Research Article

Received: 8 July 2016 Revised: 28 September 2016 Accepted article published: 27 October 2016 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.8112

Inhibitory properties of bambara groundnut protein hydrolysate and peptide fractions against angiotensin-converting enzymes, renin and free radicals

Abimbola K Arise,[a,b] Adeola M Alashi,[b] Ifeanyi D Nwachukwu,[b] Sunday A Malomo,[b] Rotimi E Aluko[b] and Eric O Amonsou[a*]

Abstract

BACKGROUND: An increased rate of high blood pressure has led to critical human hypertensive conditions in most nations. In the present study, bambara protein hydrolysates (BPHs) obtained using three different proteases (alcalase, trypsin and pepsin) and their peptide fractions (molecular weight: 10, 5, 3 and 1 kDa) were investigated for antihypertensive and antioxidant activities.

RESULTS: Alcalase hydrolysate contained the highest amount of low molecular weight (LMW) peptides compared to pepsin and trypsin hydrolysates. LMW peptides fractions (<1 kDa) exhibited the highest inhibitory activity against angiotensin-converting enzyme (ACE) for all the enzymes hydrolysates. For renin inhibition, alcalase hydrolysate showed the highest inhibition at 59% compared to other hydrolysates and their corresponding membrane fractions. The antioxidant power of bambara protein hydrolysates and peptide fractions was evaluated through the inhibition of linoleic acid peroxidation and ABTS scavenging activity. Among the hydrolysates, alcalase exhibited the highest inhibition of linoleic acid oxidation. Furthermore, all BPHs were able to scavenge $ABTS^{\bullet+}$ to a three-fold greater extent compared to the isolate.

CONCLUSION: BPH and LMW peptide fractions could potentially serve as useful ingredients in the formulation of functional foods and nutraceuticals against high blood pressure and oxidative stress.

Keywords: bambara; protein hydrolysate; renin; membrane ultrafiltration; angiotensin-converting enzyme; antioxidant activity

INTRODUCTION

Peptides with antihypertensive properties have received increasing attention in recent times. An increased rate of high blood pressure has led to human critical hypertensive conditions in most nations and is responsible for approximately 45–51% of total global deaths.[1] Hypertension is one of the primary risk factors for the development of several cardiovascular diseases, including coronary heart diseases, heart failure, stroke, peripheral arterial disease and renal failure.[2] The renin–angiotensin system (RAS) plays a vital role in blood pressure regulation, with renin and angiotensin-converting enzyme (ACE) being the main regulators that control the RAS pathway.[3,4] Renin is synthesized in the kidneys and is then released into the blood circulatory system where it cleaves the N-terminal region of angiotensinogen to produce a decapeptide, angiotensin (Ang)-I,[5] which circulates in the blood until its C-terminal dipeptide residue is cleaved by ACE to form an octapeptide Ang-II (a potent vasoconstrictor). Angiotensin-II also induces the release of aldosterone, thereby increasing the plasma sodium concentration and water retention, which leads to high blood pressure. ACE is also known to hydrolyze bradykinin, a potent vasodilator, thus leading to an inability of the blood vessels to relax adequately following contraction.[4] Consequently, by inhibiting ACE activity, the formation of angiotensin-II and degradation of bradykinin will be reduced, leading to blood pressure reduction. Moreover, simultaneous inhibition of both ACE and renin activities could provide a better blockade of the RAS compared to inhibition of either enzymes alone.[6] Free radicals have been implicated in the metabolic disorders that precede oxidative stress, which is a cause and consequence of hypertension. The results of oxidative stress in the body thus induce cardiovascular and renal damage with an associated increase in blood pressure. Therefore, some therapies based on free radicals scavenging activity have been suggested to be useful in the management of vascular damage-associated diseases such as hypertension.[4]

* *Correspondence to: EO Amonsou, Department of Biotechnology and Food technology, Durban University of Technology, Durban. E-mail: eamonsou@dut.ac.za*

a *Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, PO Box 1334, Durban 4001, South Africa*

b *Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, MB, R3T 2 N2, Canada*

Nutritional factors play a significant role in the prevention and treatment of hypertension. Therefore, the ability to use natural plant peptides for treating or regulating high blood pressure is attractive. ACE-inhibitory peptides have been obtained from plant proteins, including rice,[7] soybean[8] and peanut,[9] which shows that plant proteins are good sources of bioactive peptides. Apart from ACE inhibition, some studies have also demonstrated that food-derived peptides could inhibit the activity of renin and they also possess free radical scavenging activities.[10–12] For example, < 1 kDa peptides of kidney bean protein hydrolysate have been reported to possess both antioxidant and antihypertensive activities.[13] Concurrent inhibition of ACE and renin during antihypertensive therapy by food-derived peptides could potentially produce better blood pressure-lowering effects than inhibition of ACE activity alone.[14]

Bambara groundnut is an under-utilized and neglected crop of African origin. It is the third most important legume seed after groundnut (*Arachis hypogeal*) and cowpea (*Vigna unguiculata*) in Africa[15,16] and could be considered as a potential alternative source of plant protein. Bambara protein is high in lysine (6.1 g 100 g sample^{-1}) and its amino acid composition is comparable to other commonly consumed legumes such as soybean.[17,18] These legumes have been used in the development of numerous products such as in the functional food and nutraceutical industries.[19] Both the technological and functional properties of bambara protein have been studied.[16,17] Studies on the functional and antioxidative properties of bambara protein have been limited to a hydrolysate produced using a single digestive protease, with few reports on activities of peptides from different proteases.[19] The use of different proteases for enzymatic digestion is important for determining the specific enzyme or group of enzymes that produces peptides with the best bioactive properties. In addition, information on the RAS enzyme-inhibitory activities of bambara protein and the characteristics of their peptides is lacking in the literature.

