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Carbohydrate Moieties Contribute Significantly to the Physicochemical Properties of French Bean 7S Globulin Phaseolin

Aiko Kimura,[†] Mary Rose G. Tandang-Silvas,[†] Takako Fukuda,[†] Cerrone Cabanos,[†] Yasuhiro Takegawa,[‡] Maho Amano,[‡] Shin-Ichiro Nishimura,[‡] Yasuki Matsumura,[§] Shigeru Utsumi,[†] and Nobuyuki Maruyama*

[†]Laboratory of Food Quality Design and Development, Graduate School of Agriculture, Kyoto University, Uji, Kyoto, Japan, [‡]Graduate School of Advanced Life Science, Frontier Research Center for Post-genome Science and Technology, Hokkaido University, Sapporo, Japan, and [§]Laboratory of Quality Analysis and Assessment, Graduate School of Agriculture, Kyoto University, Uji, Kyoto, Japan

We have previously reported that the solubility of French bean 7S globulin (phaseolin) at low ionic strength and its emulsifying stability are remarkably high compared with those of 7S globulins prepared from other plant species, including soybean (Kimura et al. *J. Agric. Food Chem.* **2008**, *56*, 10273−10279). In this study, we examined the role of carbohydrate moieties in the properties of phaseolin. Three preparations of phaseolin were analyzed: (i) N7S, prepared from defatted seed meal and having intact carbohydrate moieties; (ii) R7S, expressed in *E. coli* and lacking N-linked glycans; and (iii) EN7S, having partial N-linked glycans after treatment with Endo H. The solubilities of N7S and EN7S were much higher than that of R7S at a low ionic strength (μ = 0.08). N7S exhibited good emulsifying ability under the conditions examined, but R7S did not. In terms of emulsion stability, an emulsion of R7S separated into two phases after 1 h at μ = 0.01, 0.08, and 0.5, whereas the emulsion of N7S was stable for 5 days at μ = 0.01 and for at least 10 days at μ = 0.08 and 0.5. The emulsion stability of EN7S was comparable to that of N7S under most conditions examined. These results indicate the carbohydrate modifications are necessary for the good solubility, emulsifying ability, and emulsion stability of phaseolin. Further, a structural analysis of the carbohydrate moieties indicates that truncated carbohydrate moieties are sufficient for conferring these physicochemical properties to phaseolin.

KEYWORDS: Phaseolin; carbohydrate moiety; solubility; emulsifying ability

INTRODUCTION

Seed storage proteins are an important protein source for humans and livestock. Among the legume seed proteins, soybean proteins are mostly utilized for the manufacture of processed foods. The physicochemical properties of soybean proteins have been more extensively studied than those of other seed proteins, and these studies have significantly contributed to the increase in their utilization for food production (*1*, *2*).

In general, legume seeds contain storage proteins such as 7S and/or 11S globulins. We compared the physicochemical properties of 11S and/or 7S globulins from French bean (*Phaseolus vulgaris* L.), fava bean, cowpea, and pea with those from soybean and found that French bean 7S globulin (phaseolin) exhibits remarkably high solubility at low ionic strength and emulsion stability (*3*). 7S globulins from soybean, cowpea, mungbean, Azuki bean, and French bean are N-glycosylated. The α and α′ subunits of soybean 7S globulin (β-conglycinin) and phaseolin are N-glycosylated at 2 positions (*4*). The 2 carbohydrate groups occur closer together in phaseolin than in β-conglycinin (*3*). Consequently, the carbohydrate moieties form a cluster in phaseolin but not in the α and α′ subunits of β-conglycinin.

The effects of the carbohydrate moieties of β-conglycinin on its structure−function relationships have been examined in detail (*5*,*6*). The carbohydrate moieties contribute to the solubility of all subunits and to the emulsifying ability of the β subunit. Further, the carbohydrate moieties affect the association of β-conglycinin subunits in response to heat treatment. These observations suggest that the carbohydrate moieties play an important role in the physicochemical properties and functions of 7S globulins.

Escherichia coli does not possess an N-glycosylation system (*7*). Therefore, we can prepare non-N-glycosylated phaseolin by using an *E. coli* expression system. Endo H is an enzyme that cleaves the carbohydrate moiety within the chitobiose core of N-linked glycoproteins (*8*). In this study, we employed these tools in order to prepare phaseolins lacking N-linked glycans (using the *E. coli* expression system) and phaseolins with truncated N-linked

*Corresponding author: Nobuyuki Maruyama Laboratory of Food Quality Design and Development, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan. Telephone: +81-774-38-3763. Fax: +81-774-38-3761. E-mail: marunobu@kais.kyoto-u.ac.jp.

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glycans (by treatment with Endo H). Comparison of the solubility and emulsion stability of native, recombinant, and Endo H-treated phaseolins indicates that the carbohydrate groups are necessary for the remarkably high solubility and emulsion stability of phaseolin. Structural analysis of the carbohydrate moieties of phaseolin indicates that Endo H treatment removed only $Man_5GlcNAc_2$, $Man_6GlcNAc_2$, and $Man_7GlcNAc_2$, resulting in a polypeptide with Xyl-$Man_3GlcNAc_2$ at position N1 or with $Man_8GlcNAc_2$ or $Man_9GlcNAc_2$ at position N2. Therefore, 1 carbohydrate group in a cluster is sufficient to exhibit these properties of phaseolin.

MATERIALS AND METHODS

Construction of Expression Plasmids for Phaseolin. The full-length cDNA encoding the phaseolin β subunit was generated by RT-PCR using primers based on the nucleotide sequence (accession number, X03004). Total RNA was isolated from developing French bean cotyledons according to Shirzadegan et al. (*9*). mRNA was transcribed into cDNA by the primer 5′-CGCGGATCCGGTACCCTGCAGGTCGACTTTTTTTT-TTTTTTTTT-3′, which contains the region complementary to poly(A) and 4 restriction enzymes sites. The primers (5′-ATGAT-GAGAGCAAGGGTTCCAGTCC-3′) and (5′-CGCGGATCC-TATTCAGTACACAAATG-3′) corresponding to the N- and C-termini, respectively, were used. The primer for the C-termini contained a *Bam*HI site next to the stop codon. The PCR product was subcloned into pBlurScript. Further, the PCR product corresponding to the mature region was blunted and then digested with *Bam*HI. The resultant DNA fragment was inserted into the *Nco*I (filled-in) and *Bam*HI sites of the pET-21d vector (Novagen, USA) to construct the expression plasmid pEPha.

