Role of Disulfide Bonds upon the Structural Stability of an Amaranth Globulin

Oscar F. Castellani, E. Nora Martínez, 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

Analysis of globulin-P, the polymerized amaranth globulin, gave a low amount of free sulfhydryls (10.2 ± 0.5 μmol/g) from which 7 ± 1 μmol/g was buried inside the molecule. In addition, its disulfide content was high (51 ± 1 μmol/g) and similar to soybean 11S globulin content. The more exposed disulfide bridges were found to be stabilizing polymers, whereas the less reactive bridges were either linking P_{20} and P_{30} polypeptides or forming intrachain linkages. It was found that the buried bonds participate in the stabilization of folded polypeptides and the quaternary structure of the globulin. In turn, the dissociation of polymers and disruption of the quaternary structure by the action of 2-mercaptoethanol reverted upon removal of the reducing agent. This demonstrates that the polymerized state and the quaternary structure of the molecules are most favorable from the thermodynamic point of view. The similar content of SH and SS in globulin-P and globulin-S found in this laboratory suggests that the differences between these proteins may be ascribed to other compositional differences.

Keywords: *Amaranth globulins; sulfhydryl and disulfide groups; protein structure; calorimetry*

INTRODUCTION

Amaranth proteins, with a well-balanced amino acid composition rich in lysine and sulfur-containing amino acids (Bressani, 1989), have a good potential as a food ingredient to complement cereal and leguminous proteins.

In food proteins, sulfhydryl and disulfide groups are very important because they determine protein functionality to a considerable extent. On the one hand, intramolecular disulfide bridges add rigidity and stability to the protein structure, making them less capable to form foams, films, and emulsions, systems in which surface properties are developed (Kinsella and Phillips, 1989). On the other hand, the molecular rigidity and the capacity to form intermolecular disulfide bridges during food processing may contribute to both the protein functionality in gel formation and gel, film, foam, and emulsion stabilization (Damodaran, 1989; Monahan et al., 1995).

Legumins (11S globulins) possess quaternary structure consisting of two or six subunits with the A−SS−B generalized structure, where A (acidic polypeptide) and B (basic polypeptide) are linked by disulfide bridges (Derbyshire et al., 1976). Glycinin (soybean 11S globulin) SH and SS groups have been intensively studied because of their role in stabilizing protein conformation (Kim and Kinsella, 1986; Draper and Catsimpoolas, 1978; Hoshi and Yamauchi, 1983; Wolf, 1993) and, besides, owing to their participation in thermal protein denaturation and aggregation (Zarins and Marshall, 1990; Yamagishi et al., 1981; Wolf, 1993). Glycinin has a low value of surface SH groups, since the majority of Cys residues are involved in SS bonds. Some of these bonds are linking A and B polypeptides, while others may appear as intrachain SS bonds in the acidic polypeptide and as intermolecular bonds stabilizing glycinin polymers (Briggs and Wolf, 1957; Wolf, 1993).

The main amaranth globulins are the saline-soluble globulin (Glb-S) characterized as a 11S type globulin (Segura-Nieto et al., 1994; Romero-Zepeda and Paredes-López, 1996; Chen and Paredes-López, 1997) and the highly polymerized globulin (Glb-P). This latter fraction was first characterized as albumin-2 by Konishi et al. (1991), but it was shown in subsequent work that its structural characteristics and physicochemical properties were similar to those of 11S globulins (Martínez et al., 1997; Castellani et al., 1998). Most of the Cys residues of Glb-S are involved in disulfide bridges required to maintain the quaternary structure, although their cleavage does not mainly affect the protein secondary structure (Marcone and Yada, 1997). Concerning Glb-P, values of SH and SS groups and their role in the stabilization of the protein structure are unknown. Nevertheless, Glb-P molecules have been reported as being composed of dimeric subunits linked by disulfide bonds, since their polymers are stabilized—at less partially—by SS linkages (Martínez et al., 1997).

The main objective of this work was to determine the role of disulfide interactions in the structural stability of Glb-P. To this end, we examined the number of SH and SS groups of Glb-P and how the reduction with 2-mercaptoethanol (2-ME) affects some structural properties of this protein.

MATERIALS AND METHODS

Protein Isolation. Amaranth protein fractions were isolated from hexane-defatted flour obtained from *Amaranthus hypochondriacus* seeds (Mercado cultivar) harvested at the Experimental Station of the Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), Chapingo, México. Globulins were prepared according to the procedure already

* To whom correspondence should be addressed (fax +54-221-4890741; e-mail mca@nahuel.biol.unlp.edu.ar).

