JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

JUNE 2000
VOLUME 48, NUMBER 6

Soy Glycinin: Influence of pH and Ionic Strength on Solubility and Molecular Structure at Ambient Temperatures

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This study describes the relationship between the solubility of glycinin, a major soy protein, and its structural properties at a quaternary, tertiary, and secondary folding level under conditions representative for food products. When the ionic strength is lowered from 0.5 to 0.2 or 0.03, the basic polypeptides shift more to the exterior of the glycinin complex, as determined at pH 7.6 by labeling solvent-exposed lysines, supported by the study of the proteolytic action of clostripain on glycinin. This structural reorganization caused the pH of minimal solubility to shift to higher values. Ultracentrifugational analysis shows that at pH 7.6 and an ionic strength of 0.5 glycinin forms hexameric complexes (11S), whereas at pH 3.8 and at an ionic strength of 0.03 glycinin exists as trimers (7S). Intermediate situations are obtained by modulation of pH and ionic strength. The observed quaternary dissociation correlates with an increased amount of nonstructured protein at a secondary level and with changes in tertiary folding as determined using circular dichroism. Tryptophan fluorescence shows no significant structural changes for different ionic strengths but demonstrates a more tightly packed fluorophore environment when the pH is lowered from 7.6 to 3.8.

Keywords: *Soy; glycinin; solubility; pH; ionic strength; protein structure*

INTRODUCTION

Soy proteins are applied in a wide range of food products. Despite all of the research performed in the past decades, much is still unknown about the behavior of soy proteins at a molecular level under conditions present in food. Generally, the pH of food products ranges from pH 3 to 7, and the ionic strength varies from 0.02 to 0.2, whereas the majority of soy protein studies have been carried out at pH 7.6 at an ionic strength of 0.5, as it is known that soy proteins are soluble under these conditions. Furthermore, it is known that lowering the ionic strength and pH affects soy protein structure, which in some cases seems to be related to changes in the functional behavior in food, such as solubility (Peng et al., 1984). Good protein solubility generally correlates with "optimum" gelation, emulsifying, and foaming activity [see, for example, Kinsella (1979)].

This research focuses on glycinin, a major storage protein in soybeans. Glycinin represents ~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

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10.1021/jf9908695 CCC: $19.00
Published on Web 05/05/2000

pair of acidic and basic polypeptides is encoded by a single gene and cleaved post-translationally (Tumer et al., 1981). The solubility of glycinin in dilute Tris buffer is minimal around pH 5.5 according to Thanh and Shibasaki (1976) and from pH 4.3 to 6.0 according to Yagasaki et al. (1997). Furthermore, it is known that the solubility of glycinin depends strongly on ionic strength (Eldridge and Wolf, 1967; Thanh and Shibasaki, 1976; Yagasaki et al., 1997). It has been shown that pH and ionic strength can also influence glycinin molecular structure. At pH 7.6 and at an ionic strength of 0.5 glycinin is mainly present in a hexameric form of 360 kDa with a sedimentation coefficient of 11S (Badley et al., 1975). Lowering the ionic strength to 0.01 at pH 7.6 causes glycinin to dissociate from the 11S form mainly into the 7S form (Wolf and Brigs, 1958; Utsumi et al., 1987), believed to be the trimeric form, which has a more nonstructured conformation (Utsumi et al., 1987). Wolf et al. (1958) found that at pH 3.8−2.2 the glycinin complex is present in the 7S and/or 3S form. Tertiary unfolding (Catsimpoolas et al., 1969; Koshiyama, 1972) and secondary unfolding (Koshiyama, 1972) at low pH values (<3) also have been reported.

This work presents a detailed description of the influence of both the ionic strength and the pH on glycinin in a detailed way at all different protein structural levels under conditions relevant for food products.

MATERIALS AND METHODS

Isolation of Glycinin. Glycinin was purified from Williams 82 soybeans (harvest 1994). Broken soybeans were milled into particles with a diameter of 0.5 mm. The milling was performed in the presence of solid CO_2 (volume ratio soybean/CO_2 = 2:1) to prevent heat denaturation of soy protein. After defatting with hexane at 20 °C, glycinin was extracted and purified according to the method of Thanh and Shibasaki (1976), except that the purification procedure was performed at 20 °C. The purified glycinin (in 35 mM potassium phosphate buffer, pH 7.6, with 0.4 M NaCl and 10 mM 2-mercaptoethanol) was stored at −20 °C at 12 mg/mL. The purity of glycinin was determined by SDS−PAGE under reducing and nonreducing conditions using 10−15% gradient gels in a Pharmacia Phast System according to the instructions of the manufacturer. The protein bands were stained using Coomassie Brilliant Blue. Glycinin purity was estimated to be >95% by densitometric analysis of the gel.

