International Food Research Journal 19(2): 651-656 (2012)

Solubilization, amino acid cmposition and electrophoretic characterization of Conophor nut (*Tetracarpidium conophorum*) proteins

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Abstract: Defatted flour was prepared from whole conophor nut by cold (4°C) acetone extraction. Albumins and globulins were subsequently prepared from the defatted flour by salt extraction. Effects of solubilizing agents and pH on protein solubility were evaluated. Amino acid composition and electrophoretic properties of the flour and protein fractions were also determined. Among the several solubilizing agents tested, the most effective protein solubilizer was 0.1 M NaOH and the least effective was distilled deionized water. Conophor proteins were least soluble at pH 6 and were comprised mostly of globulins (59.50%) and glutelins (33.82%) fractions. The salt extract was separated into true albumins (41.86%) and true globulins (58.14%). Hydrophobic amino acids dominated the amino acid composition of both true albumins and globulins fractions while histidine was found to be the limiting amino acid. Total proteins and globulins were dominated by two polypeptides with estimated molecular weights (MWs) of 31 200 and 59 600. True albumins were composed of only one major polypeptide with estimated molecular weight of 31 200.

Keywords: Conophor nut, solubility, protein fractionation, amino acid composition, electrophoresis

Introduction

In developing countries, the high cost of animal proteins has made the search for cheap and abundant sources of proteins with desirable functional and nutritional properties highly imperative. In recent years, research attention has focused majorly on vegetable proteins as sources of low- cost proteins to supplement human diets. Several authors have demonstrated the potential application of oilseed proteins such as peanut protein concentrate (Wu *et al*., 2009), mustard proteins (Alireza and Bhagya, 2009) and lesser known leguminous seed fractions (Ogunwolu *et al*., 2009) for product formulation and food fortification particularly for developing countries (Wu *et al*., 2009).

Due to increasing market demands on protein ingredients, underutilized oilseeds are now receiving considerable attention. Conophor nut (*Tetracarpidium conophorum*) is one of the neglected oil seeds in Nigeria but with great potential for increased utilization. The conophor plant (*Tetracarpidium conophorum*), commonly called the African walnut, is a perennial climbing shrub found in the moist forest zones of Sub-Sahara Africa (Enujuigha, 2003). It is indigenous to South-Western parts of Nigeria and is also grown in the Western Cameroon where it is known as Kaso or Ngak (Adesioye, 1991; Vivien and Faure, 1996). The proximate composition and fatty acid analysis of *Tetracarpidium conophorum* revealed that the nut contains about 5.9% crude fibre, 2.8% ash and about 46–49% fat on a dry weight basis (Ogunsua and Adebona, 1983; Enujuigha, 2003; Odoemelam, 2003). The fatty acid analysis of the oil showed that linolenic acid constituted 66% of the fatty acids. Polyunsaturated fatty acids accounted for over 80% of the fatty acids (Ogunsua and Adebona 1983). Conophor nut is equally a rich source of protein containing 20 – 24% protein on a dry weight basis (Ogunsua and Adebona, 1983; Ige *et al.,* 1984; Ogunsua, 1987; Adebona *et al.,* 1988; Adesioye 1991; Odoemelam, 2003). After defatting, the resulting cake contains about 35 – 40% protein (Ogunsua and Adebona, 1983; Ogunsua 1987). The amino acid composition of the nut has also been investigated by Ogunsua (1988) who reported that conophor nut contains essential amino acids in adequate amount for nutrition except methionine and that two unusual amino acids α-aminobutyric acid and ϒ-amino butyric acid were also present. Oyenuga *et al.* (1971) reported that conophor nut contained low level of methionine (0.6 g/16 g N) but high cystine (2.4 g/16 g N).

