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Evidence confirming the existence of a 7S globulin-like storage protein in *Amaranthus hypochondriacus* seed

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Abstract

The 7S amaranth globulin ('conamaranthin') was successfully identified, isolated and purified from the seed of *Amaranthus hypochondriacus* K343 and physico-chemical properties determined. The 7S globulin was found to exist as a hetero-oligomer with a molecular weight of 186.0 KDa and to be composed of a variety of eight non-convalently linked subunits namely: 90.1, 70.9, 40.0, 37.4, 35.2, 31.2, 23.6, and 15.6 KDa. Structural analysis of this protein using far-UV circular dichroism (CD) revealed that it possessed low levels of α-helical and very high β-sheet secondary fractions whereas near-UV CD measurements revealed that its aromatic amino acids were highly motile within its tertiary structure. The globulin was found to undergo a larger surface charge density change with decrease in pH below its isoelectric point of 5.2–5.8 than with increase in pH above this point. An acid-induced dissociation of its subunits was also noted below its determined isoelectric point. Immunochemical investigation of the 7S globulin with the anti-11S amaranth globulin antibody revealed an approximate 30% homology with its 11S amaranth globulin 'Amaranthin' counterpart and to be due to structural not sequential epitope recognition. In summary, this study was instrumental in demonstrating that there are substantial differences between the physico-chemical properties of the amaranth's 7S and 11S globulins.

1. Introduction

Archaeological and anthropological studies conducted in Peru, Mexico and the United States have shown that the production of amaranth grain was important as early as 8000–4000 B.C.E. Production of amaranth grain peaked in Central America during the Mayan and Aztec periods and then experienced a catastrophic decline due to a legislated ban placed upon its production in 1519 by the Spanish conqueror Cortez (Saunders & Becker, 1985; Bressani, 1989). Since the time of this ban, the consumption/production of amaranth grain as a popular food crop lay largely dormant, until its recent resurgence due in part to its recognized agronomic and nutritional properties (Lehmann, 1996; Coimbra & Salema, 1994; Saunders & Becker, 1985). Agronomically, amaranth species (which are dicotyledonous C_4 plants) can be and are successfully grown world wide from the tropics through to semiarid regions due to its environmental hardiness and ability to grow in places where other more conventional grains such as cereals cannot grow (Fadel, Pond, Harrold, Cavert, & Lewis, 1996; Lehmann, 1996; Bressani, 1989; Saunders & Becker, 1985). Nutritionally, based on essential amino acid composition, amaranth grain has been assigned an exceptionally high chemical score of 73 in the recommended FAO/WHO scoring patterns (Bressani, 1989). In fact, when amaranth grain is processed under conditions which do not damage the availability of essential amino acids, its protein quality is very close to that of casein (Bressani, 1989; Mendoza & Bressani, 1987). Some research has also shown that the protein quality (PER) of amaranth grain increases significantly on wet cooking as compared to the raw grain (Bejosano & Corke, 1998; Bressani, 1989).

Since the lysine-rich salt-soluble 11S globulin ('amaranthin') has been shown to be the main storage protein found in amaranth grain (Bressani & Garcia-Vela, 1990; Konishi, Fumita, Ikeda, Okuno, & Fuwa, 1985) the above nutritional attributes have been ascribed in part to this particular protein. Although the 11S globulin together with its water-soluble albumin counterpart (albumin-1) have been purified and well investigated (Marcone, Niekamp, Le Maguer, & Yada, 1994; Marcone & Yada, 1991, 1992; Konishi, Horikawa, Oku,

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Azumaya, & Nakatini, 1991; Konishi & Yoshimoto, 1989; Konishi et al., 1985) little else is known about the other (minor) amaranth proteins found in the seed. Since it is well known that storage proteins of dicotyledonous seeds are generally found occurring as two major fractions, i.e. 11S and 7S (11.57–12.6S and 10S in amaranth)(on the basis of their sedimentation behaviors), little is yet known about the amaranth 7S globulin (10S globulin) except what has been briefly reported by Segura-Nieto, Vázquer-Sánchez and Rubio-Velazquez (1992) and Barba de la Rosa, Paredez-López and Guoguen (1992) in their studies of the major amaranth proteins.

It was therefore the purpose of this study to identify, isolate and purify the amaranth 7S globulin with the objective to characterize it and compare it to the other studied amaranth proteins.

2. Materials and methods

2.1. Materials

Non-heat treated milled seed of *Amaranthus hypochondriacus* K343 (the major raw source of the amaranth globulin used throughout the studies) marketed under the trade name Ambake was purchased from American Amaranth Inc. (Bricelyn, MI). Soybean seed (*Corsoy 79*) was the kind gift of the Harrow Research Station (Harrow, Ontario, Canada). Chromatographic materials, i.e. Sephacryl™ S-300 Superfine gel permeation media and Mono-Q 5/5 anion-exchange column and high molecular weight calibration proteins, were products of Pharmacia LKB (Montreal, PQ, Canada).

All other reagents and chemicals were of AR or HPLC grade from Sigma Chemical Co. (St. Louis, MO, USA), Aldrich (Milwaukee, WI, USA) or Fisher Scientific (Toronto, ON).

2.2. Determination of protein concentration

Protein concentration was determined using the Bio-Rad protein dye-binding assay (modified Bradford) with bovine serum albumin (BSA) as the standard (Bio-Rad Laboratories, 1989). Analyses were performed in triplicate. Crude protein was also measured by a modified semi-micro Kjeldahl procedure of the American Association of Cereal Chemists (AACC, 1983). A nitrogen to protein conversion factor of 5.85 was used in the calculation (Becker et al., 1981).

