

ISOLATION AND CHARACTERIZATION OF AMARANTIN, THE 11S AMARANTH SEED GLOBULIN

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ABSTRACT

*The 11S globulin of seed storage protein of *Amaranthus hypochondriacus*, termed amarantin, was isolated. This fraction was evaluated for its purity by chromatographic techniques and ultracentrifugation. The 11S globulin was analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) without and with prior reduction of disulfide bonds, and by nondenaturing-PAGE. It exhibited an electrophoretic behavior similar to that of 11S-like proteins in other materials. Its apparent relative molecular weight was estimated to be 389 kDa by gel filtration chromatography at low ionic strength. Ultracentrifugation of the freeze-dried extract gave a sedimentation coefficient of 11S.*

INTRODUCTION

Two of the major classes of storage proteins in legume and some nonlegume seeds are termed 7S and 11S based on their sedimentation coefficients. Legumins, or 11S globulins, are hexamers with molecular weights of 300 to 400 kDa, consisting of two opposed hexagonal rings, each containing three hydrophobically associated pairs of disulfide-linked acidic (29–35 kDa) and basic (18–28 kDa) subunits (Peng *et al.* 1984; Ko *et al.* 1993). Vicilins, or 7S globulins, are glycoproteins with molecular weights of 150–200 kDa composed of six different combinations of three subunits: α (57 kDa), α' (58 kDa), and β (42 kDa), associated via noncovalent interactions (Utsumi and Kinsella 1985; Ko *et al.* 1993). The

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widespread occurrence of 11S and 7S type storage globulins in angiosperm seeds has been recognized and accepted (Wolf 1980).

Amaranth seed, due to its high protein content, is now the subject of many investigations as a potential food source. The storage globulins make up approximately 20% of total seed protein and have been the focus of most studies for physical characterization (Segura-Nieto *et al.* 1994). Amaranth proteins appear to be made of subunits, which may be disrupted under a variety of conditions (Pernollette and Mossé 1983; Gorinstein *et al.* 1991; Barba de la Rosa *et al.* 1992b). Konishi *et al.* (1985) suggested that amaranth globulins could be an oligomeric protein which dissociates into monomers at alkaline pH. This oligomeric protein has a sedimentation constant of 12.7S. Barba de la Rosa *et al.* (1992b) showed that amaranth globulins are composed of 11S-like (12.7S) and 7S-like (8S) fractions.

Sodium chloride, sodium phosphate and ammonium bicarbonate solutions, along with temperature treatments and isoelectric precipitations have been used to extract the 11S globulin-rich fraction, medicagin from alfalfa seeds (Stuart *et al.* 1988; Koleva *et al.* 1992; Lai *et al.* 1992), and legumin from common bean and green peas (Deshpande and Damodaran 1989). Other 11S globulins isolated by these methods include broad bean legumin (Pavlovskaya *et al.* 1992), quinoa chenopodin (Brinegar and Goundan 1993), and soybean glycinin (Saio and Watanabe 1973; Wolf 1980; Brooks and Morr 1985; Fukushima 1991ab; Sessa 1992).

Methods mentioned in the publications above have not been used for amaranth 11S globulin. In this work, a simple procedure is described for the extraction of the 11S globulin fraction from amaranth seeds, termed amarantin. The composition of the 11S globulin fraction was investigated by electrophoresis, gel filtration chromatography and ultracentrifugation to establish the nature of its constituents.

MATERIALS AND METHODS

Amaranth Samples

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

Flour was obtained by grinding whole seeds in an analytical mill (Tekmar, West Germany) and sieving through an 80-mesh screen. The flour was defatted with hexane by stirring a 10% (w/v) suspension 24 h at 4°C. The extracted flour was then air-dried at room temperature. Whole and defatted flours were stored at 4°C until used.