In spite of its high nutritional value, conophor nut proteins have found limited applications in the food processing industry due to lack of research information. Research efforts on *Tetracarpidium conophorum* have focused mainly on proximate

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composition, nutrient composition, fatty acid analysis, functional characteristics and food fortification of the whole nut (Ogunsua, 1987; Adebona *et al.,* 1988; Enujuigha, 2003). Unlike other oil-bearing seeds such as cashew nut proteins (Ogunwolu *et al*., 2009; Wu *et al*., 2009; Sathe, 1994), almond proteins (Sathe, 1993), walnut proteins (Sze-Tao and Sathe, 2000) whose proteins have been fully characterized, detailed characterization of conophor nut proteins to our knowledge has not been achieved. This work therefore aimed at fractionating conophor nut proteins and investigating the solubility characteristics, amino acid and polypeptide composition of the protein fractions with a view to providing preliminary information on possible use of conophor proteins as functional ingredients and food supplements.

Materials and Methods

Collection of materials

Fresh conophor nuts were obtained from the neighbouring villages around Ile-Ife, Osun State, Nigeria in July-September, 2008. The nuts were washed with tap water to remove extraneous materials, packaged in polythene bags and stored at -20^{0}C for further use. All chemicals used were obtained from either Fisher Scientific (Oakville, ON, Canada) or Sigma Chemicals (St. Louis, MO).

Preparation of defatted flour

Defatted conophor flour was prepared using a modified method of Sathe (1994). Whole uncooked conophor nuts were shelled manually and rinsed with tap water. The shelled nuts were comminuted using mortar and pestle to obtain coarse flour. The coarse flour obtained was then ground using Marlex Excella grinder (Marlex Appliances PVT, Daman) and the resulting flour was subsequently defatted with cold (4°C) acetone (flour to solvent ratio 1:5 w/v) with constant magnetic stirring provided. The slurry was then filtered through a Whatman No. 1 filter paper. The residue was re-extracted twice in a similar fashion. The defatted flour was desolventized by drying in a fume hood and the dried flour was finally ground in a Marlex Excella grinder (Marlex Appliances PVT, Daman) set at highest speed to obtain homogenous defatted flour. The defatted flour obtained was stored in an air-tight plastic bottle and kept at 4^{0}C until further use.

Separation of albumins and globulins

The procedure described by Sathe (1994) was employed with some modifications. Defatted flour (10 g) was extracted with 1 M NaCl at 4^{0}C for 4 h with constant magnetic stirring. The sample was centrifuged (7,000 g, 4^{0}C, 30 min) and the supernatant obtained was extensively dialysed against distilled deionized water at 4^{0}C (48 h, five changes, 4 litres each). The molecular weight (MW) cut-off of the dialysis tubing (Spectrum Laboratories Inc., CA) was 6000-8000. Samples were then centrifuged (7,000 g, 4^{0}C, 30 min) and the supernatant and precipitate were separately collected. The precipitate was redissolved in 0.1M NaCl, reprecipitated with 4 volumes of water and centrifuged (7,000 g, 4^{0}C, 30 min) two more times. The supernatant was dialyzed against water as above and the combined precipitate (true globulins) and supernatant (true albumins) lyophilized.

Protein solubility

Effect of solvent

The method of Sathe (1994) was employed for the evaluation of the effect of different solvents on solubilization of conophor proteins with slight modifications. Briefly, a 50 mg sample of the defatted flour was extracted with 1 ml of appropriate solvents at room temperature (20^{0}C) for 60 min with vortexing every 10 min. At the end of the extraction period, samples were centrifuged (20^{0}C, 13 000 g) using an IEC micromax centrifuge (Thermo Electron Corporation, Milford, MA) for 15 min and the supernatant was analyzed for protein content.

Effect of pH on protein solubility

Defatted flour (2 g) was extracted with 50 ml of 0.1N NaOH at 20^{0}C for 60 min with constant stirring on a magnetic stirrer (VWR, Henry Troemner LLC, USA). The sample was then centrifuged (Sorvall RC-6, USA) at 7,000 g, 4^{0}C for 30 min and the supernatant was filtered through a Whatman No. 1 filter paper. The filterate was diluted with 3 volumes of distilled deionized water and the pH of aliquots (9 ml) was adjusted to the desired value with 1 N NaOH or 1 N HCl. After pH adjustment, the sample was magnetically stirred for an additional 30 min, centrifuged (7,000g, 4^{0}C, 30 min), the supernatant made up to volume (11 ml) with water and analyzed for soluble protein content (Sathe, 1994).