2.3. Isolation and purification of the 7S amaranth globulin

2.3.1. Sample preparation

Amaranth and soybean meals (passing through a 60-mess screen) were defatted with either cold HPLC grade acetone or hexane in a ratio of 1:10 (w/v), (flour/solvent). The mixtures were mechanically stirred in sealed 2-1 glass containers for 36 h at −20°C. This was followed by three additional extractions with either cold acetone or hexane and the defatted meals dried under a stream of pure nitrogen. The defatted meals were immediately stored desiccated at −20°C until required for further study.

2.3.2. Protein extraction

The salt-soluble proteins (i.e. 7S and 11S globulins) of both the amaranth and soybean seed were extracted from their respective defatted meal by a modification of the method of Konishi et al. (1985) and Marcone, Kakuda and Yada (1998a). A 3.0-g sample of meal was extracted at 4°C for 18 h with 40 ml of a 32.5/2.6 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.50, containing 0.4 M NaCl and 0.02% (w/v) NaN_3 under mechanical stirring. The meal/buffer mixture was centrifuged at 10,000 ***g*** for 20 min at 4°C in a L8-70M Ultracentrifuge (Beckman Instruments, Mississauga, ON, Canada). In the case of the amaranth meal the supernatant containing both the 7S and 11S globulins was filtered following centrifugation through a Millipore 0.2 μ sterile filter and immediately applied to the gel filtration column.

2.3.3. Gel filtration chromatography

A Sephacryl™ S-300 Superfine (2.5 × 95 cm) gel filtration column with a molecular weight fractionation range of 10,000–1,500,000 Da was packed and equilibrated with three bed volumes of the above extraction buffer, i.e. a buffer composed of 32.5/2.6 mM K_2HPO_4/KH_2PO_4, pH 7.5, containing 0.4 M NaCl and 0.02% NaN_3 at an isocratic flow rate at 38.7 ml/h with a LKB Bromma, Model 2132 Microperpex Peristaltic Pump (LKB, Sweden). Blue dextran was used to measure the void volume. A standard molecular weight calibration curve was generated using the following proteins: ferritin (440,000 Da), catalase (232,000 Da), aldolase (158,000 Da), and cytochrome c (12,400 Da).

A 5.0 ml sample containing 30 mg of the crude globulin extract was loaded on to the gel filtration column. Protein fractions were eluted from the column at room temperature (22°C). Five-ml fractions were collected with a Bio-Rad Model 2110 Fraction Collector (Bio-Rad, Mississauga, ON, Canada). Absorbances at 280 nm were measured with a UV-visible recording spectrophometer Model UV-260 (Shimadzu Corp., Kyoto, Japan). Protein elution profiles were also measured using the Bio-Rad protein dye-binding procedure mentioned earlier (Bio-Rad Laboratories, 1989, USA). Protein peaks were individually pooled and dialysed using a 12–14,000 Da membrane against distilled water (six changes × 4 litres). The resulting retenates were lyophilized and stored desiccated at −20°C under an argon blanket until further study.

2.3.4. Anion-exchange chromatography

Anion-exchange chromatography was performed using a fast protein liquid chromatograph (FPLC) equipped with two P-500 pumps, LCC-500 PLUS/CI control unit and UV-280 detector (Pharmacia LKB, Montreal, PQ, Canada).

Two hundred-μl samples containing approximately 5 mg/ml of protein eluting from the gel filtration column were loaded onto a Mono-Q™ 5/5 anion-exchange column (Pharmacia LKB, Montreal, PQ, Canada) which had been equilibrated with a 20.0 mM Tris–HCl buffer, pH 8.0 (buffer A).

A gradient was established from 100% buffer A to 100% buffer B (20.0 mM Tris–HCl, pH 8.0, 1M NaCl). The flow rate was held constant at 1.0 ml/min. Protein elution patterns were monitored at A_{280} and AUFS = 0.01. The protein peak containing the amaranth 7S globulin was pooled and dialyzed as described above. The resulting retentate was lyophilized and stored desiccated at −77°C under an argon blanket until further study.

2.4. Evaluation of purity/characterization of the isolated 7S amaranth globulin

2.4.1. Sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE)

The purified globulins were reconstituted to 3 mg/ml in a 10 mM Tris/HCl buffer, pH 8.0, containing 1 mM EDTA, 2.5% (w/v) SDS, 0.1% (w/v) bromophenol blue and with and without, 5.0% (v/v) β-mercaptoethanol. One-μl samples were applied directly onto PhastGel gradient polyacrylamide (PAA) 8–25% SDS–PAGE gels (Pharmacia, LKB, Montreal, PG, Canada). Separations were performed using a PhastSystem Separation and Control and Development Unit (Pharmacia LKB, Montreal, PQ, Canada) according to the manufacturer's instructions. Gels were stained with PhastGel Blue R (a Coomassie R-350 dye). Linear plots of the log molecular weight versus relative mobility (Rm) were prepared with phosphorylase b (94,000 Da), bovine serum albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), soybean trypsin inhibitor (20,100 Da), and α-lactalbumin (14,000 Da) as standards.

2.4.2. Native-PAGE electrophoresis

The purified 7S amaranth globulin was reconstituted to 3 mg/ml in a 20 mM phosphate buffer, pH 7.50, and 1 μl aliquots were loaded onto PhastGel 7.5% polyacrylamide (PAA) gels with native buffer strips. The separations and subsequent scannings were performed as described earlier.

2.4.3. Amino acid analysis

Two hundred-μl samples containing 0.4 mg/ml of purified 7S amaranth globulin were lyophilized and then hydrolyzed in 200 μl of 5.7 M HCl for 24, 48 and 72 h at 108°C in vacuo. Phenol (0.2% w/v) was added as an O_2 scavenger. The samples were then neutralized with 2.0 N NaOH, dried in vacuo, and redissolved in 400 μl of buffer. Similar samples were also oxidized with performic acid before the 24 h HCl-hydrolysis to estimate cystine and cysteine as cysteic acid. Analysis was performed by loading 25-μl portions of hydrolysed-neutralized samples on to the column.

