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Structural analysis of globulins isolated from genetically different *Amaranthus* hybrid lines

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The main storage protein of *Amaranthus* species, the oligomeric salt-soluble globulin was isolated and purified to homogeneity from six genetically different *Amaranthus* lines and compared with a previously investigated line i.e., *Amaranthus* K343. Physico-chemical analyses revealed that some heterogeneity existed with respect to the overall molecular weight of the globulins tested, as did differences in the molar ratio of their constituent (individual) subunits. Amino acid compositions of these proteins were found to be very similar and characteristically high in aspartic/asparagine and glutamic/glutamine amino acids. Circular dichroic studies revealed that all globulins shared similar secondary structural conformations characterized by low α-helical and high β-sheet contents. Although internal conformations were found to be very highly conserved, tertiary structural analysis revealed that substantial differences existed in the arrangement/proximity and exposure of aromatic amino acids on globulin surfaces.

INTRODUCTION

Recent investigations of the lysine-rich, salt-soluble amaranth globulin has shown it to be of exceptional nutritional quality (Bressani & Garcia-Vela, 1990; Mora-Escobedo *et al.*, 1990) as well as possessing excellent heat stable emulsifying properties (Konishi & Yoshimoto, 1989). It is for these reasons that amaranth seed, with its high content of this protein, is now the subject of many investigations as a potential food source (Romero-Zepeda & Paredes-Lopez, 1996; Gorinstein *et al.*, 1996).

The development of grain amaranth as a new field crop has been aided by the fact that there is a great deal of genetic variation available within the cultivated species (Proceedings of the Fourth National Amaranth Symposium Perspectives on Production Processing and Marketing, 1990). This broad range of genetic variability expressed within amaranth species provides readily accessible genetic resources for plant breeders to develop improved lines, and has been paramount to the assembly of the 1374 entries of amaranth by the Rodale Research Station (PA, USA) and to the USDA collection housed at Ames, Iowa (1988, Rodale Amaranth Germplasm Catalog).

Unfortunately, it is not yet known if the physico-chemical properties of the globulin varies between amaranth lines, which could potentially affect their desired nutritional as well as functional properties. Undoubtedly, such information would provide additional insights into the breeding of new technologically superior amaranth lines for food purposes. It was, therefore, the purpose of this study to compare the structural properties of globulins isolated from six currently available amaranth lines grown in the United States, with the globulin isolated from *Amaranthus hypochondriacus* K343, which has been extensively studied in recent years (Marcone & Yada, 1991, 1992; Marcone *et al.*, 1994).

MATERIALS AND METHODS

Materials

Non-heat treated amaranth seeds were the kind gift of the Agriculture Canada Delhi Research Station (Delhi, Ontario) and the Rodale Research Station's Germplasm Collection Laboratory (PA, USA). The lines tested included K436 (*A. cruentus* hybrid), MT-3 (*A. cruentus* selection), K433 (*A. hypochondriacus* X *A. hybridus*), K432 (*A. hyprochondriacus* X *A. hybridus*), K266 (*A. cruentus* hybrid), K283 (*A. cruentus* hybrid) and K343 (*A. hypochondriacus* X *A. hydridus*). The seeds were ground and material passing through a 60 mesh screen was used for further study.

The Sephacryl™ S-300 superfine gel permeation media and high molecular weight calibration proteins

were products of Pharmacia LKB (Montreal, PQ). Gradient 8–25% and 7.5% polyacrylamide (PAA) gels and calibration proteins were also products of Pharmacia LKB (Montreal, PQ).

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

Proximate analysis of seeds

Proximate analysis was performed as prescribed in the official standard methods of the American Association of Cereal Chemists, Inc. (1983). Analyses were performed in triplicate.

Sample preparation

The previously ground seeds were defatted with cold HPLC grade acetone in the ratio of 1:10 (w/v), (flour/acetone). The mixture was mechanically stirred in a sealed 2–1 glass container for 36 h at −20°C. This was followed by three additional extractions with cold acetone. The pooled extracts were dried under a stream of pure nitrogen gas or alternatively under reduced pressure. The defatted meal was immediately stored desiccated at −20°C until required.

Determination of protein concentration

Protein concentrations were determined as previously described by Marcone and Yada (1991).