The coding sequence for phaseolin was also transferred to a glutathione S-transferase (GST) fusion vector, pGEX-6p-1. The primers (5′-CGC**GGATCC**GCCACTTCACTCCGGGAG-3′ and 5′-CGC**GAATTC**TCAGTACACAAATGCACCCTTTC-TTCCC-3′) were used for amplifying the nucleotides encoding phaseolin. The boldfaced sequences are *Bam*H1 and *Eco*R1 restriction sites. The PCR product was digested by *Bam*H1 and *Eco*R1. The resultant DNA fragment was inserted into the *Bam*H1 and *Eco*R1 sites of pGEX-6p-1 to construct the expression plasmid pGEXpha.

Sequences were confirmed by the dideoxy sequencing method using an ABI Prism 3100 DNA analyzer (Applied Biosystems, USA).

Expression and Purification of Recombinant Phaseolin. *E. coli* strains—HMS174(DE3), BL21(DE3), AD494(DE3), and Origami-(DE3)—were transformed with pEpha. Forty microliters of fully grown cultures were used to inoculate fresh 4 mL volumes of LB media and cultured at 37 °C. At A_{600} = 0.4−0.6, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. The recombinant proteins were expressed by incubating the culture at 20 °C for 15−24 h. Harvested cells were resuspended (15 mg of cells/mL of buffer) in buffer A (35 mM sodium phosphate (pH 7.6), 0.4 M NaCl, 1 mM EDTA, 0.1 mM *p*-amidinophenyl-methylsulfonyl fluoride (*p*-APMSF), 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 0.02% NaN_3, and 10 mM β-mercaptoethanol) and disrupted by sonication on ice.

The *E. coli* strain BL21(DE3) was transformed with pGEX-pha. One milliliter of fully grown cells was used to inoculate 500 mL of fresh LB media for culture at 37 °C. At A_{600} = 0.4−0.6, IPTG was added to a final concentration of 1 mM. The cells were further incubated at 20 °C for 24 h to allow the expression of recombinant proteins. Harvested cells were resuspended in PBS buffer (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.3), 0.1 mM *p*-APMSF, 1.2 μM leupeptin, 0.2 μM pepstatin A, and 1 mM DTT) and disrupted by sonication on ice. After centrifugation, the supernatant was applied to a Glutathione Sepharose 4B column (GE Healthcare Bio Science), and the recombinant GST-tagged phaseolin was eluted in the elution buffer (0.2 M NaCl, 50 mM Tris-HCl (pH 8.0), and 10 mM reduced glutathione). After removal of the GST tag by PreScission Protease (GE Healthcare Bio Science), the protein was applied to a gel filtration column (HiPrep 26/60 Sephacryl S-200 HR column; GE Healthcare Bio Science). The fractions containing the peak of recombinant phaseolin were further purified on a Mono Q HR 10/10 column (GE Healthcare Bio Science).

Purification of Native Phaseolin. Isolation of native phaseolin from French bean was done as described by Kimura et al. (*3*). Protein extracted from each defatted seed meal using buffer B (30 mM Tris-HCl (pH 8.0), 10 mM β-mercarptoethanol, 1 mM EDTA, 0.1 mM *p*-APMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, and 0.02% NaN_3) was precipitated with ammonium sulfate of 55−80% saturation to obtain crude native phaseolin. For further purification, the crude phaseolin fractions were applied to a gel filtration column (Hi-Prep 26/60 Sephacryl S-300 HR; GE Healthcare Bio Science) using buffer A as the mobile phase.

Purification of Soybean β-Conglycinin from Soybean Seeds. β-Conglycinin was prepared according to the procedure described by Nagano et al. (*10*).

Glycosidase—Treatment of Native Phaseolin from Seeds. Native phaseolin (3.0 mg/mL) was dialyzed into the reaction buffer (0.45 mM NaCl, 50 mM sodium citrate, pH 5.6) and treated with Endo H (New England Biolabs, USA) for 48 h.

Protein Measurement. Protein concentrations were determined using a Protein Assay Rapid Kit (Wako, Osaka, Japan) with bovine serum albumin as the standard.

Analysis of Self-Assembly. Self-assembly by correct folding was analyzed using a HiPrep 26/60 Sephacryl S-300 HR column (GE Healthcare Bioscience) at pH 7.6 and μ = 0.5 (35 mM sodium phosphate (pH 7.6), 0.4 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM *p*-APMSF, 1.2 μM leupeptin, 0.2 μM pepstatin A, and 0.02% (w/v) NaN_3) at a flow rate of 1.0 mL/min, as described previously (*4*).

Thermal Stability. The thermal denaturation profiles of the samples were studied by differential scanning calorimetry (DSC) experiments using a MicroCal MC-2 ultrasensitive microcalorimeter (MicroCal Inc.) at a rate of 1 °C/min, as described previously (*4*). Protein samples of 0.5 mg/mL were prepared in buffer A.

Surface Hydrophobicity. Surface hydrophobicity was measured by hydrophobic column chromatography using Phenyl Sepharose 6 Fast Flow and Butyl Sepharose 4 Fast Flow columns (GE Healthcare Bio Science), as described previously (*5*, *6*). Samples were dialyzed against buffer A containing 2.3 M ammonium sulfate and applied on columns equilibrated with the same buffer. The samples were eluted with a linear gradient of 2.3−0 M ammonium sulfate over a period of 80 min at a flow rate of 0.25 mL/min.

Solubility as a Function of pH. The protein solutions (0.8 mg/mL) were kept at 4 °C for 18 h at various pH values and at the following ionic strengths (ionic strength was calculated from the salt compositions of buffers) of 0.5, 0.08, and 0.006 and then centrifuged at 4 °C (10,000g × 20 min). The pH solubility profiles were obtained by measuring the protein concentrations in the supernatants (i.e., the soluble fractions) using a Protein Assay Rapid Kit (Wako, Osaka, Japan). The percentage of protein

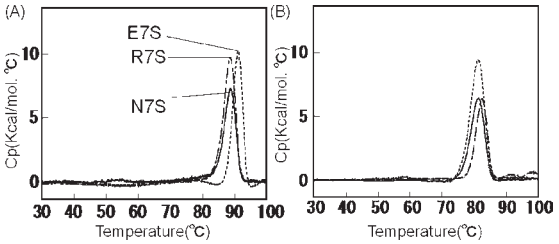

Figure 1. Differential scanning calorimetry scans of N7S, EN7S, and R7S.

solubility was calculated as the ratio of soluble protein to total initial protein.

