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Original article

Amino acid profile, protein digestibility, thermal and functional properties of Conophor nut (*Tetracarpidium conophorum*) defatted flour, protein concentrate and isolates

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Summary Functional properties, amino acid compositions, *in vitro* protein digestibility, electrophoretic and thermal characteristics of conophor defatted flour (CDF), conophor protein concentrate (CPC), isoelectric protein isolate (CII) and neutral protein isolate (CNI) were evaluated. The isolates (CII and CNI) showed significantly lower ($P < 0.05$) water and oil absorption capacities, emulsifying and gelling capacities, but higher emulsion stability and foaming capacity. *In vitro* protein digestibility, enthalpy and denaturation temperature varied between 52.28% and 73.4%, 1.62–4.04 J g^{-1} protein and 79.7–89.3 °C, respectively. The native proteins were comprised of subunits with molecular weights ranging between 15.3 and 129.3 kDa. The major amino acids in all the samples were aspartic acid, glutamic acid and arginine, whereas the percentages of essential amino acids in CDF, CPC, CII and CNI were 39.35%, 40.46%, 44.54% and 46.04%, respectively. Conophor protein products could be used as functional ingredients in food formulations and for enriching low quality protein diets.

Keywords Amino acid composition, conophor nut, conophor protein isolate, differential scanning calorimetry, foaming capacity, functional properties, *in vitro* digestibility.

Introduction

In developing countries, the supply of animal proteins for use as food and food ingredients is inadequate, which has necessitated the quest for cheap and abundant sources of protein with desirable functional and nutritional properties. In recent years, many plants have attracted a great deal of interest as sources of low-cost proteins to supplement human diets. Among the various sources of vegetable proteins considered as food ingredients are peanuts and soybeans. Several authors have demonstrated the potential application of oilseed proteins such as peanut protein concentrate (Wu *et al.*, 2009), mustard proteins (Alireza & 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 utilisation. The whole seed contains 40% oil, 5.9% crude fibre, 2.8% ash and 20–24% protein and amino acid analysis shows that all the essential amino acids are present in adequate amounts for human nutrition (Ogunsua & Adebona, 1983; Ogunsua, 1988).

Research on *T. conophorum* have focused mainly on chemical composition and fatty acid profile of the whole seed (Ogunsua, 1987). Efforts have also been made at utilising the whole flour as wheat substitute in bread making (Adebona *et al.*, 1988). Effects of food processing on nutrient and anti-nutrient composition of the whole nut (Enujuigha, 2003) and functional properties of the whole flour have equally been examined (Odoemelam, 2003). However, conophor nuts have found limited applications in the food processing industry because the only product that has been prepared and investigated so far is the whole flour. To increase its utilisation, there is the need to process the whole flour into high protein products such as protein concentrate and isolates, and then examine the suitability of these

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products for use as functional ingredients and food supplements. However, the ultimate success of utilising any plant protein as food ingredients depends largely on its functional and nutritional properties. The functional properties of proteins, such as solubility and emulsifying activities, are in turn highly dependent on many factors such as pH and type and amount of salt present. The present study therefore aimed to investigate the influence of pH and salt concentration on some functional properties of conophor defatted flour (CDF), protein concentrate and isolates. We also evaluated amino acid composition, *in vitro* protein digestibility, polypeptide composition and thermal characteristics of the protein products with a view to providing scientific information on possible utilisation of these products in food formulation and enrichment.

Materials and methods

Fresh conophor nuts were collected 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, and the clean shelled nuts were packaged in polythene bags and stored at −20 °C for further use. All chemicals used were obtained from Fisher Scientific (Oakville, ON, Canada) and Sigma Chemicals (St. Louis, MO, USA).

Preparation of conophor defatted flour, concentrates and isolates

Conophor defatted flour was prepared using the method of Sathe (1994) except that the nuts were first shelled, and de-shelled seeds were first comminuted manually using mortar and pestle and then further ground using Marlex Excella grinder (Marlex Appliances PVT., Daman). Conophor protein concentrate (CPC) was prepared by a modification of the method described by Cheftel *et al*. (1985). A flour to water ratio of 1:20 was stirred on a magnetic stirrer for 10 min, and the resultant slurry was adjusted to pH 6.0 and centrifuged at 4000 ***g*** for 30 min. The precipitate was washed twice with distilled water, adjusted to pH 7.0, centrifuged at 4000 ***g*** for 30 min and then lyophilized as the protein concentrate.

