ELSEVIER

Food Chemistry 51 (1994) 287–294

0308-8146/94/$7.00

Purification and characterization of the physicochemical properties of the albumin fraction from the seeds of *Amaranthus hypochondriacus*

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(Received 3 December 1993; accepted 3 February 1994)

The major albumin fraction (i.e. albumin-1) of *Amaranthus hypochondriacus* was isolated and purified to homogeneity, employing a protein solubilization step followed by gel filtration chromatography. The albumin was found to possess a molecular mass of 133400 Da and to be composed of a number of homogeneous low molecular weight subunits, thereby classifying the albumin as a homo-oligomer. The subunits were found to be held together by secondary forces (e.g. hydrogen bond, hydrophobic interactions) with no evidence of any covalent disulfide linkages. Physicochemical tests on the charge profile of the oligomer using electrophoretic isoelectric focusing, electrophoretic titration and zeta potential analysis indicated a pI of 7·50, below which the oligomer was substantially positively charged. Secondary structure analysis revealed that the albumin possessed relatively high levels of β-sheet but very little discernable tertiary structure, as evidenced by relatively low near-UV (i.e. 240–320 nm) circular dichroic band intensities.

INTRODUCTION

Very few edible plants of importance today have had comparable historical/cultural and/or spiritual origins to those of the Amaranthaceae family (Saunders & Becker, 1985). Archaeological and anthropological studies conducted in Peru, Mexico, and the United States have shown that the production of amaranth grain was of importance as early as 8000–4000 BC and was readily incorporated into a variety of religious beliefs and ceremonies. Production of amaranth grain peaked in central America during the Mayan and Aztec periods and then experienced a catastrophic decline due to a legislative ban placed on its production by the Spanish conqueror Cortez in 1519 (Sauders & Becker, 1985; Bressani, 1989).

The resurgence of amaranth grain today stems from its well documented nutritional value, i.e. its high lysine, tryptophan and sulfur amino acid content, in contrast to cereals and legumes (e.g. soybean, navy beans and black beans) which are deficient but widely consumed in developing countries (Teutonico & Knorr, 1985; Paredes-López *et al.*, 1988; Bressani, 1989). More recently, its lysine-rich seed globulin has been shown to have excellent heat-stable emulsification properties, and therefore has extended its usages into more thermally processed foods (Konishi & Yoshimoto, 1989). The nutritional and rheological attributes of amaranth grain in other processed foods are presently being exploited by food manufacturers in the USA and abroad especially in the form of confectionery, cereals (Konishi & Yoshimoto, 1989) and potentially in infant formula products (Sanchez-Marroquin, 1984).

The above desirable characteristics are derived in part from its two most abundant proteins, i.e. the albumins and globulin fractions, which together constitute 46–49% of the total proteins found in the dry seed (Paredes-López *et al.*, 1988). Of these two fractions only the lysine-rich salt-soluble seed globulin has been purified and well investigated (Konishi *et al.*, 1985; Konishi & Yoshimoto, 1989; Marcone & Yada, 1991, 1992).

Very little is known or published about the albumins except for the pioneering work of Konishi *et al.* (1991), who examined the albumins in their crude (non-purified) state. Their work was instrumental in demonstrating the existence of two albumin fractions, i.e. albumin-1 which was extractable with 0·5M NaCl or water and a smaller fraction albumin-2 that was only extractable with water after exhaustive extraction of albumin-1.

The purpose of the present study was to develop a rapid method for the isolation and purification of albu-

min-1 (the major albumin) in high yield and purity as well as the elucidation of the physicochemical properties of this protein in comparison to those of its salt-soluble globulin counterpart.

MATERIALS AND METHODS

Protein isolation and purification

Albumin-1 was extracted from previously defatted non-heated milled flour of *Amaranthus hypochondriacus* K343 with a 32·5 mM K_2HPO_4 – 2·6 mM KH_2PO_4 buffer (pH = 7·50) containing 0·4 M NaCl at a ratio of 1 : 10 (w/v) for 12 h. After centrifugation at 10000 *g* for 30 min at 4°C, the supernatant was dialysed against distilled water for 72 h at 4°C. This mixture was again centrifuged as described above in order to remove precipitated salt-soluble globulin and the supernatant containing crude albumin-1 frozen at –77°C and lyophilized. The chromatographic purification was performed as follows: a Sephacryl™ S-300 superfine (Pharmacia LKB, Montreal, Canada) gel filtration (2·5 cm × 95 cm) column was packed and equilibrated with a 32·5 mM K_2HPO – 2·6 mM KH_2PO_4 buffer (pH = 7·50) containing 0·4 M NaCl and a standard curve obtained using the standard proteins: ferritin (440000 Da), catalase (232000 Da), aldolase (158000 Da) and cytochrome (12400 Da). A 5 ml sample containing 60 mg of crude albumin diluted in the above buffer was applied to the column, and proteins were eluted from the column at room temperature at a flow rate of 37 ml/h. Fractions (5 ml) were collected and protein elution profiles monitored at 280 nm. Protein fractions were individually pooled, dialysed and lyophilized and stored at –77°C under an argon blanket until further study.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