Protein fractionation

The salt-soluble proteins (globulins) were extracted from the defatted meal as described by Marcone and Yada (1991) using a 32.5 mM K_2HPO_4–2.6 mM KH_2PO_4 buffer (pH 7.50) containing 0.4 M NaCl and 0.02% NaN_3.

Purification of the seed storage globulin

The globulins were purified to homogeneity by gel filtration and anion-exchange chromatography and stored desiccated at −77°C under an argon blanket until further study, as previously described by Marcone and Yada (1991).

Sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE)

The purified globulins were reconstituted to 3 mg ml^{-1} 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% 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 Zeineh Videophoresis II electrophoresis reporting integrator program (ERIS) from Biomed Instruments Inc. (Fullerton, CA). Linear plots of the log molecular weights vs relative mobility (R_m) 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 (14 400 Da) as standards.

Native-PAGE electrophoresis

The purified globulins were reconstituted to 3 mg ml^{-1} in a 20 mM phosphate buffer (pH 7.50) and 1 μl was applied to PhastGel™ 7.5% polyacrylamide (PAA) gels with native buffer strips. The separations and subsequent scannings were performed as described above.

Amino acid analysis

Two hundred-microliter samples containing 0.4 mg ml^{-1} of each purified globulin were lyophilized 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 NaOH, dried *in vacuo*, and redissolved in 400 μl of buffer. Analysis was performed by applying 25-μl portions of hydrolysed–neutralized samples onto the column.

Amino acid measurements were performed on a Beckman System Gold Amino Acid 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. Analyses were performed in triplicate.

Far-UV circular dichroic spectroscopy

Circular dichroic measurements were performed in the far-UV (190–250 nm) on 0.1 mg ml^{-1} protein/buffer solutions (32.5 mM K_2HPO_4–2.6 mM KH_2PO_4), pH 7.50, using a Jasco J-600 Spectropolarimeter (Japan Spectroscopic Co. Ltd., Tokyo, Japan) with a cell pathlength of 0.1 cm under constant nitrogen purge at 20°C. Secondary structure was determined using the Jasco Protein Secondary Structure Estimation Program (Japan Spectroscopic Co.). Analyses were performed in triplicate.

Near-UV circular dichroism spectroscopy

Circular dichroic measurements were performed 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 a cell pathlength of

Table 1. Proximate analysis of seed from different lines of *Amaranthus* spp.

Line	% moisture[a]	% ash[a]	% protein[a]	% fat[a]	% CHO[b]
K343	9.6 (±0.2)	3.1 (±0.1)	16.4 (±0.2)	9.6 (±0.2)	61.3
MT-3	9.6 (±0.1)	3.0 (±0.1)	16.1 (±0.2)	8.03 (±0.1)	63.3
K432	10.2 (±0.3)	2.7 (±0.1)	16.0 (±0.3)	8.00 (±0.1)	63.1
K433	9.8 (±0.2)	3.1 (±0.2)	16.5 (±0.3)	9.0 (±0.2)	61.6
K436	9.2 (±0.1)	3.0 (±0.1)	16.2 (±0.2)	8.04 (±0.1)	63.6
K266	9.6 (±0.1)	3.1 (±0.1)	15.9 (±0.2)	7.70 (±0.1)	63.7
K283	9.6 (±0.1)	3.1 (±0.0)	16.0 (±0.1)	8.13 (±0.1)	63.1

[a]The average of three replications (± standard deviation).
[b]Carbohydrate (CHO) by difference.

1.0 cm. A protein concentration of approximately 1.0 mg ml^{-1} in protein/buffer solutions of 32.5 mM K_2HPO_4–2.6 mM KH_2PO_4, pH 7.50 with NaCl added to a final ionic strength of 0.5 was used in the determinations. Analyses were performed in triplicate.

RESULTS AND DISCUSSION

Representative samples of raw seed material were subjected to proximate analysis (Table 1). Results obtained from these analyses indicated that the primary components of all seed material, although originating from genetically different amaranth lines, were comparable with previously published data presented by Becker *et al.* (1981).

