Study of the Charge Profile and Covalent Subunit Association of the Oligomeric Seed Globulin from *Amaranthus hypochondriacus*

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The physicochemical properties of the oligomeric salt-soluble seed globulin from *Amaranthus hypochondriacus* were examined in relationship to its surface charge and subunit association. The globulin was found to undergo substantial change in surface charge density as a function of pH. Aliphatic hydrophobicity was greatest in the pH range 4.5–6.8 and was attributed to changes in tertiary structure resulting in exposure of interior aliphatic amino acids. The p*I* of the globulin was determined to be 4.80, which corresponded to the point of highest aliphatic surface hydrophobicity. The globulin was composed of specific subunits linked through disulfide bridges to form dimers. Two distinct sedimenting populations indicating the occurrence of different hybrids or differing polymorphic forms of the globulin were observed.

INTRODUCTION

The lysine-rich salt-soluble globulin has been shown to be the main seed storage protein in *Amaranthus hypochondriacus* (Konishi et al., 1985; Bressani and Garcia-Vela, 1990). An earlier study (Marcone and Yada, 1991) indicated that the globulin existed in two heterogeneous forms with respect to molecular mass, i.e., a major and a minor form of 337 000 and 398 000 daltons (Da), respectively. The latter was found to exist as a polymeric species derived in part from a sulfhydryl–disulfide interchange reaction. The former, which is the more abundant species, however, existed as a hetero-oligomer consisting of a tetrameric and hexameric moiety held together by weak secondary forces and was readily dissociated under alkaline pH conditions.

Renewed interest in the amaranth globulin stems not only from its well-documented nutritional value (Bressani and Garcia-Vela, 1990; Mora-Escobeda et al., 1990) but also from its recent recognition as an excellent heat-stable emulsifier (Konishi and Yoshimoto, 1989). Although pertinent information on the hydrophobicity of the globulin now exists (Konishi and Yoshimoto, 1989), it is the combination of both hydrophobicity and charge frequency that constitutes the two main physical features having the greatest influence on such functional properties as solubility (Konishi and Yoshimoto, 1989; Bigelow, 1967). It was, therefore, the purpose of this study to examine the surface charge characteristics of the molecule and to further examine its structure in relation to its covalent subunit association.

EXPERIMENTAL METHODS

Protein Isolation and Purification. Non-heat-treated milled flour of *A. hypochondriacus* K343 was purchased from American Amaranth Inc. (Bricelyn, MI) and the globulin isolated and purified to homogeneity by gel filtration and anion-exchange chromatography and stored desiccated at −20 °C under an argon blanket until further study as previously described (Marcone and Yada, 1991).

Determination of Protein Concentration. Protein concentrations were determined as previously described (Marcone and Yada, 1991).

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). A pH gradient was generated in the first dimension for 150 V h. The gel was then rotated by 90° in the bed and 3 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).

Zeta Potential. Zeta potential measurements were made at different pH values, i.e., 3–9, according to the method of Hayakawa and Nakai (1985) using a Laser Zee meter (Model 501, Pen Kem Inc., Bedford Hills, NY). Buffers used were either citrate–phosphate or sodium phosphate of 0.01 ionic strength (μ) depending on the pH of the determination.

Five milliliters of a 0.08% (w/v) protein solution and 150 μL of 3,3′-dimethylbiphenyl (Aldrich, Milwaukee, WI) were emulsified with the use of a Tekmar Tissumizer (Tekmar Co., Cincinnati, OH) with a Tekmar Model TR-10 power unit set at an output of 35 for 30 s. Aliquots (0.5 mL) of the protein/carrier emulsion were added to 40 mL of the appropriate buffer and measurements determined in triplicate. Readings were corrected for temperature as indicated by the manufacturer.

UV Spectroscopy. Absorbance scans were generated using a Shimadzu UV-260 recording spectrophotometer (Tekscience, Mississauga, ON), between the wavelengths of 240 and 310 nm. Protein was dissolved in $\mu = 0.01$ phosphate buffers at pH values of 5.50 and 7.50 to a concentration of 0.8 mg/mL, filtered, and allowed to equilibrate for 30 min at 22 °C prior to spectroscopy. Scans were performed in triplicate.