Prior to each experiment, the purified glycinin was dialyzed (Visking 8/32 tubings, Medicell, London, U.K.) at 20 °C against pH 7.6 buffers of the desired ionic strength. The $I = 0.5$ buffer consisted of 35 mM potassium phosphate and 0.4 M NaCl, the $I = 0.2$ buffer of 35 mM potassium phosphate and 0.1 M NaCl, and the $I = 0.03$ buffer of 10 mM potassium phosphate. For experiments carried out at pH 3.8 and 5.2, the pH of the sample was subsequently adjusted using HCl. Consequently, for pH 3.8 the ionic strength was increased by ∼0.015 M for $I = 0.5$ and 0.2 and by ∼0.008 M for $I = 0.03$, on the basis of the amount of HCl needed to lower the pH to 3.8.

Determination of Solubility. The pH of glycinin solutions (0.6 mg/mL) in pH 7.6 buffer at $I = 0.5$, 0.2, or 0.03 was lowered by adding different amounts of HCl stock solutions (0.05−5 M) to obtain final pH values ranging from 7.6 to 2.5 with ∼0.2 pH unit intervals. After incubation of the glycinin samples for 16 h at 20 °C, the samples were centrifuged for 5 min at 15800*g* and 20 °C (precipitate consists of particles >∼0.5 μm as was determined using the Stokes equation). The protein concentration of the supernatants was determined in triplicate using the Bradford assay (Bradford, 1976) using BSA as a standard. Prolonged incubation of the samples for 16 h at 20 °C did not result in any proteolytic digestion of the material at any of the conditions studied as analyzed by SDS−PAGE (results not shown).

Fluorescein Isothiocyanate (FITC) Labeling. Labeling experiments of glycinin were performed at pH 7.6 only, because at acidic pH FITC is not reactive to ϵ-amino groups (Stark, 1970). It has been reported that the surface SH content of glycinin depends on the method of glycinin preparation (Wolf, 1993). For our material no free SH groups could be detected at pH 7.6 (unpublished results) using the Ellman reagent (Ellman, 1959).

FITC (Fluka, 46950) was dissolved in the appropriate buffer ($I = 0.5$, 0.2, and 0.03 at pH 7.6) containing 10% ethanol. The FITC solution was incubated with the glycinin solutions (12 mg/mL) in a molar ratio of 1:4.2 (volume ratio = 1:10) during 16 h at 20 °C. Next, cysteine was added to the mixture (molar ratio of glycinin/cysteine = 1:12.5) to quench the reaction. After 2 h, the solution was dialyzed (Visking 8/32 tubings) against the appropriate buffer to isolate the labeled glycinin. Next, the labeled glycinin samples were denatured in a 10 mM potassium phosphate buffer (pH 6.6) containing 6 M urea and 20 mM 2-mercaptoethanol to dissociate the acidic and basic polypeptides. The denatured samples were applied onto an HPLC system (Spectra Physics, San Jose, CA) using a mono Q 5/5 column (Pharmacia Biotech, Uppsala, Sweden) and eluted at 1 mL/min. The basic polypeptides (no contamination with acidic polypeptides) were eluted with the buffer mentioned above. The fraction bound to the column (the acidic polypeptides; containing <5% basic polypeptides) was eluted by adding 1 M NaCl to the elution buffer. The absorbance at 280 nm (A_{280}) (SpectraSYSTEM UV 3000) and fluorescence (SpectraSYSTEM FL3000; excitation at 493 nm and emission at 522 nm) were detected simultaneously. Measurements were carried out in a range in which fluorescence intensity depended linearly on protein concentration (data not shown). The absorbance at 280 nm (A_{280}) was corrected for the expected difference in molar extinction coefficient between the acidic and basic polypeptides (factor = 1.8). This factor was based on the average amount of aromatic amino acids of the basic and acidic polypeptides as was calculated from the amino acid composition of the different glycinin isoforms (Scallon et al., 1987; Nielsen et al., 1989; Sims and Goldberg, 1989; Thanh et al., 1989; Cho and Nielsen, 1989). The fluorescence signal of the basic polypeptides was multiplied by a factor of 2.5 because the basic polypeptides possess less reactive groups than the acidic ones. The factor of 2.5 was based on the amount of lysine present in the acidic and basic polypeptides (Scallon et al., 1987; Nielsen et al., 1989; Sims and Goldberg, 1989; Thanh et al., 1989; Cho and Nielsen, 1989), as arginine is not expected to be labeled significantly at pH 7.6 because of its pK_a of ∼11. The ratio between the corrected fluorescence integrated peak area of acidic and basic polypeptides and the corresponding corrected integrated absorbance at 280 nm was used as a measure for the relative exposure of the acidic and basic polypeptides under the different conditions. This ratio will be referred to as "the relative exposure". All experiments were carried out in duplicate.