It is well known that the structure and activity of bioactive peptides can be affected by the enzyme hydrolysis method. Therefore, it is essential to evaluate the efficiency of proteases with respect to the release of potential antihypertensive peptides from bambara proteins. Thus, the present study aimed to determine the ability of several proteases with respect to producing potential antihypertensive bambara protein hydrolysates (BPHs) from bambara protein isolate as measured using *in vitro* inhibition of ACE and renin activities. Because high oxidative stress may also contribute to high blood pressure, the antioxidant activities of the BPHs were determined using ABTS radical scavenging activity and inhibition of linoleic acid oxidation.

MATERIALS AND METHODS

Materials

Bambara groundnut seeds were obtained from Josini, KwaZulu-Natal province of South Africa. Alcalase 2.4 L, trypsin, pepsin, ACE, *N*(3[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG), 8-anilino-1-naphthalenesulfonic acid ammonium salt, captopril, L-glutathione (GSH), and 2,2-azobis(2-amidinopropane)dihydrochloride were purchased from Sigma-Aldrich (St Louis, MO, USA). A Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA), whereas other analytical grade reagents and ultrafiltration membranes (1-, 3.5- and 10 kDa molecular weight cut-offs), were obtained from Fisher Scientific (Oakvile, ON, Canada).

Preparation of bambara seed protein isolate (BPI)

Bambara flour was defatted with *n*-hexane in the ratio 1:5 (flour: solvent) for 3 h using a magnetic stirrer at a speed of 198 r.p.m. The defatted flour was air- dried in a fume hood overnight. According to the Soxhlet's procedure, the fat content of defatted bambara flour was less than 0.01%. BPI was produced from defatted bambara flour in accordancce with the method described by Adebowale *et al.*[18] with slight modifications. Briefly, defatted bambara flour was dispersed in deionized water (1:20, w/v) and the dispersion was adjusted to pH 10.0 with 2 mol L^{-1} NaOH to solubilize the proteins. The resultant dispersion was stirred at 37 °C for 2 h followed by centrifugation (7000 × *g* at 4 °C) for 45 min. The residue was discarded and the soluble proteins in the supernatant were subjected to isoelectric precipitation (pH 5.0) with the addition of 2 mol L^{-1} HCl. Thereafter, the mixture was centrifuged (7000 × *g* at 4 °C) for 45 min. The resultant precipitate was re-dispersed in deionized water and adjusted to pH 7.0 with 2 mol L^{-1} NaOH. The mixture was subsequently freeze-dried to obtain the BPI powder; protein content was determined by the modified Lowry method.[20]

Preparation of BPHs and fractions

BPI was hydrolysed using three food grade enzymes at an enzyme substrate ratio of 1:100 for 4 h to obtain BPHs. The following hydrolysis conditions were used: alcalase (pH 8.0 and 50 °C), pepsin (pH 2.0 and 37 °C) and trypsin (pH 8.0 and 37 °C). The pH was maintained for each hydrolysis process using either 1 mol L^{-1} NaOH or 1 mol L^{-1} HCl as appropriate with continuous stirring, with the temperature being maintained using a thermostat. After the 4-h digestion period, the enzymes were inactivated by adjusting the reaction mixture to pH 4.0 with 2 mol L^{-1} HCl or NaOH followed by heating and holding at 90 °C for 15 min. The undigested proteins were precipitated by centrifugation at 8000 × *g* for 60 min. A portion of the supernatant containing target peptides was freeze dried to obtain BPH, whereas the remaining portion was passed through ultrafiltration membranes with molecular weight cut-off (MWCO) of 1, 3, 5 and 10 kDa in a stirred ultrafiltration cell (Amicon Corp., Danvers, MA, USA). Supernatant was first passed through the 1 kDa membrane and the retentate passed through 3 kDa. The 3 kDa retentate was passed through a 5-kDa membrane, with the retentate then passed through a 10 kDa membrane. The permeate from each MWCO membrane (< 1, 1–3, 3–5 and 5– 10 kDa) was collected, lyophilized and stored at –20 °C until needed for further analysis. The protein contents of the freeze-dried BPH and membrane fractions were also determined using the modified Lowry method as described by Markwell *et al.*[20]

Analytical methods

Analysis of molecular weight distribution of BPHs

Molecular weight (MW) distribution of BPH peptides was determined using an AKTA FPLC system (GE Healthcare, Montreal, PQ, Canada) equipped with a Superdex™ Peptide 10/300 GL column (10 × 300 mm) and uktraviolet detector ($\lambda = 214$ nm). A 100-µL aliquot of the sample (5 mg mL^{-1} in 50 mM phosphate buffer, pH 7.0 containing 0.15 mol L^{-1} NaCl) was loaded onto the column and elution was performed at room temperature using the phosphate buffer at 0.5 mL min^{-1} flow rate. Molecular weight was determined from a plot of log MW versus elution volume of standard proteins (cytochrome C, 12 kDa; aprotinin, 6.5 kDa; vitamin B_{12}, 1.85 kDa; glycine, 0.075 kDa).

$$K_{av} = \frac{V_e - V_o}{V_c - V_o}$$

where K_{av} is the partition coefficient, V_e is the elution volume, V_c is the column volume and V_o is the void volume

Amino acid composition analysis

The amino acid profiles of BPI and BPH samples were determined using a high-performance liquid chromatography system. Bambara proteins and its hydrolysates were hydrolysed with 6 mol L^{-1} HCl at 116 °C for 24 h prior to chromatographic analysis, in accordance with the method of Bidlingmeyer *et al.*[21] The cysteine and methionine contents were determined after performic acid oxidation,[22] whereas tryptophan content was determined after alkaline hydrolysis.[23] The digests were separated on a cationic column (4.6 × 150 mm) using a gradient of sodium citrate buffers (pH 3.45 and pH 10.85) at a flow rate of 0.45 mL min^{-1}.

