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Characterization of Amaranth Globulins by Ultracentrifugation and Chromatographic Techniques

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Globulins were extracted from *Amaranthus hypochondriacus* and studied by ultracentrifugation and chromatographic techniques. Ultracentrifugation of freeze-dried globulins gave sedimentation constants of 10 S and 12.7 S. Gel filtration chromatography of globulins gave a molecular weight of 166 000. This protein was fractionated and characterized by ultracentrifugation, electrophoresis, and ion-exchange and affinity chromatography. The major globulin fraction appeared to be 10 S, which showed association/dissociation phenomena. It also exhibited an electrophoretic behavior similar to that of 7 S, whereas 12.7 S presented some characteristics in common with 11 S proteins (e.g., patterns unchanged and changed by a reducing agent, respectively). Affinity chromatography was the best method for 10 S purification.

INTRODUCTION

There are two major classes of legume storage proteins termed 7 S and 11 S after their sedimentation coefficients. Each class of protein is preserved across species, and even certain nonlegume species possess such proteins (e.g., oats; Peterson, 1978). The 7 S is a trimeric glycoprotein (170–141 kDa) composed of six different combinations of three subunits, α (57 kDa), α' (58 kDa), and β (42 kDa) associated via hydrophobic interactions (Utsumi and Kinsella, 1985). The 11 S consists of two opposed hexagonal rings each containing three hydrophobically associated pairs of disulfide-linked acidic (37–35 kDa) and basic (20–18 kDa) subunits (Peng et al., 1984).

It has been reported that globulins are one of the main protein fractions from amaranth proteins (Paredes-López et al., 1988; Mora-Escobedo et al., 1990), but little is known about the properties of this fraction. In an early work, Konishi et al. (1985) suggested that amaranth globulins could be an oligomeric protein which could dissociate its monomers at alkaline pH levels and reported a sedimentation constant of 12.7 S. Recent studies suggest that amaranth globulins could be composed of 7 S- and 11 S-like fractions (Barba de la Rosa et al., 1992).

The 7 S- and 11 S-rich fractions have been prepared and purified from pea and soybean proteins by different chromatographic techniques: gel filtration, ion-exchange (Gueguen et al., 1984; Iwabuchi et al., 1991), and affinity chromatography (Kitamura et al., 1974).

In this paper the composition of the globulin fraction of amaranth proteins was investigated in detail by ultracentrifugation and different chromatographic techniques to establish the nature of its constituents.

MATERIALS AND METHODS

Amaranth Samples. Mature seeds of *Amaranthus hypochondriacus* (Mercado cultivar, waxy type) were harvested at the Experimental Station of the Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), Chapingo, Mexico.

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Flour was obtained by grinding whole seeds in a ball mill (Prolabo) and attaining an average particle size of about 100 μm.

Protein Isolation. A slurry of amaranth flour in 0.1 M Na_2HPO_4 buffer, pH 7 (1 g of flour/10 mL), was stirred for 1 h at room temperature to extract the crude protein. After extraction, the mixture was centrifuged at 9000*g* for 20 min and the supernatant termed "total extract" was utilized for purification of globulins. Total extract was dialyzed for 5 days at 4 °C against distilled water and centrifuged, and the precipitated fraction termed "crude globulin" was then freeze-dried.

Ultracentrifugation. Ultracentrifugation was carried out in a Beckman 15-65B ultracentrifuge using a SW 40 Ti rotor. A 0.5-mL protein sample (100 mg of freeze-dried globulin was partially solubilized in 5 mL of Na_2HPO_4 buffer and centrifuged) was layered on the top of an isokinetic sucrose gradient (5–20% w/v) established in the Na_2HPO_4 extraction buffer and centrifuged for 21 h at 218000*g*. Protein separation was detected by a UV detector (Pharmacia) with a 280-nm filter. Standards with known sedimentation constants were used for calibration: lysozyme (1.9 S), bovine serum albumin (4.4 S), γ-globulin (7.0 S), and catalase (11.2 S) (Gueguen and Barbot, 1988).

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the method of Laemmli (1970). Protein samples (1 mg of protein/mL) were dissolved in 0.1 M Tris-HCl, pH 6.8, and 2% SDS. Reduction of disulfide bridges was performed by 2-mercaptoethanol (2-ME) (5% v/v) at 100 °C for 2 min. Electrophoresis was conducted at a constant current of 20 mA per gel for 2–3 h. After electrophoresis, the gel was fixed with trichloroacetic acid (12.5% w/v) for 30 min and stained overnight by addition of Coomassie Brilliant Blue G250 in a final concentration of 0.25%. Destaining was achieved by washing the gel during 2 h with ethanol/water/acetic acid (4.5/4.5/1 by vol) and then overnight with a solution of acetic acid (5% v/v). The molecular weights of protein subunits were calculated using the following standard proteins: phosphorylase B (94 000), bovine serum albumin (67 000), ovalbumin (43 000), trypsin inhibitor (20 100), and lactalbumin (14 400) kDa).

