Common Molecular Features among Amaranth Storage Proteins

E. Nora Martínez, Oscar F. Castellani, and M. Cristina Añón*

Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), 47 y 116 (1900) La Plata, Argentina

The structure of albumin 2 protein fraction of amaranth was investigated. It was formed by several major polypeptide subunits of molecular masses of 52.3 ± 0.8, 54 ± 2, and 56 ± 1 kDa. The former and the latter subunits were composed of a peptide of molecular mass between 31 and 38 kDa linked by S−S bonds with another peptide of molecular mass between 19 and 23 kDa. The 54 kDa subunit together with the 31−38 and 19−23 kDa subunits formed S−S-linked aggregated polypeptides. Lyophilized albumin 2 was highly polymerized, having a complex monomer component with a molecular mass of 300 ± 10 kDa. The polymers were partially stabilized by SS linkages. Because of these structural characteristics, albumin 2 was very similar to amarantin except for the presence of the 54 kDa subunit and its tendency to polymerize. Two components were obtained by gel filtration of globulin fraction. The major one exhibited heterogeneity of species and showed some common features with albumin 2. The minor component eluted at a lower volume and also showed heterogeneity, with a main species of 7S and a minor one of 12S. Their major peptides had molecular masses of 78, 72, 39, 30, and 20 kDa similar to the 7S type globulin. Its size, larger than that of amarantin, is different from a 7S type globulin, but the possibility of becoming polymerized or having a shape quite different from that of a sphere cannot be dismissed.

Keywords: *Amaranth; globulin; albumin 2; protein structure; gel filtration; electrophoresis*

INTRODUCTION

The major protein fractions of amaranth seeds are—according to the Osborne classification—albumin, globulin, and glutelin. Even though there is no general agreement, most papers state that albumin is shown to be present in the largest amount, followed by glutelins, with globulins appearing in the third place (Segura-Nieto et al., 1994). Since seed storage proteins are found in the largest amounts (Fukuyima, 1991), it is striking that the albumin fraction, which usually accounts for the biologically active proteins, is found in that highest amount in amaranth seeds, although albumins have also been described as reserve proteins in some plants (Higgins, 1984). In amaranth, however, two types of albumin have been described (Konishi et al., 1991): albumin 1, removed with water and/or saline solutions; and albumin 2, extracted with water after the flour has been treated with saline solutions to remove albumin 1 and globulins. Because albumin 2 resists treatment with Pronase, which digests albumin 1, it has been suggested that the albumin 2 fraction is located in more protected sites, perhaps associated with protein bodies, which would account for its role as a storage protein. Because of its unique solubility characteristics, the reported differences of the main fractions (Segura-Nieto et al., 1994) could result from the sequence in which the solvents had been used; therefore, the albumin 2 fraction would appear as included in either the globulin or the glutelin fraction (Konishi et al., 1992).

Whereas albumin 1 has been described by work in several laboratories (Marcone et al., 1994a; Segura-Nieto et al., 1992; Mora-Escobedo et al., 1990), information about albumin 2 is scarce. On the other hand, the globulin fraction has been thoroughly studied (Segura-Nieto et al., 1994), and a major globulin of the 11S type, amarantin (Konishi et al., 1985; Marcone et al., 1991, 1992; Barba de la Rosa et al., 1992; Romero-Zepeda and Paredes-López, 1996), has been described, together with a minor fraction of the 7S leguminous type (Barba de la Rosa et al., 1992; Segura-Nieto et al., 1992). The major globulin, with a dodecameric quaternary structure—similar to that ascribed to soy 11S globulin (Marcone et al., 1994b)—is made up, similarly to these globulins, by acid subunits (A) of molecular masses in the range of 30−40 kDa and basic subunits (B) of about 20 kDa (Segura-Nieto et al., 1994). Furthermore, other monomeric peptides, which are not included in this category because of their higher or lower molecular weight, have been reported (Segura-Nieto et al., 1994). Like the 11S globulins, peptides A and B would be bound by disulfide bonds producing intermediate subunits, although no clear identification of the intermediate subunits occurring in amarantin has been achieved as yet. Marcone et al. (1992) have shown the presence of intermediate subunits with molecular masses of 46 and 75 kDa, unlike those found in other laboratories. These intermediate subunits did not account for all the described A and B subunits, which, in turn, would integrate the molecule by means of noncovalent interactions.

The minor globulin was identified by sedimentation in a sucrose gradient with a range of 8.05−9.2S (Segura-Nieto et al., 1994; Barba de la Rosa et al., 1992). Like soy 7S globulin, it is retained by concanavalin A (Barba de la Rosa et al., 1992) and its constituent peptides are nondissociated by means of 2-mercaptoethanol (2-ME).

This paper includes studies about the albumin 2 fraction, contributing to its characterization. Moreover, further information about both globulin fractions is included, thus leading to a better understanding of their structures.

* Author to whom correspondence should be addressed (fax +54-21-890741; e-mail mcanon@isis.unlp.edu.ar).