ACE inhibition assay

The ability of BPH and peptide fractions to inhibit *in vitro* activity of ACE was measured in accordance with the method of Girgih *et al.*[10] with slight changes, using FAPGG as the substrate. Briefly, 1 mL of 0.5 mmol L^{-1} FAPGG (dissolved in 50 mmol L^{-1} Tris_HCl buffer containing 0.3 mol L^{-1} NaCl, pH 7.5) was mixed with 20 µL of ACE (1 U mL^{-1}, final activity of 20 mU) and 80 µL of sample dissolved in the same buffer. The final concentration of samples was 1 mg mL^{-1} based on protein content. The rate of decrease in absorbance at 345 nm was recorded for 2 min at room temperature. The buffer was used instead of sample solutions in the blank experiment. ACE activity was expressed as the rate of reaction (ΔA min^{-1}) and inhibitory activity was calculated as:

$$\text{ACE inhibition } (\%) = \left(\frac{\Delta A \text{ min}^{-1}_{\text{blank}} - \Delta A \text{ min}^{-1}_{\text{sample}}}{\Delta A \text{ min}^{-1}_{\text{blank}}} \right) \times 100$$

where $\Delta A \text{ min}^{-1}{}_{\text{sample}}$ and $\Delta A \text{ min}^{-1}{}_{\text{blank}}$ represent absorbance changes in the presence and absence of the BPH or peptide fractions respectively.

Renin inhibition assay

In vitro inhibition of human recombinant renin activity was investigated using the Renin Inhibitor Screening Assay Kit in accordance with the method described by Li and Aluko.[11] Briefly, for the reaction (i) 20 µL of substrate, 160 µL of assay buffer and 10 µL of Milli-Q water (Millipore, Billerica, MA, USA) was added to the background wells; (ii) 20 µL of substrate, 150 µL of assay buffer and 10 µL of Milli-Q water was added to the blank walls; and (iii) 20 µL of substrate, 150 µL of assay buffer and 10-µL samples were added to the inhibitor wells. The final sample concentration was 1 mg mL^{-1} based on protein content. The reaction was initiated by adding 10 µL of renin enzyme to blanks and sample wells before incubating for 15 min at 37 °C in a fluorometric microplate reader (Spectra MAX Gemini; Molecular Devices, Sunnyvale, CA, USA). The kinetics (10 min at intervals of 2 min) of the fluorescence intensity was recorded using an excitation and emission wavelengths of 340 and 490 nm, respectively. The percentage renin inhibition was calculated:

$$\text{Renin inhibition } (\%) = \left(\frac{\text{FI of blank well} - \text{FI of sample well}}{\text{FI of blank well}} \right) \times 100$$

Inhibition of linoleic acid oxidation

Linoleic acid oxidation was measured using the method described by He *et al.*[4] Samples with a final concentration of 1 mg mL^{-1} were dissolved in 1.5 mL of 0.1 mol L^{-1} sodium phosphate buffer (pH 7.0). A 1-mL aliquot of 50 mmol L^{-1} linoleic acid (dissolved in 95% ethanol) was added to the samples and blank (buffer). The mixtures were incubated at 60 °C under dark conditions for 7 days. The degree of linoleic acid oxidation was measured every 24 h during the incubation period. A 100-µL aliquot of the assay mixture above was transferred into a reaction tube to which 4.7 mL of 75% (v/v) ethanol, 100 µL of 30% (w/v) ammonium thiocyanate and 100 µL of 0.02 mol L^{-1} ferric chloride dissolved in 1 mol L^{-1} HCl were added. After shaking and incubating at room temperature for 3 min, 200 µL of each sample was transferred into a clear-bottom 96-well plate and the absorbance was measured at 500 nm using a spectrophotometer. An increase in absorbance value implies an increase in the level of linoleic acid oxidation. The percentage inhibition of linoleic acid was calculated using the equation:

$$\text{Inhibition of linoleic acid } (\%) = \left(1 - \left(\frac{A_S}{A_b} \right) \times 100 \right)$$

where A_s and A_b are the absorbance of sample and blank, respectively.

ABTS radical scavenging activity

This assay is based on the percentage inhibition of the peroxidation of ABTS radical, which is observed as a discoloration of the blue green colour (734 nm). The reaction was carried out in accordance with a previously described method[24,25] with slight modifications. Briefly, $ABTS^{\bullet+}$ was prepared by dissolving 7 mmol L^{-1} ABTS and 2.45 mmol L^{-1} potassium persulphate in phosphate-buffered saline (PBS), pH7.4, allowing this to stand in the dark for 16 h to generate the ABTS radical cation ($ABTS^{\bullet+}$). For the analysis, the $ABTS^{\bullet+}$ stock was diluted using PBS buffer and equilibrated at 30 °C to an absorbance of 0.7 ± 0.02 at 734 nm. Trolox was dissolved in 80% ethanol. The antioxidant capacity was measured by mixing 200 µL of samples with 2 mL of $ABTS^{\bullet+}$ solution and the decline in absorbance was observed for 5 min. Appropriate blanks were run for each sample and the radical scavenging capacity was compared with that of Trolox (6.25–200 µmol L^{-1}) and results were expressed as mmol L^{-1} Trolox equivalent (TE) g $sample^{-1}$ on the protein equivalent basis. The percentage $ABTS^{\bullet+}$ scavenged was calculated using the equation:

$$ABTS^{\bullet+}\text{scavenged } (\%) = \left(\frac{\text{Ai-Af}}{\text{Ai}} \times 100 \right)$$

where A_i and A_f are the initial and final absorbance of the sample, respectively.

The effective concentration that scavenged 50% of the free radicals (EC50, $ABTS^{\bullet+}$) was calculated for each sample by non-linear regression from a plot of percentage $ABTS^{\bullet+}$ scavenged versus sample concentration (1–10 mg mL^{-1}).

Statistical analysis

All assays were conducted in triplicate and analysed by one-way analysis of variance. The means were compared using Duncan's multiple range test. $P < 0.05$ was considered statistically different.