Solubility as a Function of Ionic Strength. The protein solutions (0.8 mg/mL) were dialyzed in a buffer (0.5 M NaCl, 10 mM NaPi, 1 mM EDTA, 0.1 mM *p*-APMSF, 1.2 μM leupeptin, 0.2 μM pepstatin A, and 0.02% (w/v) NaN_3) and adjusted to the final ionic strength for measurement. They were kept at 4 °C for 18 h and then centrifuged at 4 °C (10,000g × 20 min). The solubility profiles were obtained by measuring the protein concentration in the supernatant (i.e., as the soluble fraction) using a Protein Assay Rapid Kit (Wako, Osaka, Japan). The percentage of protein solubility was calculated as the ratio of soluble protein to total initial protein.

Emulsifying Ability and Stability. Samples were analyzed as described previously (*5*,*6*). Protein solutions (1.5 mL of 0.5 mg/mL) at pH 7.6 and at ionic strengths of 0.5, 0.1, 0.08, 0.03, 0.01, and 0.006 were homogenized and sonicated with 0.25 mL of soybean oil. Each sample was measured at least thrice, and a representative typical pattern was presented.

The emulsions were kept at room temperature without agitation and visually observed after 1 h, 20 h, and 3, 5, 10, and 18 days to assess stability.

Glycan Analysis of Phaseolin by Glycoblotting and MALDI-TOF-MS. Native phaseolin (10 μg), with or without Endo H treatment, was digested with 1 μg mass spectrometry-grade trypsin (Wako, Osaka, Japan) at 37 °C for 7 h in 20 μL of 20 mM ammonium bicarbonate buffer (pH 7.8). Then, glycans of native phaseolin (5 μg) were released by treating with 0.5 mU glycopeptidase A from *Almond* (Seikagaku Co., Tokyo, Japan) at 37 °C for 18 h in 20 μL of 100 mM acetate buffer (pH 4.0). Glycans released by glycopeptidase A and Endo H were analyzed according to the previously reported glycoblotting technology (*11*−*13*) and MALDI-TOF MS. The released glycans (from 5 μg phaseolin) were briefly spiked with A2amide glycan (an internal standard, 20 pmol) and enriched by BlotGlyco H beads (Sumitomo Bakelite Co., Tokyo, Japan). The blotted glycan was recovered upon adding the aminooxy-functionalized labeling reagent, aoWR. The excess reagent (aoWR) was removed using a HILIC microelution plate (Waters, Milford, MA). An aliquot (2.5 μL) of the recovered aoWR-derivatized glycan was mixed with 0.5 μL of 2,5-dihydroxybenzoic acid (DHB; 10 mg/mL in 30% acetonitrile) and subjected to MALDI-TOF MS analysis using an Ultraflex II time-of-flight mass spectrometer (Bruker Daltonics) controlled by the Flex Control 3.0 software package. MS spectra were obtained using a reflectron mode with an acceleration voltage of 25 kV, a reflector voltage of 26.3 kV, and a pulsed ion extraction of 160 ns in the positive ion mode. The spectra obtained were the results of signal averaging of 4000 laser shots. All peaks were picked by FlexAnalysis 3.0 using the SNAP algorithm that fits isotopic patterns to the matching experimental data.

Glycopeptide Analysis of Phaseolin by LC/ESI-MS. The tryptic peptides of native phaseolin with or without Endo H treatment were analyzed using an UltiMate 3000 nano-HPLC system (Dionex Co., Sunnyvale, CA) connected to a 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer equipped with a NanoSpray source (Applied Biosystems/MDS Sciex, Toronto, Canada). The HPLC and MS systems were both controlled using Analyst software 1.4.1. Samples (phaseolin 25 ng) were directly loaded on a C18 reversed-phase column (MonoCap for fast-flow, 0.05 mm i.d. × 250 mm; GL Sciences Inc., Tokyo, Japan) in a column oven at 40 °C and eluted with an acetonitrile gradient (0−42% acetonitrile in 65 min, followed by a

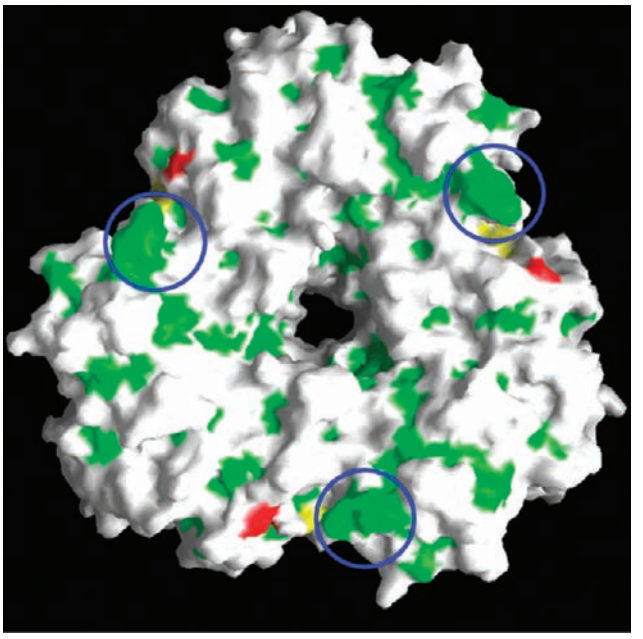

Figure 2. Distribution of hydrophobic residues on the molecular surface of French bean 7S. Ala, Val, Leu, Ile, Met, Pro, Phe, Tyr, and Trp are in green, and Asn with N-linked glycans are in red (Asn228; position N1) and yellow (Asn317; position N2).

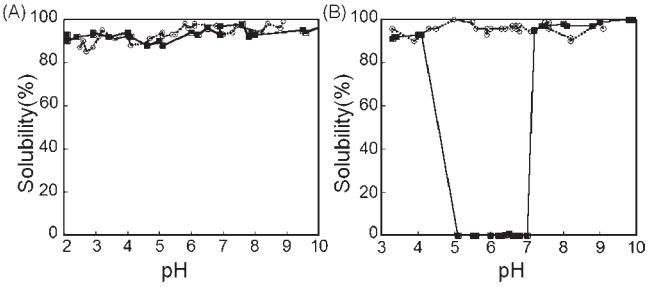

Figure 3. Dependence of the solubility of N7S and R7S on pH at ionic strength: (**A**) 0.5; (**B**) 0.08; ○, N7S; ■, R7S.

