Composition and Structural Characterization of Amaranth Protein Isolates. An Electrophoretic and Calorimetric Study

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Amaranth protein isolates were prepared by (a) extraction at different alkaline pHs and precipitation at pH 5 and (b) extraction at pH 9 and precipitation at different pHs. The isolates were compared with protein fractions. DSC analysis showed that albumin-1 was composed of several species of T_d below 80 °C and ΔH below 4.0 J/g. Glutelin had two species of different T_d. Both, globulin and albumin-2, had a main component of T_d of 94 °C and ΔH of 19.7 ± 3.2 J/g. The thermal behavior and composition of isolates prepared by method a depended on the extraction pH. The isolate extracted at pH 8 was mainly composed of albumin-1 and globulin, whereas at pHs 9, 10, and 11, albumin-2 and glutelin were also present. The increase of the extraction pH led to a decrease in the thermal stability of proteins from pH 8 on and to a decrease in ΔH at pH 11. With method b, different isolates were obtained. At pH 6 and 7, most of the albumin-2 and some of the globulins precipitated, whereas at pHs 4 and 5, all protein fractions precipitated. Acidification at pH 5 and lower lead to denaturation of the proteins, which was only partially reversed by returning to pH 7.

Keywords: *Protein isolate; amaranth proteins; calorimetry; protein structure*

INTRODUCTION

Amaranth is an ancient, cereal-like crop with high seed yield. Seed protein content is also high (Bressani, 1989), and its essential amino acid balance is better than those of cereals and legumes (National Academy of Science, 1984; Teutonico and Knorr, 1985). Therefore, amaranth proteins are one of the more promising food ingredients, capable of complementing cereal or legume proteins. There is plenty of scope to use amaranth protein isolates in food formulation.

The main protein fractions in amaranth grain are albumins, globulins, and glutelins, which differ in their solubilities. The specific amino acid composition of each fraction and some of their molecular characteristics were reported by Segura-Nieto et al. (1994). The proportions of the different fractions in a protein isolate and their particular functional and nutritional properties depend on the preparation method used. In this regard, several different techniques were studied to obtain amaranth protein isolates (Paredes-López et al., 1988; Soriano-Santos and Córdoba-Salgado, 1995), but as the objective was to improve yield, the previous contributions did not deal with the molecular characterization of the isolates, a subject that deserves attention.

In spite of the high nutritional value of amaranth proteins, their utilization as a food ingredient depends largely on their functional properties, which are related to structural characteristics. Marcone et al. (1992, 1994) reported studies on amaranth globulin molecular structure; they were carried out on the saline-soluble proteins, which represent only 25% of the total globulin fraction (Konishi et al., 1989). Amaranth proteins are very difficult to completely dissolve in aqueous solvents without using extreme conditions; as these, in turn, would change their conformation, there are serious problems for studying the structural characteristics of the whole proteins. In this sense, the differential scanning calorimetry (DSC) technique seems to be particularly suitable because it does not require previous solubilization of the sample. With this method, structural characteristics can be indirectly analyzed by measuring parameters associated with thermally induced changes in protein conformation. Gorinstein et al. (1996) have already done DSC measurements of amaranth globulin in solid state to study the effect of denaturants.

The object of the present work was (1) to analyze the effect of extraction and precipitation conditions in the preparation method of amaranth protein isolates and (2) to study the composition and structural characteristics of the protein isolates by electrophoresis and DSC.

MATERIALS AND METHODS

Plant Materials. The seeds of *Amaranthus hypochondriacus* (commercial cultivar) used in this work were grown at the Experimental Station of the Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), Chapingo, México and donated to our laboratory in Argentina.

Flour Preparation. Whole seeds were ground in a Udy mill, mesh 1 mm and screened by 10xx mesh. The flour so obtained was defatted for 24 h with hexane in a 10% (w/v) suspension under continuous stirring, air-dried at room temperature, and finally stored at 4 °C until used. Protein content of the flour, as determined by the Kjeldhal method, was 17.0% (w/w).

Preparation of Protein Isolates. The isolates were prepared according to the following techniques: (a) extraction at different pHs and precipitation at pH 5 and (b) extraction at pH 9 and precipitation at different pHs.

(a) Extraction at Different pHs and Precipitation at pH 5. The proteins were extracted at pHs 8, 9, 10, and 11. To this end, at each pH level, the flour was suspended in water (10% w/v), and the pH adjusted to the required value by adding 2 N NaOH (during this period the suspension maintained the required pH without further adjustment). The suspensions were stirred for 30 min at room temperature and then centrifuged at 9000*g* for 20 min. The supernatants were adjusted to pH 5 with 2 N HCl after which centrifugation at 9000*g* for 20 min was carried out at 4 °C. The precipitates were resuspended in water, neutralized with 0.1 N NaOH, and

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freeze-dried. The isolates so obtained were termed I8, I9, I10, and I11, according to the original extraction pH.

(b) Extraction at pH 9 and Precipitation at Different pHs. Aliquots of the supernatant of the extraction at pH 9 of method a were adjusted to pHs 3, 4, 5, 6, and 7 with 2 N HCl. Part of each of the fresh precipitates was separated for DSC studies, and the remaining part was suspended in water, neutralized with 0.1 N NaOH, and freeze-dried. The fresh precipitates were termed Pp3, Pp4, Pp5, Pp6, and Pp7, and the freeze-dried ones P3, P4, P5, P6, and P7.

Protein Fractionation. The sequential extraction of albumins and globulins was carried out according to method B of Konishi et al. (1991), with some modifications. Each extraction step was performed in three stages (60, 30, 30 min) at room temperature with a ratio of 10 mL of solvent/g of meal. Between the stages, the extraction residue was separated by centrifugation at 9000*g* for 20 min. The first solvent was water, the second 32.5 mM K_2HPO_4/2.6 mM KH_2PO_4 (pH 7.5) containing 0.4 M NaCl, and the third water.

Albumin-1. It was extracted in the first extraction step and precipitated by adjusting to pH 5 with 2 N HCl. The precipitate (albumin-1 fraction) was resuspended in water, neutralized, and freeze-dried as described before.

Globulin. In the second step, we extracted globulins by dialyzing (molecular mass cutoff at 12 000) the supernatants from the saline extraction, at 4 °C against deionized water for 4 days with three changes of dialysate each day. The dialysis tube content was centrifuged at 9000*g* for 20 min, and the pellet (globulin fraction) freeze-dried.

Albumin-2. This fraction was obtained in the third step, the residue of globulin extraction being extracted with water. After centrifugation, the supernatants showed an opalescence that precipitated after adjusting to pH 6 with 2 N HCl. No further precipitate was observed in a subsequent adjustment to pH 5. The precipitate (albumin-2 fraction) was resuspended in water, neutralized with 0.1 N NaOH, and freeze-dried. In these extractions, the yields were in agreement with those presented by Konishi et al. (1991).