RESULTS AND DISCUSSION

Protein content and yield of membrane fractions

Bambara protein isolate had 80.2% protein content (wet weight basis). The protein content and yield (i.e. the amount of protein recovered from the fractionation process for each membrane filtration) of BPHs and fractions shows that the protein content increased with increasing peptide size for all the enzymes (Table 1). The yield reflects the amount of peptides that can be obtained from a known quantity of raw materials and is an important parameter for technology adoption and commercialization. This is because a higher yield provides a better cost/benefit ratio than a lower yield, which enhances the profitability of protein hydrolysate production. The trend observed for protein content was reversed for protein yield because lower yields were obtained as the peptide molecular weight increased, except for alcalase peptide size 1–3 kDa, which gave a higher yield (41.8%) compared to its < 1 kDa peptide (21%). This is expected because there was a strong positive correlation between all protein content and the yield for all fractions. All the BPHs had protein content ≥ 73.5%. However, alcalase hydrolysate had the highest protein content (93.5%) followed by trypsin (80.5%) and pepsin (73.5%). Furthermore, alcalase BPH had higher yield (79.5%) compared to trypsin and pepsin BPHs. The result suggests that alcalase is a more effective protease in releasing peptides from bambara proteins. The > 68% yields obtained for the hydrolysates indicate that most of the proteins were susceptible to enzymatic hydrolysis and could be converted into peptide products, which would be economically beneficial for industrial purposes. Pepsin BPH had the lowest yield (68.5%), which is probably a result of the nature of enzyme because pepsin is an endoprotease and is most efficient at cleaving bonds involving the aromatic amino acids phenylalanine, tryptophan and tyrosine. This specificity could have limited the rate of bambara protein hydrolysis by pepsin and hence lowered the hydrolysate yield. The ratio of the percentage protein content to yield was higher for trypsin BPH and its fractions compared to both alcalase and pepsin BPHs. The percentage yield of protein hydrolysate is not commonly reported in literature; therefore, an adequate comparison could not be made.

Table 1. Protein content (PC) and yield of bambara protein hydrolysates and peptide fractions obtained from ultrafiltration membrane separation

Proteases	Hydrolysates		< 1 kDa		1–3 kDa		3–5 kDa		5–10 kDa	
	PC (%)[a]	Yield (%)[b]	PC (%)[a]	Yield (%)[b]	PC (%)[a]	Yield (%)[b]	PC (%)[a]	Yield (%)[b]	PC (%)[a]	Yield (%)[b]
Alcalase	93.5 ± 0.5 efg	79.5 ± 0.5 j	89.0 ± 0.7 de	21.0 ± 1.0 g	91.0 ± 0.6 de	41.8 ± 0.2 h	91.5 ± 0.5 def	14.0 ± 0.1 f	96.0 ± 1.0 fg	11.9 ± 0.2 de
Trypsin	80.5 ± 0.5 c	76.0 ± 1.0 i	75.5 ± 1.5 b	7.1 ± 0.2 c	76.0 ± 2.0 bc	4.7 ± 0.1 ab	78.0 ± 2.0 bc	5.6 ± 0.2 b	93.0 ± 1.5 def	4.1 ± 0.1 a
Pepsin	73.5 ± 0.7 b	68.5 ± 0.5 h	67.5 ± 0.5 a	12.3 ± 0.1 e	88.0 ± 0.8 d	13.6 ± 0.2 f	88.5 ± 1.0 d	11.9 ± 0.1 de	97.5 ± 0.5 g	10.7 ± 0.1 d

[a] Weight of protein in a hydrolysate expressed as a ratio of the weight of protein in the starting material (Bambara protein isolate).
[b] Weight of protein in a fraction expressed as a ratio of the weight of protein in the respective hydrolysate.
Values are the mean ± SD ($n = 3$). Means with different lowercase letters in column are significantly different ($P < 0.05$).

Molecular size distribution of bambara protein hydrolysates

The size exclusion chromatogram of BPHs illustrates differences between the molecular weight distribution of BPI and its protease hydrolysates (Fig. 1). The specificity of pepsin endoprotease activity is reflected in the chromatogram as shown by the high peak intensity (C and D) of MW ranging between 19.6 and 6 kDa. By contrast, these two peak intensities were not observed in the chromatogram of alcalase and trypsin hydrolysates. Trypsin proteolysis is even more specific because it is restricted to peptide bonds formed by arginine or lysine only; hence, the larger peptide size of the BPH compared to pepsin and alcalase BPHs. However, bambara alcalase hydrolysates had a larger range of low molecular weight (LMW) peptides as observed (peak E), whereas bambara trypsin hydrolysates had a peak (B) similar to that of the isolate (peak A) with a significant overlap between both peaks A and B. The results reflect the higher proteolytic activity and non-specificity of alcalase (acts randomly), which is consistent with the higher BPH peptide yield shown in Table 1. Low MW peptides possess a stronger effect on the ACE inhibitory activity of protein hydrolysates compared to high MW peptides because they are easily absorbed and reach the active sites to inhibit ACE activity without further digestion.[26] Therefore, bambara alcalase hydrolysates

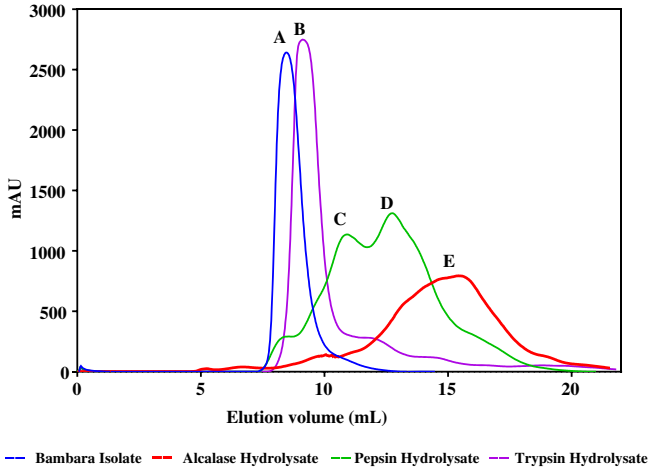

Figure 1. Gel-permeation chromatograms of bambara protein isolate and hydrolysates after passage through a Superdex™ Peptide12 10/300 GL column.

may have high potential for use as ingredients to formulate anti-hypertensive products.

