Structural Modifications of an Amaranth Globulin Induced by pH and NaCl

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The influence of pH and NaCl on the structure of globulin-P, the polymerizable amaranth 11S type globulin, was studied by differential scanning calorimetry, gel filtration, and gradient sedimentation. At $\mu = 0.54$, the protein is stable for pH ranging from 5 to 9 but becomes rapidly unfolded as pH decreases below 5. For pH values above 9, globulin-P denatures more gradually than in acidic medium, and it also dissociates into subunits, which are possibly less thermostable. At pH 6.5 or 8.5 and low sodium chloride concentrations ($\mu \leq 0.01$), dialyzed globulin-P destabilizes, yielding species of lower thermal stability. The increase in NaCl concentration up to 0.1 M induces folding of globulin-P toward a more stable structure. Above 0.1 M NaCl, increasing the ionic strength up to $\mu = 0.5$ elevates the denaturation temperature (T_d) and denaturation enthalpy (ΔH). From $\mu = 0.1$ to 0.5 the content of soluble globulin-P polymers decreases, possibly owing to protein insolubilization. Above 0.5 M, NaCl shows a stabilizing effect reflected by increasing T_d, whereas ΔH stays constant; this effect is similar to that found by other authors in some storage proteins.

Keywords: *Amaranth; globulin; protein structure; gel filtration; ultracentrifugation; pH effect; ionic strength effect*

INTRODUCTION

Amaranthus hypochondriacus is a C-4 plant the grains of which contain an important amount (17% w/w) of proteins (Teutónico and Knorr, 1985), with a well-balanced amino acid composition (National Research Council, 1984; Bressani, 1989). Albumin, one of the major amaranth seed protein fractions, consists of cytoplasmic globular proteins likely to be involved in different biological functions (Konishi et al., 1991). Seed storage proteins are rarely found as albumin but mostly as globulin and/or glutelin fractions (Segura-Nieto et al., 1994). The major globulin component, the 11S type, is removed from the flour in two subfractions of similar size. One of them, the globulin studied in many reports, is easily obtained with neutral saline solutions (Marcone et al., 1994; Segura-Nieto et al., 1994), whereas the other requires several treatments before being extracted and was named globulin I (2) by Chen and Paredes López (1997) or albumin-2 by Konishi et al. (1991) and Martínez et al. (1997). Konishi et al. (1991) compared the relative amount of the latter subfraction with albumin-1 and globulin and obtained the following relative proportions: albumin-1:globulin:albumin-2, 1:0.55:0.67. Although having a similar polypeptide composition, albumin-2 possesses higher denaturation enthalpy (determined by calorimetry), lower solubility in neutral aqueous solutions, and higher tendency to polymerize than salt soluble globulin (Martínez et al., 1997). Thus, owing to the last property, we shall identify this subfraction as globulin-P (polymerizable globulin).

Plant protein structure and, therefore, functionality depend largely on the ionic strength and pH of the medium. The variation in these conditions may disturb or even disrupt protein quaternary, tertiary, and secondary structure (Peng et al., 1984; Damodaran and Kinsella, 1982; Damodaran, 1989a; Wagner and Guéguen, 1995; Guéguen et al., 1988; Sripad and Narasinga Rao, 1987), and these modifications may lead to new and different functional properties (Damodaran, 1989b; Kinsella and Phillips, 1989).

Being one of the major amaranth protein fractions, globulin-P is still little known and its conformational changes induced by changes of pH and ionic conditions have not been studied until now. Knowledge of the structural behavior of globulin-P in different media will be valuable for using this protein as a new ingredient in formulated foods and will provide a better understanding of the interactions that stabilize its conformations.

MATERIALS AND METHODS

Materials. Seeds of *Amaranthus hypochondriacus* (Mercado cultivar) were harvested at the Experimental Station of the Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), Chapingo, Mexico, and donated to our laboratory in Argentina. Flour was obtained by grinding whole seeds in an Udy mill (Facultad de Agronomía, Universidad Nacional de La Plata, Argentina), 1 mm mesh and screened by 10 xx mesh. It was defatted for 24 h with hexane in a 10% (w/v) suspension under continuous stirring, then air-dried at room temperature, and stored at 4 °C until used. Protein content of the flour as determined by the Kjeldhal method according to AOAC methods (AOAC, 1984) was 17.0% (w/w) on dry weight basis (N × 5.85) (Becker et al., 1981; Segura Nieto et al., 1994).

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Protein Isolation. Globulin-P was extracted according to the method described elsewhere for albumin-2 preparation (Martínez et al., 1997). Briefly, the isolation was carried out as follows: flour was treated three times with water for albumin (albumin-1) extraction and three times with 32.5 mM K_2HPO_4/2.6 mM KH_2PO_4 (pH 7.5)/0.4 M NaCl (buffer A) for globulin extraction. Globulin-P was then extracted by treating the last residue three times with water in a ratio of 10 mL of water to 1 g of meal. After each treatment, the extraction residue was separated by centrifugation at 9000*g* for 20 min. All of the preceding steps were conducted at room temperature. The supernatants containing globulin-P (as soluble and dispersed protein) were adjusted to pH 6 with 2 N HCl, yielding a precipitate that was resuspended in water, neutralized with 0.1 N NaOH, and freeze-dried.

Dialyzed globulin-P was prepared by resuspending the precipitate in water and dialyzing against 200 volumes of distilled water with 0.02% NaN_3 for 7 days at 4 °C with three changes a day. Once dialysis was finished, the suspension was freeze-dried.

Differential Scanning Calorimetry (DSC). The runs were performed in a Polymer Laboratories (Rheometric Scientific Ltd., U.K.) calorimeter fitted with Plus V 5.41 software. Calibration was carried out at a heating rate of 10 °C/min by using the standards (Rheometric Scientific Ltd.) indium pro-analysis (p.a.), lauric acid (p.a.), and stearic acid (p.a.).