Circular Dichroism Spectroscopy (CD). Circular dichroism measurements were carried out in the near-UV (240–320 nm) at 20 °C under constant nitrogen purge using a Jasco J-600 spectropolarimeter (Japan Spectroscopic Co. Ltd., Tokyo) with cell path length of 10 mm. A protein concentration of approximately 1.0 mg/mL in phosphate buffers of pH 5.50 and 7.50 with NaCl added to a final ionic strength of 0.5 was used in the determinations.

Extinction Coefficient. The extinction coefficient $E_{280}^{0.1\%}$ of the purified globulin was determined according to the gravimetric procedure of Ellis and Bell (1988). Measurements were performed in triplicate.

Surface Hydrophobicity (So). Both aliphatic and aromatic surface hydrophobicities of the globulin were determined using *cis*-parinaric acid (CPA) and 1-anilino-8-naphthalenesulfonate (ANS), respectively, as a function of pH according to the methods of Hayakawa and Nakai (1985) and Akita and Nakai (1990) using protein concentrations of between 0.001 and 0.029% (w/v) in the buffers described earlier. Excitation wavelengths of 355 and 380 nm and emission wavelengths of 415 and 475 nm for CPA and

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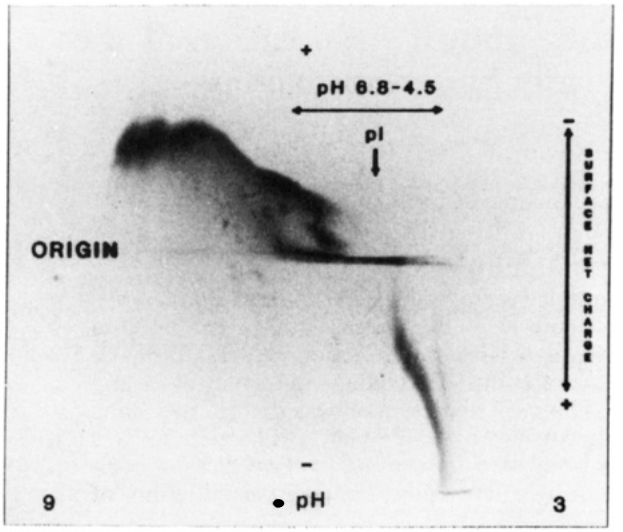

Figure 1. Electrophoretic titration (pH–mobility) curve of the oligomeric seed globulin from *A. hypochondriacus* between pH 3 and 9.

ANS were used, respectively. Intrinsic fluorescence was performed with an excitation wavelength of 355 and emission of 415 nm.

Analytical Ultracentrifugal Analysis. Sedimentation velocity measurements were performed at 20 °C with a Beckman Model E analytical ultracentrifuge at 44 000 rpm (140600*g*) equipped with a A_{280} photoelectric scanner. Samples were dissolved in and dialyzed against a 32.5 mM K_2PO_4–2.6 mM KH_2PO_4 buffer (pH 7.50) containing 0.4 M NaCl and 0.02% (w/v) NaN_3.

Sodium Dodecyl Sulfate–Polyacrylamide Electrophoresis (SDS–PAGE). The purified globulin was reconstituted to 3 mg/mL in a 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 2.5% (w/v) SDS, and 0.1% (w/v) bromophenol blue, with (5%) and without β-mercaptoethanol, as described by Bhushan and Redding (1989).

One-microliter aliquots were applied to PhastGel gradient polyacrylamide (PAA) 8–25% SDS–PAGE gels (Pharmacia LKB). Separations were performed using a PhastSystem separation and control and development unit (Pharmacia LKB) according to the manufacturer's instructions. Gels were subsequently scanned using a Zeineh Model SLR-540-XL soft laser scanning densitometer interfaced with a Zelneh Videophoresis II electrophoresis reporting integrator program (ERIS) from Biomed Instruments Inc. (Fullerton, CA). Linear plots of the log molecular weight vs relative mobility (R_m) were prepared with phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 000) as standards.