Proteolytic Digestion. Clostripain (Sigma, C7403) was dissolved in the appropriate buffer ($I = 0.5$, 0.2, or 0.03 at pH 7.6). Ten microliters of enzyme solution (0.01 mg/mL) was reacted with 100 μL glycinin samples (4 mg/mL) in buffers with 0.5, 0.2, and 0.03 ionic strengths at pH 7.6 in the presence of 1 mM $CaCl_2$ for 30 min at 30 °C. After the addition of EDTA (molar ratio of EDTA/Ca^{2+} = 10:1), the samples were analyzed by reduced SDS−PAGE (12% polyacrylamide gels) using the Mini-PROTEAN II system (Bio-Rad, Hercules, CA) according to the instructions of the manufacturer using Coomassie Brilliant Blue for staining. The amount of digested acidic and basic polypeptides was calculated by quantification of the protein bands by densitometric analysis.

Ultracentrifugation Experiments. To determine sedimentation coefficients of the glycinin samples, 5−20% sucrose step gradients (four steps; 12 mL total volume) were prepared in buffers with $I = 0.5$, 0.2, and 0.03 at both pH 7.6 and 3.8. Prior to the experiments the gradients were allowed to diffuse to linearity during 24 h at 4 °C. Glycinin samples (0.3 mL, 4

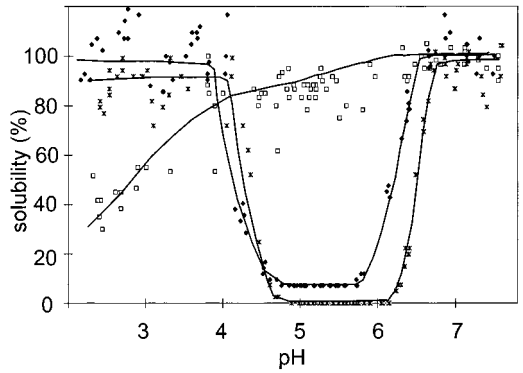

Figure 1. pH-dependent solubility profiles of glycinin [I = 0.5 (□), 0.2 (◆), 0.03 (∗)] determined after incubation of 0.6 mg/mL samples for 16 h at 20 °C.

mg/mL) in I = 0.5, 0.2, and 0.03 buffers at pH 7.6 and 3.8 were loaded on top of the gradient. Next, the tubes were centrifuged in a Beckman L60 centrifuge at 186000g (at r_{max}) for 16 h at 20 °C. After ultracentrifugation, the gradient was fractionated in 0.5 mL aliquots of which the absorbance at 280 nm was measured. The experiments were performed in duplicate. Svedberg (S) values were estimated after calibration of the gradient using proteins with known S values (11.2S for γ-globulin, 7S for katalase, 4.4S for BSA, 2.5S for trypsin, and 1.78S for ribonuclease).

Fluorescence Spectroscopy. Fluorescence spectra of 0.2 mg/mL glycinin samples in pH 7.6 and 3.8 buffers at different ionic strengths (0.5, 0.2, and 0.03) were recorded on a Perkin-Elmer luminescence spectrometer LS 50 B at 20 °C. Excitation was at 295 nm, and the emission spectrum was recorded as the average of three spectra from 300 to 450 nm using a scan speed of 50 nm/min and a resolution of 0.5 nm. Both the excitation and emission slit were set at 2.5 nm.

