

Denaturant-Induced Conformations of Globulins

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The effects of denaturation of amaranth globulins (A-G) by urea, guanidine hydrochloride (GuHCl), and sodium dodecyl sulfate (SDS) were studied. Progressive unfolding of globulins was measured as a function of fluorescent light intensity, peak response, and shift in the maximum emission wavelength. Decreases in fluorescence intensity and shift in maximum emission wavelength (from 341.5 to 355.0 nm) were shown using the fluorescence of tryptophan at 295 nm. Kinetic measurements showed that the decrease of fluorescence intensity was faster with SDS than with GuHCl and exhibited a double exponential decay pattern. Differences in secondary and tertiary structures during denaturation were observed, and changes in α -helical content and position of aromatic groups were calculated. The A-G were compared with quinoa globulins (Q-G). Comparison of relative structural stability of native globulins by intrinsic fluorescence and circular dichroism measurements showed that A-G are relatively stable in comparison with Q-G and bovine γ -globulins.

Keywords: *Amaranth; conformation; denaturation; globulins; proteins; quinoa; structure*

INTRODUCTION

The globulins represent the main storage proteins in legumes, oats, tubers, amaranth, and quinoa (Wright, 1987; Gorinstein et al., 1991; Paredes-López et al., 1994; Segura-Nieto et al., 1994). There are several published studies on the isolation and purification of amaranth (Gorinstein et al., 1991; Segura-Nieto et al., 1994), soybean and pea (Wright, 1987), and quinoa (Becker and Hanners, 1990; Brinegar and Goundan, 1993) globulins. These studies are based mostly on electrophoretic patterns, molecular weights, amino acid compositions, and sedimentation coefficients, and partly on physicochemical and functional properties, such as solubility, hydrophobicity, and emulsifying activity (Romero et al., 1985; Barba de la Rosa et al., 1992; Marccone and Yada, 1992; Gorinstein, 1993; Paredes-López et al., 1994; Gorinstein et al., 1996).

Amaranth (*Amaranthus*) and quinoa (*Chenopodium quinoa*) belong to the class of pseudo-cereals. Quinoa (11.0–13.7%), like amaranth (14.8–16.6%), contains more protein and a better balanced amino acid composition than the major cereals (Risi and Galway, 1984; Romero et al., 1985; Gorinstein et al., 1991; Brinegar and Goundan, 1993). There are mainly three species of amaranth that produce nutritive seeds with potential to become a cereal-like grain crop: *Amaranthus* (*A. caudatus*, *A. cruentus*, and *A. hypochondriacus*). The *A. cruentus* species have the lowest crude protein content (13.2–18.2%) compared with *A. caudatus* (17.6–18.4%) and *A. hypochondriacus* (17.9%). The lysine content of the amaranth species is relatively high (3.2 to 6.4%)

compared with that found among the most common cereals (2.2 to 4.5%), and the sulfur amino acid concentration (2.6 to 5.5%) is higher than that of the most important legumes (1.4%), such as pea, beans, soybeans, etc. These characteristics make amaranth a promising crop as a food or source of dietary proteins (Bressani and Garcia-Vela, 1990; Gorinstein et al., 1991; Paredes-López et al., 1994; Segura-Nieto et al., 1994).

According to our investigations and literature data (Risi and Galway, 1984; Becker and Hanners, 1990; Gorinstein et al., 1991, 1996; Barba de la Rosa, 1992), amaranth and quinoa have different proportions of salt-soluble proteins (albumins and globulins). Most of the results show that albumins and globulins represent 39.1–82.0% and 74.1–79.9% of the total seed proteins for amaranth and quinoa, respectively. Albumins are the main fraction, and globulins constitute only 13.7–18.1% of the total seed proteins, depending on the different extraction procedures used (Bressani and Garcia-Vela, 1990; Gorinstein et al., 1991; Barba de la Rosa et al., 1992; Segura-Nieto et al., 1994). The ratio between albumins and globulins in amaranth varies from 0.48 to 3.6. The globulin fraction from amaranth contained some heterooligomers with high molecular weight (MW) of 50–73 kDa that might be related to the 7S type; polypeptides with an average MWs of 31.5–38.0 and 20–27 kDa that can be related to the 11S type; and some other components with MWs of <18 kDa (Gorinstein et al., 1991, 1996; Barba de la Rosa et al., 1992; Marccone and Yada, 1992; Segura-Nieto et al., 1994). Globulins from quinoa had polypeptides with MWs of 8, 22–39, and 50 kDa. The polypeptides in the 8–9 kDa group are similar in size to the 2S type. The 11S type of seed proteins (Brinegar and Goundan, 1993) was represented by two classes of polypeptides (30–40 and 20–25 kDa).