Slab gel electrophoresis was also performed using the SDS–PAGE system of Laemmli (1970) without β-mercaptaethanol on 6.5% polyacrylamide running gels. The bands were carefully excised after visualization with a nonfixative copper chloride staining (Lee et al., 1987) and quantitatively recovered by electroelution in a 0.04/0.004 M Tris–acetate buffer (pH 8.40) using an ISCO Model 1750 concentrator (Canberra Packard, Toronto, ON). Electrophoretic elution was carried out at 12 °C for 8 h at 12 W per the manufacturer's instructions.

RESULTS AND DISCUSSION

Electrophoretic titration data for the globulin showed a characteristic precipitation in the pH range 4.5–6.8 (Figure 1), which was consistent with the spectroturbidity work performed by Konishi et al. (1985). Further examination of the titration data revealed that maximum insolubility occurred in the pH range 4.5–6.0 and that partial precipitation occurred between pH 6.0 and 6.8. This partial precipitation could be attributed to the protein existing in differing ionizable states (Haff et al., 1983).

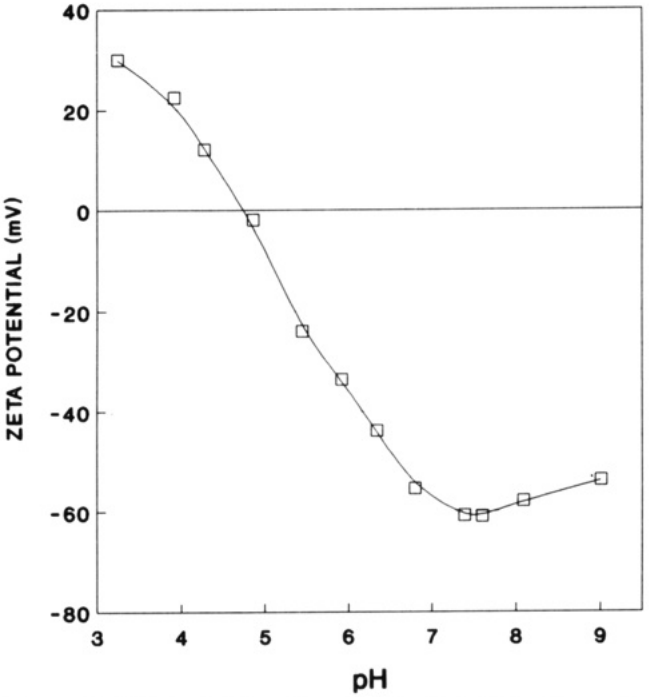

Figure 2. Effect of pH on the zeta potential of the oligomeric seed globulin from *A. hypochondriacus*.

Conversely, the observed increase in solubility as demonstrated in the electrophoretic titration curve below pH 4.5 could be attributed to the p*K* values of 4.25 and 3.86 of the ionizable side chains of the glutamic and aspartic acid residues, respectively, and the increase in positive charge arising from the amino acids such as lysine, arginine, and histidine.

Although no direct reading of the isoelectric point could be made, i.e., the point where the titration curve crosses the origin (zero mobility plane), the steep slopes of the curve were indicative of a large surface charge density change with change in pH (Haff et al., 1983).

Zeta potential measurements demonstrated a dramatic increase in negativity with increasing pH (Figure 2). This result was consistent with the titration data and with earlier findings which showed that the globulin contains high levels of acidic amino acids, namely aspartic and glutamic acids (Marcone and Yada, 1991). Since proteins display essentially the same mobility as free molecules as they do when absorbed on a carrier (Hiemenz, 1986), extrapolation from the zeta potential curve indicated an isoelectric point (p*I*) of 4.80 (zero zeta potential) for the globulin. The latter is consistent with the p*I* reported by Konishi et al. (1985), who used a spectroturbidimetric method. In addition, the above result is consistent with the well-known fact that many proteins show minimum solubility at zero zeta potential (Hayakawa and Nakai, 1985; Nakai and Li-Chan, 1988).

