Heat Denaturation of Soy Glycinin: Influence of pH and Ionic Strength on Molecular Structure

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The 7S/11S glycinin equilibrium as found in Lakemond et al. (*J. Agric. Food Chem.* **2000**, *48*, xxxx−xxxx) at ambient temperatures influences heat denaturation. It is found that the 7S form of glycinin denatures at a lower temperature than the 11S form, as demonstrated by a combination of calorimetric (DSC) and circular dichroism (CD) experiments. At pH 7.6, at which glycinin is mainly present in the 11S form, the disulfide bridge linking the acidic and the basic polypeptides is broken during heat denaturation. At pH 3.8, at which glycinin has dissociated partly into the 7S form, and at pH 5.2 this disruption does not take place, as demonstrated by solubility and gel electrophoretic experiments. A larger exposure of the acidic polypeptides (Lakemond et al., 2000) possibly correlates with a higher endothermic transition temperature and with the appearance of an exothermic transition as observed with DSC. Denaturation/aggregation (studied by DSC) and changes in secondary structure (studied by far-UV CD) take place simultaneously. Generally, changes in tertiary structure (studied by near-UV CD) occur at lower temperatures than changes in secondary structure.

Keywords: *Soy; glycinin; pH; ionic strength; heat denaturation; protein structure*

INTRODUCTION

Soy proteins are widely applied in food products. Generally, food products containing soy are heated to inactivate antinutritional factors, for food preservation reasons, and to obtain desired functional properties, such as solubility or textural properties. Glycinin is one of the major soy proteins, representing ∼30% of total protein in soybeans. It is composed of an acidic (∼38 kDa) and a basic polypeptide (∼20 kDa) (Staswick et al., 1981), linked by a single disulfide bridge, except for the acidic polypeptide A_4 (Staswick et al., 1984). Each pair of acidic and basic polypeptides is encoded by a single gene and cleaved post-translationally. Generally, multiple genes encoding for glycinin are present in each soy species (Tumer et al., 1981).

Several studies have been reported on glycinin heat denaturation. Koshiyama et al. (1980/1981) and Hashizume et al. (1975) found that at pH 7.6 the heat denaturation temperature of glycinin was influenced by ionic strength. Koshiyama et al. (1980/1981) found no indications for a correlation between the denaturation temperature and the protein structure at ambient temperatures. Furthermore, according to Yamagishi et al. (1987) and Hashizume and Watanabe (1979), at I = 0.5 and pH 7.6 the acidic polypeptides were present in the soluble fraction after heat treatment, whereas the basic polypeptides were found in the precipitate. However, these studies do not describe the effect of heat treatment of soy glycinin at low pH, whereas, as demonstrated in the preceding paper, pH has a dominant influence on the structural properties of glycinin. At ambient temperatures at pH 7.6 glycinin forms hexameric complexes (11S), whereas at pH 3.8 glycinin is mainly present in trimeric complexes (7S) and more pronounced at lower ionic strength. The dissociation into the 7S form seems to correlate with changes in secondary and tertiary structure as described in Lakemond et al. (2000). Furthermore, at pH 7.6, when the ionic strength is lowered from 0.5 to 0.03, the basic polypeptides shift more to the exterior of the glycinin complex. This altered arrangement of acidic and basic polypeptides seems to influence solubility. In this work the relationship is studied between the structural properties of glycinin at ambient temperatures modulated by pH and ionic strength and its heat denaturation.

MATERIALS AND METHODS

Sample Preparation. Glycinin was purified from Williams 82 soybeans (harvest 1994) as described in Lakemond et al. (2000). Prior to an experiment, the purified glycinin was dialyzed at 20 °C against pH 7.6 phosphate buffers of ionic strengths of 0.5, 0.2, and 0.03. The pH and ionic strength conditions studied are similar to those used in the preceding paper to allow direct comparison. For experiments carried out at pH 3.8 and 5.2, the pH of the sample was subsequently adjusted using HCl.