Glutelin. To obtain the glutelin fraction, two extractions of the residue of albumin-2 were performed with 0.1 M borate buffer (pH 10) at room temperature. This step was followed by dialysis against water at 4 °C or precipitation at pH 6 at room temperature, with a further dialysis against deionized water at 4 °C. The dialyzing tube content was centrifuged, and the pellets so obtained (glutelin fraction) were freeze-dried. The thermal and electrophoretic behavior of the proteins obtained by the two techniques (i.e., with and without precipitation at pH 6) was similar. Concerning the extracting agent, it must be pointed out that, although the addition of SDS and β-mercaptoethanol would have increased the yield (Barba de la Rosa et al., 1992a; Paredes-López et al., 1993), we used borate buffer alone instead to preserve the structural characteristics of proteins.

The protein content of isolates and fractions was measured according to the Lowry method (Lowry et al., 1951). The freeze-dried isolates were solubilized in 1 N NaOH before protein determinations. In this case the standard protein solution also contained NaOH and the Lowry reagent was modified (Stoscheck, 1990). Bovine serum albumin was used as standard.

Electrophoresis. All gels were run in minislabs (Bio-Rad Mini Protean II Model).

Dissociating, Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). The runs were carried out in the following continuous buffer system: 0.125 M Tris-HCl pH 6.8/1% (w/v) SDS for the stacking gel; 0.375 M Tris-HCl, pH 8.8/1% (w/v) SDS for the separating gel, and 0.025 M Tris-HCl/0.192 M glycine/1% (w/v) SDS, pH 8.3 for the running buffer (Laemmli 1970). The separating gels were prepared with 10% (w/v) acrylamide. Protein samples (10 mg/mL) were dissolved in 0.125 M Tris-HCl, pH 6.8/20% (v/v) glycerol/1% (w/v) SDS/0.05% (w/v) bromophenol blue and centrifuged at 15800*g* for 5 min; the supernatants were used to load the gel (4−6 μL/lane). The electrophoretic runs were conducted for 1 h at a constant voltage of 200 V. The molecular weights of polypeptides were calculated by using the following protein standards: bovine serum albumin (66 000); ovalbumin (45 000); glyceraldehyde-3-phosphate dehydrogenase (36 000); carbonic anhydrase (29 000); trypsinogen (24 000); trypsin inhibitor (20 000) or phosphorylase *b* (94 000); bovine serum albumin (67 000); ovalbumin (45 000); carbonic anhydrase (30 000); trypsin inhibitor (20 100); α-lactalbumin (14 400).

Nondenaturing Polyacrylamide Gel Electrophoresis (Native-PAGE). These tests were performed using the same buffer system as that of SDS−PAGE, but without SDS, in 4−7.5% (w/v) acrylamide gradient gels. Protein samples (20 mg/mL) were dissolved in 0.75 M Tris-HCl, pH 8.8, 20% (v/v) glycerol/0.05% (w/v) bromophenol blue and centrifuged at 15800*g* for 5 min. The supernatants were separated and used to load the gel (4−6 μL/lane).

The gels obtained by both electrophoretic techniques were fixed and stained with 0.1% R-250 Coomasie Brilliant Blue in water/methanol/acetic acid (5:5:20) for 12 h and destained later with 25% (w/v) methanol and 10% (w/v) acetic acid.

Differential Scanning Calorimetry. DSC measurements were performed in a Polymer Laboratories (Rheometric Scientific) calorimeter. The equipment was calibrated at a heating rate of 10 °C/min by using indium, lauric acid, and stearic acid (p.a.) as standards.

As sample holder, hermetically sealed aluminum pans containing 12−16 mg of protein fractions or isolates suspended in water (25% w/v) were used; a double empty pan was employed as reference. The capsules were scanned at 10 °C/min in the range of 30−130 °C. After each run, the pans were punctured and their dry matter content determined by leaving the pans overnight in an oven set at 105 °C. The denaturation temperature (T_d) and enthalpy of transition (ΔH) were obtained by analyzing the thermograms with a Software Plus V5.41. The ΔH value was calculated from the area below the transition peaks. In the thermograms showing more than one peak, the total enthalpy (ΔH_T) was calculated from the area below all peaks with a straight baseline drawn from the beginning of the first peak to the end of the last peak.

Analysis of Results. Isolates were prepared at least in triplicate, and as a minimum, duplicate analyses of each isolate were performed. The data so obtained were statistically evaluated by variance analysis (ANOVA). The comparison of means was done by the least significant difference (LSD) test at a significance level (α) of 0.05.

RESULTS AND DISCUSSION

Amaranth Proteins Extracted at pHs 8, 9, 10, and 11 and Precipitated at pH 5. These isolates, hereafter referred to as I8, I9, I10, and I11, according to the extraction pH, had a protein concentration in the range 80−90 g of protein/100 g of isolate. The yield, expressed as grams of isolate per 100 g of flour, increased with the extraction pH from 4.9 ± 1.1 at pH 8 to 12.4 ± 1.2 at pH 11. These results coincide with those of Paredes López et al. (1988) and Soriano-Santos and Córdoba-Salgado (1995).

Native-PAGE. The amaranth proteins extracted were initially analyzed by native-PAGE and compared to the protein fractions obtained as described in Materials and Methods; the results are shown in Figure 1. The profile of fractions (Figure 1b) show globulins (lane 3) running in a main band, with $R_m = 0.32$. In turn, albumin-1 (lane 1) exhibits bands of different mobility mainly in the 0.25−0.35 R_m range, whereas albumin-2 (lane 2) shows abundant protein unable to penetrate the gel, and four low-mobility bands (R_m 0.21, 0.13, 0.06, and 0.02), indicating species of large size or low charge. On the other hand, glutelins (lane 4) present two bands, of R_m 0.20 and 0.30.

