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Ionic Strength and pH-Induced Changes in the Immunoreactivity of Purified Soybean Glycinin and Its Relation to Protein Molecular Structure

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This study examined the immunogenic response of glycinin under varying conditions of pH and ionic strength using enzyme-linked immunosorbent assay. Differential scanning calorimetric (DSC) analysis and Fourier transform infrared spectroscopy (FTIR) were used to investigate the conformational changes induced as a result of these conditions, and the correlation with the changes observed in glycinin immunoreactivity were determined. A highly purified glycinin obtained by isoelectric precipitation followed by native preparative continuous flow electrophoresis was used for these studies. Purity was confirmed by two-dimensional-polyacrylamide gel electrophoresis and mass spectroscopy. DSC and FTIR results suggest that glycinin immunoreactivity is affected by changes in the tertiary and secondary packing of the protein, when flexibility, stability, and accessibility of certain substructures are modified. Aggregation and/or increased compactness of glycinin subcomponents could have potentially prevented epitopes from reacting with the IgG antibodies.

KEYWORDS: Soybeans; glycinin; 11S protein; immunoreactivity; antigenicity; protein conformation; structure; food allergy; food processing

INTRODUCTION

Soybeans are included in the "big eight" foods that are believed to be responsible for 90% of all allergenic reactions. Soy allergy is particularly of concern because of the extensive use of soy ingredients in processed foods. Growing interest in the utilization of soybeans is partly due to the high nutritional value and excellent functional properties of soy proteins. The major proteins in soy are the salt-soluble globulins, glycinin (320−360 kDa) and β-conglycinin (180 kDa) (*1*). Both proteins have been identified as major soy allergens (*2*−*4*). Glycinin is of particular interest to the food industry because it accounts for up to 35−40% of total soybean proteins (*5*) and is a major determinant of the nutritional quality and functionality of soybeans.

Glycinin is a heterogeneous protein with a polymorphic subunit composition, which varies among different cultivars (*6*). Each of the glycinin subunits (58−69 kDa) can be dissociated under reducing conditions into acidic (A; 31−45 kDa) and basic (B; 18−20 kDa) polypeptide chains (*5*). Presently, five major subunits have been characterized (*7*), namely, A1aB2 (G1), A1bB1b (G2), A2B1a (G3), A3B4 (G4), and A5A4B3 (G5). The acidic polypeptide A5 has an exceptionally low molecular mass of 10 kDa. The currently accepted model of native glycinin is a hexamer consisting of two layers of trimers. Each trimer has its three acidic and three basic polypeptides paired and held together by disulfide and hydrogen bonds, with acidic and basic alternating (*8*). The crystal structures of a proglycinin A1aB1b homotrimer (*9*) and a mature glycinin A3B4 homohexamer from a soybean mutant line (*10*) were recently unveiled by X-ray analysis. These studies revealed that like other members of the cupin superfamily, glycinin subunits are folded into two jelly roll β-barrel domains and two helix domains. This structure consists of a series of antiparallel β-sheets associated with α-helices, which form a cavity (a cup, from where the term cupin was derived) and a binding site for hydrophobic ligands. This β-barrel motif is a conserved structure among many major food allergens (*11*). Reports on the secondary structure of glycinin have been, however, inconsistent (3−25% α-helices, 25−56% β-sheets, 21−42% β-turns, and 7.1−66% random coils) (*12*−*15*). These contradictions may have resulted from differences in the analytical techniques used, computation, conditions of analysis (sample preparation and treatment), sample storage conditions, and glycinin purity. Numerous studies have, indeed, shown that the glycinin molecular structure is strongly modulated by its environment. Lakemond et al. (*16*)

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looked at the glycinin secondary structure using circular dichroism (CD) under different conditions of pH and ionic strength (pH 7.6 and 3.8 at ionic strengths of 0.5, 0.2, and 0.03) and found that glycinin consisted predominantly of α-helices at pH 7.6 and that the amount of nonstructured protein increased when the pH was lowered to 3.8, which also correlated with the quaternary dissociation of glycinin molecule. In another study, Kim et al. (*17*) found that the CD profiles of glycinin were not significantly altered over a wide range of pH values, even in acidic and basic conditions, but that increasing the salt concentration at pH 8.0 resulted in an increase in the percentage of α-helix in glycinin. It is well-known that CD spectroscopy is very sensitive to changes in the α-helix, but the presence of NaCl greatly interferes with the results due to the UV absorbance of chloride ions (*18*). Thus, much is still unknown on how glycinin is affected by its environmental pH and ionic strength conditions, and also, very little is known of how such glycinin conformational modulation affects its antigenicity.

The purpose of the present study was, therefore, first, to purify glycinin to eliminate interferences from other soy antigens and, second, to examine the specific immunogenic response of glycinin under varying conditions of pH and ionic strength. Differential scanning calorimetric analysis (DSC) and Fourier transform infrared spectroscopy (FTIR) were used to investigate the conformational changes induced as a result of these conditions, and the correlations with the changes observed in glycinin immunoreactivity were determined.