Circular Dichroism (CD) Spectroscopy. Far- and near-UV CD spectra of 0.6 and 1.2 mg/mL glycinin samples, respectively, were recorded at a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) in 0.5, 0.2, and 0.03 ionic strengths at pH 7.6 and 3.8 at 20 °C. For near-UV CD measurements quartz cells with a path length of 10 mm were used, whereas for far-UV CD measurements quartz cells with a path length of 0.2 mm were used. The scan interval for near-UV CD was 350−250 nm and for far-UV CD measurements, 260−190 nm. Spectra were recorded as averages of 25 spectra using a scan speed of 100 nm/min, a bandwidth of 1 nm, a response time of 64 ms, and a step resolution of 0.5 nm.

RESULTS

Solubility of Glycinin. Although many studies on soy protein have been performed at pH 7.6 and I = 0.5, this investigation was aimed at measuring changes in the solubility of glycinin when the ionic strength and pH were lowered to conditions more representative for food products. From Figure 1 it can be seen that glycinin solubility at 20 °C depends strongly on ionic strength and pH. At I = 0.5 and pH 7.6 all glycinin is soluble (100%), but the solubility gradually decreases from 100 to 30% when the pH is lowered from 6.5 to 2.5. The precipitation of glycinin below pH 3.8 is not instantaneous but requires several hours of incubation. At ionic strengths of 0.2 and 0.03 the solubility profiles show one minimum; at I = 0.2 ∼95% of the protein precipitates in the pH range from 5.8 to 4.7, whereas at I = 0.03 complete precipitation occurs between pH 6.2 and 4.7. In the latter case the precipitation occurs almost immediately after adjustment of the pH. Glycinin is completely soluble below pH 3.8 at both I = 0.2 and 0.03.

To investigate whether the observed solubility behavior relates to differences in molecular organization of glycinin, experiments described in the following sections were performed at the three ionic strengths at both pH 7.6 and 3.8. These particular pH values were chosen because they represent the solubility maxima of glycinin. No pH values at solubility minima were chosen because the spectroscopical techniques used require the material to be soluble.

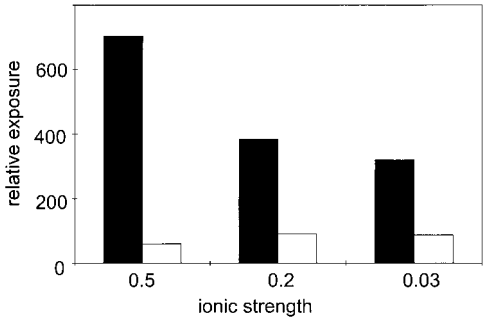

Figure 2. Relative exposure (for definition, see Materials and Methods) at pH 7.6 of acidic (black bars) and basic polypeptides (white bars) after labeling of glycinin with FITC at different ionic strengths at pH 7.6.

Structural Arrangement of Acidic and Basic Polypeptides within Glycinin. To investigate whether the exposure of acidic and basic polypeptides in the glycinin molecule could explain the different solubility curves, FITC labeling experiments were performed at pH 7.6. As explained under Materials and Methods, this probe is expected to label under these conditions merely lysines present at the surface of the molecule, which makes it possible to detect conformational rearrangements of acidic and basic polypeptides in the glycinin complex. Figure 2 shows that the relative exposure of both basic and acidic polypeptides depends on the ionic strength. The acidic polypeptides are exposed ∼12 times more than the basic ones at I = 0.5. The relative exposure at I = 0.2 and 0.03 of the acidic polypeptides is about half of that at I = 0.5, whereas the relative exposure of the basic polypeptides increases by a factor of ∼1.5. When the ionic strength is lowered to 0.2 and 0.03, the acidic polypeptides are still 4 times more exposed than the basic ones. These results clearly indicate a rearrangement of the polypeptides within the glycinin molecule, modulated by ionic strength.