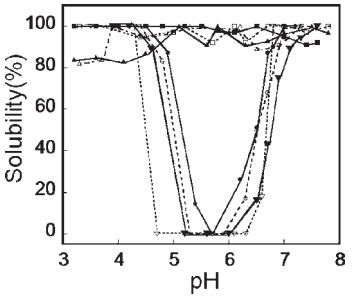

Figure 4. Dependence of the solubility of N7S (solid line; closed symbol) and EN7S (dashed line; open symbol) on pH at the ionic strengths 0.5 (■, □), 0.1 (▲, △), 0.03 (●, ○), and 0.006 (▼, ▽).

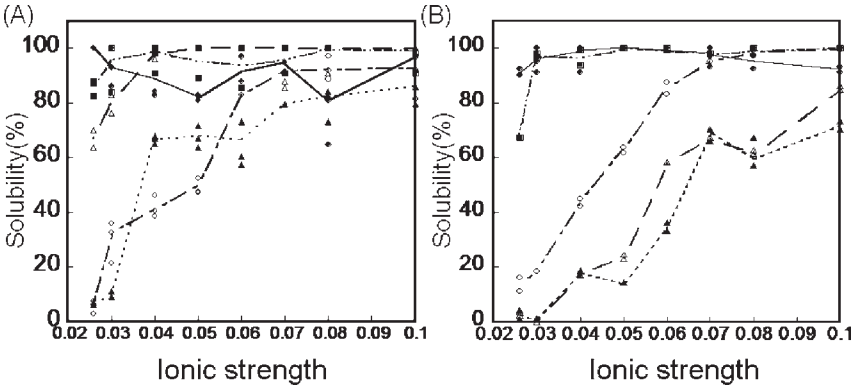

Figure 5. Dependence of the solubility of N7S (**A**) and EN7S (**B**) on ionic strength at the pH values 5.0 (●), 5.5 (○), 6.0 (▲), 6.5 (△), and 7.0 (■).

5-min wash with 70% acetonitrile) containing 0.1% formic acid at a flow rate of 500 nL/min. Mass spectra were recorded in the information dependent acquisition (IDA) mode with an enhanced MS scan (EMS), an enhanced resolution scan (ER), and an enhanced product ion scan (EPI) for the 2 most intense ions. The total cycle time for this method was 2.4 s. The ESI settings were as follows: interface heater temperature, 150 °C; ion source and curtain gas, 18 and 10, respectively; capillary voltage, +3000 kV; declustering potential, 50. The obtained MS/MS spectra were searched against the Mass Spectrometry Protein Sequence Database (MSDB) to assign peptide sequences using the Mascot search engine (Matrix Science, London, U.K.).

RESULTS AND DISCUSSION

Preparation of Three Types of Phaseolin. *E. coli* strains—HMS174(DE3), BL21(DE3), AD494(DE3), and Origami-(DE3)—were transformed with the expression plasmid pEPha. All hosts did not show high-level expression in a small-scale experiment (data not shown). Therefore, we replaced the pET expression vector with pGEX . BL21(DE3) cells were transformed with the expression plasmid, pGEXpha, which resulted in relatively high expression (recombinant protein constituted about 20% of total proteins). The expressed recombinant protein was purified to near homogeneity by affinity column chromatography. The eluate fractions containing recombinant phaseolin were designated as R7S. We confirmed that R7S has no carbohydrate moiety (data not shown).

Native phaseolin from the French bean was purified from extracts of defatted seed meal and designated as N7S. N7S treated with Endo H was designated as EN7S. The mobility of EN7S, by SDS-PAGE, was found to be between those of R7S and N7S (data not shown). N7S consisted of a minor band and a major band which correspond to the 2 differentially glycosylated formas of phaseolin (*14*). This indicates that the carbohydrate moieties of N7S were partially removed by Endo H.

Self-assembly. Previously, we showed that gel filtration chromatography is ideal for examining the self-assembly of proteins into trimers (*15*). To assess self-assembly of N7S, EN7S, and R7S into trimers, they were applied onto a gel filtration column, Sephacryl S-200 HR. The elution times for N7S, EN7S, and R7S were 120.7 min, 121.0 min, and 124.6 min, respectively (data not shown). The elution time of R7S was slightly longer than those of N7S and EN7S, indicating that the carbohydrate moieties of N7S and EN7S affect the elution times and that R7S self-assembled into trimers in a manner similar to N7S and EN7S.

Thermal Stability. The DSC profiles of N7S, EN7S, and R7S at $\mu = 0.5$ and 0.08 are shown in **Figure 1**. The T_m values of N7S, EN7S, and R7S were 88.6 °C, 88.5 °C, and 90.0 °C at $\mu = 0.5$ (**Figure 1A**), respectively, and 81.5, 81.9, and 82.5 °C at $\mu = 0.08$ (**Figure 1B**), respectively. These indicate that the thermal stabilities of N7S, EN7S, and R7S are very similar to each other, and the carbohydrate moiety of phaseolin does not affect this property. We have previously demonstrated that the carbohydrate moieties of β-conglycinin do not affect thermal stability (*5*). The destabilization effected by deglycosylation depends on the original carbohydrate content (*16*). β-Conglycinin and phaseolin have 1 or 2 carbohydrate moieties per subunit. This carbohydrate content of 7S globulins might be too small to influence thermal stability.

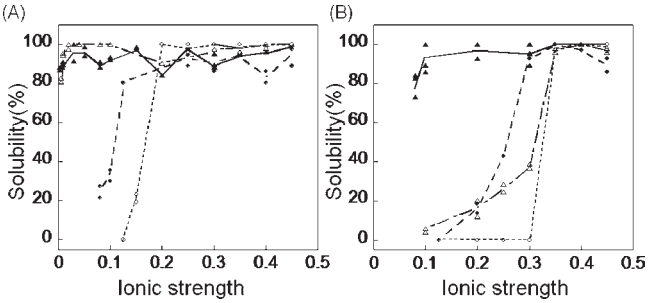

Figure 6. Dependence of the solubility of N7S and R7S on ionic strength at pH 7.6 (**A**) and pH 6.0 (**B**) compared with that of β-conglycinin β-native homotrimer and the recombinant homotrimer. Symbols: ▲, N7S; △, R7S; ●, native β homotrimer; ○, recombinant β homotrimer.

Table 1. Emulsifying Activity of the Protein Samples at Various Ionic Strengths

	mean droplet diameter (μm)					
sample	$\mu = 0.006$	$\mu = 0.01$	$\mu = 0.03$	$\mu = 0.08$	$\mu = 0.1$	$\mu = 0.5$
N7S	4.33/0.3[a]	1.78/0.09	2.51/0.5	2.04/0.4	2.51/0.1	2.71/0.2
R7S		10.6/0.2		12.1/0.3		11.4/0.2
EN7S	9.57/0.1		3.83/0.1		2.31/0.01	

[a] Standard error.