Conophor protein isolates were prepared by a slight modification of the method described by Chavan *et al*. (2001). A flour to water ratio of 1:20 was stirred on a magnetic stirrer for 10 min, and the pH of the medium was adjusted to 10.0 and stirred for 4 h at constant pH. The slurry was centrifuged (Sorvall RC-6; Thermo Scientific, Waltham, MA, USA) at 7000 ***g*** for 30 min at 4 °C, and the supernatant was collected and filtered through a Whatman No. 1 filter paper. The supernatant was divided into two portions, each was adjusted to pH 6.0, and the mixture was centrifuged as above. The first precipitate was lyophilized and referred to as isoelectric protein isolate (CII). The second precipitate was washed twice with distilled water, then re-suspended in distilled water and adjusted to pH 7.0 and freeze–dried as the neutralized protein isolate (CNI). All samples were kept at −4 °C in tightly sealed containers for further use.

Composition analysis

Protein content of samples was determined using the modified Lowry method (Markwell *et al*., 1978). Moisture, ash, crude fibre, crude fat and carbohydrate contents (by difference) of samples were determined using standard methods of analysis (AOAC, 1990).

Physical and functional properties determination

Bulk density was determined using the method described by Okezie & Bello (1988). Water absorption capacity (WAC) was determined by a modified method of Sathe & Salunkhe (1981). The weight of sample used was 2 g, and the centrifugation was carried out at 4000 ***g*** for 20 min. Water absorption was expressed as percentage increase of the sample weight. Oil absorption capacity (OAC) was determined following the method described by Lin & Zayas (1987). The method of Coffman & Garcia (1977) was adopted in the determination of least gelation concentration (LGC). Foam capacity (FC) was determined by dispersing 20 mg protein sample in 20 mL of distilled water and homogenised for 60 s using Polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland) at 4026 ***g***. The percentage ratio of the volume increase to that of the original volume of protein solution was calculated and expressed as FC or whippability (Chavan *et al*., 2001).

Effect of pH and NaCl concentration on emulsifying activity index and emulsion stability index

To study the effect of pH and salt concentration on emulsifying activity index (EAI), the method described by Wanasundara & Shahidi (1997) was followed with some modifications. The protein sample (50 mg) was dispersed in 0, 2.0 or 4.0 mL of a 10.24% (w/v) NaCl solution to make a final NaCl concentration of 0.0, 0.35 or 0.70 M. The pH was then adjusted with 1 M HCl or 1 M NaOH, followed by addition of distilled water to reach a 10 mL volume. The protein solution was mixed with 5 mL of pure canola oil, and the mixture was homogenised using Polytron PT 3100 homogenizer (Kinematica AG) at 4026 ***g*** for 60 s. Fifty microlitres of an aliquot of the emulsion was transferred from the bottom of the centrifuge tube after homogenisation, and mixed with 5 mL of 0.01% sodium dodecyl sulphate (SDS) solution. The absorbance of the diluted solution was measured at 500 nm using spectrophotometer (50

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Bio UV-Visible Spectrophotometer; Varian, Melbourne, Australia). The EAI was expressed as interfacial area per unit weight of protein ($m^2\ g^{-1}$). The emulsions were allowed to stand for 10 min at room temperature and the emulsion stability index (ESI) was determined as described above for EAI and expressed as a percentage of the initial EAI (Aluko & Yada, 1993).

