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Functional and Antioxidative properties of Bambara groundnut *(Voandzeia subterranea)* protein hydrolysates

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Abstract

The purpose of this study was to investigate the chemical composition, functionalities and antioxidant properties of the Bambara groundnut protein concentrate (BPC) and hydrolysates (BPHs) prepared from defatted groundnut flour, using Alcalase with various degrees of hydrolysis (DH) (10, 20 and 30%). The BPC and BPHs had high 62.75 to 66.83% protein contents and contained high amounts of the essential amino acids leucine and lysine. The BPHs exhibited excellent solubility over the wide 3 to 9 pH range, and the solutions had good thermal stability (80ºC for 30 min) at various concentrations of NaCl (50 to 300 mM). The interfacial properties of the BPC and the BPHs were governed by both concentration and DH. For the antioxidant properties, DPPH radical scavenging and metal chelating activities of the BPHs increased with DH ($p< 0.05$), whereas ABTS radical scavenging and reducing power were decreasing with DH ($p< 0.05$). This study demonstrated that the BPHs prepared using Alcalase provide functional peptides with antioxidant properties.

Introduction

Bambara groundnuts, also known in Thai as "Thua Rang", are non-traditional seeds, but are now cultivated as a promising crop in the southern part of Thailand to contribute as a locally cultivated produce. An important attribute of the Bambara groundnuts is their tolerance to drought and poor soil, being very adaptable to hot temperatures, but also tolerant to rainfall, while having relatively good resistance to pests and diseases (Collision *et al*., 2000). The Bambara groundnut seeds are rich in proteins with about 18 to 24% content, and have a good balance of essential amino acids with relatively high proportions of lysine and methionine (Eltayeb *et al*., 2011). Elegbede (1998) and Stephens (2003) noted that Bambara groundnut protein contains higher amounts of the essential amino acid methionine than other grain legumes. For this reason, the protein isolates from Bambara groundnut could be used to complement other grain proteins in order to provide a blanched amino acid profile.

Enzyme technology has proven important in modifying food protein functional properties, and may find applications in a broad spectrum of food ingredients. Enzymatic hydrolysis tends to have three distinct effects on proteins, by decreasing of molecular weight, by increasing the number of ionizable groups, as well as by exposing previously inaccessible hydrophobic groups (Panyam and Kilara, 1996). These effects can effectively modify the conformation and structure of proteins, improving solubility, surface properties, and gel properties (Zhao *et al*., 2011). The extent of hydrolysis greatly affects the properties of the resulting hydrolysate, and is described by the degree of hydrolysis (DH) defined as the percentage of peptide bonds cleaved. Proteins modified by enzymatic hydrolysis have a wide range of applications that depend on the molecular size, the structure, and the specific amino acid sequence. These include use as ingredients in the preparation of formulated foods and as source of nitrogen in enteral diets suitable for hospitals, as well as use in hypoallergenic infant formulas, in dietetic food, and in sports drinks (Clemente, 2000). In addition, enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides (Thiansilakul *et al*., 2007a). Numerous peptides derived from hydrolyzed plant proteins have antioxidant activity (Zhao *et al*., 2011). The proteins from Bambara

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groundnuts and their enzymatic modifications might have capability to contribute in the development of a new class of formulated foods, having both functional properties and bioactivity. Therefore, this study aimed to produce protein hydrolysates from the Bambara groundnut proteins, with a range of DHs from enzymatic hydrolysis by Alcalase, and to characterize their functional properties (solubility, thermal stability, foam and emulsion properties) and antioxidant properties (ABTS and DPPH radical scavenging, metal ion chelating and reducing power).

Materials and Methods

Enzyme and chemicals

Alcalase 2.4 L (declared activity of 2.4 AU/g) was purchased from Sigma-Aldrich. It is an endopeptidase prepared from a strain of *Bacillus lincheniformis*. 2, 4, 6-trinitrobenzenesulphonic acid (TNBS) (CID: 11045), 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (CID: 74358), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-Sulfonic-acid) diammonium salt (ABTS) (CID: 9570474), Iron (II) chloride tetrahydrate (Ferrous chloride) (CID: 16211588), and 3-(2-Pyridyl) – 5,6 – diphenyl- 1,2,4 – triazine - 4′,4′′, disulfonic acid sodium salt (Salt ferrozine) (CID: 23662871) were purchased from Sigma Chemical Co. (St. Louis, MO). Potassium persulfate (CID: 24412) and Sodium dodecyl sulphate (SDS) (CID: 3423265) were procured from Bio-Rad Laboratories (Hercules, CA). All reagents were analytical grade.

Preparation of Bambara groundnut protein concentrates (BPC)

Bambara groundnut seeds were purchased from a local market in Pattani province, Thailand. The seeds were washed until subjectively clean, and were left overnight in clean water. The seeds were manually dehulled, then dried in an oven at 55±1°C for 24 h, ground to a powder, and passed through a 60-mesh sieve. The resulting powder was defatted by extraction with chloroform:methanol (9:1) at 1:10 powder:solvent ratio, in a mixer set at 100 rpm, at room temperature for 6 h. The defatted solids in suspension were filtered onto Whatman No. 4 filter paper with vacuum assist. The defatted powder was air-dried in a fume hood at room temperature overnight, to remove residual solvent, and powdered by using to obtain flour which was taken as defatted Bambara groundnut flour.

The BPC was prepared from defatted Bambara groundnut flour by isoelectric precipitation method as described by Yu *et al*. (2007). Defatted flour was dispersed in deionized water (1:10, w/v) and the dispersion was adjusted to pH 10 with 1 and 6 N NaOH. This alkaline dispersion was gently stirred at room temperature (28±2°C) for 1 h, then centrifuged at 9000 g for 15 min in a high speed centrifuge (Model HARRIER 15/80 Bench Top Refrigerated Centrifuge, Sanyo, Japan). The pellet was discarded and the supernatant adjusted to pH 4.5 with 1 and 6 N HCl, and then was centrifuged at 8000 g and 15°C for 15 min. The precipitate was re-dispersed in deionized water, then stirred and adjusted to pH 7.5 with 1 N NaOH. This solution was lyophilized to the protein concentrate (BPC) using FD8-Cool Safe Advance freeze dryer (Scanvac, Denmark).