Enriched 7S and 11S glycinin fractions were obtained from a glycinin sample at I = 0.5 and pH 3.8, where about equal amounts of 7S and 11S were present (Lakemond et al., 2000). The fractions were separated on a 5−20% sucrose gradient as described in Lakemond et al. (2000) and subsequently dialyzed against potassium phosphate buffer (I = 0.5, pH 3.8) at 20 °C to remove sucrose. To investigate the purity and stability of the sample, the enriched 7S and 11S glycinin fractions were

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again analyzed by ultracentrifugation on a sucrose gradient after 8 h. The results demonstrated that the obtained 7S and 11S glycinin fractions had a cross-contamination of maximally 10−20%. The protein concentration used in this work was <1.5%, at which concentration no gelation of the protein solution occurs (Yamauchi et al., 1991).

Differential Scanning Calorimetry (DSC). DSC thermograms were recorded on a micro DSC III (Setaram, Caluire, France) using 0.9 mL vessels. Glycinin (0.9 mL; 3 mg/mL) at different ionic strengths and pH values was heated from 20 to 115 °C at a heating rate of 1 K/min and subsequently cooled to 20 °C at the same rate. To investigate the reversibility of denaturation, a subsequent heating/cooling cycle was performed. The transition temperature (T_t) is defined as the temperature at the minimum/maximum heat flow of the endothermic/exothermic transition. Enthalpies were calculated using the Setaram software, based on integration of the area of the transitions. The detection limit for transitions was 84 $\mu J\ g^{-1}\ K^{-1}$.

Circular Dichroism (CD) Spectroscopy. Far- and near-ultraviolet (UV) CD temperature scans of glycinin samples at different pH values and ionic strengths were recorded on a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) at temperatures ranging from 20 to 98 °C with a heating rate of 1 K/min. The protein concentration used for near-UV CD measurements was 1.2 mg/mL and for far-UV CD measurements, 0.2 mg/mL, using cells with path lengths of 10 and 1 mm, respectively. Temperature scans were recorded using a step resolution of 0.2 °C and a response time of 8 s. Detection wavelengths were 285 and 215 nm for near-UV and far-UV CD, respectively. Spectra recorded at $I = 0.5$ and pH 7.6 at different temperatures showed that at these wavelengths the ellipticity was most sensitive to temperature (results not shown).

In addition, far-UV CD spectra of 0.6 mg/mL of glycinin were recorded at 20 °C as described previously (Lakemond et al., 2000).

Determination of Glycinin Solubility. Glycinin samples (12 mg/mL) at various ionic strengths and pH conditions were heated from 20 to 98 °C at a rate of 1 K/min, kept at 98 °C for 30 min, and subsequently cooled to 20 °C. Next, the glycinin samples were centrifuged for 5 min at 20 °C at 15800*g*. The protein content of the supernatants was determined in triplicate using the Bradford assay (Bradford, 1976) using BSA (A-4503, Sigma, St. Louis, MO) as standard.

Gel Electrophoresis. The protein composition of the supernatants and precipitates obtained after heat treatment of glycinin at the various ionic strength and pH conditions was determined using reduced and nonreduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) on a Phast System (Pharmacia, Uppsala, Sweden) according to the instructions of the manufacturer. Gradient gels (10−15%) were used, which were stained with Coomassie Brilliant Blue and calibrated with low molecular weight markers ranging from 14 to 94 kDa (Pharmacia).

Ultracentrifugation Experiments. To determine the sedimentation coefficients of the glycinin fraction that remained soluble after heat treatment, ultracentrifugation of sucrose gradients was performed as described in the preceding paper. Samples (0.3 mL) were loaded on top of the sucrose gradient [$I = 0.5$, 0.2, and 0.03 at pH 7.6 (3 mg/mL) and $I = 0.03$ at pH 3.8 (4.5 mg/mL)].