Protein solubility

To study the effect of pH and salt concentration on solubility, a 50 mg sample was dispersed in 36 mL of 0.1 N NaOH containing 0, 0.74 or 1.48 g of NaCl to make a final NaCl concentration of 0.0, 0.35, or 0.70 M, respectively. The mixture was stirred on a magnetic stirrer for 30 min, centrifuged (Sorvall RC-6) at 7000 ***g*** for 10 min and then filtered through Whatman No. 1 filter paper. The pH of the filtrate (25 mL) was adjusted to the desired value with 2 N HCl or NaOH. The solution was then made up to 50 mL and stirred for another 10 min. An aliquot of the mixture was centrifuged at 12 500 ***g*** for 10 min. The centrifuged solution was then diluted with three volumes of distilled water, and the protein content was determined using the modified Lowry method (Markwell *et al.*, 1978).

In vitro digestibility

In vitro digestibility of proteins was determined using pepsin-pancreatin enzyme systems according to the method of Saunder *et al.* (1973) with minor modifications. A 250 mg sample was suspended in 15 mL of 0.10 M HCl containing 1.5 mg of pepsin, followed by gentle shaking for 1 h at 37 °C. The resultant solution was then neutralised with 0.50 M NaOH and treated with 4 mg pancreatin (from porcine pancreas, activity equivalent to 4 × US Pharmacopeia) in 7.5 mL of phosphate buffer (0.10 M, pH 8.0). The mixture was shaken for 24 h at 37 °C in a water bath shaker, and the undigested solids were separated by centrifugation, washed with distilled water and air dried. The undigested residue was then extracted with 0.1 N NaOH, and the soluble protein content was determined (Markwell *et al.*, 1978). Protein digestibility was obtained by the following equation:

$$\%\ \text{in vitro digestibility} = \frac{(\mathrm{I} - \mathrm{F})}{\mathrm{I}} \times 100$$

where I is the protein content of sample before digestion and F is the protein content of sample after digestion.

Amino acid analysis

Amino acid composition was determined using S433 Amino Acid Analyzer (SYKAM, Eresing, Germany). Samples were freeze-dried and then hydrolysed for 24 h at 110 °C with 6 N HCl. After hydrolysis, the samples were freeze-stored in sodium citrate buffer at pH 2.2. When ready for analysis, a 50 μL of the hydrolysate was directly injected into the analyser. 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 polyacrylamide gel electrophoresis and sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) were carried out on the defatted flour, concentrates and isolates using the PhastSystem Separation and Development units according to the manufacturer's instructions (GE Healthcare, Montreal, QC, Canada). For native PAGE, each sample (20 mg) was dispersed in 10 mM Tris-HCl buffer (pH 8.0), containing 0.2 M NaCl, 0.01% bromophenol blue (dye) and 1 mM EDTA and centrifuged at 15 000 ***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^{-1} 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 (MW) standards (Fisher BioReagents protein marker), ranging from 14.4 to 116 kDa was also loaded onto the same gradient gel.

Differential scanning calorimetry

The thermal properties of CDF, concentrates and isolates were investigated using a differential scanning calorimetry (DSC) Q200 (TA Instruments, New Castle, DE, USA). The procedure of Li-Chan & Ma (2002) was employed, and analyses were conducted in triplicate. One milligram (protein basis) of each sample was accurately weighed into the aluminium pan, and 10 μL of distilled water was added. The pan was hermetically sealed and then heated from 25 to 140 °C at a rate of 10 °C min^{-1}. A sealed empty pan was used as the reference. The onset temperature (T_m) denaturation temperature (T_d), enthalpy of denaturation (ΔH) and cooperativity, represented by the width at half-peak height ($\Delta T_{1/2}$), were computed from the thermograms using the universal Analysis program, Version 1.9 D (TA Instruments).

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Statistical analysis

With the exception of amino acid analysis, all experiments were conducted 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, CA, USA) was employed.