Preparation of Bambara groundnut protein hydrolysates (BPHs)

Different DHs of BPH were prepared from the BPC using Alcalase. The BPC was suspended in distilled water at 10 mg/ml protein concentration. The mixture was adjusted to the near optimal pH 8 using 1 N NaOH. The obtained solutions were conditioned by incubation at 50°C for 10 min, prior to enzymatic hydrolysis. The hydrolysis reaction was started by the addition of Alcalase at levels of 1 or 3% (w/w; solid matter) and the reaction was conducted at 50°C up to 360 min. Samples (1 ml) were taken at designated times (0, 10, 20, 40, 60, 90, 120, 150, 180, 240, 300 and 360 min), mixed with 1 ml of 1% SDS solution (90°C), and then placed into a 90°C water bath for 10 min to solubilize the sample and to inactivate the enzymes. The degree of hydrolysis (DH) was determined as described by Benjakul and Morrissey (1997). Based on the initial DH results, a sufficient hydrolysis time was selected as 360 min to give high DHs for further study.

The effects of enzyme concentration on the DH of BPC were evaluated. A BPC solution (10 mg/ml) was prepared and adjusted to specific conditions as previously described. Various amounts of Alcalase, (0.5%, 1%, 2.5%, 5%, 7.5% and 10%, w Enzyme/w protein solid matter) were added in the pre-incubated (50°C for 10 min) BPC suspensions and mixed thoroughly. The hydrolysis was allowed to proceed for the selected 360 min duration, and the enzyme was inactivated by heating the slurry to 90°C for 10 min. Then the DH of hydrolysates was determined. Log10 of enzyme amount vs. % DH was plotted and fit by regression. The enzyme amounts required for DH 10, 20 and 30% were calculated from the fit, and then used in the preparation of BPHs. The resulting BPHs were clarified by centrifuging at 4,500 g for 20 min at 4ºC, to remove insoluble debris, and then lyophilized to BPH. The BPH samples were stored in polyethylene bags at 4ºC until analyses.

Determination of DH

The DH of the protein was analyzed according to the method of Benjakul and Morrissey (1997). A BPH sample (125 µl) was added to the mixture of 2.0 ml of 0.2 M phosphate buffer at pH 8.2 and 1.0 ml of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a temperature controlled water bath (WB-22, Memert, Schwach,, Germany), at 50ºC for 30 min in dark. To terminate the reaction, 2.0 ml of 0.1 M sodium sulfite was added. The mixture was cooled for 15 min at room temperature (25-27ºC), and absorbance was measured at 420 nm (Genesys 10S UV-Vis spectrophotometer, Thermo Fisher Scientific, USA). The content of α-amino acid was expressed in terms of L-leucine. The DH was calculated as follows:

$$DH = [(L_t-L_0)/ (L_{max}-L_0)] \times 100$$

where L_t is the amount of α-amino acid released at time t, L_0 is the amount of α-amino acid in the original BPH solution, and L_{max} is the total α-amino acid in original BPH solution obtained after acid hydrolysis with 6 N HCl at 100ºC for 24 h.

Proximate analysis

BPC and BPHs were analyzed for moisture, protein, lipid and ash contents following AOAC methods 950.46, 928.08, 960.39 and 920.153, respectively (AOAC, 2000).

Amino acid analysis

Amino acids in BPC and a selected BPH (20% DH) were determined. The hydrolysate (40 mg) was dissolved in 6 N HCl (15 ml). Then 100 µl sample solutions were taken and derivatived using EZ:Faast™-Amino acid analysis Kit. The amino acids were separated by HPLC-MS. (1100 SL, Agilent, CA, USA) at 35ºC, using gradient mobile phase: eluent A (10 mM Ammonium formate in water) and eluent B (10 mM Ammonium formate in methanol). The amounts of amino acids were calculated, based on the peak areas in comparison with that of the standard. Each amino acid content was expressed as a percentage of total amino acids in the sample.

Determination of solubility

Solubility was tested at different pH levels following the method of Nalinanon, *et al*. (2011) with slight modifications. A sample (10 mg) was dispersed in 8 ml of deionized water and pH of the mixture was adjusted to 3, 4, 5, 6, 7, 8 or 9, with either 1 N HCl or 1 N NaOH. The mixture was stirred at room temperature for 30 min. The solution was made up to 10 ml by distilled water, previously adjusted to the same pH as the sample solution, prior to centrifugation at 5000 g for 15 min. Protein content in the supernatant was determined using the Lowry method (Lowry *et al*., 1951), using bovine serum albumin as a standard. Total protein content in the sample was determined after solubilization of the sample in 0.5 M NaOH. The protein solubility was calculated as follows:

Solubility (%) = [(protein content in supernatant)/ (total protein content in sample)] x 100

Determination of turbidity

A protein solution (1 mg protein/ml) was prepared by slowly dissolving the BPC or BPH in 0.1 M phosphate buffer at pH 7.0, with various concentrations of NaCl (0, 25, 50, 10, 200 and 300 mM). The solution was stirred at room temperature and left for 30 min to complete the hydration. The solution placed in a test tube was then heated at 80ºC for 30 min in a temperature-controlled water bath and cooled in an iced bath for 10 min. The resulting solution was mixed well and placed in a cuvette with 1 cm light path, and turbidity was determined by measuring the optical density (OD) at 400 nm, at room temperature using a Genesys 10S UV-Vis spectrophotometer.