Protein fractionation

Defatted flour (10 mg) was sequentially extracted at room temperature (20^{0}C) for 1 h (vortexing every 10 min) with 1 ml of each of: water; 1 M NaCl; 70% (v/v) ethanol and 1 N NaOH. At the end of each extraction the slurry was centrifuged (12,000 g, 15 min, 20^{0}C) and the supernatant used for further analysis (Sathe, 1994).

Protein determination

Soluble protein content of samples was determined by the modified Lowry method (Markwell *et al.,* 1978). The amount of protein in samples was determined from a standard curve using bovine serum albumin as the standard protein.

Amino acid analysis

Amino acid composition was determined using S433 Amino Acid Analyzer (SYKEM, Germany). Samples were freeze- dried and then hydrolyzed for 24 h at 110ºC with 6N HCl (Blackburn, 1978). After hydrolysis, the samples were stored frozen in sodium citrate buffer at pH 2.2. When ready for analysis a 50 µl of the hydrolysate was injected for analysis. Tryptophan was determined separately by hydrolysis of the sample with sodium hydroxide. Cysteine and methionine were determined after performic acid oxidation prior to hydrolysis in 6 N HCl, and measured as cysteic acid and methionine sulphone, respectively (Blackburn, 1978).

Native and sodium dodecyl sulphate (SDS) – polyacrylamide gel electrophoresis (PAGE)

Native polyacrylamide gel electrophoresis (PAGE) was carried out on the defatted flour, globulins and albumins while sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on the defatted flour, globulins, albumins and the fractionated proteins using the PhastSystem Separation and Development units according to the manufacturer`s instructions (Pharmacia LKB, Montreal, Canada). For native PAGE, each sample (40 mg) was dispersed in 0.1 M sodium phosphate buffer, pH 8.0, containing 0.01% bromophenol blue (dye), centrifuged at 15000 g for 10 min and 1 µl of the supernatant was loaded onto an 8-25% gradient gel. For SDS-PAGE, sample concentration was 10 mg/ml in 10 mM Tris-HCl buffer (pH 8.0) containing 0.03% EDTA, 0.01% bromophenol blue (dye) and 2.5% (w/v) SDS. SDS-PAGE, under reducing conditions, was similar except for the addition of 5% (v/v) β-mercaptoethanol. All SDS-PAGE samples under reducing and non reducing conditions were heated in boiling water for 5 min, cooled to room temperature and centrifuged (16,000 g) for 10 min and an aliquot 1 µl) of the supernatant was loaded onto the 8-25% gradient gel. A similar aliquot of molecular weight standards (Fisher BioReagents protein marker) containing β-lactosidase (116,000), bovine serum albumin (66,200), Ovalbumin (45,000), lactate dehydrogenase (35,000), restriction endonuclease Bsp981 (25,000), β-lactoglobulin (18,400) and lysozyme (14,400) was also loaded onto the same gradient gel.

Statistical analysis

With the exception of amino acid analysis which was performed once, all experiments were conducted at least in triplicate. Data are reported as means ± standard deviation. Analysis of variance (ANOVA) was performed and differences in mean values were evaluated using Tukey`s test at $P<0.05$. One-way ANOVA with Turkey`s post test procedures of GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA) was employed.

Results and Discussion

Effect of solvent on protein solubility

Protein solubility data as influenced by different solvents for conophor defatted flour is presented in Table 1. Three of the solvents: 0.1 M NaOH, 0.1 M Na_2CO_3 and 0.1 M Tris-HCl were effective in solubilizing conophor nut proteins as more than 75% of the protein was solubilized. However, 0.1 M NaOH was the most effective protein solubilizer (96.06%), while distilled deionized water was the least effective (19.81%). The results suggest that effective extraction of conophor proteins will require the use of solvents alkaline or acidic in solution since neutral solvents including distilled water were ineffective in solubilizing conophor proteins. Prickly pear flour and concentrate proteins were most soluble at extreme alkali and acidic solutions (Nassar, 2008). The results of this study were in agreement with earlier studies on protein solubility of other oil bearing seeds. Cashew nut proteins and walnut proteins were found to be most solubilized by 0.1 M NaOH and least solubilized by distilled water (Sathe, 1994; Sze-Tao and Sathe, 2000).