MATERIALS AND METHODS

Materials. Defatted soybean flakes (Nutrisoy untoasted 7B flakes) were obtained from ADM (Decatur, IL). Ultrafiltration membranes (10 kDa molecular cutoff) were from Millipore Co. (Bedford, MA). High molecular and low molecular weight calibration kits were from Amersham Pharmacia Biotech (Uppsala, Sweden). Bio-Rad Econo Pac DG-10 desalting columns, precast (10−20 and 4−15%) gradient polyacrylamide Tris-HCl gels, Coomassie Blue R-250, ReadyPrep 2D-clean up kit, Immobilized pH Gradient (IPG) ready strips, precast Criterion XT IPG+1 bis-tris gel (4−12%), and XT-MES running buffer were all purchased from Bio-Rad Laboratories (Hercules, CA). Waters SEP-PAK C18 cartridges were from Waters Co. (Milford, MA). All others chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation and Purification of Glycinin. Glycinin was first isolated from defatted soybean flakes, as reported in Achouri et al. (*19*). Briefly, defatted soybean flakes were dispersed in water at a ratio of 1:10, and the pH was adjusted and maintained at 8.5 with 2 N NaOH and stirred for 2 h at 55 °C. The slurry was then centrifuged at 4 °C for 30 min at 14300*g*. The pH of the clarified supernatant was adjusted to 6.8 with 2 N HCl, left to stand overnight at 4 °C for 18 h, and then centrifuged at 14300g for 30 min at 4 °C. The precipitate obtained was dialyzed against deionized water for 48 h at 4 °C. The protein precipitate [glycinin-rich (GR) fraction] was neutralized to pH 7.0 with 2 N NaOH, frozen with liquid nitrogen, and freeze-dried. Further purification of the GR fraction was achieved by native preparative continuous flow electrophoresis using a Bio-Rad 491-prep cell (Bio-Rad). The GR fraction (800 mg) was resolved with a 10.5 cm separating gel (6% acrylamide) in a 37 mm internal diameter column and eluted with 25 mM Tris-HCl buffer (pH 8.3) containing 0.192 M glycine at a constant flow rate of 1 mL/min and a current of 12 W. Collected fractions were analyzed by gel electrophoresis, and those corresponding to glycinin were pooled and concentrated by ultrafiltration using a 10 kDa molecular cutoff membrane. The concentrated glycinin fraction was desalted three times successively using an Econo Pac DG-10 column and labeled as purified glycinin (PG).

Analytical One-Dimensional (1D) and Two-Dimensional (2D) Gel Electrophoresis. Analytical 1D sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and native polyacrylamide gel electrophoresis (native-PAGE) were carried out on precast 10−20 and 4−15% gradient polyacrylamide Tris-HCl gels, respectively, using the Bio-Rad Criterion cell at a constant voltage (200 V). The gels were stained with Coomassie brilliant blue R-250 and scanned using Bio-Rad GS-690 calibrated imaging densitometer (Bio-Rad) and analyzed with the Multi-Analyst/PC Analysis Software (Bio-Rad). Amersham Pharmacia Biotech low molecular (14.4−97 kDa) and high molecular (66−669 kDa) weight calibration kits were used as molecular markers for SDS- and native-PAGE, respectively.

Two-dimensional gel electrophoresis using isoelectric focusing (IEF) on IPG Ready strips (first dimension) and SDS-PAGE (second dimension) was performed as follows: Aliquots from the GR and PG fractions (0.5 mg of protein) were treated with the Bio-Rad ReadyPrep 2D-clean up kit as described by the supplier. The obtained protein pellets were resuspended in 150 μL of IPG rehydration solution {8 M urea, 2% 3-[(3-sholamidopropyl)dimethylammonio]-1-propanesulfonate, 50 mM dithiothreitol (DTT), and 0.2% ampholytes (pH 3−10), and 0.001% Bromphenol Blue}. The sample solutions were then used to rehydrate IPG strips (11 cm, pH 3−10) for 16 h. The strips were focused on a Protean IEF cell (Bio-Rad) using the rapid ramp program with an end voltage of 8000 V and a focusing time of 35000 V h. Subsequent to IEF, the IPG strips were equilibrated for 10 min in an equilibration buffer (6 M urea, 2% SDS, 0.375 M tris-glycine buffer, 20% glycerol, and 30 mM DTT) and then transferred to a second equilibration buffer (6 M urea, 2% SDS, 0.375 M tris-glycine buffer, 20% glycerol, and 135 mM iodoacetamide) where they were allowed to equilibrate for 10 min. The strips were placed on top of a 4−12% Criterion XT IPG+1 bis-tris gel and sealed with warm 0.5% agarose. The gels were run at 200 V using XT-MES running buffer and stained with Coomassie brilliant blue R-250.

Liquid Chromatography/Electrospray Ionization−Mass Spectrometry (LC/ESI-MS) Analysis of Tryptic Glycinin Digest. The GR and PG fractions were reduced, and trypsin was digested [enzyme/protein ratio of 1/25 (w/w)] using the method of Stone et al. (*20*). The digested samples were desalted by solid-phase extraction on Waters SEP-PAK C18 cartridges and eluted with 100% acetonitrile. Eluates were vacuum-dried and dissolved to a concentration of 5 pmol/μL in a 1:1 (v/v) mixture of water and methanol containing 0.1% formic acid. LC/ESI-MS/MS analysis was performed using a Hewlett-Packard 1050 series HPLC system (Agilent, Santa Clara, CA) interfaced with a Finnigan LCQ Classic Mass Spectrometer equipped with an ESI source (Thermo Scientific, Waltham, MA). For LC analysis, 10 μL of sample was injected on a reverse-phase Jupiter 4 μm Proteo column (4 μm; 2 mm × 150 mm) (Phenomenex, Torrance, CA) and separated using a linear water−acetonitrile gradient (60−85% acetonitrile in 5 min, followed by a 10 min wash with 98% acetonitrile) containing 0.1% formic acid and 0.001% TFA. The flow rate was set at 200 μL/min, and a split ratio gave a final flow rate of 10 μL/min before MS ionization. ESI-MS/MS analysis was performed in a positive ion mode with three scan events: event 1, full scan with mass range *m*/*z* 350−2000; event 2, dependent MS/MS, most intense ion from event 1; and event 3, dependent MS/MS, second most intense ion from event 1. The ion source voltage was set at 4.5 kV and a capillary heater temperature of 150 °C. The BioWorks program (Thermo Scientific) and Fasta database were used for protein identification.