Protein Isolation and Quantitation

Total globulins were isolated by the technique described by Mora-Escobedo *et al.* (1990). Defatted flours (10 g dry basis) were first extracted by mild shaking with 100 ml of 0.5 M NaCl. The suspension was centrifuged and decanted. Then, a second extraction was carried out with 50 ml of deionized water. The two supernatants were combined and the residue was discarded. The extracted samples were dialyzed (mol. wt. cut-off at 6–8 kDa, Spectrapor Spectrum, Medical Industries, Inc., Los Angeles, CA) at 4C against deionized water until a constant conductivity was reached. The contents of the dialysis tubes were centrifuged at $10,000 \times g$ for 30 min. The supernatant was discarded and pellet (total globulin fraction) was freeze-dried.

To isolate the amarantin, we used a modification of the procedure reported by Saio and Watanabe (1973). Defatted flour was stirred for 2–3 h at room temperature in 10 mM calcium chloride (CaCl_2 ; 1:10, w/v). The suspension was centrifuged at $11,000 \times g$ for 20 min and the supernatant was discarded. The residue, resuspended with ten times its weight of warm water (40C), was adjusted to pH between 8.0–8.5, stirred for 2 h and then centrifuged at $11,000 \times g$ for 20 min. This residue was discarded and the supernatant was adjusted to pH 7.0 and stored at 4C for 5 h. This mixture was centrifuged at $1800 \times g$ for 20 min at 4C. The resulting pellet was resuspended with water (1:10, w/v), stirred for 30 min at 4C and again centrifuged at $9000 \times g$ for 10 min at 4C. This protein pellet was freeze-dried (Fig. 1).

Protein content ($N \times 5.85$) was determined according to the AOAC procedure number 14.026 (1984).

Denaturing Polyacrylamide Gel Electrophoresis

All gels were run using minislabs (Hoefer Scientific Instruments SE200, San Francisco, CA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using a 15% acrylamide separating gel and a 2.5% acrylamide stacking gel. Acrylamide monomer to bisacrylamide ratio was 37.5:1. Freeze-dried amarantin samples were dissolved in 0.1 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol and 0.01 mg/ml bromophenol blue. Reduction of disulfide bonds was performed with 2-mercaptoethanol (ME; 5%, v/v) by heating at 95C for 2 min. Electrophoresis was conducted at a constant current of 20 mA per gel (0.75 mm thickness) for 2–3 h.

After electrophoresis, the proteins on the gel were fixed with trichloroacetic acid (12.5%, w/v) for 30 min and then stained overnight by addition of Coomassie Brilliant Blue G250 to a final concentration of 0.25%. Destaining was achieved by washing the gel for 2 h with methanol/acetic acid/water (1:4.5:4.5, v/v/v) and followed by an overnight rinse with a solution of acetic acid (5%, v/v).

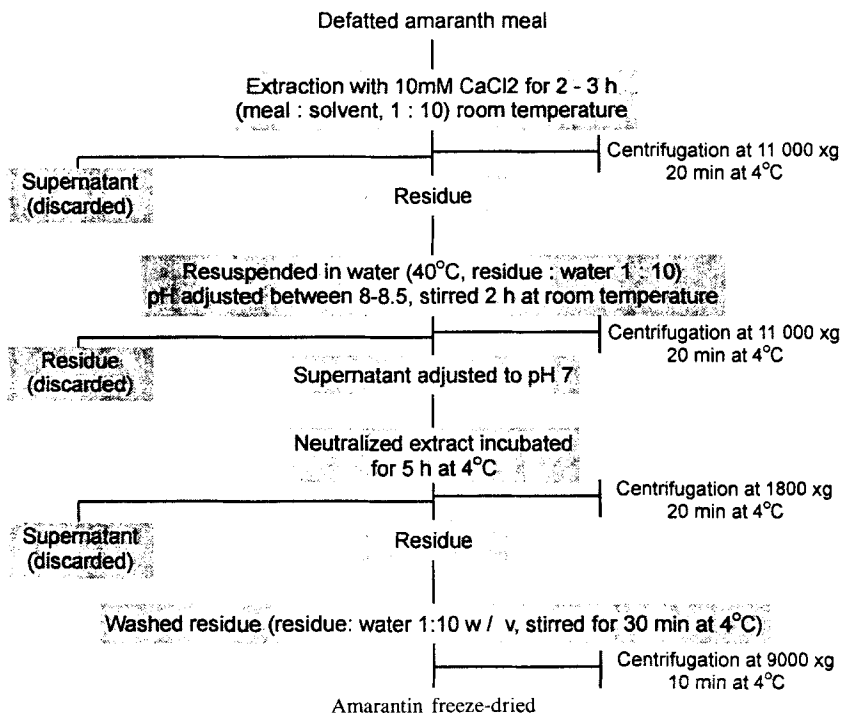


FIG. 1. PROTEIN ISOLATION OF AMARANTIN FRACTION

Molecular weight of amarantin samples were calculated with the following standard proteins: carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) and phosphorylase B (97.4 kDa).

