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Food Chemistry 51 (1994) 319–324

0308-8146/94/$7.00

Solubilization and electrophoretic characterization of cashew nut (*Anacardium occidentale*) proteins†

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(Received 22 October 1993; accepted 24 January 1994)

The major proteins in cashew nut (*Anacardium occidentale*) were found to be soluble in aqueous solvents. Of the total solubilized proteins, water, 1 M NaCl, 70% ethanol, and 1 M NaOH-soluble proteins respectively accounted for 45·6, 42·4, 0·4, and 11·7%. The true albumin to globulin ratio was 2·0. Cashew nut proteins had a minimum solubility at pH 5·0. Among the protein solubilizing agents tested, 0·1 M NaOH was the most effective solubilizer of cashew proteins. Electrophoretic analyses indicated that the cashew nut protein composition was dominated by a single multimeric protein. This dominant protein was composed of two major kinds of polypeptides linked together via disulfide bond(s). The estimated molecular weights (MWs) of subunit polypeptides were 30 000–32 000 and 20 000–22 000. Amino acid analyses of the albumins and globulins indicated methionine to be the first limiting amino acid in both fractions. Hydrophobic, acidic, basic, and uncharged polar amino acids accounted for 34·39, 25·87, 19·77 and 19·97% of total albumins while the corresponding percentages for the globulins were 34·25, 26·14, 18·93 and 20·68, respectively.

INTRODUCTION

Tree nuts enjoy worldwide acceptance and are valued for their sensory and nutritional attributes. They are typically high in fat (45–70%, w/w) and protein (20–25%, w/w) and are therefore energy-rich foods. Among tree nuts, cashew nut (*Anacardium occidentale*) is an oilseed with excellent consumer acceptance and therefore has a great potential for increased utilization in foods. The global production of cashew nuts in 1988 was 475 928 metric tonnes (approximately 1 047 million pounds), with Asia, South America, and Africa accounting for 42·92, 30·00, and 26·41% respectively of the total production. India (34·25%) and Brazil (30·00%) led the world cashew nut production in 1988 (FAO, 1988).

Cashew nuts provide 7·76 kcal/g, 21% protein, 48% fat and are also a good source of many minerals such as phosphorus, potassium, magnesium, and iron (Fetuga *et al.*, 1974). Although cashew nut chemical composition and nutritional quality have been investigated (Piva *et al.*, 1971; Fetuga *et al.*, 1974; Toschi *et al.*, 1993), detailed characterization of cashew nut proteins has not yet been accomplished. The purpose of this investigation was to evaluate the cashew nut proteins for their solubility, electrophoretic characteristics, and amino acid composition.

†Presented in part at the Annual Meeting of Food Technologists, 1–5 June 1991, Dallas, TX; and the 8th World Congress of Food Science and Technology, 29 September–4 October 1991, Toronto, Canada.

MATERIALS AND METHODS

Materials

Unprocessed cashew nuts were obtained from a local grocery store. Electrophoresis chemical sources were the same as reported earlier (Sathe, 1993). Other chemicals were from either Sigma Chemical Co., St. Louis, MO or Fisher Scientific Company, Orlando, FL.

Methods

Preparation of flour

Cashew nuts were ground in a Waring Blender (approximately 40 mesh) and defatted with cold (4°C) acetone (flour to solvent ratio 1 : 5, w/v) with constant magnetic stirring provided. The slurry was filtered through a filter paper (vacuum filtration). The residue was re-extracted twice in a similar fashion. The defatted flour was dried in a fume hood and the dry flour was ground in a blender (as above) to obtain

a homogeneous defatted flour. The defatted flour was stored at −20°C in an air-tight plastic bottle until further use.

Protein solubility

Effect of solvent. To evaluate the ability of common solvents to solubilize cashew nut proteins, defatted flour (100 mg) was extracted with 1 ml of appropriate solvent at room temperature (25°C) for 30 min with vortexing every 5 min. At the end of the extraction period, samples were centrifuged in a Beckman table-top microcentrifuge at 25°C, 13 600*g* for 10 min and the supernatant analyzed for protein content.

Effect of pH on protein solubility. Defatted flour (2 g) was extracted with 20 ml of 0·1 N NaOH at 25°C for 30 min with constant magnetic stirring provided. The samples were then centrifuged at 12 600*g* for 10 min at 4°C, and the supernatant was filtered through a filter paper. The filtrate was diluted with 9 volumes of distilled deionized (DIDI) water and the pH of aliquots (5 ml) was adjusted to the desired value with 1 N NaOH or 1 N HCl. After pH adjustment, the samples were magnetically stirred for an additional 30 min, centrifuged (12 600*g*, 15 min, 4°C), the supernatant made up to volume (6 ml) with DIDI water and analyzed for soluble protein.