Surface Hydrophobicity. Surface hydrophobicity of proteins is related to their physicochemical properties, such as emulsifying ability, forming ability, and solubility (*17*, *18*). We employed 2 columns (phenyl sepharose and butyl sepharose) to judge the surface hydrophobicity. The elution times for R7S were longer than those for N7S on both columns: butyl sepharose: N7S, 36.5 min, R7S, 59.4 min, and EN7S, 37.9 min; phenyl sepharose: N7S, 52.0 min, R7S, 76.2 min, and EN7S, 53.8 min. In contrast, the elution times of EN7S were very similar to those of N7S. These results indicate that carbohydrate moieties affect the surface

hydrophobicity of N7S. Phaseolin has 2 possible N-glycosylation sites, designated N1 (red) and N2 (yellow) in **Figure 2**. There is a relatively large hydrophobic patch (shown as a blue circle in **Figure 2**) on the surface near the carbohydrate moieties. The finding that N7S and EN7S display lower hydrophobicity than R7S suggests that the carbohydrate groups probably cover the hydrophobic patch. The N-glycosylated native form of mungbean vicilin also has a lower hydrophobicity than that of the recombinant form (*19*). Thus, it is likely that the carbohydrate groups on 7S globulins influence their hydrophobicity.

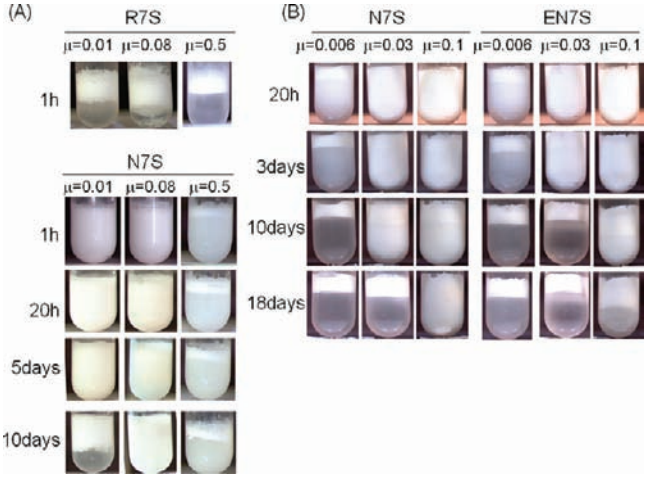

Figure 7. Emulsion stability. (**A**) Time-dependence of the emulsion stability of N7S and R7S at μ = 0.01, 0.08, and 0.5. (**B**) Emulsion stability of N7S versus EN7S at μ = 0.006, 0.03, and 0.1 after 18 days at room temperature.

Solubility. The solubility of proteins is the most important determinant of physicochemical properties such as gelation, emulsification, and foaming (*20*, *21*). The solubilities of N7S and R7S were examined at μ = 0.5 and μ = 0.08 (**Figure 3**). All samples were completely soluble at μ = 0.5 and at the pH values examined (**Figure 3A**). In contrast, R7S, which does not have any carbohydrate moiety, was insoluble between pH 5.0 and 7.0 at μ = 0.08 (**Figure 3B**). These results indicate that the carbohydrate moieties of phaseolin play an important role in its solubility at pH values near its isoelectric point at μ = 0.08. The carbohydrate moieties of mungbean vicilin and β-conglycinin also contribute to their solubility at low ionic strength (*5*−*19*). These findings indicate that the carbohydrate moieties of 7S globulins play an important role in preventing aggregation at low ionic strength.

In a preliminary experiment, we found that the solubility of EN7S is similar to that of N7S. Therefore, we examined the differences in solubility between N7S and EN7S as a function of pH at various ionic strengths (**Figure 4**). The solubility profiles for EN7S and N7S were similar at all ionic strengths. At μ = 0.006, EN7S was insoluble between pH 4.7 and 6.3—a slightly broader pH range than that seen for N7S. Considering solubility as a function of ionic strength at pH values of 5.0, 5.5, 6.0, 6.5, and 7.0 (**Figure 5**), we found that the solubility profiles of EN7S and N7S at pH = 5.0, 5.5, 6.0, and 7.0 were similar. However, N7S

