Effects of Dissociation, Deamidation, and Reducing Treatment on Structural and Surface Active Properties of Soy Glycinin

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Different soluble protein derivatives were prepared from soy glycinin by means of progressive dissociation, deamidation, and later reducing thermal treatment. Dissociation was achieved at 4 °C in low ionic strength or mild acid conditions (0.1 M HCl). For deamidation, mild acid treatment of glycinin was performed at 70 °C for 1.5–9 h. Deamidation levels (1.7–25.0%) increased linearly with time of heating. These results were compared to those obtained by alkaline deamidation. The reducing thermal treatment led to both soluble and insoluble polypeptides. Greater soluble protein yields were obtained when this treatment was applied to previously deamidated glycinins. The structural properties and surface activity of native and modified glycinin were studied. Dissociation, deamidation, and reduction resulted in structural changes on glycinin (increased surface hydrophobicity, decreased molecular size, and isoelectric pH) which enhanced adsorption of proteins at the air–water interface. In contrast to native glycinin, the influence of ionic strength on surface behaviors is rather limited when the protein is both deamidated and reduced.

Keywords: *Soybean; 11S protein; glycinin; deamidation; chemical modification; functional properties*

INTRODUCTION

Glycinin is one of the major globulins of soybean seeds. Belonging to the 11S-type protein family, it is characterized by a hexameric structure $(AB)_6$, organized in a close packed globular conformation, with a molecular weight around 360 000. The quaternary structure of this protein, constituted of six AB subunits, is stabilized by electrostatic and hydrophobic interactions, as well as by disulfide bridges between the A and B polypeptides (Peng et al., 1984).

The functional properties of this protein, as emulsifying or foaming agents for food formulation, have been extensively studied (Kinsella, 1979) and were shown to be limited by its globular structure, characterized by a low surface hydrophobicity and a limited molecular flexibility (Kim, 1985). Appropriate modifications of the structure could consequently improve these properties. For example, surface behaviors of 11S proteins could be enhanced by controlled dissociation of the oligomeric structure and partial unfolding of the polypeptides (Subirade et al., 1992). Breakdown of the native conformation by reducing disulfide bonds or by succinylation also markedly increases solubility, viscosity, and surface activity of 11S proteins (Kim and Kinsella, 1987a,b; Schwenke et al., 1993). However, most of the modifications used either are developed at the laboratory scale and cannot be applied for industrial purposes or employed chemical reagents that are not allowed in food industry.

Besides chemical modifications, the dissociation of glycinin could also be induced by varying environmental factors such as ionic strength, pH, or temperature (Peng et al., 1984). At low ionic strength and acidic or slightly alkaline pH conditions, partial or total dissociation of glycinin into 7S and 2S components is observed. Simultaneous unfolding of the polypeptide chains could occur (Koshiyama, 1972; Eldridge and Wolf, 1967). Mild acid treatments have been used to dissociate soy, oat, and sunflower protein isolates, leading to improvement of solubility, foaming, and emulsifying properties (Matsudomi et al., 1985; Ma and Khanzada, 1987; Claughton and Pearce, 1989).

In the present study, the objectives were to study the relationships between the structural and functional changes of glycinin resulting from progressive dissociation, deamidation, and reducing treatments. The effects of structure modification on solubility and surface behaviors were especially investigated.

Glycinin appears to be an interesting protein for such studies, according to its oligomeric structure and also to the very different physicochemical characteristics of the constitutive polypeptides, acidic and hydrophilic for the A type, basic and hydrophobic for the B type. Moreover, these differences are very interesting to exploit for applied purposes. However, the poor solubility of the B polypeptides appeared to be a real limitation for functional uses. Consequently, one of the goals of this study was to produce soluble B polypeptides.

MATERIALS AND METHODS

Chemical reagents of analytical grade were obtained from Merck and Sigma Chemical Co. Seeds of soybean variety Alaric, obtained from the Station de Génétique et d'Amélioration des Plantes (INRA, Montpellier, France), were employed. The seeds were dehulled, ground, and defatted by Soxhlet extraction with hexane. The remaining solvent was removed from the defatted flour by evaporation at room temperature.

All solutions were prepared with Millipore water and with added 1 mM benzamidine as protease inhibitor and 0.02% sodium azide as antimicrobial agent, even those used for dialysis.

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0021-8561/95/1443-1993$09.00/0

Table 1. Treatments and Nomenclature of Protein Samples

no.	sample	modification	nomenclature
I	crude glycinin	none	$(AB)_6$
II	crude glycinin dialyzed against water	dissociation at low ionic strength	$(AB)_6$*
III	crude glycinin treated in cold 0.1 M HCl for 9 h and neutralized	dissociation and unfolding in cold acidic medium	AB_0
IV	crude glycinin treated in 0.1 M HCl solution at 70 °C during 1.5, 3, 4.5, 6, and 9 h and neutralized	dissociation, unfolding, deamidation and limited hydrolysis in hot acidic medium	$AB_{1.5}$ AB_3 $AB_{4.5}$ AB_6 AB_9
V resulting	soluble fraction from sample III submitted to reducing thermal treatment at pH 8	idem III plus cleavage of disulfide bonds; separation from the B polypeptides as insoluble fraction	A_0
VI resulting	soluble fractions from samples (IV) submitted to reducing thermal treatment at pH 8	idem IV plus cleavage of disulfide bonds; deamidated and free polypeptides (A and B)	A_3B_3 A_6B_6 A_9B_9
VII resulting	soluble fraction from deamidated glycinin in alkaline medium (0.2 M HCO_3^- or 0.2 M PO_4^{3-}, pH 8, at 100 °C during 5 h) submitted to a following reducing thermal treatment at pH 8	idem VI	AB_{CO_3} AB_{PO_4}

Preparation of Glycinin. Crude glycinin was prepared from defatted soy flour according to the procedure of Thanh and Shibasaki (1976) as adapted by Kim and Kinsella (1987a), with slight modifications.