Amino acid composition of BPI and BPHs

The amino acid composition of BPI and BPHs is shown in Table 2. The similarities in the amino acid compositions, especially some of the hydrophobic amino acids compared to BPI suggest that the protein hydrolysis process did not have a negative effect on the amino acid composition of the hydrolysates. Hydrolysates obtained from alcalase and trypsin contained higher concentrations of hydrophobic amino acid (HAA) compared to pepsin hydrolysate (Table 2). HAA have been reported to act as antioxidants by increasing the solubility of peptides in lipids, which facilitates better interaction with free radicals.[27] Similar values of HAA have been reported for rapeseed protein hydrolysates[28] and canola hydrolysates.[25] Generally, BPI and BPHs contained low levels of methionine and cysteine, which is typical of legume proteins that are usually deficient in sulphur-containing amino acids.[29] Furthermore, the amino acid compositions of all the hydrolysates revealed that they have high levels of Glu, Asp, Arg and Leu. Previous reports have indicated that acidic amino acids such as Glu and Asp have strong antioxidant effects as a result of the presence of excess electrons that can be donated during interaction with free radicals.[14,25] Furthermore, threonine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine and lysine values were also found to be higher than the FAO/WHO recommendations for animal protein (Table 1). The result shows that bambara proteins are of high nutritional quality and may be used as protein source in the human diet.

ACE-inhibitory activities of BPHs and membrane fractions

The ACE-inhibitory activities of BPHs and peptide fractions showed similar trends as evident in Fig. 2. The activity of peptide fractions for all the hydrolysates was clearly molecular weight-dependent. The LMW peptides (< 1 kDa) showed a higher percentage of ACE-inhibitory activity than the high molecular weight (HMW) peptides (5 – 10 kDa).This observation is in line with previous study of Grimble *et al.*,[30] which demonstrated that smaller peptides are better absorbed than larger size peptides. Generally, all peptides fractions and BPHs showed a higher percentage (above 57%) of

Table 2. Amino acid composition of bambara protein isolates and hydrolysates (g/100 g sample)

Amino acid	Isolate	Trypsin	Pepsin	Alcalase	FAO/WHO 1991
ASP	9.0	8.1	7.4	7.5	
THR	5.2	5.1	4.1	5.1	3.4
SER	4.7	4.2	3.7	4.1	
GLU	14.4	12.4	11.8	12.3	
PRO	4.1	3.7	2.3	4.3	
GLY	2.4	2.2	1.9	2.1	
ALA	2.7	2.4	1.5	2.3	
CYS	0.3	0.3	0.2	0.3	
VAL	4.1	3.9	3.6	3.9	3.5
MET	0.7	0.5	0.3	0.6	
ILE	3.5	3.6	3.3	3.8	2.8
LEU	7.0	6.8	6.7	7.5	6.6
TYR	2.7	2.4	1.8	2.7	1.1
PHE	5.1	4.5	4.1	4.9	6.3
HIS	2.7	2.3	2.1	2.4	1.9
LYS	6.1	5.9	5.8	6.1	5.8
ARG	5.8	5.1	4.6	5.1	
TRP	0.5	0.9	0.5	0.6	
HAA	30.7	29.0	24.3	30.1	
PCAA	14.6	13.3	12.5	13.6	
NCAA	23.4	20.5	19.2	19.9	
AAA	8.3	7.8	6.4	8.2	

Combined total of hydrophobic amino acids: alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine (HAA). Positively-charged amino acids: arginine, histidine, lysine (PCAA). Negatively-charged amino acids: ASX and GLX (NCAA). Aromatic amino acids: phenylalanine, tryptophan, and tyrosine (AAA).

ACE-inhibitory activity. The highest ACE inhibition at 93.9% was achieved by alcalase peptide fraction < 1 kDa, whereas trypsin peptide fraction 5 – 10 kDa showed minimum inhibition at 59.6%. Among the hydrolysates, alcalase hydrolysate showed the highest inhibition at 70.1%. Hydrophobic and bulky amino acids have been reported as a structural requirement for ACE-inhibitory peptides.[31] Thus, the high hydrophobic and aromatic amino acids of alcalase hydrolysate may have contributed to the high ACE-inhibitory activity. Similar results were reported for rapeseed[4] and peanut protein isolate and its alcalase hydrolysate at different degrees of hydrolysis.[32] In addition, this result also suggests that LMW peptides fractions were more active at inhibiting ACE activity compared to HMW peptides. The results are inconsistent with data from studies carried out by Zhu *et al.*,[33] which showed that the best ACE-inhibitory activity was attributed to LMW fractions. Similar ACE-inhibitory activity behaviours of peptides were also reported for cowpea hydrolysates, kidney bean peptide fractions and alcalase derived peptides from azufrado beans.[13,34,35] The alcalase BPH ACE inhibition obtained in the present study (93.9%) is greater than 80% obtained for alcalase kidney bean hydrolysate[13] and slightly higher than 89.4% reported for alcalase rapeseed protein hydrolysate.[36]

Renin-inhibitory activities of BPHs and membrane fractions

The type of enzyme used significantly affected the renin inhibitory potential of bambara protein hydrolysates (Fig. 3).