For those runs intended to test the effect of pH, 20% w/w suspensions of globulin-P were prepared in 0.2 M Na salt buffers: 0.17 M $C_6H_8O_7$/0.03 M $C_6H_7O_7^-$ (pH 2.3); 0.08 M $C_6H_8O_7$/0.12 M $C_6H_7O_7^-$ (pH 3.1); 0.015 M $C_6H_8O_7$/0.15 M $C_6H_7O_7^-$/0.035 M $C_6H_6O_7^{2-}$ (pH 4.1); 0.11 M $C_6H_7O_7^-$/0.09 M $C_6H_6O_7^{2-}$ (pH 4.7); 0.06 M $C_6H_7O_7^-$/0.14 M $C_6H_6O_7^{2-}$ (pH 5.1); 0.18 M $H_2PO_4^-$/0.02 M HPO_4^{2-} (pH 6.3); 0.12 M $H_2PO_4^-$/0.08 M HPO_4^{2-} (pH 7.5); 0.132 M H_3BO_3/0.068 M $H_2BO_3^-$ (pH 8.8); 0.046 M H_3BO_3/0.154 M $H_2BO_3^-$ (pH 9.7); 0.128 M HCO_3^-/0.072 M CO_3^{2-} (pH 10.1); 0.03 M HCO_3^-/0.17 M CO_3^{2-} (pH 11.0); 0.163 M $H_2BO_3^-$/0.037 M HBO_3^{2-} (pH 12.2). When necessary, NaCl was added as required for each solution to reach 0.54 ionic strength (μ). Concerning the experiments devised to test the effect of ionic strength, 20% w/w suspensions (pH 6) of dialyzed globulin-P were prepared in NaCl solutions of 0.005, 0.01, 0.03, 0.05, 0.11, 0.5, 1, and 2 M. After preparation, all suspensions were allowed to rest for 40 min at room temperature. DSC samples consisted of hermetically sealed aluminum pans filled with 12−14 mg suspensions; these were run at a rate of 10 °C/min from 300 K (27 °C) to 400 K (127 °C), and a double, empty pan was used as reference. After each run, the dry matter content was determined by puncturing the pans and exposing them to 107 °C overnight; the dry protein was calculated by subtracting the weight of the salts added with the solutions from the dry matter. The denaturation parameters were calculated with the equipment software, the denaturation temperature (T_d) being taken as the value corresponding to the transition peak maximum; the value of $\Delta T_{1/2}$ was obtained as the peak width at half peak height, whereas the transition enthalpy (ΔH) values were calculated from the area below the transition peaks.

At least three analyses were performed for each sample to obtain the mean value and standard deviation of each parameter.

Ultracentrifugation. Ultracentrifugation was performed in an Optima TL, Beckman ultracentrifuge, using the TLS-55 swinging bucket rotor at 50000 rpm for 5 h at 10 °C. Samples of 0.1 mL were layered on top of 10−30% (w/w) sucrose linear gradients in the same buffer as the samples. Calibration was carried out using catalase (11.20S) as standard, which was run in 35 mM phosphate buffer (pH 7.5) when low ionic strength samples were analyzed and in 35 mM phosphate buffer (pH 7.5) plus enough NaCl to reach $\mu = 0.54$ when samples of high ionic strength were run. Gradient UV profiles were measured with a Gilson LC detector model 111 and recorded with a Gilson N2 recorder. Fractions of 0.1 mL were collected.

Chromatography. Globulin-P and dialyzed globulin-P were analyzed by chromatography at room temperature in a Superose 6B HR 10/30 column using a Pharmacia LKB, FPLC system. Samples contained 4 mg of protein in 200 μL of each of the following pH 8.5 buffers: 0.035 M K_2HPO_4 buffer with sufficient NaCl to reach a μ value of 0.54; 0.035 M K_2HPO_4 buffer ($\mu = 0.08$), and 0.0035 M K_2HPO_4 buffer ($\mu = 0.008$). Elution was done with the same buffer as that used in the sample, at a flow rate of 0.2 mL/min; 0.3 mL fractions were collected, and elution profile (absorbance at 280 nm) was obtained. The column was calibrated with Blue dextran (V_0) and the following proteins were used as standards: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), and alcohol dehydrogenase (150 kDa). The calibration curve obtained from duplicate measurements was

$$\ln \mathrm{MM} = 11.05 - 0.40V_e \qquad (r = -0.91)$$

where V_e is the elution volume in mL and MM is the molecular mass in kDa.

Fractions from the corresponding peaks were used for electrophoretic analysis.

At least three replicates of each sample were analyzed by ultracentrifugation and chromatography so that the sedimentation coefficients and estimated molecular masses that will appear in the text are the corresponding mean values.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Linear gradient separating gels (6−12% in polyacrylamide) were run in minislabs (Bio-Rad Mini Protean II model). Runs were carried out according to the method of Laemmli (1970) as modified by Petruccelli and Añón (1994). The molecular masses of the polypeptides were calculated using the following protein standards: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). Protein samples were prepared by mixing 3 volumes of chromatographic or sedimentation fractions with 1 volume of concentrated sample buffer. Gels were colored with silver stain (Blum et al., 1987).

Solubility of Globulin-P. Samples of freeze-dried globulin-P were dispersed in buffers of pH ranging from 2.3 to 11.0 (the same buffers as those used in the calorimetric analysis) with a protein concentration of 1.0% w/v and kept at room temperature for 2 h with periodic stirring (every 15 min) in a Thermolyne Maxi-Mix II mixer set at maximum speed. They were then centrifuged at 18000*g* for 30 min to determine protein in the supernatants according to the Lowry method (Stoschek, 1990). Solubility was expressed as 100 × protein content in the supernatant/total protein content (g%). Bovine serum albumin was used as the standard protein.