Defatted flour (100 g) was suspended in 2 L of 0.03 M Tris-HCl buffer, pH 8, containing 2 mM 2-mercaptoethanol. The dispersion was stirred for 2 h at 24 °C, adjusted to pH 8 periodically, and then centrifuged at 4800 rpm for 30 min at 20 °C (Centrifuge Jouan K 110). The supernatant was once again centrifuged at 9700 rpm for 30 min at 20 °C (Centrifuge Sorvall, rotor GS3). The clarified supernatant was adjusted to pH 6.4 with 2 N HCl, stirred overnight at 2 °C, and then centrifuged at 9700 rpm for 30 min at 4 °C.

Glycinin precipitate was washed exhaustively with 0.03 M Tris-HCl buffer, pH 6.5, and then dispersed in 0.03 M Tris-HCl buffer, pH 8. While stirring until the protein was fully dissolved, the pH of the protein dispersion was maintained at pH 8 by adding 1 N NaOH. The glycinin solution was dialyzed against 1% $(NH_4)HCO_3$, pH 8, at 4 °C and then frozen with liquid nitrogen and freeze-dried. The enriched glycinin sample contained more than 90% glycinin as determined by PAGE and affinity chromatography on concanavalin A Ultrogel. The nomenclature of the sample thus obtained is $(AB)_6$, as representative of the hexameric nonmodified glycinin.

Preparation of Modified Glycinins. *Dissociation by Low Ionic Strength.* Aqueous solutions (10 mg/mL) of crude glycinin were dialyzed against water (adjusted to pH 8 with NH_3) at 4 °C during 3 days and then freeze-dried. The nomenclature used for this sample was $(AB)_6$*.

Dissociation and Deamidation in Acidic Medium. Dissociation and acid deamidation were carried out as summarized in Table 1. Glycinin lost its hexameric structure, leading to dissociated AB subunits, more or less deamidated by the acidic treatment. The resulting protein derivatives were called AB_0, $AB_{1.5}$, AB_3, $AB_{4.5}$, AB_6, and AB_9, the index being related to the duration of the acidic treatment, from 0 to 9 h.

Reducing Thermal Treatment. The acid-treated samples (AB_0, AB_3, AB_6, and AB_9) were solubilized at 2.5–5 mg/mL in 0.03 M Tris-HCl buffer, pH 8, with 0.2% $Na_2S_2O_5$ as reducing agent. The protein solutions were then heated at 100 °C during 5 min. After the treatment, solutions were cooled and centrifuged at 10000*g* during 15 min. The supernatants were dialyzed against water and then freeze-dried. The nomenclature of the soluble proteins thus obtained is A_0, A_3B_3, A_6B_6, and A_9B_9, indicating that the disulfide bridge between A and B constitutive polypeptides has been reduced.

Dissociation and Deamidation in Alkaline Medium plus Reducing Thermal Treatment. Deamidation of crude glycinin was also performed using the alkaline conditions of Shih (1991) to reach a high degree of deamidation. Solutions of crude glycinin (10 mg/mL) in 0.2 M $NaHCO_3$ or NaH_2PO_4 (adjusted to pH 8 with NaOH) were heated at 100 °C during 5 h and reduced as previously. The nomenclature of samples corresponding to the present treatment is AB_{CO_3} and AB_{PO_4}, respectively.

Summarized information on treatments and nomenclature of the resulting protein samples are presented in Table 1.

Protein Determination. Protein concentration was determined using the biuret method described by Gornall et al. (1949). The Lowry method (Lowry et al., 1951) was used as an alternative for diluted solutions. Calibration of both methods was performed by using bovine serum albumin and purified pea legumin (11S-type protein) as standard proteins.

Degree of Deamidation. The degree of deamidation was determined by comparing the amount of ammonia released by a moderated protein hydrolysis (Matsudomi et al., 1985) for the modified and nonmodified glycinin samples. Twenty milligrams of sample was dispersed in 5 mL of 3 M H_2SO_4 and incubated during 3 h at 110 °C in sealed tubes. The ammonia released is measured according to the colorimetric method of Berthelot on the filtered hydrolyzed sample (Godon and Loisel, 1981).

Degree of Hydrolysis. The extent of peptide bond hydrolysis was determined by the increase of α-amino groups measured by the trinitrobenzenesulfonic acid (TNBS) method (Adler-Nissen, 1979). Complete peptide bond hydrolysis (100%) was achieved by hydrolysis with 6 N HCl at 110 °C for 24 h. The degree of hydrolysis was expressed as a percentage of the value obtained after this treatment.

Polyacrylamide Gel Electrophoresis (SDS–PAGE). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out after reduction of the protein by β-mercaptoethanol (5% v/v), as described by Laemmli (1970). Gel dimensions were 105 × 85 × 1.5 mm (Midget of Touzard and Matignon system, Model HE 6410, France), and acrylamide concentrations were 15% and 5.5% for separation and stacking gels, respectively. After electrophoresis, the gel slabs were fixed by trichloroacetic acid (12.5%) for 30 min and stained overnight by addition of Coomassie Blue G-250 (125 mg/100 mL of methanol/water 1:1). Destaining was achieved with water/ethanol/acetic acid (4.5:4.5:1 by vol) and then with a solution of acetic acid (5%).

Gel Filtration. An exclusion–diffusion Superose 12 HR column connected to an LKB HPLC was used; 200 μL of protein solution (1 mg/mL) was injected into the column equilibrated either with 0.035 M phosphate/0.4 M NaCl buffer, pH 7.6 (ionic strength $\mu = 0.5$) (standard phosphate buffer) (Thanh and Shibasaki, 1976), or with 0.02 M phosphate buffer, pH 7.6 ($\mu = 0.057$). Elution was performed at 0.5 mL/min.