Table 1. Conophor nut protein solubility* as influenced by solubilizing agents

Solvent	Soluble protein (%)
Distilled deionized water	19.81 ± 0.21^{a}
0.1 M NaOH	96.06 ± 1.89^{b}
0.1 M Potassium phosphate buffer (pH 7.5)	51.07 ± 0.15^{c}
0.1 M Sodium phosphate buffer (pH 7.5)	53.38 ± 2.39^{e}
0.1 M Tris-HCl (pH 8.1)	76.21 ± 2.32^{d}
0.1 M Na_2SO_4	54.75 ± 1.27^{c}
0.1 M Na_2CO_3	87.80 ± 0.21^{e}
0.1 M NaCl	66.76 ± 2.22^{f}

*Values reported are means ± standard deviation of triplicate determinations. Mean values bearing different superscript roman letters are significantly ($P < 0.05$) different from one another.

Effect of pH on protein solubility

Solubility profile of conophor nut proteins is depicted in Fig. 1. Conophor nut protein exhibited minimum solubility at three different pH values of 6.0 (6.40%), 5.0 (10.36%) and 4.0 (11.23%). This indicates that conophor nut proteins are probably acidic in nature and that the isoelectric point of

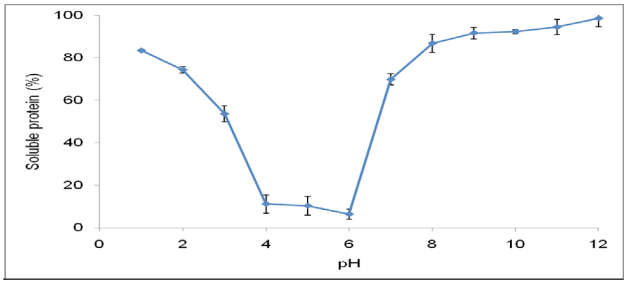

Figure 1. Solubility profile of conophor nut proteins

conophor proteins was in the range of 4-6. According to Sorgentini and Wagner (2002), the occurrence of minimum solubility near the isoelectric point is due primarily to both the net charge of peptides, which increase as pH moves away from the isoelectric point, and surface hydrophobicity that promotes the aggregation and precipitation via hydrophobic interactions. Moving away from pHs of minimum solubility, protein solubility increased significantly ($p<0.05$) at both acidic and alkaline pH values. Alkaline pH was however more effective in solubilizing conophor nut proteins compared to acidic pH. Since extreme alkali pH is detrimental to protein quality due to lysine destruction (Sathe, 1994), pH 10 rather than 12 was considered optimum to solubilize conophor nut proteins effectively. The results of this work compared favourably with the influence of pH on cashew nut protein concentrate (Ogunwolu *et al.,* 2009), peanut protein concentrates (Wu *et al.,* 2009) and gingerbread plum (Amza *et al.,* 2011).

Protein fractionation

The results of protein fractionation showed that the globulins (59.50% of total protein) and glutelin (33.82% of total protein) fractions accounted for the major portion of conophor nut proteins (Table 2). Albumins comprised only a small portion (6.36%) of the total soluble protein. Walnut solubilized proteins have been reported to contain majorly of glutelins fractions (70.1%) while globulin and albumin fractions accounted for relatively small portions of the total solubilized proteins (Sze-Tao and Sathe, 2000). The reduced solubility of walnut proteins in water and dilute salt solutions was attributed to the presence of phenolic compounds and its subsequent binding to proteins (Sze-Tao and Sathe, 2000). The low solubility of conophor nut proteins in water may possibly be an indication of the presence of phenolic compounds soluble in aqueous solvents. For salt soluble proteins, the true albumins in conophor nut accounted for 41.86% while the true globulins content was 58.14%. Almond proteins were reported to contain 95% true albumins (Sathe, 1993) and 64.74% true albumins were obtained in cashew nut (Sathe, 1994) which was considered higher than the value obtained for conophor true albumins in the present study.