Figures 1 and 2 depict the typical Sephacryl™ S-300 gel filtration chromatograms obtained for each of the various crude globulin preparations originating from the six lines analyzed. Typical of all six globulin preparations a strong UV-absorbing, turbid fraction (labelled fraction 1) was observed in each chromatogram at or near the void volume $K_{av} = 0$. Further biochemical investigation of this fraction revealed that all preparations were typically composed of high levels of carbohydrates (>45%), as well as unidentified high molecular weight components.

Of the total protein eluted, 78, 79, 81, 79, 78 and 77% was recovered from the amaranth globulin peaks marked '2b' of MT-3, K436, K266, K432, K433 and K283 lines, respectively, and were identified as the 11S seed storage globulin (Figs 1 and 2). The K_{av}'s for these respective globulins were 0.455 for MT-3, 0.477 for K436 and K266, 0.5 for K432 and K433 and 0.614 for K283 and corresponded to 381 000, 366 000, 350 000 and 273 000 Da, respectively. Although some heterogeneity with respect to overall molecular weight was observed among the globulins, results were in close agreement with the value of 337 500 Da obtained from *Amaranthus* K343 (Marcone & Yada, 1991, 1992; Marcone *et al.*, 1994).

Closer examination of the above-mentioned gel filtration chromatograms also revealed a characteristic well resolved protein peak labelled '2a' proceeding the

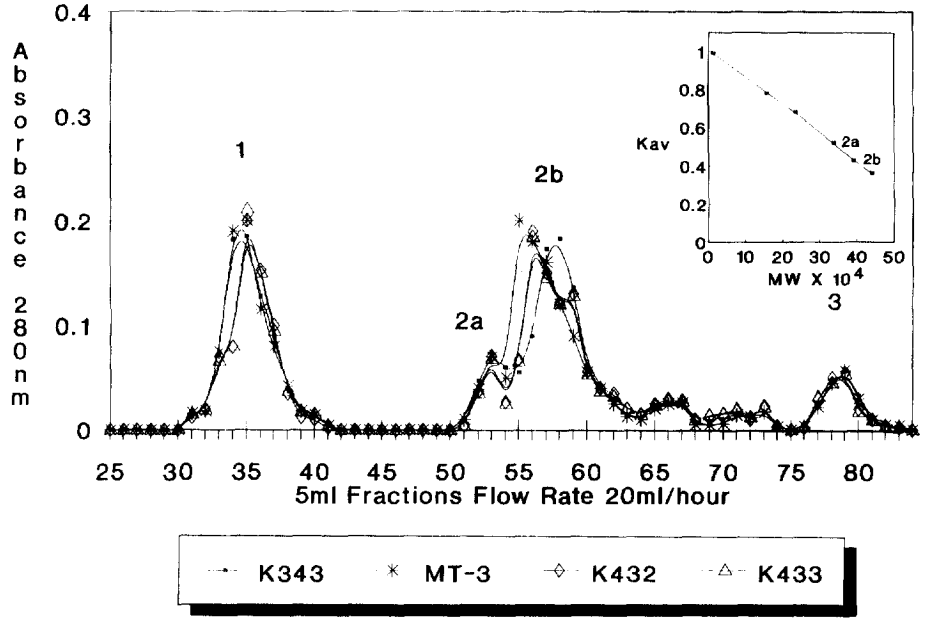

Fig. 1. Sephacryl™ S-300 gel filtration chromatogram of crude globulin extract (25 mg injection) for *Amaranthus* lines (K343, MT-3, K432 and K433).