Fluorescence Spectroscopy. Fluorescence spectra of 0.2 mg/mL glycinin samples were recorded on a Perkin-Elmer luminescence spectrometer LS 50 B as described previously (Lakemond et al., 2000).

RESULTS

In this work we investigated how pH and ionic strength influence solubility and molecular structure during heating under conditions relevant to food systems. The conditions studied are $I = 0.5$, 0.2, and 0.03 at pH 7.6, 5.2, and 3.8; the condition $I = 0.5$ and pH 7.6 is included for comparison to the literature.

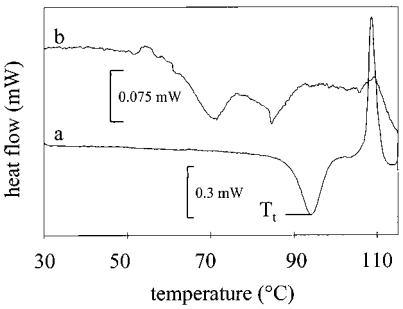

Figure 1. DSC thermograms of glycinin at $I = 0.5$, pH 7.6 (a), and $I = 0.03$, pH 3.8 (b), recorded at a rate of 1 K/min.

Table 1. Transition Temperatures of Glycinin at $I = 0.5$, 0.2, and 0.03 at pH 7.6, 5.2, and 3.8 As Measured by DSC and Far-UV CD Temperature Scanning at 215 nm and the Enthalpy of the Transitions As Determined by DSC

		T_t (°C)				enthalpy (J/g)	
I (M)	pH	DSC endo	DSC exo	far-UV CD	near-UV CD	endo-therm	exo-therm
0.5	7.6	94	109	>98	>98	41	−42
	5.2	96	92	nd[a]	nd	18[b]	−16
	3.8	85	73	70/83	61/89	10[b]	−19
0.2	7.6	87		89	54/80	37	
	5.2	92		nd	nd	22	
	3.8	67/82		68−81	67/79	30[b]	
0.03	7.6	78		78	73	39	
	5.2	58/88		nd	nd	53[b]	
	3.8	71/85		72−82	52/66	25[b]	

[a] nd, not determined. [b] Different transitions are not well separated.

Denaturation Temperatures of Glycinin. To determine if pH and ionic strength influence the denaturation temperature of glycinin, DSC experiments were performed. Figure 1 shows DSC thermograms of glycinin for two typical examples. From these thermograms the transition temperatures of glycinin were determined as the temperature at which the heat flow is maximal (Table 1). The highest transition temperatures at a particular ionic strength are found at pH 5.2. The transition temperatures at each pH decrease generally when the ionic strength is lowered. For $I = 0.2$ and 0.03 endothermic transitions are observed, and remarkably, at pH 3.8 and 5.2 and $I = 0.03$ two endothermic transitions are present. The enthalpy of the transitions is also presented in Table 1. For $I = 0.5$ and 0.2 the highest enthalpies are observed at pH 7.6, whereas for $I = 0.03$ the highest enthalpy is observed at pH 5.2.

At $I = 0.5$ both exothermic and endothermic transitions are observed. Interestingly, at pH 7.6 the exothermic transition is observed at a higher temperature than the endothermic transition, whereas at pH 5.2 and 3.8 the opposite is found. This exothermic transition was not observed when 10 mM 2-mercaptoethanol was present in the sample (results not shown). The position of this exothermic transition depends strongly on the heating rate (results not shown). All transitions found are irreversible as in the second heating scan no transitions could be observed at all conditions studied (results not shown).

Secondary and Tertiary Folding of Glycinin as a Function of Temperature. CD temperature scans were recorded to determine if changes in secondary and tertiary structure play a role in glycinin denaturation.