Separation of albumins and globulins. To determine the relative proportion of water-soluble (albumins) and salt soluble (globulins) proteins, 5 g of defatted flour was extracted with 1 M NaCl at 25°C for 30 min with constant magnetic stirring and samples centrifuged (12 600*g*, 10 min, 25°C). The supernatant was then extensively dialyzed against distilled deionized water at 4°C (48 h, six changes, 5 liters each). The molecular weight (MW) cut-off of the dialysis tubing was 6000–8000. Samples were then centrifuged (12 600*g*, 10 min, 4°C) and the supernatant (true albumins) and precipitate (true globulins) collected. The precipitate was dissolved in a minimum amount of 1 M NaCl, reprecipitated with 5 volumes of cold (4°C) DIDI water and centrifuged (12 600*g*, 10 min, 4°C) three more times. The supernatant was dialyzed against DIDI water as above and the combined supernatant (albumins) and precipitate (globulins) lyophilized.

Protein fractionation. Defatted flour (100 mg) was sequentially extracted at room temperature (25°C) for 1 h (vortexing every 10 min) with 1 ml of each of: DIDI water; 1 M NaCl: 70% (v/v) ethanol; and 1 N NaOH. At the end of each extraction the slurry was centrifuged (15 000*g*, 10 min, 25°C) and the supernatants used for further analysis.

Protein determination

Soluble protein content of samples was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard protein. A standard curve was prepared in appropriate solvent for every assay.

Electrophoresis

Nondenaturing and nondissociating (NDND)–polyacrylamide gel electrophoresis (PAGE) was done as according to Andrews (1986) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was done by the method of Fling and Gregerson (1986). Details of gel electrophoreses were the same as described earlier (Sathe, 1993).

Amino acid analysis

Amino acid composition was determined using Waters Pico-Tag Column Amino Acid Analyzer (Waters Chromatography Division, Milford, MA). A sample (0·5 mg) was hydrolyzed in 600 μl of 6 N HCl in the presence of nitrogen (18 h, 110°C) and 4 μl was injected for analysis. Tryptophan content was determined by the colorimetric method of Spies and Chambers (1948).

Statistical analysis

All experiments were done at least in duplicate. Data are reported as mean ± standard error of mean (SEM). When appropriate, data were analyzed for significant differences using Fisher's LSD (protected test) as described by Ott (1977).

RESULTS AND DISCUSSION

Protein solubility

Among several solvents tested, 0·1 M NaOH was the most effective protein solubilizer while DIDI water and 0·2 M sodium acetate buffer (pH 4·5) containing 0·25 M NaCl were the least effective (Table 1). Protein solubility data are consistent with the protein solubility profile (Fig. 1) in that, as the pH of the extraction medium increased above 7·0, the solvent was efficient in solubilizing the proteins. Based on protein solubility profile, cashew nut proteins were least soluble (14·63 ± 0·73%) at pH 5·0, indicating that the proteins were acidic in nature. On either side of this pH the protein solubility increased significantly. Alkaline pH was slightly more effective in solubi-

Table 1. Cashew nut protein solubility as influenced by solubilizing agent

Solvent	Protein solubility[a]
Distilled deionized water	12·05 ± 0·48
1·0 M NaCl	19·95 ± 0·45
0·1 M Tris-HCl (pH 8·1)	22·16 ± 0·31
0·1 M Na_2CO_3	22·56 ± 0·77
0·1 M $NaHCO_3$	21·50 ± 1·19
0·1 M Na_2SO_4	20·90 ± 1·04
0·1 M Sodium phosphate buffer (pH 7·5)	21·45 ± 1·50
0·1 M NaOH	25·73 ± 1·52
0·1 M Potassium phosphate buffer (pH 7·5)	21·18 ± 0·37
0·2 M Sodium acetate buffer (pH 4·5) containing 0·25 M NaCl	12·95 ± 0·79
LSD[b] (α = 0·05)	1·37

[a]Data are expressed as mean ± SEM (n = 4) soluble protein (mg protein/100 mg defatted flour).
[b]Fisher's least significant difference. The differences between two means exceeding this value are significant.