Concerning the profile of isolates (Figure 1a), the electrophoretic pattern of I8 (lane 1) shows a band with higher, as well as bands with lower, mobility than that of the main band of $R_m = 0.27$ (band a). As the

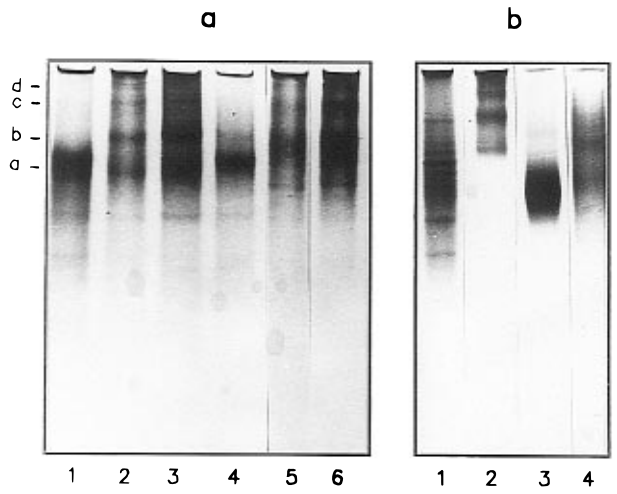

Figure 1. Native-PAGE patterns. (a) Amaranth protein isolates obtained by extraction at different pHs and precipitation at pH 5: lane 1, I8; lane 2, I9; lane 3, I10; lane 4, I11; lane 5, IΔ8−9; lane 6, IΔ9−10. (b) Amaranth protein fractions: lane 1, albumin-1; lane 2, albumin-2; lane 3, globulin; lane 4, glutelin.

extraction pH increases (I9 and I10; lanes 2 and 3), the low-mobility bands of R_m 0.17, 0.09, and 0.02 (bands b, c, and d) become much more noticeable. In the profile of I11 (lane 4), there is an intermediate situation because the main band is at $R_m = 0.23$ while those of the less mobile species are either much weaker or not observed at all. If the residue obtained after the extraction at pH 8 is re-extracted at pH 9 (IΔ8−9; lane 5), and the residue of the extraction at pH 9 is similarly re-extracted at pH 10 (IΔ9−10; lane 6) the isolates so obtained are such that their profiles are similar to those of I9 and I10 but with a predominance of low-mobility bands.

The R_m of band a of I8 is similar to the main band of albumin-1 and globulin, which suggests a selective extraction of those fractions at pH 8. The presence of low-mobility bands in I9 and I10 correlates with the prevalence of these bands in IΔ8−9 and IΔ9−10. As albumin-2 also shows low-mobility bands, these results suggest that this protein (and perhaps also glutelin, whose electrophoretic profile partially overlaps those of globulins and albumin-2) requires pH values higher than 8 to be soluble. Although, in I11, the extraction was more effective in quantitative terms, it exhibits less protein species than I9 or I10. This fact may be caused by the possible denaturing action of the alkaline medium on the protein (Privalov and Khechinashvili, 1974; Hermansson, 1978; Harwalkar and Ma, 1987; Arntfield and Murray, 1981). The unfolded protein, in turn, may form aggregates during precipitation and/or freeze-drying (Arntfield et al. 1990), which could be insoluble in the sample buffer, so remaining in the pellet after the centrifugation stage performed before the electrophoretic analysis (see Materials and Methods).

SDS−PAGE. The isolates and protein fractions were also analyzed by SDS−PAGE, and the results are shown in Figure 2. In the pattern of albumin-1 (Figure 2b, lane 1), peptides of low molecular mass (below 30 kDa) predominate, though there are some components of intermediate molecular mass (between 30 and 60 kDa) in agreement with previous studies (Konishi et al., 1991; Segura-Nieto et al., 1994; Marcone et al., 1992). Notwithstanding, protein is also present at the top of the gel, and this correlates with the results of Segura-Nieto et al. (1992), which show that aggregates settled to the bottom of the tube when albumin-1 was sedimented in a sucrose gradient. In albumin-2 (lane 2), the more important bands range from 51.2 ± 1.6 to 55.8 ± 1.9 kDa, and the peptides of high (above 56 kDa) and intermediate molecular mass (∼30 kDa) were less abundant. These data are in agreement with those of Konishi et al. (1991) except for protein above 56 kDa, which was not reported by the above-mentioned authors and observed in the present work. In globulins (lane 3, Figure 2b), the electrophoretic profile shows a band distribution similar to that already described by Segura-Nieto et al. (1994). There are components of high molecular mass (76.7 ± 3.8 kDa and above 80 kDa), those of 53.0 ± 1.9 and 57.2 ± 1.7 kDa already observed in albumin-2, and polypeptides of intermediate (41.2 ± 1.9, 37.3 ± 1.4, 34.1 ± 1.4, and 32.4 ± 1.3 kDa) and of low molecular mass (≤20 kDa). As in the work of Barba de la Rosa et al. (1992b), we observed that the band of 76.7 kDa belong to a minor, 7S-type component (data not shown). The polypeptide pattern of glutelins (lane 4, Figure 2b) is similar to that of a combination of globulins and albumin-2. It shows bands of 52 and 57 kDa, of intermediate values (between 30 and 45 kDa), and abundant protein above 80 kDa. As the extraction of these fraction was performed under nonextreme conditions (without SDS and β-mercaptoethanol), it could have been considerably contaminated with globulins and albumins-2.

By comparing the profiles of the isolates with those of the partially purified fractions, we observed that in I8 (lane 1, Figure 2a) the low molecular mass peptides, typical of albumin-1, are in higher proportion. There are, also, intermediate molecular mass peptides (present in all fractions) and a band of 76.8 ± 3.7 kDa as in globulins. These result suggest that, at pH 8, the extracts contain a high proportion of albumin-1. In the other isolates, I9, I10, I11, IΔ8−9, and IΔ9−10 (Figure 2a, lanes 2, 3, 4, 5, and 6, respectively), the higher contents of polypeptides of 53.0 ± 2.0 and 56.5 ± 1.8 kDa and of those of high molecular mass suggest that albumins-2, globulins, and glutelins are extracted mainly at a pH greater than 8. As the band of 76.8 ± 3.7 kDa is hardly detected in isolates IΔ8−9 and IΔ9−10 (Figure 2a, lanes 5 and 6), it can be assumed that most of the globulin component containing that peptide has been extracted at pH 8.

I11 (Figure 2a, lane 4), shows a lower proportion of high molecular mass species, in agreement with the decrease in low-mobility species shown in native-PAGE. The IΔ10−11 profile (Figure 2a, lane 7) shows protein that only penetrate the stacking gel and very a low content of the previously described peptides. These results suggest that the protein extracted at a pH above 10 is mostly composed of high molecular mass aggregates which are soluble in the sample buffer containing SDS.

Proteins Extracted at pH 9 and Precipitated at pHs 3, 4, 5, 6, and 7 (Isolates P3, P4, P5, P6, and P7, Respectively). The effect of precipitation pH was also analyzed, and the protein yields obtained for the isolates are shown in Figure 3. Maximum protein yield was significantly higher ($LSD_{0.05}$) at pH 3−5 than at greater pH values, in agreement with the results of Paredes López et al. (1988).