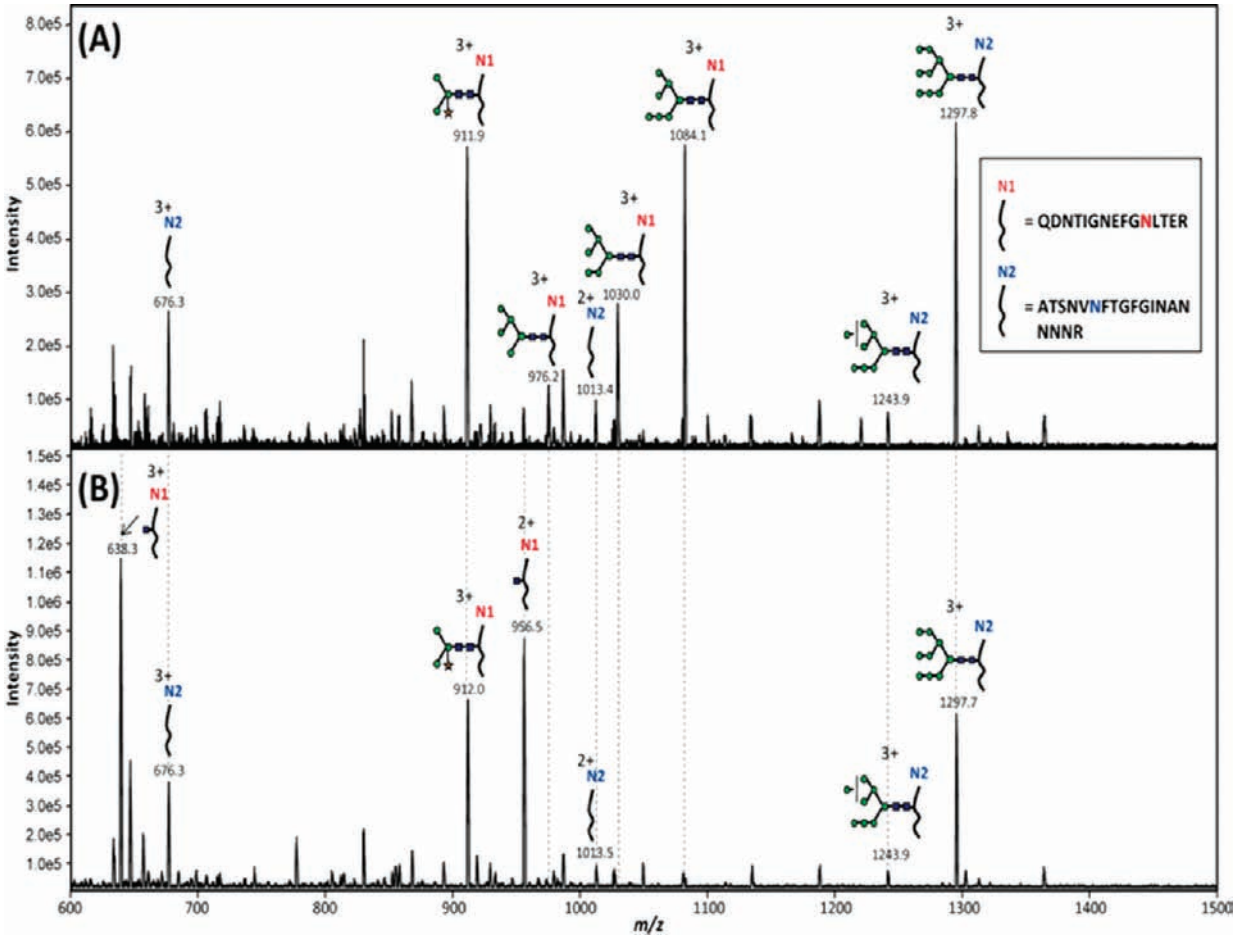

Figure 8. LC/ESI-MS spectra showing the tryptic *N*-glycopeptide profiles of N7S (**A**) and EN7S (**B**). Mass spectra were accumulated from retention times of 33.5−37.0 min. Annotated peaks were detected as doubly (2+) or triply (3+) protonated ions, and their structures were identified by MS/MS analysis as discussed in **Figure 9**. The amino acids are represented in their single letter codes.

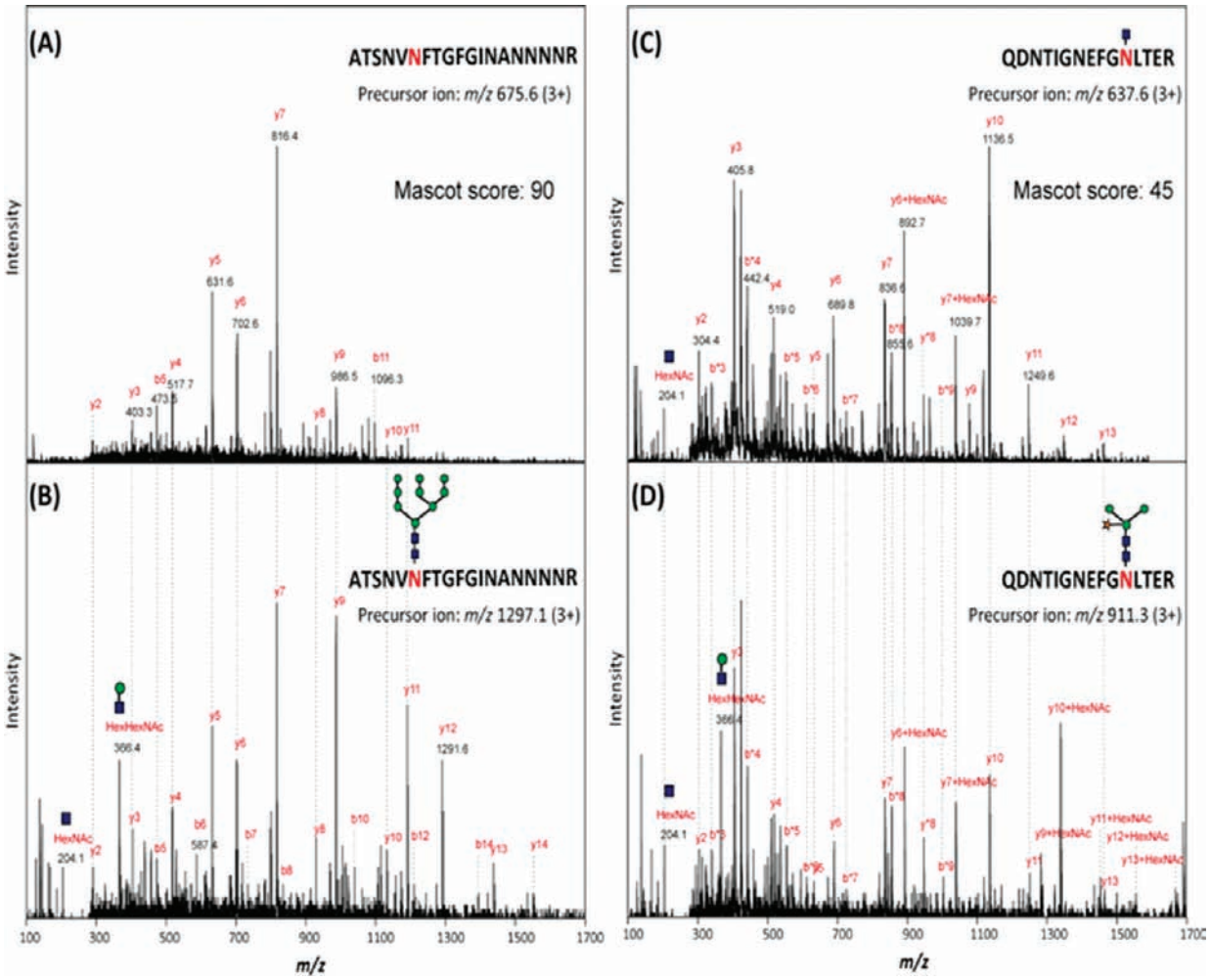

Figure 9. LC/ESI-MS/MS identification of glycopeptides on the 2 glycosylation sites of phaseolin. (**A** and **B**): Comparison of MS/MS spectra of nonglycosylated peptide and glycopeptides with the same peptide sequence. (**C** and **D**): Comparison of MS/MS spectra of peptides carrying 1 GlcNAc after Endo H treatment and glycopeptides with the same peptide sequence. Accurate charge state and *m/z* information of the precursor ion was obtained by an enhanced resolution scan.

exhibited 20−60% higher solubility than EN7S at pH 6.5. These results show that although the truncated carbohydrate groups remaining in EN7S are sufficient to conger similar solubility to that of N7S under most conditions, a loss of solubility of EN7S does become evident under specific conditions (e.g., at pH 6.5).