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MATERIALS AND METHODS

Materials. Seeds of *Amaranthus hypochondriacus* (commercial cultivar) were harvested at the Experimental Station of the Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), Chapingo, México, and donated to our laboratory in Argentina. Flour was obtained by grinding whole seeds in a Udy mill, mesh 1 mm, and screened by 10 × mesh. Flour was then defatted for 24 h with hexane in a 10% (w/v) suspension under continuous stirring, air-dried at room temperature, and stored at 4 °C until used. The protein content of the flour, as determined by Kjeldhal method, was 17.0% (w/w).

Protein Isolation. *Albumin 2* and *globulin* were extracted according to method B of Konishi et al. (1991), with some modifications. Each extraction step was performed in three stages (60, 30, 30 min) at room temperature with a ratio of 10 mL solvent/g of meal. Between the stages, the extraction residue was separated by centrifugation at 9000*g* for 20 min at room temperature. The first solvent used was water; in that step albumin 1 was extracted.

Globulin was extracted in the second step, treating the residue of the first stage with 32.5 mM K_2HPO_4−2.6 mM KH_2PO_4 (pH 7.5)/0.4 M NaCl (buffer A). After being centrifuged, the supernatant was adjusted to pH 5 with 2 N HCl, and globulins were precipitated. The precipitate was dissolved in buffer A for further chromatography.

Albumin 2. This fraction was obtained in the third step by extracting with water the residue remaining after globulin extraction. After centrifugation, the supernatants showed an opalescence that precipitated after adjustment to pH 6 with 2 N HCl. No further precipitate was observed in a subsequent change to pH 5. The precipitate (**albumin 2** fraction) was suspended in water, neutralized with 0.1 N NaOH, and freeze-dried. Albumin 2 was also isolated according to the same procedure, but using water containing 20 mM 2-ME as the extraction solvent in the third step. This nonlyophilized fraction was termed **albumin 2M**.

To compare results, albumin 2 isolation was also carried out according to method B of Konishi et al. (1991). Protein was determined according to a modified Lowry method (Stoschek, 1990) using bovine albumin as standard protein.

Gel Filtration Chromatography. A 2 mL sample of precipitated globulin dissolved in a small amount of buffer A (approximately 20 mg) was applied on a Sephacryl S-300 (1.6 × 100 cm) column and eluted with buffer A at room temperature, at a flow rate of 15 mL/h. Fractions of 1.2 mL were collected. Absorbances at 280 nm were measured with a Beckman DU 650 spectrophotometer. The column was calibrated using blue dextran to determine void volume (V_0) and the following standard proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa). Those fractions corresponding to peaks described under Results were pooled and used for ultracentrifugation studies.

Albumin 2 was chromatographed in a Superose 6 HR 10/30 column equilibrated with 33.3 mM K_2HPO_4−1.7 mM KH_2PO_4 (pH 8.5) (buffer B) using a Pharmacia FPLC system. Approximately 3 mg of albumin 2 in buffer B was injected. Elution was performed with buffer B at room temperature at a flow rate of 0.2 mL/min; 0.3 mL fractions were collected. Elution profile (absorbance at 280 nm) was thus obtained. Column was calibrated with blue dextran (V_0) and the standard proteins thyroglobulin (669 kDa), ferritin (440 kDa), β-amylase (200 kDa), and alcohol dehydrogenase (150 kDa). Fractions from the corresponding peaks were used for electrophoretic analysis.

Molecular masses of globulin components were calculated from runs in both systems (Sephacryl S-300 and FPLC), and those of albumin 2 components from runs in FPLC. Data from at least three different runs were obtained.

Ultracentrifugation. Ultracentrifugation was performed in a Beckman TL Optima ultracentrifuge using a TLS-55 rotor. Samples of 0.1 mL were layered on top of 10−30% (w/v) sucrose linear gradients in buffer A and centrifuged at 10 °C for 5 h at 50 000 rpm. Sedimentation constants of the peaks were calculated as indicated by Martin and Ames (1961) using catalase as standard protein with known sedimentation constant (11.20 S). Mean values from three different runs are reported. Since only one standard protein could be used, the calculated sedimentation coefficients are approximated. Gradient UV profile was measured with a Gilson LC detector Model 111 and recorded with a Gilson N2 recorder. Fractions of 0.1 mL were collected.

Electrophoresis. All gels were run in minislabs (Bio-Rad Mini Protean II Model).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Two-Dimensional SDS→SDS+2-ME. Runs were carried out according to Laemmli's method (1970) as modified by Petruccelli and Añón (1994). In both cases a linear gradient separating gel was used (6−12% in polyacrylamide). The following continuous buffer system was used: 0.375 M Tris-HCl (pH 8.8), 0.1% (w/v) SDS for the separating gel; 0.025 M Tris-HCl, 0.192 M glycine, and 0.1% (w/v) SDS (pH 8.3) for the running buffer; and 0.125 M Tris-HCl (pH 6.8), 20% (v/v) glycerol, 1% (w/v) SDS, and 0.05% (w/v) bromophenol blue as sample buffer. Some samples were run under reducing conditions, as follows: Samples were boiled for 1 min in sample buffer containing 5% 2-ME. All samples—reduced and nonreduced—were then centrifuged at 15800*g* for 5 min, and the supernatants were used to load the gels (30−40 μg of protein/lane when stained with Coomassie Brilliant Blue stain or 1 μg of protein/lane when stained with silver stain). Prior to the second electrophoresis, each first-dimension slab gel portion was treated with 10 volumes of SDS buffer composed of 62.5 mM Tris-HCl (pH 6.8), 1% SDS, 0.2 M 2-ME, and 20% sucrose for 30 min at 55 °C with two changes of solution. The treated gel was placed on the top of the second-dimension SDS slab gel. Electrophoretic runs were performed for 1 h at a constant 200 V voltage. The molecular masses of polypeptides were calculated from at least three different runs by 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); and α-lactalbumin (14.4 kDa). Protein samples were prepared by dissolving freeze-dried protein with sample buffer or mixing 3 volumes of chromatographic or sedimentation fractions with 1 volume of concentrated sample buffer.