The noted drop in negativity or electrical potential of the globulin (Figure 2) could result from nonhomogeneous charge distribution brought about by the dissociation of the oligomer into its hexameric and tetrameric moieties at alkaline pH. A similar leveling off and slight drop in the electrophoretic titration curve was also observed (Figure 1) and may be attributed to the availability of charged amino acids brought about by any protein unfolding induced at alkaline pH (Donovan, 1976).

In light of previous work which examined the surface hydrophobic characteristics of the globulin (Konishi and Yoshimoto, 1989) and the above results (i.e., charge), it is postulated that localized sites (clusters) of hydrophobic

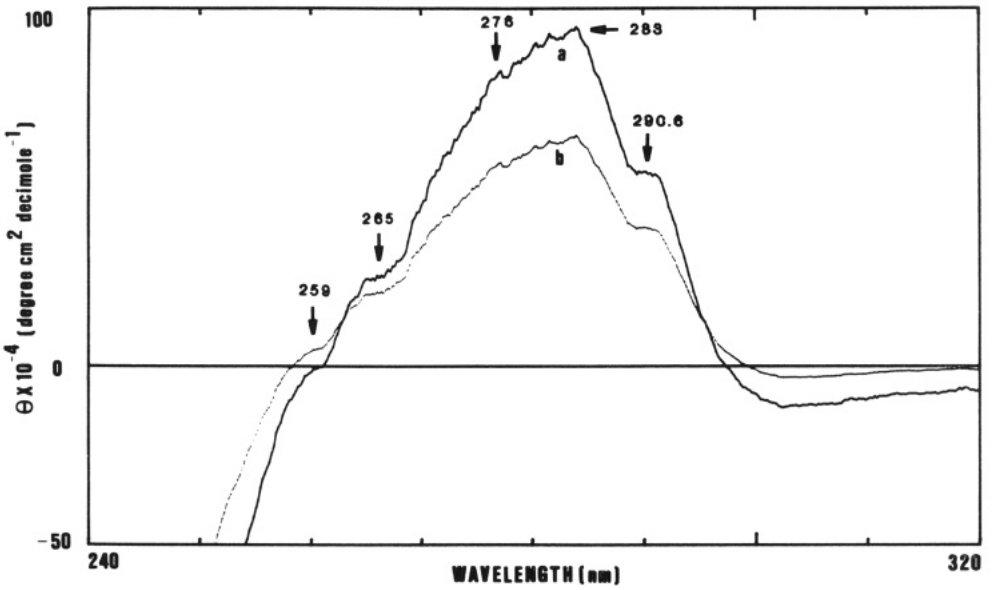

Figure 3. Near-UV CD spectral scans of globulin at pH (a) 5.50 and (b) 7.50.

groups as well as charged groups exist on the surface of the molecule. The above-noted precipitation in the pH range 4.5–6.8 could then be attributed to the regions or clusters of hydrophobic residues on the molecule's surface suppressing any regions of surface charge, and thereby causing hydrophobic association and subsequent aggregation of the molecules. To verify if surface hydrophobicity was indeed changing as a function of pH, two fluorescent probes, *cis*-parinaric acid (CPA) and 1-anilino-8-naphthalenesulfonate (ANS), were used over the above range. The ANS probe proved unsuccessful in measuring aromatic hydrophobicity, probably due to the large amount of surface charge groups which have been reported to restrict the binding of this probe (Yada, 1984). In fact, the derived extinction coefficient for this protein was quite low, i.e., $E_{280}^{0.1\%} = 0.562 \pm 0.005$, indicating that the protein did not contain high levels of surface aromatic amino acids. UV absorption spectra at pH 5.50 revealed a slight but consistent blue shift (to shorter wavelengths) by 1.8 nm as compared to the spectrum at pH 7.50, suggesting subtle changes in the tertiary structure of the protein.

Since near-UV CD spectra reflect changes in the tertiary structure of protein (Strickland, 1974) brought about by the interaction and orientation of the aromatic rings of tyrosine, tryptophan, and phenylalanine with other amino acid moieties in the protein, near-UV CD spectra of the globulin were investigated at pH 5.50 and 7.50 (Figure 3). Identification of the fine structure of the individual aromatic groups became difficult in view of the low intensities of CD spectral scans. Tentative identification of the fine structure of phenylalanine at both pH values was made at 259 and 265, whereas the 0–0-cm^{-1} ^1L_b of tryptophan was assigned to 290.6 nm although the 0+850-cm^{-1} ^1L_b band was barely visible. Tyrosine showed its characteristic 0+800-cm^{-1} band at 276 nm with the 0–0-cm^{-1} ^1L_b band of tryptophan being identified at 291 nm but lacked the 850-cm^{-1} ^1L_b band at 283 nm.