Determination of emulsifying properties

The emulsifying activity index (EAI) and the emulsion stability index (ESI) were determined according to the method of Pearce and Kinsella (1978), with slight modifications. Soybean oil (2 ml) and protein hydrolysate solutions (1, 5, 10 and 30 mg protein/ml, 6 ml) were homogenized at a speed of 20,000 rpm for 1 min using a high-speed homogenizer (Model T25 basic; IKA Labortecnik, Selangor, Malaysia). An aliquot of the emulsion (50 µl) was pipetted from the middle section of the container at 0 and 10 min after homogenization, and subsequently diluted 100-fold using 0.1% sodium dodecyl sulphate (SDS) solution. The mixture was mixed thoroughly for 10 s using a vortex mixer (G-560E, Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY). A500 of the resulting dispersion was measured using a spectrophotometer (Model Genesys 10S UV-Vis, Thermo Fisher Scientific, USA). The EAI and the ESI were calculated by the following formulae:

$$EAI\ (m^2/g) = (2\times 2.303\times A_{500}\times DF)/l\Phi C$$

where A is the absorbance at 500 nm, DF is the dilution factor (100), l is the path length of cuvette

(m), Φ is the oil volume fraction and C is the protein concentration in aqueous phase (g/m^3), and

$$ESI\ (min) = A_0 \times t/(A_0-A_{10})$$

where t is 10 min, A_0 is the absorbance at 500 nm immediately after emulsification and A_{10} is the absorbance at 500 nm immediately after 10 min.

Determination of foaming properties

Foam expansion (FE) and foam stability (FS) of each hydrolysate solution were determined as described by Shahidi *et al*. (1995), with slight modifications. A sample solution (2 ml) with 1, 5, 10 or 30 mg protein/ml was transferred into a 100-ml cylinder. These solutions were homogenized at 16,000 rpm for 1 min at room temperature. The samples were allowed to stand for 0 and 60 min. FE and FS were then calculated using the following equations:

$$FE\ (\%) = (V_T/V_0) \times 100$$
$$FS\ (\%) = (V_t/V_0) \times 100$$

where V_T is the total volume after whipping, V_0 is the original volume before whipping, and V_t is the total volume after relaxing at room temperature for 60 min.

Determination of ABTS radical scavenging activity

ABTS radical-scavenging activity was determined by ABTS assay, as described by Arnao *et al*. (2001) with a slight modification. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities, and allowing them to react for 12 h at room temperature. Fresh ABTS solution was prepared for each assay. A 150 µl sample solution (5 mg/ml) was mixed with 2850 µl of ABTS solution and the mixture was left at room temperature for 2 h in the dark. The absorbance was then measured at 734 nm using a spectrophotometer. The blank was prepared in the same manner, except that distilled water was used instead of the sample. The ABTS radical-scavenging activity was calculated according to the following equation:

$$\text{ABTS radical-scavenging activity (\%)} = [(B-A)/B] \times 100$$

where A is A_{734} of sample, and B is A_{734} of blank

Determination of DPPH radical scavenging activity

The DPPH radical-scavenging activity was determined by a DPPH assay, as described by Shimada *et al.* (1992). To 1.5 ml of sample solution (5 mg/ml), 1.5 ml of 0.15 mM DPPH in 95% ethanol was added. The mixture was mixed vigorously, and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured 517 nm using Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). The blank was prepared in the same manner, except that distilled water was used instead of the sample. The DPPH radical-scavenging activity was calculated according to the following equation:

$$\text{DPPH radical-scavenging activity (\%)} = [(B-A)/B] \times 100$$

where A is A_{517} of the sample, and B is A_{517} of the blank

Determination of chelating activity on Fe^{2+}

The chelating activity on Fe^{2+} was measured by the method of Boyer and McCleary (1987), with a slight modification. A 4.7 ml solution sample (5 mg/ml) was mixed with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature. The absorbance was then read at 562 nm; the blank was prepared in the same manner except with distilled water, and the activity was calculated as follows:

$$\text{Chelating activity (\%)} = [(B-A)/B] \times 100$$

where A is A_{562} of the sample, and B is A_{562} of the blank.

Determination of reducing power

The reducing power was determined according to the method of Benzie and Strain (1996), with a slight modification. A 200 µl sample at 5 mg/ml was mixed with 1 ml of 0.2 M phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide. The mixture was incubated at 50ºC for 30 min, followed by the addition of 1 ml of 10% tricholroacetic acid. To an aliquot (1 ml) of this reaction mixture, 1 ml of distilled water and 200 µl of 0.1% $FeCl_3$ were added. The absorbance of the resultant solution was read at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Statistical analysis

All experiments were run in triplicates, and all analyses were also conducted in triplicates. Statistical analysis was performed with one-way analysis of variance (ANOVA). Mean comparisons were carried out with Duncan's multiple range test (Steel and

Table 1. Chemical compositions of the Bambara groundnut protein concentrate (BPC) and the Bambara groundnut protein hydrolysates (BPHs)

Compositions*	BPC	BPHs		
		10%DH	20%DH	30%DH
Protein (% dry basis)	66.83 ± 0.84[d#]	64.47±0.90[b]	65.78 ± 0.45[c]	62.72 ± 0.41[a]
Lipid (% dry basis)	2.28 ± 0.21[c]	1.56 ± 0.10[b]	3.77 ± 0.20[d]	0.76 ± 0.28[a]
Ash (% dry basis)	1.66 ± 0.18[a]	4.58 ± 0.31[d]	4.25 ± 0.04[c]	4.09 ± 0.06[b]
Total carbohydrate[†] (%)	29.23 ± 0.48[b]	29.39 ± 0.81[b]	26.2 ± 0.36[a]	32.43 ± 0.24[c]
Crude fiber (% dry basis)	ND	ND	ND	ND

* Values are given as mean ± SD from triplicate determinations.
†Total carbohydrate was calculated by difference.
#Different superscripts (a-b) in the same row indicate statistically significant differences ($p< 0.05$)
ND = non detected

Torrie, 1980).