Results and discussion

Proximate composition

The results of proximate composition are shown in Table S1. The moisture contents ranged from 3.07% to 7.38% with CDF and CNI exhibiting the highest and lowest moisture contents, respectively. CII had the highest ash content (5.08%) followed by CNI (4.71%), and the lowest was recorded in CDF (3.62%). The high ash contents in CII and CNI may be attributed to salt formation during protein precipitation at the isoelectric point. There was no significant difference ($P < 0.05$) in the lipid contents of CII (6.57%) and CNI (6.53%), but the lipid contents in these samples were significantly higher ($P < 0.05$) than for CDF (5.36%) and CPC (5.76%). This may be due to concentration of lipid in the protein fractions. A similar observation has been reported for beach pea protein isolates (Chavan *et al.*, 2001). The amount of protein in CNI (80.5%) and CII (80.0%) were significantly higher ($P < 0.05$) than for CDF (36.4%) and CPC (45.6%). The protein contents of CNI and CII compared well with those of bambarra groundnut native protein concentrates (79.43%) (Lawal *et al.*, 2007), heat processed peanut isolates (84.20%) (Kain *et al.*, 2009), but lower than those of heat coagulated mustard protein isolates (95%) (Alireza & Bhagya, 2009) and safflower protein isolates (90.1%) (Ulloa *et al.*, 2011).

Functional properties of conophor defatted flour, conophor protein concentrate, isoelectric protein isolate and neutral protein isolate in water

Bulk density

The results of bulk density and functional properties of the defatted flour, concentrate and isolates in water are presented in Table S2. The bulk density varied between 0.16 and 0.66 g mL^{-1} with CNI and CII exhibiting similar bulk density (0.66 g mL^{-1}) which was higher than those of CDF (0.39 g mL^{-1}) and CPC (0.16 g mL^{-1}). The bulk density of CNI and CII was higher than those of cashew nut protein isolate (0.31 g mL^{-1}) and sesame protein isolate (0.169 g mL^{-1}) obtained by Ogunwolu *et al.* (2009) and Kanu *et al.* (2007), respectively. The bulk density of CDF was higher than that of defatted peanut meal (Amza *et al.*, 2011), but lower than the bulk density of defatted cashew nut protein concentrate (Ogunwolu *et al.*, 2009). The high volume per gram of protein material is important in relation to its packaging. Increase in bulk density is desirable in that it offers greater packaging advantage, as a greater quantity may be packed within a constant volume (Fagbemi, 1999).

Water absorption capacity

The WAC varied from 194.1% to 564.4% with CPC exhibiting the highest capacity and CII the lowest capacity to absorb water. The WAC of CDF, CPC, CII and CNI was significantly different ($P < 0.05$) from one another. It has been reported that water binding capacity of proteins is a function of several parameters; including size, shape, steric factors, conformational characteristics, hydrophilic–hydrophobic balance of amino acids in the protein molecules as well as lipids, carbohydrates and tannins associated with proteins (Chavan *et al.*, 2001). The high WAC exhibited by CPC may be related to its high polysaccharide content following the removal of soluble carbohydrates (oligosaccharides) during its preparation. The WAC of conophor isolates were significantly lower than those of soy (289%) and sesame protein isolates (302%) as reported by Kanu *et al.* (2007), but compared well with those of cashew nut protein isolate (220%) and peanut protein isolate (135%) reported by Ogunwolu *et al.* (2009) and Wu *et al.* (2009), respectively. The water absorption capacities of CDF (412%) and CPC (564.4%) were superior to those of prickly pear seed flour (316%) and prickly pear protein concentrate (471%) as reported by Nassar (2008). The water absorption characteristics of flour are represented by its ability to associate with water under conditions where water is limited, such as doughs and pastes (Singh, 2001). The results obtained suggest that conophor flour and its proteins may be incorporated into aqueous food formulations, especially those involving dough handling.

Oil absorption capacity

The defatted flour, concentrates and isolates showed significant differences ($P < 0.05$) in their abilities to absorb oil. OAC ranged between 120% and 422%. The OAC of CDF and CPC was superior to those of pra seed flour (Choonhahirun, 2010) and defatted peanut meal (Amza *et al.*, 2011). Low-density protein powders with a small particle size have been reported to adsorb and/or entrap more oil than do high-density protein powders. Low fat absorption capacity exhibited by conophor isolates may be related to its high density and large particle size. The results of OAC for CII and CNI were comparable to those of mustard protein hydrolysate

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(Alireza & Bhagya, 2009) and bambara protein isolate (Eltayeb *et al.*, 2011). OAC is important because oil acts as flavour retainer and increases the mouth feel of foods (Aremu *et al.*, 2007). The higher OAC exhibited by CDF and CPC suggested that these products may find useful applications as flavour retainers and meat extenders.