RESULTS

Effect of pH. The polymerized globulin (globulin-P) was analyzed by DSC at different pH values in the range of 2−12, with a constant ionic strength of 0.54 (see Materials and Methods). The corresponding denaturation temperatures (T_d), representative thermograms, and the denaturation enthalpies (ΔH) are depicted in parts A, B, and C of Figure 1, respectively. The results indicate that between pH 6 and 8, this protein presents its maximum stability (highest T_d and ΔH). Nevertheless, stability differences are minor in the broader pH range of 5.0−8.5. The corresponding thermograms, for example, that at pH 7.5 (Figure 1B), shows one endotherm of high T_d ($T_{d2} = 375.9 \pm 0.5$ K) and low $\Delta T_{1/2}$ (7.1 ± 0.1 K), suggesting the presence of compact, ordered structures of similar thermal stabilities. At pH <5.0 (e.g., pH 4.1) there is a sharp T_d decrease of 16 K (Figure 1A) and a 70% drop in ΔH with respect to the preceding values (Figure 1C) that indicates protein denaturation. At these low pH values, the endotherms are wider ($\Delta T_{1/2} = 18 \pm 1$ K, pH 4.1, Figure 1B), reflecting loss of cooperativity in the denaturation

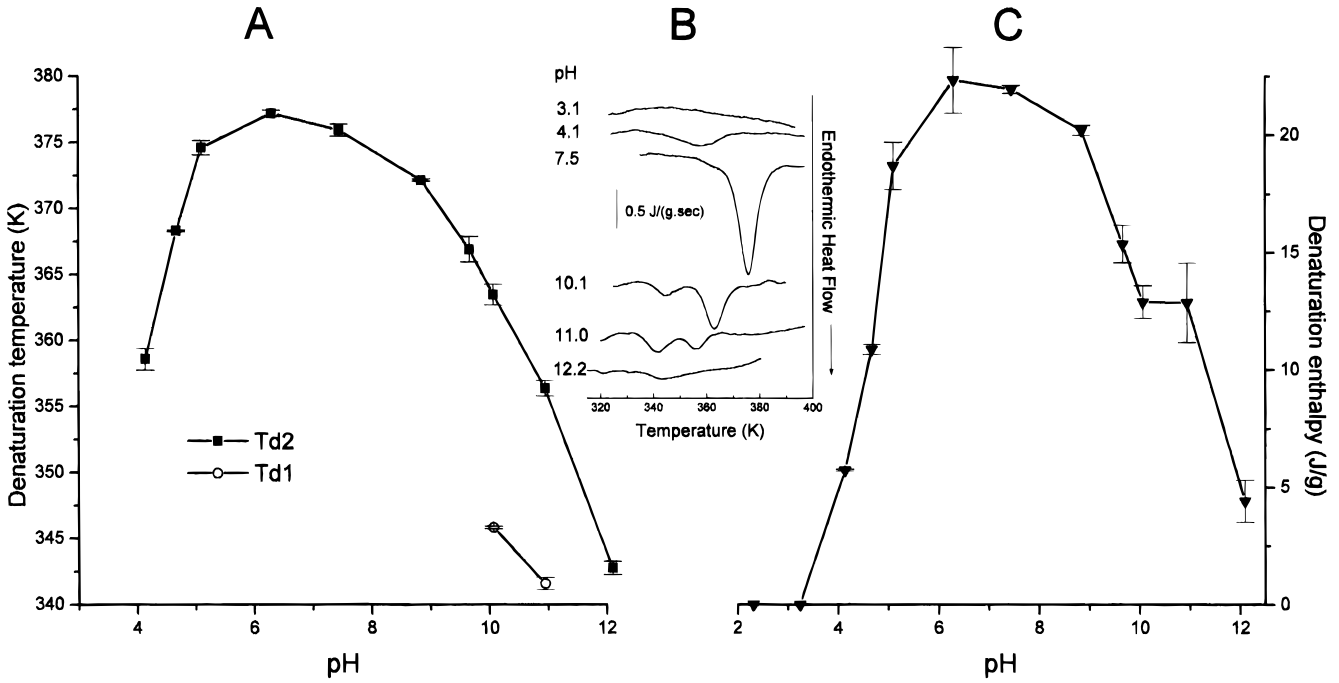

Figure 1. (A) Effect of pH on the denaturation temperature (T_d) of globulin-P at $\mu = 0.54$. T_{d2} is the denaturation temperature of the main endotherm; T_{d1} is the denaturation temperature of the minor endotherm at pH 10.1 and 11. (B) Representative thermograms at pH values ranging from 3.1 to 12.2. (C) Influence of pH on denaturation enthalpy (ΔH) of globulin-P.

process. This is possibly caused by the presence of more open structures resulting from charge repulsion. Neither endothermic transition was detected at pH 3.1 or below (Figure 1B), indicating a maximum degree of protein unfolding. At alkaline pH values, and apart from the decrease of T_d and ΔH, the thermograms show a new endotherm of lower T_d ($T_{d1} = 345.8 \pm 0.1$ K, Figure 1A), the area of which enlarges at increasing pH values (Figure 1B, pH 10.1 and 11.0). Thus, higher pH values induce conformational changes in globulin-P and the appearance of new structures with lower thermal stability. The gradual unfolding of the structures (regardless of T_d value) occurring for increasing pH is evidenced by the decrease in ΔH at pH 12.2 (Figure 1B,C).