Gel Filtration Chromatography. A 7-mL sample of the total extract (7 mg of protein/mL) was applied to a Sephacryl S-300 (Pharmacia) (2.5 × 83 cm) packed column and eluted with 0.1 M Na_2HPO_4 buffer, pH 7, at 42 mL/h. Fractions of 4.2 mL were collected. The elution profile was recorded by following the absorbance at 280 nm (UV–vis scanning spectrophotometer, Phillips Pye Unicam, Ltd). The following standard proteins were used for calibration: thyroglobulin (669 kDa), aldolase (158 kDa), and albumin (67 kDa). The globulin peak was concentrated in an ultrafiltration cell using a PM-10 membrane (Amicon). After

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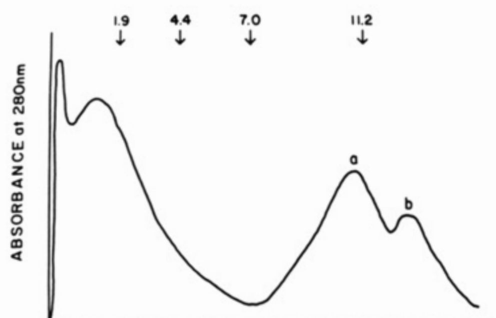

Figure 1. Sucrose density gradient ultracentrifugation of freeze-dried amaranth globulins. Peak a, 10 S fraction; peak b, 12.7 S fraction (arrows show standard values).

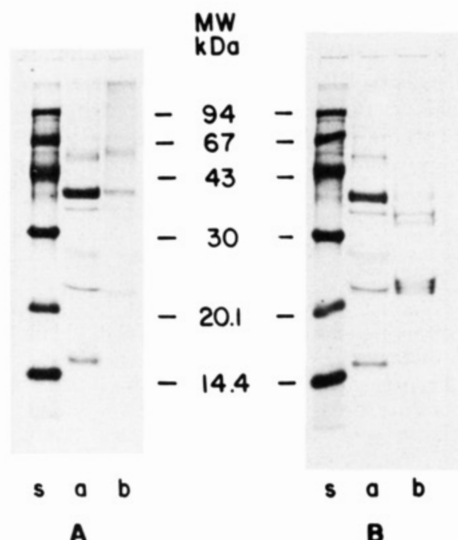

Figure 2. Electrophoretic patterns of protein samples collected under peaks a and b of Figure 1. A, absence of 2-mercaptoethanol (ME); B, presence of 2-ME. s, standard reference proteins.

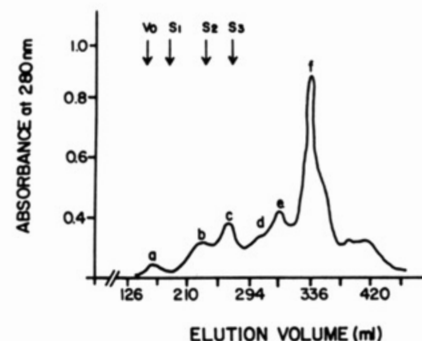

Figure 3. Gel filtration pattern of the total extract sample from amaranth flour. Vo, void volume; S_1, thyroglobulin (669); S_2, aldolase (158); S_3, albumin (67 kDa).

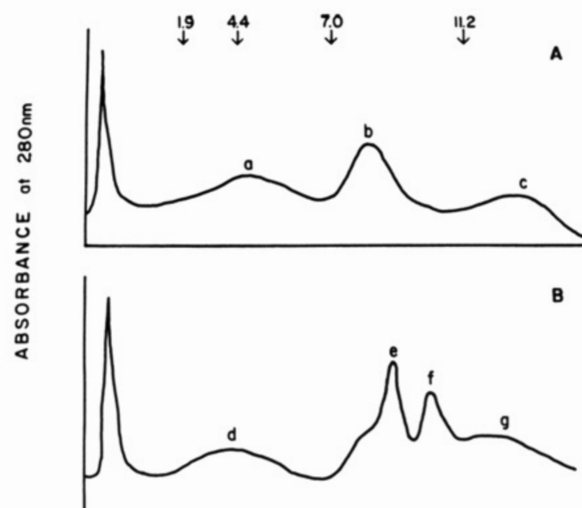

Figure 4. Ultracentrifugation profile of a protein sample collected under peak b of Figure 3. A, without concentration; B, with concentration (arrows show standard values).

concentration, the fraction was used for ion-exchange chromatography and for ultracentrifugation studies.