Proteins were detected at the column outlet at 280 nm. The column was calibrated by using the following MW standards: dextran blue (2×10^6), aldolase (158 000), thyroglobuline

(669 000), ferritin (440 000), BSA (67 000), ovalbumin (45 000), anhydrase carbonic (29 000), lysozyme (14 400), and L-glycyl-lysine (239.7).

Ultracentrifugation. The ultracentrifugation was performed in a 5–20% sucrose density gradient established in the standard phosphate buffer, using a Beckman XL-70 type ultracentrifuge equipped with a swing-out rotor (Type SW 40 TI) at 30 000 rpm during 16 h at 20 °C. For calibration, catalase (11.2S), γ-globulin (7.0S), BSA (4.4S), and lysozyme (1.9S) were used. The sedimentation coefficients of the samples were determined according to the method of Martin and Ames (1961). The proteins were dissolved in standard phosphate buffer, and 0.5 mL of the solution at a concentration of 6 mg/mL was brought onto the top of the sucrose gradient.

Surface Hydrophobicity. Surface hydrophobicity was determined, as described by Kato and Nakai (1980), using 1-anilino-8-naphthalenesulfonate (ANS) as hydrophobic probe. Two milliliters of protein solutions (0.001–0.1 mg/mL) in standard phosphate buffer was added with 40 μL of ANS (8.0 mM in 0.1 phosphate buffer, pH 7.6). The resulting fluorescence intensity was measured with a Kontron spectrofluorometer. The excitation and emission wavelengths were 374 and 485 nm, respectively. The initial slope (S_0) of the FI vs protein concentration (milligrams per milliliter) plot, calculated by linear regression analysis, was used as an index of the protein surface hydrophobicity.

Solubility as a Function of pH. The samples were solubilized in water at pH 8 (adjusted with NaOH) at a concentration range of 3–5 mg/mL. Aliquots of the resulting solutions were taken, and the pH was adjusted to values ranging from 7.5 to 2.0 by adding 1–0.1 N HCl. After centrifugation, protein concentrations were determined in the supernatant by the biuret method. Solubility was expressed as percentage of the total protein content in the sample.

Determination of Adsorption Behavior at Air–Water Interfaces. The surface tension γ (millinewtons per meter) was measured at 20 °C according to the Wilhelmy Plate technique using a Prolabo tensiometer. The γ–time curves were recorded. The tensiometer trough was filled with 25 mL of 0.1 M phosphate buffer, pH 8.0, and a platinum plate (1 cm × 2 cm × 0.05 cm) was carefully put at the air–water interface. Between each use, the platinum plate was carefully cleaned by rinsing it successively with standard buffer, purified water, and acetone. Just before use, all traces of organic contaminants were burned by putting the plate into the flame. The protein samples were solubilized at pH 7.6 into four solutions of different ionic strengths: 0.002 M phosphate (μ = 0.0057), 0.02 M phosphate (μ = 0.057), 0.1 M phosphate (μ = 0.285), and standard phosphate buffer (μ = 0.5). These protein solutions (1–5 mg/mL) were injected at final concentrations of 0.02 and 0.08 mg/mL. The protein sample in the lower phase was mixed by a moderate and short (10 s) magnetic stirring.

The surface pressure ($\pi_5 = \gamma_0 - \gamma_5$) after 5 min of adsorption was kept as a measure of the adsorption rate during the first step of adsorption, according to the procedure of Kim (1985). To confirm Kim's studies in our case, π_5 was plotted as a function of $\Delta\gamma/\Delta\sqrt{t}$ for all the samples studied in the present work. The slope $\Delta\gamma/\Delta\sqrt{t}$ was calculated from the linear portion of the γ vs $\sqrt{t}$ curve according to the procedure of Tornberg (1978).

As the correlation between $\Delta\gamma/\Delta\sqrt{t}$ and π_5 was very satisfying ($\pi_5 = 8.835\Delta\gamma/\Delta\sqrt{t}$, r = 0.96, number of samples 53), π_5 values were used in the present study as a significant parameter for following the effect of modification on the adsorption rate of glycinin derivatives, in the first step of the adsorption procedure.

RESULTS AND DISCUSSION

Influence of the Various Treatments on the Physicochemical Properties of Glycinin. *Effect of Dialysis at Low Ionic Strength and Cold Acidic Treatment.* Gel filtration chromatography in standard phosphate buffer (μ = 0.5) shows that crude glycinin sample

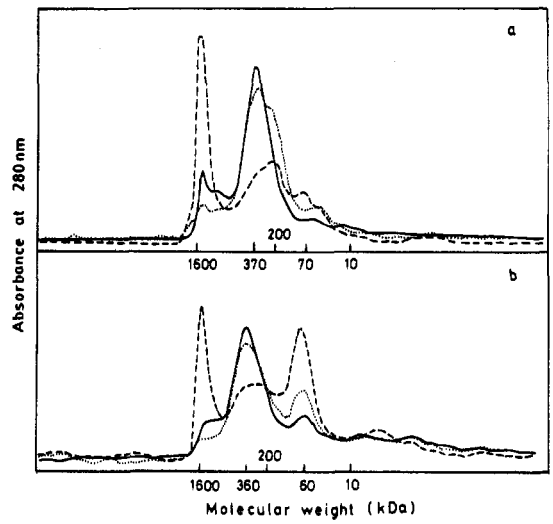

Figure 1. Gel filtration profiles of native and dissociated glycinin samples on Superose 12 HR columns, using (a) standard phosphate buffer, pH 7.6 (μ = 0.5), and (b) 0.02 M phosphate buffer, pH 7.6 (μ = 0.057), as eluant. Flow rate = 0.5 mL/min. Samples: $(AB)_6$ —; $(AB)_6$* ···; $(AB)_0$ - - -.