The purified albumin was reconstituted to 5 and 10 mg/ml in a 10 mM Tris-HCl buffer of pH 8·0 containing 1 mM EDTA, 2·5% (w/v) SDS, 5·0% (v/v) β-mercaptoethanol, and 0·1% (w/v) bromophenol blue, and then boiled for 5 min.

Samples (1 μl) were applied to PhastGel gradient polyacrylamide 8–25% SDS-PAGE gels (Pharmacia, LKB). Separation was performed using a PhastSystem Separation and Control and Development Unit (Pharmacia, LKB, Montreal, PQ) according to the manufacturer's instructions (Pharmacia Technical File 110). Gels were stained with PhastGel Blue R (a Coomassie R-350 dye). Linear plots of the log molecular weight versus relative mobility (*Rm*) were constructed.

Native-PAGE electrophoresis

The purified albumin was reconstituted to 10 mg/ml in a 25 mM phosphate buffer of pH = 7·50 and 1 μl applied on to PhastGel 7·5% polyacrylamide gels with native buffer strip. The separations and staining were performed as described by the manufacturer (Pharmacia Technical File 120).

Isoelectric focusing electrophoresis (IEF)

The purified albumin was reconstituted to 10 mg/ml in a 25 mM phosphate buffer of pH = 7·50 and 1 μl applied on to PhastGel 3/9 IEF gels as described by the manufacturer (Pharmacia Technical File 100).

Titration curves

Titration curves were performed by the two-dimensional technique described in the Phast System Technical File 100 using IEF 3/9 gels (Pharmacia LKB, Montreal, PQ). A pH gradient was generated in the first dimension for 150 Vh. The gels were then rotated 90° in the bed and 3·5 μl of a 10 mg/ml protein solution was applied in the second dimension perpendicular to the pH gradient. The voltage was again applied as described in the Phast System Development Technique File 200 (Pharmacia LKB, Montreal, PQ).

Circular dichroism spectroscopy (CD)

Circular dichroic measurements were performed in the far-UV (190–250 nm) on 0·01% protein–buffer solutions (32·5 mM K_2HPO_4–2·6 mM KH_2PO_4), pH = 7·5, using a Jasco J-600 Spectropolarimeter (Japan Spectroscopic Co. Ltd, Tokyo) with a cell pathlength of mm. Secondary structure was determined using the Jasco Protein Secondary Structure Estimation Program (Japan Spectroscopic Co.). Tertiary structure analysis was performed under similar (240–320 nm) conditions except that 1·0 mg/ml was used in the above buffer containing 0·4 M NaCl and measured in a 10 mm cuvette.

Amino acid analysis

Samples (800 μl) containing 0·1 mg/ml of the purified albumin were lyophilized and then hydrolysed 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 concentrated performic acid before a 24 h HCl-hydrolysis in order to estimate cystine and cysteine as cysteic acid. Analysis was performed by applying 30 μl portions of hydrolysed–neutralized samples on to the column.

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

Extinction coefficient

The extinction coefficient $\varepsilon_{280}^{0.1\%}$ of the purified albumin was determined according to the gravimetric procedure

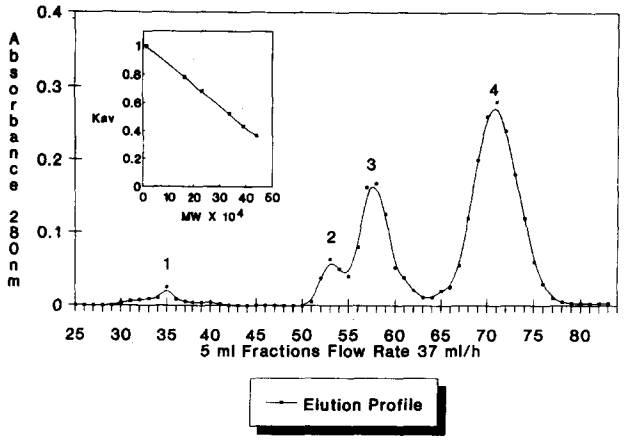

Fig. 1. Sephacryl™ S-300 gel filtration chromatogram of crude albumin (60 mg injected) from *Amaranthus hypochondriacus*. Flow rate was 37 ml/h with a 32·5 mM K_2HPO_4 – 2·5 mM KH_2PO_4 buffer (pH = 7·50) containing 0·4 M NaCl.

of Ellis and Bell (1985). Measurements were performed in triplicate.