Nondenaturing Polyacrylamide Gel Electrophoresis (Native−PAGE). These tests were performed using the same buffer system as that of SDS−PAGE, but without sodium dodecyl sulfate, in 4−7.5% (w/v) acrylamide linear gradient gels. Samples were prepared either by dissolving freeze-dried protein in sample buffer (0.75 M Tris-HCl, pH 8.8, 20% (v/v) glycerol, and 0.05% (w/v) bromophenol blue) or by mixing 3 volumes of chromatographic or gradient fractions with 1 volume of sample buffer. Gels were loaded with 30−40 μg of protein/lane when stained with Coomassie Brilliant Blue or 1−3 μg of protein/lane when stained with silver stain.

Two-Dimensional Polyacrylamide Gel Electrophoresis UREA→SDS. First dimension was carried out using the same buffer system as native−PAGE, with the additions of 6 M urea to the separating buffer and 6 M urea and 5% 2-ME to the sample buffer (4−7.5% gradient in polyacrylamide). Samples were heated in a boiling water bath for 1 min. Treatment of first-dimension slab portion and second-dimension run were performed as in two-dimensional SDS→2-ME+SDS. Molecular mass protein standards were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa).

The gels were fixed and stained with Coomassie Brilliant Blue stain or silver stain.

Differential Scanning Calorimetry. DSC measurements were performed in a Polymer Laboratories (Rheometric Scientific) calorimeter. The equipment was calibrated at a heating rate of 10 °C/min by using indium, lauric acid, and stearic acid (p.a.) as standards.

Hermetically sealed aluminum pans were prepared to contain 12−14 mg of albumin 2 suspended in buffer B (20%

Table 1. Solubility of Crude Albumin 2 in Different Solvents

solvent	s^a (%)	μ^b	pH^c
H_2O	1.7 ± 0.1		6.5
buffer A	6.6 ± 0.7	0.5	7.5
buffer B	55.9 ± 8.7	0.08	8.5

[a] g of dissolved protein/100 g of total protein. [b] Ionic strength calculated from buffer concentration. [c] pH of the solution.

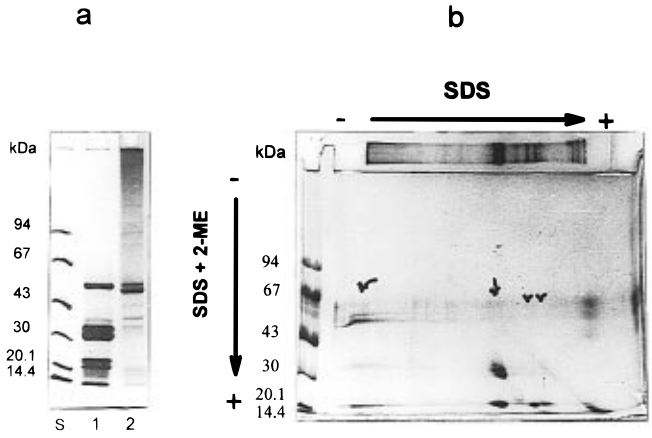

Figure 1. Electrophoretic patterns of crude albumin 2. (a) SDS−PAGE: S, standard reference proteins; 1, presence of 2-ME; 2, absence of 2-ME. (b) Two-dimensional electrophoresis: first dimension, absence of 2-ME; second dimension, presence of 2-ME. Standard reference proteins were included in the second dimension. Coomassie Brilliant Blue stain was used.

w/v); a double empty pan was employed as reference. Capsules were heated from 30 to 130 °C at a rate of 10 °C/min. After each run, the pans were punctured, and their dry-matter content was determined by leaving the pans overnight in an oven at 105 °C. The denaturation temperature (T_d) and enthalpy of transition (ΔH) were obtained by analyzing the thermograms with Software Plus V5.41. The ΔH value was calculated from the area below the transition peaks.

Solubility of Albumin 2. Samples of freeze-dried albumin 2 were dispersed in distilled water, buffer A, or buffer B (1.0% protein w/v) and stood at room temperature for 2 h with periodic (every 15 min) agitation with a Thermolyne Maxi-Mix II mixer at maximum speed. Afterward, they were centrifuged at 18000*g* for 30 min and protein was determined from 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 standard protein.

RESULTS

Albumin 2. As described by Konishi et al. (1991), the albumin 2 fraction is extracted with water after extraction of globulins by saline treatment of flour. The same procedure, with some modifications (see Materials and Methods), was used in our laboratory, leading to a higher yield of albumin 2, which is extracted as a fine nonsedimenting suspension after centrifuging at 9000*g* for 20 min. This fraction is more soluble in pH 8.6 phosphate buffer, $\mu = 0.08$, than in pH 7.6 phosphate, $\mu = 0.5$, and is insoluble in water (Table 1).