The important role of legume and amaranth globulins as functional agents in food products has also been shown (Konishi and Yoshimoto, 1989; Marccone and Yada, 1992). Renewed interest in amaranth was based

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not only on its well-documented nutritional value (Bresani and Garcia-Vela, 1990; Segura-Nieto et al., 1994), but also on its property to serve as a heat-stable emulsifier (Konishi and Yoshimoto, 1989; Marcone and Yada, 1992).

It is difficult to follow the conformational changes of large proteins, such as globulins, in food systems because of problems with solubilities and lack of biological activities (Arntfield et al., 1987; Gruen et al., 1987). Differential scanning calorimetry (DSC), circular dichroism (CD), and intrinsic fluorescence (IF) techniques were used to show the denaturation effects of pH, temperature, and denaturants on fababean proteins, vicilin, pea seed albumin, amaranth, and lupin proteins (Lilley, 1986; Arntfield et al., 1987; Gruen et al., 1987; Gorinstein et al., 1996). There also are investigations showing comparisons among soybean, oats, and amaranth in relation to nutritional, structural, and functional properties (Gorinstein, 1993; Marcone and Yada, 1992; Segura-Nieto et al., 1994). To date, there is no information on the relative stability and structure of cereal-like plant proteins, such as amaranth and quinoa, which can be used as dietary proteins in food systems.

The present study focuses on the denaturant-induced secondary and tertiary structural changes that occur during amaranth globulin (A-G) denaturation as determined by CD spectroscopy. Measurements of fluorescence intensity plus wavelength of the peak output response and kinetic studies were also used to monitor the unfolding of globulins from the native structure to random coil. Quinoa globulins (Q-G) and bovine γ -globulins (γ -G) were compared with A-G.

MATERIALS AND METHODS

Materials. Guanidine hydrochloride (GuHCl), urea, sodium dodecyl sulfate (SDS), 2-mercaptoethanol (2-ME), and bovine γ -G were reagent grade chemicals from Sigma Chemical Co. Deionized water was used throughout.

Sample Preparation. Whole mature seeds of amaranth [*Amaranthus (A. caudatus (cau)* and *A. hypochondriacus (hyp)*] and quinoa (*Chenopodium quinoa*) were investigated. Amaranth and quinoa were ground in a mill with a 60-mesh screen and defatted in a Soxhlet extractor with a ratio of *n*-hexane (boiling point 69 °C) to sample of 30:1 (v/w) for 10 h. The meal was stored at 4 °C after removal of *n*-hexane. Amaranth and quinoa albumins (Alb) and globulins (G) were extracted with 0.5 M NaCl from the meal (1 g) with a solvent: sample ratio of 10:1 (v/w) and vigorously shaken for 1 h at 4 °C. The extracts were centrifuged at 10000*g* for 10 min. Each step was repeated twice according to the method of Landry and Moureaux (1970). Then, globulins were separated from albumins by dialysis against deionized water for 3 days at 4 °C with cellulose tubing (Sigma) that had a MW cutoff of 2000 (dialysate was changed three times a day). Then, the pellet (globulins) was resuspended in 10 mL of deionized water and freeze-dried and stored at 4 °C until time of use. This dry fraction of globulins was used for denaturing and fluorescence studies. Freeze-dried globulins were completely dissolved in 0.01 M phosphate buffer, 0.4 M NaCl, and 0.02% sodium azide (pH 7.2). The denaturant concentrations (M) were as follows: 0, 2, 3, and 8 for urea; 0, 1, 3, 5, and 6 for GuHCl; and 0, 0.001, 0.01, 0.1, 0.4, and 0.6 for SDS. Protein assays were performed by the Lowry method (Lowry et al., 1951).

Amino Acid Analysis. Globulins were analyzed by the procedure of Spackman et al. (1958). Freeze-dried samples, with and without previous oxidation with performic acid, were hydrolyzed with 6 N HCl in sealed tubes for 66 h at 110 °C. The vacuum-dried hydrolysate was applied onto a Beckman 120C automatic amino acid analyzer (Beckman Instruments International, S. A., Geneva, Switzerland) for determination of amino acid composition. For tryptophan determination,

samples were hydrolyzed with 4 N LiOH for 20, 24, 28, 36, and 40 h at 110 °C, and then treated with 6 N HCl for 22 h at 110 °C (Lucas and Sotelo, 1980).