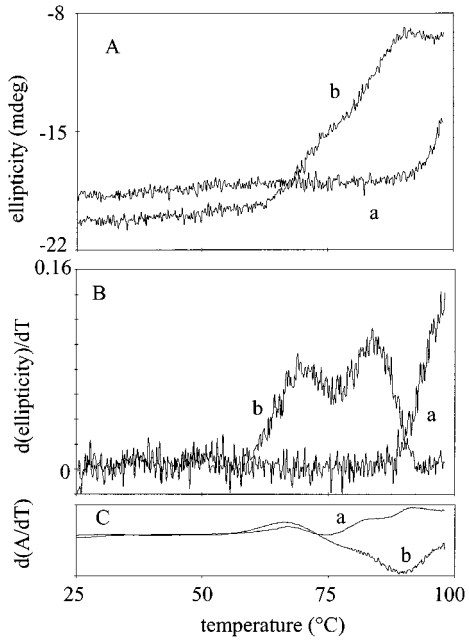

Figure 2. Far-UV CD temperature scans (A) of glycinin at $I = 0.5$, pH 7.6 (a), and $I = 0.5$, pH 3.8 (b), recorded at 215 nm at a heating rate of 1 K/min and the first derivative of the heating curves (B) and the first derivatives of the signal as measured by the applied voltage of the CD photomultiplier (C).

The pH 5.2 conditions could not be studied because of precipitation of the protein at ambient temperatures, whereas the CD technique requires the protein to be soluble. Figure 2A shows the recorded far-UV CD temperature scans at $I = 0.5$ at pH 7.6 and 3.8 to give typical examples. The transition temperatures are determined by taking the first derivative of the temperature scans (Figure 2B) and are presented in Table 1. At pH 3.8 and $I = 0.5$ two subsequent transitions are observed, whereas at $I = 0.2$ and 0.03 transitions over a broad temperature range are observed. At $I = 0.5$ and pH 7.6 the first derivative does not show a clear maximum because it was not possible to heat above 98 °C. Generally, the transitions observed with far-UV CD are found at temperatures comparable to those at which the transitions are observed with DSC.

In all samples, except at $I = 0.03$ and pH 3.8, precipitation of the protein is observed after heating, as indicated by an increase of the turbidity of the sample. A decrease in ellipticity at higher temperatures can therefore be attributed to changes in secondary structure as well as to precipitation. The temperatures at which aggregation/precipitation appears are detected by the applied voltage for the CD photomultiplier signal, which is related to the optical density of the sample. Figure 2C displays the first derivative of these turbidity signals. Because the turbidity curves do not entirely coincide with changes in the ellipticity, these CD curves display to some extent changes in the secondary structure of the material.

Similar experiments have been performed in the near-UV region by monitoring a spectral region that was shown previously to be sensitive to tertiairy interactions (results not shown). The observed transition temperatures are presented in Table 1. Apart from the condition $I = 0.2$ and pH 7.6, no changes in tertiary structure could be observed prior to aggregation/precipitation.

Thermal Denaturation of 11S versus 7S Glycinin. To explain the nature of the double-endothermic transition observed for glycinin at particular conditions (Figure 1; Table 1), glycinin was heated at $I = 0.2$ and pH 3.8 up to 72 °C to obtain partly denatured and precipitated glycinin (only the transition at the lowest denaturation temperature occurred). Subsequent ultracentrifugational analysis of this sample no longer showed a 7S peak, whereas the 11S peak was mainly intact (results not shown). This demonstrates that 7S glycinin denatures at a lower temperature than 11S glycinin.

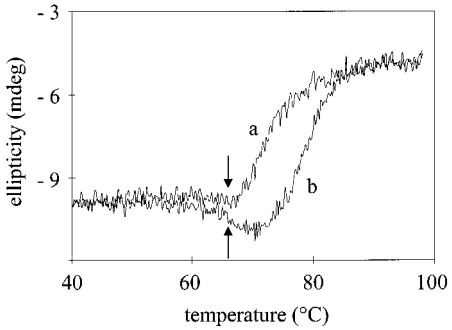

Figure 3. Far-UV CD temperature scans of 7S glycinin (a) and 11S glycinin (b) at $I = 0.5$, pH 3.8, recorded at 215 nm at a heating rate of 1 K/min.