Nondenaturing Polyacrylamide Gel Electrophoresis

Nondenaturing-polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli (1970) using a 7% acrylamide separating gel and a 2.5% acrylamide stacking gel. Acrylamide monomer to bisacrylamide ratio was 37.5:1. Freeze-dried amarantin samples were dissolved in 0.1 M Tris-HCl, pH 6.8, containing 10% glycerol and 0.01 mg/ml bromophenol blue. Electrophoresis, fixing, staining and destaining were conducted as described in the previous section.

Bovine albumin monomer (66 kDa) and dimer (132 kDa), and jack bean urease trimer (272 kDa) and hexamer (545 kDa) were used as molecular weight references.

Gel Filtration Chromatography

A 5-ml sample of the amarantin (20 mg of amarantin/ml) was applied to a Sephacryl S-300 (Pharmacia) packed column (2.5×83 cm). Elution was carried out with 0.1 M NaCl, 50 mM Tris-HCl, pH 8.0, at a flow rate of 37 ml/h, at 7°C, 15°C and 20°C. Fractions of 5 ml were collected. The elution profile was recorded by following the absorbance at 280 nm (UV-visible LKB 2138 Uvicord S. Detector, Bromma, Sweden). Protein peaks were individually pooled and freeze-dried. The following standards were used for column calibration: carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), β -amylase (200 kDa), apoferritin (443 kDa), thyroglobulin (669 kDa) and Blue Dextran (2000 kDa).

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 amarantin were partially solubilized in 1 ml of 0.1 M NaCl, containing 50 mM Tris-HCl, at pH 8.0) was layered on top of an isokinetic sucrose gradient (5–20%, w/v) established in 0.1 M NaCl, 50 mM Tris-HCl, pH 8.0, and centrifuged for 21 h at $218,000 \times g$. Protein separation was monitored by a UV detector (Pharmacia) with a 280 nm filter. Standards with known sedimentation constants were used for calibration: lysozyme (1.9S), bovine serum albumin (4.4S), γ -globulin (7S) and catalase (11.2S) (Gueguen and Barbot 1988).

RESULTS AND DISCUSSION

Protein Isolation

Figure 1 shows the most important steps followed for amarantin isolation. The phenomena that 11S selectively precipitates from the supernatant with calcium salt was recognized in earlier studies (Saio and Watanabe 1973). The residue left after extracting the amaranth meal with 10 mM CaCl_2 at room temperature was resuspended at pH 8–8.5 at 40°C, then centrifuged. The supernatant was neutralized and stored at 4°C for 5 h. The precipitate was washed and freeze-dried. This protein fraction, termed amarantin, was obtained with 94.4% protein content (Table 1).

Protein extracted showed that globulins and amarantin represent 20.5 and 18.6% of the total seed protein, respectively (Table 1). Thus, globulins are mostly composed by amarantin.

TABLE 1.
MASS BALANCE FOR PROTEIN EXTRACTED FROM DEFATTED AMARANTH SEED
FLOUR (DRY BASIS)

Material / fraction	Weight (g)	Protein content (‰)	Total protein (‰)
Defatted flour	100	14.7 + / - 0.4	100
Total globulins	3.1 + / - 0.0	97.0 + / - 0.1	20.5 + / - 0.1
Amarantin	2.9 + / - 0.0	94.4 + / - 1.2	18.6 + / - 0.2

^aThree repetitions average +/— standard deviation

Polyacrylamide Gel Electrophoresis

Without reduction, SDS-PAGE of the amarantin fraction gave three main bands at 39, 55 and 62 kDa (Fig. 2). Additionally, the 11S-like globulin showed some