Since intense CD bands for the 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 globulin would indicate that they are either distant from one another (Yada and Nakai, 1986), highly motile (Strickland, 1974), or in low quantities. The appearance of a negative CD band for phenylalanine (259 nm) at pH 5.50 would indicate a change in the relative position of two or more aromatic amino acids and may be the cause of the observed blue shift in UV spectroscopy. The general increase in the intensity or amplitude of the spectra at pH 5.50 combined with the appearance of a negative band for phenylalanine is indicative of a change in tertiary structure.

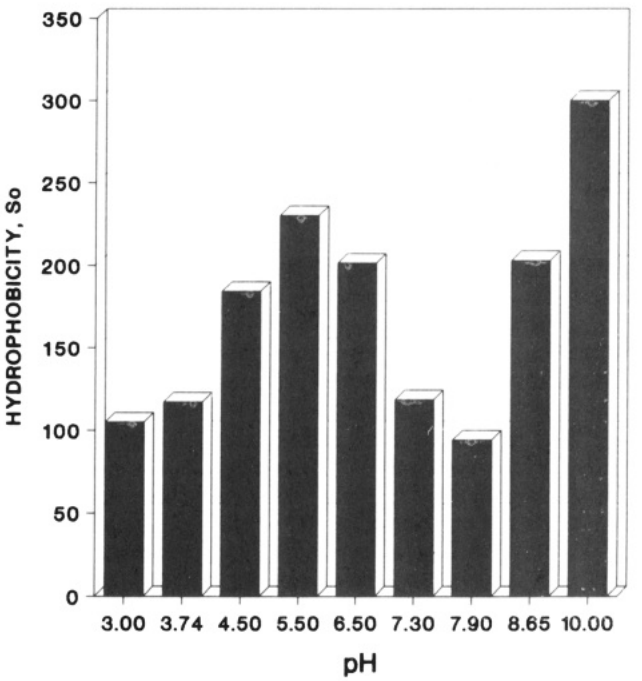

Figure 4. CPA surface hydrophobicity of the globulin at differing pH values.

Unlike the aromatic amino acids, the less hydrophobic aliphatic amino acids are found both at the surface and in the interior of the molecule due to their smaller size and greater elasticity (Akita and Nakai, 1990). CPA, a measure of aliphatic hydrophobicity, showed a local maximum (pH 5.50) in the region of precipitation, i.e., pH 4.5–6.8 (Figure 4). A similar profile was also found for intrinsic fluorescence spectra, which is also an indicator of hydrophobicity (data not shown). Although only aromatic hydrophobicity has been correlated with protein insolubility (Hayakawa and Nakai, 1985), the observed precipitation may have been caused by a combination of subtle changes in tertiary structure arising from changes in the environment around the aromatic amino acids coupled with changes in the level