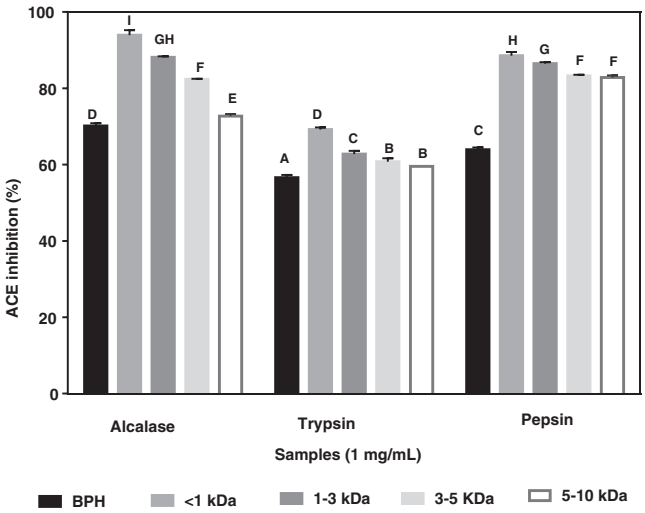

Figure 2. Inhibition of ACE by enzymatic bambara protein hydrolysate and membrane ultrafiltration fractions at a concentration of 1 mg mL^{-1}. Error bars (mean ± SD, $n = 3$) with different alphabets have mean values that are significantly different ($P < 0.05$).

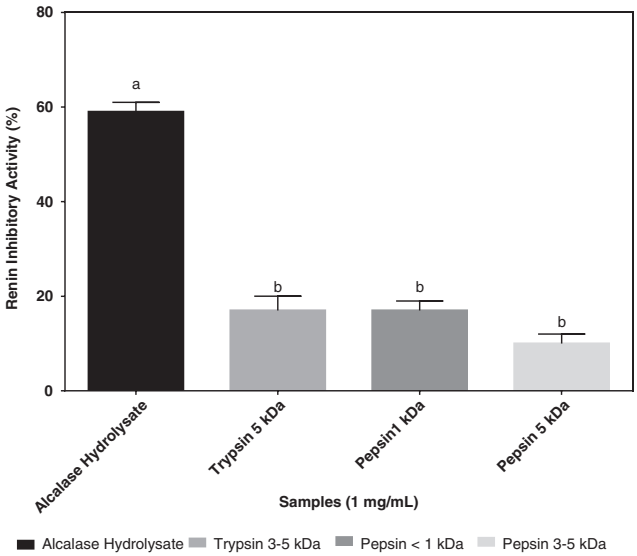

Figure 3. Inhibition of renin by enzymatic bambara protein hydrolysate and membrane ultrafiltration fractions at a concentration of 1 mg mL^{-1}. Error bars (mean ± SD, $n = 3$) with different alphabets have mean values that are significantly different ($P < 0.05$).

No renin inhibitory activity was observed for pepsin and trypsin hydrolysates. This result agrees with previous studies showing that renin activity was dependent on the type of proteolytic treatment of the substrate protein.[37] Renin inhibition was significantly ($P < 0.05$) higher for alcalase hydrolysate. The ultrafiltration peptide fractions did not show a similar trend to the unfractionated hydrolysate (Fig. 3). Alcalase fraction did not show any level of renin inhibition. This same trend was observed for *Palmaria palmata* protein hydrolysate from alcalase in which there was a loss in renin inhibition activities of the hydrolysates.[38] Among the peptides, trypsin 5–10 kDa, pepsin < 1 kDa and 5–10 kDa were the fractions that are able to inhibit renin. Trypsin 5–10 kDa and pepsin < 1 kDa showed higher renin inhibition compared to other peptide fractions It is interesting to note that the 59% renin inhibition obtained for alcalase hydrolysate (1 mg mL^{-1}) in the present study is higher than the 44.5% obtained for a flaxseed protein hydrolysate administered at 7.5 mg mL^{-1}.[31] In addition, the 59% inhibitory value is also higher than 23% value reported for an alcalase *Palmaria palmata* protein hydrolysate.[38]

Inhibition of linoleic acid peroxidation

In lipids such as free and ester forms of polyunsaturated fatty acids, lipid peroxidation is considered to proceed via radical mediated abstraction of hydrogen atoms from methylene. This initiates a sequence of reactions that generates aldehydes, ketones and other potentially toxic substances. As a result of this, inhibition of lipid peroxidation is an important indicator for measuring the antioxidant activity of peptides. The lipid peroxidation inhibition activities of GSH, BPI, BPHs and their membrane fractions were evaluated at 1 mg mL^{-1} using a linoleic acid system. The results obtained after 7 days of incubation indicated that the addition of peptide inhibitors was effective in decreasing linoleic acid oxidation up umtil day 5 of the incubation for most of the samples, except for alcalase peptide 1–3 kDa, which was effective for the whole 7 days (Fig. 4). The samples generally exhibited a significantly higher percentage inhibition ($P < 0.05$) on day 2 compared to day 5. This may be attributed to the gradual loss in their ability to adequately reduce the linoleic peroxyl radical with continuous incubation as a result of the slow formation of peroxidation of linoleic acid in the system. However, BPHs showed better linoleic acid oxidation inhibition compared to peanut protein hydrolysates, which only showed inhibition activity at higher concentrations.[39] The unhydrolysed BPI showed the least effective inhibition (53.3%) after day 5 of incubation, whereas alcalase hydrolysate showed the highest inhibition (82.7%) after 5 days of incubation. These results are comparable to those of previously reported studies from hempseed[10] and rapeseed protein hydrolysates,[40] which also showed effective inhibition of linoleic acid oxidation. In addition, our results showed that the BPHs had superior inhibition of linoleic acid oxidation compared to alcalase digests of wheat gluten that lost inhibitory activity after 3 days at a higher concentration of 4 mg mL^{-1}.[33]