The influence of alkalinization on globulin-P structure was also tested by sucrose gradient ultracentrifugation and gel filtration. Figure 2 exhibits a characteristic UV (280 nm) profile after sedimentation at pH 8.5 and $\mu = 0.54$. The graph shows a peak of light material, below 2S, composed of polypeptidic subunits (shown in the SDS−PAGE pattern, inset of Figure 2) that are probably released during the isolation. The graph also shows an 8S species, a major band of 12S, and, in lower amounts, a 15S species. This profile is similar to those described for the 11S type amaranth globulin (Romero-Zepeda and Paredes-López, 1996; Barba de la Rosa et al., 1996; Chen and Paredes-López, 1997; Martínez et al., 1997) and other plant globulins (Wagner and Guéguen, 1995; Wolf, 1993; Wolf and Nelsen, 1996), for which the 11S−12S species are the hexameric molecules and the 7S−8S component the trimeric subunits. Except for its trend to polymerization, globulin-P shares many of the structural characteristics of 11S globulins (Martínez et al., 1997); therefore, it may be considered that, like legumins, 12S and 8S species correspond to unitary molecules and subunits, respectively, whereas the 15S component is a polymer. This hypothesis is supported by the fact that the three components share similar SDS−PAGE profiles as illustrated in the inset of Figure 2.

The characteristic sedimentation profile of globulin-P at pH 11.0 and $\mu = 0.54$ (Figure 2) shows a main band of 8S and a minor band of ≈3S. The broadening in the major band toward the bottom of the gradient suggests the presence of species of higher sedimentation constant. This profile, showing the absence of polymers and the predominance of subunits over units, suggests that alkaline conditions promote globulin partial dissociation. Such dissociation may include the splitting of 12S monomers into 8S subunits along with further loss of quaternary structure in some protein molecules. This latter results in the generation of smaller particles reflected in the 3S sedimentation band. In agreement with these results, dissociation of salt soluble globulin at alkaline pH was also observed by several authors (Konishi et al., 1985; Marcone and Yada, 1991).

Gel filtration UV profiles are shown in Figure 3. At pH 8.5 and 0.54 ionic strength four fractions are detected, namely, large aggregates (1500 ± 100 kDa), dimeric forms (508 ± 65 kDa), unitary molecules (250 ± 53 kDa), and free polypeptides (40 ± 11 kDa). This profile is mostly like that obtained at pH 8.5 and $\mu = 0.08$ (Martínez et al., 1997) except in that the polymer content is greatly diminished for higher μ (the influence of ionic strength will be analyzed below). At pH 11.0 the elution profile changes (Figure 3), showing a major fraction with an estimated molecular mass of 154 ± 30 kDa and smaller quantities of 292 ± 78 and 82 ± 10 kDa fractions. The SDS−PAGE patterns of these three fractions are similar, as shown in the inset of Figure 3.

The chromatographic 154 kDa fraction (Figure 3, pH 11.0) may belong to the 8S subunits obtained by ultracentrifugation (Figure 2, pH 11.0), whereas the 292 and 82 kDa fractions would be the 12S molecules and 3S particles, respectively. Therefore, chromatographic and sedimentation results agree and evidence the

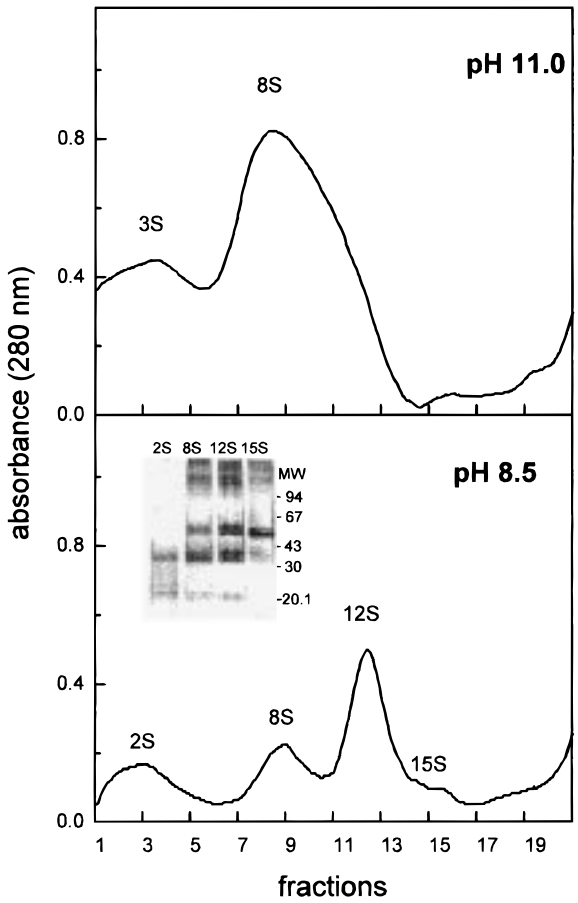

Figure 2. Sedimentation patterns of globulin-P at pH 8.5 (0.035 M K_2HPO_4 buffer, enough NaCl to achieve $\mu = 0.54$) and pH 11 (0.200 M $NaHCO_3$ buffer, $\mu = 0.54$). The sedimentation coefficients of the major species are indicated in the graph. (Inset) SDS−PAGE profile from 2S, 8S, 12S, and 15S fractions obtained at pH 8.5; molecular weight standards are indicated on the right side.

dissociation of globulin-P in alkaline medium. The molecular masses estimated from the pH 11.0 chromatographic profile do not coincide with those obtained in the pH 8.5 chromatography. In this regard, calorimetric results showed that the proteins experience conformational changes at alkaline pH which may modify their hydrodynamic behavior.