Table 2. Composition of Precipitates (P) and Supernatants (S) of Glycinin after Heat Treatment at $I = 0.5$, 0.2, and 0.03 at pH 7.6, 5.2, and 3.8 As Determined by Nonreduced SDS−PAGE

I (M)	pH	solubility (%)	S/P	A polypeptides	B polypeptides	AB subunits	aggregates (>100 kDa)
0.5	7.6	53 ± 4	S	+	−	−	+
			P	−	−	−	+
	5.2	9 ± 8	P	−	−	−	+
	3.8	4 ± 1	P	+	+	+	+
0.2	7.6	52 ± 3	S	+	−	−	+
			P	−	−	−	+
	5.2	2 ± 8	P	−	−	−	+
	3.8	5 ± 6	P	−	−	−	+
0.03	7.6	54 ± 1	S	+	−	−	+
			P	−	−	−	+
	5.2	1 ± 1	P	−	−	−	+
	3.8	72 ± 6	S	+	+	+	+

Because at $I = 0.5$ and pH 3.8 a comparable amount of 7S and 11S glycinin is present (Lakemond et al., 2000), this condition was used to obtain enriched 7S and 11S fractions as described under Materials and Methods. Far-UV CD temperature scans of these enriched fractions were recorded (Figure 3). The enriched 7S and 11S fractions underwent a single transition at 73 and 80 °C, respectively. This demonstrates that the denaturation profile of the total glycinin fraction is composed of a separate 7S and 11S contribution (compare Figure 2A). Because the protein concentrations of the enriched 7S and 11S fractions were too low for DSC analysis and protein concentration techniques could not be used because of the danger of modifying protein structure, these experiments were omitted.

Solubility and Aggregate Formation of Acidic and Basic Polypeptides after Heat Treatment. To study to which extent glycinin precipitates at different conditions after heat treatment, the solubility of the supernatant, obtained by centrifugation, was determined (Table 2). SDS−PAGE analysis under reduced and nonreduced conditions was performed to determine whether acidic and/or basic polypeptides are present in the supernatants and precipitates obtained after heat treatment and to determine the involvement of disulfide

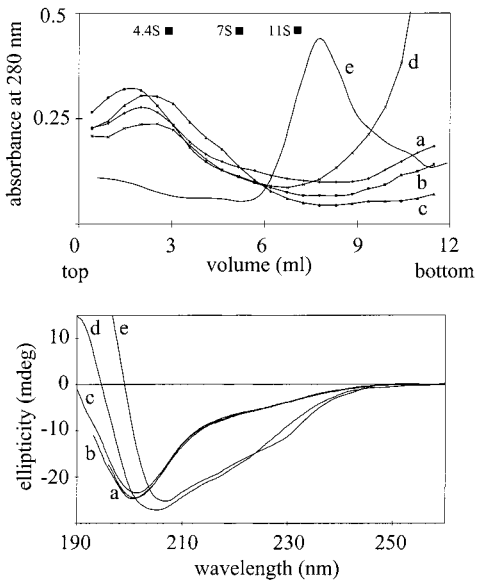

Figure 4. Protein elution profiles of supernatants of heat-denatured glycinin after ultracentrifugation in 5−20% sucrose density gradients (A) and far-UV CD spectra (B) at pH 7.6 at $I = 0.5$ (a), 0.2 (b), and 0.03 (c), at $I = 0.03$ M at pH 3.8 (d), and as nonheated glycinin at $I = 0.5$ and pH 7.6 (e) (■ = standard proteins).

bridges (results not shown). The results of the nonreduced SDS−PAGE are qualitatively presented in Table 2. Whereas the solubility of glycinin is 100% at pH 7.6 before heating, ∼50% of the protein remains soluble after heating at all ionic strengths. Only acidic polypeptides are present in the supernatants at pH 7.6, partly in aggregates that could be dissociated upon reduction in SDS. The precipitates at pH 7.6 contain mainly basic polypeptides, although also acidic polypeptides are present (corresponding to ∼10−20% of the amount present before heat treatment). These polypeptides are all present in SDS as large aggregates containing intermolecular S−S bridges.