Fluorescence. Fluorescence was measured with a model FP-770, Jasco-Spectrofluorometer (Japan Spectroscopic Co. Ltd., Hachioji City, Japan). Samples were measured at 30 °C, and diluted to 0.015% protein (w/v) with 0.01 M phosphate buffer, 0.4 M NaCl, and 0.02% sodium azide (pH 7.2); this procedure kept the absorbance at <0.1 in a 1-cm path length and allowed a linear interpretation of concentration based on relative fluorescence intensity. Absorbance values were determined with a Uvikon 930 spectrophotometer (Kontron AG Instruments, Zürich, Switzerland). Fluorescence emission spectra of all proteins were measured at excitation wavelengths of 274 and 295 nm and recorded over the frequency range from the excitation wavelength to a wavelength of 450 nm. The percentage of protein denaturation (%D) was determined by eq 1:

$$(\%D) = (I_0 - I_1)/I_0 \times 100 \quad (1)$$

In eq 1, I_0 and I_1 are the fluorescence intensities of native and denaturated proteins, respectively. The %D was measured after incubation of globulins with denaturant for 1 h. All data were determined in triplicate for each experimental condition. Rate constants for denaturation were calculated (Zemser et al., 1994).

CD Spectra. The CD spectra were measured with a Jasco J-600 spectropolarimeter (Japan Spectroscopic Company, Ltd., Japan) with a 1-cm quartz cell at room temperature under constant nitrogen purge. Solutions (0.03 mg/mL) of globulins were prepared by dissolving the lyophilized powder in 0.01 M phosphate buffer, 0.4 M NaCl, and 0.02% sodium azide (pH 7.2). The absorbances of all solutions were kept at <1.0 (Matsuura and Manning, 1994; Zemser et al., 1994). Denaturation of globulins was performed with 6 M GuHCl, 0.1% SDS, and with 50 mM 2-ME. These conditions were chosen on the basis of our previous results obtained from another biological system (i.e., ovalbumin; Zemser et al., 1994). The CD spectra represented an average of eight scans collected in 0.2-nm steps at a rate of 20 nm/min over the wavelength ranges 180–250 nm of far-UV (FUV) and 240–320 nm of near-UV (NUV). The CD spectra were baseline corrected, and the data are presented as the mean residue ellipticities (Θ). Helical content of globular globulins was obtained from the following equations:

$$\% \alpha - \text{helix} = (-[\Theta]_{222\text{nm}} - 2340)/(30\,300 - 2340) \times 100 \quad (2)$$

$$\% \alpha - \text{helix} = (-[\Theta]_{208\text{nm}} - 4000)/(33\,000 - 4000) \times 100 \quad (3)$$

In eqs 2 and 3, $\Theta_{222\text{nm}}$ and $\Theta_{208\text{nm}}$ are residue ellipticities at 222 and 208 nm, respectively (Chen et al., 1972; Yang et al., 1986).

RESULTS AND DISCUSSION

Amino Acid Composition of Globulins. The main subject for investigation was crude globulins from A-G. Crude globulins from Q-G were used for comparison. The amount of tryptophan (g of amino acid/100 g of protein) varied in amaranth seed from 1.0 to 1.4 and in the quinoa seed from 0.9 to 1.1. For amaranth and quinoa seeds, phenylalanine was determined in conjunction with tyrosine and varied from 7.1 to 9.1 and from 6.2 to 7.4 (g of amino acid/100 g of protein), respectively (Risi and Galway, 1984; Becker and Hanners, 1990; Paredes-López et al., 1994). The results of amino acid analysis of A-G and Q-G are shown in Table 1. Special treatment was used, as mentioned in Materials and Methods, to prevent the destruction of tryptophan (Lucas and Sotelo, 1980). The amount of tryptophan (g of amino acid/100 g of protein) in ama-

Table 1. Amino Acid Composition of Amaranth and Quinoa Globulins^a

amino acid	species of amaranth			quinoa
	<i>caudatus</i>	<i>hypochondriacus</i>		
Trp	1.4 ± 0.1 ^b	0.6 ± 0.3 ^b	nd ^d	nd ^d
Tyr	2.4 ± 0.2	2.9 ± 0.2	4.3 ^c	2.9 ^c
Phe	3.7 ± 0.2	4.5 ± 0.2	5.0	4.0

^a Mean values (g of amino acid/100 g of protein) of triplicates ± standard deviation. ^b From Gorinstein et al. (1991). ^c From Barba de la Rosa et al. (1992). ^d Not determined. ^e From Brinegar and Goundan (1993).

ranth samples varied from 0.6 to 1.4 (Gorinstein et al., 1991). Tyrosine was higher than the amount of tryptophan in A-G (Bressani and Garcia-Vela, 1990; Gorinstein et al., 1991; Barba de la Rosa et al., 1992). The amount of tyrosine and phenylalanine was slightly lower in Q-G than in A-G samples (Brinegar and Goundan, 1993). The data of other amino acids were omitted because the fluorescence measurements were based only on tryptophan, tyrosine, and phenylalanine contents.