Amino acid measurement was performed on a Beckman System Gold Amino Acid Analyzer (Beckman Instruments, Mississauga, ON, Canada) component system (126AA pump), 235 column heater, 231 post-column reactor, 166 programmable detector, and 506 autosampler with ninhydrin detection at 570 nm. Analyses were performed in triplicate.

2.4.4. Near- and far-UV circular dichroism spectroscopy (CD)

Circular dichroism measurements were performed in the near-UV (240–320 nm) on 1.0 mg/ml globulin/buffer solutions (32.5/2.6 mM K_2HPO_4/KH_2PO_4) pH 7.5 containing 0.4 M NaCl, using a Jasco J-600 spectropolarimeter (Japan Spectroscopic Co. Ltd., Tokyo, Japan) with a cell pathlength of 1.0 cm. Circular dichroic measurements were also carried out in the far-UV (190–250 nm) using the above instrumentation and with a cell pathlength of 1 mm. Secondary structure fractions were tabulated using the Jasco Protein Secondary Structure Estimation Program (Japan Spectroscopic Co., Tokyo, Japan) which is based on the algorithm of Chang, Wu and Yang (1978) and the data base of Hennessey and Johnson (1981). Analyses were performed in triplicate with 6 scan per replication.

2.4.5. Micro-differential scanning calorimetry (DSC)

DSC measurements were performed on a MC-2D instrument (Upscan Ultrasensitive Differential Scanning Calorimeter) with twin 1.2 ml total-fill tantalum cells. Data was collected using the Origin DSCITC Data Collection Software V.1.1 and analysed using the Origin Version 2.9 DSC Program (Micro Cal, Inc., Northampton, MA, USA) at a heating rate of 1.37°C/min from 30 to 118°C (under 20 psi pressure). Crude globulins of amaranth and soybean were diluted and dialysed against 32.5/2.6 mM K_2HPO_4/KH_2PO_4 buffer pH 7.5, containing 0.4 M NaCl to a concentration of 0.05%. The identical buffer served as the reference. Analyses were performed in duplicate.

2.4.6. Titration curves

Titration curves were performed by the two-dimensional technique described in PhastSystem Technical File 100 using IEF 3–9 gels (Pharmacia LKB, Montreal, PQ, Canada). A pH gradient was generated in the first dimension for 150 V h. The gel was then rotated by 90°

and 3.5 μl of a 3 mg/ml protein solution applied in the second dimension perpendicular to the pH gradient. The voltage was again applied for an additional 40 V h. Coomassie staining was performed as described in PhastSystem Development Technique File 200 (Pharmacia LKB, Montreal, PQ, Canada).

2.4.7. Extinction coefficient

The extinction coefficient ${}^{\xi}{}_{280}{}^{0.1}$ of the purified globulin was determined according to the gravimetric procedure of Ellis and Bell (1985). Analyses were performed in triplicate.

2.4.8. Immunochemical analysis

Immunochemical analysis of the 7S globulin was performed essentially as described by Marcone, Kakuda and Yada (1998b) using an ELISA method employing the expressed anti-amaranth 11S globulin polyclonal antibody.

2.4.9. Assessment of immunoreaction with unfolded protein

The purified 7S amaranth globulin (10 μg/ml) was dissolved in 1 ml of 8 M urea in PBS, 0.1 ml β-mercaptoethanol and 0.04 g of SDS and allowed to stand at room temperature for 1 h, and then boiled for 10 min as described by Deutsch (1976). Immunochemical analyses were performed as described above.

3. Results and discussion

Efforts aimed at isolating the sought after 7S amaranth globulin together with its more abundant 11S globulin counterpart using standard high ionic strength extraction buffers followed by their co-precipitation with dialysis against distilled water did not result in high yields of the 7S globulin. The low recovery of the 7S globulin using this method could probably be ascribed to the higher solubility of the 7S globulin verse the 11S globulin at the lower ionic strengths occurring during dialysis leading to the 7S globulin remaining primarily in the supernatant and not co-precipitating with the 11S globulin.

In order to surmount this obstacle and thereby quantitatively isolating the 7S globulin from the amaranth seed meal, freshly extracted salt-soluble globulins (i.e. 7S and 11S) were applied directly to the gel filtration column omitting the dialysis step. Fig. 1 illustrates the typical gel filtration elution chromatogram obtained from this particular type of crude globulin isolation. The turbid, strong UV-absorbing fraction (labelled fraction 1) with a Kav = 0 (i.e. void volume) was typically found to contain both high levels of nucleic acids and carbohydrate as well as other non-identifiable high molecular weight components (data not shown). The observation of the occurrence of this characteristic turbid peak in gel filtration studies had been previously