Emulsifying activity index

Data on EAI show that CDF had the highest (40.70 m^2 g^{-1}) EAI followed by CPC (27.15 m^2 g^{-1}) and CII (24.42 m^2 g^{-1}), whereas CNI had the lowest (20.17 m^2 g^{-1}). The EAI of all the samples were significantly different ($P < 0.05$) from one another. The decrease in EAI observed in this study with increase in protein agreed with the report of Ogunwolu *et al.* (2009) who observed that the emulsifying capacity of proteins tends to decrease as protein concentration is increased in cashew nut proteins. Tsai *et al.* (1972) explained that as protein concentration is decreased, a greater degree of unfolding of polypeptides occurs during the shearing involved in the emulsifying process, and this is aided by hydrophobic association of the peptide chains with the lipid droplets, so that the net result is that, a much greater volume of surface area of protein is made available, and emulsifying efficiency is enhanced. This explanation is further corroborated by the reports of Aremu *et al.* (2006) and Oladele & Aina (2007) that emulsifying activities were closely associated with protein surface hydrophobicity. The ESI on the other hand followed a reverse order with CNI showing the highest stability (86.6%) and CDF showing the lowest stability (25.11%). A similar observation has been reported for cashew nut protein isolate, concentrate and defatted flour with ESI increasing with increase in protein concentration (Ogunwolu *et al.*, 2009).

Least gelation concentration

Wu *et al.* (2009) reported that protein gels were composed of three-dimensional matrices or intertwined networks, partially associated with polypeptides, in which de-ionised water was entrapped. It plays a major role in the preparation of many foods, including various dairy products, coagulated egg white, various heated, comminuted meat or fish products. The ability of conophor proteins to form a stable gel was found to decrease with increase in protein concentration. The CII and CNI exhibited the highest LGC (20%) followed by CPC (10%), whereas CDF showed the lowest LGC and therefore the best gelation capacity. The results suggest that non-protein constituents, especially polysaccharides may have contributed to the superior gelation ability of the low-protein conophor products. The higher gelation concentrations of CII and CNI appear to be in conformity with globular nature, which could limit protein–protein interactions required for gel formation. Although the proteins were heated, it is possible that there was inadequate protein unfolding necessary for increased protein network formation. The difference in gelation concentration of the defatted flour, concentrate and isolates may be attributed to the relative ratios of different constituents such as proteins, carbohydrates and lipids, that make up the flours and also the interactions between such components.

Foam capacity and foam stability

Protein concentration has pronounced effect on FC and stability. The FC of the samples increased with increase in protein concentration. The FC of CII (50%) was higher than those of CNI (38.33%), CPC (24%) and CDF (35%), and these values were significantly different ($P < 0.05$) from one another. As protein concentration is increased, the formation of interfacial protein membranes at the air–water interface is increased, and this enhances encapsulation of air bubbles. According to Damodaran (1997), the FC and stability were enhanced by greater protein concentration, because this increases the viscosity and facilitates the formation of a multilayer, cohesive protein film at the interface. The higher FC of CII when compared with CNI may be attributed to the unfolding of protein molecules at isoelectric pH into more flexible structures that form foams better than the more compact molecules. The FC of the flour and concentrate are, however, comparable to those of pra seed flour (32.6%) reported by Choonhahirun (2010), defatted peanut flour and aqueous alcohol bis-leach peanut protein concentrate (Wu *et al.*, 2009). The foam capacities of the isolates are comparable to the values of 79% and 76% reported for sesame protein isolates and soy isolates, respectively, by Kanu *et al.* (2007). The results suggest that conophor proteins may not function well as whipping agents in food formulations.