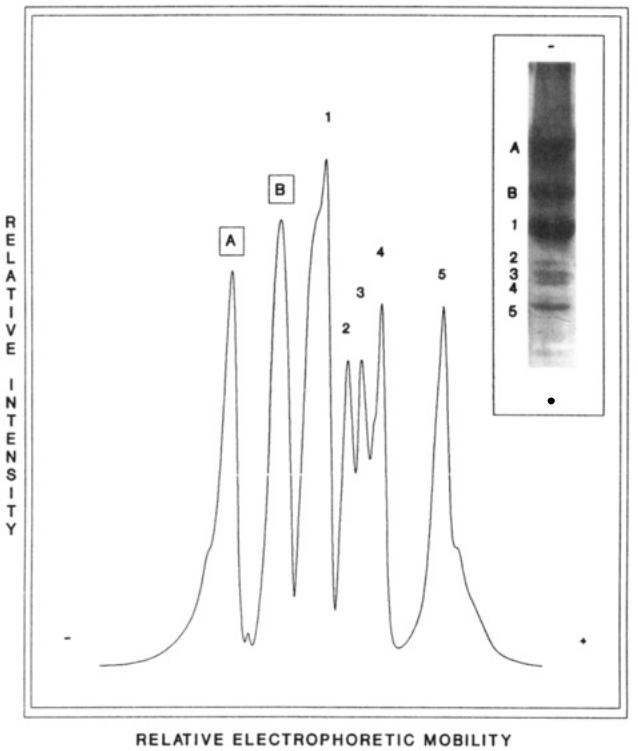

Figure 5. Densitometric scans of 8–25% SDS–PAGE gel of the oligomeric seed globulin from *A. hypochondriacus* performed in the absence of a reducing agent and photographic inlay of a homogeneous 6.5% slab gel performed in a similar manner.

of aliphatic hydrophobicity brought about by conformational changes exposing the more hydrophobic core. It is interesting to note that CPA measurements correlated well with protein functionality, i.e., with the emulsification activity vs. pH data reported by Konishi et al. (1989).

In an effort to better understand the intrinsic interactions that exist between the subunits of the oligomer, with particular interest in the arrangement of disulfide bonds in the subunit association, SDS–PAGE was performed under nonreducing conditions. Two higher molecular weight intermediate subunits of molecular mass of 75 500 and 46 500 Da (not present under the corresponding reducing conditions) were revealed and designated bands A and B, respectively (Figure 5). The bands were carefully excised from preparative 6.5% gels, after their visualization with a nonfixative copper chloride staining, and quantitatively recovered by electroelution.

Further investigation revealed that the 46 500-Da subunit (band B) was in fact composed of a 37 500- and 14 500-Da subunits linked covalently through disulfide bonds to form a dimer. Whereas (band A) the 75 500-Da subunit was composed mainly of a dimer of 37 500-Da subunits, traces of a 67 000-Da subunit (Figure 6) were also found (Table I). Interestingly, the 67 000-Da subunit was found to be a disguising subunit in the polymerized form of this protein designated 2A in a previous study (Marcone and Yada, 1991).

The cross-linking of constituent units through covalent disulfide bonds into dimers would have the effect not only of giving extra stability to the globulin to environmental perturbations (Schulz and Schirmer, 1979) but also of reducing the degree of oligomeric hybridization and consequently the number of quaternary species possible (Klotz et al., 1975). Analysis of the purified globulin by analytical ultracentrifugation revealed the existence of two distinct populations of molecules which may be due to the ability of the sedimentation velocity technique to