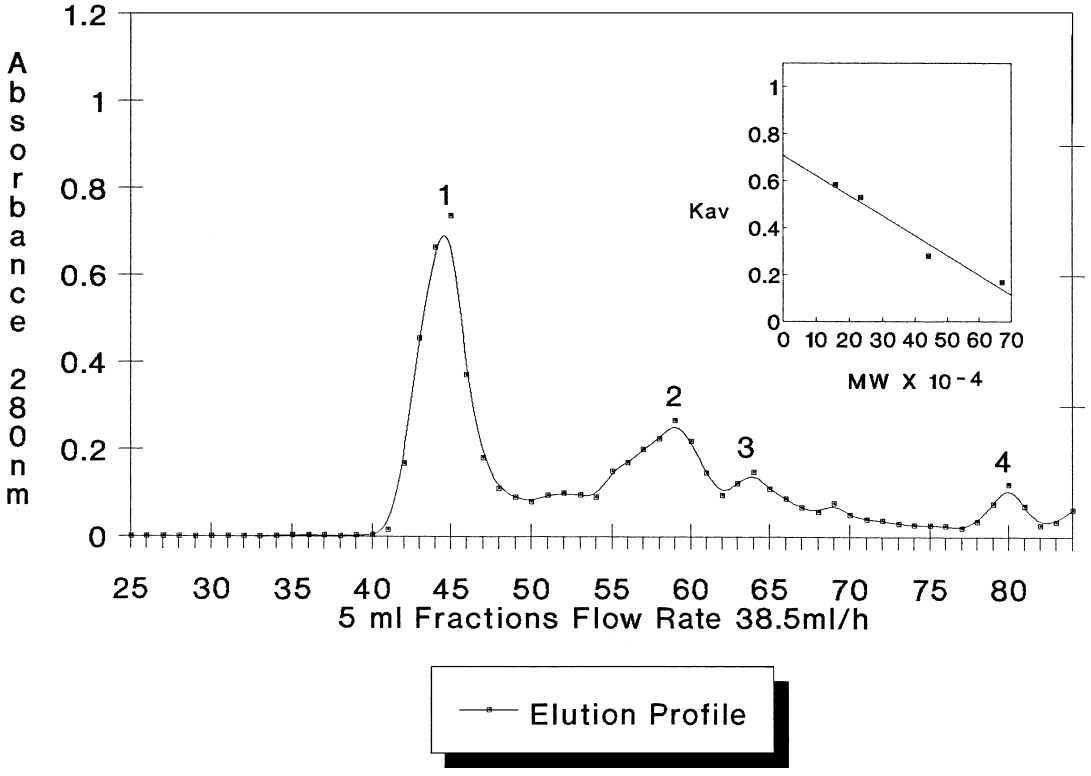

Fig. 1. Sephacryl® S-300 Superfine gel filtration chromatogram of the crude amaranth globulin extract (30 mg/5 ml protein injection). Flow rate was 38.7 mml/h with a 32.5/2.6 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.50) containing 0.4 M NaCl and 0.02% NaN_3.

reported by Madhusudhan and Singh (1983), Marcone and Yada (1991) and Marcone et al. (1998a) for a variety of other seed storage globulin preparations.

Physico-chemical examination of the major protein peak (labelled fraction 2 and constituting approximately 31.6% of the total protein injected) enabled its identification as the 11S amaranth seed globulin having a Kav = 0.417 and corresponding to a molecular weight of 343,000 Da. This molecular weight is in very close agreement with the molecular weight 337,000 Da previously determined by Marcone and Yada (1991) for this protein. The determined molecular weights of the subunits from this protein was also in good agreement with those determined by Marcone and Yada (1991) for the 11S globulin (Fig. 2, Table 1).

The next eluting peak (labelled fraction 3 and constituting approximately 12.3% of the total protein injected) with a Kav = 0.556 and a corresponding molecular weight of 186,000 Da was identified as the sought after 7S amaranth globulin.

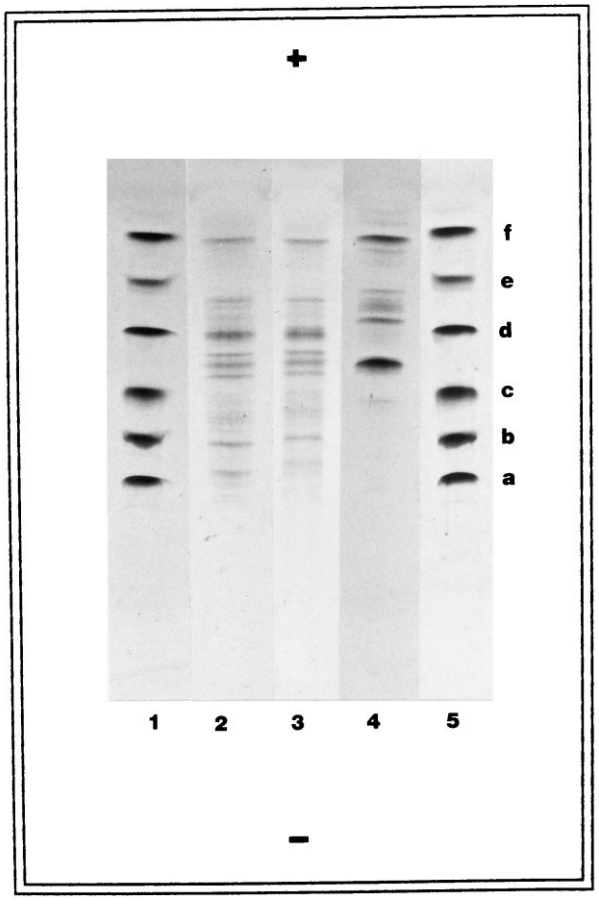

Fig. 2. SDS–PAGE (reducing) (1 μl of 3 mg/ml protein solutions) applied to a gradient 8/25 PhastGels (Pharmacia, LKB). Lanes 1 and 5: standards: (a) phosphorylase b, 94,000 Da; (b) bovine serum albumin, 67,000 Da; (c) ovalbumin, 43,000 Da; (d) carbonic anhydrase, 30,000 Da; (e) soybean trypsin inhibitor, 20,100 Da; (f) α-lactalbumin, 14,400 Da. Lane 2: purified 7S globulin (non-reduced); lane 3: purified 7S globulin (reduced); lane 4: purified 11S globulin (reduced).