Effect of pH and NaCl concentration on protein solubility

Protein solubility of CDF, CPC, CII and CNI as a function of pH and NaCl concentration is shown in Fig. S1a–d, respectively. In general, in the absence of NaCl (distilled water), all the samples demonstrated large variations over a wide range of pH. In distilled water, minimum solubility of CDF (6.58%), CPC (8.45%), CII (15.09%) and CNI (17.98%) was obtained at pH 6, 5, 4 and 5, respectively, indicating their isoelectric points (pI). Solubility rapidly increased below and above their pI, and alkaline pH was more effective than acidic pH in solubilising the proteins. These observations have been reported previously for African locust bean (Lawal, 2004). According to Sorgentini & 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

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hydrophobic interactions. In 0.35 and 0.70 M NaCl, protein solubility of CDF and CPC at isoelectric point significantly increased ($P < 0.05$) with increasing NaCl concentration compared with solubility in distilled water. On either side of these points (higher acidic and alkaline regions), protein solubility decreased relative to 0.00 M NaCl concentration. The protein solubility of the isolates in the presence of NaCl was generally lower than solubility in the absence of NaCl at all pH values, and may be due to a decrease in water activity that diminishes the solubilising interactions between water and polar protein groups resulting in the 'salting-out' of the protein from solutions. CII and CNI exhibited a decrease in protein solubility with increasing NaCl concentration around their pI. At pH lower than the pI, solubility of the isolates in the presence of 0.70 and 0.35 M NaCl decreased and increased, respectively, but as pH increased above pI, solubility was improved. The decrease in protein solubility at pH lower than the pI could be explained by the predominant electrostatic screening of the positively charged protein and/or by adsorption of chloride ions by the protein (Retailleau *et al.*, 1997). However, changes in protein solubility are caused mainly by anions, and the effects from cations are minimal (Reis-Kautt & Ducruix, 1989). This may explain why the effect of NaCl on protein solubility was less apparent at pH higher than the pI, the region where the proteins were negatively charged. A similar observation has been reported for cowpea globulin isolate (Aluko & Yada, 1995), lentil protein isolates (Suliman *et al.*, 2006) and beach pea protein isolates (Chavan *et al.*, 2001).

Effect of pH and NaCl concentration on emulsifying activity index and emulsion stability index

Emulsifying activity index of conophor proteins (CDF, CPC, CII and CNI), measured as a function of pH and NaCl concentration is presented in Fig. S2a–d. At low pH (2–6), the EAI of all samples in 0.00 M NaCl decreased with increasing pH with the lowest EAI obtained around the isoelectric regions (pH 6) and thereafter, increased with increasing pH. The lowest EAI obtained for CDF, CPC, CII and CNI were 81.95, 24.80, 14.48 and 10.39 $m^2 g^{-1}$, respectively, and the differences were significant ($P < 0.05$) except between the isolates. The highest EAI of CDF (454.2 $m^2 g^{-1}$), CPC (406.9 $m^2 g^{-1}$), CII (583.3 $m^2 g^{-1}$) and CNI (556.8 $m^2 g^{-1}$) were obtained at pH 12. Some studies have suggested that good emulsifying activity of a protein is related to its high solubility (El Nasri & El Tinay, 2007), and others have shown that the pH-emulsifying properties profile of various proteins resembles the pH-solubility profile (Ogunwolu *et al.*, 2009). Proteins generally have been shown to be poor emulsifiers at isoelectric pH, owing to their low solubility, poor hydration and lack of electrostatic repulsive forces (Damodaran, 1997). At pH 6, sodium chloride at 0.35 and 0.70 M concentrations increased EAI of CDF and CPC compared to the samples without sodium chloride and on eiter sides of this pH except pH 12 where EAI significantly rose, EAI decreased. These results where EAI first rose and then fell with addition of NaCl are in accordance with the observation of Chavan *et al.* (2001). The increase in EAI with pH increase might suggest that droplet size decreased as the pH increased beyond the isoelectric point. The low EAI at high pH and high salt concentration may be due to the increased repulsive forces resulting in poor rheological properties of the interfacial film.