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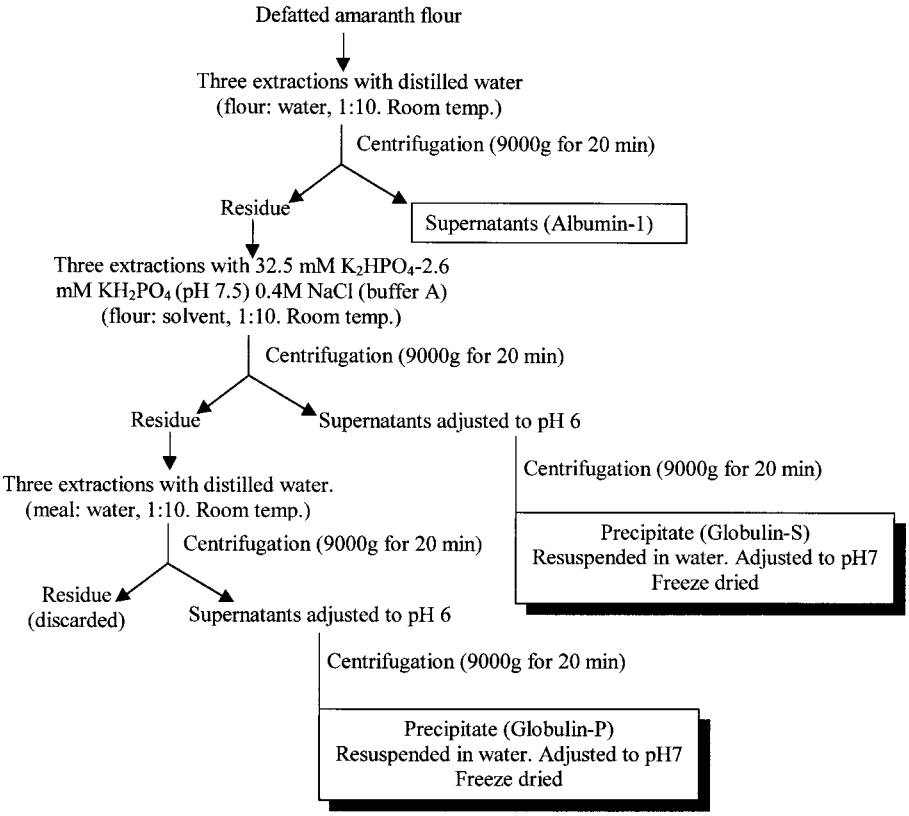

Figure 1. Isolation of amaranth protein fractions.

described (Castellani et al., 1998), as shown in Figure 1. The flour was successively treated 3 times with water for albumin extraction, 3 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-S (Glb-S) extraction, and 3 times with water for globulin-P extraction. All treatments were done at room temperature in a ratio of 10 mL of solution to 1 g of flour, and after each treatment the extraction residue was separated by centrifugation at 9000 *g* for 20 min at room temperature. Globulin-S and globulin-P were isolated from the corresponding supernatants by precipitation at pH 6 using 2 N HCl. The precipitates were resuspended in water, neutralized with 0.1 N NaOH, and freeze-dried. Globulin-P yield was 37 ± 5 mg/g of flour.

Differential Scanning Calorimetry (DSC). Measurements were carried out in a Polymer Laboratories (Rheometric Scientific) calorimeter driven with the Plus V 5.41 software. Calibration was carried out at a heating rate of 10 °C/min by using (Rheometric Scientific Ltd.) indium proanalysis (p.a.), lauric acid p.a., and stearic acid p.a. as standards.

Hermetically sealed aluminum pans were prepared to contain 12−14 mg of protein suspension (20% w/v), a double empty pan being employed as a reference. Globulin-P suspensions in 33.3 mM K_2HPO_4−1.7 mM KH_2PO_4 (pH 8.5) (buffer B), buffer B with 2-mercaptoethanol (2-ME) at several concentrations, and buffer B with ethanol at different concentration were analyzed after being left for 30 min at room temperature. Capsules were heated from 30 to 130 °C at 10 °C/min. Once each of the runs was over, the pans were punctured to determine their dry matter by leaving them overnight in an oven at 105 °C. Calorimetric denaturation parameters were calculated using the software mentioned above, with the denaturation temperature (T_d) being taken as the value corresponding to the transition peak maximum and the denaturation enthalpy (ΔH) values calculated from the area below the transition peaks.

At least three replicates were performed for each sample to obtain the mean value and a measure of the statistical dispersion of each parameter.

Chromatography. Globulin-P in buffer B or in buffer B with 0.020 or 0.570 M 2-ME was analyzed by chromatography at room temperature in a Superose 6B HR 10/30 column using a Pharmacia LKB, FPLC System. Samples (4 mg of protein) were eluted with the same buffer as that used in the sample or with buffer B at a flow rate of 0.2 mL/min. 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), alcohol dehydrogenase (150 kDa), albumin (66 kDa), cytochrome C (12.4 kDa), and aprotinin (6.5 kDa). The calibration curve obtained from duplicate measurements was

$$\ln MM = 12.90 - 0.54V_e \; (r = -0.97)$$

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

Electrophoresis. All gels were run in minislabs (BioRad Mini Protean II model) using the procedure already described (Martínez et al., 1997).

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and two-dimensional SDS → SDS + 2-ME were performed with 6−12% (w/v) acrylamide linear gradient separating gels and the following continuous buffer system: 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.

In two-dimensional electrophoresis, the first dimension slab gel portion was treated with 10 volumes of treatment buffer composed of 62.5 mM Tris-HCl, pH 6.8, 1% SDS, 20% sucrose with 0.2 M 2-ME, for 30 min at 55 °C with two changes of solution.

The following protein molecular mass standards were used: 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).

Nondenaturing polyacrylamide gel electrophoresis (native-PAGE) and the first dimension of two-dimensional native → SDS were carried out in 4−7.5% (w/v) acrylamide linear gradient gels at pH 8.3 with the same buffer