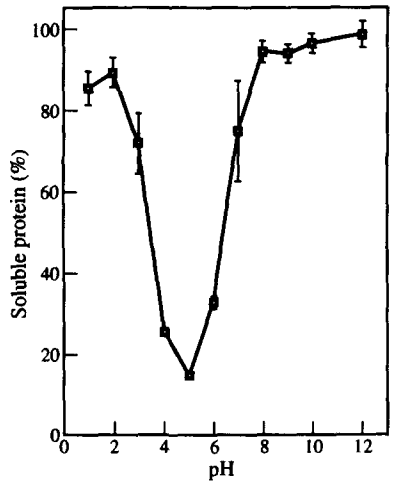

Fig. 1. Solubility profile of cashew nut proteins. Duplicate extractions were subjected to pH adjustments. The protein content of each supernatant was analyzed in duplicate. Data points on the curve are therefore mean ± SEM (vertical bars) of four determinations.

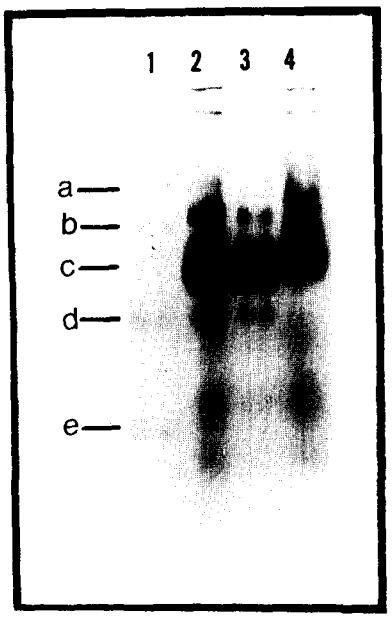

Fig. 2. NDND–PAGE of cashew nut proteins. The defatted nut meal was extracted with 0·1 M Tris-HCl (pH 8·1) buffer (1 : 10, w/v) at room temperature (25°C) for 30 min, centrifuged (12 600*g*, 10 min, 25°C), and the supernatant used for electrophoresis. True albumins and globulins were separated by subjecting 1·0 M NaCl extract (prepared in the same way as Tris-HCl extraction) to dialysis against DIDI water (48 h, 6 changes). Samples were then centrifuged (12 600*g*, 4°C, 10 min) and the supernatant (true albumins) and residue (true globulins) were lyophilized and stored at −20°C until further use. This is a 3–30% linear gradient acrylamide gel (acrylamide : bis = 37 : 1, w/w) with 90 mM Tris, 80 mM boric acid, 2·5 M Na–EDTA (sodium–ethylenediamine tetra-acetic acid), pH 8·5. Running buffer was 90 mM Tris, 80 mM boric acid, 2·5 mM Na–EDTA, pH 8·4. Gels were run at constant current (8 mA/gel, 18 h) with cooling (15°C) provided by running tap water. Proteins were dissolved in sample buffer (0·45 M Tris, 0·14 M boric acid, 12·5 mM Na–EDTA mixed with 1 volume of glycerol) containing 0·001% bromophenol blue as the tracking dye. Protein standards (Pharmacia high molecular weight (HMW) kit) were thyroglobulin (669 000), ferritin (440 000), catalase (232 000), lactate dehydrogenase (140 000), and albumin (67 000). Lane: 1, Pharmacia HMW standards; 2, Tris-HCl (0·1 M, pH 8·1) extract; 3, true albumins; 4, true globulins. Cashew nut protein load in each lane was 100 μg.

lizing proteins compared to acidic pH (98·82 ± 2·95% solubilized protein at pH 12 compared with 89·29 ± 3·57% at pH 2). At pH 8·0, solubilized protein was 94·68 ± 2·52%. Raising the pH above 8 did not increase protein solubility significantly. Since extreme alkali pH is detrimental to protein quality (e.g. lysine destruction), pH 7–8 is optimum to solubilize cashew nut proteins effectively. The cashew nut proteins were quite soluble in aqueous solvents (Table 2). Water and 1 M NaCl soluble proteins (45·59 and 42·37% respectively) accounted for 87·96% of the total soluble proteins. Glutelins comprised a small but significant portion (11·68 ± 1·21%) of the total soluble proteins. The solubility of cashew nut proteins in aqueous solvents is comparable to that of almond proteins (≥ 95% water-soluble proteins) reported earlier (Sathe, 1993). However, unlike almond proteins, the true albumins in cashew nuts were only 64·74% (compared to 95% in almonds) and also contained significant amount (32·27 ± 3·90% of soluble protein) of true globulins.