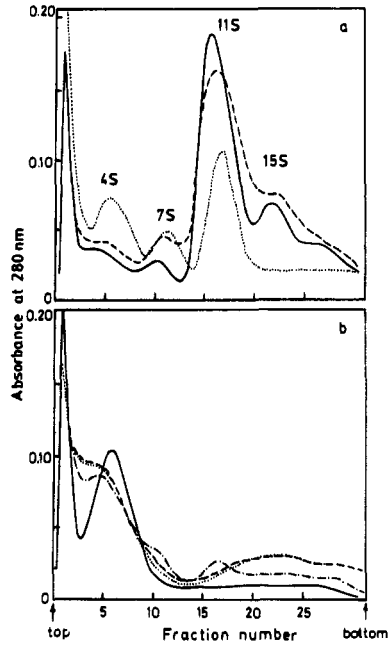

Figure 2. Ultracentrifugation patterns of glycinin samples: (a) $(AB)_6$ —, $(AB)_6$* - - -, AB_0 ···; (b) A_0 —, $AB_{1.5}$ — · —, AB_3 - - -, $AB_{4.5}$ ···.

(AB_6) is mainly composed by three species of apparent molecular weight around 1 600 000, 370 000, and 60 000 (Figure 1a), corresponding to aggregate (15S), hexameric (11S), and monomeric (4S) forms, as seen by ultracentrifugation (Figure 2). They represent 21.9%, 71.5%, and 6.5% of the whole protein sample, respectively. At lower ionic strength (0.02 M phosphate, μ = 0.057), the 11S form remained around 70%, but the aggregate proportion decreased, whereas the dissociated 4S forms slightly increased (Figure 1b). These results are in good agreement with earlier studies on the variation of glycinin structure with salt conditions (Eldrich and Wolf, 1967; Thanh and Shibasaki, 1976), which have shown partial dissociation of glycinin at low ionic strength.

By dialysis against water [$(AB)_6$*] or after acidic treatment at 4 °C (AB_0), moderate modifications of the glycinin structure were obtained. This dissociating

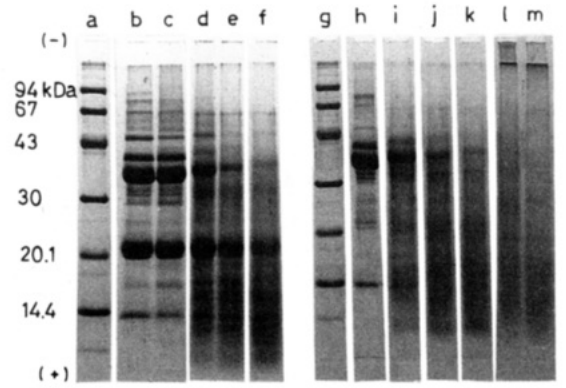

Figure 3. SDS–PAGE patterns of glycinin samples: (a and g) markers; (b) $(AB)_6$; (c) AB_0; (d) AB_3; (e) AB_6; (f) AB_9; (h) A_0; (i) A_3B_3; (j) A_6B_6; (k) A_9B_9; (l) AB_{PO_4}; (m) AB_{CO_3}.

Table 2. Extent of Deamidation of 0.1 N HCl–70°C Treated Glycinin

treatment	duration (h)	samples	% of deamidation[a]
0.1 HCl–70°C	1.5	AB1.5	1.7 (0.1)
	3	AB3	5.9 (0.2)
	4.5	AB4.5	10.6 (0.5)
	6	AB6	16.1 (0.7)
	9	AB9	25.0 (1,1)
0.2 M NaHCO3 – 100 °C	5	$ABC0_3$	34.6 (1.7)
0.2 M NaH_2PO_4 – 100 °C	5	AB_{PO_4}	22.5 (1.1)

[a] Average of four assays corresponding to duplicate determinations on replicated samples. The standard deviation is given in parentheses.

effect of dialysis was confirm to lead to a decrease of the aggregates (6.7%) and an increase of 7S and 4S conformations (28.2%) (Figure 2a), characterized by molecular weights around 200 000 and 60 000, respectively (Figure 1). After cold acidic treatment [$(AB)_0$], a drastic decrease of the 11S form occurred, leading to dissociated components 7S and 4S (39.3% and 16.5%, respectively) (Figure 3a). By HPLC, components characterized by a high apparent molecular weight (1 600 000) were also observed, although no aggregates were seen from ultracentrifugation results. This main peak, eluted at the void volume of the column, may correspond to soluble aggregates, characterized by a high hydrodynamic volume. These aggregates were not detected by ultracentrifugation because they probably sedimented at the bottom of the tube. Comparison of parts a and b of Figure 1 shows they tended to dissociate at lower ionic strength into components of 60 000 MW, close to that of AB subunits. This aggregation which mainly occurred when the sample was treated in cold HCl, may result consequently from noncovalent interactions between AB subunits dissociated by the treatment.

Effect of Deamidation. The deamidation degree of glycinin increases with the time of acidic treatment (Table 2), as it was previously observed on other types of proteins (Popineau et al., 1988; Claughton and Pearce, 1989; Shih, 1991). Compared to acidic treatments, alkaline treatments for similar reaction times lead to higher deamidation levels. Deamidation levels of 22.5% and 34.6% were obtained after 5 h in phosphate and bicarbonate conditions, respectively, instead of 16.1% after 6 h acidic treatment.