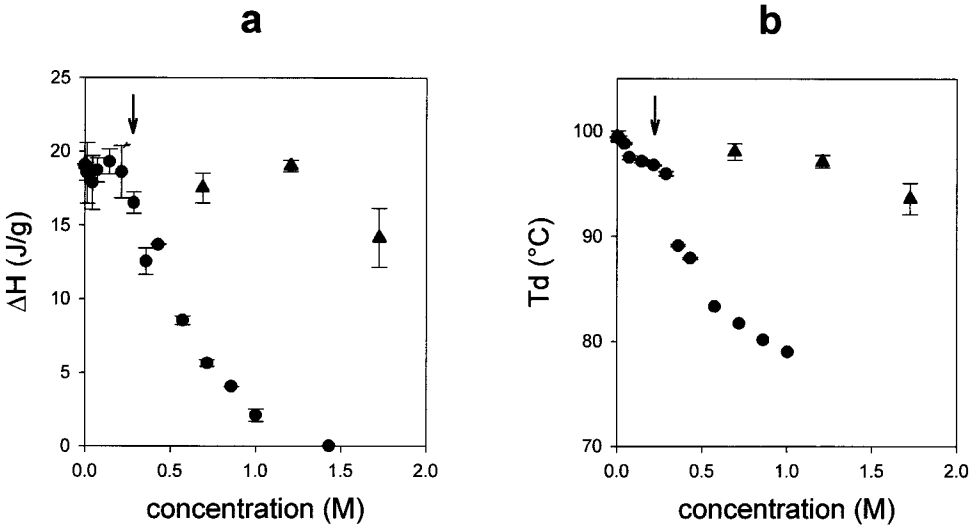

Figure 2. Effect of 2-mercaptoethanol (●) and ethanol (▲) on the DSC characteristics of globulin-P in buffer B: (a) denaturation enthalpy (ΔH), (b) denaturation temperature.

system as that used for SDS−PAGE but without SDS. The second dimension of **native → SDS** was performed by the same method as **SDS → SDS + 2-ME** but using the treatment buffer lacking 2-ME.

All gels were fixed and stained with Coomasie Brilliant Blue Stain.

Samples. Freeze-dried globulin-P (4 mg) was suspended in buffer B (200 μL) containing 2-ME at different concentrations from 0.007 to 1 M and after vortexing kept for 30 min at room temperature. Samples were prepared by mixing equal volumes of treated suspensions and sample buffers. Some SDS−PAGE samples were prepared by dissolving freeze-dried globulin-P in sample buffer with different concentrations of 2-ME. In some cases, suspensions were heated for 1 min at 100 °C.

Sulfhydryl and Disulfide Group Determination. The procedure described by Beveridge et al. (1974) using Ellman's reagent was used to measure SH groups. For total free sulfhydryl determination, protein (10 mg) was dissolved in 1 mL of 0.086 M Tris, 0.09 M Gly, 0.004 M EDTA, 8 M urea buffer (pH 8.0). After 1 h, the sample was centrifuged at 16 000 *g* and 20 °C for 20 min. The supernatant (0.6 mL) was mixed with 8 μL of Ellman's reagent (4 mg/mL of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) in methanol). After 15 min, the absorbance was measured at 412 nm and a molar extinction coefficient of 13 600 M^{-1} cm^{-1} was used. For testing the free exposed SH groups, the buffer used was the same but without urea. The protein concentration was determined by the Lowry method (Lowry et al., 1951). Bovine serum albumin dissolved in the same medium as that of the samples was used as the standard protein. At least three determinations were made for each protein fraction.

Disulfide groups were measured according to the method of Thannhauser (Thannhauser et al., 1984) by mixing the protein fraction in buffer B (10 mg/mL) with NTSB reagent (disodium 2-nitro-5-thiosulfobenzoate in 0.2 M Tris pH 9.5, 0.1 M Na_2SO_3, 10 mM EDTA, and 3 M guanidinium thiocyanate) prepared just before use. After 30 min in the dark, the absorbance was determined at 412 nm and a molar extinction coefficient of 13 600 M^{-1} cm^{-1} was used. The number of disulfide groups was calculated according to Petruccelli and Añón (1995). The protein concentration was determined according to the Biuret method (Gornall et al., 1949), using bovine albumin as the standard. At least triplicate determinations were carried out.

RESULTS

Sulfhydryl and Disulfide Content of Globulin-P. The mean free sulfhydryl group value of globulin-P in pH 8.0 Tris buffer was 3.4 ± 0.6 μmol/g, whereas the SH content of this protein, after denaturation with 8 M urea, was found to be 10.2 ± 0.5 μmol/g. This result indicates that 7 ± 1 μmol/g of SH groups are not reactive in pH 8.0 Tris buffer, so they may be protected inside the protein. Analysis of total half cystines in denatured globulin-P gave a mean value of 113 ± 1 μmol/g, which, together with the data shown above, verifies that for this protein most Cys residues are involved in disulfide bridges.

These results are equivalent to those obtained with partially purified globulin-S showing 2 ± 1 μmol/g free sulfhydryl groups in pH 8 Tris buffer, 7 ± 1 μmol/g free sulfhydryl groups in 8 M urea, and 112 ± 5 μmol/g total half cystines.

Calorimetric Analysis of Globulin-P with 2-ME. The changes of ΔH and T_d of globulin-P heated in the presence of 0−1.5 M 2-ME are shown in Figure 2a and b, respectively. It can be observed that 2-ME concentrations below 0.3 M (see arrow in the figure) do not change ΔH values ($\alpha = 0.05$) or seriously modify the T_d, which shows a slight decrease, suggesting that protein structures were hardly destabilized. On the contrary, for 2-ME concentrations above 0.3 M, a pronounced decrease in both ΔH and T_d is found (Figure 2a,b), indicating a progressive unfolding of globulin-P. A similar effect of 2-ME was observed on 11S globulins from oat (Harwalkar and Ma, 1987) and soybean (Zarins and Marshall, 1990). To assess the possibility that 2-ME, acting as a monohydric alcohol, destabilized the proteins by weakening the hydrophobic interactions, globulin-P was analyzed in the presence of different concentrations of ethanol. The corresponding ΔH and T_d values (Figure 2a,b) did not show any significant ($\alpha = 0.05$) change until the ethanol concentration was 1−1.5 M, concentrations for which thermal parameters did become smaller. As the destabilizing effect of 2-ME on globulin-P is much higher than that of ethanol, one can conclude that the ability of 2-ME to reduce disulfide bridges has a strong effect on globulin-P structure. Therefore, these results suggest that disulfide bonds contribute significantly to globulin-P structural stabilization. This is in agreement with observations by Marcone and Yada (1997) on amaranth globulin-S and with Kinsella (1982) reports on other plant proteins.