Ion-Exchange Chromatography. A DEAE-Sepharose CL-6B (Pharmacia) column was equilibrated with 50 mM Tris-HCl buffer, pH 8.5, and 10 mL of total extract (7 mg of protein/mL) was applied. The salt gradient (0–0.5 M NaCl) was eluted during 12 h at a flow rate of 1 mL/min. Fractions of 1 mL were collected.

Affinity Chromatography. A concanavalin A–Sepharose CNBr (Pharmacia) column (1.5 × 12 cm) was washed with 2.6 mM KH_2PO_4 + 32.5 mM K_2HPO_4 + 0.4 M NaCl + 0.02% sodium azide + 10 mM 2-ME at pH 7.6. The protein sample (10 mg of protein/mL) was then added to the column and eluted with the buffer at a flow rate of 5 mL/h at room temperature. Adsorbed protein was eluted with the same buffer containing 0.25 M methyl α-D-mannoside. Fractions of 1 mL were collected (Kitamura et al., 1974).

RESULTS

Ultracentrifugation of the freeze-dried globulin fraction showed sedimentation constants around 1.2, 10, and 12.7 S (Figure 1). Peaks a and b correspond to 10 and 12.7 S, respectively. The low molecular weight proteins at 1.2 S might correspond to albumin contamination (Konishi et al., 1985; Gorinstein et al., 1991). Without reduction electrophoresis of the 10 S fraction gave six main bands at 52, 37, 34, 28, and 23 kDa and a doublet around 15–16 kDa (Figure 2Aa). As well, most of these bands appeared in the 12.7 S fraction but in different concentrations (Figure 2Ab). This similarity was interpreted to be a result of the relatively poor resolution between these two peaks; additionally, the 10- and 12.7-S peaks showed some polypeptides at the top of the gel. In nonreductive conditions Mori and Utsumi (1979) reported that the 11 S globulin fraction could lead to the formation of different intermediary subunits of high molecular weight. The fact that the electrophoretic pattern of the main bands of 10 S components was not modified in reductive conditions (Figure 2Ba) led to the supposition that this 10 S fraction, apparently without disulfide links subject to reduction, might correspond to a 7 S-type oligomeric protein (Utsumi and Kinsella, 1985). On the other hand, upon reduction of the 12.7 S globulin new bands appeared at 33–32 and 24–23 kDa (Figure 2Bb). The molecular weights of the constituent polypeptides were indeed very close to those of some 7 S- and 11 S-type proteins (e.g., pea vicilin, soybean glycinin; Kitamura et al., 1976; Peng et al., 1984; Gueguen et al., 1988).

The higher sedimentation constant values might result from aggregation during the preparation procedure. For this reason, characterization of these proteins by gel filtration, from a fresh total extract, was performed directly on the eluate without dialyzing or freeze-drying.

Figure 3 illustrates gel filtration elution patterns of total extract. Previous electrophoretic patterns (data not shown) had shown that the peak with a molecular weight around 166 000 (Figure 3b) corresponded to the globulin fraction. Ultracentrifugation profiles of this peak, before and after a 2-fold concentration step, are shown in Figure 4. It clearly appears that the protein concentration induced the aggregation of some components. Before concentration, peaks sedimenting around 3.8, 7.2, and 11.9 S were obtained (Figure 4, peaks Aa–c, respectively),

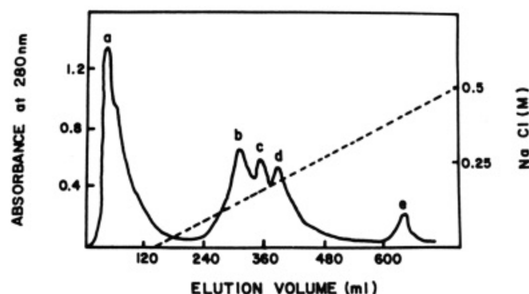

Figure 5. Fractionation for 12 h with a DEAE-Sepharose Cl-6B column of total extract sample from amaranth flour. (—) absorbance at 280 nm; (- - -) NaCl concentration.