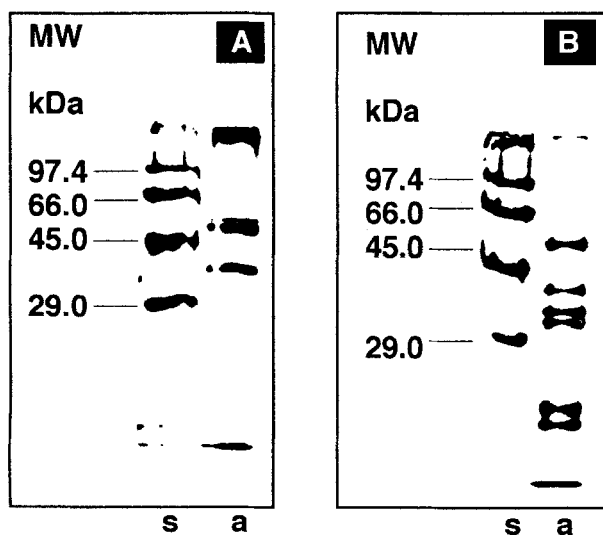


FIG. 2. ELECTROPHORETIC PATTERNS OF AMARANTIN (THE 11S-LIKE GLOBULIN FRACTION) BY SDS-PAGE (A) WITHOUT 2-ME; (B) WITH 2-ME; (s) STANDARDS AND (a) 11S GLOBULIN FRACTION

polypeptides at the top of the gel. With reduction, the amarantin fraction gave doublets at 16–19 and 33–36 kDa, and two bands at 41 and 55 kDa, respectively. Since the electrophoretic pattern of the bands was modified in reductive conditions this fraction behaved similarly to that of the 11S-type protein soybean glycinin (Peng *et al.* 1984; Utsumi and Kinsella 1985; Fukushima 1991ab) and quinoa chenopodin (Brinegar and Goundan 1993). The electrophoretic patterns and molecular weights of the constituent polypeptides of amarantin fraction (Fig. 2), without and with reduction, were similar and even with the same doublets to those from the globulin fraction from *Amaranthus hypochondriacus* described by Konishi *et al.* (1985), Mora-Escobedo *et al.* (1990) and Barba de la Rosa *et al.* (1992a). Moreover, nondenaturing polyacrylamide gel electrophoresis showed a clear and unique band of amarantin (Fig. 3).

Gel Filtration Chromatography

Different elution profiles were observed when the gel filtration chromatography was carried out at 7°C and 15°C (results not shown), compared to that at 20°C (Fig. 4). In agreement with this observation, elution profiles for soybean 11S globulin have been reported to be different when gel filtration chromatography was run at 4°C due to protein cryoprecipitation (Wolf 1980; Peng *et al.* 1984; Rasyid *et al.* 1992).

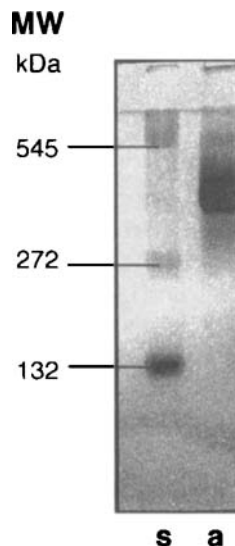


FIG. 3. ELECTROPHORETIC PATTERNS OF AMARANTIN FRACTION BY NONDENATURING-PAGE (s) STANDARDS AND (a) 11S-LIKE GLOBULIN FRACTION