Effect of Sodium Chloride. The thermal behavior of dialyzed globulin-P (see Materials and Methods) was analyzed at pH 6.5 for sodium chloride concentrations ranging from 0 to 2 M. The thermograms were grouped in three ionic strengths ranges (Figure 4A): I, low (μ = 0−0.01); II, intermediate (μ = 0.01−0.1); and III, high (μ = 0.1−2). Thermograms belonging to the first range show two small and wide endotherms suggesting the presence of partially unfolded proteins with different thermal stabilities and low cooperativities during thermal denaturation. T_d values are $T_{d1} = 344.0 \pm 0.7$ K for the smaller endotherm and $T_{d2} = 364.8 \pm 0.8$ K in the larger. T_d and ΔH (13 ± 1 J/g) remain constant over this low ionic strength range (0−0.001) (Figure 4B). At ionic strengths >0.01 (range II) the T_d values of the smaller endotherm begin to increase with sodium chloride concentration, approaching the value of the major endotherm. At $\mu = 0.03$, ΔH also increases and the endotherms join, forming only one transition peak (Figure 4B). This unified endotherm is wide ($\Delta T_{1/2}$ = 14 ± 4 K) and may indicate either the presence of species with similar but not coincident thermal stability or, else, only one species with open structure and low cooperative thermal denaturation. In range II, T_d and ΔH keep constant at 366.0 ± 0.8 K and 14.5 ± 0.9 J/g, respectively (Figure 4B), whereas from 0.1 to 0.5 M NaCl, the behavior is different: T_d and ΔH increase, strongly suggesting that the protein structure stabilizes as μ increases (Figure 4B). The ΔH value at $\mu = 0.5$ (17.3 ± 0.4 J/g) does not show any important change for higher ionic strengths, unlike T_d, which keeps increasing up to $\mu = 2.0$.

As in this study of the effect of pH, we also added here the analysis of the soluble fraction by ultracentrifugation and gel filtration. Given that globulin-P has a low solubility at pH 6.5 (31 ± 1 g %), the tests were carried out in pH 8.5 buffer media in which the protein is more soluble (71 ± 5 g %), and the results were compared with those from calorimetric analysis. We tested this fraction also by DSC in the same conditions as those used in sedimentation and chromatography, namely, pH 8.5 and ionic strengths of 0.008 (range I), 0.08 (range II), and 0.54 (range III). To this end, for the two lower ionic strengths (μ = 0.008 and 0.08) NaCl was replaced by phospate buffer, whereas at $\mu = 0.54$, sodium chloride and phosphate buffer were mixed. As illustrated in Figure 5 thermograms are equivalent (similar T_d and ΔH) to those obtained at pH 6.5 in the presence of NaCl (Figure 4). These results suggest that globulin-P stability at $\mu < 0.1$ is not affected when NaCl is replaced by pH 8.5 phosphate buffer, nor is it altered at $\mu > 0.5$ by the presence of 0.035 M phosphate buffer (pH 8.5).

Figure 6 exhibits the gel filtration chromatograms of dialyzed globulin-P for the three ionic strengths. The profile at $\mu = 0.08$ reveals the predominance of high molecular mass polymers and the presence of monomeric molecules of 275 ± 33 kDa, that is, a result similar to that shown by the nondialyzed globulin-P (Martínez et al., 1997). In contrast, polymers are absent in the 0.54 ionic strength profile, which does exhibit major fractions corresponding to monomeric molecules and polypeptide subunits (Figure 6). This profile is rather different from that shown by nondialyzed globulin-P in the same ionic strength (Figure 3, pH 8.5) and may indicate irreversible alterations in globulin-P during dialysis. Both profiles at $\mu = 0.54$, pH 8.5 (Figures 6 and 3), show low optical density peaks, reflecting the low solubility of globulin-P in these conditions. Therefore, at high salt concentration ($\mu = 0.54$) it may be assumed that most of the proteins become aggregated and insoluble, leaving soluble only the monomeric molecules and polypeptides shown on the profile. The low ionic strength ($\mu = 0.008$) chromatogram (Figure 6) shows a main peak at a low elution volume, corresponding to an estimated molecular mass of 1184 ± 120 kDa, along with a smaller peak eluting at even lower volumes. This result at low salt concentrations may indicate interactions between monomeric molecules with formation of soluble aggregates.

The effect of salt concentration in globulin-P was also studied by gradient sedimentation. The sedimentation profile for $\mu = 0.08$ (Figure 7) shows a higher amount of 13S and fast sedimentation proteins, indicating that unitary molecules and polymers are the main components in this medium. On the other hand, at $\mu = 0.54$ (Figure 7) the major species are the 2S polypeptidic subunits belonging to slow elution chromatographic