Preparation of Glycinin Solutions with Different pH Values and Ionic Strengths (*I*). A series of 10 mM citrate-phosphate buffer solutions at various pH values (2.2, 2.8, 6.1, and 7.2) were prepared. Intermediate pH values from 3 to 6 could not be studied due to the very poor solubility of glycinin at these pH values. The desired ionic strengths were achieved by addition of NaCl to the various pH citrate-phosphate buffer solutions (0.4 and 0.1 M NaCl for $I = 0.5$ and $I = 0.2$, respectively). Citrate-phosphate buffers with no addition of salt were taken as $I = 0.01$. PG was added to the different ionic strengths and pH buffer solutions at the desired concentration as determined by Bio-Rad protein microassay (Bio-Rad) using bovine serum albumin as standard.

Enzyme-Linked Immunosorbent Assay (ELISA) Assessment of Glycinin Immunoreactivity. A modified procedure of Tepnel Biosystems indirect competitive enzyme immunoassay (Tepnel BioSystems Ltd., Deeside, Flintshire, United Kingdom) was used to assess the

binding of glycinin to specific IgG. To examine the effects of pH and ionic strength on glycinin in its native state, the samples were not subjected to denaturation and renaturation as stated in the kit assay procedure. Glycinin solutions at different ionic strengths and pH values were diluted with Tepnel immunoassay sample diluent to a concentration of 0.06 mg of protein/mL and placed in soy protein-coated plastic microwells along with a specific rabbit anti-soy serum. The competitive reaction was allowed to proceed for 20 min at 25 °C; unbound material was washed out five times with Tris-buffered saline solution. The amount of rabbit anti-soy bound to the coated well was determined by reaction with peroxidase-conjugated anti-rabbit globulin (20 min at 25 °C). The microwells were washed as previously, and bound peroxidase was detected with a 3,3′,5,5′-tetramethylbenzidine procedure at 450 nm using a Bench Mark Plus Microplate Reader (Bio-Rad) controlled by Microplate Manager Software (Bio-Rad). Triplicate analyses were performed for each sample.

DSC. Calorimetric measurements were carried out using a 2910 Modulated DSC (TA Instruments, Inc., New Castle, DE). The instrument was calibrated using indium as standard. PG was dispersed (10% w/v) in 10 mM citrate-phosphate buffer solutions at various pH values (2.2, 2.8, 6.1, and 7.2) and ionic strengths (0.5, 0.2, and 0.01). Aliquots (20 μL) of the solutions were hermetically sealed in aluminum DSC pans and heated at a scan rate of 5 °C/min under helium through the range of 20 to 120 °C. A pan filled with 20 μL of the corresponding buffer was used as a reference. Data were analyzed using the Universal Analysis TA Software. Transition temperatures (T_d, maximum temperature of denaturation; $\Delta T_{1/2}$, width of peak at half-height) and enthalpies (ΔH) (areas below the endothermic curves in Joules per gram of dry weight) were measured. All samples were run in triplicate.

FTIR. For FTIR analysis, citrate-phosphate buffer solutions of various pH values (2.2, 2.8, 6.1, and 7.2) and ionic strengths (0.5, 0.2, and 0.01) were prepared using D_2O. D_2O was used instead of H_2O because of its greater transparency in the infrared region of interest (between 1600 and 1700 cm^{-1}). PG was dissolved (100 mg/mL) in the different D_2O buffers 1 day before analysis to allow for hydrogen−deuterium exchange. Aliquots (10 μL) of each sample were held in an IR cell between two CaF_2 windows, with a 25 μm path length, and infrared spectra were recorded at 25 °C with an FTS 3000 Bio-Rad FTIR spectrometer equipped with a deuterated triglycine sulfate detector (Bio-Rad). The spectrometer was purged with dry air for 10 min before recording the spectrum. A total of 256 scans were averaged at 4 cm^{-1} resolution. The signal-to-noise ratio was >20000:1. Deconvolution was performed using the Merlin FTIR software (version 1.2) with a bandwidth of 13 cm^{-1} and a narrowing factor of 2.5 (*21*). Throughout this study, the FTIR spectrum of glycinin in plain D_2O was recorded and used, unless otherwise specified, as a control against which spectra at modified pH and ionic strength conditions were compared.

Statistical Analysis. ELISA and DSC data were statistically evaluated by one-way analysis of variance using the PRISM software, version 3.02 (Graph Pad Software, Inc. San Diego, CA). Significant differences between means were determined by the Tukey's Multiple Comparison Test procedure at the 5% significance level.

RESULTS AND DISCUSSION

Glycinin Purification. In the present investigation, it was of importance to use a highly purified natural soybean glycinin, since previous studies showed differences in the structure and physicochemical properties between mature glycinin from natural seeds and glycinin that was produced either in vitro (*22*) or from mutant soybean lines (*23*).

The purification of soybean glycinin using isoelectric point (pI) precipitation, followed by preparative continuous flow gel electrophoresis, resulted in a clear separation of glycinin from the other soybean proteins. Native-PAGE analysis of the collected fractions revealed protein bands corresponding to glycinin in both its 11S and 7S forms (**Figure 1A**). To confirm that the 7S forms were glycinin and not β-conglycinin, SDS-PAGE was performed and the results revealed bands corresponding only to glycinin subunits (**Figure 1B**). The fractions corresponding to the most PG (I, II, and III) were pooled and named the PG fraction and were used for the remainder of the study. The purity of glycinin (acidic and basic polypeptides) as determined by densitometry was about 100% based on the total sum of protein bands that were resolved and detected in the PG fraction by SDS-PAGE analysis (**Figure 1B**). Purity was further analyzed by 2D-PAGE and LC/ESI-MS analysis of PG tryptic digest. The PG 2D gel (**Figure 2**) revealed spots corresponding to glycinin acidic and basic polypeptides only.