Native-PAGE. The electrophoretic profiles of the isolates (Figure 4) show that, as the precipitation pH increases, the lower mobility bands become more important (increase their intensity). In the profile of P3

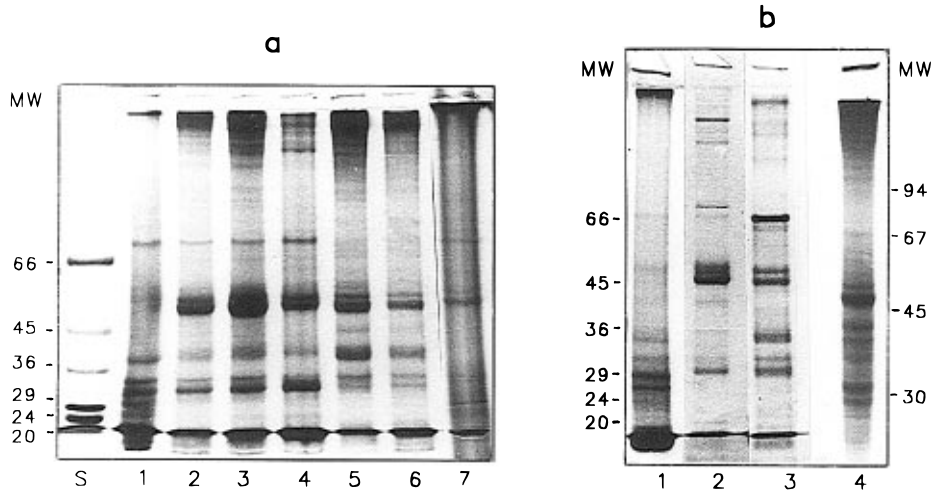

Figure 2. SDS−PAGE patterns. (a) Amaranth protein isolates obtained by extraction at different pHs and precipitation at pH 5: lane S, standard molecular weight proteins; lane 1, I8; lane 2, I9; lane 3, I10; lane 4, I11; lane 5, IΔ8−9; lane 6, IΔ9−10; lane 7, IΔ10−11. (b) Amaranth protein fractions: lane 1, albumin-1; lane 2, albumin-2; lane 3, globulin; lane 4, glutelin. Lanes 1−3 have standard molecular weights (MW) on the left side, and lane 4 has standard molecular weight (MW) on the right side.

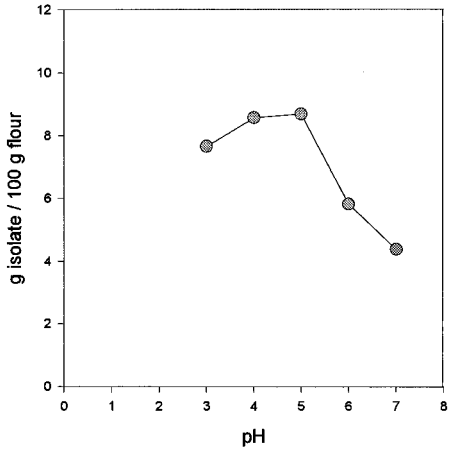

Figure 3. Yield (g of isolate/100 g of defatted flour) of protein isolates obtained by extraction at pH 9 and precipitation at different pHs, as a function of pH of precipitation. $LSD_{0.05}$ = 18.11.

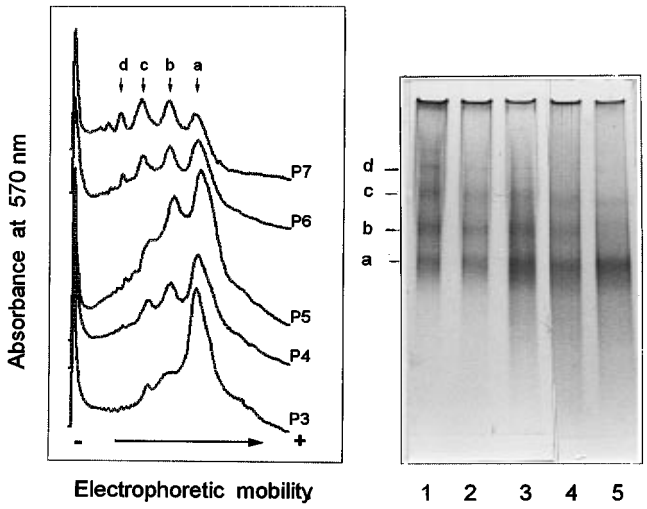

Figure 4. Native-PAGE of amaranth protein isolates obtained by extraction at pH 9 and precipitation at different pHs. Gel (lane 1, P7; lane 2, P6; lane 3, P5; lane 4, P4; lane 5, P3) and corresponding densitometries.

(Figure 4, lane 5 and densitometry), the major band is a whereas in the profiles of P4 and P5 (Figure 4, lanes 4 and 3, and densitometries) besides band a, bands b

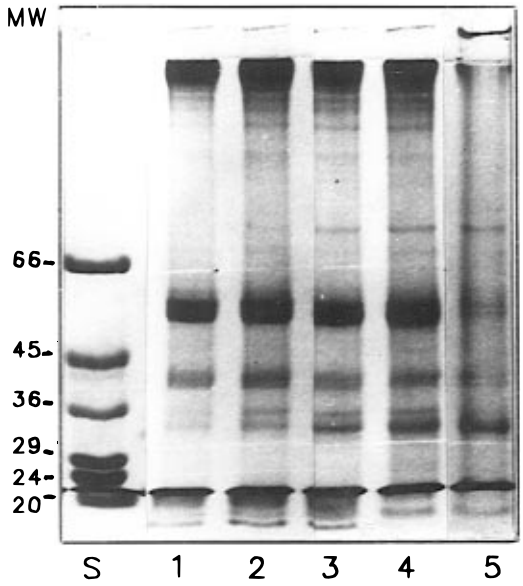

Figure 5. SDS−PAGE of amaranth protein isolates obtained by extraction at pH 9 and precipitation at different pHs. Lane S, standard molecular weight proteins; lane 1, P7; lane 2, P6; lane 3, P5; lane 4, P4; lane 5, P3.

and c are also important. Comparison of these patterns with those obtained for the partially purified fractions (Figure 1b) suggests the presence of albumin-1 and globulin proteins in P3 and albumin-1, albumin-2, globulin, and glutelin species in P4 and P5. In P6 and P7 (Figure 4, lanes 2 and 1, and densitometries), low-mobility bands are more intense than in the other isolates, suggesting that P6 and P7 contain albumin-2 and globulin proteins in greater proportion. These results correlate with the published values of pI = 5 for globulin (Konishi et al., 1985; Marcone et al., 1991) and pI = 5.8 for albumin-2 (Konishi et al., 1991), which indicate that globulin precipitates mostly at pH 5 and albumin-2 at pH 6.