The SDS−PAGE profile of albumin 2 (Figure 1a, lane 2) shows major peptides of 52.3 ± 0.8 and 56 ± 1 kDa molecular mass; polypeptides of molecular mass >80 and <40 kDa have also been found. This peptidic composition is similar to that of the 11S type gobulin or amarantin (Romero-Zepeda and Paredes-López, 1996; Segura-Nieto et al., 1994; this paper). Similarly to the behavior of amarantin subunits, the peptides of 52.3, 56, and >80 kDa are dissociated by the presence of 2-ME producing peptides of 54 ± 2, 38 ± 1, 33.1 ± 0.5, 31.2 ± 0.5, 23.6 ± 0.3, 21.0 ± 0.3, 19.3 ± 0.8, and 14.6 ± 0.5 kDa (Figure 1, lane 1). Bidimensional electrophoresis (Figure 1b, straight arrows) indicates that each of the 52 and 56 kDa subunits is formed by one peptide in the range of 31−38 kDa linked by disulfide bridges with one peptide of 19−24 kDa, in the same way as the A−B subunits of 11S globulins. It is also shown (Figure 1b, arrowheads) that some peptides of 39 and 43 kDa—minor bands in the nonreduced profile—are also made up by peptides linked by S−S bridges. The former is composed by two peptides of 19−24 kDa and the latter by a peptide of 19−24 kDa and another of approximately 14.6 kDa. This result correlates with the presence of dimers of basic subunits reported for soy globulins in different conditions (Mori et al., 1979; Utsumi et al., 1980; Hoshi and Yamauchi, 1983b). High molecular weight peptides (Figure 1b, curved arrow) consist of 31−38 and 19−24 kDa peptides—like those forming the 52 and 56 kDa subunits—and the 54 kDa peptide that would indicate that the latter peptides form aggregates involving disulfide bridges. Aggregates consisting of intermediate subunits linked by disulfide bonds have also been found in soy 11S globulin (Wolf, 1993). In accordance with these electrophoretic results, the monomeric peptide of 54 kDa is not released by SDS, but is found, instead, forming part of the higher molecular weight polypeptide aggregates. We were unable to detect this peptide in the purified globulin; instead, we have found it as part of aggregates in preparations of cryoprecipitated globulins or globulins precipitated at pH 6 (results not shown). On the other hand, Romero-Zepeda and Paredes-López (1996) reported the presence of a 55 kDa peptide in the SDS−PAGE profile of reduced amarantin, in the same way a monomer of 50 kDa in the SDS−PAGE profile of the reduced 11S globulin of quinoa was found (Brinegar and Goudan, 1993).

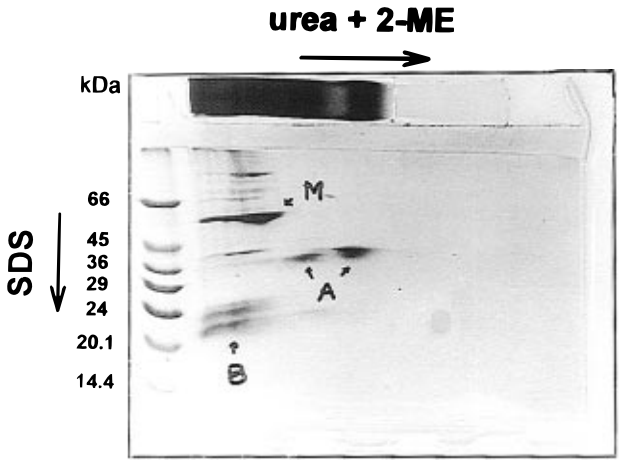

Figure 2. Two-dimensional electrophoresis: first dimension; urea−PAGE, albumin 2 was in the presence of 6 M urea and 5% 2-ME; second dimension, SDS−PAGE. Standard reference proteins were included. Coomassie Brilliant Blue stain was used.

Albumin 2 in 6 M urea and 5% 2-ME exhibited a two-dimensional electrophoretic profile (Figure 2) in which 31−33 kDa spots (spots A) could come from higher mobility bands in the first dimension than the bands corresponding to the 22 kDa spots (spot B). In the first dimension, proteins are in a medium with urea and 2-ME; therefore, 19−24 and 31−34 kDa polypeptides run as free molecules, according to their charge/mass ratio. Since 31−36 kDa polypeptides have a higher