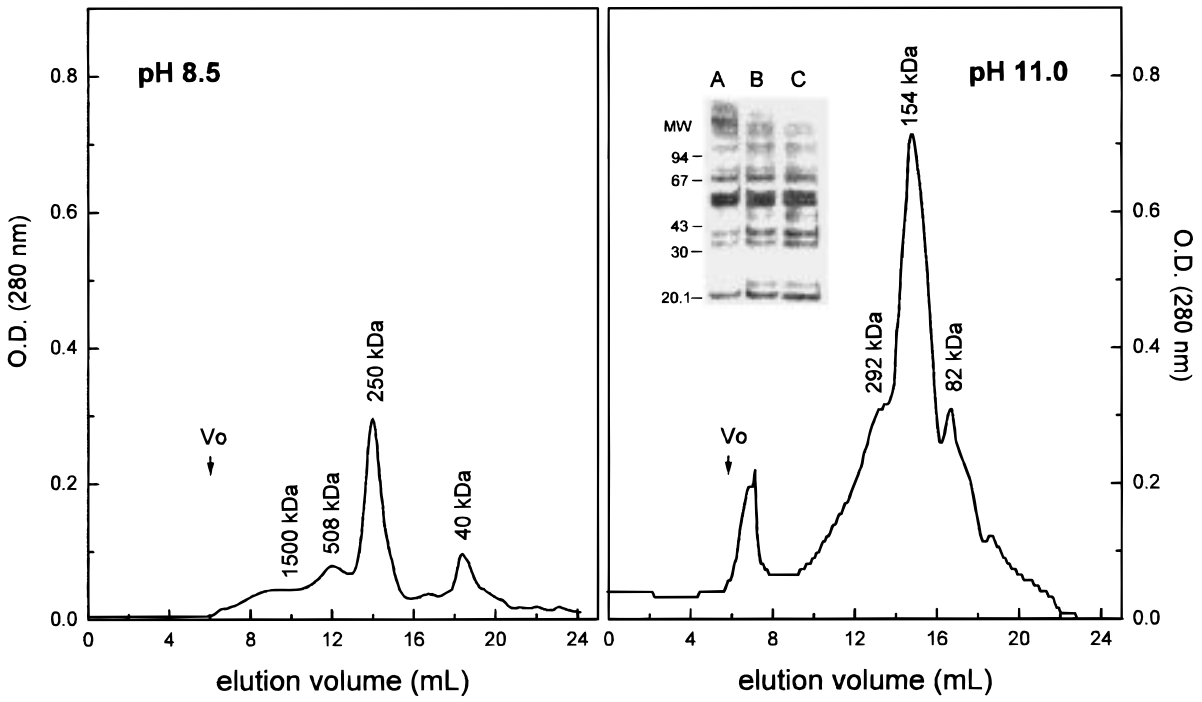

Figure 3. Gel filtration elution patterns of globulin-P at pH 8.5 (0.035 M K_2HPO_4 buffer, enough NaCl to achieve $\mu = 0.54$) and pH 11 (0.200 M $NaHCO_3$ buffer, $\mu = 0.54$). V_0 is the void volume, and the estimated molecular masses for the species are indicated above the corresponding peak. (Inset) SDS−PAGE profile from fractions eluted at pH 11.0: 292 kDa (A), 150 kDa (B), and 82 kDa (C); molecular weight standards are indicated on the left side.

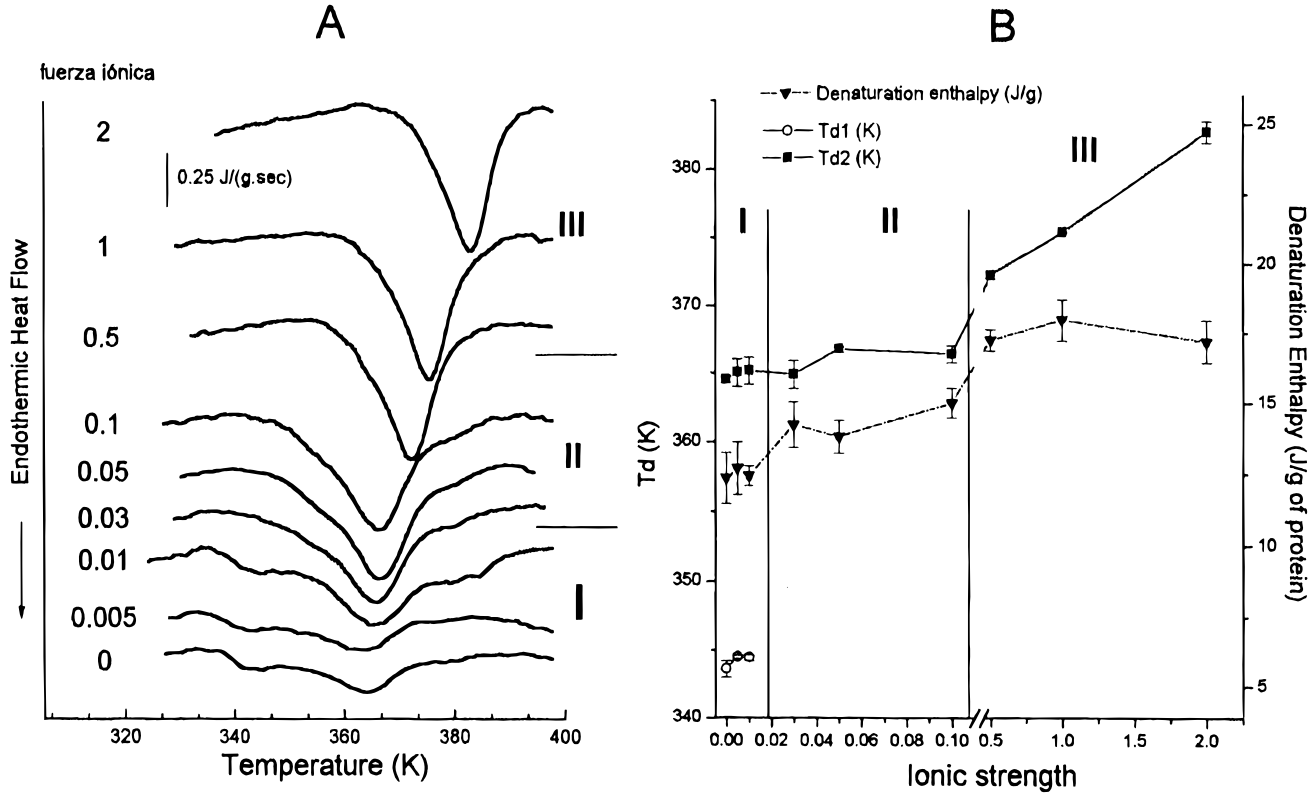

Figure 4. Effect of ionic strength on the thermal behavior of dialyzed globulin-P (pH 6.5): (A) thermograms; (B) denaturation enthalpy (ΔH) and denaturation temperature (T_d) as a function of ionic strength. Roman numerals indicate the three ionic strength regions described in the text.

fraction and 12S molecules included in the fast elution chromatographic fraction. These results are in agreement with the chromatographic data shown above.