β-Conglycinin has 3 subunits: α, α′, and β. The β subunit comprises only the core region similar to phaseolin, whereas the α and α′ subunits contain the extension regions in addition to the core region (*4*). We have reported that the β subunit of β-conglycinin is insoluble above pH 5.0 (*5*, *6*), in contrast to our observation that R7S was soluble above pH 7.0 at $\mu = 0.08$; these findings indicated differences in solubility existed between the 2 proteins. To examine the difference in detail, we compared the solubilities of the β-conglycinin β subunit and phaseolin at pH 6.0 and 7.6 under various ionic strengths (**Figure 6**). The native form of the β-conglycinin β subunit was purified from soybean seeds, and the recombinant β-conglycinin β subunit was generated using an *E. coli* expression system developed previously (*5*, *6*). The solubilities of native and recombinant β-conglycinin β subunits were found to decrease at $\mu = 0.2$ and 0.15, respectively, at pH 7.0 (**Figure 6A**). N7S and R7S were completely soluble at all ionic strengths examined at pH 7.0. On the other hand, R7S showed higher solubility than the recombinant β-conglycinin β subunit at $\mu = 0.1-0.3$ and pH 6.0. These findings indicate that phaseolin without carbohydrate moieties has a higher solubility than the β-conglycinin β subunit without carbohydrate moieties and suggest that the protein portion of phaseolin, in addition to the carbohydrate moiety, may also contribute to solubility.

Emulsifying Ability and Emulsion Stability. The emulsification properties of proteins are one of the most important physicochemical properties for food processing. We assessed the emulsifying abilities of the phaseolin samples by measuring the sizes of their emulsions—the smaller the size, the better the emulsifying ability. We prepared emulsions of N7S, EN7S, and R7S at various ionic strengths (**Table 1**). N7S showed good emulsifying ability under all ionic strengths examined here ($\mu = 0.06$, 0.01, 0.03, 0.08, 0.1, and 0.5). R7S exhibited reduced emulsifying ability compared with N7S at $\mu = 0.01$, 0.08, and 0.5. These results indicate that the carbohydrate moieties play an important role in the emulsifying ability of phaseolin. On the other hand, EN7S, which contains truncated carbohydrate groups, showed an emulsifying ability comparable with that of N7S at $\mu = 0.03$ and 0.1, although it exhibited slightly reduced emulsifying ability at $\mu = 0.006$.

We further examined the emulsion stability of the phaseolin samples (**Figure 7**). The emulsion of R7S separated into 2 phases after 1 h at $\mu = 0.01$, 0.08, and 0.5, whereas the emulsion of N7S was stable for 5 days at $\mu = 0.01$ and during at least 10 days at $\mu = 0.08$ and 0.5. These results indicate that the carbohydrate moieties have an important influence on both emulsifying ability

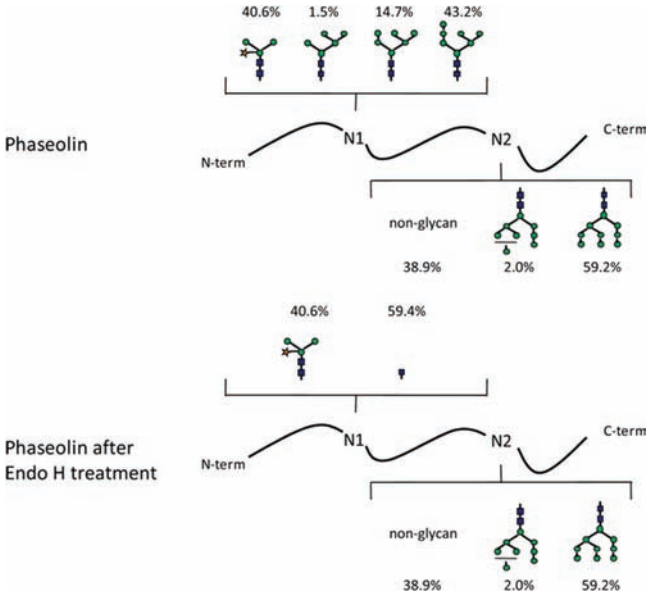

Figure 10. Summary of carbohydrate moieties of N7S (phaseolin) and EN7S identified by mass spectrometry.

and emulsifying stability. In comparison, the emulsion stability of N7S was very similar to that of EN7S under the conditions examined (**Figure 7B**), with only a slight difference observed after 18 days. These results indicate that the carbohydrate moiety of EN7S is sufficient for it to exhibit high emulsion stability under most conditions.

The emulsion stability of the native form of the β-conglycinin β subunit is low, even without removal of its carbohydrate groups (data not shown). Thus, the protein portion of phaseolin, in addition to its glycosylation, might play a role in emulsifying ability as well as solubility.

Carbohydrate Moiety Structure of EN7S. Phaseolin is encoded by a small gene family and divided into 2 highly homologous classes, α and β (*22*). Polypeptides of both classes are synthesized as either of 2 glycoforms, containing 1 or 2 glycan chains (N1 and N2 in **Figure 2**) (*14*). For polypeptides having only 1 glycan chain, the glycan is the complex type. For polypeptides having 2 glycan chains, both chains are the high-mannose type (*14*).

We analyzed the carbohydrate moiety of EN7S in detail. First, we examined the carbohydrate moiety from N7S by treatment of EndoH (data not shown). The treatment by Endo H produced $Man_5GlcNAc_2$, $Man_6GlcNAc_2$, and $Man_7GlcNAc_2$, which means that Endo H did not remove $Man_8GlcNAc_2$, $Man_9GlcNAc_2$, and Xyl-$Man_3GlcNAc_2$ of N7S. Next, we examined the glycopeptides of N7S and EN7S by tryptic digestion and analyzed several peaks by MS/MS methods (**Figure 8**). The peptide in both N7S and EN7S was identified as ATSNVNFTGFGINANNNNR by searching against the MSDB database using the Mascot search engine (**Figures 8** and **9A**). It contains the tripeptide consensus sequence for N-glycosylation (underlined; N-X-S/T, where X can be any amino acid except proline; sequences including position N2). The MS/MS spectrum of a triply protonated ion (m/z 1297.1) shows glycan specific b-type fragment ions (HexNAc; m/z 203, HexHexNAc; m/z 366) and similar b/y-type fragment ions of the peptide with extensive glycan degradations (**Figure 9B**). In addition to this characteristic MS/MS spectrum, a mass difference of the precursor ion between peptide and glycopeptides indicates that this glycopeptide was ATSNVNFTGFGINANNNNR + $Man_9GlcNAc_2$. This indicates that both N7S and EN7S exist as glycosylated and non-glycosylated forms at position N2.