The arrangement of the acidic and basic polypeptides in the complex was alternatively studied by testing their accessibility to proteolytic action by clostripain, resulting in degradation products of approximately 15 and 25 kDa using SDS−PAGE under reducing conditions (results not shown). At an ionic strength of 0.5 and 0.2, 85 ± 1% (n = 5) of the acidic polypeptides are degraded, whereas at an ionic strength of 0.03 only 62 ± 4% (n = 5) of the acidic polypeptides are degraded. This means that the relative exposure of the acidic polypeptides has decreased by a factor of 1.4. The basic polypeptides are not affected significantly by the presence of clostripain at any of the tested ionic strengths. This could be either due to the fact that the overall relative exposure of the acidic polypeptides is higher than that of the basic polypeptides at all ionic strengths or due to the interference of electrostatic repulsions between the basic polypeptides and the Ca^{2+} required for clostripain action. Qualitatively these data support the FITC labeling results.

Quaternary Folding of Glycinin. To study possible differences in the quaternary folding of glycinin, ultra-

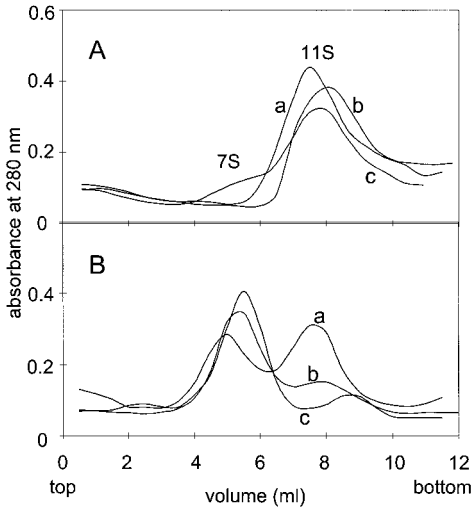

Figure 3. Protein elution profiles using 5−20% sucrose density gradients after ultracentrifugation at pH 7.6 (A) and 3.8 (B) [I = 0.5 (a), 0.2 (b), 0.03 (c)].

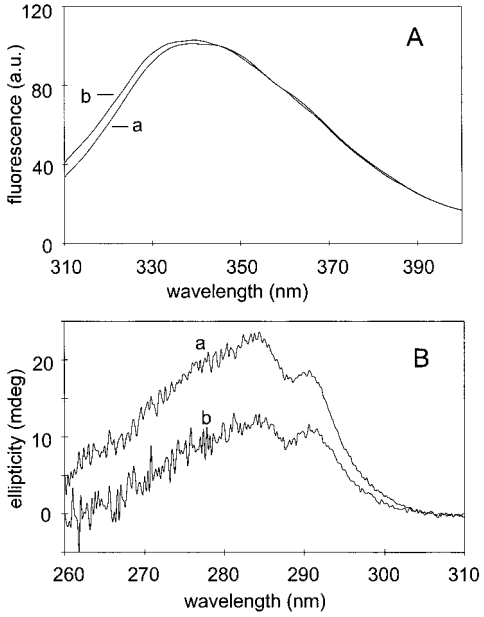

Figure 4. Tertiary folding of glycinin at I = 0.5 M at pH 7.6 (a) and 3.8 (b) studied with tryptophan fluorescence spectroscopy (A) and near-UV CD spectroscopy (B).

centrifugation experiments were performed (Figure 3). At pH 7.6 and an ionic strength of 0.5 or 0.2 glycinin has a sedimentation coefficient of 11S, as estimated by calibration with proteins with a known Svedberg coefficient. A fraction with a higher Svedberg coefficient, probably the 15S fraction as described by Wolf and Nelsen (1996), also seems to be present at I = 0.5. At I = 0.03, next to an 11S fraction, a 7S fraction, representing 15−25% of all protein, could be observed. At pH 3.8 and I = 0.5 about half of the glycinin molecules are present in the 7S form, whereas at I = 0.2 and 0.03 glycinin is predominantly present in the 7S form.