Zeta potential

Zeta potential measurements were performed at different pH values, i.e. 3–9, according to the method of Marcone and Yada (1992) using a Laser Zee Meter (Model 501, Pen Kem Inc., Bedford Hills, NY) using 0·1% (w/v) protein solutions. Buffers used were either citrate-phosphate or sodium phosphate of 0·01 ionic strength (μ) depending on the pH of the determination.

RESULTS AND DISCUSSION

Figure 1 depicts a typical elution profile obtained for the crude albumin preparation by gel filtration. Unlike previous purification studies of the amaranth globulin (Konishi *et al.*, 1985; Marcone & Yada, 1991) and other seed storage globulins like soybean (Okubo & Shibasaki, 1967; Marcone *et al.*, 1994) and linseed (Madhusudhan & Singh, 1983), no major strong, turbid, UV-absorbing fraction (i.e. peak 1) was observed near the void volume, K_{av} = 0. This result indicated that the crude albumin preparation was virtually free of the larger molecular weight components (contaminants) customarily present in the above globulin preparations.

Further examination of the chromatogram demonstrated the presence of two other peaks (labelled 2 and 3) which had K_{av} of 0·43 and 0·52 corresponding to apparent molecular weights of 398000 and 337000 Da, respectively, and which were believed to be those of the two heterogeneous forms of the previously studied salt-soluble globulin (Marcone & Yada, 1991). SDS-PAGE confirmed that these proteins were the salt-soluble globulin in question (data not shown) and were a major contamination (33% of the total protein) present in the crude albumin preparation. Any further work by researchers on the characteristics of the crude albumin preparation should take into consideration the presence of the contaminating globulin.

The largest fraction (64% of the total protein in the chromatogram, 23·7 ± 2·0 mg/g defatted flour), i.e. peak 4, with a K_{av} of 0·82, was observed as a well resolved peak and was identified as the albumin having a molecular weight of 133400 Da. It should be noted that this protein was 2·5 times smaller than its previously purified globulin counterpart.

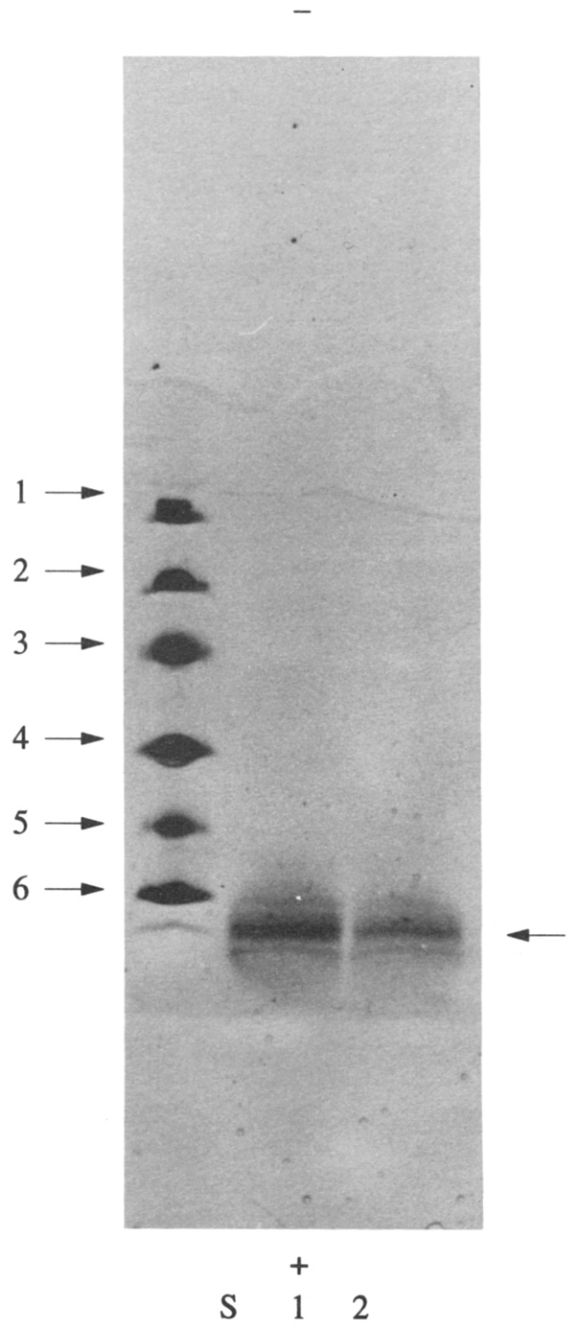

Fig. 2. SDS-PAGE of purified albumin. Lane 1, 10 mg/ml; lane 2, 5 mg/ml applied to gradient 8/25 PhastGels (Pharmacia LKB); Lane S, standards (1) phosphorylase b, 94000 Da, (2) bovine serum albumin, 67000 Da, (3) ovalbumin, 43000 Da, (4) carbonic anhydrase, 30000 Da, (5) soybean trypsin inhibitor, 20100 Da, α-lactalbumin, 14400 Da.