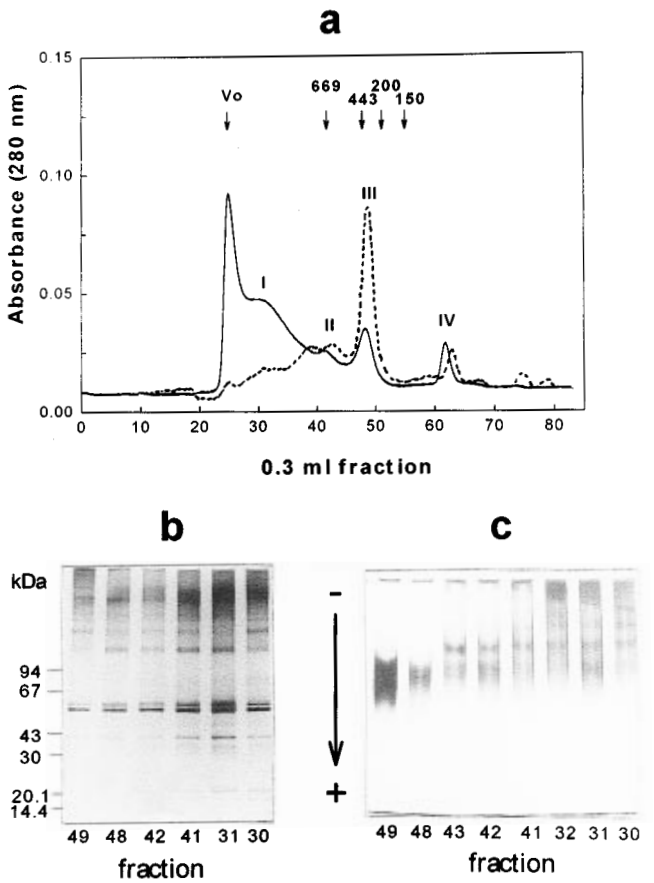

Figure 3. (a) Gel filtration−FPLC profile of albumin 2: solid line, albumin 2 dissolved in buffer B; dashed line, albumin 2M (isolated in the presence of 20 mM 2-ME) dissolved in buffer B with 20 mM 2-ME. Arrows indicate either void volume (V_0) or elution volumes of standard proteins (molecular masses in kDa). (b) SDS−PAGE of chromatographic fractions. (c) Native−PAGE of chromatographic fractions. Silver stain was used.

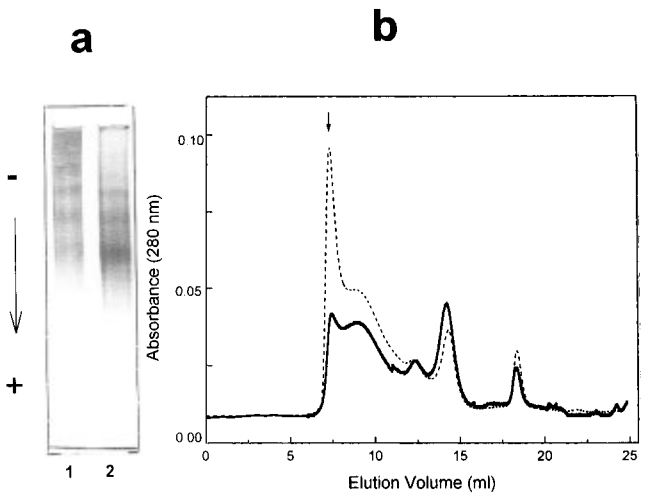

Figure 4. (a) Native−PAGE of crude albumin 2: 1; absence of 2-ME; 2, presence of 20 mM 2-ME. Coomassie Brilliant Blue stain was used. (b) Gel filtration FPLC patterns: dashed line, albumin 2 dissolved in buffer B; solid line, albumin 2 dissolved in buffer B with 20 mM 2-ME. Arrow indicates void volume (V_0).

mass than 19−24 kDa polypeptides, the only reason for a greater mobility is ascribed to their having a much higher negative charge. These characteristics are similar to those already described for amaranth 11S type globulin (Segura-Nieto et al., 1994); thus, these subunits are called A and B. The bidimensional profile also shows the monomer peptide of 54 kDa (spot M) with intermediate mobility.

Analysis of albumin 2 by gel filtration showed four absorption peaks at 280 nm (Figure 3a): a wide peak I, involving a range of elution volumes including the void volume; a peak II, the molecular mass of which—as estimated from its elution volume—was 611 ± 37 kDa; peak III, with an estimated molecular mass of 301 ± 10 kDa. As determined by SDS−PAGE, peak IV contained peptides of very low molecular weight (results not shown), whereas the rest of the fractions (Figure 3b) had the same peptidic composition (major peptides of molecular masses of 52.3 and 56 kDa). Conversely, the fractions exhibited different profiles with native−PAGE (Figure 3c). Fractions corresponding to peak III showed profiles with only one band; those of peak II exhibited two bands: the above-mentioned band and another of lower mobility; whereas profiles of peak I fractions showed, besides the already mentioned bands, several bands of higher intensity and lower mobility. Both electrophoretic and chromatographic results suggest that the lower the mobility of the bands in the profiles of native−PAGE, the greater the size of the corresponding molecules. Taking into account that all of them have polypeptides of similar molecular masses, the differences in size would result from the presence of polymers of different number of a single molecule eluted in peak III. According to its molecular mass, peak II would be formed by dimers.

The chromatographic profile of nonlyophilized albumin 2 extracted in the presence of 20 mM 2-ME (albumin 2M) is shown in Figure 3a (dashed line), in which polymers are found in lower amounts. When this same fraction was dialyzed against water, a protein was obtained that, once dissolved in buffer B, exhibited a chromatographic profile identical to that of the lyophilized fraction. This would indicate that polymers had been formed during dialysis. The presence of 20 mM 2-ME in a solution of lyophilized albumin 2 in buffer B produced a slight decrease of the polymers of higher molecular size, as evidenced by the profile in native−PAGE (Figure 4a) and the chromatographic profile (Figure 4b). The latter results suggest that disulfide bonds are involved in maintaining the polymerized aggregates.