After heating at pH 5.2, at which pH solubility was very low before heating at $I = 0.2$ and 0.03, almost all material appears in the precipitate, which consists of large aggregates that are linked by S−S bridges, as in SDS no individual acidic and basic polypeptides could be observed.

At pH 3.8 and $I = 0.5$ and 0.2 all protein precipitates, where as at $I = 0.03$ ∼75% of the protein remains soluble after heat treatment. At $I = 0.5$ and 0.2 the precipitate consists predominantly of large aggregates in SDS. In contrast to the precipitate at $I = 0.5$, at $I = 0.2$ no covalently linked acidic and basic polypeptides are found. In the supernatant $I = 0.03$ acidic as well as basic polypeptides were present, both as aggregates and as individual acidic (A) or basic (B) polypeptides or as AB molecules in the presence of SDS.

Quaternary, Tertiary, and Secondary Structure of Soluble Acidic Polypeptides Obtained after Heat Treatment. To examine the molecular structure of the nonprecipitated polypeptides in the supernatants obtained after heat treatment, ultracentrifugational analysis (Figure 4A), tryptophan fluorescence spectroscopy, and CD spectroscopy (Figure 4B) were performed. Only the proteins in the supernatants obtained after heating at pH 7.6 at all three ionic strengths and at pH 3.8 at $I = 0.03$ were studied, because at the other pH/I combinations >90% of the protein has precipitated after heat treatment.

Figure 4A shows that all ultracentrifugational profiles demonstrate two major populations. At pH 7.6 the acidic polypeptides present in the supernatants are present partly as large (soluble) aggregates and partly as oligomers and monomers (4S or smaller). The AB complexes found in the supernatant at pH 3.8 and $I = 0.03$ (Table 2) are apparently present as larger aggregates or as (oligomers of) the individual AB molecule and not as an 11S/7S form.

Tryptophan fluorescence spectra of the supernatants of glycinin demonstrated that the difference in λ_{max} after heating compared to the λ_{max} of the spectra recorded before heating differs significantly for the various conditions (pH 3.8/$I = 0.03$, ∼1 nm; pH 7.6/$I = 0.5$, ∼0.5 nm; pH 7.6/$I = 0.2$, ∼2 nm; pH 7.6/$I = 0.03$, ∼1.5 nm). In all cases the local environment of the tryptophans is more polar after heating than before.

The CD spectrum of the supernatant of glycinin obtained after heat denaturation at $I = 0.03$ and pH 3.8 (Figure 4B) has a different shape (zero-crossing around 196 nm) from the spectra of the soluble glycinin fraction obtained at pH 7.6 at $I = 0.5$, 0.2, and 0.03 (zero crossing below 190 nm). From comparison with reference spectra (Johnson, 1990) it can be concluded that the amount of random coil at $I = 0.03$ at pH 3.8 is the lowest of all conditions studied. For comparison also the spectrum of nonheated glycinin at $I = 0.03$ at pH 3.8 is shown in Figure 4B. This spectrum resembles most the spectrum at $I = 0.03$ at pH 3.8 after heat treatment, indicating that at this ionic strength and pH secondary structure has changed less than for the soluble polypeptides at pH 7.6 at all ionic strengths.

DISCUSSION

This study was performed to investigate the influence of pH and ionic strength on glycinin solubility and structure during and after heat treatment. It was determined whether glycinin behaves differently under conditions used frequently in the literature ($I = 0.5$ and pH 7.6) in comparison to conditions more representative for food systems ($I = 0.02$−0.2 and pH 3−7).