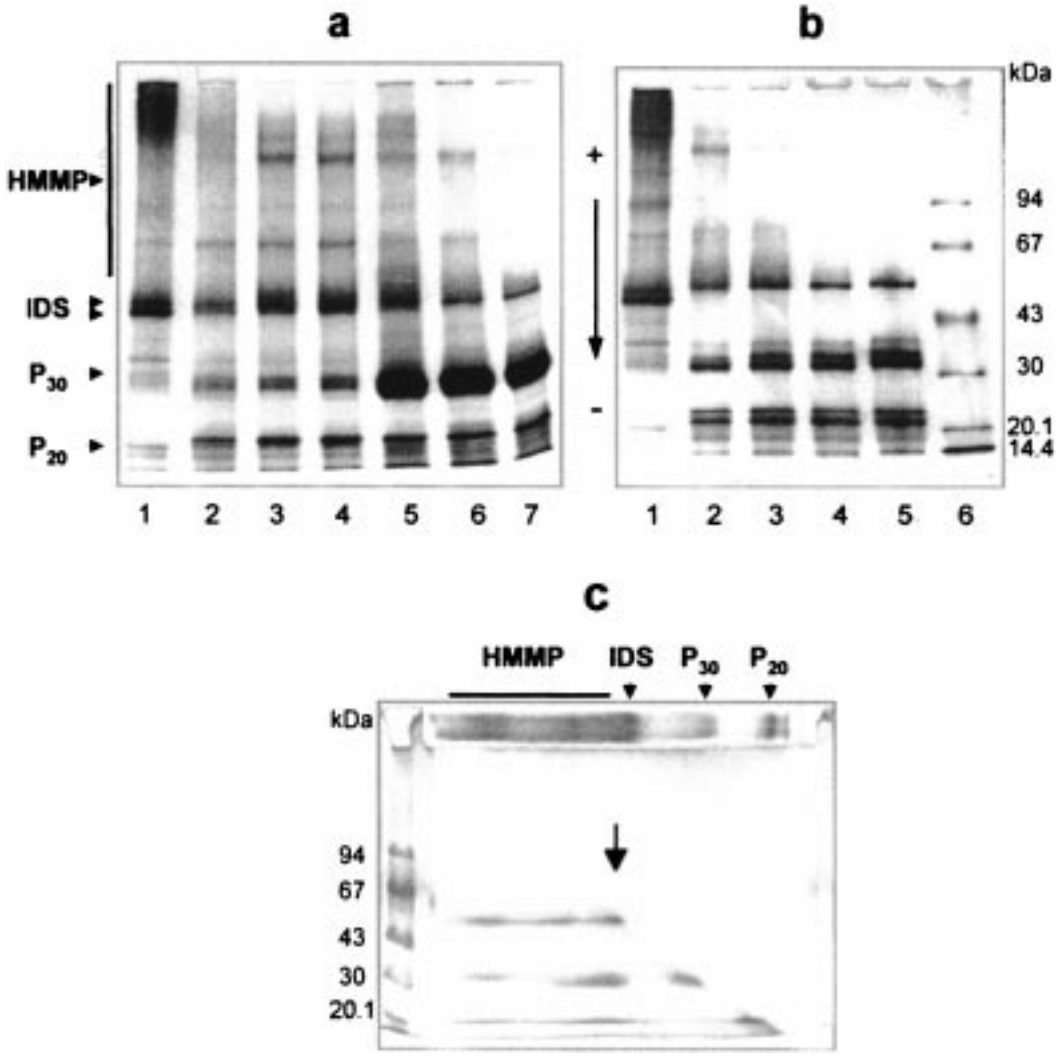

Figure 3. (a) SDS−PAGE of globulin-P in buffer B (lane 1) and in buffer B treated with 2-ME: 0.007 M (lanes 2 and 5), 0.014 M (lanes 3 and 6), and 0.043 M (lanes 4 and 7). Samples of lanes 5, 6, and 7 were treated at 100 °C for 1 min. (b) SDS−PAGE of globulin-P in sample buffer (lane 1) and in sample buffer with 2-ME: 0.014 M (lane 2), 0.072 M (lane 3), 0.143 M (lane 4), and 0.72 M (lane 5). (c) Two-dimensional SDS → SDS + 2-ME-PAGE of globulin-P in buffer B with 0.014 M 2-ME.

Electrophoretic Analysis of Globulin-P in the Presence of 2-ME. Globulin-P in buffer B was treated with 2-ME at different concentrations (0.007, 0.014, and 0.043 M) for subsequent SDS−PAGE analysis. Globulin-P sample with no 2-ME added (Figure 3a,b, lanes 1) presents high molecular mass polypeptides (HMMP in the figure) and the 52 and 56 kDa intermediate dimeric subunits (IDS in the figure) as main components. The profiles also show a low quantity of polypeptides whose molecular masses were around 30 (polypeptides P_{30}) and 20 kDa (polypeptide P_{20}). The profiles of the 2-ME-treated proteins (Figure 3a, lanes 2, 3, and 4) display an increase in the intensity of the P_{30} and P_{20} bands, suggesting that some intermediate dimeric subunits were dissociated. In addition, in the HMMP zone, the very low mobility bands are less intense while the bands that migrate faster, for example, bands of 77 and 134 kDa, are more intense, suggesting that some high molecular mass polypeptides have also been dissociated. For this test it must be borne in mind that after the 2-ME treatment, the protein solutions were diluted to one-half their original concentration with SDS-containing sample buffer. In these conditions, the proteins were unfolded and the reducing agent, although diluted, had access to disulfide bonds that were inside the protein in buffer B.