Characterization of the purified albumin on SDS-PAGE under reducing conditions (Fig. 2) demonstrated the presence of a single low molecular weight protein. Although the molecular weight for the protein was determined by extrapolating beyond the limits of the standard curve (i.e. 94000 to 14400 Da), an apparent molecular weight of 12000 Da was estimated. The albumin could, therefore, be classified as a homo-oligomer composed of a repeating low molecular protomer unit and is thought to be composed of approximately 12 subunits (i.e. molecular weight of the oligomer divided by the molecular weight of the monomer). This contrasts with the globulin which was shown to exist as a hetero-oligomer composed of a non-equimolar ratio of five dissimilar polypeptide chains (Marcone & Yada, 1991). When the albumin oligomer was run on SDS-PAGE in the absence of β-mercaptoethanol, no change occurred in the observed electrophoretic pattern (data not shown), thus indicating that it did not possess any interchain disulfide linked subunits (e.g. dimers) which had been shown to be an integral part of the architecture of the amaranth globulin (Marcone & Yada, 1992). In addition, the above results would indicate that secondary forces were responsible for holding the monomers that make up the oligomer together.

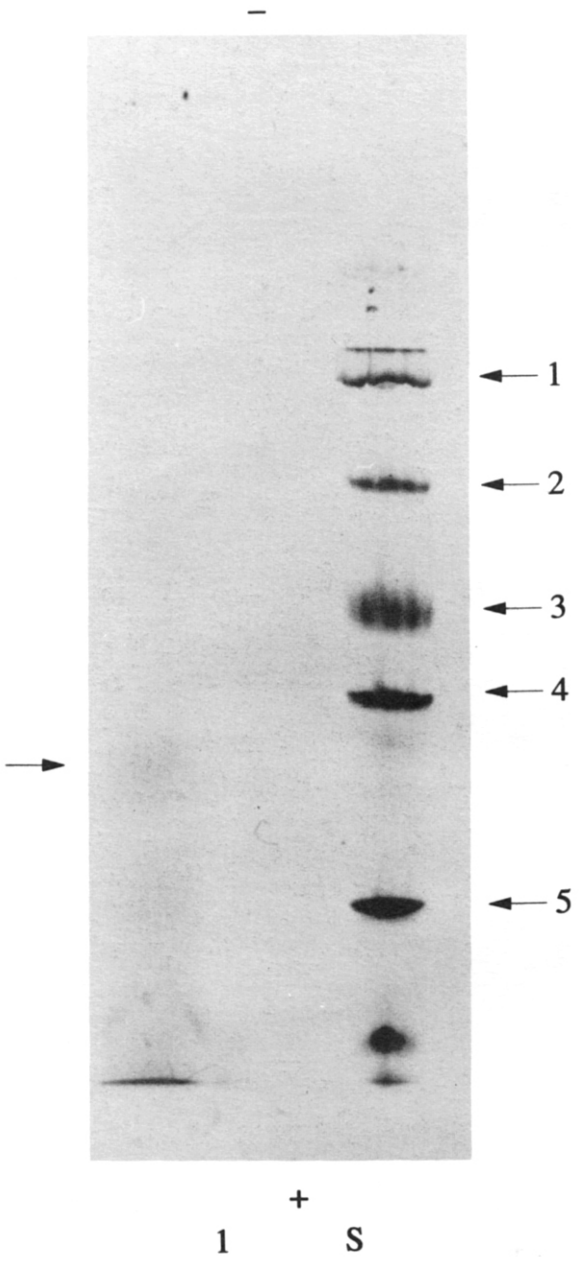

Fig. 3. Native-PAGE (pH = 8·8) (1 μl of 20 mg/ml) of purified albumin. Lane 1, applied to gradient 8/25 PhastGels (Pharmacia LKB); lane S, standards (1) thyroglobulin, 669000 Da, (2) ferritin, 440000 Da, (3) catalase, 232000 Da, (4) lactate dehydrogenase, 140000 Da, (5) bovine serum albumin, 67000 Da.