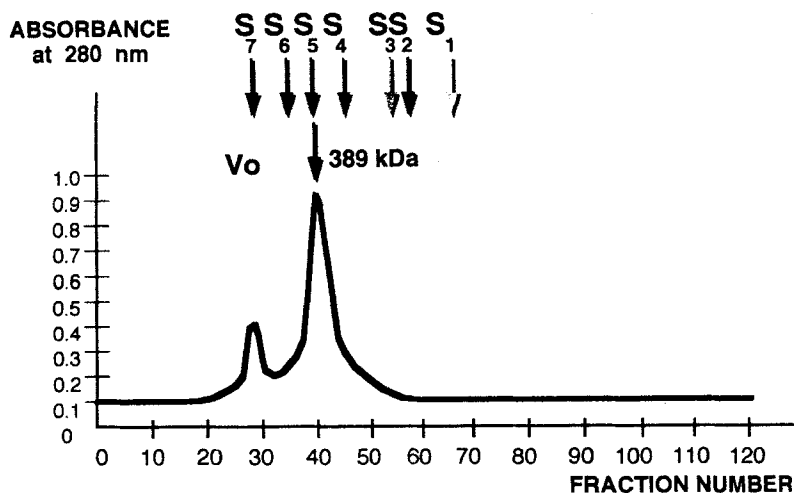


FIG. 4. GEL FILTRATION CHROMATOGRAPHY PATTERN OF AMARANTIN (THE 11S-LIKE GLOBULIN FRACTION) ELUTED AT 20°C

Protein markers and their corresponding molecular weights were as follows: S1, carbonic anhydrase (29 kDa); S2, bovine serum albumin (66 kDa); S3, alcohol dehydrogenase (150 kDa); S4, α -amylase (200 kDa); S5, apoferritin (443 kDa); S6, thyroglobulin (669 kDa); S7, Blue Dextran (2000 kDa).

The amarantin fraction had an apparent molecular weight of 389 kDa. This result for amarantin is also within the calculated molecular weight of other 11S-type globulins reported by Brinegar and Goundan (1993) for quinoa chenopodin (320 kDa), by Danilenko *et al.* (1993) for broad bean legumin (360 kDa), and by Konishi *et al.* (1985) for amaranth globulins (440 kDa).

Ultracentrifugation

Ultracentrifugation of the freeze-dried amarantin showed sedimentation constants around 1.4S, 8.5S, and 11.0S (Fig. 5). Saio and Watanabe (1973) showed that the protein precipitated by calcium ion was mainly 11S globulin; under those conditions about one third of the 2S and one half of the 7S fraction were also precipitated. In our work the principal fraction was 11S protein. The proteins of low molecular weight at 1.4 S might correspond to albumin contamination. It has been observed that many 2.0S albumins show sedimentation constants between 1.2S and 2.0S (Konishi *et al.* 1985; Barba de la Rosa *et al.* 1992b; Dey and Mandal 1993; Kolivas and Gayler 1993).

In summary, our results suggest that amarantin, the 11S globulin from amaranth seed, is a member of the highly conserved 11S storage globulin family. Further biochemical studies are pending.

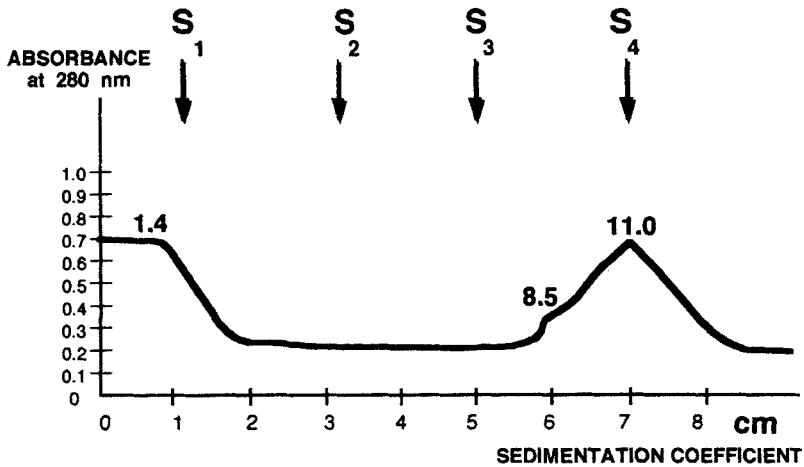


FIG. 5. ESTIMATION OF THE SEDIMENTATION COEFFICIENT OF AMARANTIN FRACTION FROM GLOBULIN SOLUTION

The protein markers and their corresponding sedimentation coefficients were as follows: S1, lysozyme (1.9S); S2, bovine serum albumin (4.4S); S3, α -globulin (7.0S); S4, catalase (11.2S).

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