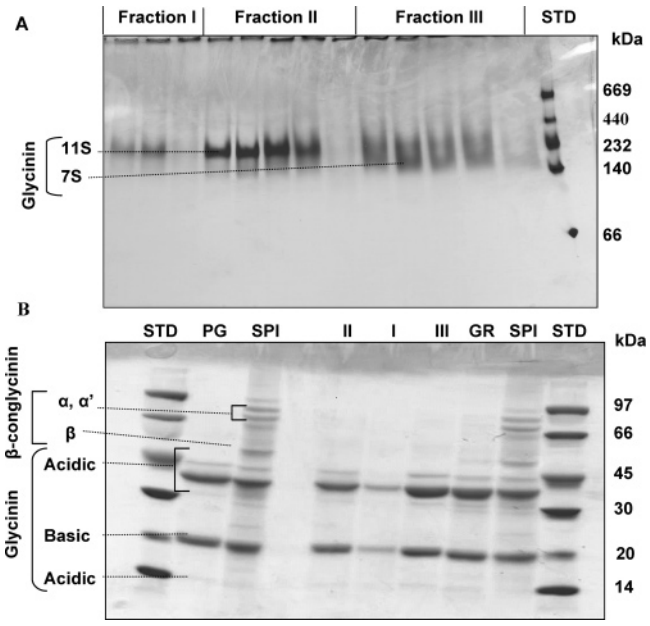

Figure 1. Native (**A**) and SDS-PAGE (**B**) of glycinin fractions. Fractions I–III, glycinin fractions collected during preparative electrophoresis; PG, pooled purified glycinin fractions; GR, glycinin-rich fraction; SPI, soy protein isolate; and STD, molecular weight markers.

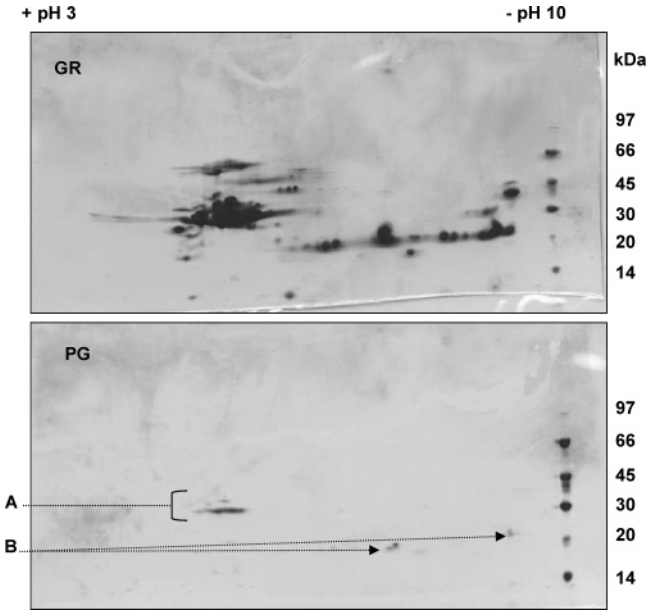

Figure 2. 2D-PAGE analysis of GR and PG fractions. (**A**) Glycinin acidic polypeptides and (**B**) glycinin basic polypeptides.

Table 1. Identification of Purified Soybean Glycinin by LC/SI-MS/MS Analysis

protein identified: soybean glycinin G1 (precursor)

number of amino acids: 495
monoisotopic mass: 55672.6
isoelectric point: 5.86

MAKLVFSLCF	LLFSGCCFAF	SSR**EQPQQNE**	**CQIQKLNALK**	PDNRIESEGG	LIETWNPNNK
PFQCAGVALS	RCTLNRNALR	RPSYTNGPQE	IYIQQGKGIF	GMIYPGCPST	FEEPQQPQQR
GQSSRPQDRH	QKIYNFREGD	LIAVPTGVAW	WMYNNEDTPV	VAVSIIDTNS	LENQLDQMPR
R**FYLAGNQEQ**	**EFLKYQQEQG**	**GHQSQK**GKHQ	QEEENEGGSI	LSGFTLEFLE	HAFSVDKQIA
KNLQGENEGE	DKGAIVTVKG	GLSVIKPPTD	EQQQRPQEEE	EEEEDEKPQC	KGKDKHCQRP
RGSQSKSRRN	GIDETICTMR	LRHNIGQTSS	PDIYNPQAGS	VTTATSLDFP	ALSWLRLSAE
FGSLRKNAMF	VPHYNLNANS	IIYALNGRAL	IQVVNCNGER	VFDGELQEGR	VLIVPQNFVV
AARSQSDNFE	YVSFKTNDTP	MIGTLAGANS	LLNALPEEVI	QHTFNLKSQQ	ARQ**IKNNNPF**
KFLVPPQESQ	KRAVA				

protein coverage

sequence of identified peptides	MH^+	% mass	amino acid position	% amino acid
EQPQQNECQIQK	1472.68	2.65	24–35	2.42
LNALKPDNR	1040.59	1.87	36–44	1.82
FYLAGNQEQEFLK	1586.79	2.85	182–194	2.63
YQQEQGGHQSQK	1417.65	2.55	195–206	2.42
GAIVTVK	687.44	1.23	253–259	1.41
VFDGELQEGR	1149.55	2.06	401–410	2.02
SQSDNFEYVSFK	1450.65	2.61	424–435	2.42
NNNPFK	733.36	1.32	476–481	1.21
FLVPPQESQK	1172.63	2.11	482–491	2.02
totals	9176.42	16.48	78	15.76