The effects of pH and salt concentration on ESI of CDF, CPC, CII and CNI are presented in Fig. S3a–d respectively. ESI, in the absence of NaCl, decreased at the initial stage (pH 2–6) and thereafter increased with increasing pH in all the samples. The minimum ESI for CDF (20.73%), CPC (19.79%), CII (16.84%) and CNI (34.60%) were obtained at around their isoelectric pH, and the maximum ESI was obtained at pH 12 for all the samples. The inclusion of NaCl in the medium generally reduced ESI at all pH values except for CDF and CPC, where significant ($P < 0.05$) increases were observed at pH 4 and 6 that coincidentally happened to be their isoelectric points. It is also noteworthy that except at pH 4 and 6 for CDF and CPC, the decrease in ESI was more pronounced at higher salt concentration than at lower concentration at all the pH values. The low ES at low pH and salt concentration has been reported earlier by Chavan *et al.* (2001) and was attributed to increased interactions between the emulsified droplets, as net charge on the protein is decreased by the presence of chloride ions. The subsequent increase in Coulombic repulsion between neighbouring droplets and increased hydration of the charged protein molecules may lower interfacial energy and retard droplet coalescence (Chavan *et al.*, 2001) that may account for high ESI obtained at higher pH and ionic strength. These results are in agreement with literature values for cashew nut flour, protein concentrate and isolate (Ogunwolu *et al.*, 2009).

In vitro digestibility

The results of *in vitro* digestibility of conophor proteins with pepsin-pancreatin enzyme systems revealed that digestibility increased with increasing protein concentration. The digestibility values varied from 52.28% to 73.47% with CNI exhibiting the highest digestibility and CDF the lowest (Table S2). There were significant differences ($P < 0.05$) among the samples except for the isolates (CII and CNI) where the difference was insignificant. The result of protein digestibility of defatted flour obtained in this study was higher than that of uncooked soybean flour (30.5%) and lower than

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that of soybean meal (76.08%) as reported by Maha *et al*. (2009) and Ali *et al*. (2009), respectively. Reduced digestibility of low-protein flours may be due to interference with enzymatic activities by the high levels of non-protein compounds, especially polysaccharides. The digestibility of conophor isolates was lower than those of beach pea protein isolate (80.6–82.6%; Chavan *et al*., 2001) and flaxseed protein isolates (90%; Wanasundara & Shahidi, 1997).

Amino acid composition

The amino acid composition of CDF, CPC, CII and CNI is presented in Table S3. The results showed that polar uncharged amino acid residues dominated the amino acid profile of CDF (32.52%) and CPC (31.33%) followed by hydrophobic amino acid residues (28.93% and 29.98% respectively), whereas hydrophobic amino acid residues were the dominant amino acids in CII (33.68%) and CNI (35.4%), followed by acidic and polar uncharged amino acid residues that contributed almost equally to their amino acid composition. The acidic amino acids contributed significantly higher ($P < 0.05$) amounts to the overall profile than the basic amino acids in all the samples. The percentages of essential amino acids in CDF, CPC, CII and CNI were 39.35%, 40.46%, 44.54% and 46.04%, respectively, and the ratios of essential to non-essential amino acids were 0.65, 0.68, 0.80 and 0.85, respectively. 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 results demonstrate that CDF and CPC contain glycine in higher proportion, but all the samples are rich in arginine, aspartic acid and glutamic acid.

Electrophoresis

Native PAGE separates proteins based on their negative charges without protein denaturation. Based on these profiles, the conophor proteins from the defatted flour and concentrates soluble in 10 mM Tris-HCl buffer (pH 8.0) were dominated by one type of protein, whereas the isolates (CII and CNI) were equally dominated by one type of protein. Two other proteins appeared to be present in insignificant amounts in all the samples (data not shown). SDS-PAGE electrophoregrams under non-reducing and reducing conditions are depicted in Fig. S4a and b, respectively. Under non-reducing conditions (Fig. S4a), CDF, CPC, CII and CNI exhibited similar banding patterns containing about seven polypeptides with estimated MW ranging between 15 300 and 129 300. Two major polypeptides with estimated MWs of 32 000 and 55 800 were the major polypeptides in all the samples. Polypeptides with estimated MWs of 15 300 and 25 700 were also present in significant amounts. In the presence of 5% β-mercaptoethanol (Fig. S4b), samples were resolved into four polypeptides each with estimated MW ranging between 25 700 and 63 500. Two major polypeptides (estimated MWs of 25 700 and 32 000) were present in all the samples. The disappearance of two polypeptides of MWs 87 800 and 129 300 following addition of β-mercaptoethanol suggested that these polypeptides contained intra-disulphide bonds as evidenced by the appearance of only two polypeptides (MWs 43 500 and 63 500) before the major polypeptide of MW 32 000. It may be concluded that each of these polypeptides was most likely to be a dimer with each subunit being about 43 500 and 63 500 in size, joined presumably by one or more disulphide bond(s). In contrast, the 32 000 polypeptide does not contain disulphide bonds.