At low salt concentration ($\mu = 0.008$, Figure 7) the 13S is the major species, accompanied by minor species of higher (18S) and lower (2S) sedimentation constant. In this condition, the molecular masses estimated by gel filtration (e.g., 1184 kDa) and the corresponding sedimentation constant (13S) do not correlate, suggesting that the shapes of the particles are highly nonspherical. In this regard, a sphere having a mass of 1184 kDa would have had a sedimentation constant of

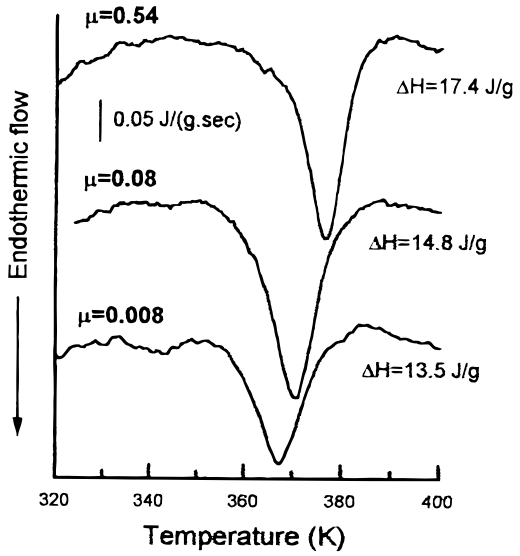

Figure 5. Thermograms of dialyzed globulin-P at pH 8.5, μ = 0.54 (0.035 M K_2HPO_4 buffer, enough NaCl to achieve μ = 0.54), μ = 0.08 (0.035 M K_2HPO_4 buffer, pH 8.5), and μ = 0.008 (0.0035 M K_2HPO_4 buffer, pH 8.5). Corresponding denaturation enthalpies (ΔH) are shown on the right side.

30S, much higher than that shown. This observation is also supported by the decrease of denaturation enthalpy observed by DSC at low ionic strengths (Figure 4B) and suggests a partial unfolding of the proteins. The 13S value for a partially unfolded structure suggests a molecular mass higher than that of the unitary molecules, so it can be assumed they are aggregated.

DISCUSSION

The molecular characteristics of amaranth globulin-P fraction resemble those of 11S globulins (Martínez et al., 1997), and the results of the present study indicate that its stability depends on pH, in agreement with previous works for other legumins such as soybean glycinin (Hermansson, 1986) and oat and fababean globulins (Harwalkar and Ma, 1987; Arntfield and Murray, 1981). Therefore, at 0.54 ionic strength, this protein is highly stable against thermal denaturation for pH in the range 5−8.5, an interval that includes its pI (pI = 5−6; Konishi et al., 1991). Toward both pH limits, the protein loses stability with partial unfolding as reflected by the decrease of T_d and ΔH and the increase of $\Delta T_{1/2}$. These changes are possibly caused by a net charge gain that generates electrostatic repulsions. However, according to the calorimetric results, denaturation in alkaline conditions is more gradual than in acidic medium, and the results of chromatography and sedimentation reveal protein dissociation into trimeric subunits during denaturation. These subunits may be more sensitive to heat than the undivided molecules, being among the species of lower thermal stability in calorimetry. Alkaline dissociation was also observed in the salt soluble subfraction of amaranth globulin (Konishi et al., 1985; Marcone and Yada, 1991), in soybean glycinin (Catsimpoolas et al., 1969; Peng et al., 1984), and in pea legumin (Guéguen et al., 1988). In agreement with the results of this study for alkaline pH, Petruccelli and Añón (1996) have found that soybean glycinin thermograms also show an additional endotherm assigned to dissociation.

The large decrease of the thermal parameters observed for pH <5 suggests that carboxylic residues influence greatly the stability of this protein structure. At acidic pH values placed below the pI, some 11S globulins undergo denaturation with dissociation into trimeric subunits and A−B polypeptides (Guéguen et al., 1988; Sripad and Narasinga Rao, 1987; Koshiyama, 1972). The rapid globulin-P denaturation occurring for decreasing pH and the low solubility observed at μ = 0.54 and pH 6.5 or below (at pH 5.0 the solubility is 9.3 ± 0.4 g % and at pH 3.1 is 5.1 ± 0.7 g %) suggest that globulin-P experiences complete dissociation and aggregation at very acid pH values. A similar behavior was also observed by Carbonaro et al. (1997) for other legume proteins.

As is known, protein structural stability depends strongly on the ionic conditions of the medium (Damodaran and Kinsella, 1982; Damodaran, 1989a; Arntfield et al., 1986; Yamauchi et al., 1991). The present work shows that at low NaCl concentrations ($\mu \leq$ 0.01)

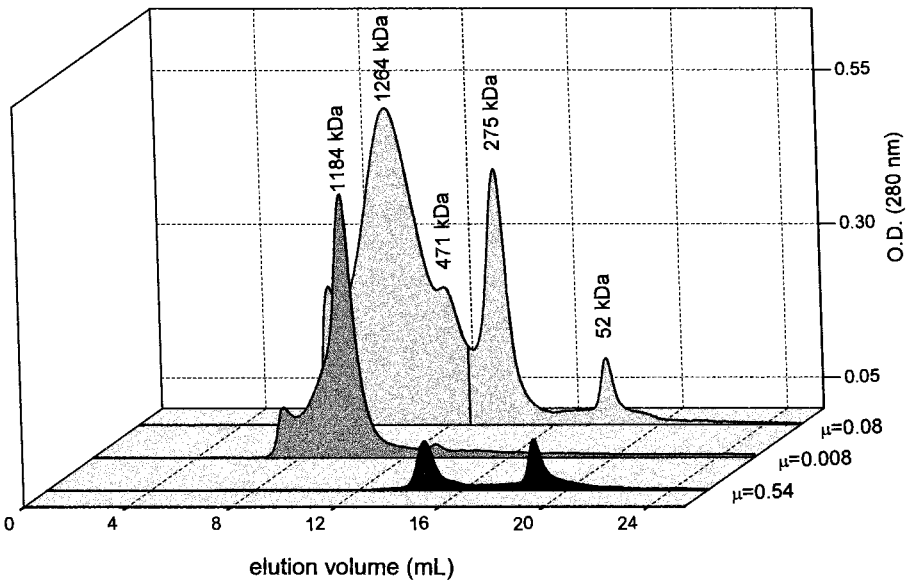

Figure 6. Gel filtration elution profiles of dialyzed globulin-P at pH 8.5, μ = 0.54 (0.035 M K_2HPO_4 buffer, enough NaCl to μ = 0.54), μ = 0.08 (0.035 M K_2HPO_4 buffer, pH 8.5), and μ = 0.008 (0.0035 M K_2HPO_4 buffer, pH 8.5). Some of the molecular masses estimated are included.