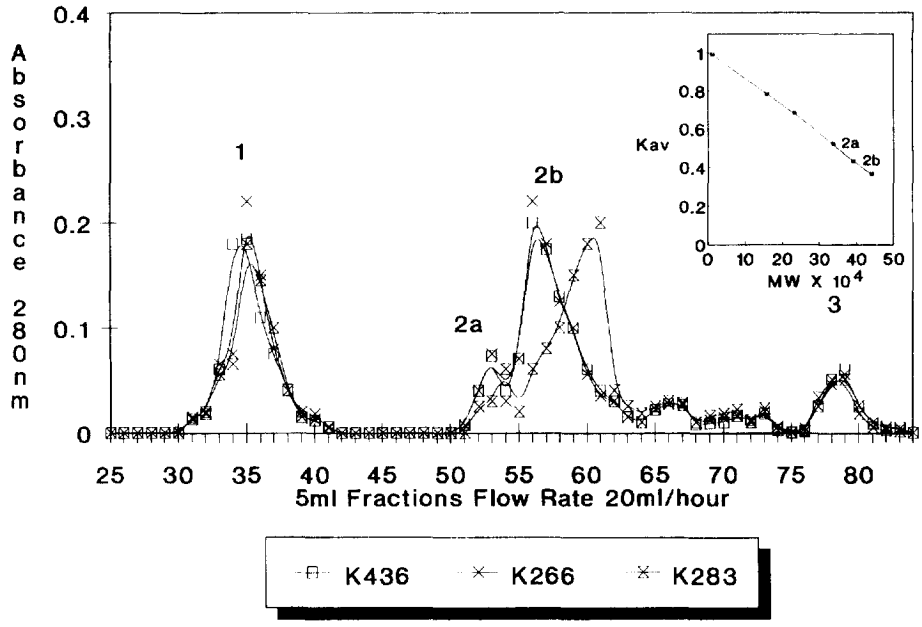

Fig. 2. Sephacryl™ S-300 gel filtration chromatogram of crude globulin extract (25 mg injection) for *Amaranthus* lines (K436, K283 and K266).

characteristic 11S globulin peak (2b). These fractions were thought to be composed of aggregates of polymerized protein and were found to possess similar subunit profiles as their non-polymerized 11S globulin counterpart (data not shown). This fraction was believed to be caused by a sulfhydryl–disulfide interchange reaction (Marcone & Yada, 1991) and thought to contribute to the undesirable insolubility, turbidity and increased viscosity of globulin solutions (Briggs & Wolf, 1957; Circle *et al.*, 1964; Lillford & Wright, 1981; Hoshi *et al.*, 1982).

Electrophoretic characterization

In order to determine the level to which the subunit composition of the non-polymerized globulin was conserved between each of the six lines, SDS–PAGE was performed on each of the purified globulins (i.e. fraction 2b) (Fig. 3). Results indicated that the globulins obtained from each line existed as hetero-oligomers composed of a non-equimolar ratio of five polypeptide chains namely: 37 500, 31 500, 26 500, 20 500 and 14 500 Da. Although all globulins were composed of the same type of subunits (mentioned above) there were substantial differences in the relative amounts of each of the subunits found in each protein (particularly in the amounts of the 37 500 Da subunit present). The previously described heterogeneity in the overall molecular weights of the globulins (273 000–366 000 Da) could, therefore, be attributed to the differing molar ratio of individual subunits just noted.

When the purified amaranth globulins were subjected to native–PAGE, a minor protein band of slightly higher relative electrophoretic mobility was found to

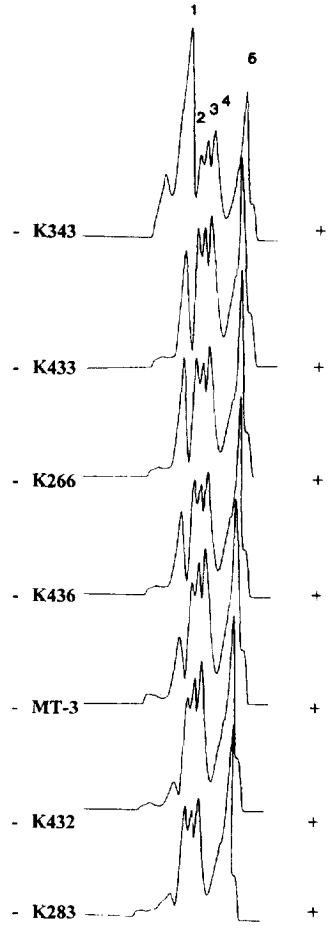

Fig. 3. Densitometric scans of SDS–PAGE performed on purified seed globulins from *Amaranthus* lines (K343, K433, K266, K436, MT-3, K432 and K283).