Intrinsic Fluorescence Properties of Globulins.

In our studies, the fluorescence properties of A-G and Q-G were measured directly on the basis of the amounts of the three amino acids tryptophan, tyrosine, and phenylalanine. Changes in maximum emission wavelength and fluorescence intensity of these amino acids reflect alterations in protein structure because of different denaturing factors. The amounts of chromophores in the investigated proteins are sufficient to avoid inclusion of fluorescent probes (Zemser et al., 1994). The emission maxima of tryptophan, tyrosine, and phenylalanine are 348, 303, and 282 nm, respectively. According to the content of tryptophan, tyrosine, and phenylalanine in globulins (Table 1), at least two emission peaks are expected in the fluorescence spectra of these amino acids because of the existence of tryptophan and tyrosine. Khan et al. (1980) observed that tyrosine has a lower extinction coefficient than tryptophan. The energy transfer also shows the dominance of the tryptophan fluorescence response. The presence of even one tryptophan residue in globulins gives a spectrum with a single peak of tryptophan emission in the interval 331–342 nm at excitation wavelengths of both 295 and 274 nm (Arntfield et al., 1987). This result indicates that excitation at 274 or 295 nm results in the emission spectra of native globulins with a single emission peak. Replacement of the excitation wavelength of 274 with 295 nm resulted in a decrease of relative fluorescence intensity that correlated with a decrease in tryptophan emission and almost complete absence of tyrosine fluorescence (Khan et al., 1980). Tryptophan appears to be the only aromatic amino acid absorbing light at this radiation wavelength. At the excitation wavelength of 295 nm (Figure 1A), the spectra were typical for tryptophan content, but the peak of A-G was displaced to a longer wavelength (341.5 nm), demonstrating a more polar environment of the tryptophan residues than quinoa proteins. The spectrum of quinoa showed a maximum emission peak at ~333.5 nm. Amaranth proteins may demonstrate remarkably high contents of phenylalanine plus tyrosine (Figure 1B, curve a), whereas quinoa proteins appear to contain slightly lower contents (Figure 1B, curve b). At an excitation wavelength of 274 nm, shoulders indicating the presence of tyrosine were seen in globulins from quinoa (Figure 1B, curve b).

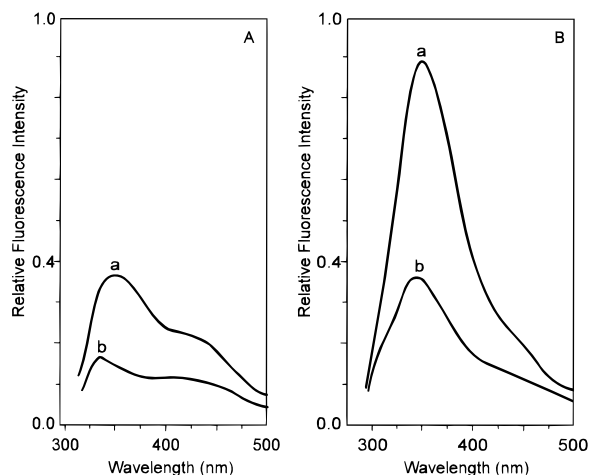


Figure 1. Fluorescence emission spectra of globulins from (a) *Amaranthus hypochondriacus* and (b) quinoa. Excitations (nm) at 295 and 274 on A and B, respectively.

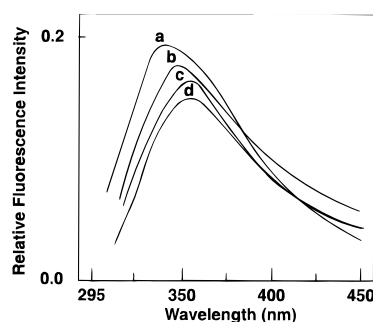


Figure 2. Dependence of the fluorescence emission of globulins from *A. hypochondriacus* on urea concentrations. Excitation at 295 nm. Key: (a) 0 M; (b) 2 M; (c) 3 M; and (d) 8 M urea concentrations.