Progressive hydrolysis of A polypeptides occurred after 6 h of deamidation treatment, whereas B polypeptides hydrolysis is rather limited (Figure 3a). However, the degree of hydrolysis is lower than 2% even for AB_9 and AB_{PO_4}, which contained a relatively high amount

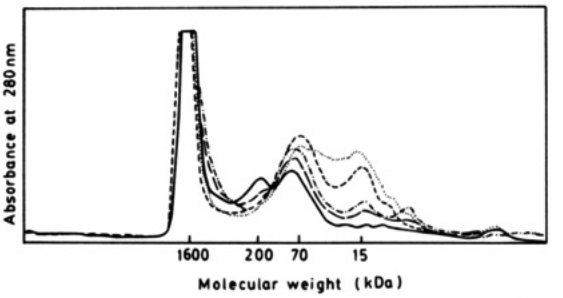

Figure 4. Gel filtration profiles of deamidated glycinin samples on a Superose 12 HR column, using standard phosphate buffer, pH 7.6 (μ = 0.5), as eluant. Flow rate = 0.5 mL/min. Samples: $AB_{1.5}$ —; AB_3 – – –; $AB_{4.5}$ -•-; AB_6 - - -; AB_9 •••.

of low MW peptides. From a comparison of SDS–PAGE patterns of AB_9, AB_{CO_3}, and AB_{PO_4} (Figure 3), it was observed that most of the A and B polypeptides have disappeared after alkaline deamidation, in contrast to acid deamidation for which most of the B polypeptides are preserved; this signifies that B polypeptides are greatly hydrolyzed as A polypeptides in alkaline deamidation conditions.

Gel filtration of acidic deamidated glycinins reveals the absence of a peak around 360 000 corresponding to the hexameric form (Figure 4). In addition, it could be seen that 100 000–200 000 peaks tend to disappear for the higher degrees of deamidation. Besides, species characterized by low MW in the range 15 000–90 000 and by high apparent MW (1 600 000) were increasing with the degree of deamidation. Ultracentrifugation patterns of these samples show that 2–4S forms are predominant (Figure 2b), without species of high sedimentation coefficient characterizing aggregated proteins. This difference between ultracentrifugation and gel filtration profiles confirmed the previous data on AB_0 and could be similarly explained. The polypeptides of MW comprised in the range 10 000–40 000 should result from partial hydrolysis according to SDS–PAGE studies (Figure 3).

Effect of Reducing Treatment. The reducing thermal treatment at pH 8 of nondeamidated glycinin provided approximately 50% of soluble protein (A_0). In agreement with Damodaran and Kinsella (1982), results from SDS–PAGE (Figure 3h) showed that A_0 sample has an enhanced content in A polypeptides, whereas B polypeptides were almost completely eliminated in the insoluble centrifuged pellet. When the reducing thermal treatment was applied to previously deamidated glycinins, higher yields of protein solubilization were obtained. They varied from 65% to 80% for 6–16% deamidation degrees and increased close to 100% for deamidation degrees above 22%. These results are mainly explained by the lower isoelectric pH of the deamidated B polypeptides. According to calculations based on the amino acid composition of the B polypeptide and taking into account the proportion of glutaminyl and asparaginyl on the p*K* values of each residue, the isoelectric pH values should diminish approximately from 8.5 (without deamidation) to 6.3, 5.20, and 4.57 for deamidation percentages around 10%, 20%, and 30%, respectively. Comparison of the electrophoresis patterns of AB_3 and A_3B_3 and of AB_6 and A_6B_6 confirmed that most of the B polypeptides remained in solution in the reduced samples.

From gel filtration (Figure 5a), it was confirmed that A_0 is mainly composed of A polypeptides with molecular weight around 40 000. However, components charac-

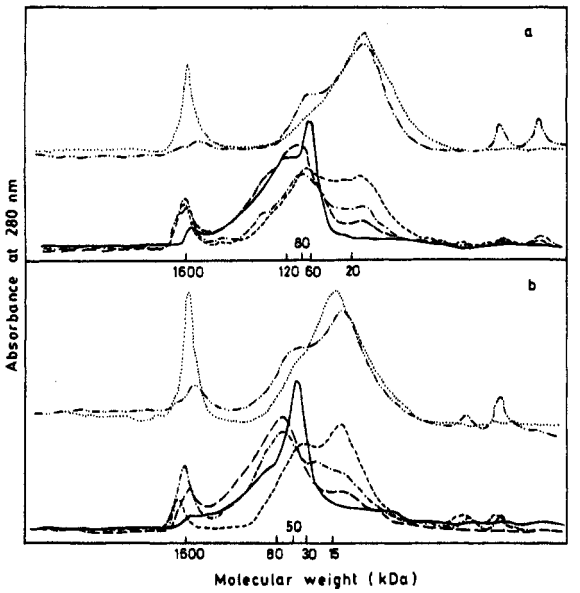

Figure 5. Gel filtration profiles on Superose 12 HR of the glycinin derivatives recovered in the supernatant after reduction of dissociated–deamidated glycinin: (a) standard phosphate buffer, pH 7.6, $\mu = 0.5$; (b) 0.02 M phosphate buffer, pH 7.6, $\mu = 0.057$. Flow rate = 0.5 mL/min. Samples: A_0 —; A_3B_3 – – –; A_6B_6 - • -; A_9B_9 - - -; AB_{PO_4} — • • —; AB_{CO_3} • • •.