SDS−PAGE. In P7 and P6 (Figure 5, lanes 1 and 2), the 53.0 ± 2.0 and 56.5 ± 1.8 kDa polypeptides (the main peptides of albumins-2) predominate over those of low or intermediate molecular mass. Both P4 and P5 (Figure 5, lanes 4 and 3) exhibit all the bands of the four fractions, while the intermediate molecular mass (∼35 kDa) peptides predominate in P3 (Figure 5, lane

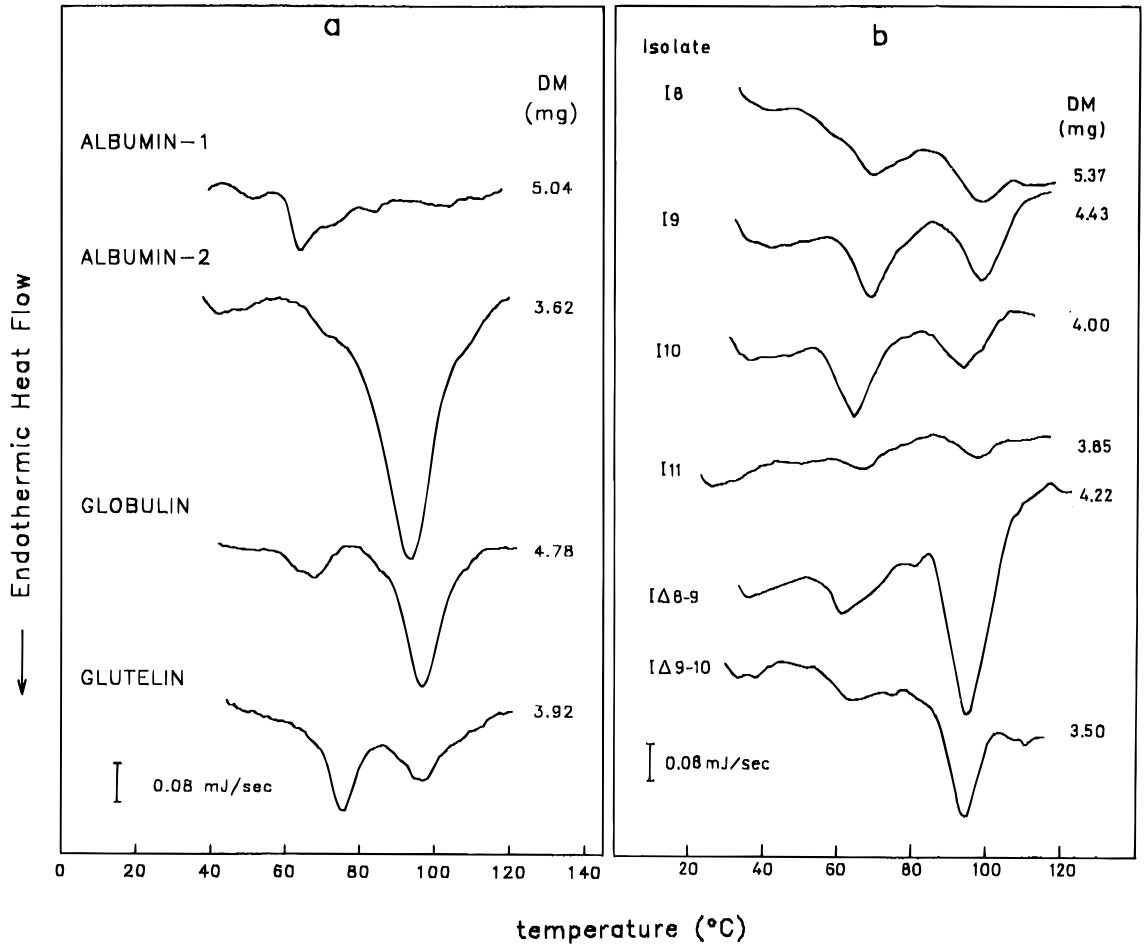

Figure 6. DSC thermograms of 25% dispersion of the sample in distilled water. Heating rate 10 °C/min. DM, corresponding dry matter. (a) Amaranth protein isolates obtained by extraction at different pHs and precipitation at pH 5. (b) Amaranth protein fractions.

5). Although there are globulin peptides in P6 and P7 [Marcone et al. (1992) have observed partial precipitation of globulins at such pH levels], the 76.8 ± 3.7 kDa precipitates at a pH lower than 6, since it is present in P5, P4, and P3.

These results are in agreement with those of native-PAGE. Both of them suggest a preferential precipitation of albumin-2 at pHs 6 and 7 and of globulin and albumin-1 at pH 5 and lower.

Differential Scanning Calorimetry. This technique allows the thermally induced unfolding of protein molecules to be detected. By analyzing DSC thermograms, the denaturation temperature (T_d) and enthalpy (ΔH) are determined. The T_d, temperature of the transition peak, is a measure of the thermal stability of a given protein, while ΔH, calculated from the area below the transition peak, correlates with the extent of ordered secondary structure of a protein (Koshiyama et al., 1981).

In order to compare the thermal behavior of isolates with the protein fractions, DSC was applied to partially purified globulin, albumin-1, albumin-2, and glutelin fractions (Figure 6a). The thermogram of albumin-1 shows low ΔH endothermal transitions of different T_d, the main endotherm having a T_d of 64 °C. The thermogram of globulins shows two transition peaks. The major endotherm would belong to the already described 12S fraction (Konishi et al., 1985; Marcone et al., 1991; Barba de la Rosa et al., 1992b). Its high denaturation temperature, T_d = 94 °C, similar to that of other 11S globulins [soybean T_d = 92 °C, broadbean T_d = 94 °C; sunflower T_d = 95 °C (Ma and Hawalkar, 1991)], indicates that this protein is thermostable, which reflects a high number of hydrophobic interactions (Myers, 1990). The minor endothermal peak, of lower T_d, can be assigned both to components in low proportions accompanying globulin 12S [Barba de la Rosa et al. (1992b), data not shown] and to contaminant albumin-1.

The thermogram of the albumin-2 fraction presents only one endothermal transition, suggesting that this fraction consists essentially of one protein species or else of several species of the same thermostability. Its T_d of 94 °C indicates, as in globulins, a high thermal stability.

The glutelin fraction presents two endotherms with transition temperatures (T_d) of 70 and 96 °C, indicating the presence of proteins with different thermal stability. The thermal behavior of different preparations of this fraction was not alike, as reflected by the relation between the areas below each endotherm. The values of the ratio, defined as r = (area of lower T_d endotherm)/(area of higher T_d endotherm), ranged between 1.5 and 0.25. In this regard, the higher T_d endotherm may correspond to globulins and/or albumin-2 contaminating each preparation to varying degrees.