Table 2. Thermal Properties of PG at Different pH and Ionic Strength (I) Conditions

pH	I[a]	T_d (°C)[b]	ΔH (J/g)[c]	$\Delta T_{1/2}$ (°C)[d]
6.1	0.5	98.26 a ± 0.05	24.28 a ± 1.90	5.54 a ± 0.15
	0.2	93.23 b ± 0.17	22.47 a,b ± 1.03	6.69 b ± 0.11
	0.01	91.94 c ± 0.07	18.05 b ± 1.16	7.20 c ± 0.19
7.2	0.5	97.76 d ± 0.09	20.66 a,b ± 2.66	6.08 d ± 0.03
	0.2	91.49 e ± 0.12	17.82 b ± 2.92	6.41 b ± 0.07
	0.01	90.13 f ± 0.02	18.93 b ± 0.29	7.85 e ± 0.02

[a] I, ionic strength. [b] T_d, temperature of denaturation. [c] ΔH, enthalpy of denaturation. [d] $\Delta T_{1/2}$, peak width at half-height. Different letters indicate statistically significant differences among samples within the same column ($p < 0.05$). Data are means ± standard deviations of triplicates.

LC/ESI-MS/MS analysis of tryptic digest of GR fraction (data not shown) identified glycinin G1 (precursor) and β-conglycinin α-subunit as first and second hits, with six unique peptides each and a protein coverage of 13.53 and 8.60%, respectively. Soybean trypsin inhibitor was also detected. For the PG digest, glycinin was the only protein identified, confirming its high purity. As a first hit, the glycinin G1 (precursor) was identified with nine unique peptides and a protein coverage of 16.48% (**Table 1**).

Structural Modifications in Glycinin at Various pH and Ionic Strength Conditions. *DSC.* Glycinin thermograms (not shown), at both acidic pH values of 2.2 and 2.8, over the three investigated ionic strengths, exhibited no endothermic peak, suggesting a possible molecular denaturation. These results are in agreement with those of Kim et al. (*17*), who reported a flat thermogram of glycinin at a pH below 3. At pH 6.1 and pH 7.2 and over the range of ionic strength investigated, glycinin exhibited one single endothermic transition with a maximum between 90 and 100 °C. At these pH values, DSC data (**Table 2**) showed a significant ($p < 0.001$) increase (+7 °C) in the temperature of denaturation (T_d) with increasing ionic strength (i.e., from $I = 0.01$ to 0.5), suggestive of an increase in thermostability. The stabilizing effect of salt may be explained by the neutralization of the charged amino acid groups with counterions (especially Na^+), which would have neutralized the negative charges of the protein, thus reducing intra- and/or intermolecular electrostatic repulsions and possibly increasing the hydrophobic interactions that contribute to stabilize protein structure (*24*).

At the same ionic strength, the temperature of denaturation at pH 6.1 was found to be significantly higher ($p < 0.01$) than at pH 7.2, confirming that protein stability was greater near the pI (*25*). Glycinin demonstrated the greatest stability at pH 6.1 and $I = 0.5$, with a T_d of 98.26 °C. The denaturation enthalpy (ΔH), however, did not significantly vary over the whole range of ionic strengths studied, suggesting that once denaturation has started the presence of a higher concentration of ions has little effect on the forces (e.g., hydrogen and disulfide bonds) that stabilize glycinin conformation. The index of cooperativity ($\Delta T_{1/2}$) increased when the ionic strength decreased ($p < 0.05$), without significantly affecting the enthalpy of denaturation. Similar results have been observed with other plant globulins (*26*, *27*). It has been suggested that at low ionic strength, some glycinin molecules (11S) tend to dissociate into trimers (7S); the 11S and 7S forms would, thus, denature in slightly different ways (*28*), which could result in a widening of the thermal transition peak.

FTIR Spectroscopy. FTIR spectra of glycinin at various pH and ionic strength conditions are presented in **Figures 3** and **4**. From the locations and relative intensities of the infrared bands, it is evident that the secondary structure of soybean glycinin is predominantly β-sheets [1636 cm^{-1} (*29*)] and random coils structures [1643 cm^{-1} (*31*)] with few α-helices, in view of the absence of a defined band at 1650 cm^{-1}, which is generally attributed to an α-helix structure (*30*). These observations support the grouping of glycinin into the class of β-sheet proteins, as is the case for most plant globulins (*31*).

The FTIR spectra in **Figures 3** and **4** are presented to allow the assessment of the effect of pH on the secondary structure of glycinin at a specific ionic strength and vice versa. At pH