Results and Discussion

Effects of Alcalase concentration and hydrolysis time on Bambara groundnut protein concentrate hydrolysis

The DH changes of BPH as affected by Alcalase concentration and hydrolysis time are now assessed (data not show). As expected, the DH of BPH increased with hydrolysis time, indicating gradual release of peptide fragments during hydrolysis. The rate of the hydrolysis was faster during the first 30 min of hydrolysis, and slower thereafter. This reduction of hydrolysis rate might be due to the limited amount of substrate, as the short chain peptides formed might not be further cleaved by Alcalase. The reduction of hydrolysis rate might also hypothetically be attributed to the decrease in peptide bonds available for hydrolysis, the decrease in enzyme activity, or product inhibition (Safari *et al*., 2009). For the same hydrolysis time, a higher amount of enzyme gave a higher DH ($p< 0.05$). The highest DHs after 360 min hydrolysis were 12.07% and 19.33% for 1% and 3% Alcalase used, respectively. During 180 min of hydrolysis, a gradually increasing rate with time was found for 3% Alcalase, while a very low hydrolysis rate was obtained for 1% Alcalase. In general, legume seeds are rich in protease inhibitors (Stephens, 2003), so contamination by an indigenous protease inhibitor in Bambara groundnut might also have influenced the Alcalase activity to some degree. The hydrolysis time using in this study is similar to that of buckwheat (*Fagopyrum esculentum* Moench) protein treated by Alcalase (Tang *et al.*, 2009). In the present study, the 360 min hydrolysis time was selected for further study. Various amounts of Alcalase were added to hydrolyze BPC for 360 min. When log (Alcalase concentration) and DH were plotted, a linear relationship was observed (data not show). From this regression, y= 16.594x+11.087, the extract concentration (exp x) of Alcalase required to hydrolyse BPC to obtain designated DHs (y = 10, 20 or 30%) were calculated.

Chemical compositions

The proximate compositions of Bambara groundnut flour were also determined to characterize it as a raw material. The flour contained carbohydrate, protein and lipid as the major constituents with contents 54.12%, 17.43% and 13.18% (dry constituent per fresh weight of flour), respectively. The high carbohydrate content indicates that Bambara groundnut has a very high energy content, so it can serve as an energy source in food. The protein level of the flour (17.43%) is also significant for its nutritive value. The 13.18% oil content of Bambara groundnut flour is low compared to soya beans (19.6%) (Belitz *et al*., 2009). With defatting the protein contain of the flour increased to 35.64%, whereas moisture, lipid, ash and carbohydrate contents decreased to 8.97, 6.94, 4.32 and 44.30%, respectively, indicating that the removal of lipids served to concentrate the protein in Bambara groundnut flour. From the defatted flour, proteins were extracted, precipitated and then lyophilized into a powder called BPC. Proximate compositions of the BPC and the BPHs are shown in Table 1. The chemical composition of the obtained BPC was as follows: protein 66.83%, lipid 2.28%, total carbohydrate 29.23% and ash 1.66%. The proximate compositions of the freeze-dried BPH samples are also shown in Table 1. The samples had high 62.72 to 65.78% protein content and could be considered for protein supplements to humans. The protein contents of BPHs (dried solid form) decreased with % DH, which might be due to

Table 2. Amino acid compositions of the Bambara groundnut protein concentrate (BPC) and the Bambara groundnut protein hydrolysate with DH 20%, prepared using Alcalase. The last column shows recommended minimum contents of essential amino acids in human nutrition

Amino acids	Content (g/ 100g)		
	BPC	BPH (20%DH)	FAO/WHO (1991)
Alanine	3.90	5.04	
Arginine	7.86	7.15	
Aspartic acid	8.75	8.31	
Cystine	0.06	0.00	
Glutamic acid	20.37	20.09	
Glycine	2.90	3.13	
Histidine*	6.00	4.93	1.90
Isoleucine*	3.96	4.63	2.80
Leucine*	8.29	9.28	6.60
Lysine*	8.72	9.33	5.80
Methionine*	0.23	0.09	
Phenylalanine*	6.03	5.68	
Proline	4.96	4.95	
Serine	4.68	4.36	
Threonine*	2.87	2.91	3.40
Tryptophan*	0.72	0.23	1.10
Tyrosine	5.25	4.49	
Valine*	4.45	5.40	3.50
Methionine+Cystine	0.29	0.09	2.5
Phenylalanine+Tyrosine	11.28	10.17	6.3
Total essential amino acids	41.26	42.47	33.9
Total non-essential amino acids	58.74	57.53	

*Essential amino acid

the increase in solid matter from enzyme addition, but overall this variation was small. In addition, these apparent differences in protein content might also be due to the loss of non-protein nitrogen (Sindaykengera and Xia, 2006). The lipid contents of BPHs might be affected by the loss of lipids with insoluble protein on centrifugation, but the case with 20%DH still had about 1.5% higher content than that the BPC. The high ash contents (4.09-4.58%) of BPHs were mainly caused by Na^+ and Cl^- ions provided by pH adjustment before enzymatic hydrolysis. According to some prior studies, a high ash content is a drawback limiting the applications of a protein hydrolysate (Shahidi *et al.*, 1995; Thiansilakul *et al.*, 2007a).

Amino acid compositions

The amino acid compositions of the BPC and the selected BPH with 20% DH are shown in Table 2. Both the BPC and the 20%DH BPH were rich in glutamic acid, lysine, leucine and aspartic acid, these being the top ranked amino acids by fraction of total. The hydrophobic amino acids, such as leucine (9.28%), phenylalanine (5.68%), valine (5.40%) and isoleucine (4.63%) may contribute to the bitterness of the hydrolysate. FitzGerald and O'Cuinn (2006) reported that bitterness of protein hydrolysates is associated with the release of peptides containing hydrophobic amino acid residues. In terms of the essential amino acids (see the last column of Table 2), both BPC and 20%DH BPH were rich in lysine and leucine but poor in tryptophan. Glutamic acid and aspartic acid were the major non-essential amino acids. These results are similar to those of Mune *et al.* (2001), and appear common to most vegetable proteins. The BPC and the 20%DH BPH had mostly higher total essential amino acids than the FAO/WHO (1991) reference pattern, except for threonine, tryptophan and total sulphur amino acids. Thus, the BPC and 20%DH BPH satisfied the FAO/WHO requirements. In nutritional use the BPH has potential as a dietary protein supplement with otherwise poorly balanced dietary protein, as with those cereals that are rich in tryptophan and sulfur amino acids.