Examination of the last gel filtration peak (labelled fraction 4) with a Kav =∼ 1.0 was identified as the water-soluble albumin previously identified by Marcone et al. (1994).

3.1. Anion-exchange chromatography

Following the described gel filtration chromatography, the isolated 7S globulin (i.e. from fraction 3) was further subjected to anion-exchange chromatography for further purification (Fig. 3). Examination of the typical chromatogram obtained for this second chromatographic purification step revealed the presence of a single major peak having a retention time of 11.2 min (i.e. eluted at 0.37 M NaCl). Since anion-exchange chromatography did in fact show that only one major protein species was present, it could then be concluded that gel filtration chromatography was an excellent first step in eliminating the other undesirable proteins present in the intial crude globulin preparation. Tabulation of the purification yield data would indicate that of the 95.1 mg of crude globulin extracted per gram of defatted meal, 12.3% was present in the form of the 7S globulin. For further physico-chemical analysis of the major protein peak eluting from the anion exchange column was used as the source of the purified 7S globulin under investigation.

3.2. Electrophoretic protein characterization

3.2.1. SDS–PAGE

Further characterization of the purified 7S amaranth globulin by SDS–PAGE (performed under reducing

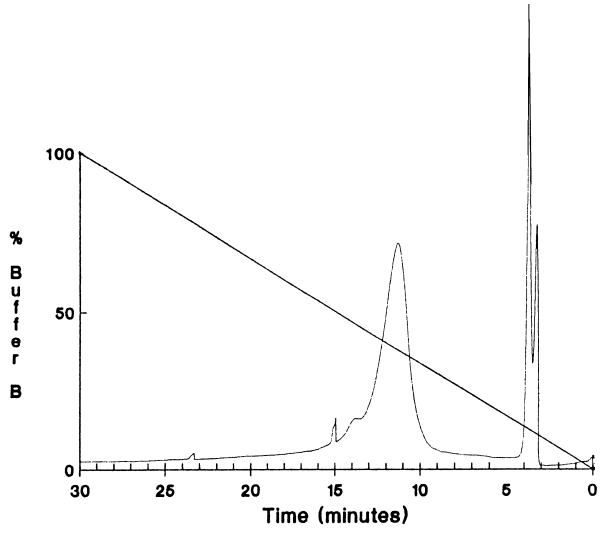

Fig. 3. Fast protein liquid chromatogram (FPLC) of purified 7S amaranth globulin from a Sephacryl® S-300 superfine gel filtration chromatography run on a Mono-Q 5/5 anion-exchange column. Flow rate was 1.0 ml/min using a buffer gradient starting with 100% buffer A (20.0 mM Tris–HCl buffer pH 8.0) to 100% buffer B (20.0 mM Tris–HCl buffer pH 8.0 containing 1.0 M NaCl) over 30 min.

condition) revealed that it was composed of eight subunits and could therefore be classified as a hetero-oligomer (Fig. 2). The molecular weights determined for these subunits are reported in Table 1 and are in excellent agreement with the total number and molecular weights determined by Barba de la Rosa et al. (1992) for this protein.

It is interesting to note that the 7S globulin in contrast to its 11S counterpart is composed of subunits having a wider molecular weight range (i.e. ~90–15 kDa versus ~37–15 kD for the 11S globulin) (Fig. 2). Further inspection of this data reveals that in fact very few subunits are shared in common between both types of these globulins indicating that they are very distinct/different proteins within the amaranth seed on a purely structural basis.

In order to further elucidate the nature and type of the bond forces holding the above mentioned subunits together in its hetero-oligomeric type structure, the purified 7S globulin was again subjected to SDS–PAGE but in the absence of β-mercaptoethanol (Fig. 2). Results indicate that all of its subunits were held together strictly by non-covalent forces (bonds) by Barba de la Rosa et al. (1992). These results further support its classification as a 7S globulin since 7S globulins are classically recognized as being devoid of disulfide bridges (Barba de la Rosa et al., 1992).

In contrast to the 7S globulin, the 11S globulin ('Amaranthin') (like most other 11S globulins from dicotyledonous plants) had been shown to be held together by specific disulfide linked intermediary subunits which are thermodynamically favourable to its overall structure (Table 2) (Marcone & Yada, 1992; Marcone et al., 1998a).

3.2.2. Native-PAGE

Further characterization of the purified 7S amaranth globulin by native-PAGE indicated that it possessed a molecular weight of approximately 200,000 Da which closely corresponded with the value of 186,000 Da previously determined by gel filtration chromatography in this study.

It is interesting to note that the 7S globulin did not undergo an alkaline induced dissociation under this particular electrophoretic technique although it had been previously determined that the majority of attractive forces between its subunits were weak secondary (non-covalent) forces, i.e. hydrogen, hydrophobic and electrostatic. The 11S amaranth globulin, like many other dicotyledonous globulins, typically undergo this type of dissociation although they possess some covalent (disulfide bonds between subunits) which help strut or support the overall molecule (Marcone et al., 1998a). The possibility therefore exists that the 7S amaranth globulin, unlike its 11S counterpart, has stronger internal bond forces holding subunits together in the molecule, or alternatively have larger amounts of asparagine and glutamine rather than having a larger quantity of aspartic or glutamic acids (between subunits) which are ionizable at alkaline pH, causing electrostatic repulsion and dissociation of the protein.

3.2.3. Electrophoretic titration curve analysis

In order to obtain further information about the 7S globulin, particularly about its surface properties, namely surface net charge, electrophoretic isoelectric titration was performed.