Figure 3c shows the two-dimensional SDS → SDS + 2-ME profile of globulin-P in buffer B with 0.014 M 2-ME. It can be observed that HMMP are composed of P_{30} and P_{20} polypeptides and the monomeric subunit of 54 kDa, as described in previous work for untreated globulin-P (Martínez et al., 1997). The 54 kDa monomeric subunit was not found as a free polypeptide in untreated globulin-P (Martínez et al., 1997) and now is present as a free polypeptide together with IDS in the 2-ME-treated samples. This fact is demonstrated in the two-dimensional profile (Figure 3c, arrow) where proteins from the 50−60 kDa region (IDS) of the first dimension migrate in the second dimension as three spots, one on the diagonal corresponding to the monomeric subunit and two under the diagonal corresponding to the P_{30} and P_{20} polypeptides coming from IDS dissociation.

When samples with 2-ME were heated at 100 °C for 1 min, an increase was observed in the intensity of the P_{30} and P_{20} bands of the profiles (Figure 3a, lanes 5, 6, and 7) along with a decrease in the HMMP bands, suggesting that more disulfide bonds were broken.

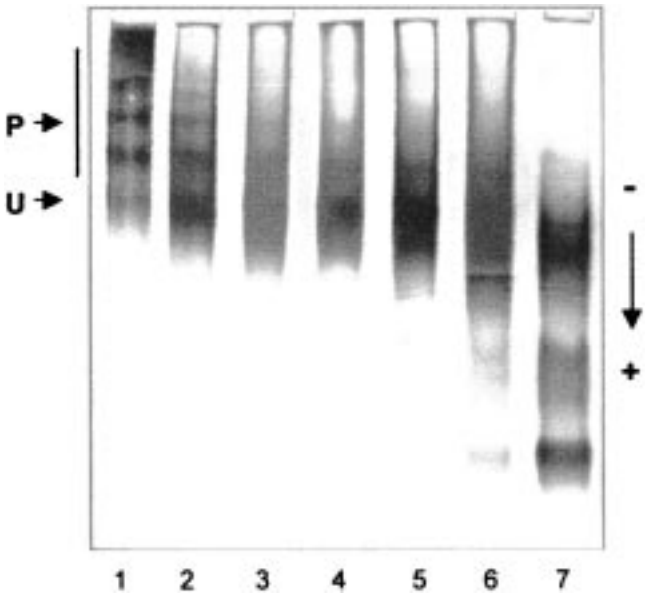

Figure 4. Native-PAGE of globulin-P in buffer B (lane 1) and in buffer B with 2-ME at the following concentrations: 0.014 M (lane 2), 0.072 M (lane 3), 0.143 M (lane 4), 0.358 M (lane 5), 0.573 M (lane 6), and 1 M (lane 7).

Treatments with 2-ME were also applied on globulin-P in sample buffer containing SDS. Results indicated that at 2-ME concentrations of 0.014 and even 0.072 M (Figure 3b, lanes 2 and 3), a few HMMP stabilized by disulfide bridges still remained unbroken and that complete cleavage is reached with 0.43 and 0.72 M 2-ME, as shown by the corresponding profiles (Figure 3b, lanes 4 and 5) in which only the P_{20}, P_{30}, and 54 ± 1 kDa polypeptides are displayed. These results suggest that one condition to attain the cleavage of all disulfide bridges of globulin-P is the presence of a denaturing agent and 2-ME at high concentration.

Globulin-P treated with 2-ME was also analyzed by native-PAGE (Figure 4). The profile without 2-ME (lane 1) shows several bands from which those of lower mobility (P bands) correspond to polymerized molecules and the one that migrates faster (U band) comprises unitary molecules of 300 kDa (Martínez et al., 1997). In the presence of less than 0.3 M of 2-ME (lanes 2, 3, and 4) polymers degrade, as evidenced by the progressive decrease in the number of P bands and the increase in the U band intensity. In the presence of 2-ME, the bands shown by the profiles display a slightly higher mobility compared with those of the control, and this may be caused by the appearance of the negative charges of the new S^- groups. From 0.35 M 2 ME on (lanes 5−7), the bands shown migrate faster than the U band, suggesting that some molecules unfold to release free polypeptides.

The profiles of the samples with 0.014, 0.57, and 1 M 2-ME were analyzed in a second dimension with SDS−PAGE (Figure 5b−d) and then compared with the 2-ME free control (Figure 5a). This latter profile shows that all native-PAGE bands display the same polypeptidic composition in the second dimension, with more intense bands belonging to the low-mobility HMMP and to the IDS (about 50−60 kDa), as already shown in the one-dimension SDS−PAGE profile (Figure 3, lane 1). In the HMMP zone of the second dimension profiles, the bands of samples treated with 0.014 and 0.57 M 2-ME (Figures 4b,c) show higher mobility compared to the control (Figure 5a); the behavior is similar to that of the SDS−PAGE profiles of globulin-P with 2-ME in buffer B (Figure 3a).