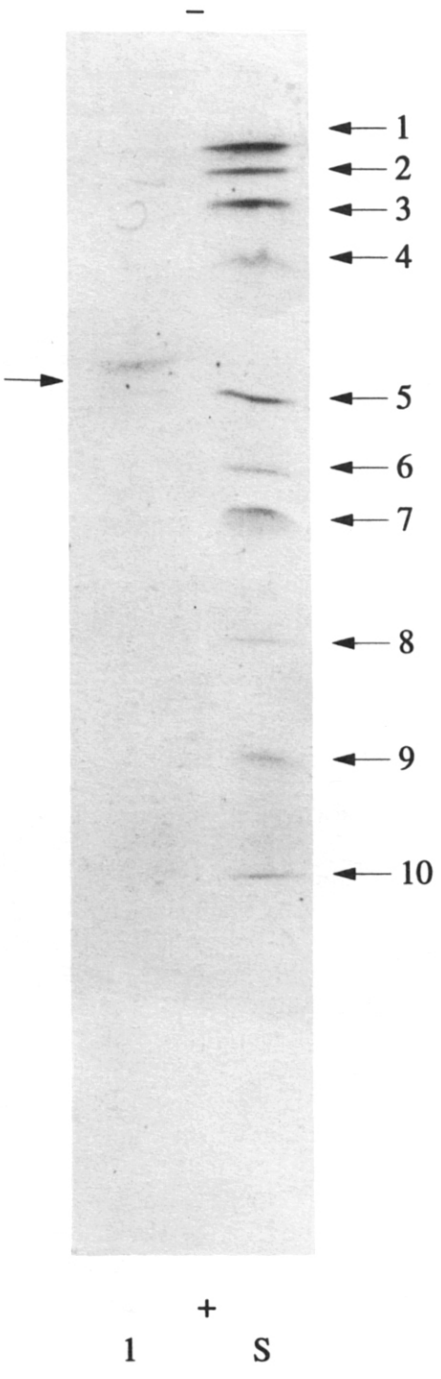

Fig. 4. IEF-PAGE (1 μl of 20 mg/ml) of purified albumin. Lane 1, applied to 3/9 PhastGel IEF (Pharmacia LKB); lane S, standards (1) trypsinogen, 9·30, (2) lentil lectin, 8·65, (3) lentil lectin, 8·45, (4) lentil lectin, 8·15, (5) horse myoglobin, 7·35, (6) horse myoglobin, 6·85, (7) human carbonic anhydrase B, 6·55, (8) bovine carbonic anhydrase B, 5·85, (9) β-lactoglobulin A, 5·20, (10) soybean trypsin inhibitor, 4·55.

Table 1. Circular dichroic secondary structure fractions of the purified seed alubumin of *Amaranthus hypochondriacus*

Fraction[ab]	
α-Helix	12·2 (±0·7)
β-Sheet	41·1 (±1·5)
β-Turn	14·2 (±0·4)
Random coil	32·6 (±0·4)

[a]Expressed as a percentage of the total.
[b]Results are the mean values (± standard deviation) of two replications (six scans per replicate).

Native-PAGE analysis (Fig. 3) demonstrated that the oligomer was susceptible and did undergo an alkaline-pH-induced dissociation, a property which is also a characteristic of the amaranth globulin (Konishi *et al.*, 1985; Marcone & Yada, 1991), and other storage proteins like the llS soybean globulin (Kitamura *et al.*, 1974; Marcone *et al.*, 1994) and the peanut globulin (Cater *et al.*, 1957). This dissociation is in agreement with the high levels of glutamic acid found by amino acid analysis of the purified albumin (Table 1). Under the high pH conditions (i.e. pH 8·8) that the Native-PAGE is run, the secondary carboxylic acid group of this amino acid would be negatively charged and the resultant electrostatic repulsion between non-covalently (disulfide) subunits described above would give rise to this dissociation. Native-PAGE further demonstrated that the major band had an apparent molecular weight of 126000 Da which further confirmed the molecular weight obtained using gel filtration chromatography. It must be noted, however, that molecular weight determinations by Native-PAGE takes into consideration both molecular size as well as native charge, therefore, these factors may account for the minor discrepancy between the values obtained by the two methods used.