Figure 6b shows the thermograms of isolates obtained by extraction at different pHs. In all isolates, two endotherms are observed, the T_d values of which are between 70 and 73 °C for the first and between 94 and 100 °C for the second. There is a small decrease of the T_d of both endotherms as the extraction pH of the isolate

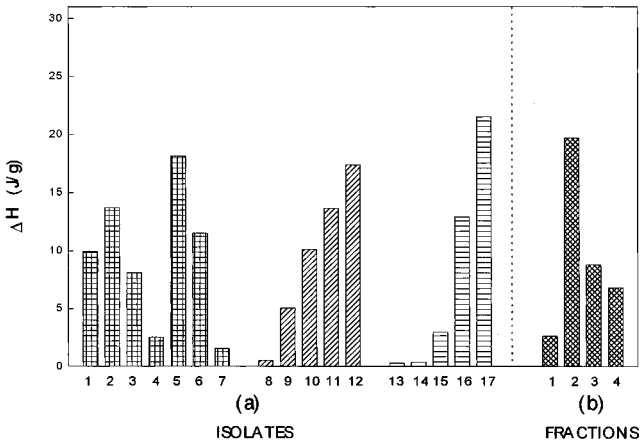

Figure 7. Enthalpy value (ΔH) of isolates and protein fractions. Panel a, isolates. (group a, 1−7) Protein isolates obtained by extraction at different pHs and precipitation at pH 5: 1, I8; 2, I9; 3, I10; 4, I11; 5 IΔ8−9; 6 IΔ9−10; 7, IΔ10−11. $LSD_{0.05} = 5.36$. (group a, 8−12) Protein isolates obtained by extraction at pH 9 and precipitation at different pHs: 8, P3; 9, P4; 10, P5; 11, P6; 12, P7. $LSD_{0.05} = 3.58$. (group a, 13−17) Fresh protein precipitates obtained by extraction at pH 9 and precipitation at different pHs: 13, Pp3; 14, Pp4; 15, Pp5; 16, Pp6; 17, Pp7. $LSD_{0.05} = 1.78$. Panel b, fractions. (group b, 1−4) Amaranth protein fractions: 1, albumin-1; 2, albumin-2; 3, globulin; 4, glutelin. $LSD_{0.05} = 3.87$.

increases. By comparison of these thermograms with those of the protein fractions (Figure 6a) it is assumed that all fractions, but mainly albumins-2 and globulins, contribute to the second (of higher T_d) endotherm, while only albumins-1, glutelins, and the minor components of globulins would contribute to the first. For the isolates, it must be emphasized that the observed area below the first (of lower T_d) endotherm is higher than those of several hypothetical estimates, based on consideration of the isolates composed in different proportions of the above-mentioned fractions. The area below the first endotherm of albumin-1 and globulin is very small; therefore, the main contribution would be that from glutelin, and it is unlikely that isolates (at least I8 and I9) contain a large proportion of this fraction. Therefore, as a result of the alkaline treatment followed by isoelectric precipitation, we cannot discard the possibility of irreversible conformational changes in some molecules that leads to a decrease in their thermal stability. In this regard, for isoelectric isolates of fababean, Murray et al. (1985) observed a decrease in T_d and ΔH as a consequence of pH manipulation.

Figure 7 shows the ΔH of protein fractions and isolates. Such ΔH is, in fact, the result of a combination of endothermal reactions such as disruption of hydrogen bonds (Privalov and Khechinashvili, 1974) and exothermal processes, including protein aggregation and breakup of hydrophobic interactions (Jackson and Brandts, 1970; Arntfield and Murray, 1981). In this regard, the low ΔH value of albumins-1 (Figure 7b, lane 1) could indicate a low bonding energy conformation, a high proportion of hydrophobic interactions (less likely because of its low T_d), or aggregation. The denaturation enthalpy of globulin (Figure 7b, lane 2) is somewhat low if compared to those of other storage proteins [soybean globulin, 25 J/g; broadbean globulin, 23 J/g (Ma and Harwalkar, 1991); oat globulin, 26.33 J/g (Harwalkar and Ma, 1987)]. This can be due to the fact that the fraction studied is a partially purified preparation of globulins with some albumin-1. The ΔH of albumin-2 (Figure 7b, lane 3) is similar to those of other storage proteins (Ma and Harwalkar, 1991; Harwalkar and Ma, 1987) and much higher than those of the remaining amaranth proteins. Such a high ΔH value may indicate native conformation stabilized by a greater number of hydrogen bonds, though it may well be the result of a lower tendency of albumin-2 to aggregate than the other protein fractions. As partially denatured proteins require less heat to complete denaturation, the low denaturation enthalpy of glutelin (Figure 7b, lane 4) can be attributed to partial denaturation of this fraction induced by the extraction conditions (alkaline pH). This behavior can be also explained as having a low bonding energy conformation or by considering that, in the thermal transitions, there are some exothermic processes such as the formation of aggregates.

The denaturation enthalpies of the isolates are included in Figure 7a. Isolate IΔ8−9 (Figure 7a, lane 5), showed the higher ΔH value, significantly different ($P_{0.05}$) from the other enthalpy values except that of I9 (Figure 7a, lane 2). In the thermogram of IΔ8−9 (Figure 6), the second endotherm, which possibly was caused by the thermal transition of globulins and albumins-2 (fractions of higher ΔH), has the largest area (Figure 6b). These results are consistent with those obtained by electrophoresis, which suggested that most of the albumin-2 and globulin proteins were extracted at a pH above 8. The presence of glutelins (whose ΔH is lower than that of albumins-2), and perhaps a partially denaturing effect of the alkaline pH, would explain the decrease in the ΔH value of IΔ9−10 (Figure 7a, lane 6) with respect to that of IΔ8−9 (Figure 7a, lane 5). Ma and Hawalkar (1991) have observed that pH values above 8 have a destabilizing effect in plant proteins, with a decrease in T_d and ΔH. We also observed a decrease of T_d in both endotherms as the extraction pH increases (Figure 6).

In I11 and IΔ10−11 (Figure 7a, lanes 4 and 7) the ΔH values are significantly lower ($P_{0.05}$) than in the other isolates, which suggest a higher denaturation degree.

The thermograms and enthalpy values of the isolates obtained by extraction at pH 9 and precipitation at different pH are shown in Figures 8a and 7. The ΔH value is significantly higher ($P_{0.05}$) for P7 isolate than for P3, P4, and P5 (Figure 7a, lanes 12, 8, 9, and 10) while P6 (Figure 7a, lane 11) shows an intermediate ΔH value, not significantly different from those of P5 and P7. These results correlate with those arrived at by electrophoresis suggesting the presence of a higher proportion of albumin-2 in P6 and P7. As the precipitation pH decreases (P5, P4, and P3), the area of the first endotherm increases (Figure 8a), at the same time as the ΔH values decrease (Figure 7a, lanes 10, 9, and 8). This may suggest that the proteins of lower thermal stability and lower denaturation enthalpy (albumins-1 and minor components of globulins) precipitate mostly in this pH range. Alternatively, the decrease in the ΔH could have been caused partially by low-pH denaturation. This is particularly valid in P3 since its thermogram shows very small thermal transitions besides a marked decrease in ΔH. The increase of positive charges at lower pH could induce unfolding of protein molecules. In this regard, Ma and Harwalkar (1991) have observed that, as in alkaline treatments, pH values lower than 5 also have a denaturing effect on several plant proteins.