ABTS radical scavenging activity

Figure 5 shows that the ABTS• + scavenging ability (EC_{50}) for peptide was significantly ($P < 0.05$) better (< 25 μg mL^{-1}) compared to that of BPI (84 μg mL^{-1}). This is because scavenging potency is inversely related to peptide EC_{50} value. However, GSH had the lowest ($P < 0.05$) EC_{50} value of 1.8 μg mL^{-1}, which indicates highest radical scavenging potency among all the tested samples. Therefore, the BPHs have superior ABTS• + scavenging ability compared to the unhydrolysed BPI. The higher activity observed for the hydrolysates compared to the isolates showed that antioxidant peptides were released from BPI during enzyme hydrolysis. This is an indication that the peptide was able to donate hydrogen atoms for ABTS• + reduction. Generally, among the hydrolysates, trypsin hydrolysate was found to be a better ABTS• + scavenger with a low value of 22 μg mL^{-1} compared to alcalase and pepsin hydrolysates. In addition, trypsin having < 1 kDa and 1–3 kDa fractions with a value of 19.8 μg mL^{-1} was the most effective ($P < 0.05$) ABTS• + scavenger. The values obtained for the alcalase hydrolysate in the present study are similar to those reported for alcalase hydrolysed amaranth protein (22.4 μg mL^{-1}) and cocoa seed protein[41,42] but higher than those

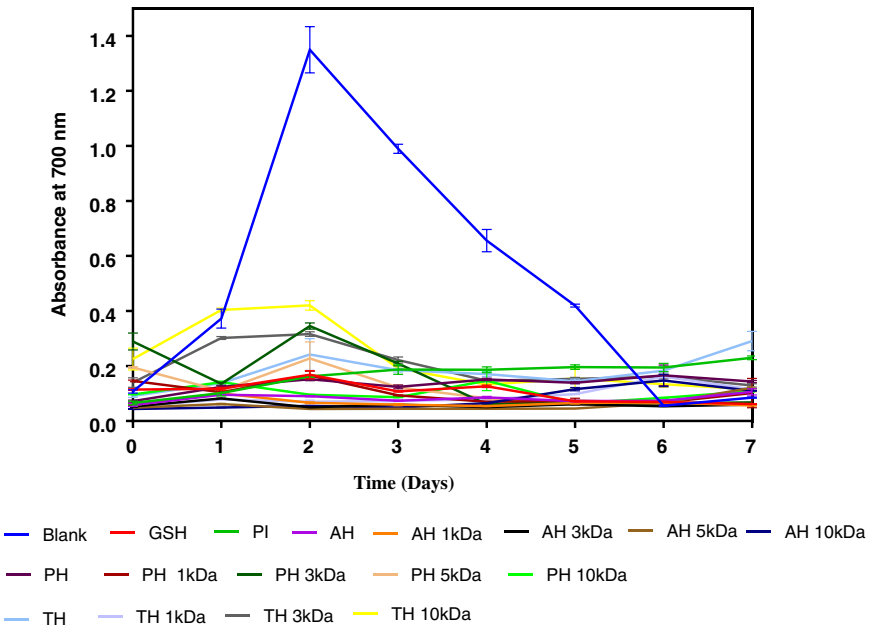

Figure 4. Inhibition of linoleic acid oxidation of GSH, BPI, hydrolysates (alcalase, AH; pepsin, PH; trypsin, TH) and membrane ultrafiltration peptide fractions measured over 7 days at 500 nm.

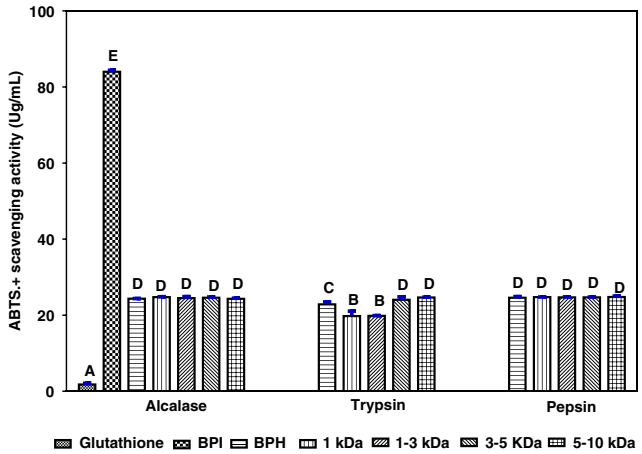

Figure 5. The effective concentration that scavenged 50% (EC_{50}) values for $ABTS^{\bullet+}$ scavenging activity values of BPI, BPHs and membrane ultrafiltration peptide fractions. Error bars (mean ± SD, $n = 3$) with different alphabets have mean values that are significantly different ($P < 0.05$).

reported for canola alcalase hydrolysates.[25] In general, there is no significant difference between the BPHs and peptide fractions.

CONCLUSIONS

Fractions with MW < 1 and 1–3 kDa exhibited significantly higher ($P < 0.05$) inhibition, as well as the ability to inhibit the peroxidation of linoleic acid and scavenge ABTS•+. Therefore, for each enzyme hydrolysate, the fractions with < 1 and 1–3 kDa peptide sizes showed high potential as antihypertensive and antioxidant peptides, suggesting that LMW peptides could be considered in the development of functional foods and nutraceutical ingredients. These results are consistent with previous research indicating that smaller peptides exhibit better bioactivity because of the higher possibility for an increased rate of intestinal absorption (without structural degradation) and entry into cells compared to the larger sized peptides. Therefore, *in vivo* tests will be required to confirm the observed enzyme inhibition and antioxidant properties of the BPHs and peptide fractions.

ACKNOWLEDGEMENTS

We thank the African Union Commission for their financial support, as well as the National Research Foundation of South Africa for providing research grant support. The research program of Dr R. E. Aluko is funded through a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant. The technical assistance of Temitola Awosika in Dr Aluko's laboratory is also appreciated.