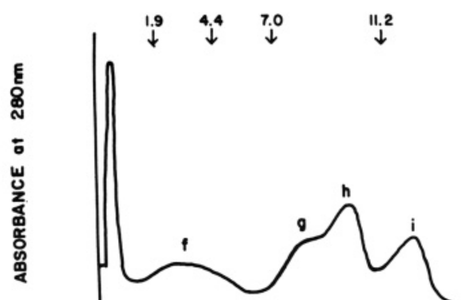

Figure 6. Ultracentrifugation profile of a protein sample collected under peak d of Figure 5 (arrows show standard values).

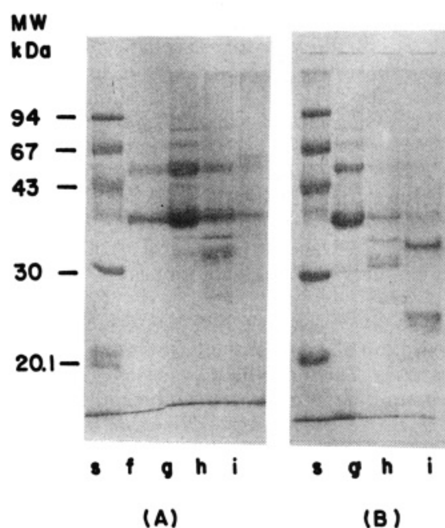

Figure 7. Electrophoretic patterns of protein samples collected under peaks g–i of Figure 6. A, absence of 2-mercaptoethanol (ME); B, presence of 2-ME. s, standard reference proteins.

whereas peaks at 7.9, 9.4, and 10.9 S were observed after concentration (Figure 4, peaks Be–g, respectively).

To improve the separation of the globulin fraction from the total extract, ion-exchange chromatography was used and samples were eluted for 12 h. The profile of this extract showed a nonadsorbed peak plus four adsorbed peaks (Figure 5). Fractions adsorbed were eluted at 0.13, 0.17, 0.20, and 0.41 M NaCl (peaks b–e, respectively). By electrophoresis of these samples (data not shown), it was learned that the fraction at 0.20 M corresponded to the globulin fraction. After concentration of this peak, ultracentrifugation showed four peaks with sedimentation constants of 2.8, 7.4, 9.7, and 12.4 S (Figure 6, peaks f–i, respectively). By electrophoresis under nonreductive conditions (Figure 7A) peaks f and g gave the same main bands at 72, 67, 52, and 38 kDa; peak g showed some additional bands; peak h was composed of major bands at 52, 38, 34, 32, and 25 kDa; and peak i showed bands at 58 and 38 kDa plus faint bands at 52, 28, and 23 kDa. After

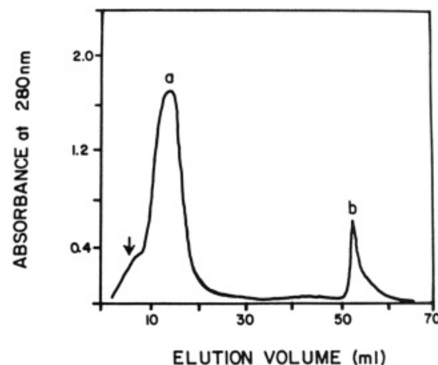

Figure 8. Affinity chromatography profile of a protein sample collected under peak b of Figure 3.

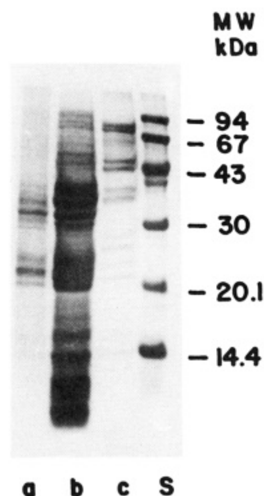

Figure 9. Electrophoretic patterns at reductive conditions of protein samples collected under the shoulder and peaks a and b of Figure 8. a, sample under the shoulder; b, sample under peak a; c, sample under peak b. s, standard reference proteins.

reduction with 2-ME (Figure 7B), there were practically no changes on peaks f (pattern not shown)–h, whereas peak i underwent more noticeable changes. This peak showed major bands at 33 and 24 kDa.