Tertiary Folding of Glycinin. To determine differences in the tertiary interactions within the glycinin molecule, tryptophan fluorescence spectra were recorded at pH 7.6 and 3.8 at I = 0.5, 0.2, and 0.03. Only the spectra recorded at I = 0.5 (normalized at 345 nm) are shown (Figure 4A), to give typical examples. Fluorescence spectroscopy shows at I = 0.5 a shift of ∼2 nm of λ_{max} to lower wavelengths when the pH is lowered from

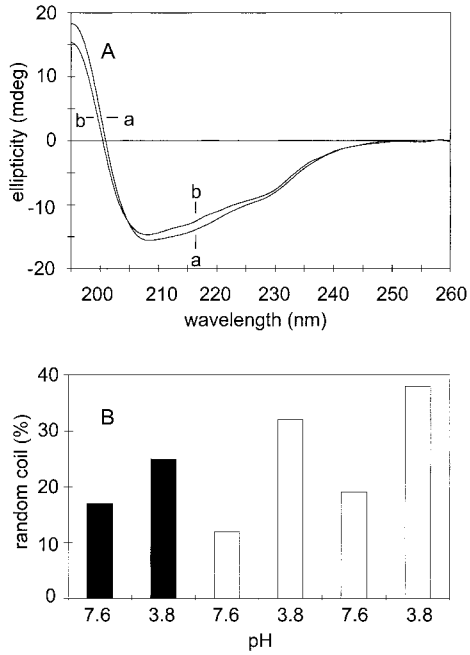

Figure 5. Far-UV CD spectra of glycinin at I = 0.5 at pH 7.6 (a) and 3.8 (b) (A) and the estimated amount of nonstructured glycinin as a function of pH and ionic strength (B) [I = 0.5 (black bars), 0.2 (gray bars), 0.03 (white bars)].

7.6 to 3.8, demonstrating that the local environment of tryptophan is changed into a more apolar one. A similar effect, although smaller (∼1 nm), can also be observed at I = 0.2 and 0.03. No significant changes in λ_{max} can be observed when the ionic strength is varied either at pH 7.6 or at pH 3.8 (results not shown).

All recorded near-UV CD spectra (Figure 4B) show positive ellipticity between 250 and 300 nm, with a maximum at 285 nm and a resolved shoulder at 291 nm. The near-UV CD spectra of samples of different ionic strengths all show a comparable shape at each particular pH. When the ionic strength is lowered at pH 7.6, a decrease of 20% in intensity can be observed when the spectrum at I = 0.5 is compared with that recorded at I = 0.03. At pH 3.8 a similar trend is observed when the ionic strength is lowered, although it is less pronounced than at pH 7.6 (∼10% decrease of intensity). Such a decrease in intensity of the near-UV CD bands generally points at a destabilization of the protein tertiary structure (Vuilleumier et al., 1993). When the pH is lowered from 7.6 to 3.8 at all three ionic strengths, the total intensity of the CD spectra decreases significantly, ∼35−45%. Furthermore, the ratio between the intensity at 285 and 291 nm decreases slightly. Because tryptophan generally absorbs at higher wavelengths than tyrosine in the 280−295 nm region (Vuilleumier et al., 1993), this could suggest that the local environment of the tyrosines is more destabilized than that of the tryptophans when the pH is lowered. These results confirm those found with fluorescence spectroscopy. It is concluded that the tertiary folding of glycinin depends more strongly on pH than on ionic strength.

Secondary Folding of Glycinin. The secondary structure of glycinin was studied by far-UV CD (Figure 5). Only the results at I = 0.5 are presented in Figure 5A to give typical examples. All far-UV CD spectra exhibit a negative extreme around 208 nm and a zero-crossing around 200 nm. Spectra could not be recorded below 195 nm due to high concentrations of chloride ions

in the $I = 0.2$ and 0.5 buffers. On the basis of comparison with reference spectra (Johnson, 1990), glycinin predominantly consists of α-helical structures. Using curve-fitting procedures with reference spectra (de Jongh and de Kruiff, 1990), the secondary structure content was estimated. Figure 5B presents the amount of nonstructured protein for the various conditions. The amount of nonstructured protein increases significantly when the pH is lowered from 7.6 to 3.8. Furthermore, at pH 3.8 the amount of nonstructured protein also increases when the ionic strength is lowered, whereas at pH 7.6 such a correlation is not observed.

DISCUSSION

This study was undertaken to determine glycinin structure under conditions (pH, ionic strength) used frequently in the literature in comparison to conditions more representative for food systems. Its structural properties at different folding levels are related to the solubility.