Albumin 2 and albumin 2M were analyzed by DSC (Figure 5). Both preparations showed a single denaturation endotherm with a denaturation temperature (T_d) of 99.7 °C. This indicates that polymers—main components of albumin 2—and monomers—major components of albumin 2M—have the same thermal stabilities. In the same way, albumin 2 and albumin 2M exhibited similar denaturation enthalpies (ΔH) (Figure 5). Since at the high T_d (99.7 °C) hydrophobic interactions are negligible (Privalov and Khechinashvili, 1974), this result suggests that monomer and polymer conformations have similar amounts of hydrogen bonds. This might imply that these interactions are not important in the polymer consolidation.

Globulins. Chromatographic profile of globulin precipitated at pH 5 is shown in Figure 6. The material eluted with the void volume (first peak) contained a low amount of protein, making its analysis difficult. Peaks I and II were components of globulins, whereas peaks eluting at larger volumes (electrophoretic results not shown) corresponded to contamination with albumin 1. Peak II, the largest, was the already described ama-

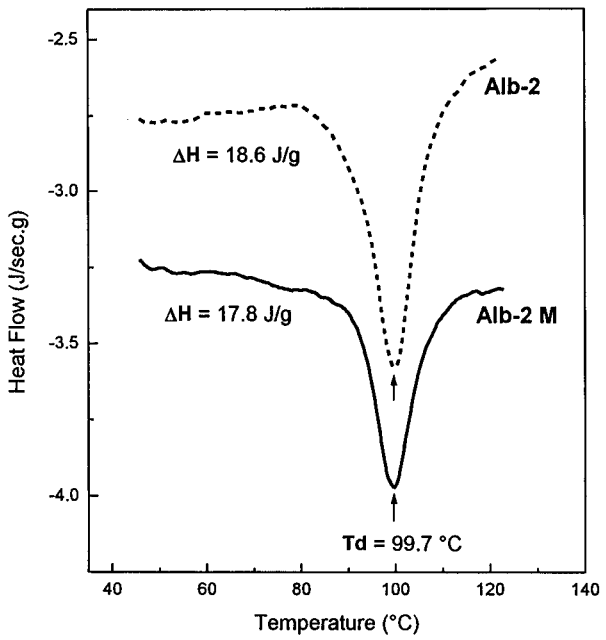

Figure 5. DSC thermograms of albumin 2 in buffer B: albumin 2 (dashed line); albumin 2M (isolated in presence of 20 mM 2-ME) (solid line). ΔH, denaturation enthalpy, and T_d, denaturation temperature values, are indicated.

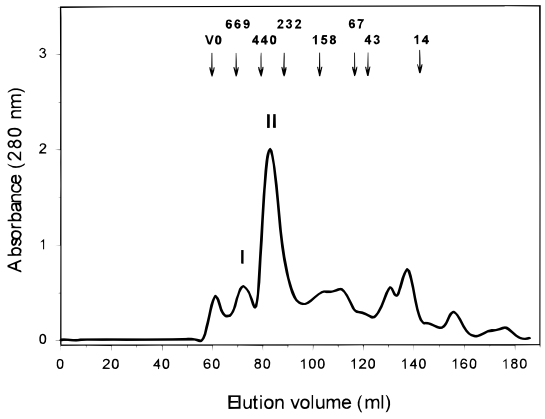

Figure 6. Sephacryl S-300 gel filtration profile of crude globulin. Arrows indicate either void volume (V_0) or elution volumes of standard proteins (molecular masses in kDa).

rantin (Romero-Zepeda and Paredes-López, 1996; Segura-Nieto et al., 1994); its apparent molecular mass, estimated from its elution volume in several runs, was 302 ± 78 kDa, a value similar to that reported by other authors (Segura-Nieto et al., 1994). By sedimentation in a sucrose gradient, under the same salt conditions as that used in chromatography (pH 7.5, $\mu = 0.5$) this component appeared heterogeneous (Figure 7II), with a 13S species (Figure 7II, peak d) and another of 9S (Figure 7II, peak c). Species of different sedimentation constants, derived from globulin isolated by gel filtration, had been already reported (Barba de la Rosa et al., 1992; Marcone et al., 1992) The SDS−PAGE profile of the 13S species (Figure 8, lanes d), with major bands at 52 ± 1, 56.0 ± 0.8, and 39.6 ± 0.6 kDa, was in accordance with that shown above for albumin 2. These peptides were dissociated by 2-ME in subunits of 31−36 and of 19−24 kDa (results not shown). In the SDS−PAGE pattern of the 9S species (Figure 8a, lanes c), peptides of 39.6 ± 0.6 and 16 kDa were those found in the greatest amounts. These two, 9S and 13S, globulin species might be composed of molecules of the same Stokes radius but different shapes. The minor globulin,