Table 2. Fluorescence Properties^a of Native and Denatured Amaranth (A-G) and Quinoa (Q-G) Globulins

protein	I_{274}^b	I_{295}^c	%D ^d
A-G + 0 M urea	0.48 ± 0.020	0.20 ± 0.047	0
A-G + 2 M urea	0.40 ± 0.030	0.18 ± 0.004	10
A-G + 3 M urea	0.38 ± 0.009	0.17 ± 0.025	15
A-G + 8 M urea	0.35 ± 0.011	0.15 ± 0.008	25
Q-G	0.36 ± 0.025	0.17 ± 0.005	0
Q-G + 8 M urea	0.22 ± 0.015	0.14 ± 0.009	18

^a Mean values of triplicates ± standard deviation. ^b Fluorescence intensity at 274 nm. ^c Fluorescence intensity at 295 nm. ^d Denaturation.

Urea-Induced Denaturation. The fluorescence emission spectra of A-G showed a maximum at 341.5 nm (Figure 2, curve a). This peak is typical for tryptophan-containing proteins and agrees with the reported results of Arntfield et al. (1987). Curves b, c, and d in Figure 2 show a decrease of relative fluorescence intensity with increasing urea concentration. The %D values with 2, 3, and 8 M urea were 10, 15, and 25%, respectively (Table 2). The fluorescence of denatured globulins was measured at excitation wavelengths of both 295 (Figure 2) and 274 nm (Table 2). When the concentration of urea increased, the fluorescence intensity decreased gradually from 8 to 2 M. At the 2 M concentration, the fluorescence intensity decreased and shifted to the higher wavelength. With the concentration of 3 M urea, a shoulder that is characteristic of tyrosine fluorescence appeared at 308 nm. At the same time, the emission peak that belongs to tryptophan fluorescence shifted from 341.5 to 351.5 nm. This wavelength changed slightly up to the addition of 8 M

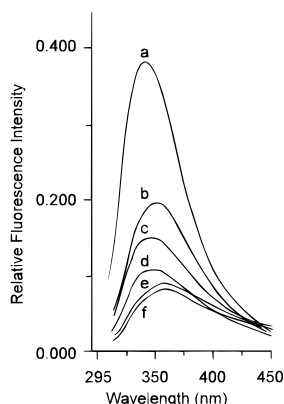


Figure 3. Dependence of the fluorescence emission of globulins on 8 M urea concentration, with excitation at 295 nm. Intensities of: (a) standard native bovine γ -globulin (γ -G); (b) γ -G with urea; (c) native globulins from *A. caudatus* (A-Gcau); (d) native globulins from *A. hypochondriacus* (A-Ghyp); (e) A-Gcau with urea; and (f) A-Ghyp with urea.

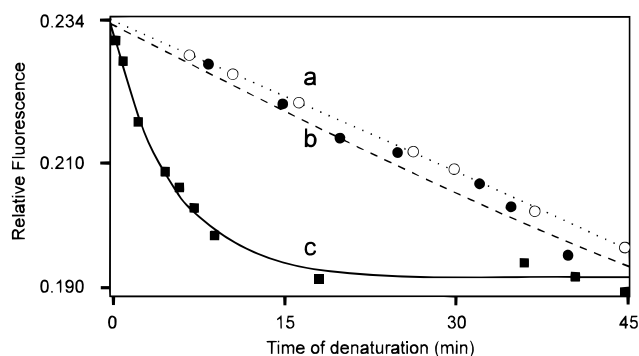


Figure 4. Time course of the fluorescence transitions of A-G in (a) 3 M GuHCl; (b) 6 M GuHCl; and (c) 0.1 M SDS. Determinations were at 30 °C, and excitation was at 295 nm.

urea. The appearance of a shoulder was a result of a ceasing of energy transfer from excited tyrosine to tryptophan residues in globulins during unfolding. The distance between the tryptophan and tyrosine residues was increased. According to Khan et al. (1980), a shift of tryptophan residues towards an increase in polarity of the microenvironment around the aromatic amino acids was attributed to a decrease in energy in the maximum of emission and quenching of fluorescence intensity. At a wavelength of 295 nm, there was no contribution of tyrosine fluorescence; therefore, the extent of %D was calculated with this excitation wavelength. The results in Figure 2 indicate that maximum denaturation was achieved at 8 M urea. The effects of 8 M urea on the degree of denaturation (%D) of γ -G, *A. caudatus* globulin (Acau-G), and *A. hypochondriacus* globulin (Ahyp-G) are 49.5, 40.0, and 27.6%, respectively (Figure 3). The difference in the extent of denaturation between the two varieties of A-G may be explained by the differences in amounts of amino acids and by the sulfur bridges existing in such proteins. γ -G showed less stability to urea than the two amaranth species (Acau-G and Ahyp-G).