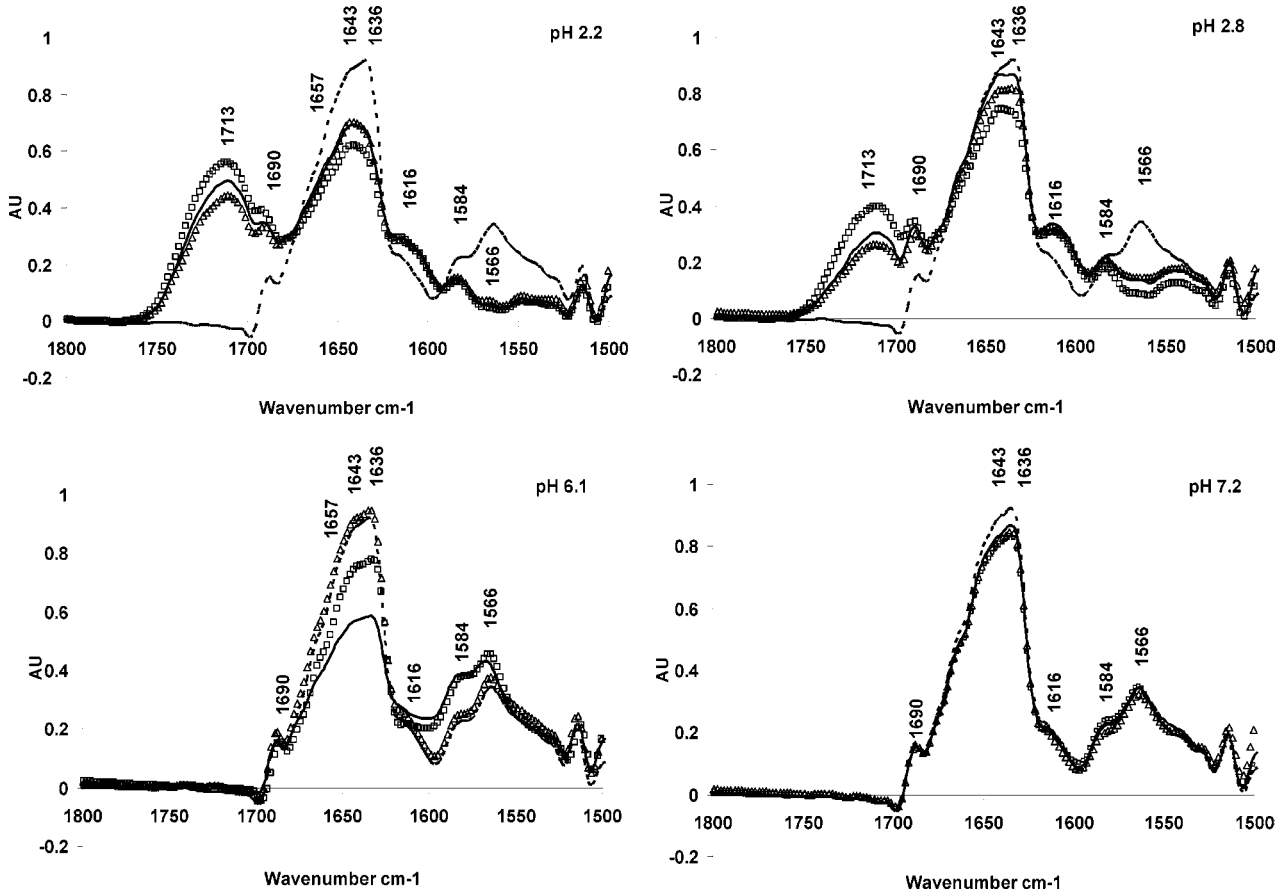

Figure 3. FTIR spectra of PG at various pH values. Key: $I = 0.5$, □; $I = 0.2$, —; $I = 0.01$, △; and D_2O (with no pH and ionic strength adjustments), ---.

2.2, major changes in glycinin structure (**Figure 3**) were observed for the three ionic strengths, when compared to that observed in D_2O (neutral pH). The large decrease in the intensity of the β-sheet band at 1636 cm^{-1}, coupled with the transition toward the random coil band at 1643 cm^{-1} and the concomitant increase in the intensity of the 1690 cm^{-1} band [associated with the onset of protein unfolding (*32*)], are all indicative of glycinin unfolding. Such an unfolding could have resulted from electrostatic repulsion emanating from the net positive charge of polypeptide chains at low pH and the consequent break-up of hydrogen bonds and hydrophobic interactions (*33*). The abrupt loss in the intensity of the amide II band (1600−1480 cm^{-1}) further indicates a higher H−D exchange of buried −NH groups (*34*) as a result of greater solvent accessibility, thereby confirming the changes in glycinin folding state. In addition to unfolding, the slight increase in the intensities of the intermolecular β-sheets bands at 1616 and 1682 cm^{-1} indicates some protein association/aggregation. There is also the apparition of a major peak at 1713 cm^{-1} that has been associated with the vibration of the protonated COOH as a result of an acidic environment (*35*). Contrary to the DSC analysis, which showed complete absence of a denaturation peak, FTIR analysis further revealed that at these pH and ionic strengths conditions (pH 2.2, $I = 0.01$, 0.2, and 0.5), some secondary structures were still conserved. Goto et al. (*36*) have previously demonstrated that many small globular proteins are significantly unfolded at low pH due to charge−charge repulsion emanating from multiple positively charged ammonium groups and that the addition of salt resulted in substantial refolding leading to the formation of folding intermediates with properties similar to those of a molten globule state. Some of these proteins in the presence of salts at acidic conditions transformed directly to the molten globule conformation (*37*). This conformation is usually characterized by a relatively high content of secondary structures and a largely disordered tertiary structure (*38*). The glycinin structure observed at the acidic pH 2.2 may, thus, represent such a molten globule state. Interestingly, glycinin appears to be in a less ordered and structured form at high ionic strength ($I = 0.5$), as compared to lower ionic strengths ($I = 0.01$ and $I = 0.2$), as suggested by the increased intensity of the 1690 cm^{-1} band (unfolding). Such behavior could find an explanation in the work of Nishimura et al. (*39*) who reported that the rate of protein refolding under acidic pH conditions was much slower at high salt concentrations than at low salt concentrations.