Functional properties

Protein solubility

Solubility is usually the first functional property determined during the development and testing of new protein ingredients (Zayas, 1997). It is related to other functional properties and can be increased by hydrolysis (Klompong *et al.*, 2007). Figure 1A shows solubilities of the BPC and the BPHs with various DHs, in the pH range from 3 to 9. The solubilities were generally lowest around pH 4 to 5. Those high molecular weight proteins or peptides remaining after hydrolysis precipitated close to the isoelectric point (pI), which was reached at these pH values, and the BPHs had more than twice the solubility of the BPC (unhydrolysed protein). The solubility increased with DH mainly because of reduction in the molecular weight and increased number of polar

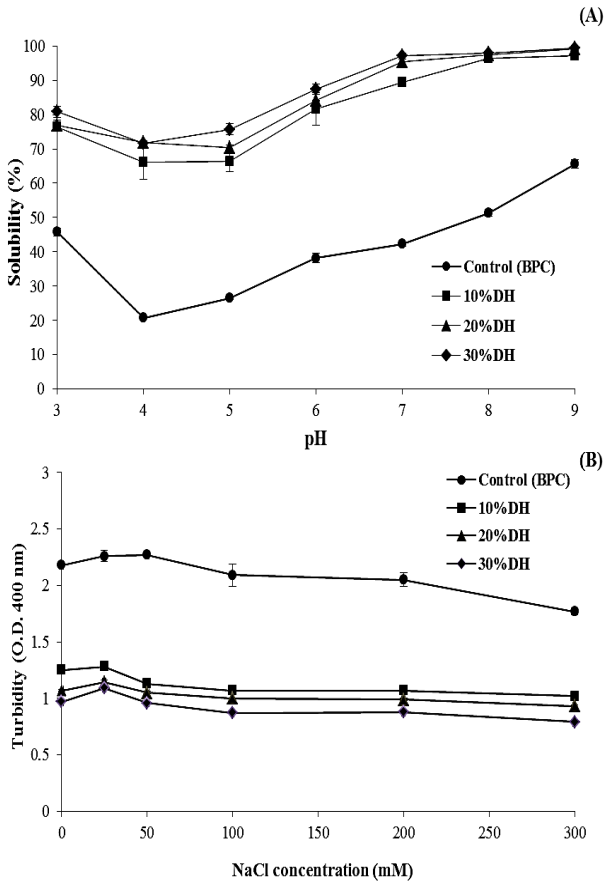

Figure 1. Protein solubility profiles of the BPC, and the BPHs with varying DH, across a range of pH values (A). Turbidity of 5 mg protein/ml for the BPC and the BPHs at various NaCl concentrations after heating at 80ºC for 30 min (B). Mean values are shown with standard deviation error bars from triplicate measurements

groups (Nielsen, 1997). All the BPHs had better than 65% solubility over a wide pH range, relative to the maximal solubility of each. At pH 4 to 7, solubilities of the BPHs increased with DH, and their differences disappeared around pH 8 to 9. This agrees with the findings of Klompong *et al*. (2007) and Nalinanon *et al*. (2011), who reported that hydrolysates had excellent solubilities at high degrees of hydrolysis. Gbogouri *et al.*, (2004) point out that the small peptides are expected to have proportionally more polar residues, with the ability to form hydrogen bonds with water, and this augments solubility. Enzymatic hydrolysis potentially also affected hydrophobicity, as well as the polar and the ionisable groups of the protein hydrolysates (Mutilangi *et al*., 1996). The balance of hydrophilic and hydrophobic forces is crucial to solubility (Gbogouri *et al.*, 2004). The high solubility over a wide pH range suggests that the BPHs can be applied widely in formulated food systems.

Thermal stability

Thermal instability is manifested by the formation of large coagulums of heat-denatured protein or peptide molecules, and the light scattering effect of these coagulums can be observed optically as turbidity. The turbidity values reflect the varying rates of protein aggregation combined with the aggregate size distribution. Thermal stability of the BPC and BPHs was characterized by determining turbidity (A_{400}) of the protein solution with varied NaCl concentrations, after heating at 80ºC for 30 min. Turbidity observations for the BPC and the BPHs are shown in Figure 1B. At a fixed NaCl concentration, the BPHs had dramatically lower turbidities than the BPC, and the trend was decreasing with DH ($p< 0.05$). This indicates that the BPHs are more tolerant to thermal treatment than the BPC in the presence of NaCl, regardless of its concentration. The turbidities of the BPC and the BPHs increased with NaCl concentrations from 0 to 25 mM. The NaCl promotes heat induced aggregation of proteins, such as whey protein (Majhi *et al*., 2006). The effects of low ionic strength on the thermal aggregation of Bambara groundnut protein may explained by effects on the protein structure that expose hydrophobic groups. Similarly, Xiong (1992) showed that, at pH 6.0, addition of up to 20 mM NaCl accelerated whey protein isolate aggregation when heated from 60 to 90ºC. In our case, the protein solution turbidity seemed to decrease beyond 50 mM NaCl concentration. A high ionic strength might impact the charge distribution in the side chains, reducing charge repulsion that prevents thermal aggregation of the protein. Von Hippel and Schleich, (1969) suggest that the ability of electrolytes to influence the conformation and stability of proteins depends on the concentration and/or ionic strength of the salt.