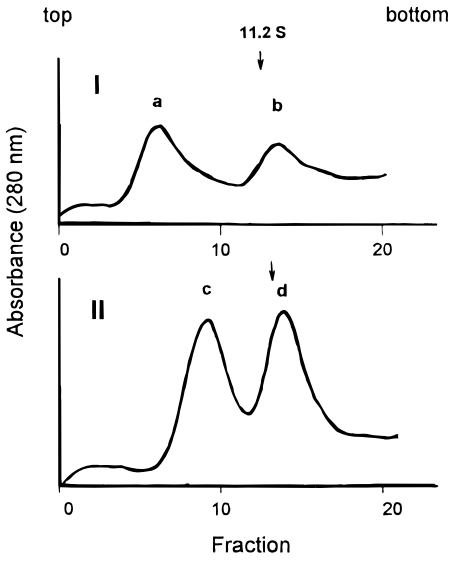

Figure 7. Ultracentrifugation profiles of pooled samples under peaks I and II of Figure 6. Arrows show catalase standard value 11.2S.

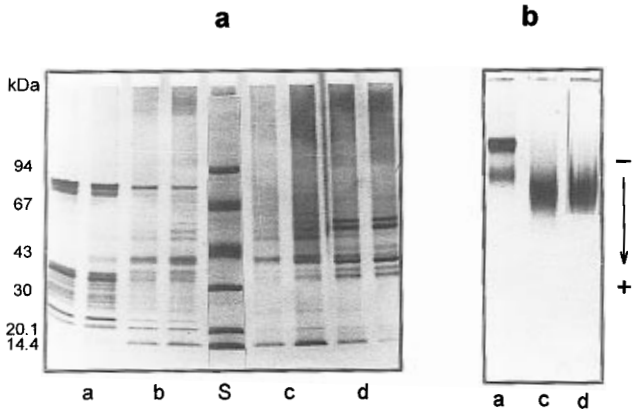

Figure 8. Electrophoretic patterns of fractions from sedimentation gradients of Figure 7: (a) SDS−PAGE patterns of two fractions of each peak (a−d); (S standard proteins which molecular masses are indicated on the left side); (b) native−PAGE patterns of peaks a, c, and d.

eluting at a lower volume than amarantin (peak I, Figure 6), showed elution volumes in Sephacryl S-300 and Superose 6HR columns having a greater dispersion than amarantin, its estimated mean molecular mass being 450 ± 153 kDa. It also exhibited heterogeneity when sedimented in a sucrose gradient (Figure 7I), showing a 7S species (Figure 7I, peak a) and a 12S species. The 12S species, which was found in the lowest proportion, showed a higher contamination with amarantin, as expected according to the chromatographic profile, and its most important peptides were those of 79 ± 2 and 39.5 ± 0.8 kDa (Figure 8a, lanes b). The 7S species consisted of 20.0 ± 0.5−21.7 ± 0.7, 31.0 ± 0.5−33.1 ± 0.6, and 71 ± 1−78 ± 2 kDa peptides (Figure 8a, lanes a), not dissociated by 2-ME (results not shown). Because of its subunit sizes and its behavior during sedimentation, this globulin was similar to the 7S globulin of legumes (Derbyshire et al., 1976**)**. By means of native−PAGE, the 7S species of the minor globulin (Figure 8b, lane a) exhibited two bands, a broad band migrating more slowly than the other, narrow band. Otherwise, both species of amarantin showed only one band, with a mobility higher than those of the minor globulin (Figure 8, lanes c and d). In comparison with amarantin, the lower mobility of 7S globulin could

result from differences of charge or size. With regard to soy globulins, 7S also has a mobility lower than 11S (Hill and Breidenbach, 1974), in this case, resulting from charge differences.

DISCUSSION

The term albumin 2 applied to the fraction studied here results from the similarity between the method of extraction used in our laboratory and that described by Konishi et al. (1991). However, both fractions exhibit different characteristics. Differences in the preparation procedures could result in the different solubilities in water, but they are unable to explain the differences in peptide composition. Morover, we have observed that simultaneous preparations of albumin 2, according to both the technique described by Konishi et al. (1991) and that described above, exhibited the same electrophoretic profile, except for the fact that the fraction prepared according to Konishi et al. (1991) contained a lower proportion of peptides of more than 80 kDa. Albumin 2 described in this paper showed a subunit composition similar to that observed in 11S type globulin or amarantin, containing mainly peptides of 31−38 kDa linked by disulfide bridges with 19−23 kDa peptides with a lower negative charge at pH 8.3. In addition, albumin 2 contained a higher amount of a monomer peptide of 54 kDa, found in high molecular weight aggregates, in which it was attached by disulfide bonds. The monomeric molecule of albumin 2 had a molecular mass of around 300 kDa, similar to that of amarantin, but, at variance with the latter, albumin 2 contained a high proportion of polymers of high molecular mass. The presence of dimers and larger aggregates had also been observed in preparations of 11S globulins of different plants (Catsimpoolas et al., 1969; Mori and Utsumi, 1979; Wolf et al., 1962; Wolf and Nelsen, 1996), disulfide bonds being described as one of the forces involved in their maintenance. As shown in the work described here, disulfide bonds were involved in the stabilization of the albumin 2 polymers. It is difficult to evaluate to which extent either the polymers or aggregates were present as such in the flour or seeds and to which extent resulted from the preparation technique. It is a well-known fact that the precipitation and freezing followed by lyophilization steps—procedures leading to concentration of the protein—may stimulate aggregate formation (Wolf and Nelsen, 1996; Hoshi and Yamauchi, 1983a). Our results showed that 20 mM 2-ME prevented the formation of some of the polymers or dissociated some of those already present in the seed. This compound also decreased the polymers present in the lyophilized samples. Albumin 2M, having a low amount of polymers, showed a strong tendency to polymerization, as indicated by aggregation after dialysis. Similarity of both thermal stability (T_d) and denaturation enthalpy (ΔH) of the polymerized protein, and that containing mainly monomers, suggests this would not occur in this case due to the presence of aggregation resulting from denaturation. Furthermore, this protein had the same thermal stability as the globulins (Martínez and Añón, 1996).