In addition, in the second dimension profile of globulin-P with 0.57 M 2-ME (Figure 5c), the P_{30} and P_{20} bands are more intense, suggesting that some disulfide bonds linking those polypeptides have been cleaved in this condition but also that the polypeptides are still associated by secondary forces. The profiles with 0.57 and 1 M 2-ME demonstrate that the bands moving faster in native-PAGE are polypeptides whose molecular masses are about 30 kDa (P_{30}). These bands have different mobility in native-PAGE but their similar molecular masses indicate that they differ in charge, similar to the acidic polypeptides of 11S globulins (Derbyshire et al., 1976; Utsumi and Mori, 1980; Utsumi et al., 1981). Spots of the corresponding free P_{20} polypeptides were not present in these two-dimension profiles. It might be speculated that the behavior of free P_{20} polypeptides is similar to that of the basic polypeptides of soybean glycinin, which are highly hydrophobic polypeptides that aggregate easily (German et al., 1982; Damodaran and Kinsella, 1982). Likewise, P_{20} amaranth polypeptides may have formed large aggregates which, owing to its size, cannot penetrate the native gel.

The slower native band of globulin-P treated with 1 M 2-ME (Figure 5d) shows polypeptides of 50−60 kDa and the two 77 and 134 kDa HMMP bands as main components in the second dimension. As calorimetric results showed that a great degree of unfolding has taken place in this condition, we cannot ensure whether these polypeptides have formed some kind of open structure.

Gel Filtration Analysis of Globulin-P with 2-ME. The profile of globulin-P analyzed by gel filtration (Figures 6 and 7, profile C) shows, as already reported (Martínez et al., 1997), the main components I, II, and III corresponding to polymers (3000 kDa), dimers (650 kDa), and unitary molecules (300 kDa), as well as a minor component IV possibly composed of dissociated polypeptides. This profile also shows that high molecular mass polymers are in a larger amount than the other components. By contrast, in globulin-P treated with 0.020 M 2-ME (Figure 6, profile A), components II and III predominate and there is a lower amount of large polymers, indicating, in accordance with electrophoretic results, that some polymers have been disrupted. When 2-ME is separated from the medium (elution buffer without 2-ME, Figure 6, profile B), the increase in components I and II shown in the profiles suggests the partial reformation of polymers.

In the profile of globulin-P treated with 0.57 M 2-ME (Figure 7, profile A), polymers and unitary molecules are almost absent, with the species of molecular masses lower than 55 kDa as the main components. This result, as well as those from electrophoresis, indicate quaternary structure disruption at 0.57 M 2-ME. The amount of polypeptides released is larger in the chromatographic profile than it is in the native-PAGE pattern, which shows a higher amount of unitary molecules. This difference may be ascribed to methodological reasons as in the electrophoresis the reducing agent may be diluted during the run.

The gel filtration profile A (Figure 7) also shows a UV absorbance peak in the void volume region that should not be assigned to larger polymers because the smaller polymers, i.e., those retained by the column, are absent. Considering the possibility mentioned above that re-

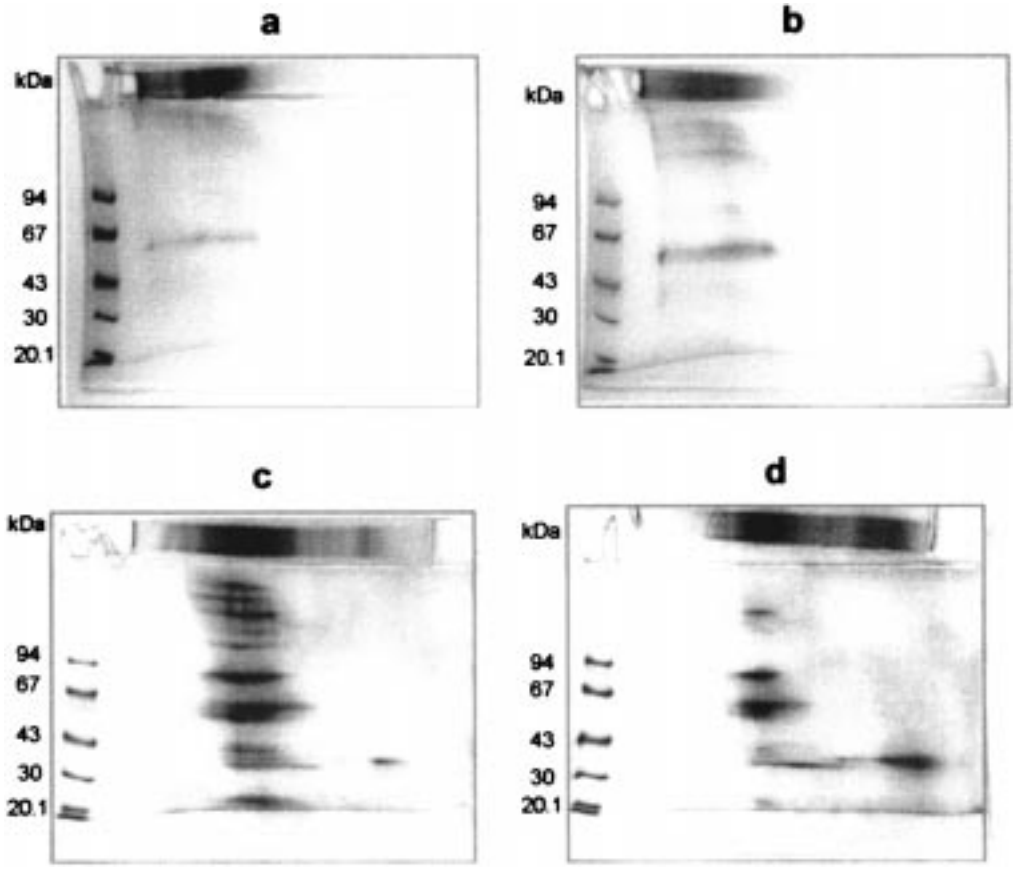

Figure 5. Two-dimensional native → SDS−PAGE of globulin-P in buffer B (a), and globulin-P in buffer B with 2-ME: 0.014 M (b), 0.573 M (c) and 1 M (d).