Electrophoresis

NDND gel electrophoretic patterns of total proteins, true albumins, and true globulins are shown in Fig. 2.

Table 2. Cashew nut protein fractionation

Solubility fraction	Soluble protein (%) Mean ± SEM
Distilled deionized water (albumins)[a]	45·59 ± 1·67
1·0 M NaCl (globulins)[a]	42·37 ± 0·86
70% Ethanol (prolamins)[a]	0·36 ± 0·01
1·0 M NaOH (glutelins)[a]	11·68 ± 1·21
True albumins[b]	64·74 ± 2·65
True globulins[b]	32·27 ± 3·90

[a]Data expressed as per cent of the total solubilized proteins.
[b]Data expressed as per cent of the total soluble proteins in 1·0 M NaCl.

NDND–PAGE separates proteins based on their negative charge without protein denaturation. Based on these profiles the cashew nut proteins soluble in 0·1 M Tris-HCl [tris(hydroxymethyl)aminomethane-HCl] pH 8·1 buffer were dominated by one type of protein (lane 2). The true albumins and true globulins were also dominated by single type of protein (lanes 3 and 4 respectively). The albumins had more net negative charge and therefore migrated slightly faster than the globulins. When the fractionated proteins were analyzed using SDS–PAGE in the absence of 2% β-mercaptoethanol (β-ME) a much more complex pattern was observed (Fig. 3). The total proteins, albumins and globulins (lanes 3, 4, and 5 respectively) had similar banding patterns with estimated MWs ranging over 12 880–98 860. Polypeptides with estimated MWs of 57 540, 55 210, 51 290, 29 170, 23 990, 22 910 and 12 880

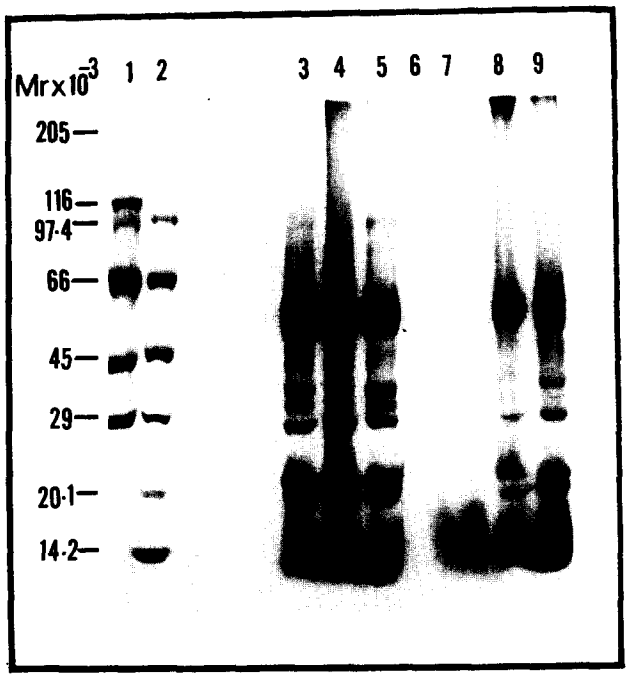

Fig. 3. SDS–PAGE (8–25% linear acrylamide gradient) analysis of cashew nut protein fractions in the absence of β-ME. Defatted meal was sequentially extracted with DIDI water, 1·0 M NaCl, 70% ethanol, and 1·0 M NaOH (meal to solvent ratio 1 : 10 (w/v) in each case) at 25°C for 1 h with vortexing every 10 min. The samples were centrifuged (15 000 *g*, 10 min, 25°C) and protein content of the samples was determined by the method of Lowry *et al.* (1951). Tris-HCl (0·1 M, pH 8·1) extract, true albumins, and true globulins were prepared as described in Fig. 2 legend. Samples were mixed with sample buffer without 2% β-ME. The protein standards did have 2% β-ME. Lane: 1, Sigma HMW standards containing myosin heavy chain (205 000), β-galactosidase (116 000), phosphorylase b (97 400), bovine serum albumin (66 000), ovalbumin (45 000), and carbonic anhydrase (29 000); 2, Pharmacia low molecular weight (LMW) standards containing phosphorylase b (97 400), bovine serum albumin (66 000), ovalbumin (45 000) carbonic anhydrase (29 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 200); 3, Tris-HCl (0·1 M, pH 8·1) extract (100 μg); 4, DIDI water extract (100 μg); 5, 1·0 M NaCl extract (100 μg); 6, 70% ethanol extract (32·5 μg); 7, 1·0 M NaOH extract (100 μg); 8, true albumins (100 μg); 9, true globulins (100 μg).