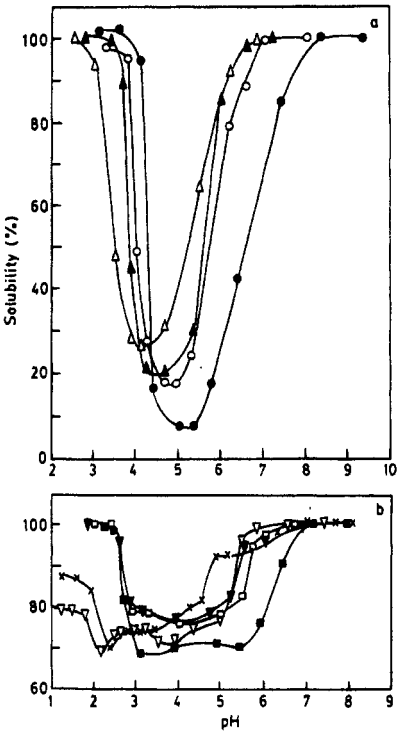

Figure 6. Effect of pH on water solubility of (a) native and deamidated glycinins and (b) proteins recovered in the supernatant after reduction of deamidated glycinin. Samples: $(AB)_6$ ●; AB_3 ○; AB_6 ▲; AB_9; △; A_0 ■; A_3B_3 □; A_6B_6 ▼; A_9B_9 ▽; AB_{PO_4} ×.

terized by higher apparent molecular weight (80 000–100 000) were also detected. These components drastically decreased when the ionic strength of the eluting buffer was lower (Figure 5b), leading to an increase of the peak eluted around 40 000. It may be concluded that these components with an apparent molecular weight around 80 000–100 000 are in fact A polypeptides aggregated or characterized by a partially unfolded conformation and a higher hydrodynamic volume.

For a limited degree of deamidation (A_3B_3 and A_6B_6) the main peak is eluted for an apparent MW of 100 000 when the standard buffer is used as eluting buffer. As previously observed for A_0, this peak is shifted toward lower molecular weight (60 000–80 000) when the elution was performed at lower ionic strength (Figure 5b); according to previous SDS–PAGE results, this peak may correspond to A polypeptides. The difference between elution volumes for A_0 (≈40 000) and deamidated A polypeptides was interpreted as an increase of the hydrodynamic volume due to the increased number of charges after deamidation. The increased density of charges for deamidated polypeptides may induce their unfolding by electrostatic repulsion effects between the different regions of the polypeptide chain. In the elution profile of these samples, a shoulder was also detected around 20 000, especially for A_6B_6, as well as peaks eluted around 10 000. From SDS–PAGE results, they correspond to B polypeptides and partially hydrolyzed derivatives, respectively.

For a higher degree of deamidation (A_9B_9), low MW components drastically increased, in relation with the increased hydrolysis of the proteins.

In agreement with the electrophoretic pattern, A_9B_9 and AB_{PO_4}, which are characterized by close deamidation degree, exhibited also similar elution profiles. This may suggest that the polypeptide molecular weight profiles of these samples are mainly ruled by the degree of hydrolysis which drastically rose when the degree of deamidation increased: the higher is this degree, the higher is the hydrolysis. This was also confirmed by the higher hydrolysis rate of the carbonate deamidated sample, characterized by a deamidation degree of 34.6%.

For each sample, components characterized by a high apparent molecular weight (1 600 000) were eluted at the void volume of the gel filtration column. However, comparison of the elution profiles to those of deamidated samples shows the proportion of aggregates was considerably decreased by the reducing treatment. Cleaving the intermolecular S–S bonds between the A and B polypeptides completely changes the structure of the AB subunits. That should drastically modify the equilibrium of hydrophobic interactions and limit the aggregation phenomena.

Functional Characterization of Crude and Modified Glycinins. *Solubility.* Increasing the net charge of the protein and shifting the isoelectric pH from 5.4 (native glycinin) to 4.9, 4.5, and 4.2 for deamidation degrees of 5.9%, 16.1% and 25%, respectively, improved the solubility of the protein in the pH range 6–7 (Figure 6a). Besides, the solubility of each sample in the isoelectric pH region increased, resulting probably from the partial protein hydrolysis (Figure 6a). The solubility profiles of the samples treated by a reducing thermal treatment are drastically modified (Figure 6b). The proteins remaining in the supernatant after reduction of the deamidated samples (60–100% of the total proteins) exhibited a very high solubility above 70% whatever was the pH. The better solubility characteristics were reached for A_3B_3 and A_6B_6. Higher degree of deamidation (A_9B_9, AB_{PO_4}) led to lower solubility in acidic pH.

Surface Behaviors. The kinetic adsorption at the air–water interface was followed as a main parameter of the ability of protein to form emulsions or foams (Graham and Phillips, 1979). It has been shown that the rate constant of adsorption at the beginning of the process was very dependent on the conformation state

of 11S-type proteins and that this parameter was of great importance for explaining emulsifying and foaming properties of proteins (Damodaran and Song, 1988; Subirade et al., 1992).

The equilibrium surface pressure (π_e) did not differ significantly among the various samples; 20.3 mN/m was obtained for native glycinin and 22.5 mN/m as a maximum value for deamidated proteins. In contrast, significant differences among samples can be observed in the rate of adsorption, especially in the first step of the process.

By following the first steps of interfacial adsorption for the native, dissociated, and deamidated glycinin derivatives, higher rates of adsorption were observed as the dissociation–deamidation degrees increased (Figure 7a). The more efficient samples to decrease rapidly the surface tension were AB_6 and AB_9. After reduction of disulfide bridges, these surface behaviors were considerably improved for all samples, which exhibited very close properties to each other despite their relatively large differences in A and B polypeptide composition. A_0, which is mainly composed of A-type polypeptides, is not so different from A_6B_6, which contained B polypeptides, or from A_9B_9, which was characterized by a relatively high degree of hydrolysis (Figure 7b). Moreover, it appeared from the results obtained with both alkaline deamidated samples and A_9B_9 that partial hydrolysis has a rather negative effect on surface activity.

The more efficient samples for decreasing surface tension γ were A_3B_3 and A_6B_6. The improved surface behaviors of deamidated as well as of deamidated and reduced samples could be explained by their faster diffusion to the interface and easier anchorage into the interfacial layer. Due to their lower molecular weight, they diffused more quickly to the interface; due to their partial unfolding, which induced a higher flexibility of the polypeptides, they anchored in the interfacial layer more easily.