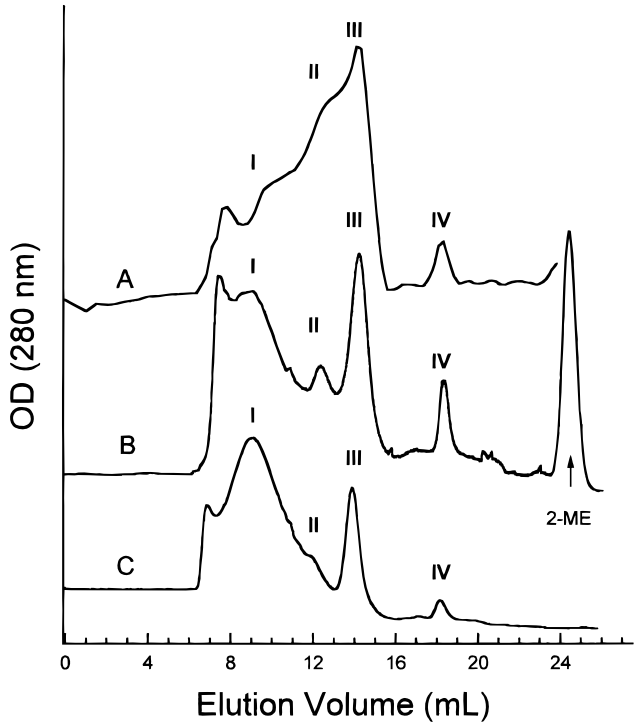

Figure 6. Gel filtration FPLC of globulin-P in buffer B + 0.020 M 2-ME eluted with buffer B + 0.020 M 2-ME (A), globulin-P in buffer B with 0.020 M 2-ME eluted with buffer B (B), and globulin-P in buffer B eluted with buffer B (C). Elution peak of 2-ME is marked in (B).

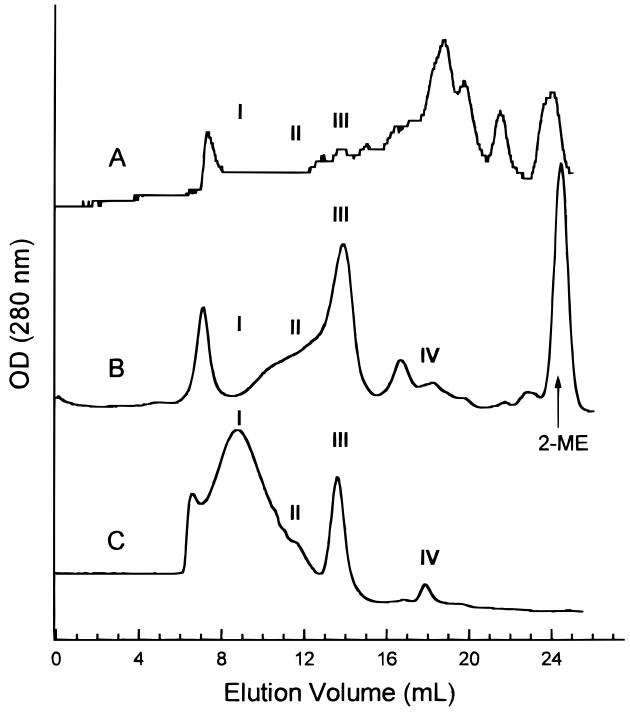

Figure 7. Gel filtration FPLC of globulin-P in buffer B + 0.573 M 2-ME eluted with buffer B + 0.57 M 2-ME (A), globulin-P in buffer B + 0.573 M 2-ME eluted with buffer B (B), and globulin-P in buffer B eluted with buffer B(C).

leased P_{20} polypeptides are in the form of aggregates, the peak may be ascribed to them.

The presence of a major component III and absorbance in the component II region when elution was carried out without the reducing agent (Figure 7, profile B) indicate that unitary molecules and some polymers

or aggregates have been reconstituted after removal of 2-ME. This profile also shows the presence of low molecular mass components, suggesting that not all the polypeptides released by the action of 0.57 M 2-ME had reassociated. These results are in agreement with those of Marcone and Yada (1997) who reported the partial reformation of intermediary subunits of dissociated amaranth globulin-S after removing the reducing agent.

DISCUSSION

According to our results, most Cys residues form disulfide bonds (103 ± 1 μmol/g). The low amount of free sulfhydryl groups found in globulin-P, a characteristic of many proteins (Thornton, 1981), is in agreement with data for salt-soluble amaranth globulin determined in this laboratory (9.6 ± 1 μmol/g) and determined by Marcone and Yada (1997) (9.6 μmol/g). The majority of free sulfhydryls of globulin-P are inside the protein molecule, as in the case of globulin-S data measured in this work, and similar results were found again by Marcone and Yada (1997).