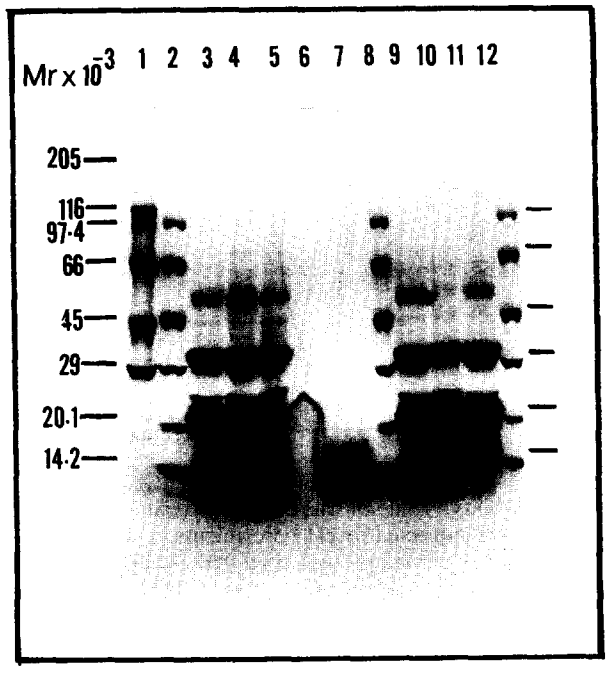

Fig. 4. SDS–PAGE analysis of cashew nut proteins in the presence of 2% β-ME. Samples described in the Fig. 3 legend were used for these gels. The gels were 8–25% linear acrylamide gradient. Lane: 1, Sigma HMW standards; 2, Pharmacia LMW standards; 3, Tris-HCl (0·1 M, pH 8·1) extract; 4, DIDI water extract; 5, 1·0 M NaCl extract; 6, 70% ethanol extract; 7, 1·0 M NaOH extract; 8, Pharmacia LMW standards; 9, true albumins; 10, true globulins; 11, Tris-HCl (0·1 M, pH 8·1) extract; 12. Pharmacia LMW standards. Protein loads were exactly the same as in Fig. 3.

were the major polypeptides in all these samples (subjectively judged, based on band width and intensity). Polypeptides with estimated MWs of 36 310, 32 739, 21 380 and 18 840 were also present in significant amounts. Prolamins (lane 6) and glutelins (lane 7) respectively had one polypeptide each with approximate MW 26 300 for prolamins and one diffuse band for glutelins in the MW range 12 000–22 000. The true albumins (lane 8) and true globulins (lane 9) also had similar polypeptide banding patterns. The major difference between the two was that the true albumins had few polypeptides that either failed to enter the separating gel or barely migrated into the separating gel. These polypeptides were of high MWs (> 205 000). These data when coupled with those from Fig. 2 would suggest that the total cashew proteins, although composed of few species, contained several polypeptides linked together by hydrogen bonds and hydrophobic interactions. When these same protein fractions were electrophoresed using SDS–PAGE in the presence of 2% β-ME (Fig. 4), the major polypeptides had estimated MWs of 54 080, 31 990, 24 830, 22 910, 21 380, 19 410, 14 130 and 10 720. The major difference between the true albumins and true globulins was that the polypeptide with estimated MW of 54 080 was barely visible in true globulins but was quite prominent in true albumins (indicated by the solid circle in lane 9). The differences and similarities in the polypeptide composition of the true albumins and true globulins were further illustrated by two-dimensional gel electrophoretic analyses (Fig. 5). The major distinguishing features with respect to differences in polypeptides were that the true albumins contained a polypeptide with estimated MW of 49 890 (indicated by an arrowhead) while true globulins contained several small MW polypeptides of MWs ≤ 14 200 (indicated by arrowheads). Two-dimensional gel electrophoresis data clearly demonstrate that both the true albumins and the true globulins are primarily composed of similar polypeptides (indicated by solid circles) with estimated MWs of 30 000–32 000 and 20 000–22 000 although they do contain several other polypeptides. The charge heterogeneity observed in both the true albumins and globulins and the charge differences between these two protein fractions must therefore arise due to a unique combination of subunit