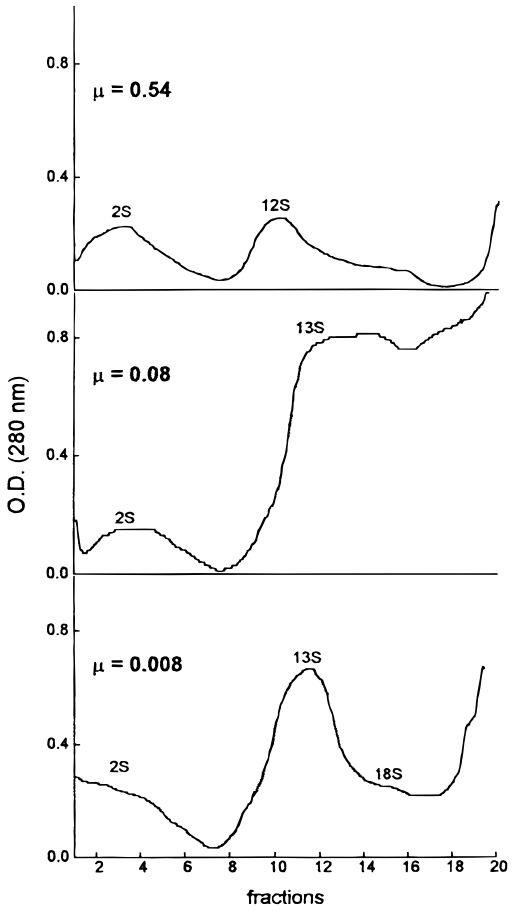

Figure 7. Sedimentation profiles of dialyzed globulin-P at pH 8.5, $\mu = 0.54$ (0.035 M K_2HPO_4 buffer, enough NaCl to $\mu = 0.54$), $\mu = 0.08$ (0.035 M K_2HPO_4 buffer, pH 8.5), and $\mu = 0.008$ (0.0035 M K_2HPO_4 buffer, pH 8.5). The sedimentation coefficients of the major species are also included.

dialyzed globulin-P structure becomes unstable, generating species of lower thermal stability. Disagreement between the sedimentation coefficients and gel filtration molecular masses estimated in low ionic strength suggests partial unfolding in globulin-P with possible formation of aggregates. Given the conditions of the medium where they were formed, these aggregates may be stabilized predominantly by ionic interactions. This behavior agrees with a report by Guéguen et al. (1988) that pea legumin forms insoluble aggregates at pH 7 and low ionic strengths. Other 11S globulins also have lower T_d values at low NaCl concentrations (Arntfield et al., 1986; Hermansson, 1986; Harwalkar and Ma, 1987; Zheng et al., 1993). In addition, Wagner and Guéguen (1995) have reported partial dissociation in soybean glycinin dialyzed against water. On the basis of our results at low ionic strengths and after dialysis, we can say that globulin-P is likely to experience dissociation with subsequent aggregation of subunits. Undivided molecules and subunits, or their respective aggregates, may have had different thermal stabilities, which is why they display two endotherms per thermogram.

The increase of NaCl concentration up to 0.1 M induces globulin-P to fold toward a more stable structure. At $\mu = 0.1$ globulin-P consists of a high proportion of polymers and a lower amount of 280 kDa unitary molecules, all of similar thermal stability. At low ionic strengths, the stabilizing effect of increasing NaCl concentrations would be of electrostatic nature, as already proposed for other proteins (von Hippel and Schleich, 1969; Arntfield et al., 1986) causing a "salting in" effect. The resulting rise in solubility is reflected by the higher UV absorbance of chromatographic and sedimentation bands at $\mu = 0.08$ compared to the results obtained at $\mu = 0.008$ (Figures 6 and 7, respectively).

Above $\mu = 0.1$, thermal stability increases with NaCl concentration, as observed in other plant proteins (Arntfield et al., 1986; Hermansson, 1986; Harwalkar and Ma, 1987; Zheng et al., 1993). However, unlike in such proteins, the ΔH of globulin-P also increases up to $\mu = 0.5$. The increase of T_d values suggests that hydrophobic interactions play an important role in stabilizing the protein structure owing to the ion-specific lyotropic effect of chloride ions expected to take place in this ionic strength range (Damodaran and Kinsella, 1982; Damodaran, 1989a). Such hydrophobic interactions may induce a more compact structure, with formation of new hydrogen bonds having endothermic disruption that would make the ΔH increase up to $\mu = 0.5$. By contrast, for ionic strengths >0.5, the exothermic process of protein aggregation is likely to take place during thermal denaturation and so would oppose the increase of the ΔH, keeping the resulting enthalpy change constant.

The increase of saline concentration above $\mu = 0.1$, at which chloride ions produce a chaotropic effect, would also cause aggregation of globulin-P, which becomes partially insoluble. Therefore, owing to this proposed mechanism and to calorimetric results, it is concluded that the aggregates might be stabilized by hydrophobic interactions.

At $\mu = 0.54$, the nondialyzed globulin-P has a larger amount of polymers than the dialyzed globulin-P (Figures 3 and 6, respectively), and this would suggest that dialysis induces some irreversible conformational change, which, at this saline concentration, promotes aggregation.

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