Further analysis of the albumin oligomer on electrophoretic isoelectric focusing gels revealed a single band which further confirmed the homogeneity (purity) of the isolated albumin and demonstrated that only one isogenic form of the oligomer exists in the seed (Fig. 4). Isoelectric focusing indicated a pI of 7·50 for the oligomer. Previous investigation of the amaranth globulin by zeta potential analysis revealed that the globulin had a pI of 4·80 (Marcone & Yada, 1992). The difference in pI between the albumin and globulin fractions indicates that their surface charge and surface composition were quite different. In the work of Konishi *et al.* (1991), a pI of 4·5 to 7·5 for the albumin was reported and was probably due to other proteins

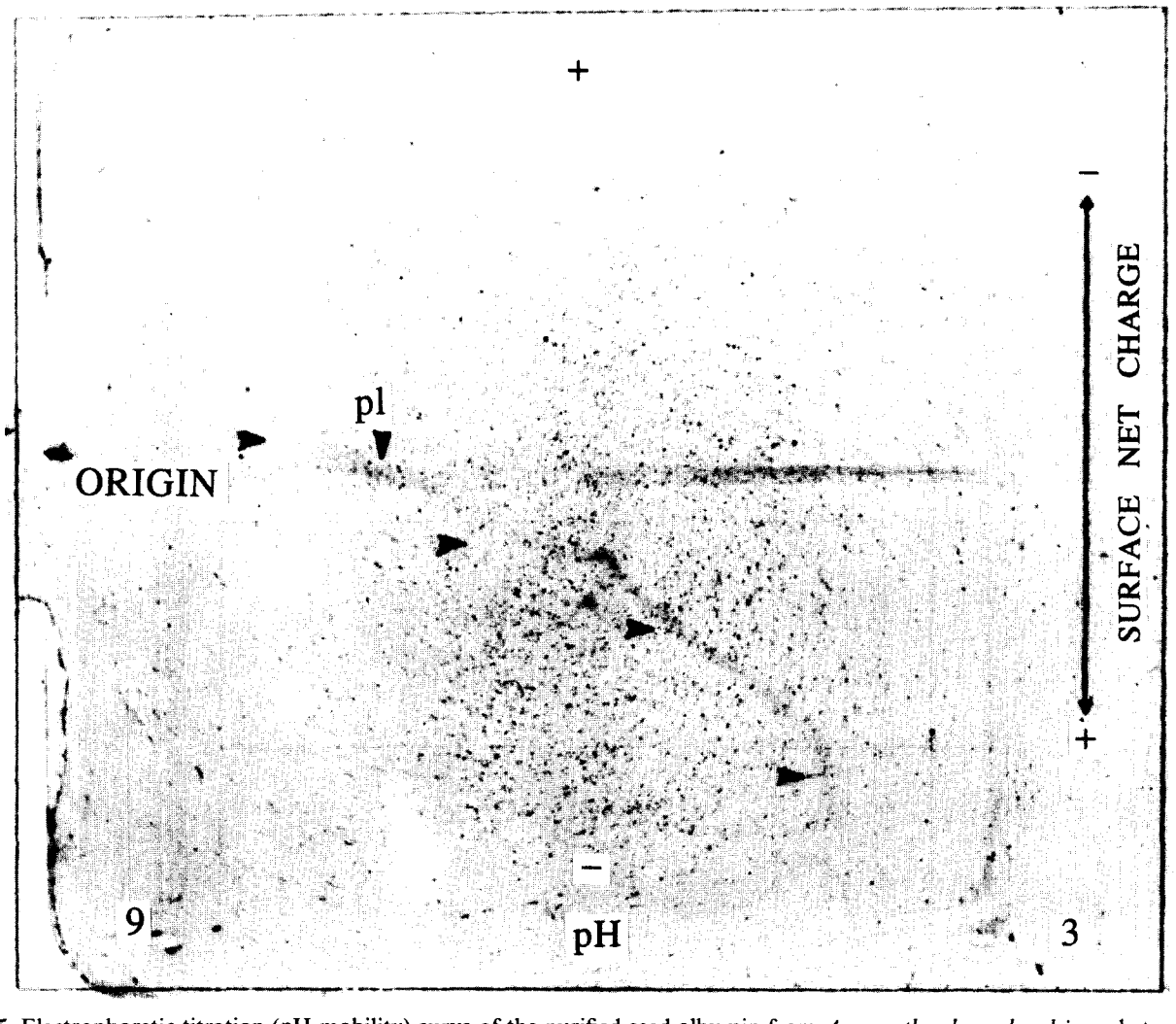

Fig. 5. Electrophoretic titration (pH-mobility) curve of the purified seed albumin from *Amaranthus hypochondriacus* between pH 3 and 9.