To study the pH effect on the conformation of the protein precipitates, the isolates obtained by precipitation at different pHs (Pp3, Pp4, Pp5, Pp6, Pp7), were

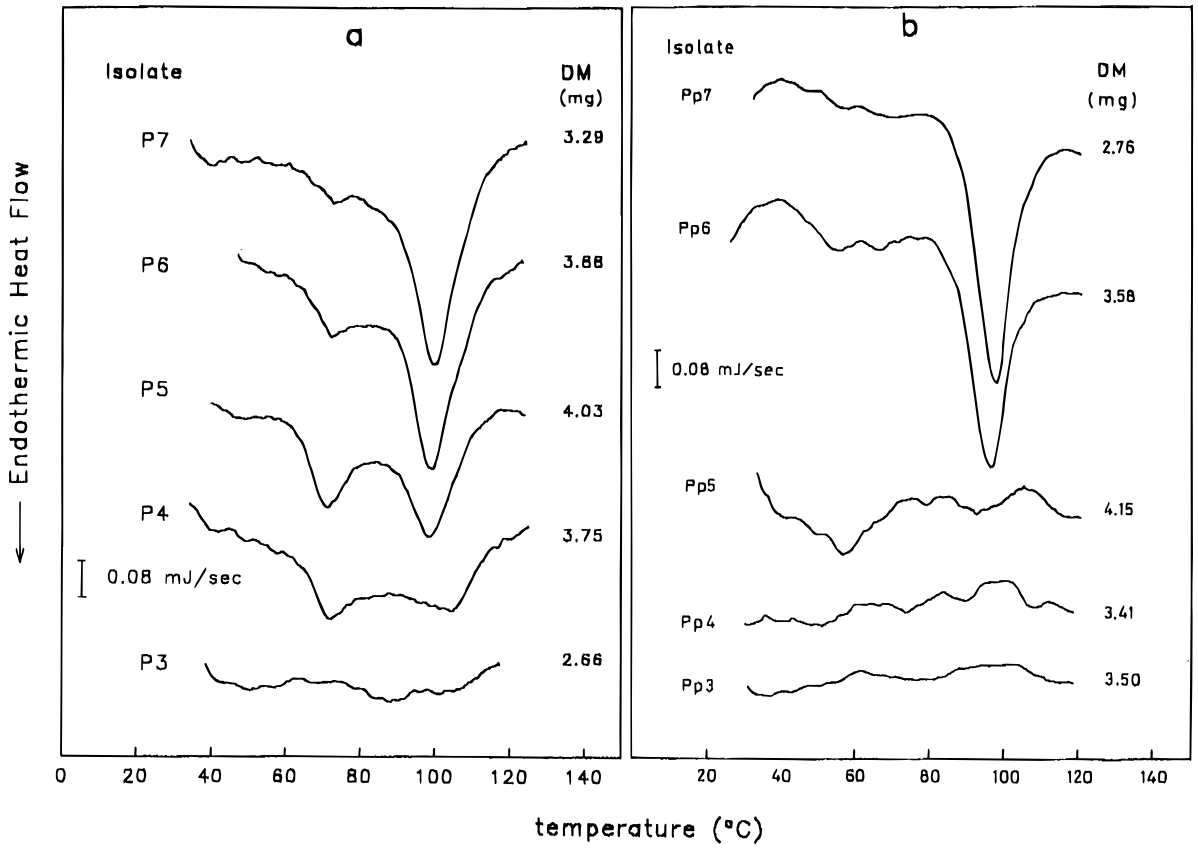

Figure 8. DSC thermograms of 25% dispersion of the sample in distilled water. Heating rate 10 °C/min. DM, correponding dry matter. (a) Amaranth protein isolates obtained by extraction at pH 9 and precipitation at different pHs. (b) Fresh protein precipitates obtained by extraction at pH 9 and precipitation at different pHs.

analyzed by DSC before being neutralized and freeze-dried. The corresponding denaturation enthalpies and thermograms are shown in Figures 7 and 8b. The enthalpies of Pp4 and Pp5 (Figure 7a, lanes 14 and 15) are significantly lower ($P_{0.05}$) than the corresponding freeze-dried isolates run at pH 7 (P4 and P5; Figure 7a, lanes 9 and 10) and this suggests that the proteins exposed at pH $\leq$5 undergo different denaturation degrees, which partially reversed as soon as the pH was increased to 7. The thermograms of precipitates Pp3 and Pp4 show very small and broad thermal transitions, possibly due to a high degree of unfolding caused by repulsion between positive charges. In precipitate Pp5, the thermogram shows the first widening endotherm with a larger area (T_d = 57 °C) and another endotherm at higher temperature of smaller area. These results suggest the existence, at pH 5, of two populations of protein molecules. One with the same thermal stability as that at pH 7 and the other much less thermostable, possibly due to weakening of the protein structure. The peak widening shown by both endotherms evidences a loss of cooperativity in the thermal denaturation process, which could be caused by dissociation of oligomeric forms. Marcone et al. (1992) had reported conformational changes at pH 5.5 of amaranth globulin 11.53S. The behavior of amaranth proteins at pH 5 (a value that coincides with the p*I* of some of them) is different from that of other plant proteins (soybean, oat, fababean), which show maximum stability at this pH (Hermansson, 1978; Arntfield and Murray, 1981; Hawalkar and Ma, 1987). The partial unfolding at low pH may not be totally reversible when the pH returns to 7, an alternative that would be reflected by a relatively larger area of the first endotherm of the corresponding freeze-dried isolates. The freeze-drying process may also introduce conformational changes in these isolates. Arntfield et al. (1990) have observed a reduction in ΔH after freeze-drying a 20% slurry of fababean protein isolate.

CONCLUSIONS

The composition and structural characteristics of amaranth protein extracted at various alkaline pHs and precipitated at pH 5 depend on the extraction pH. The results here obtained suggest that albumin-1 and part of globulin are mostly extracted at pH 8, whereas, at pH above 8, albumin-2, the remaining globulin, and glutelin are also present. The increase of the extraction pH seems to induce conformational changes in the proteins, as evidenced in the thermal analysis by a decrease of the denaturation temperature at pH higher than 8 and by a decrease in ΔH at pH 11.

The composition of amaranth protein isolates could be modified by varying the precipitation pH. At pHs 6 and 7, there is a selective precipitation of albumin-2 and of some of the globulins, whereas at pHs 4 and 5, all protein fractions precipitate. Acidification at pH 5 and lower seems to cause conformational changes in the proteins, which are only partially reversed when the pH returns to 7.