GuHCl-Induced Denaturation. The denaturation of A-G with addition of GuHCl is shown in Figure 4 and in Table 3. The relative fluorescence intensity decreased with addition of GuHCl at the excitation wavelength of 295 nm. Denaturation of A-G reached 16% at 3 M GuHCl and was slightly changed to 18% at 6 M GuHCl. The difference between the addition of 3 and 6 M GuHCl was only in the shift of the peak. At 3 M GuHCl, the extent of denaturation was similar to that

Table 3. Fluorescence Properties^a of Amaranth Globulins^b

de-naturant	concn of denaturant, M	%D	shift, nm	K , min ⁻¹ ^c	K , min ⁻¹ ^d
GuHCl	3	16	352.5	4.16×10^{-3}	— ^e
	6	18	356.5	6.14×10^{-3}	—
SDS	0.1	19	344.0	1.75×10^{-1}	1.17×10^{-4}

^a Mean values of triplicates; the standard deviation was 10% of the values. ^b Emission spectrum of native globulin has maximum at 341.5 nm. ^{c,d} Rate constants of first and second phases of denaturation, respectively. ^e —, Not determined.

with the same concentration of urea. At 8 M urea, the shift (353.5 nm) was lower than at 6 M GuHCl where the maximum of emission was at 356.3 nm (Table 3; Figure 2, curve d; and Figure 3, curve f). These results explain the lower stability of the globulins for GuHCl than for urea. These data, which are characteristic of the tertiary structure of globulins with urea and GuHCl, agree with the results of Lilley (1986) on the reactivity of free sulfhydryl (SH) groups in globulins. At 6 M GuHCl, all SH groups of globulins reacted; however, the unfolding of protein with urea is not sufficient to expose free SH groups buried in the interior of the protein.

SDS-Induced Denaturation. The influence of addition of SDS to the protein was different from that of urea and GuHCl, as observed by the decrease in relative fluorescence intensity and the denaturation achieved at low concentrations of SDS (Figure 4 and Table 3). The shift in the emission maximum wavelength corresponding to tryptophan fluorescence was only at 344 nm. GuHCl and urea induce transition by interfering with the hydrophobic regions in the interior as well with the hydrogen-bonding pattern involved in the polar regions of the peptide chain in the protein molecule. In contrast, SDS interacts mostly with the hydrophobic bonds. This interaction permits an increased development of the helix (Meyer and Kauzmann, 1962); the two different mechanisms of interaction are also reflected in the fluorescence behavior. Interaction between protein and detergent arises from the electrostatic interaction between positively charged groups and hydrophobic interactions.

Kinetic Studies of Denaturation. The rate constants for globulin denaturation with GuHCl and SDS are shown in Table 3. These data were generated at a fixed wavelength of maximal shift. The progressive decrease of fluorescence intensity, which is consistent with unfolding, was more intensive with SDS than with GuHCl. Rate constants confirm the stronger effect of detergent than of denaturants. Transition from the native to the denatured state occurred in two separate stages only with SDS (Table 3). This unfolding proves the existence of a stable intermediate conformation. The two-state model shows that globular proteins, after disappearance of nearly all the α -helix, still have a large part of folded protein molecule (Sarkar and Doty, 1966). These molecules become unfolded during the subsequent denaturation (Gryczynski et al., 1988).

CD Studies. The CD spectra of globulins from amaranth and quinoa in the FUV region are shown in Figure 5. Polypeptide chains can assume only a limited number of stable structures; they are α -helix, the interchain hydrogen bonded β -structure, and a fully extended parallel or antiparallel arrangement of peptide chains (Chen et al., 1972; Gruen et al., 1987; Matsuura and Manning, 1994). Optical activity of α -helix in FUV permits the use of CD studies for investigation of