The FTIR spectra of glycinin at pH 2.8 were in some aspects different from those at pH 2.2. **Figures 3** and **4** show that at pH 2.8 ($I = 0.2$ and $I = 0.01$), the intensity of the β-sheet band (1636 cm^{-1}) did not decrease to the same extent as compared to pH 2.2 conditions. Similarly, the transition toward unordered structure (1643 cm^{-1}) was less marked at pH 2.8 ($I = 0.2$ and $I = 0.01$) than at pH 2.2 ($I = 0.2$ and $I = 0.01$). The intensity of the amide II band at pH 2.8 was also higher than at pH 2.2, indicating an incomplete deuteration. The intermolecular β-sheet bands at 1616 and 1682 cm^{-1} were also slightly more intense at pH 2.8 than at pH 2.2. These results suggest that at pH 2.8 and at low to intermediate ionic strength conditions, glycinin

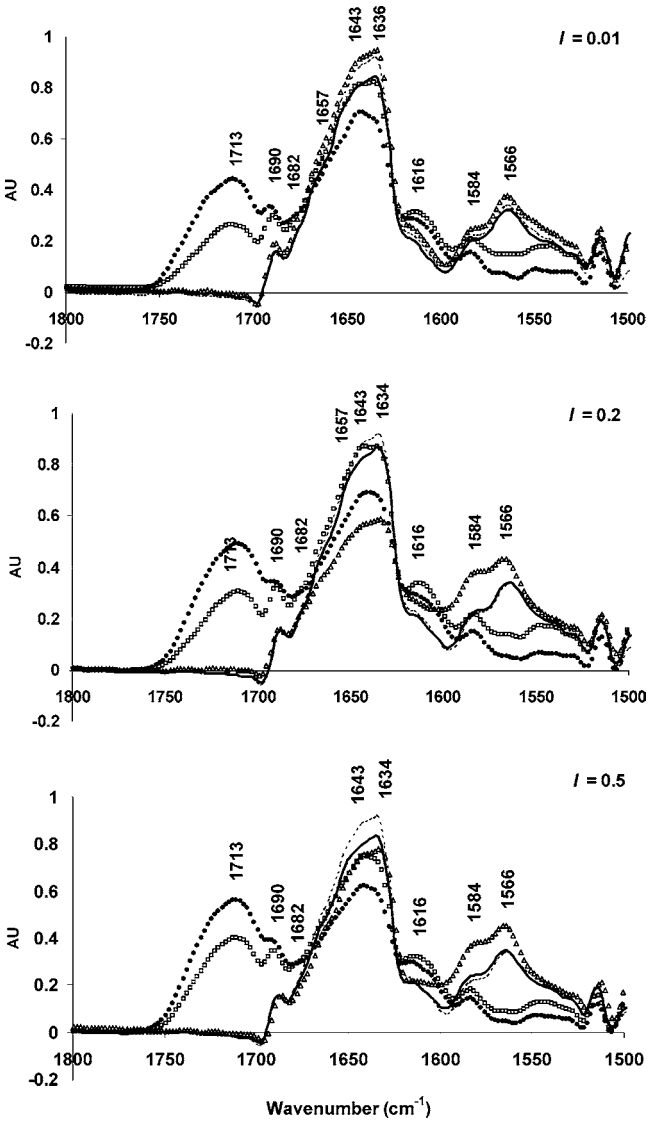

Figure 4. FTIR spectra of PG at various ionic strengths (*I*). Key: pH 2.2, ●; pH 2.8, □; pH 6.1, △; pH 7.2, —; and D_2O (with no pH and ionic strength adjustments), –.

has a more ordered structure with more intact nativelike secondary structures (higher β-sheets content), despite an incipient aggregation. Several studies have reported that the formation of protein folding intermediates (molten globules) observed at acidic conditions could be either marginally stable, highly disordered conformations, or stable states with nearly native structures (*40*, *41*). It has also been shown that aggregation could take place between these nativelike folding intermediates (*42*, *43*). High ionic strength ($I = 0.5$), at pH 2.8, as at pH 2.2, resulted in glycinin being in a less ordered and folded state as compared to lower ionic strengths, as indicated by the more pronounced transition toward random coil (band at 1643 cm^{-1}), and the lower intensity of the amide II band (more H−D exchange) (**Figure 3**).

At pH 6.1, major differences in glycinin secondary structure were observed as the ionic strength was varied (**Figure 3**). At pH 6.1 and $I = 0.01$, glycinin FTIR spectrum was very similar to that obtained in D_2O, suggesting very little denaturation. At pH 6.1, glycinin is closer to its isoelectric point [pH 5.67 (*27*)] and is, therefore, more likely to be in its globular native state. At $I = 0.2$, the FTIR spectrum revealed an abrupt loss in glycinin β-sheet secondary structure (net decrease in the intensity of the band at 1636 cm^{-1}), which was even greater than that observed at acidic conditions (pH 2.2). The transition toward unordered structure (1643 cm^{-1}) was, however, not observed. At higher ionic strength ($I = 0.5$), this decrease was much less pronounced. At $I = 0.2$, there was also a greater increase in the intensity of the intermolecular β-sheets band at 1616 cm^{-1} as compared to $I = 0.5$. Another notable difference at pH 6.1 (**Figure 3**) was the higher intensities of the bands at 1584 and 1566 cm^{-1} in the amide II region at both $I = 0.2$ and $I = 0.5$ as compared to $I = 0.01$, suggesting poor accessibility to deuteration of certain secondary structures under these higher ionic strength conditions. It has been reported that low concentrations of salt at neutral pH decrease the level of intramolecular electrostatic repulsion resulting in protein stabilization (*39*), whereas at high salt concentrations, the major effects result from changes in water structure and preferential exclusion of the ions from the vicinity of the protein (*44*). Ion-induced effects on water structure, in addition to affecting hydrogen bonding (*45*), can also affect hydrophobic interactions within the protein (*46*), resulting in the burial of certain amino acid residues into the protein hydrophobic core and slowing down hydrogen exchange rates. Such conformational changes would result in a more compact and less flexible structure (*39*). Furthermore, it has been suggested that the effects of salts on electrostatic interactions largely affect α-helices, whereas salt effects on hydrophobic interactions mostly affect β-sheets (*47*), which have the tendency of being deeply buried within the polypeptide chain (*48*). At pH 7.2, glycinin had a highly ordered secondary structure that was not significantly affected by the addition of salt (**Figures 3** and **4**).