Foaming properties

Foam expansion (FE) and foam stability (FS) as functions of concentration (1-30 mg/ml) are depicted in Figs. 2A and 2B, for the BPC and the BPHs (10-30% DH), respectively. The FE increased with protein concentration in all cases. At 1 and 5 mg/ml of hydrolysate the DH had no consistent effect on FE, and at 1 mg/ml of hydrolysate the highest FE was observed at 20%DH ($p<0.05$). At 10 and 30 mg/ml of hydrolysate the FE decreased with DH ($p< 0.05$). This is possibly due to the smaller peptides being less aligned at the air–water interface (Nalinanon *et al*., 2008). The FE of the BPC was significantly improved by hydrolysis, and the maximum FA occurred at DH of 10% with 10 and 30 mg/ml of concentration used. This could be attributed to the production of amphiphilic peptides which can migrate to the air-water interface. During formation

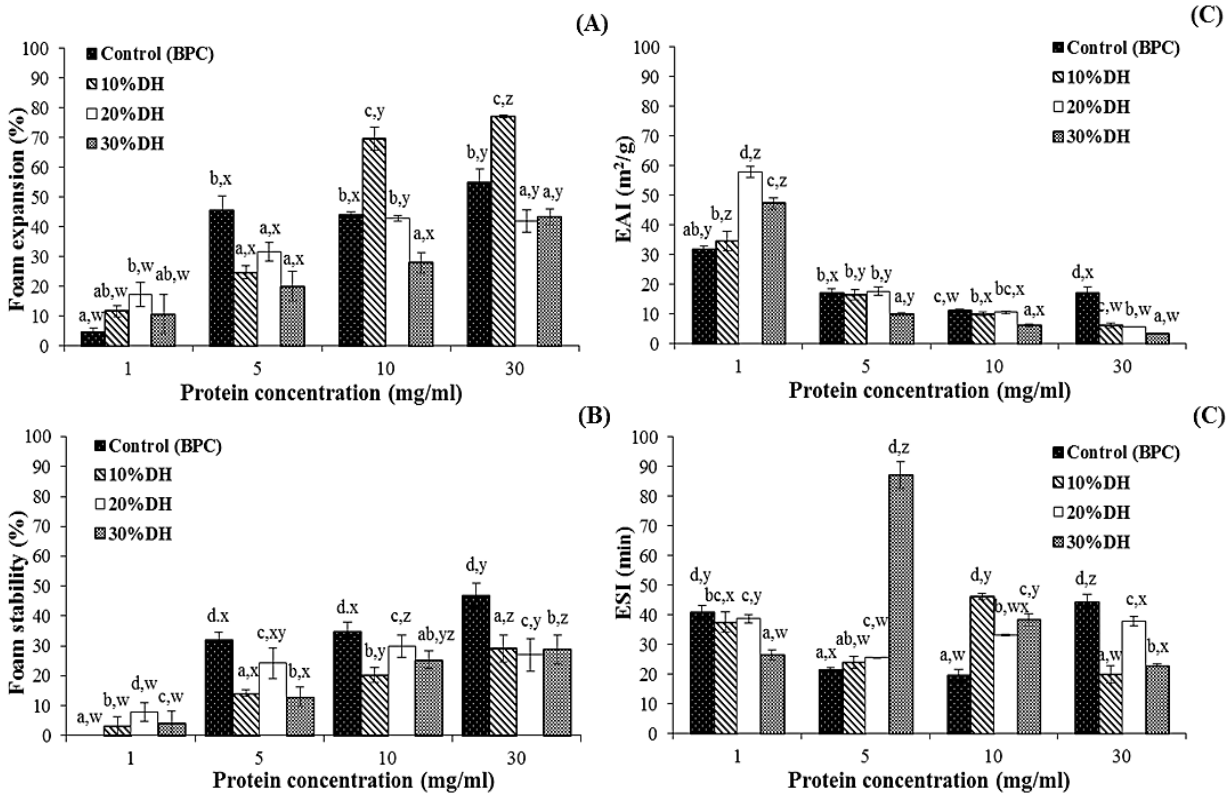

Figure 2. Foaming expansion (A), foam stability (B), emulsion ability index (C) and emulsion stability index (D) of the BPC and the BPHs across a range of protein concentrations. Mean values are shown with standard deviation error bars from triplicate measurements. Different characters (a-d) labeling the bars, within one concentration, indicate statistically significant differences ($p< 0.05$). Different characters (w-z) indicate significant differences ($p< 0.05$) within the same DH.

of foam, the peptides are rapidly adsorbed at the newly created air-water interface during bubbling, and undergo unfolding and molecular rearrangement at the interface; they foam better than proteins that adsorb slowly and resist unfolding at the interface (Thiansilakul *et al.*, 2007b).

The FE after whipping for 30 min (at room temperature) was monitored to indicate FS of the BPC and the BPHs (Figure 2B). In all cases the FS improved with concentration. The highest FS of BPH was either at 30 mg/ml or at 10 mg/ml, the latter with 20%DH, with statistical significance ($p<0.05$). The density and stability of foams increased with protein concentration, because this increased the interfacial film thickness (Zayas, 1997) and its strength; the more stable foams are likely also stiffer. The BPHs had less stable foam than BPC. This suggests that the low molecular weight peptides and the free amino acids from BPH could not maintain their orientation at the interface, instead of forming a thick, cohesive, and viscoelastic film that would prevent the foams from collapsing. Nevertheless, also the BPC had very poor FS at 1 mg/ml due to its small number of protein molecules and the lack of their orientation at the interface. In summary, the molecular properties affecting the foam capacity and stability of the BPC and the BPHs were different. The maximun foam expansion of BPH occurred at a relatively low DH (10%), and the minimum foam stability also occurred in the same sample. Studies of FE with wheat gluten hydrolysate have also shown maximum foam expansion at a relatively low DH (Adler-Nissen and Olsen, 1997). The molecular wight distribution is important, because low molecular weight peptides and amino acids tend to destabilize the foam. Foam formation has three conceptual stages: 1) the soluble globular protein diffuses to the air/water interface, concentrates and reduces surface tension, 2) the proteins unfold at the interface with the orientation of polar moieties toward the water; as a result of unfolding, there is an orientation of hydrophilic and hydrophobic groups towards the aqueous and non-aqueous phase, and 3) polypeptides interact to form a film with possible partial denaturation and coagulation (Zayas, 1997). The hydrolysis of Bambara grounut protein may have produced a range of peptides with altered hydrophobicity, charge balance and conformation compared to the native molecules. The reduction of molecular weight increases the rate of diffusion to the interface, but hinders the flexible forming of a stable interface, affecting the foamability properties negatively.