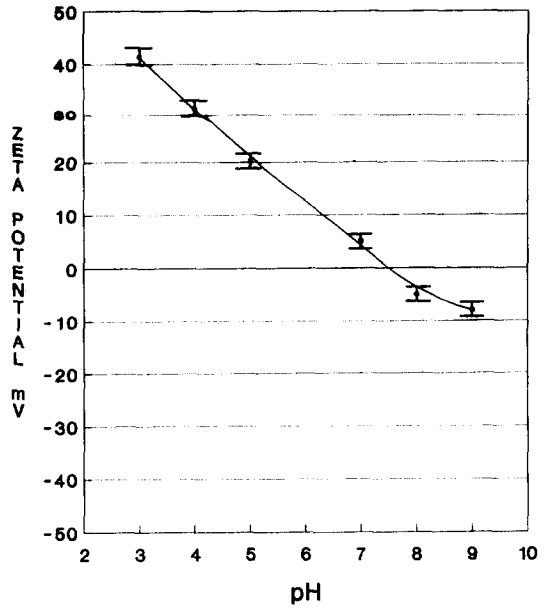

Fig. 6. Effect of pH on the zeta potential of the purified seed albumin from *Amaranthus hypochondriacus*. Error bars indicate the standard deviation of three determinations.

(possibly the globulin as shown earlier which has a pI of 4·80) being present in their crude preparation.

Since charge frequency is considered to be one of the main physical features having the greatest influence on a protein's functional properties (Bigelow, 1967), electrophoretic titration curves and zeta potential analysis were used to examine the surface charge characteristics of the albumin. Previous electrophoretic titration work on the 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 mobility plane, did demonstrate steep slopes which were indicative of a large surface charge density change with change in pH. In the present study a different profile from that of the globulin was observed, i.e. much shallower slopes indicative of a smaller surface charge density change with change in pH (Fig. 5). It was noted that the albumin was much more positively charged at most pH values than its globulin counterpart which was highly negatively charged at the same pH values.

Electrophoretic titration of the albumin also gave a pI of 7·50 (the point where it crosses the origin), and therefore confirms the pI value obtained by one-dimensional isoelectric focusing previously described. Zeta potential analysis also confirmed the above findings obtained by both electrophoretic titration and isoelectric focusing (Fig. 6).

In order to study further the surface properties of the oligomer, tertiary structure analysis using circular dichroism was performed (Fig. 7). Results demonstrated that major differences existed between the albumin and globulin proteins. 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 from the albumin would indicate that they are either distant from one another (Yada & Nakai, 1986) or highly motile compared to the globulin which comparatively speaking gave more intense CD bands (Marcone & Yada, 1992). The derived extinction coefficient for the purified albumin was $\varepsilon_{280}^{0.1\%} = 0.750 \pm 0.05$, which was higher than 0·562 for the globulin despite the fact that amino acid analysis indicated lower levels of aromatic amino acids for the albumin as compared to the globulin. Together, these results indicate that the aromatic acids are more highly motile in the albumin protein.

Analysis of secondary structure spectral data indicated that the oligomer possessed relatively high levels of β-sheet secondary structure (Fig. 8, Table 1). The amaranth globulin had also been shown to possess relatively high levels of β-sheet, i.e. 57·6% (Marcone & Yada, 1991). β-Sheet has a tendency to be buried in the interior of proteins (Cid *et al.*, 1982) and can indicate that some commonality may be shared by the two oligomers.

Amino acid analysis revealed major differences in the amino acid profiles between the purified albumin and globulin proteins (Table 2). The albumin was shown to

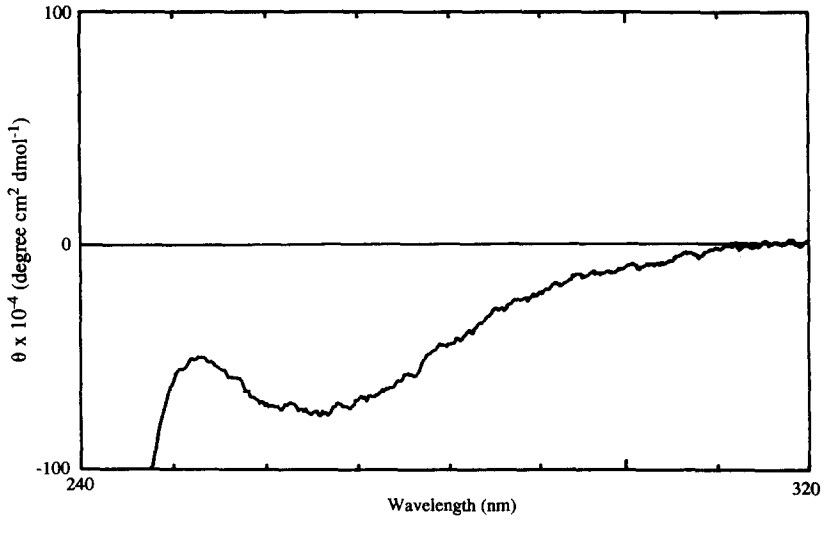

Fig. 7. Near-UV CD spectral scan of the purified seed albumin from *Amaranthus hypochondriacus*.