The globulin-P disulfide content (51 ± 1 μmol/g) coincides with the content of partially purified globulin-S found in this work (53 ± 5 μmol/g) and is rather high compared to that reported by Marcone and Yada (1997) for purified globulin-S (33 μmol/g). As the data compared above was arrived at by different methods, these differences may be ascribed to the distinct methods used. Nonetheless, the discrepancies correlate with the differences between globulin-S structure reported by Marcone and Yada (1992) and those of globulin-P and globulin-S described in this laboratory (Martínez et al., 1997). In the first mentioned structure, monomeric subunits are more abundant than a disulfide-linked dimeric subunit whereas the other two structures are composed mainly by disulfide-linked dimeric subunits. Globulin-P disulfide content is also in the same range of glycinin disulfide content, i.e., 57.8−67 μmol/g, values calculated considering a molecular weight of 320000 and the data 37−42.9 mol/mol, as reported in the literature by Draper and Catsimpoolas (1978), Hoshi and Yamauchi (1983), and Wolf (1993). In agreement with this, the reported glycinin structure is similar to those proposed for globulin-P and globulin-S (Martínez et al., 1997; Chen and Paredes-López, 1997).

Disulfide bridges linking globulin-P polymers are some of the more exposed disulfides and are cleaved by 2-ME in concentrations below 0.3 M. These and other exposed disulfides may not take part in the structural stabilization of the molecules, as indicated by the small change in thermal stability (ΔH and T_d values) produced by less than 0.3 M 2-ME. A similar behavior was described for soybean glycinin (Wolf, 1993) whose 11S dimers are split by 0.01−0.1 M 2-ME without distorting their quaternary structure. The sulfhydryls yielded by the reduction of SS-linked polymers are highly reactive, as shown by their great propensity to reconstitute the polymers when the reducing agent is separated by gel filtration. This behavior also suggests that polymerized globulin-P is the thermodynamically more stable state in the experimental conditions used. This characteristic of globulin-P makes it different from globulin-S and other 11S globulins which, having a similar content of disulfides and similar type of subunits, present a lower amount of polymers (Martínez et al., 1997; Wolf and Nelsen, 1996). It can be speculated that, whereas some Cys residues of globulin-S could form either internal (intramolecular) disulfide bridges or disulfides bonds linking the polymers, the corresponding globulin-P Cys residues only have the chance to form disulfide bridges linking the polymers. This could happen provided the globulin-P conformation is different from those of globulin-S or other 11S globulin. The existence of conformational differences between globulin-P and globulin-S is suggested by their distinct calorimetric behavior (Martínez and Añón, 1996; Martínez et al., 1997; Gorinstein et al., 1996). Conformational differences may be caused by slight differences in composition, a subject currently being investigated.

The less exposed disulfide bridges that contribute to the structural stability of globulin-P begin to be reduced by 2-ME at concentrations above 0.3 M. Among these bonds are those linking P_{30} and P_{20} polypeptides to form the dimeric subunits. Possibly some $P_{30}-P_{20}$ linkages are broken at lower 2-ME concentrations (as shown by SDS−PAGE profiles), but we cannot discard that some SDS−PAGE results are produced by disulfide interchanges occurring when molecules are unfolded, in the presence of sample buffer with SDS. At 0.57 M 2-ME, when $P_{30}-P_{20}$ linkages and perhaps some intramolecular disulfides are cleaved, some important conformational changes occur, causing free polypeptides to dissociate out of the molecule. As it is known (Thornton, 1981), many proteins having disulfide bridges completely unfold when the disulfides are cleaved. Likewise, amaranth globulin-S quaternary structure becomes disrupted upon exhaustive SS bond cleavage (Marcone and Yada, 1997). It was also shown that the cleavage of SS-linking AB soybean glycinin intermediary subunits destroys the molecular quaternary structure. If, furthermore, glycinin intramolecular (intra-polypeptide) disulfide bridges are cleaved, the tertiary structure also results disrupted (Kim and Kinsella, 1986). By using calorimetric analysis, we found that this is the case (complete unfolding) for globulin-P with 1.4 M 2-ME. Nevertheless, at a 2-ME concentration as high as 0.57 M, in which disruption of the quaternary structure and a considerable degree of unfolding take place, the separation of the reducing agent by gel filtration leads to the reformation of some unitary molecules and polymers. These results suggest that, as demonstrated for soybean and broad bean 11S globulins (Utsumi et al., 1980), the hexameric quaternary structure of globulin-P molecules is the most favorable thermodynamically in which disulfide bridges would contribute to strengthen the molecular stability. According to these results, the destabilizing effect of 2-ME may be mainly ascribed to its reducing action. However, the changes that 2-ME causes in the dielectric constant of the medium cannot be discarded as an additional destabilizing factor.

CONCLUSION

Globulin-P SH as well as SS group contents are similar to those of partially purified amaranth globulin-S. Most globulin-P and globulin-S SH groups are found deeply inside the molecule. The value of SS groups is in the same range as that of soybean glycinin (Wolf, 1993) and is higher than that reported by Marcone and Yada (1997) for purified globulin-S. The difference may be explained by differences in the reported structures of globulin-S preparations, which, in turn, may be ascribed to dissimilar cultivars from which the seeds were extracted. Some of the globulin-P disulfide bridges

are more exposed, its main function is to stabilize polymers, whereas others, buried in the molecule, join P_{20} and P_{30} polypeptides to form dimeric subunits or establish intrachain linkages. These two last bonds participate in the structural stabilization of the molecules.

Globulin-P results described in previous works by Martínez et al. (1997) and Castellani et al. (1998) and those described in this paper give sufficient support to consider globulin-P as an 11S type globulin. Nevertheless, its higher propensity to polymerize and its lower solubility in saline solutions make globulin-P different from other 11S globulins. The conformational differences showing globulin-P as a peculiar globulin may be due to differences in composition. This subject is currently being investigated.

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