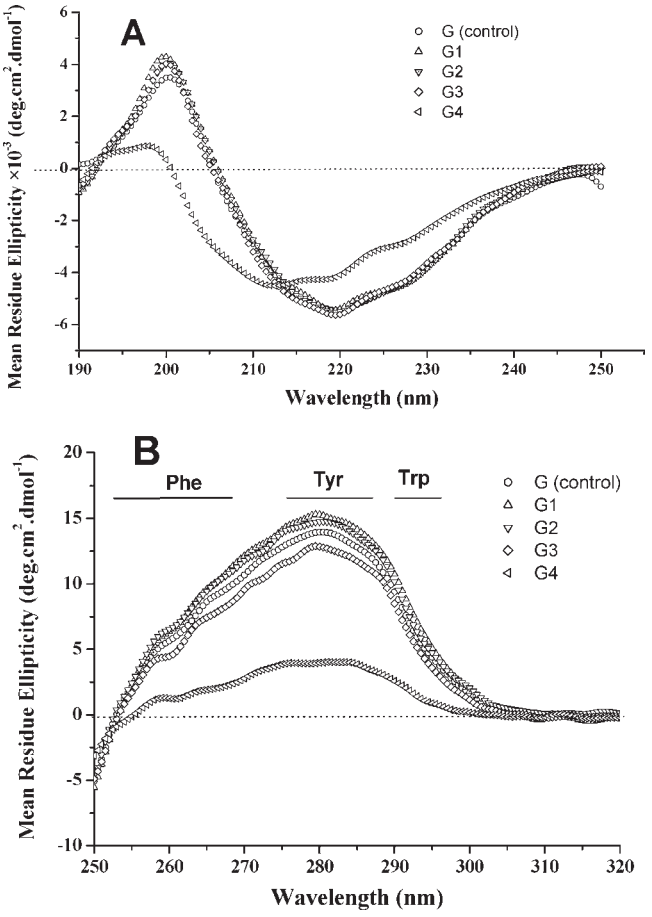

Figure 5. Far-UV (**A**) and near-UV (**B**) CD spectra of various mungbean globulins, solubilized in 10 mM phosphate buffer (pH 7.0). For the legends G, G1, G2, G3 and G4, refer to **Figure 1**.

fractionated globulins (G1-G4) is consistent with the reversal of the H_0 order (**Table 3**).

Emulsifying Ability. The emulsifying property is one of important physicochemical properties for the proteins that may directly affect the applications of the protein in food formulations. The emulsifying ability index (EAI) values of various mungbean globulins at various pHs are shown in **Figure 4**. All the samples exhibited similar EAI profiles with pH. The EAI values were minimum at pH 5.0, and gradually increased when the pH gradually deviated from this value (**Figure 4**), suggesting that the electrostatic repulsion interactions were of vital importance for the emulsifying property. The EAI profiles with the pH are basically consistent with the PS profiles (**Figure 3**), indicating a close relationship between the emulsifying ability and the protein solubility for mungbean globulins. The importance of PS on the EAI can also be well reflected as follows: G4 with much higher PS at pH 5.0 exhibited much higher EAI than various 8S globulins (**Figures 3** and **4**). Furthermore, it was observed that the extent of increase in EAI was much more evident at neutral and alkali pH than that at acidic pH (relative to the minimum). This phenomenon confirms that besides the PS (or surface charge), there are other parameters affecting the emulsifying ability of the proteins, e.g., surface hydrophobicity and conformational stability (*26*).

At pH 7.0 and 9.0, the zeta potential and PS values for various mungbean globulins were similar (**Figure 2**). However, the EAI values considerably varied with type of tested samples, and highest EAI was observed for G4, and then followed by G3, G2 and G1, and least for control (**Figure 4**). This observation is well in agreement with the H_0 data obtained at pH 7.0 (**Table 3**), indicating the importance of surface hydrophobicity for the emulsifying property.

Table 4. Secondary Structure Compositions of Various Mungbean Globulins as Calculated from Far-UV CD Spectra Using the CONTIN/LL Program in CDPro Software[a]

	secondary structure composition (%)			
protein samples	α-helix[b]	β-strand[c]	turns	random coil
G (control)	6.4 b	37.1 a	22.8 c	33.8 b
G1	4.5 d	37.5 a	23.6 b	34.3 b
G2	4.7 d	36.0 b	24.5 a	34.8 b
G3	5.2 c	36.4 b	24.4 a	34.0 b
G4	7.1 a	32.4 c	22.9 c	37.6 a

[a] Each data is the mean of duplicate measurements. Different letters (a−d) indicate a significant difference at the $p < 0.05$ level in the same column. For the legends G, G1, G2, G3 and G4, refer to **Figure 1**. [b] Combined regular and distorted α-helix. [c] Combined regular and distorted β-strands.