Table 2. Conophor nut protein fractionation*

Solubility fraction	Soluble protein (%)
Distilled deionized water (albumins)[a]	6.36 ± 0.06[ag]
1.0 M NaCl (globulins)[a]	59.50 ± 0.70[ah]
70% Ethanol (prolamins)[a]	1.32 ± 0.01[ac]
1.0 M NaOH (glutelins)[a]	32.82 ± 0.20[ad]
True albumins[b]	41.86 ± 2.14[be]
True globulins[b]	58.14 ± 0.32[bf]

*Values reported are means ± standard deviation of triplicate determinations. Mean values bearing different superscript roman letters are significantly ($P < 0.05$) different from one another. [a]Data expressed as per cent of the total solubilised proteins. [b]Data expressed as per cent of the total soluble proteins in 1.0 M NaCl.

Amino acid composition

The amino acid composition of the defatted flour and those of the albumins and globulins is presented in Table 3. The results of the amino acid composition of the defatted flour are in good agreement with earlier reports on amino acid composition of conophor nuts (Oyenuga *et al.,* 1971; Ogunsua, 1988). The hydrophobic residues dominated the amino acid composition of the true globulins, followed by uncharged polar and then acidic amino acid residues. The uncharged polar and hydrophobic amino acid residues contributed almost equally to the overall composition in true albumins, followed by acidic amino acids. The order of dominance in total proteins is uncharged polar > hydrophobic > acidic. However, in all the samples basic amino acid residues contributed the least amount. Sulphur containing amino acid residues were present in relatively high proportion in the albumin when compared to the globulins and total proteins. The total acidic amino acids in albumins (23.72%) and globulins (23.60%) were almost equal but higher than the total basic amino acids in albumins (15.00%) and globulins (15. 92%). The percentage ratio of essential to total amino acids was well above 36% which is considered adequate for an ideal protein (FAO/WHO, 2007). The present study showed that both the total proteins and the fractions contain all the essential amino acids in adequate levels when compared with the recommended amino acid pattern for an adult (FAO/WHO, 2007). Oyenuga *et al*. (1971) reported high levels of cysteine (4.90%) and tryptophan (4.45%) and abundance of glutamic and aspartic acids and arginine in conophor nut. Similarly, Ogunsua (1988) reported that conophor nut contains all the essential amino acids in adequate levels for nutrition except methionine. In this study we have considered methionine and cysteine together (FAO/WHO, 2007) and this may have been responsible for the observed difference.

Electrophoresis

The Native-PAGE profiles of the total (defatted

Table 3. Total amino acid composition (%) of conophor nut proteins

Amino acid	True albumins	True globulins	Defatted flour
Isoleucine	4.13	4.83	3.29
Leucine	6.73	7.51	6.49
Lysine	5.11	4.93	4.28
Cysteine	7.43	2.89	2.60
Methionine	1.40	1.22	1.30
Total sulphur amino acid	8.83	4.20	3.90
Tyrosine	6.23	5.53	4.13
Phenylalanine	1.30	2.10	2.66
Threonine	6.16	6.62	5.39
Tryptophan	4.77	4.98	3.49
Valine	4.64	5.03	3.77
Histidine	1.22	1.32	1.96
Total essential amino acid	49.12	47.05	39.36
Arginine	8.67	9.67	8.93
Aspartic acid + asparagine	12.79	13.19	12.13
Glutamic acid+ glutamine	10.93	10.41	11.24
Serine	5.52	6.36	9.43
Proline	4.75	5.02	4.62
Glycine	5.13	5.10	10.97
Alanine	3.09	3.20	3.32
Total non essential amino acid	50.88	52.95	60.64

flour) and the protein fractions (true albumins and true globulins) were dominated by 2 types of proteins (electrophoregram not shown). SDS-PAGE electrophoregrams of conophor proteins, true albumins, true globulins and the fractionated proteins under non-reducing and reducing conditions are shown in Figs. 2 and 3 respectively. Under non reducing conditions, total proteins, true globulins and fractionated globulins (lanes B, D and F respectively) showed similar band patterns containing about six polypeptides with estimated MWs ranging between 14,400-119,300. Two polypeptides with estimated MWs of 31,200 and 59,600 were the major polypeptides in all these samples. Polypeptides with estimated MWs of 14,400, 87,800, 91,900 and 119,300 were also visible. True albumins and fractionated albumins (lanes C and E) also showed similar band patterns containing three polypeptides with estimated MWs of 14,400, 31,200 and 59,600. The samples were however dominated by a single type of polypeptide having an estimated MW of 31,200. The prolamins (lane G) had one major polypeptide with estimated MW of 31,200. In addition both the prolamins and glutelins (lane H) showed one diffuse band each with approximate MWs in the range 12,500-16,000 for prolamins and 12,500-21,000 for glutelins. These results suggest that total conophor proteins, although composed of few species, contained several polypeptides linked together by hydrogen bonds and hydrophobic interactions.