Differential scanning calorimetry

The thermal transition characteristics of CDF, CPC, CII and CNI are listed in Table S4. CPC and CDF were observed to have a much significant ($P < 0.005$) higher enthalpy values (4.04 and 3.94 J g^{-1} protein respectively) than CII and CNI (2.39 and 1.62 J g^{-1} protein respectively) suggesting that the CPC and CDF are more resistant to heat induced denaturation than the protein isolates. The denaturation temperature (T_d) ranged between 79.7 and 89.3 °C, and there were significant ($P < 0.05$) differences among the samples. CPC exhibited the highest thermal stability followed by CDF and CII, whereas CNI was the least stable to heat-induced protein denaturation. The high T_d exhibited by CPC and CDF may be attributed to the presence of sugars and other non-protein substances. Sugars have been shown to have a protective effect on proteins against thermal denaturation (Harwalkar & Ma, 1992). The values of $\Delta T_{1/2}$ showed progressive decrease with increasing protein content. This indicates that increase in protein content favours greater cooperativity of the thermal denaturation temperature measured by $\Delta T_{1/2.}$ The DSC data suggest that denaturation of conophor proteins occur at temperatures below the boiling temperature of water.

Conclusion

In view of high water and oil absorption capacities, gelation concentration and good emulsifying properties displayed by conophor proteins, the protein flours may find suitable applications as functional ingredients in food formulations. The heat processing of conophor proteins should, however, be carried out at temperatures below boiling temperatures of water to prevent heat-induced protein denaturation that may affect conophor protein functionality. In developing countries, conophor proteins may also serve as good sources of essential

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amino acids and therefore help to enrich protein deficient or low quality protein diets.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Protein solubility profiles of conophor defatted flour (a), conophor protein concentrates (b), conophor isoelectric isolate (c) and conophor neutral isolate (d) as a function of pH and NaCl concentration.

Figure S2. Emulsifying activity index of conophor defatted flour (a), conophor protein concentrate (b), conophor isoelectric isolate (c) and conophor neutral isolate (d) as a function of pH and NaCl concentration.

Figure S3. Emulsion stability index of conophor defatted flour (a), conophor protein concentrate (b), conophor isoelectric isolate (c) and conophor neutral isolate (d) as a function of pH and NaCl concentration.

Figure S4. SDS-PAGE (8–25% linear acrylamide gradient) analysis of conophor nut protein fractions in the absence (a) and in the presence (b) of 5% β-ME. Lane: (A) Fisher Bioreagents standards 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); (B) conophor defatted flour; (C) conophor protein concentrate; (D) conophor isoelectric isolate; (E) conophor neutral isolate.

Table S1. Proximate composition (%) of defatted flour (CDF), protein concentrate (CPC), isoelectric protein isolate (CII) and neutral protein isolate (CNI).

Table S2. Some functional properties and *in vitro* digestibility of defatted flour (CDF), protein concentrate (CPC), isoelectric protein isolate (CII) and neutral protein isolate (CNI).

Table S3. Amino acid composition (%) of defatted flour (CDF), protein concentrate (CPC), isoelectric protein isolate (CII) and neutral protein isolate (CNI).

Table S4. Thermal transition properties of defatted flour (CDF), protein concentrate (CPC), isoelectric protein isolate (CII) and neutral protein isolate (CNI).

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