Table 1
SDS–PAGE molecular weight distribution of subunits of the 7S and 11S globulins from *Amaranthus hypochondriacus* K343 seed

Amaranth 7S globulin (Da)	Amaranth 7S globulin literature[a] (Da)	Amaranth 11S globulin (Da)	Amaranth 11S globulin literature[b] (Da)
90,000	72,000	–	–
70,900	67,000	37,400	37,500
40,000	52,000	28,200	31,500
37,400	38,000	24,800	26,500
35,200	34,000	22,400	20,500
31,200	32,000	14,800	14,500
23,600	25,000	–	–
15,200	–	–	–

[a] Barba de la Rosa et al. (1992).
[b] Marcone and Yada (1991).

Table 2
A comparison of the amino acid composition of the purified 7S and 11S globulins from *Amaranthus hypochondriacus* K343 seed

Amino acid	Purified 7S globulin	Purified 11S globulin[a]
Asx[b]	10.1 (±0.1)	8.76
Thr	7.2 (±0.0)	4.02
Ser	6.9 (±0.1)	6.90
Glx[c]	17.2 (±0.3)	15.29
Pro	5.3 (±1.6)	6.14
Gly	5.8 (±0.1)	8.00
Ala	15.6 (±0.4)	5.71
Cys[d]	1.6 (±0.4)	0.93
Val	5.8 (±0.1)	6.29
Met	ND	1.77
Leu	6.8 (±0.2)	6.82
Ile	3.9 (±0.1)	5.01
Tyr	0.3 (±0.0)	2.13
Phe	4.4 (±0.4)	6.78
Lys	3.7 (±0.1)	4.25
His	1.7 (±0.3)	2.62
Ary	4.5 (±0.1)	10.12
Trp	ND	ND

Reported in molar percent.
[a] Marcone and Yada (1991).
[b] Asx = Asp + Asn.
[c] Glx = Glu + Gln.
[d] Cystine and cysteine measured as cysteic acid by performic acid treatment.
Results are the mean value (±SD) of three replications.
ND, not determined.

Previous electrophoretic titration studies of the 11S globulin by Marcone and Yada (1992), although producing no direct reading of the isoelectric point (i.e. the point where the titration curve crosses the origin zero immobility plane), did demonstrate steep slopes which were indicative of a large surface charge density change with change in pH. In contrast to the 11S globulin, the 7S globulin demonstrated more shallow slopes indicative of a smaller surface charge density change with change in pH (Fig. 4). This trait was most pronounced in the neutral to alkaline pH range (i.e. 7 to 9). Similar to the titration curve of the 11S globulin, that the purified 7S globulin also demonstrated a characteristic precipitation phenomena (Fig. 4). However, in this case, it was observed in the pH range 5–3.

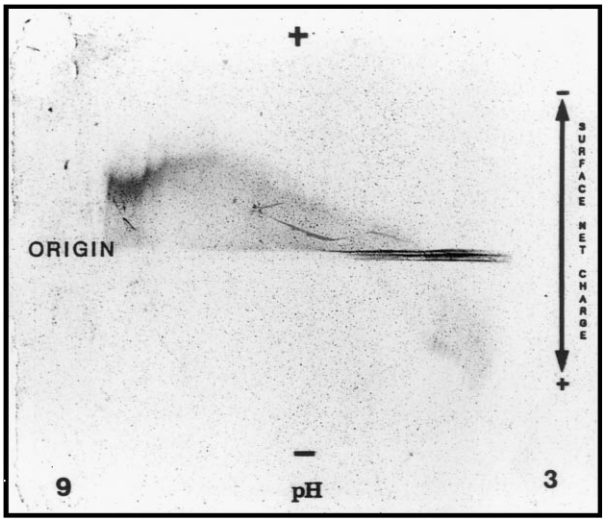

Fig. 4. Electrophoretic titration curve of the purified 7S amaranth globulin between the pH of 9 to 3 (5 mg/ml, 3.5 μl applied at origin).

Titration curve data for the 7S globulin is also consistent with previously published data which indicated that the subunits of this particular globulin could undergo an acid-induced dissociation (Barba de la Rosa et al., 1992). The evidence for the occurrence of this phenomena was readily observed by the appearance of multiple titration curves in the acidic pH range of (3.0–5.2) of the titration gel. In the acidic pH range, the 7S globulin possessed much steeper slopes than in the alkaline pH range indicating that a decrease in pH had a much greater effect on the surface charge density of this globulin than did a change at high pH. The above mentioned positive charge increase with decrease in pH could have resulted from the exposure of more basic amino acid residues due to the unfolding of the 7S globulin followed by subunit dissociation and/or to the propensity of basic amino acids being between subunit surfaces at their point of contact. In addition, the dissociation of those subunits could also result in an increase in the surface area of the subunits which would then result in the exposure of these otherwise buried amino acids at subunit/subunit interfaces. It should be again noted that this type of acid-induced dissociation was not observed for the amaranth 11S globulin and is, therefore, a distinguishing feature between the two.

3.3. Amino acid analysis

Examination of the amino acid composition of the 7S globulin indicates that similar to its 11S globulin