Our investigations show that the quaternary structure of glycinin is modulated by both the ionic strength and, more effectively, pH. Whereas at $I = 0.5$ and pH 7.6 glycinin is present in the 11S form, at $I = 0.03$ and pH 3.8 glycinin has dissociated into the 7S form (Figure 3). The dissociation of 11S into 7S seems to be correlated with significant changes at the secondary (Figure 5) and, to a lesser extent, the tertiary folding levels (Figure 4). Apparently there is no correlation between the 11S/7S ratio and the solubility at a concentration of 0.6 mg/mL, because at both conditions the solubility is 100% (Figure 1). Intermediate situations of the 11S/7S ratio can be obtained by varying the pH and ionic strength (Figure 3). Indications for such an 11S/7S dissociation/association have been reported previously (Wolf and Briggs, 1958; Wolf et al., 1958; Utsumi et al., 1987).

Although the relative exposure of the basic polypeptides increased significantly at $I = 0.03$ compared to at $I = 0.5$, the relative exposure of the acidic polypeptides at all ionic strengths was still higher than that of the basic polypeptides (Figure 2). Such a structural reorganization with changing ionic strength is qualitatively confirmed by probing the accessibility of the acidic and basic polypeptides by proteolytic digestion, as could explain the results of Kamata et al. (1982), who observed ionic strength dependent digestion of glycinin. This structural rearrangement does have a strong influence on the solubility profile (Figure 1). At $I = 0.2$ precipitation occurs between pH 5.8 and 4.7, which is in about the same range of the pI values of the acidic polypeptides (pH 4.8−5.4) (Catsimpoolas, 1969). At $I = 0.03$ this region of precipitation occurs at slightly higher pH (6.2−4.7) (Figure 1), suggesting that it shifts toward the isoelectric points of the basic polypeptides, which vary from pH 8 to 8.5 (Catsimpoolas, 1969). This correlates well with the observation that at all ionic strengths the acidic polypeptides are predominantly facing the outside of the glycinin complex (Figure 2), whereas when the ionic strength is lowered, the basic polypeptides partly displace the acidic ones from the exterior of the complex. The obtained data on the relative exposure of the acidic and basic polypeptides are in line with the model proposed by Marcone et al. (1998), which is based on the model of Plietz et al. (1983), who suggested that the basic polypeptides are present in the interior of the glycinin molecule.

The decreased solubility of glycinin below pH 3.8 (Figure 1) can be attributed to pH denaturation of the protein caused by protonation of the carboxyl groups. It is only observed at $I = 0.5$, where apparently the screening of positive charges of the salt is as efficient to overcome electrostatic repulsions. For $I = 0.5$ an additional complexity is observed. No minimum in solubility could be observed between pH 7.6 and 3.8 (Figure 1) at 20 °C, probably due to the salting-in effect. At 4 °C, however, precipitation does occur at pH 4.6 (instantaneous; 40% solubility), whereas the solubility is 100% at pH 7.6 and 80% at pH 3.8 (unpublished results). At 20 °C at $I = 0.5$ and pH 7.6 the solvent exposure of the acidic polypeptides is maximal. Generally, the preference of the basic polypeptides to reside at the inner part of the glycinin complex could be attributed to the higher content of hydrophobic amino acids in the basic polypeptides compared to that of the acidic ones (Catsimpoolas et al., 1971). When the temperature is lowered, the role of electrostatic interactions increases as the role of hydrophobic interactions decreases. This could favor the shift of the basic polypeptides to the exterior of the molecule, similar to the rearrangements induced by lowering the ionic strength at pH 7.6. To what extent this hypothesis is valid needs to be examined. Conclusively, the solubility behavior of glycinin at low pH is related to the relative arrangement of acidic and basic polypeptides at pH 7.6. The solubility profile of glycinin described in this work is in line with the results of Thanh and Shibasaki (1976), although in the latter work the ionic strength dependent shift in solubility was found at a lower ionic strength than presented here.

Because it can be expected that the observed modulation of quaternary structure and reorganization of polypeptides within the glycinin complex, as described in this work, has a strong influence on the functional properties of the protein, it is of great importance to study this protein under "food conditions" (pH 3−7 and $I = 0.02-0.2$).

ABBREVIATIONS USED

BSA, bovine serum albumin; CD, circular dichroism; FITC, fluoresceine isothiocyanate; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; UV, ultraviolet.

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Received for review August 5, 1999. Revised manuscript received February 17, 2000. Accepted February 17, 2000.

JF9908695