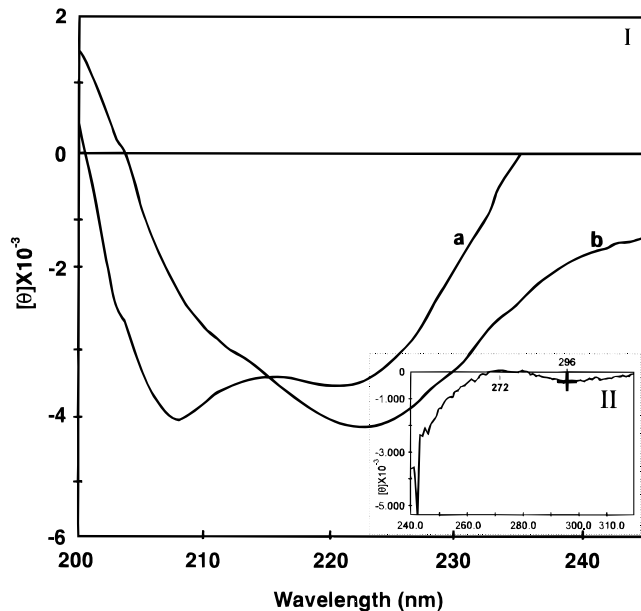


Figure 5. CD spectra of globulins: (I) FUV from (a) amaranth and (b) quinoa; (II) NUV from amaranth with 6 M GuHCl.

Table 4. Secondary Structure Compositions^a (H,^b β,^c and R^d) of Amaranth (A-G) and Quinoa (Q-G) Globulins Estimated from CD Spectra

protein	form	λ, nm ^e	[θ], deg cm ² dmol ⁻¹ f	percentage
A-G	H	208	-13110	31.4
	β	216	-11826	27.0
	R	—	—	41.6
A-G + GuHCl	H	208	-6998	10.3
A-G + SDS	H	222	-13281	36.1
Q-G	H	225	-5307	9.8
Q-G + GuHCl	H	222	-2527	0.6

^a Mean values of triplicates. ^b α-helix. ^c β-sheet. ^d Disordered structure. ^e Wavelength. ^f Residue ellipticity.

conformational changes in protein solutions, and the CD band position for various structures has been reported (Sarkar and Doty, 1966; Chen et al., 1972). Globulins from amaranth and quinoa showed bands with minima at 208 and at 225 nm, respectively (Figure 5, section I, curve b; Table 4). The CD curve of A-G also exhibited a band with a minimum at 216 nm, which represents the β-structure of the protein. The corresponding mean residue ellipticity (θ) was -11 826 deg cm² dmol⁻¹ (Table 4). If it is assumed that the molecular ellipticity for the 100% β-structure is -23 000 deg cm² dmol⁻¹ at 216 nm (Sarkar and Doty, 1966), the proportion of this structure in A-G is estimated to be 27% (Table 4). Our results correspond with those of Lilley (1986) who showed that conglutin δ₁ and δ₂ of lupins contained 38% of the α-helix conformation. The amount of α-helix in amaranth was 31.4%. Probably, crude globulins that are a mixture of 7S and 11S globulins represent α-helix, β-sheet, and unordered forms, with their different stability, as a total amount of both oligomers. Quinoa showed a low percentage of α-helical structure (Table 4). In the presence of GuHCl, the CD spectrum (Table 4) of A-G showed a decrease in magnitude of λ_{208nm}, suggesting that a significant loss in α-helix structure occurred.

The effects of 6 M GuHCl and 0.1 M SDS on the α-helical structure of A-G are shown in Table 4 and in Figure 5 (section II). Similar to the results of Lilley (1986) on *Lupinus angustifolius*, denaturation was evident at concentrations >3 M GuHCl, increased with

6 M, and was completed at 6.5 M GuHCl. With the addition of 50 mM 2-ME and 0.6 M GuHCl, only negligible α-helix remained in A-G after the reducing action of 2-ME overnight (data not shown). The overall association capacity in A-G is unusually high. Such characteristics are matched in the gelling and functional behavior of the protein. These observations are in agreement with those of Marcone and Yada (1992) who found two heterogeneous forms of globulins, a major of 337 kDa and a minor of 398 kDa. The latter exists as polymeric species and was partly a result of a sulfhydryl-disulfide interchange reaction. The former, an 11S globulin with five dissimilar subunits (37.0; 31.5; 26.5; 20.5; and 14.5 kDa), was found as a heterooligomer with a tetrameric and hexameric moiety with weak secondary forces that dissociate under alkaline pH.