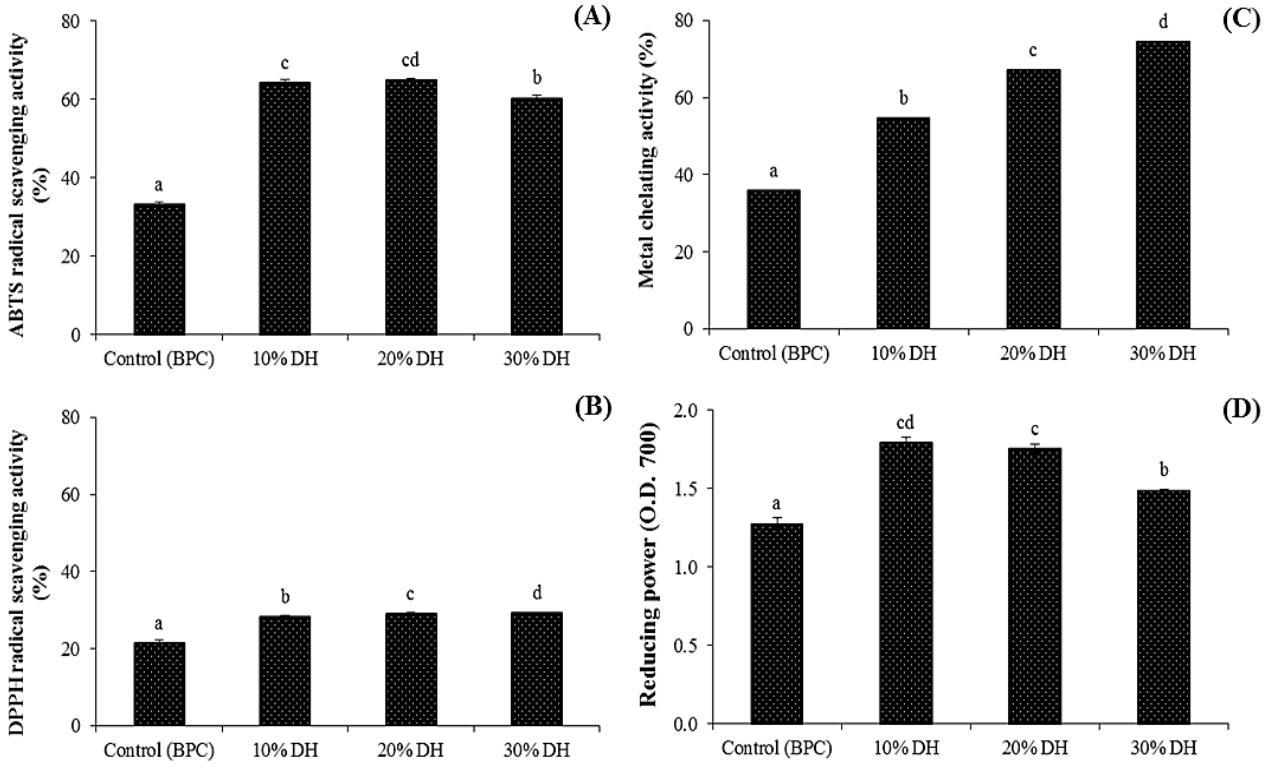

Figure 3. ABTS radical scavenging activity (A), DPPH radical scavenging activity (B), metal chelating activity (C), and reducing power (D) of the BPC and the BPHs. Mean values are shown with standard deviation error bars from triplicate measurements. Different characters (a-d) labeling the bars indicate statistically significant differences ($p< 0.05$)

Emulsifying properties

The emulsifying activity index (EAI) and emulsion stability index (ESI) of the BPC and the BPHs at various protein concentrations are shown in Figures 2C and 2D. The EAI and the ESI of BPH were in the wide ranges from 3.43 to 57.85 m^2/g and from 19.97 to 81.14 min, respectivly. The EAI of BPH decreased with the protein concentration ($p<0.05$, Figure 2C). The highest EAI was found for the BPH with 20%DH at 1 mg/ml protein concentration. At concentrations above 1 mg/ml, the EAI of the hydrolysates decreased with their DH, and the lowest EAI was found with 30% DH ($p<0.05$). The EAI estimates the ability of a protein to aid in the formation and stabilisation of a newly-created emulsion, in terms of how much it stabilizes the interface per unit weight of the protein. This is assessed quantitatively by the emulsion turbidity at 500 nm wavelength (Kinsella, 1976). Protein hydrolysates are surface-active materials and promote oil-in-water emulsions with their hydrophilic and hydrophobic groups. Intrarasirisawat *et al.* (2012) point out that extensive hydrolysis commonly yields small most likely hydrophilic peptides that cannot be adsorbed at the interface. They are instead most likely localised in the aqueous phase so that a weaker EAI results as DH is increased. Thus, the variation of EAI with DH might be associated with the peptide chain lengths. The dependence of EAI on the concentration of protein has been explained by absorption kinetics (Kinsella, 1976). At a low concentration, protein adsorption at the oil-water interface is diffusion-controlled, while at high protein concentration the activation energy barrier does not allow protein migration in diffusion-dependent manner, which leads to the accumulation of proteins in the aqueous phase (Kinsella, 1976). Thus, the proteins or peptides were most likely localised in the aqueous phase, with only a low fraction of them at the interface. Moreover, an excessive amount of protein hydrolysate might favor self-aggregation of long-chain peptides.