Affinity chromatography of peak b from total extract fractionated by Sephacryl S-300 in Figure 3 gave a shoulder (see arrow) on the full peak a, which was not adsorbed, and one adsorbed peak (Figure 8b). Electrophoresis in reductive conditions of the sample under the shoulder showed a region with various bands between 38 and 19 kDa (Figure 9a); peak a from Figure 8 showed the same bands at higher concentration plus some others of higher and lower molecular weights (Figure 9b). The adsorbed peak b from Figure 8 exhibited six doublets of bands at 70–74, 50–46, 37–35, 28–26, 23–21, and 15–14 kDa (Figure 9c). The ultracentrifugation profile of fractionated samples from Figure 8 gave sedimentation constants of 1.5 S for the shoulder (Figure 10 Aa), 8.3 S, and 12.3 S for nonadsorbed peak a (Figure 10Bb,c, respectively) and of 9.2 S for the adsorbed peak b (Figure 10Cd).

DISCUSSION

Fractions with sedimentation constants of 10 and 12.7 S from freeze-dried amaranth globulins might correspond, as judged by electrophoretic patterns, to 7 S and 11 S globulin fractions, respectively, reported for other materials (Peterson, 1978; Peng et al., 1984; Nielsen et al., 1988; Weller, 1989). After reduction with 2-ME, the electrophoretic pattern of the 10 S fraction did not change, which is classical behavior in 7 S proteins due to the lack of

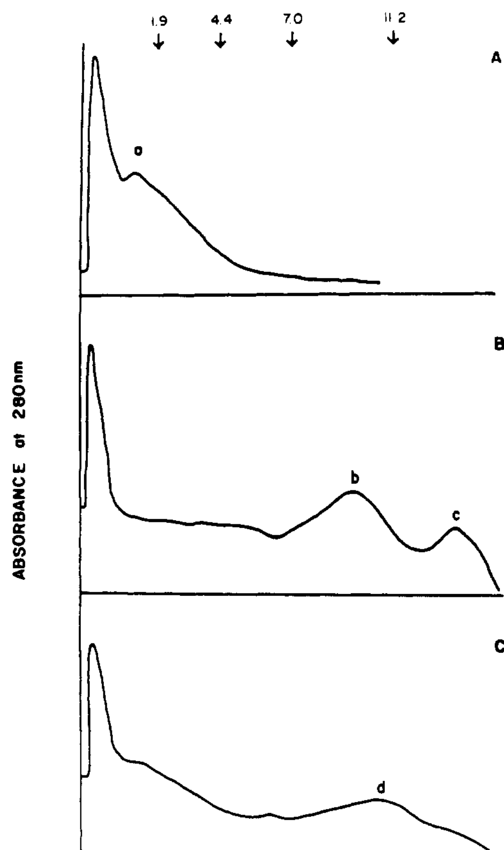

Figure 10. Ultracentrifugation profile of protein samples collected under the shoulder and peaks of Figure 8. A, sample under the shoulder; B, sample under peak a; C, sample under peak b (arrows show standard values).

disulfide bridges. On the other hand, upon reduction the 12.7 S globulin fraction changed dramatically, exhibiting components of similar molecular weight to the reported 33–32-kDa acidic and 24–23-kDa basic subunits of 11 S fractions isolated from other materials (Robert et al., 1983; Gueguen et al., 1988).

Gel filtration was not useful for the purification of 10 S and 12.7 S globulins. Kitamura et al. (1974) have found that 7 S globulin was capable of 7 S → 9 S aggregation and eluted in an earlier position than that of 11 S globulin. Shouldering of the globulin elution peak on Sephacryl S-300 was considered by Utsumi et al. (1984) to be due to heterogeneity of 7 S β-polymer by formation of soluble complexes of 7 S subunits and basic subunits of the 11 S fraction. These complexes depend largely on the electrostatic atmosphere surrounding protein molecules; formation of complexes occurs more randomly and larger complexes are formed under systems containing NaCl (Kitamura et al., 1974; Utsumi et al., 1984; Utsumi and Kinsella, 1985; Iwabuchi et al., 1991). The failure of the ion-exchange technique to separate the globulin fraction might be related to the likely association of 7 S- and 11 S-like subunits.

In conclusion, amaranth globulins were composed of two fractions, 10 S and 12.7 S, 10 S being the larger fraction. This 10 S protein showed great association/dissociation phenomena and gave sedimentation constant values of 2.8, 7.4, and 9.7 S. It exhibited in electrophoretic studies major bands at 72, 67, 52, 38, 34, 32, and 25 kDa. The best method for purification of this 7 S-like protein appeared to be affinity chromatography. The acidic subunits of the 12.7 S fraction could be associated with 10 S subunits, as was suggested by the experiments with ion-exchange chromatography. However, further studies are needed on 10 S and 12.7 S amaranth globulins to determine their biochemical and physicochemical properties.

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