Table 2. A comparison of the amino acid compositions of purified seed globulins of *Amaranthus* K343, MT-3, K432, K433, K436, K266 and K283

Amino acid	Fraction[a]						
	K343[f]	MT-3	K432	K433	K436	K266	K283
Asx[b]	8.76 (±0.25)	10.43 (±0.02)	9.94 (±0.35)	9.95 (±0.00)	10.11 (±0.16)	9.75 (±0.00)	9.97 (±0.00)
Thr[d]	4.02	4.94 (±0.01)	5.17 (±0.190)	5.11 (±0.00)	5.09 (±0.11)	5.08 (±0.03)	4.95 (±0.01)
Ser[d]	6.90	7.27 (±0.01)	6.97 (±0.05)	7.07 (±0.01)	7.22 (±0.11)	6.89 (±0.24)	7.27 (±0.02)
Glx[c]	15.29 (±0.31)	17.24 (±0.02)	16.47 (±0.14)	17.01 (±0.01)	16.68 (±0.83)	16.29 (±1.06)	17.19 (±0.04)
Pro	6.14 (±0.39)	6.59 (±0.10)	6.48 (±1.19)	5.59 (±0.20)	5.77 (±0.13)	6.00 (±0.68)	5.97 (±0.14)
Gly	8.00 (±0.14)	17.24 (±0.02)	8.87 (±0.25)	8.82 (±0.03)	8.46 (±0.23)	8.77 (±0.13)	8.43 (±0.06)
Ala	5.71 (±0.10)	6.26 (±0.04)	6.92 (±0.51)	6.83 (±0.09)	6.89 (±0.106)	6.92 (±0.64)	6.53 (±0.08)
Cys[e]	0.93 (±0.01)	NA	NA	NA	NA	NA	NA
Val[d]	6.29 (±0.09)	6.17 (±0.06)	6.14 (±0.18)	6.11 (±0.057)	6.17 (±0.14)	6.18 (±0.07)	6.29 (±0.08)
Met	1.77 (±0.05)	1.68 (±0.04)	1.67 (±0.10)	1.48 (±0.05)	1.77 (±0.22)	1.66 (±0.04)	1.59 (±0.05)
Ile[d]	5.01 (±0.04)	4.84 (±0.03)	4.58 (±0.210)	4.51 (±0.00)	4.61 (±0.10)	4.50 (±0.16)	4.46 (±0.03)
Leu	6.82 (±0.06)	7.08 (±0.01)	6.72 (±0.05)	6.46 (±0.05)	6.81 (±0.12)	6.68 (±0.01)	6.73 (±0.06)
Tyr[d]	2.13	2.40 (±0.03)	2.26 (±0.19)	2.14 (±0.00)	2.34 (±0.00)	2.27 (±0.07)	2.14 (±0.04)
Phe	6.78 (±0.26)	5.69 (±0.04)	6.68 (±0.297)	6.50 (±0.04)	6.59 (±0.15)	6.61 (±0.37)	6.49 (±0.42)
Lys	4.25 (±0.16)	3.00 (±0.04)	3.26 (±0.714)	3.58 (±0.07)	2.35 (±0.25)	3.89 (±0.30)	3.31 (±0.00)
His	2.62 (±0.15)	2.27 (±0.163)	2.40 (±0.13)	2.45 (±0.04)	2.42 (±0.04)	2.36 (±0.04)	2.63 (±0.20)
Arg	10.12 (±0.72)	5.56 (±0.01)	6.05 (±0.21)	6.16 (±0.04)	6.03 (±0.014)	5.90 (±0.13)	5.85 (±0.00)

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

Table 3. Circular dichroic secondary structure fractions of purified seed globulins of *Amaranthus* K343, MT-3, K432, K433, K436, K266 and K283

Fraction[ab]	K343	MT-3	K432	K433	K436	K266	K283
α-helix	7.8 (±1.3)	15.1 (±1.1)	2.8 (±1.0)	6.2 (±0.5)	4.4 (±0.6)	8.1 (±1.0)	2.6 (±0.3)
β-sheet	57.6 (±1.1)	34.8 (±0.9)	68.8 (±1.1)	57.9 (±1.0)	61.9 (±0.5)	52.5 (±1.1)	71.0 (±0.4)
β-turn	17.6 (±0.5)	22.4 (±1.0)	16.3 (±1.0)	20.1 (±0.8)	14.6 (±0.4)	18.6 (±1.0)	16.0 (±0.6)
Random coil	16.9 (±0.5)	27.7 (±1.5)	12.1 (±1.0)	15.8 (±0.4)	19.0 (±0.1)	20.7 (±1.1)	10.5 (±1.0)