According to these results, the composition and degree of unfolding of amaranth protein isolates could be controlled by choosing different combinations of extraction and precipitation pH.

Partially purified whole protein fractions (soluble and insoluble portions) were analyzed by means of the DSC technique. It was observed that, as in other plant storage proteins, albumin-2 and globulin are composed

of a main protein species with a high thermal stability, probably rich in hydrophobic interactions. On the other hand, albumin-1 consists of several species with different denaturation temperatures, all below 80 °C, and glutelin is formed by two species with different T_d. Among the protein fractions of amaranth, albumin-2 has the highest denaturation enthalpy. This suggests that the conformation of albumin-2 is stabilized by a higher bonding energy, since its ΔH value (19.7 ± 3.2 J/g) is similar to that of other plant proteins (Ma and Harwalkar, 1991).

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LITERATURE CITED

Arntfield, S. D.; Murray, E. D. The influence of processing parameters on food protein functionality I. Differential scanning calorimetry as an indicator of protein denaturation. *Can. Inst. Food Sci. Technol. J.* **1981**, *14* (4), 289−294.

Barba de la Rosa, A.; Gueguen, J.; Paredes-López, O.; Viroben, G. Fractionation procedures, electrophoretic characterization, and amino acid composition of amaranth seed proteins. *J. Agric. Food Chem.* **1992a**, *40*, 931−936.

Barba de la Rosa, A.; Paredes-López, O.; Gueguen, J. Characterization of amaranth globulins by ultracentrifugation and chromatographic techniques. *J. Agric. Food Chem.* **1992b**, *40*, 937−940.

Bressani, R. The proteins of grain amaranth. *Food Rev. Int.* **1989**, *5* (1), 13−38.

Harwalkar, V. R.; Ma, C. Y. Study of thermal properties of oat globulin by differential scanning calorimetry. *J. Food Sci.* **1987**, *52* (2), 394−398.

Hermansson, A. M. Physico-chemical aspects of soy proteins structure. *J. Texture Stud.* **1978**, *9*, 33−58.

Jackson, W. M.; Brandts, J. F. Thermodynamics of protein denaturation. A calorimetric study of the reversible denaturation of chymotrypsinogen and conclusions regarding the accuracy of the two state approximation. *Biochemistry* **1970**, *9*, 2294.

Konishi, Y.; Yoshimoto, N. Amaranth globulin as a heat-stable emulsifying agent. *Agric. Biol. Chem.* **1989**, *53* (12), 3327−3328.

Konishi, Y.; Fumita, Y.; Ikeda, K.; Okuno, K.; Fuwa, H. Isolation and characterization of globulin from seeds of *Amaranthus hypochondriacus L. Agric. Biol. Chem.* **1985**, *49*, 1453−1459.

Konishi, Y.; Horikawa, K.; Oku, Y.; Azumaya, J.; Nakatani, N. Extraction of two albumin fractions from amaranth grains: comparison of some physicochemical properties and the putative localization in the grains. *Agric. Biol. Chem.* **1991**, *55* (11), 1745−1750.

Koshiyama, I.; Hamano, M.; Fukushima, D. A heat denaturation study of the 11S globulin in soybean seeds. *Food Chem.* **1981**, *6*, 309−312.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.

Ma, C. Y.; Harwalkar, R. Thermal analysis of food proteins. *Adv. Food Nutr. Res.* **1991**, *35*, 317−366.

Marcone, M. F.; Yada, R. Y. Isolation, purification, and characterization of the oligomeric seed globulin from *Amaranthus hypochondriacus. Agric. Biol. Chem.* **1991**, *55* (9), 2281−2289.

Marcone, M. F.; Yada, R. Y. Study of the charge profile and covalent subunit association of the oligomeric seed globulin from *Amaranthus hypochondriacus. J. Agric. Food Chem.* **1992**, *40*, 385−389.

Marcone, M. F.; Niekamp, F. K.; LeMaguer, M.; Yada, R. Y. Purification and characterization of the physicochemical properties of the albumin fraction from the seeds of *Amaranthus hypochondriacus. Food Chem.* **1994**, *51*, 287−294.

Murray, E. D.; Arntfield, S. D.; Ismond, M. A. H. The influence of processing parameters on food protein functionality II. Factors affecting thermal properties as analyzed by differential scanning calorimetry. *Can. Inst. Food Sci. Technol. J.* **1985**, *18* (2), 158−162.

Myers, C. D. Study of thermodynamics and kinetics of protein stability by thermal analysis. In *Thermal Analysis of Foods*; Elsevier Science Publishing: New York, 1990; Chapter 2, pp 16−50.

National Academy of Science. *Amaranth: Modern prospects for an ancient Crop;* National Academy Press: Washington, DC, 1984.

Paredes-López, O.; Mora-Escobedo, R.; Odorica-Falomir, C. Isolation of amaranth proteins. *Lebensm. Wiss. Technol.* **1988**, *21*, 59−61.

Paredes-López, O.; Mendoza, V.; Mora, R. Isolation of amaranth flour proteins by fractionation procedures and sonication. *Plant Foods Hum. Nutr.* **1993**, *43*, 37−43.

Privalov, P. L.; Khechinashvili, N. N. A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study. *J. Mol. Biol.* **1974**, *86*, 665−684

Segura-Nieto, M.; Vázquez-Sanchez, N.; Rubio-Velázquez, H.; Olguín-Martínez, L. E.; Rodríguez-Néster, C. E.; Herrera-Estrella, L. Characterization of amaranth (*Amaranthus hypochondriacus* L.) seed proteins. *J. Agric. Food Chem.* **1992**, *40*, 1553−1558.

Segura-Nieto, M.; Barba de la Rosa, A. P.; Paredes-López, O. Biochemistry of amaranth proteins. In *Amaranth: Biology, Chemistry and Technology;* CRC Press: Boca Raton, FL, 1994; Chapter 5, pp 75−106.

Soriano-Santos, J.; Córdoba-Salgado, M. A. Evaluation of different methods of solubilization of nitrogen for preparation of protein concentrates from amaranth seed. *Rev. Esp. Cienc. Tecnol. Aliment.* **1995**, *35* (2), 161−177.

Soriano-Santos, J.; Iwabuchi, S.; Fujimoto, K. Solubility of Amaranth seed proteins in sodium sulphate and sodium chloride: the main factor in quantitative extraction for analysis. *Int. J. Food Sci., Technol.* **1992**, *27*, 337−346.

Stoscheck, Christa M. Quantitation of protein. In *Methods in Enzymology;* Vol 182, *Guide to Protein Purification*; Academic Press: San Diego, CA, 1990; Section II, pp 50−68.

Teutonico, R. A.; Knorr, D. Amaranth: Composition, properties and applications of a rediscovered food crop. *Food Technol.* **1985**, *39* (4), 49−61.

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