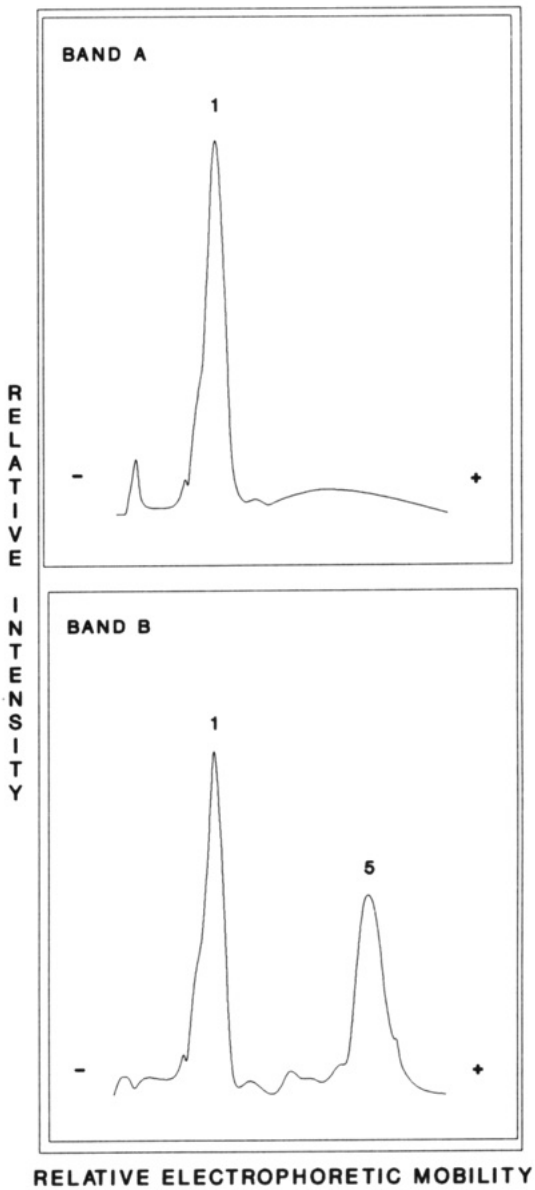

Figure 6. Densitometric scans of SDS–PAGE gels performed in the presence of a reducing agent, i.e., β-mercaptoethanol, of bands A and B extracted from the gel represented in Figure 3.

Table I. SDS–PAGE Molecular Mass (Daltons) Distribution of Subunits

band	reducing[a,b]	nonreducing[a,c]	extraction and reduction[a,b]
A	*d*	75 500	37 500 and 67 000 (trace)
B	*d*	46 500	37 500 and 14 500
1	37 500	37 500	
2	31 500	31 500	
3	26 500	26 500	
4	20 500	20 500	
5	14 500	14 500	

[a] Molecular weights reported to the nearest 500 Da (see Figure 5). [b] Performed in the presence of β-mercaptoethanol. [c] Performed in the absence of β-mercaptoethanol. [d] Band not recovered.

separate molecules of similar size but differing shapes (Chervenka, 1970) or differing quaternary structures (Figure 7). A polymorphic composition of subunits cannot be discounted. The purified globulin was shown to sediment at 11.53 S, which is consistent with the 11.7 S obtained with schlieren optics by Konishi et al. (1985) for a crude globulin preparation.

Dimerization between specific subunits in the 11S soybean globulin has already been reported (Koshiyama, 1983). Since covalent (disulfide) association between

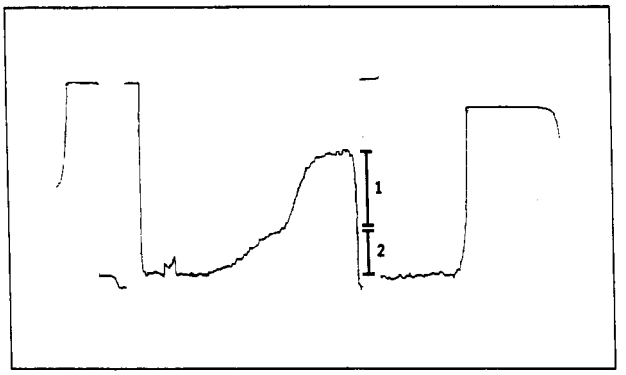

Figure 7. Analytical ultracentrifugal trace of the globulin in phosphate buffer, μ = 0.5, pH 7.50, taken at 44 000 rpm demonstrating the two globulin species.

soybean subunits has been demonstrated to occur in a nonrandom fashion and is probably governed by specific genetic traits (Staswick et al., 1983), it would be interesting to conjecture that a similar level of control also exists in the amaranth globulin.

Nonreducing SDS–PAGE further revealed that the subunits making up the hexameric and tetrameric moieties of the oligomer, which had previously been shown to undergo an alkaline-induced dissociation (Marcone and Yada, 1991), were themselves held together in part by secondary forces such as hydrogen, ionic, hydrophobic, and/or van der Waals types of interactions (Figure 5). Since oligomers are in a dynamic state of dissociation and reassociation (Schulz and Schirmer, 1979; Klotz et al., 1975), these weak secondary forces would facilitate the rapid exchange of subunits with the elimination of defective ones especially at the time of biosynthesis and assembly into a quaternary ensemble.

As with the 11S soybean globulin, a degree of variability in the composition of the amaranth globulin has already been recognized and attributed to genetic differences (Mora-Escobeda et al., 1990). It is with a better understanding of the structure and charge profile of the globulin that molecular genetic strategies may be initiated to modify the globulin's composition in efforts to improve its functional properties without decreasing its nutritive value.

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