[a]Expressed as a percentage of total.
[b]Results are the mean values (± standard deviation) of three replications.

precede the major band (data not shown). This dissociation phenomenon was postulated as being caused by electrostatic repulsion between subunits of non-covalently linked protein subunits (Wolf *et al.*, 1958; Yamagishi, 1982; Marcone & Yada, 1991). In light of the amino acid data which showed comparably high levels of glutamic/glutamine and aspartic/asparagine amino acids, repulsive forces between subunits could then result from an increase in the number of negatively charged moieties coupled with the deprotonation of the α-amino acid with increasing pH (Table 2).

Not only were the acidic type amino acids (responsible for this dissociation) found to occur in high quantity in all isolated globulins, but comparable levels of the other 15 amino acids were also found. Amino acid analysis would, therefore, indicate that they are equivalent proteins.

Secondary and tertiary structure

Figure 4 shows the circular dichroism spectra obtained from each of the purified globulins. Quantitative estimation of the relative amounts of α-helical, β-sheet, β-turn and random coil secondary structure fractions calculated from these spectra are tabulated in Table 3. Examination of these secondary structure fractions revealed that all globulins except that from MT-3 possessed generally low levels of α-helical and very high β-sheet secondary structure contents. It is worth noting that α-helical and β-sheet secondary structure fractions are commonly found deeply buried within the polypeptide chain (Scheidtmann, 1990). This observed similarity in the amounts of α-helical and β-sheet secondary structures in all globulins would imply that their interior conformations are highly conserved.

Since the near-UV CD spectra reflects the tertiary structure of proteins (Strickland, 1974) brought about by the interaction and orientation of the aromatic ring of tyrosine, tryptophan and phenylalanine with other amino acid moieties in proteins (Strickland, 1974; Yada, 1984), near-UV CD spectra were examined for all six lines (Fig. 5). The identification of the fine structure arising from the above amino acids was difficult in view of the low intensities of CD spectral scans (Fig. 5). Tentative identification of phenylalanine was made at 259 and 265 nm. Tyrosine showed its characteristic $0+800\,cm^{-1}$ band at 276 nm with the $0-0\,cm^{-1}$ ^1Lb