Far-UV and Near-UV CD Spectra. The secondary and tertiary (and even quaternary) conformations of various mungbean globulins were evaluated and compared using far-UV and near-UV CD spectra, respectively (**Figure 5**). Secondary structure elements such as α-helices and β-sheets have dichroic activity in the wavelength range from 190 to 260 nm (*27*). All the 8S globulins and the control showed similar far-UV spectra, with a prominent negative band centered at about 219 nm and a positive band at 200 nm (**Figure 5A**). These features are sufficient indicators of a highly ordered structure, most probably of the β types (*28*, *29*). The secondary structure compositions (including α-helix, β-strand, turns and random coil) of these globulins were calculated according to the CONTIN/LL program in CDPro software (*30*), and the results are displayed in **Table 4**. In the control, the secondary structure composition was 6.4% α-helix, 37.1% β-strand, 22.8% turns and 33.8% random coil. Various fractionated 8S globulins had similar secondary structure compositions, except that the α-helix contents (4.5−5.2%) were slightly lower than the control (**Table 4**).

In contrast, G4 exhibited an evidently different far-UV CD spectrum from those of 8S globulins (and the control). For this protein, two major negative bands centered at 212 and 219 nm, and a minor positive band at 198 nm, were observed (**Figure 5A**). The calculated secondary structure composition of G4 was evidently different from that of those 8S globulins (**Table 4**). In this protein, the secondary structure composition was 7.1% α-helix, 32.4% β-strand, 22.9% turns and 37.6% random coil. Much higher α-helix and random coil contents (relative to those 8S globulins) indicated that the secondary conformation of this protein was much more unordered and flexible.

The near-UV CD spectra of various 8S globulins (and control) similarly showed a prominent positive dichroic band at 280 nm and a minor shoulder band at about 261 nm (**Figure 5B**). These two bands were near-UV CD characteristics of Tyr and Phe residues, respectively (*27*). The actual shape and magnitude of the near-UV CD spectrum of a protein will depend on the number of each type of aromatic amino acid present, their mobility, the nature of their environment (H-bonding, polar groups and polarizability), as well as their spatial disposition in the protein (*27*). The differences in Tyr near-UV CD ellipticity between various 8S globulins seemed to be due to the differences in Tyr residue contents in these globulins (**Table 2**). However, this explanation is distinctly not applicable for the Phe case. In this case, the Phe residue of G3 was higher than that of G1 or G2, but the ellipticity of the former was less. Thus, the differences in Phe or Tyr ellipticity might be more possibly due to the differences in extent of protein tertiary and/or quaternary structure unfolding, or tertiary and quaternary conformational flexibility. Lower ellipticity in G3 (relative to G1 or G2) suggested

more unfolded tertiary conformation, or exposure of aromatic amino acids. This explanation is consistent with the conclusion obtained from the H_0 and ΔH data (**Table 3**).

Compared with the 8S globulins (and the control), the magnitude of the near-UV CD spectrum of G4 was considerably lower (**Figure 5B**), though the Tyr residue content in this protein was higher (**Table 3**). This indicated that the tertiary and quaternary conformation of the globulins in G4 was much more flexible and unfolded than those 8S globulins.

In conclusion, the physicochemical and conformational properties, including amino acid composition, surface net charge and hydrophobicity, free SH and SS contents, protein solubility, thermal stability and emulsifying property, as well as secondary and tertiary conformations of mungbean 8S and/or 11S globulins varied with their polypeptide constituents. The emulsifying ability of these globulins was closely dependent upon their protein solubility (or surface net charge), surface hydrophobicity and the heterogeneity of the polypeptides. The secondary and tertiary (and quaternary) conformations of 11S globulins were much nore flexible and unordered than 8S globulins (vicilins). There were no evident differences in secondary and tertiary conformations among the vicilins with different polypeptide constituents. These results can thus provide useful suggestions for possible utilization of these globulins from mungbean in the food processing industry and the research direction of protein engineering to improve their physicochemical properties. The mungbean globulins with higher 11S/7S globulin ratios or with higher polypeptide I ratios would exhibit better functionalities, e.g. solubility and emulsifying activities of industrial importance.

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