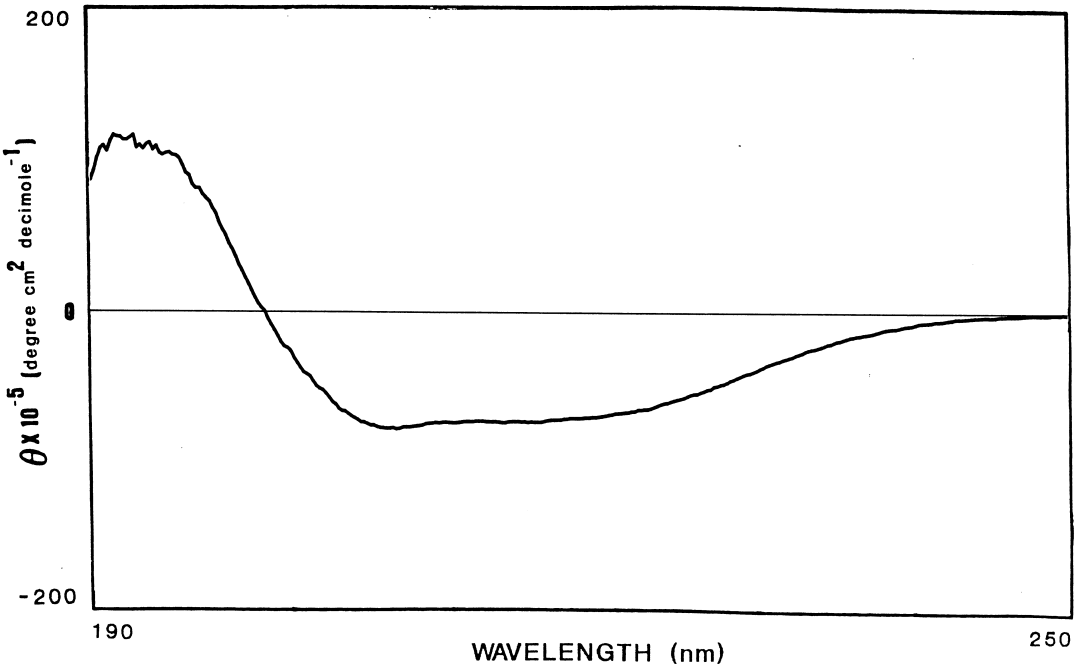

Fig. 5. Far-UV CD spectral (250–190 nm) scans of the purified 7S amaranth globulin (0.1 mg/ml solution, average of six scans per replication).

counterpart it is composed of a comparably high amount of amide (glutamic acids–glutamine aspartic acid–asparagine and arginine) (Table 2) indicating that both the 7S and 11S have a storage type role in the seed (for use at the time of germination as a nitrogen source) and may be equivalent proteins. A comparable level of other amino acids was also found.

3.4. Conformational/structural characterization

3.4.1. Secondary structural conformation

Fig. 5 illustrates the circular dichroic spectra obtained for the purified 7S globulin. Quantitative estimation of the relative amounts of α-helical, β-sheet, β-turn and random coil secondary structure fractions for this protein are tabulated in Table 3. Examination of these relative quantities revealed that the 7S globulin possessed low levels of α-helical and very high β-sheet secondary structure fractions which were comparable in quantity to those determined for the 11S globulin and reported by Marcone and Yada (1991). α-Helical and β-sheet secondary structure fraction have the tendency of being deeply buried within the polypeptide chain (Hopp & Woods, 1981, 1986). The observed similarity in the amounts of these two secondary structure may indicate that the interior conformation of both the 7S and 11S globulins are very similar or highly conserved.

Table 3
Circular dichroism secondary structure of the purified 7S globulin from *Amaranthus hypochondriacus* K343 seed

Fraction	Purified amaranth 7S globulin	Purified amaranth 11S globulin[a]
α-Helix	12.6 (±0.4)	7.8
β-Sheet	49.6 (±1.4)	57.6
β-Turn	12.2 (±1.3)	17.6
Random	24.9 (±0.4)	16.9

[a] Marcone and Yada (1998).
Results are the mean value (±SD) of three replications.

3.4.2. Tertiary structural conformation

It is very well recognized that near-UV CD spectra reflects the tertiary structure of a protein (Strickland, 1974) brought about by the interaction and orientation of the aromatic rings of tyrosine, tryptophan and phenylalanine with other moieties in the protein.

In this study, identification of the fine structure of the individual aromatic amino acid groups for 7S globulin was somewhat difficult due to the low spectra intensities of the CD spectral scans (Fig. 6). Identification of the fine structure of phenylalanine was made at 260.2 and 264.0 nm, while the 0–0-cm^{-1} ^{1}Lb and 0 + 850-cm^{-1} ^{1}Lb band of tryptophan and the 0 + 800-cm^{-1} band of tyrosine were absent.

Results demonstrate that major differences in tertiary structure existed between the 7S and 11S globulin proteins previously studied by Marcone and Yada (1992). Since intense CD bands for individual aromatic amino acids commonly occur when aromatic groups are in close proximity to one another (Yada, 1984) the absence of such intense bands for the 7S globulin would indicate that they are either distant from one another (Yada & Nakai, 1986) or highly motile compared to the 11S globulin which generally speaking gave more intense CD