Effects of pH and Ionic Strength on Glycinin-IgG Binding. The ELISA results (**Figure 5**) revealed major differences in the immunoreactivity of glycinin as a function of both pH and ionic strength, with pH having a more dominant effect than ionic strength. At the three *I* values, greater IgG binding was observed at pH 7.2 and pH 2.2 than at pH 6.1 and pH 2.8. DSC and FTIR analyses revealed that these differences in expressed immunoreactivity are the result of important changes in glycinin conformational structure. Thus, at pH 2.2, the enhanced immunoreactivity of glycinin could be related to it being in a partially unfolded state, which may have allowed the antibodies greater access to otherwise hidden epitopes. Demonte et al. (*49*) have reported a major reduction in glycinin immunoreactivity when subjected to elevated temperatures at pH 2.0 as compared to pH 7.6. Interestingly, at pH 2.2 and $I = 0.5$, glycinin exhibited a significantly ($p < 0.001$) higher immunoreactivity than at $I = 0.01$ and $I = 0.2$. At these acidic and high ionic strength conditions, the FTIR spectrum showed glycinin to be in a more unfolded state, which would result in greater exposure of shielded epitopes. Similar conformational changes were observed at pH 2.8, which would explain why glycinin also showed higher IgG binding at $I = 0.5$ as compared to $I = 0.2$ and $I = 0.01$. At pH 2.8, glycinin immunoreactivity was, however, significantly ($p < 0.001$) reduced at all ionic strengths, as compared to pH 2.2. The FTIR spectra showed differences in aggregate formation/association at these two pH values (i.e., at pH 2.8, more aggregation occurred while highly ordered secondary structures were still conserved). This aggregation could have led to steric hindrance of certain epitopes, which

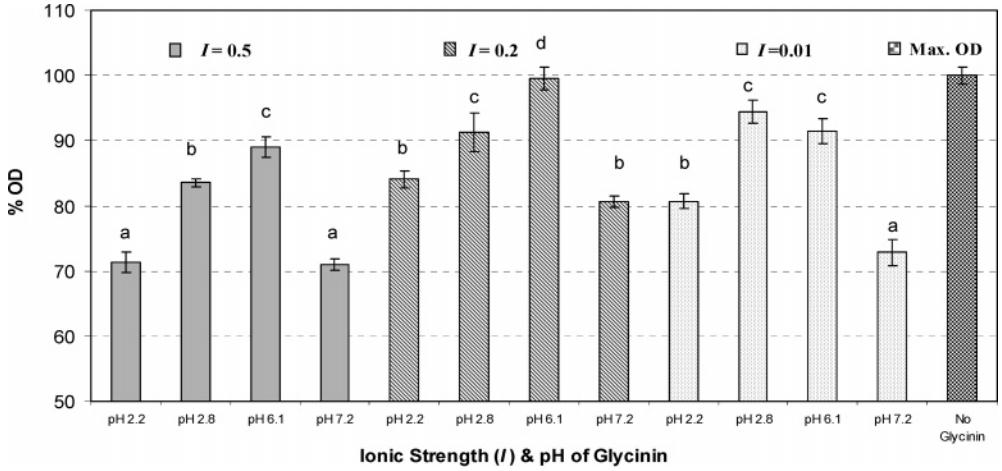

Figure 5. IgG binding of PG at different conditions of pH and ionic strength (*I*). Different letters indicate statistically significant differences among samples within the same column ($p < 0.05$). Data are means ± standard deviations of triplicates.

would explain the observed decrease in glycinin immunoreactivity at pH 2.8 (**Figure 5**).

At pH 6.1, glycinin exhibited the lowest IgG binding, particularly at $I = 0.2$, where glycinin immunoreactivity was reduced by more than 28% as compared to that observed at pH 7.2, $I = 0.5$ ($p < 0.001$). At these conditions (pH 6.1 and $I = 0.2$), the FTIR spectrum revealed an abrupt loss in glycinin β-sheet secondary structure, accompanied by an enhanced formation of intermolecular β-sheets, which would have resulted in increased molecular compactness. The major structural rearrangement in these glycinin β-sheet regions could have potentially prevented glycinin epitopes from reacting with the IgG antibodies.

For each of the ionic strength conditions studied, higher immunoreactivity was always observed at pH 7.2; this seems to relate to its highly ordered structure at this pH (as observed by FTIR), which may have allowed for optimal epitope recognition by the specific anti-soy antibodies (**Figure 5**).

Conclusion. The present investigation showed that the immunological response of soybean glycinin is strongly modulated by its conformational structure, which in turn is greatly affected by its environmental conditions. DSC and FTIR results suggest that pH- and ionic strength-induced changes in glycinin immunoreactivity are related to changes in the secondary and tertiary packing of the protein, where flexibility, stability, and accessibility of certain substructures are significantly modified. The DSC results also clearly show that pH and ionic strength significantly affect the thermal properties of glycinin. FTIR results further suggest that aggregation and/or increased compactness of glycinin subcomponents could potentially lead to either steric hindrance or modification of epitopes.

It is important to point out that immunoreactivity is dependent on the specificity of antibody−antigen interactions, which may strongly depend on the conformational state of the proteins against which the antibodies are raised. Changes in the structure of the targeted protein will inevitably influence the overall immunogenic response and may, thus, affect the sensitivity of the immunoassay. An appreciation of the interactions that occur between food components during processing and their effects on molecular structure and consequently antigenicity is essential in the development of knowledge-based strategies for controlling and manipulating allergens along the food chain.

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