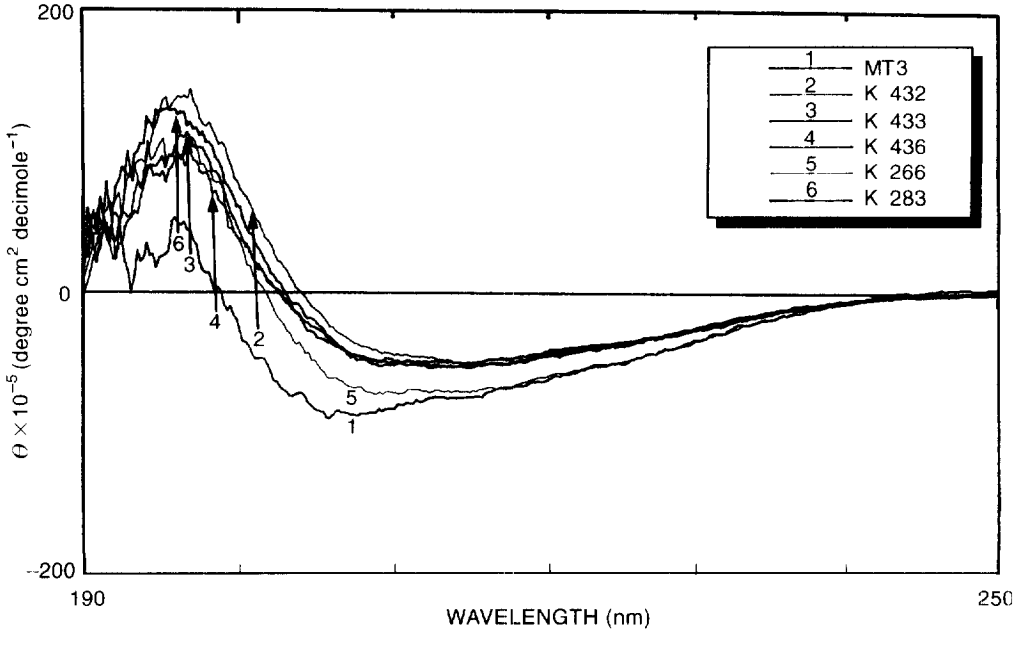

Fig. 4. Far-UV CD spectral scans (250–190 nm) of purified seed globulins from *Amaranthus* lines (MT-3, K432, K433, K436, K266 and K283).

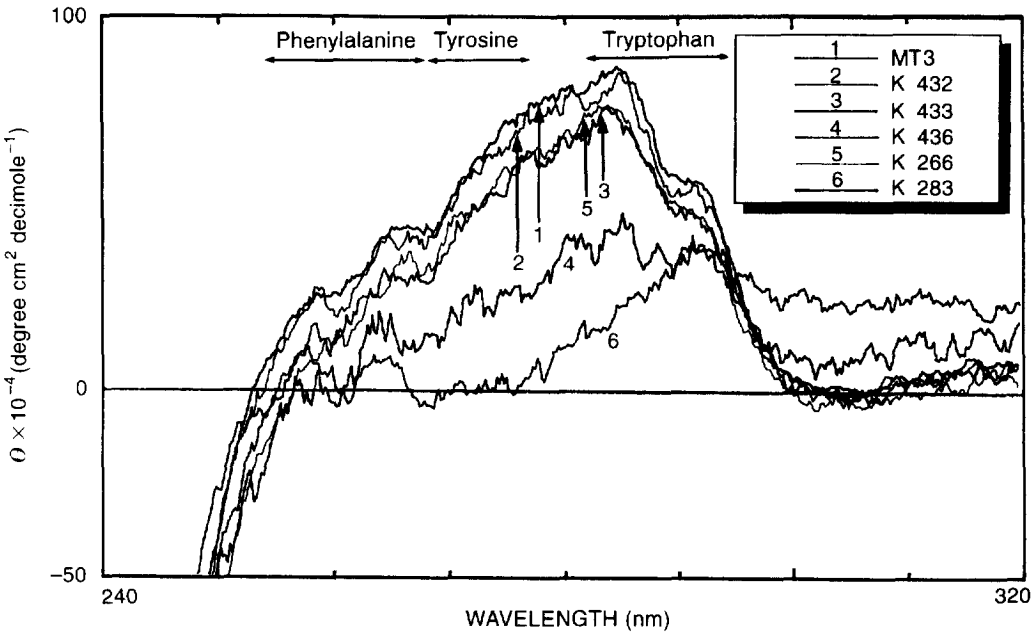

Fig. 5. Near-UV CD spectral scans (320–240 nm) of purified seed globulins from *Amaranthus* lines (MT-3, K432, K433, K436, K266 and K283).

(absorption band) of tryptophan being identified at 291 nm but lacked the 850-cm^{-1} ^{1}Lb band at 283 nm. Further examination of the spectra indicated that each globulin possessed its own characteristic near-UV CD fingerprint with the amplitude or intensity of the CD signal being the major difference. Since amino acid analysis revealed that the amounts of tyrosine and phenylalanine were comparable between lines, the above observations would indicate that substantial differences exist in the exposure, mobility, and arrangement/proximity of aromatic amino acids on the globulin surfaces (Strickland, 1974; Yada, 1984; Marcone & Yada, 1992). In addition, differences in the quaternary structure (or subunit interactions) of these proteins may have also been a contributing factor.

In conclusion, detailed characterization and comparison of seven seed globulins derived from seven amaranth lines, indicates that some heterogeneity exists with regard to their molecular weight as did differences in the molar ratio of individual subunits, despite possessing similar amino acid profiles. Structural analyses revealed that all globulins possessed low levels of α-helical and high levels of β-sheet secondary structure fractions, indicating conservation of interior conformation among them. In contrast to internal conformations, tertiary conformations indicate that very distinct surface properties exist between these globulins which may be reflected in different functional properties.

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