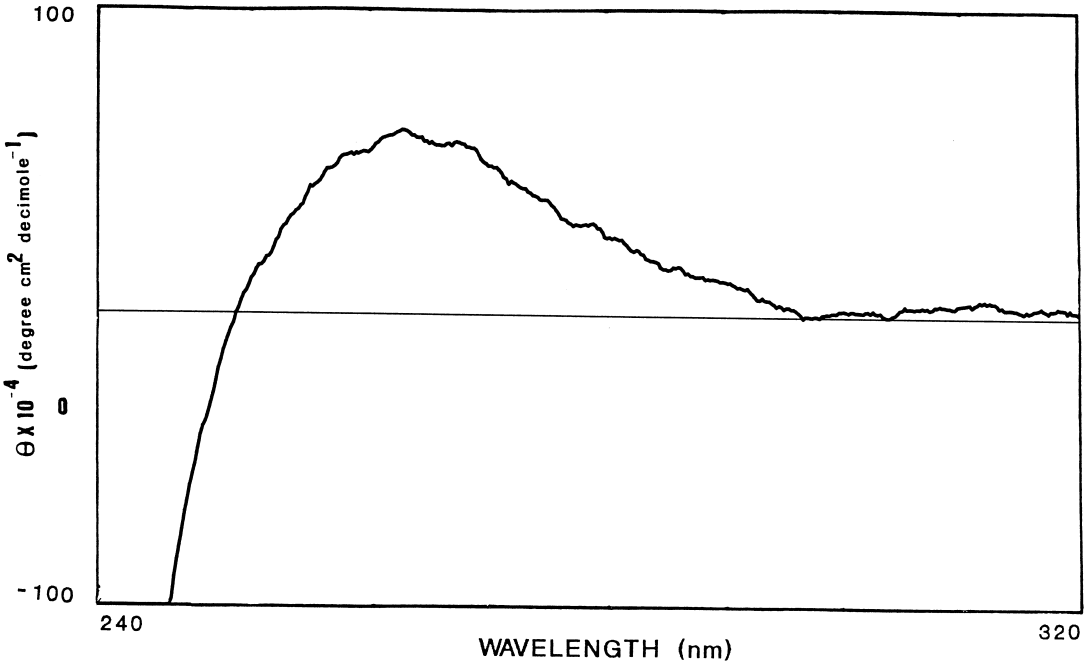

Fig. 6. Near-UV CD spectral (320–240 nm) scans of the purified 7S amaranth globulin (1.0 mg/ml solution, average of six scans per replication).

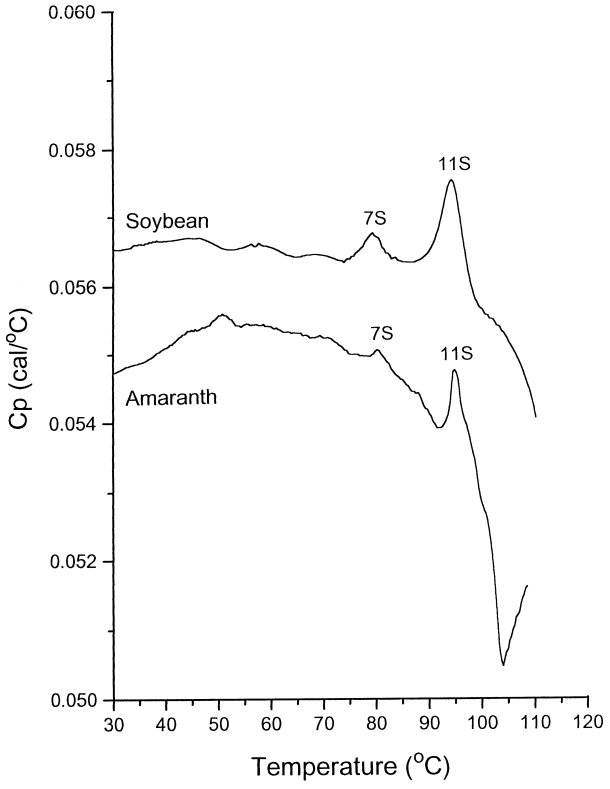

Fig. 7. Micro-differential scanning calorimetry thermograms of the crude amaranth globulin extract and the crude soybean globulin extract (i.e. extracts containing both the 7S and 11S globulins).

bands (Marcone & Yada, 1992). The derived extinction coefficient for the purified 7S globulin was $\varepsilon_{280}^{0.1\%} = 1.33 \pm 0.05$, which was higher than 0.562 determined for the 11S globulin (Marcone & Yada, 1992) despite the fact that amino acid analysis indicated lower levels of aromatic amino acids for the 7S globulin as compared to the 11S globulin. Together, these results indicate that the aromatic amino acids are more highly motile in the 7S globulin.

3.5. Thermal analysis

The thermal stability of the 7S globulin, like any other protein, results from a large number of stabilizing structural factors including amino acid composition, compact packing/protein–protein contacts, binding of metals and other prosthetic groups, as well as intramolecular interactions and linkages (Stanley & Yada, 1994). Micro-differential scanning calorimetric analysis of the crude amaranth globulin extract demonstrate two endothermic transitions with the larger one with a T_D of 94.3°C being characteristic of the 11S globulin and the smaller one with a T_D of 80.2°C usually being attributed to the 7S globulin. Analysis of a crude soybean globulin extract also gave a characteristic 11S globulin T_D of 94.4 and a 7S globulin T_D of 79.2 which is supported by other researchers (Biliaderis, 1983). It can presently be concluded that the 7S and 11S globulins exist as two distinct proteins in the amaranth seed.

3.6. Immunoanalysis

In order to determine the surface characteristics and surface homologies that the 11S and 7S amaranth may share in common, tests were performed with an expressed anti-amaranth 11S globulin polyclonal antibody (Marcone et al., 1998b). The results indicate that there was approximately 30% cross reactivity between the IgG expressed against the purified 11S amaranth globulin and the 7S globulin. On denaturation of the 7S globulin, antigenicity was completely lost. This would indicate that structural epitopes rather than sequential epitopes (amino acid sequence) are being recognized by the antibody and present on the surface of the 7S globulin.

4. Conclusions

In summary, the 7S amaranth globulin was successfully identified, isolated and purified from the seed of *Amaranthus hypochondriacus* K343 allowing for its characterization and comparison with the more well-studied and more abundant 11S globulin form. The 7S amaranth globulin was found to exist within the seed as a very distinct globulin species and to possess overall molecular weight, subunit composition and charge and immunological characteristics which distinguish it from its 11S globulin counterpart. In keeping with the present method of nomenclature for 75 globulins, the amaranth 75 globulin should be named 'conamaranthin'. Future studies will need to be performed in order to determine the extent to which varying quantities of the 7S globulin occurring due to amaranth variety may have on the overall functional and nutritional properties of amaranth grain.

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