Moreover, the higher accessibility of the hydrophobic region of the sequence for the unfolded polypeptides should also have positive effects. Accessible hydrophobicity was indeed significantly increased by these treatments (Table 3). Acid dissociation without heating (AB_0) led to a surface hydrophobicity 2 times higher than that of native glycinin by increasing the accessibility of the buried hydrophobic core of the protein. This phenomenon appeared to be increased by the mild conditions of deamidation, which raise the accessibility of some hydrophobic regions by unfolding the polypeptides. For the higher degrees of deamidation, the hydrophobicity decreased, because new charges appeared and partial hydrolysis occurred. Surface hydrophobicity of glycinin reached a maximum for a deamidation degree in the range 6–10% (Tables 2 and 3). It suggests that limited deamidation leads to a charge increase enough to induce repulsion between AB subunits and subsequent exposure of hydrophobic sites, especially those of the B polypeptides, which in turn are available to anchor at the interface. Deamidated glycinin samples at a deamidation level higher than 16% have a more hydrophilic character, which could be due to their higher net charge. It was also observed that dissociated and/or deamidated glycinins possess greater surface hydrophobicity than corresponding samples submitted to an additional reducer thermal treatment (Table 3). The lower hydrophobicity of the reduced samples could be due to the partial insolubilization of

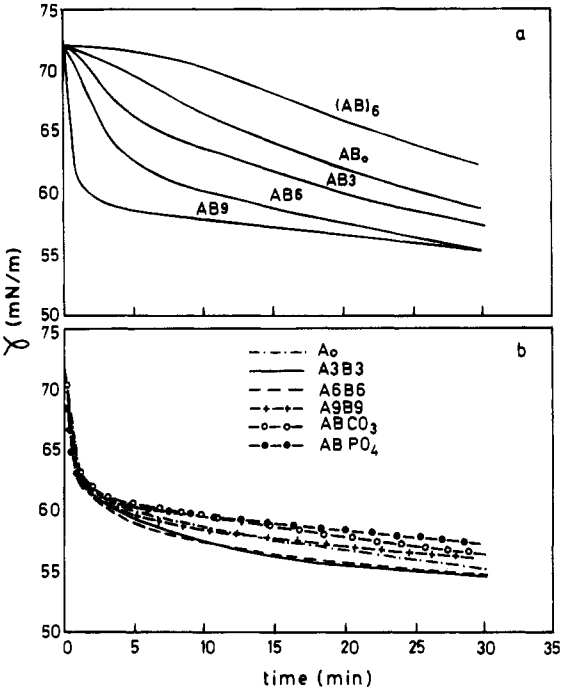

Figure 7. Kinetics of adsorption of proteins at air–water interface: (a) native, dissociated, and deamidated glycinin; (b) proteins resulting from reducer thermal treatment of dissociated–deamidated glycinin. The solubilization and measurement buffer was 0.1 M sodium phosphate, pH 8, and the protein concentration 0.02 mg/mL.

Table 3. Influence of Modification Treatments on Surface Hydrophobicity (S_0) of Glycinin

sample	S_0[a]	sample	S_0[a]
$(AB)_6$	1.53 (0.07)		
$(AB)_6$*	1.75 (0.08)		
AB_0	3.13 (0.12)	A_0	2.46 (0.1)
$AB_{1.5}$	3.70 (0.15)		
AB_3	4.28 (0.02)	A_3B_3	2.16 (0.1)
$AB_{4.5}$	4.35 (0.02)		
AB_6	4.02 (0.02)	A_6B_6	1.85 (0.08)
AB_9	3.52 (0.018)	A_9B_9	1.41 (0.08)
		AB_{PO_4}	1.80 (0.07)
		AB_{CO_3}	1.85 (0.08)

[a] Average of four assays corresponding to duplicate determinations on replicated samples. The standard deviation is given in parentheses.

the more hydrophobic B polypeptides. The variations of surface hydrophobicity are, however, rather limited when the samples are compared to each other inside each group, deamidated or deamidated and reduced, and cannot be the main factor to explain the great differences of interfacial behaviors between the samples. Molecular size of the polypeptides and unfolding should be consequently the predominant factors that ruled the interfacial adsorption procedure even if accessible hydrophobicity may also interfer. For example, if one compares the reduced samples, which all anchored very rapidly at the interface, those having greater deamidation degree and lower hydrophobicity value (A_9B_9, AB_{CO_3}, AB_{PO_4}) led to lower surface pressure.

These results, which really showed the drastic influence of molecular size and conformation on the rate of adsorption, were confirmed by studying the effects of salt concentration on these kinetics. As ionic strength has a great effect on the conformation of this protein as previously observed from gel filtration data (Figure 5), this parameter may also change significantly the surface behaviors of these modified proteins. Such