The MS/MS spectrum of another triply protonated ion (m/z 637.6) was detected in only EN7S (**Figures 8B** and **9**). This peptide was identified as QDNTIGNEFGNLTR (sequences including position N1) + HexNAc. The glycopeptides (MS/MS spectrum of the triply protonated ion; m/z 911.3) in both N7S and EN7S were identified as QDNTIGNEFGNLTR + Xyl-$Man_3GlcNAc_2$. Further, we quantified the compositions of peptides at the positions N1 and N2 based on the extent of glycosylation. These results indicate that Endo H removes $Man_5GlcNAc_2$, $Man_6GlcNAc_2$, and $Man_7GlcNAc_2$ at position N1 and that Xyl-$Man_3GlcNAc_2$ at position 1 and $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ at position 2 were not removed by Endo H (**Figure 10**). Therefore, EN7S is composed of the polypeptide with Xyl-$Man_3GlcNAc_2$ at position N1 or with $Man_8GlcNAc_2$ or $Man_9GlcNAc_2$ at position N2. These findings indicate that phaseolin with at least 1 carbohydrate moiety per subunit can exhibit the favorable physicochemical properties observed.

Conclusion. The carbohydrate moiety of phaseolin is required for good solubility, emulsifying ability, and emulsion stability of the protein. One carbohydrate moiety per subunit was found to be sufficient to confer these properties, suggesting that the clustering of 2 carbohydrate groups is not required.

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LITERATURE CITED

(1) Utsumi, S. Plant food protein engineering. In *Advances in Food and Nutrition Research*; Kinsella, J. E., Ed.; Academic Press: San Diego, CA, 1992; pp 89−208.

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

(3) Kimura, A.; Fukuda, T.; Zhang, M.; Motoyama, S.; Maruyama, N.; Utsumi, S. Comparison of physicochemical properties of 7S and 11S globulins from pea, fava bean, cowpea, and French bean with those of soybean-French bean 7S globulin exhibits excellent properties. *J. Agric. Food Chem.* **2008**, *56*, 10273–10279.

(4) 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.

(5) Maruyama, N.; Salleh, M. R.; Takashashi, K.; Yagasaki, K.; Goto, H.; Hontani, N.; Nakagawa, S.; Utsumi, S. The effect of the N-linked glycans on structural features and physicochemical functions of soybean β-conglycinin homotrimers. *J. Am. Oil Chem. Soc.* **2002**, *79*, 257–291.

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

(7) Baneyx, F.; Mujacic, M. Recombinant protein folding and misfolding in *Eschelichia coli*. *Nat. Biotechnol.* **2004**, *22*, 1399–1408.

(8) Maley, F.; Trimble, R. B.; Tarentino, A. L.; Plummer, T. H., Jr. Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. *Anal. Biochem.* **1989**, *180*, 195–204.

(9) Shirzadegan, M.; Christie, P.; Seemann, J. R. An efficient method for isolation of RNA from tissue cultured plant cells. *Nucleic Acids Res.* **1991**, *19*, 6055.

(10) Nagano, T.; Hirotsuka, M.; Mori, H.; Kohyama, K.; Nishinari, K. Dynamic viscoelastic study on the gelation of 7S globulin from soybean. *J. Agric. Food Chem.* **1992**, *40*, 941–944.

(11) Nishimura, S.-I.; Niikura, K.; Kurogochi, M.; Matsushita, T.; Fumoto, M.; Hinou, H.; Kamitani, R.; Nakagawa, H.; Deguchi, K.; Miura, K.; Monde, K.; Kondo, H. *Angew. Chem., Int. Ed.* **2005**, *44*, 91−96.

(12) Miura, Y.; Hato, M.; Shinohara, Y.; Kuramoto, H.; Furukawa, J.-i.; Kurogochi, M.; Shimaoka, H.; Tada, M.; Nakanishi, K.; Ozaki, M.; Todo, S.; Nishimura, S.-I. *Mol. Cell. Proteomics* **2008**, *7*, 370−377.

(13) Furukawa, J.-i.; Shinohara, Y.; Kuramoto, H.; Miura, Y.; Shimaoka, H.; Kurogochi, M.; Nakano, M.; Nishimura, S.-I. *Anal. Chem.* **2008**, *80*, 1094−1101.

(14) Sturm, A.; Albert Van Kuik, L.; Vliegenthart, J. F. G.; Chrispeels, M. J. Structure, position, and biosynthesis of the high mannose and the complex oligosaccharide side chains of the bean storage protein phaseolin. *J. Biol. Chem.* **1987**, *262*, 13392–13403.

(15) Fukuda, T.; Maruyama, N.; Salleh, M. R.; Mikami, B.; Utsumi, S. Characterization and crystallography of recombinant 7S globulins of Azuki bean and structure-function relationships with 7S globulins of various crops. *J. Agric. Food Chem.* **2008**, *56*, 4145–4153.

(16) Wang, C. M.; Eufemi, M.; Turano, C.; Giartosio, A. Influence of the carbohydrate moiety on the stability of glycoproteins. *Biochemistry* **1996**, *53*, 7299–7307.

(17) Nakai, S. Structure-function relationships of food proteins with an emphasis on the importance of protein hydrophobicity. *J. Agric. Food Chem.* **1983**, *31*, 676–683.

(18) Nakai, S.; Li-Chan, E. *Hydrophobic Interactions in Food Systems*; CRC Press: Boca Raton, FL, 1988.

(19) Garcia, R. N.; Adachi, M.; Tecson-Mendoza, E. A.; Bernardo, A. M. N.; Utsumi, S. Physicochemical properties of native and recombinant mungbea (Vigna radita L. Wilczek) 8S globulins and the effect of the N-linked glycans. *J. Agric. Food Chem.* **2006**, *54*, 6005–6010.

(20) Hayakawa, S.; Nakai, S. Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins. *J. Food Sci.* **1985**, *50*, 486–491.

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

(22) Slightom, J. L.; Drong, R. F.; Klassy, R. C.; Hoffman, L. M. Nucleotide sequences from phaseolin cDNA clones: the major storage proteins from *Phaseolus vulgaris* are encoded by two unique gene families. *Nucleic Acids Res.* **1985**, *13*, 6483–6498.

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