The CD profile of A-G also typified that of a protein with considerable α-helix with a stable structure due to the presence of the disulfide cross-links (Lilley, 1986; Marcone and Yada, 1992). The NUV CD spectra reflect changes in the tertiary structure of the protein (Strickland, 1974), showing the interaction and orientation of the aromatic rings of tyrosine, tryptophan, and phenylalanine with other amino acid moieties. The CD spectra of the A-G were investigated at a pH of 7.2 under native and denatured conditions with 6 M GuHCl (Figure 5). Identification of the fine structure of the aromatic groups is difficult because of the low intensities of CD structural scans. For native A-G, tyrosine showed its characteristic band at 278 nm, with an intensity of 0.001 154, and tryptophan gave its band at 281 nm with an intensity of 0.001 243. Under denaturing conditions, the tyrosine and tryptophan bands were shifted to 272 nm, with an intensity of 0.000 069 and 296 nm, with an intensity of 0.000 324, respectively. These data are slightly different from those of Marcone and Yada (1992) who showed the presence of tyrosine at 274 nm and of tryptophan at 291 nm. In our spectrum, the band of tryptophan in native protein appeared at 281 nm, which is comparable to the results of Marcone and Yada (1992) who reported the band at 283 nm. The tryptophan band for the denatured protein was assigned to 290.6 nm, according to Marcone and Yada (1992), and in our spectrum it appeared at 296 nm. The band at 281 nm can be ascribed to tryptophan vibrational fine structure, and the bands at 278 nm to tyrosine (Towell and Manning, 1994). The loss of the tryptophan vibrational band at 281 nm in the NUV CD spectrum and a shift to 296 nm, plus the loss at 278 nm and a shift to 272 nm for tyrosine during denaturation, indicate a significant reduction in tertiary structure around tryptophan and tyrosine residues. Before denaturation, the CD spectrum still exhibited vibrational tryptophan and tyrosine structures. A native tertiary structure appeared to exist because intense CD bands for the individual aromatic amino acids commonly occur when aromatic groups are in close proximity to one another. The absence of such intense bands for globulins would indicate that the aromatic groups are either distant from one another, or highly motile, or are in low quantities (Yada and Nakai, 1986). In the spectra of native and denatured A-G, the CD band for phenylalanine was not shown. Marcone and Yada (1992) explained the change in the tertiary structure of A-G by the general increase in the intensity of the spectra at pH 5.50 combined with the appearance of a negative band for phenylalanine at 259 nm (a change in the position of aromatic amino acids). The difference in CD

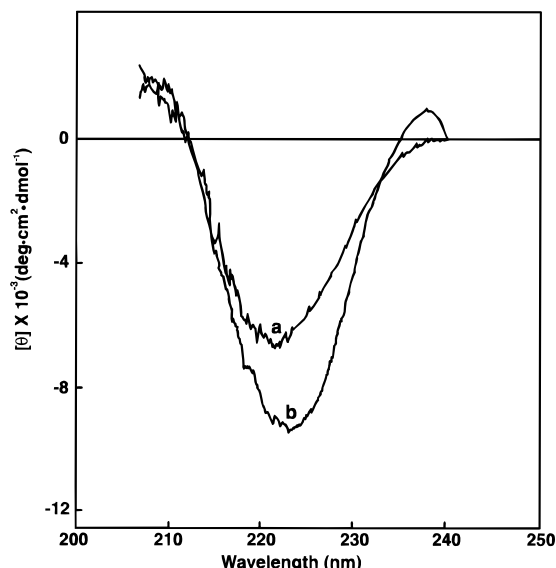


Figure 6. CD spectra of globulins from *A. hypochondriacus*: (a) native and (b) denatured with 0.1% SDS.

spectra of these globulins can be explained by the purity of the sample (crude globulins) as well as by the difference in pH. SDS denaturation of the protein (0.1% SDS) leads to an increase in α -helical content to 36.1% (Figure 6b) compared with 31.4% in the native state (Figure 6a). The spectrum does not closely resemble that of the native state and demonstrates the much more pronounced negative maximum at 222 and 208 nm that is typical of α -helix. However, at low concentrations of SDS (0.025%), a decrease in α -helical content in comparison with the native structure was observed (28.9% compared to 31.4% in the native state). At the same time, the spectrum state was similar to the high-binding protein state. It is obvious that SDS does not unfold the protein molecule at a concentration of 0.025%. Random coil undergoes transition to α -helix and β -sheet. Thus, CD spectra for native and denatured A-G showed a decrease in α -helix with GuHCl at 208 nm and a slight increase with SDS.

The CD spectra of A-G and Q-G reported for the first time in this study showed the changes in the amount of α -helix and β -sheet and in the position of aromatic amino acids. This indicates that the secondary and tertiary structures are dependent on denaturation.

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