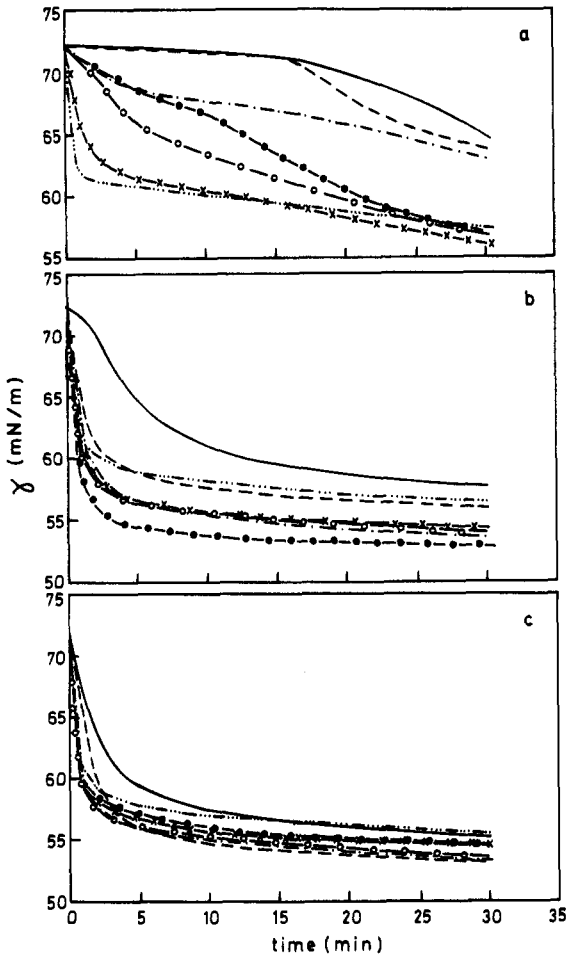

Figure 8. Kinetics of adsorption of proteins at air–water interface. Measurement conditions: 0.1 M phosphate, pH 8, buffer; protein concentration, 0.02 mg/mL. Solubilization buffers: (a) standard phosphate, pH 7.6, μ = 0.5; (b) 0.02 M phosphate, pH 7.6; (c) 0.002 M phosphate, pH 7.6. Samples: $(AB)_6$ —; AB_0 – –; AB_3 –·–; AB_9 ○; A_0 ●; A_6B_6 ×; AB_{PO_4} –···–.

effects should affect the interest of these protein derivatives for food uses.

Thus, samples were solubilized in three different phosphate buffers, pH 7.6, with different salt concentration (standard buffer, μ = 0.5; 0.02 M phosphate, μ = 0.057; 0.002 M phosphate, μ = 0.0057) and the surface tension measurements carried out in 0.1 M phosphate buffer, pH 7.6 (μ = 0.285). The objective pursued through this procedure was that differences to be found could be attributed mainly to the solubilization step and, as a consequence, to the protein state of conformation at the beginning of the adsorption process.

When the standard buffer was used as solubilizing medium, differences between native glycinin and modified derivatives were very large (Figure 8a). At lower ionic strength (Figure 8b,c) the samples exhibited closer behaviors. For 0.02 M phosphate as solubilization buffer, native glycinin is almost as efficient as modified sample for decreasing the surface tension. As previously observed (Figure 1), glycinin dissociates at low ionic strength into subunits which should diffuse and adsorb very rapidly at the interface. This confirms the predominant effect of dissociation on the adsorption rate.

From a more applied point of view, it is of great interest to observe that the effect of ionic strength of solubilization is rather limited for modified proteins,

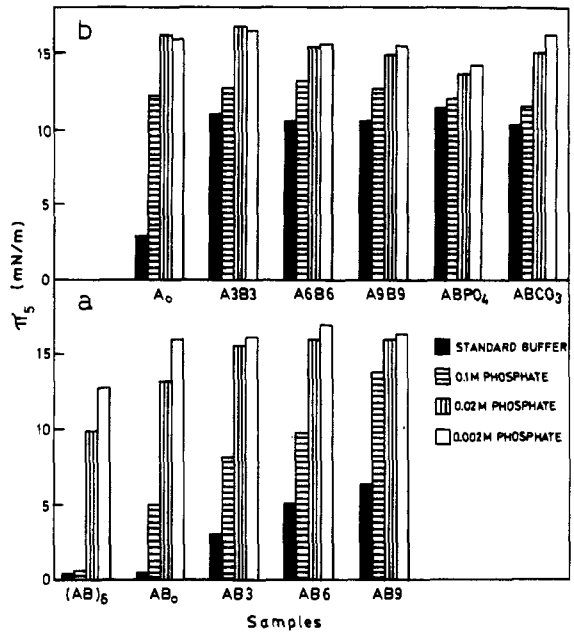

Figure 9. π_5 values (surface pressure after 5 min of adsorption) of samples solubilized in four different buffers. Measurement conditions: 0.1 M phosphate, pH 8, buffer; protein concentration, 0.02 mg/mL.

especially for the deamidated and reduced samples. A_6B_6 and AB_{PO_4} exhibited relatively constant properties whatever the ionic strength.

This phenomenon was systematically studied on the whole set of the samples, by comparing the surface pressure after 5 min of adsorption (π_5) according to the method of Kim (1985). Figure 9 confirms the positive effect of dissociation–deamidation on π_5 values. This effect became particularly evident when samples were solubilized in standard phosphate buffer. Comparison of native $(AB)_6$, dissociated AB_0, and deamidated glycinin (AB_3, AB_6, AB_9) (Figure 9a) with corresponding reduced proteins (Figure 9b) highlighted the positive effect of reducing treatment on adsorption rate. Moreover, it could also be confirmed that the reducing thermal treatment decreased the influence of the ionic strength in the solubilization buffer.

Conclusions. In conclusion, surface behaviors of soybean glycinin could be greatly improved by mild treatments which induce progressive dissociation, deamidation, and unfolding of the oligomeric structure. It results from a predominant effect of the decreased molecular size and also from the unfolding of the polypeptides and an increased surface hydrophobicity and charge. The reducing thermal treatment of deamidated glycinin led to soluble proteins (80%) for a deamidation degree higher than 16%. These reduced samples are highly soluble (>70%) in water whatever the pH conditions. They diffuse rapidly at the air–water interface after solubilization in low or high ionic strength conditions. These modifications should also lead to variations in the organization of the structure layers that may influence the stability of foams or emulsions. Thus, by combination of acidic and reducing treatments, it seems possible to prepare soluble proteins from soybean glycinin, which could be employed as efficient surface active agents in different food systems, without significant effect of salt concentration on the surface properties. Thus, the foaming rate of the proteins as well as the foam expansion should be increased by using the modified proteins. However, the foam stability, which is related to the rheological

properties of the surface layer, may not be improved. For these reasons, further studies on the foaming and emulsifying properties of these modified proteins are necessary.

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Received for review January 5, 1995. Accepted May 30, 1995.[⊗]

JF950009Y

[⊗] Abstract published in *Advance ACS Abstracts,* July 15, 1995.