The BPH with 30% DH had a dramatic maximum peak in its ESI at 5 mg/ml ($p< 0.05$) (Fig. 2D). At 5 and 10 mg/ml concentrations the BPHs had higher ESIs than the BPC control ($p<0.05$), whereas at 1 and 30 mg/ml they had the lower ESIs. The variation of ESI with both DH and concentration suggests that the molecular properties (including chain length) and the amount of BPH at the interface determined the ESI. Emulsion stability is the ability of emulsion droplets to remain dispersed without coalescing, flocculation or creaming. Emulsion stability is affected by the properties of the interfacial film. Long-term stability of an emulsion depends on the thickness and strength

of the adsorbed proteins at oil-water interface. In summary, the emulsifying properties of Bambara groundnut hydrolysate were governed by both the molecular properties and the concentration employed.

Antioxidative properties

ABTS radical scavenging activity

An ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating compounds (scavengers of aqueous phase radicals) and of chain-breaking antioxidants (scavenger of lipid peroxyl radicals) (Binsan *et al*., 2008). It can be applied to both lipophilic and hydrophilic compounds, and has been widely used as an antioxidant activity assay (Binsan *et al*., 2008). The ABTS radical is relatively stable and is readily reduced by antioxidants (Klompong *et al.*, 2009). The scavenging capacities of the BPC and the BPHs for the ABTS radical were measured and compared (Figure 3A). Unhydrolyzed BPC itself exhibited some (33.2% scavenging) antioxidant properties, but the BPH hydrolysates had clearly higher scavenging activities (60.3-64.97%). The ABTS activity was higher at 10% and 20%DH than at 30%DH ($p< 0.05$). It has been postulated that antioxidative compounds with high ABTS radical-scavenging activity are most likely hydrophilic (Nalinanon *et al*., 2011). Nalinanon *et al*. (2011) also found that hydrolysates from ornate threadfin bream muscle having above 20% DH have decreased ABTS radical scavenging. Dryakova *et al.* (2010) point out that whey peptide mixtures of high chain length (> 16 of amino groups per chain) are not as effective in scavenging ABTS as a hydrolysate with shorter chain lenghts. Thus, the peptides in our hydrolysates scavenged radicals, and can potentially retard lipid oxidation in foor formulations.

DPPH radical scavenging activity

The 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) is a stable free radical, which has an unpaired valence electron at one atom of nitrogen bridge. The scavenging of DPPH free radical is the basis of the popular DPPH antioxidant assay, which is among the most frequently employed and most accurate methods to investigate the antioxidant potential of various natural components *in vitro*. The BPHs had a DPPH radical scavenging effect dependent on DH, ranging from 21.45 to 29.29% (Figure 3B), and the highest activity was at 30%DH ($p< 0.05$). This might be governed by chain length, amino acid composition, amino side chains, and hydrophobicity (Klompong *et al.*, 2007). Some previous studies point out that a high DPPH or other radical scavenging activity for the protein hydrolysate or peptide is usually associated with highly hydrophobic amino acids or hydrophobicity (Li *et al.*, 2008). The current results indicate that the BPHs had peptides acting as electron donors and free radical scavengers, thereby terminating the chain reaction. In general, the results for DPPH and ABTS activities were similar (Figure 3A), but some differences were observed indicating that the protein hydrolysates scavenge the two different radicals, ABTS and DPPH, by differing mechanisms. In summary, the peptides in BPH exhibited different scavenging mechanisms toward the ABTS and the DPPH radicals, and may be able to reduce lipid oxidation via chain-breaking reactions.

Metal chelating activity

The metal chelating activities of the BPHs with varying DH are shown in Figure 3C. The chelating activity increased with DH, and was highest at 74.41% with 30% DH ($p<0.05$). The peptide chain length was likely essential for the chelating activity of BPH, in accordance with Interasirisawat *et al.* (2012), Nalinanon *et al.* (2011) and Klompong *et al.* (2007), who found that the chelating activity on Fe^{2+} increased with DH when determined in defatted skipjack roe, ornate threadfin bream muscle, and yellow stripe trevally protein hydrolysates, respectively. The chelation of Fe^{2+} represents the ability of hydrolysates in metal chelating (Nalinanon *et al.*, 2011). Ferrozine quantitatively forms complexes with Fe^{2+} ion. In the presence of chelating agents this complex formation is disrupted, affecting the color of the solution (Thiansilakul *et al*. 2007b). Metal ions are effective pro-oxidants that can accelerate oxidation reactions or their initiation (Thiansilakul *et al*., 2007b). Metal ions react very quickly with peroxides, as single electron donors to from alkoxyl radicals (Klompong *et al*., 2007). The protein hydrolysate prepared from Bambara groundnut had potential chelating ability towards iron. The chelation of transition metal ions by an antioxidant or antioxidative peptide would reduce the availability of pro-oxidative metal ions, and could also by this means reduce lipid oxidation.

Reducing power

The reducing power may directly reflect the activity of electron donors. The power to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) through the donation of an electron was determined, and is shown in Figure 3D for BPH as a function of DH. Absorbance at 700 nm was measured to monitor the Fe^{2+} concentration. Unhydrolysed BPC also had reducing power, although lower than BPH. The reducing power of BPH decreased with DH ($p<0.05$).

As reported by Klompong *et al.* (2007), the reducing power of yellow stripe trevally hydrolysates, prepared using Alcalase, also decreased with DH. Therefore, the BPHs possibly contained amino acids or peptides, which functioned as electron donors and reacted with free radicals to form more stable products.

Conclusions

The protein hydrolysates dervied from Bambara groundnut using Alcalase appear to be good sources of desirable peptides. They had high solubility, antioxidant activities, radical scavenging activity, reducing power, and metal chelating activity, and could serve as emulsifiers or foaming agents with antioxidative activities. The interfacial effects observed through foams and emulsions were governed by both the DH and the protein concentration. In summary, Bambara groundnut protein hydrolysates can provide functional peptides with antioxidant properties for use in food systems.

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