Heat Denaturation of Glycinin. It has been shown that both the denaturation temperature and the enthalpy vary strongly with pH and ionic strength (Table 1). The highest denaturation temperatures of glycinin at a particular ionic strength are found at pH 5.2. This is in accordance with the general opinion that globular proteins are most stable close to their apparent pI (Privalov and Khechinashvili, 1974). The enthalpies of the transitions found in this study are generally higher than those reported in the literature. At pH 7.6 enthalpies are reported ranging from 7−17 J/g for different ionic strengths (Koshiyama et al., 1980/1981; Marshall and Zarins, 1989; Bogracheva et al., 1996). Exothermic transitions, probably due to aggregation (Marshall and Zarins, 1989), are observed only at $I = 0.5$.

Denaturation/aggregation (as studied by DSC) and changes in secondary structure (far-UV CD) take place simultaneously (Table 1). Upon heating, changes in tertiary structure, as determined by near-UV CD, occur at lower temperatures than changes in secondary structure, except for $I = 0.2$ at pH 3.8 (Table 1). This is more remarkable because it is generally believed that the energy involved in these transitions is required to compensate for the increased exposure of hydrophobic sites of the protein to the solvent, as expected to occur in tertiary unfolding.

Glycinin Heat Denaturation in Relation to the 7S/11S Ratio. This work shows that the 7S/11S glycinin ratio influences heat denaturation of glycinin. At all conditions studied glycinin 7S denatures at a lower temperature than 11S glycinin. This is complementary to the results of Utsumi et al. (1987), who reported this phenomenon at $I = 0.01$ and pH 7.6 (ratio 7S/11S = 1:2). Although at pH 3.8 and $I = 0.5$ ~50% of the protein is present in the 7S form, its endothermic transition could not be detected (Table 1). This could be a combination of the fact that (1) due to its lower denaturation temperature the enthalpy of 7S glycinin is by definition lower than that of 11S, assuming that the folding state of 7S and 11S glycinin is equivalent at a tertiary and secondary folding level (Privalov and Khechinashvili, 1974); and (2) the 7S glycinin endotherm could be compensated by the exothermic transition.

In addition, at conditions at which the 11S form dominates over the 7S form (at pH 7.6), 80−90% of the acidic polypeptides remain in solution after heat treatment (Table 2). This implies a disruption of the S−S bridge and the noncovalent bonds between the acidic and basic polypeptide. This is in line with the results of Wolf and Tamura (1969), Hashizume et al. (1975), Mori et al. (1981), and Yamagishi et al. (1987). It is likely that the disruption observed at pH 7.6 does not take place at pH 5.2 and 3.8, on the basis of the presence of both acidic and basic polypeptides in the precipitate or the supernatant at these conditions (Table 2).

Glycinin Heat Denaturation in Relation to the A/B Polypeptide Arrangement. A larger exposure of acidic polypeptides (Lakemond et al., 2000), as observed in the preceding paper, could possibly correlate with a higher endothermic transition temperature. It is also remarkable that the exothermic transition could be observed only at high ionic strength at which the acidic polypeptides are exposed the most. Although the previous work (Lakemond et al., 2000) shows a correlation between the relative exposure of the polypeptides and the solubility at ambient temperature, this correlation could not be observed for the heated material.

In conclusion, the temperature at which and the mechanism by which glycinin denatures depend on pH and ionic strength and seem to be correlated to glycinin quaternary, tertiary, and secondary structure before heating. Because ionic strength and pH affect the solubility of glycinin after heating, it is important to take these parameters into account when soy proteins are applied in food. It can be expected that pH and ionic strength also affect gel formation, another important functional property in food. This will be the subject of our next studies.

ABBREVIATIONS USED

BSA, bovine serum albumin; UV CD, ultraviolet circular dichroism; DSC, differential scanning calorimetry; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; A, acidic polypeptides; B, basic polypeptides.

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