In the presence of 5% β-mercaptoethanol (Fig. 3), total proteins, true globulins and fractionated globulins were resolved into 4 polypeptides each with estimated MWs ranging between 14,400-55,700. Each of these samples showed 2 major polypeptides with one of the polypeptide (estimated MW of 31,200) present in all the samples. The disappearance of two major polypeptides of MWs 91,000 and 119,300 following addition of β-mercaptoethanol suggested that these polypeptides contained intra-disulphide bonds as evidenced by the appearance of only two polypeptides (MWs 45,900 and 59,600) above the major 31,200 polypeptide. The results suggest that each of these polypeptides was most likely a dimer with each subunit being about 45,900 and 59,600 in size, joined presumably by one or more disulphide bond(s). While the true albumins was resolved into only one major polypeptide identical in size to the major polypeptide found in all other samples, the fractionated albumins contained in addition a polypeptide of an estimated MW of 14,400. The prolamins and glutelins showed no apparent difference under reducing conditions when compared to what was obtained under non reducing conditions.

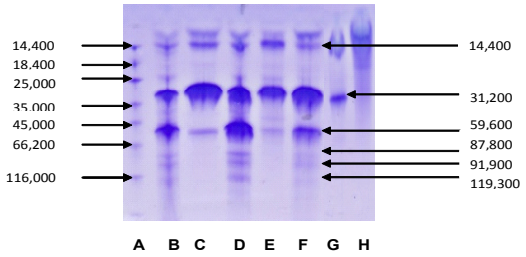

Figure 2. SDS-PAGE (8-25% linear acrylamide gradient) analysis of conophor nut protein fractions in the absence of β-ME. Lane: A, Fisher Bioreagents standards (14.4 – 116 kDa); B, defatted flour extract; C, true albumins; D, true globulins; E, water extract (fractionated albumins); F, 1.0 M NaCl extract (fractionated globulins); G, 70% ethanol extract (fractionated prolamins); H, 1.0 M NaOH extract (fractionated glutelins)

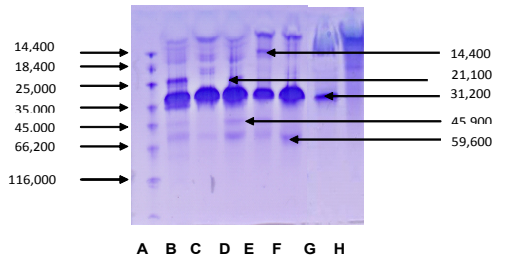

Figure 3. SDS-PAGE (8-25% linear acrylamide gradient) analysis of conophor nut protein fractions in the presence of 5% β-ME. Lane: A, Fisher Bioreagents standards(14.4 – 116 kDa); B, defatted flour extract; C, true albumins; D, true globulins; E, water extract (fractionated albumins); F, 1.0 M NaCl extract (fractionated globulins); G, 70% ethanol extract (fractionated prolamins); H, 1.0 M NaOH extract (fractionated glutelins)

Conclusion

The solubility profile of conophor proteins showed that the proteins were most soluble in aqueous solvents and at alkali pH and globulins and glutelins were the major protein fractions in conophor proteins. The protein fractions contained all the essential amino acids in adequate amount for nutrition. Conophor proteins may serve as functional ingredients and food supplements to enhance nutritional values of

low protein foods.

Acknowledgements

S.O. Gbadamosi is a recipient of the Graduate Students Exchange Program (GSEP) Grant awarded by Foreign Affairs and International Trade Canada (DFAIT). Funding for Dr. Aluko`s research program is provided by the Natural Sciences and Engineering Research Council (NSERC) and the Advanced Foods and Materials Network of Centre of Excellence (AFMnet).

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