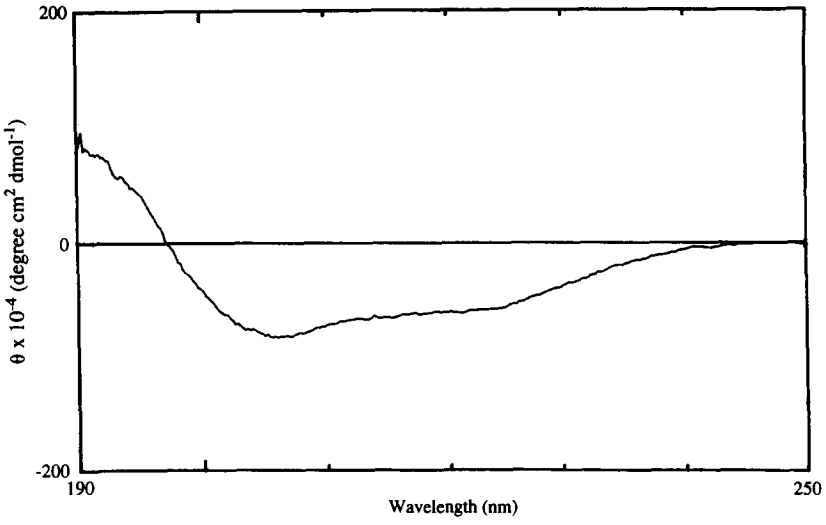

Fig. 8. Far-UV CD spectral scan of the purified seed albumin from *Amaranthus hypochondriacus*.

have high levels of the acidic amino glutamic acid like the globulin but to have comparably lower levels of lysine. This is of a nutritional concern. On the other hand, the albumin was shown to have higher levels of the sulfur containing amino acids, i.e. nine times more cysteic acid and 1·3 times more methionine than the amaranth globulin.

In the present study, the major albumin fraction (i.e. albumin-1) of *Amaranthus hypochondriacus* was isolated and purified to homogeneity employing a protein solubilization step and gel filtration chromatography, and its physicochemical properties were determined and compared to the globulin counterpart. The albumin was found to possess a molecular mass of 133400 Da and to be composed of a number of homogeneous low molecular weight subunits, thereby classifying the albumin as a homo-oligomer. The subunits were found to be held together by secondary forces (e.g. hydrogen and hydrophobic bonds) with no evidence of any covalent disulfide linkages. Physicochemical tests on the charge profile of the oligomer using electrophoretic isoelectric focusing, electrophoretic titration and zeta potential analysis indicated a pI of 7·50 below which the oligomer was substantially positively charged. Secondary structure analysis revealed that the albumin possessed relatively high levels of the β-sheet but had very little discernable tertiary structure, as evidenced by relatively low near-UV (i.e. 240–320 nm) circular dichroic band intensities. Identification of the physicochemical properties of the albumin will undoubtedly lead to a better understanding of how best to utilize these proteins to meet the best nutritional and functional properties in food. Since the solubility of water-soluble proteins is related directly to many protein functions (e.g. emulsification and foaming capacity/stability), the use of highly soluble proteins of the *Amaranthus* species would be certainly advantageous to the food industry.

Table 2. A comparison of the amino acid composition of the purified albumin and purified globulin

Amino acid	Fraction[a]	
	Globulin[b]	Albumin
Asx[c]	8·76	4·79 (0·01)
Thr[d]	4·02	3·57
Ser[d]	6·90	4·14
Glx[e]	15·29	19·12 (±0·34)
Pro	6·14	26·44 (±0·52)
Gly	8·00	8·86 (±0·18)
Ala	5·71	2·79 (±0·01)
Cys[f]	0·93	8·40 (±0·01)
Val[d]	6·29	2·20 (±0·04)
Met	1·77	2·35 (±0·04)
Lle[d]	5·01	2·01 (±0·03)
Leu	6·82	2·26 (±0·04)
Tyr[d]	2·13	1·85
Phe	6·78	1·27 (±0·04)
Lys	4·25	2·78 (±0·04)
His	2·62	1·15 (±0·06)
Arg	10·12	6·42 (±0·11)

Results are the mean values (± standard deviation) of three replications.
Tryptophan was not determined.
[a]Reported in mol.%.
[b]Results obtained from Marcone and Yada (1991).
[c]Asx = Asp + Asn.
[d]Extrapolation from 24, 48 and 72 h hydrolysis times.
[e]Glx = Glu + Gln.
[f]Cystine and cysteine determined as cysteic acid by performic acid treatment.

ACKNOWLEDGMENT

The financial assistance of the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

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