According to the characteristics described, the albumin 2 fraction appears as the 11S globulin or amarantin, containing an extra 54 kDa peptide and a higher tendency toward polymerization. Since the subunit of 54 kDa was found only in high molecular weight aggregates, its action as polymerization inducer becomes likely. This peptide could be a partially processed intermediate subunit, which for some reason had not undergone the proteolytic phenomenon that separates the basic subunit from the acid one, and might be more likely to have disulfide bonds between chains. Brinegar and Goudan (1993) have also considered the 50 kDa peptide which appears in chenopodin reduced samples as an immature intermediate subunit. On the basis of these results and taking into account the fact that a large extent of homology between globulins and amaranth glutelins has been found (Vasco-Méndez and Paredes-López, 1995), it could be thought that in amaranth the most abundant storage proteins would be the globulins. These proteins could be found in different aggregation or conformational states within the seed leading to different extractabilities or solubilities in aqueous solvents. Both 7S and 11S globulins would correspond to one type of globulin. Polymerized globulins (described here as albumin 2) and glutelins would correspond to two other types of globulins. This is in accordance with the storage protein classification on the basis of the structure of their genes, as presented by Fukuyima (1991). Further support for this notion would be mandatory, by determining the sequence homology and by proving a common genetic origin of globulin, albumin 2, and amaranth glutelins.

The 11S type globulin described in this paper shows major peptides of 52, 56, and 40 kDa. Similar results were reported by Romero-Zepeda and Paredes-López (1996) for amarantin and by Barba de la Rosa et al. (1992) for the 12.7S globulin. These peptides are dissociated by 2-ME into subunits of 31−36 and 19−24 kDa and exhibited the same bidimensional electrophoretic profile as that shown by albumin 2 (Figure 1b). On this basis, it could be speculated that the hexamer described by Marcone et al. (1994b) would be constituted mainly by intermediate subunits of molecular masses near 52 and 56 kDa. Since 16 and 39 kDa free subunits have also been found, mostly in the lower sedimentation constant species, it could be thought that those peptides result from dissociation of some other molecules. In this way, amarantin, as well as other legumins (Kitamura et al., 1980; Utsumi and Mori, 1980; Utsumi et al., 1981; 1987), would possess a certain molecular heterogeneity.

As far as the minor globulin component is considered, it was found to have a molecular mass larger than that of amarantin, by means of the gel filtration technique. It is likely that because it was found in low amount, the presence of this globulin had remained masked by dimers of the major globulin in other purifications (Marcone et al., 1991). This minor globulin component could be the same as the 8S component described by Segura-Nieto et al. (1994) or the 9S reported by Barba de la Rosa et al. (1992) and, in turn, similar to 7S globulins of legumes. If it is thought that its peptides were arranged as trimers (78−72−20, 78−72−30, or 78−72−39), as in the vicilins, its molecular mass would be close to 200 kDa. Therefore, the apparent molecular size, as shown by gel filtration, would suggest that under this saline condition this globulin was found polymerized. It is already known that, according to the ionic conditions, 7S globulins tend to dimer formation (Derbyshire et al., 1976). Moreover, its molecular mass, as estimated by gel filtration, does not correlate with the sedimentation constants observed under the same saline conditions. An alternative notion—i.e., that we are dealing here with molecules far removed from the sphere and, having a smaller size than amarantin, exhibiting a higher Stokes radius—could also be taken

into consideration. In this case, the electrophoretic mobility lower than that of amarantin, exhibited in native−PAGE, would result from charge differences, in the same way as soy 7S globulin.

CONCLUSIONS

The protein extracted with water from the residue that remains after extraction of albumin 1 and globulins from amaranth flour showed characteristics similar to those of amarantin, but having instead a difference of an extra peptide of 54 kDa and a higher tendency to polymerization. This suggests that, in fact, that protein would be the same 11S type globulin that was found in the seed under different conformational states and/or different degrees of polymerization.

According to data found in the literature and our own findings, hexameric molecules of amarantin would be mostly formed by intermediate subunits of 52 and 56 kDa. The minor globulin, similar to 7S of legumes, at pH 7.5 and ionic strength 0.5, exhibited molecular features (Stokes radius, sedimentation constant) suggesting that, being of a size similar to globulins 7S, it could be polymerized under these saline conditions or, else, that the molecular shape could not be assumed as a sphere.

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