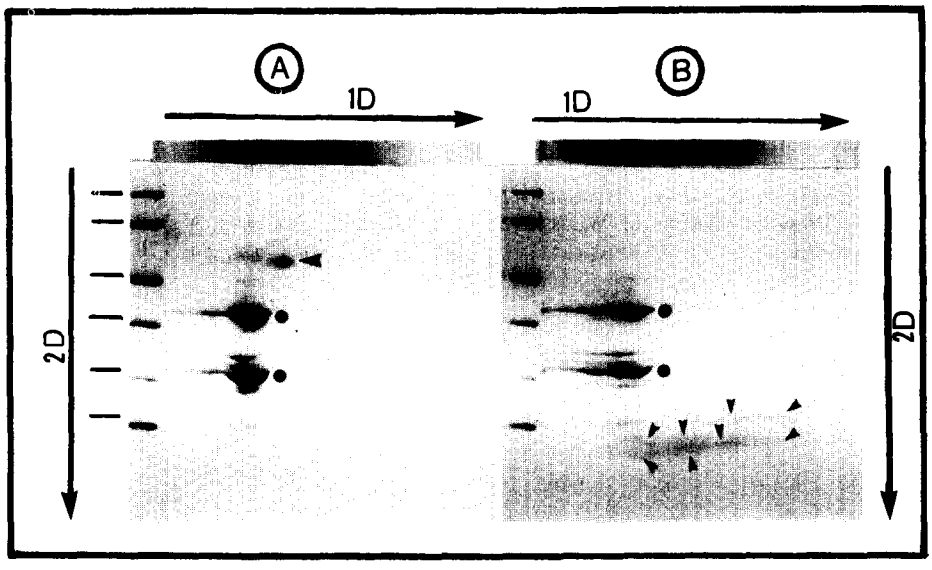

Fig. 5. Two-dimensional electrophoretic analysis of cashew nut proteins. The first dimension was NDND–PAGE (0·75 mm thick slab gels) with migration from left to right (top gel slice) followed by the second-dimension in SDS–PAGE in the presence of 2% β-ME. The reference slot in the second dimension contained Pharmacia LMW standards. (A) True albumins; (B) true globulins. Protein load used in the first dimension was 200 μg for each.

polypeptides. The native multimeric protein in cashew nuts is thus composed of two major types of polypeptides (MWs 30000–32000 and 20000–22000) and several other subunit polypeptides linked by disulfide linkages and the hydrogen bonds/hydrophobic interactions.

Amino acid composition

Amino acid composition of true albumins and globulins (Table 3) was in good agreement with the published amino acid composition of cashew kernel meal.

Table 3. Amino acid composition of cashew nut albumin and globulins[a]

Amino acid	True albumins	True globulins	Total proteins[b]
Asx	7·36	5·91	9·20
Glx	16·54	17·77	18·74
Ser	5·60	5·73	4·76
Gly	7·34	7·10	4·60
His	1·42	1·32	2·06
Arg	9·28	10·43	10·70
Thr	3·01	2·49	3·10
Ala	4·69	3·91	3·70
Pro	4·65	4·47	3·72
Tyr	2·21	2·53	2·37
Val	5·89	5·83	5·80
Met	0·64	0·90	1·40
Cys/2	0·29	0·89	0·89
Ile	3·88	3·61	3·86
Leu	7·39	7·27	6·51
Phe	3·79	3·98	3·89
Lys	7·57	5·40	4·04
Trp	0·85[c]	1·06[c]	1·37

[a]Current study; data are average of duplicate determinations and are expressed as g amino acid/100 g protein.
[b]From Fetuga *et al.* (1974), who reported data as g/16 g N and Cys/2 as cystine (1·78 g/16 g N).
[c]Colorimetric method (Spies & Chambers, 1948).

The hydrophobic residues dominated the amino acid composition of both true albumins and globulins followed by acidic amino acid residues. The uncharged polar and the basic residues contributed nearly equally in both protein fractions. Methionine was the first limiting amino acid in both protein fractions. The essential amino acid composition of cashew nut proteins is otherwise good when compared with the essential amino acid requirement of humans.

The total acidic amino acids in albumins and globulins were respectively 25·87 and 26·14% of total amino acids while the corresponding figures for the basic amino acids were 19·77 and 18·93% respectively. The difference between the acidic and basic residues for albumins and globulins was therefore 6·10 and 7·21% respectively. This would suggest that the globulins should have a greater net negative charge compared to albumins, which is contradictory to the observations from NDND–PAGE (Fig. 2) mentioned earlier. The higher net negative charge on albumins compared to globulins must therefore arise due to unique combination of constituent polypeptides.

ACKNOWLEDGEMENT

K. W. Clara Sze is thanked for her help in manuscript preparation.

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