REFERENCES

1 Malomo SA, Onuh JO, Girgih AT and Aluko RE, Structural and antihypertensive properties of enzymatic hemp seed protein hydrolysates. *Nutrition* **7**:7616–7632 (2015).
2 Sharp SI, Aarsland D, Day S, Sønnesyn H and Ballard C, Hypertension is a potential risk factor for vascular dementia: systematic review. *Int J Geriatr Psychiatry* **26**:661–669 (2011).
3 Daien V, Duny Y, Ribstein J, Du Cailar G, Mimran A, Villain M *et al.*, Treatment of hypertension with renin–angiotensin system inhibitors and renal dysfunction: a systematic review and meta-analysis. *Am J Hypertens* **25**:126–132 (2012).
4 He R, Alashi A, Malomo SA, Girgih AT, Chao D, Ju X *et al.*, Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates. *Food Chem* **141**:153–159 (2013).
5 Aluko RE, Antihypertensive peptides from food proteins. *Annu Rev Food Sci Technol* 2015;**6**:235–262.
6 Fitzgerald MA, Hypertension treatment update: focus on direct renin inhibition. *J Am Acad Nurse Prac* **23**:239–248 (2011).
7 Kang MG and Ahn BH, Characterization of new antihypertensive angiotensin I-converting enzyme inhibitory peptides from Korean traditional rice wine. *J Microbiol Biotechnol* **22**:339–342 (2012).
8 Rho SJ, Lee J-S, Chung YI, Kim Y-W and Lee HG, Purification and identification of an angiotensin I-converting enzyme inhibitory peptide from fermented soybean extract. *Process Biochem* **44**:490–493 (2009).

9 Jimsheena V and Gowda LR, Arachin derived peptides as selective angiotensin I-converting enzyme (ACE) inhibitors: structure–activity relationship. *Peptides* **31**:1165–1176 (2010).
10 Girgih AT, Udenigwe CC and Aluko RE, In vitro antioxidant properties of hemp seed (*Cannabis sativa* L.) protein hydrolysate fractions. *J Am Oil Chem Soc* **88**:381–389 (2011).
11 Li H and Aluko RE, Identification and inhibitory properties of multifunctional peptides from pea protein hydrolysate. *J Agric Food Chem* **58**:11471–11476 (2010).
12 Huang W-Y, Majumder K and Wu J, Oxygen radical absorbance capacity of peptides from egg white protein ovotransferrin and their interaction with phytochemicals. *Food Chem* **123**:635–641 (2010).
13 Mundi S and Aluko RE, Inhibitory properties of kidney bean protein hydrolysate and its membrane fractions against renin, angiotensin converting enzyme, and free radicals. *Austin J Nutr Food Sci* **2**:1008 (2014).
14 Udenigwe CC and Aluko RE, Food protein-derived bioactive peptides: production, processing, and potential health benefits. *J Food Sci* **77**:R11–R24 (2012).
15 Adegbola OBJ and Bamishaiye E, Bambara groundnut: an Under-Utilized Nut in Africa. *Adv Agric Biotechnol* **1**:60–72 (2011).
16 Arise AK, Amonsou EO and Ijabadeniyi OA, Influence of extraction methods on functional properties of protein concentrates prepared from South African bambara groundnut landraces. *Int J Food Sci Technol* **50**:1095–10101 (2015).
17 Kudre TG, Benjakul S and Kishimura H, Comparative study on chemical compositions and properties of protein isolates from mung bean, black bean and bambara groundnut. *J Sci Food Agric* **93**:2429–2436 (2013).
18 Adebowale YA, Schwarzenbolz U and Henle T, Protein isolates from bambara groundnut (*voandzeia subterranean L.*): chemical characterization and functional properties. *Int J Food Prop* **14**:758–775 (2011).
19 Thammarat K, Leena N, Punnanee S and Soottawat B, Functional and antioxidative properties of bambara groundnut (*Voandzeia subterranea*) protein hydrolysates. *Int Food Res J* **22**:1584–1595 (2015).
20 Markwell MAK, Haas SM, Bieber L and Tolbert N, A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* **87**:206–210 (1978).
21 Bidlingmeyer B, Cohen S and Tarvin T, The PICO-TAG method for amino acid determination. *J Chromatogr* **33**:93–104 (1984).
22 Gehrke CW, Wall Sr L, Absheer J, Kaiser F and Zumwalt R, Sample preparation for chromatography of amino acids: acid hydrolysis of proteins. *J Assoc Off Anal Chem* **68**:811–821 (1985).
23 Landry J and Delhaye S, Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. *J Agric Food Chem* **40**:776–779 (1992).
24 Arts MJ, Dallinga JS, Voss H-P, Haenen GR and Bast A, A new approach to assess the total antioxidant capacity using the TEAC assay. *Food Chem* **88**:567–570 (2004).
25 Alashi AM, Blanchard CL, Mailer RJ, Agboola SO, Mawson AJ, He R *et al.*, Antioxidant properties of Australian canola meal protein hydrolysates. *Food Chem* **146**:500–506 (2014).
26 Terashima M, Oe M, Ogura K and Matsumura S, Inhibition strength of short peptides derived from an ACE inhibitory peptide. *J Agric Food Chem* **59**:11234–11237 (2011).
27 Rajapakse N, Mendis E, Jung W-K, Je J-Y and Kim S-K, Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Res Int* **38**:175–182 (2005).
28 Pan M, Jiang TS and Pan JL, Antioxidant activities of rapeseed protein hydrolysates. *Food Bioprocess Technol* **4**:1144–1152 (2011).
29 Moure A, Domínguez H and Parajó JC, Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates. *Process Biochem* **41**:447–456 (2006).
30 Grimble G, Rees R, Keohane P, Cartwright T, Desreumaux M and Silk D, Effect of peptide chain length on absorption of egg protein hydrolysates in the normal human jejunum. *Gastroenterology* **92**:136–142 (1987).
31 Udenigwe CC, Adebiyi AP, Doyen A, Li H, Bazinet L and Aluko RE, Low molecular weight flaxseed protein-derived arginine-containing peptides reduced blood pressure of spontaneously hypertensive rats faster than amino acid form of arginine and native flaxseed protein. *Food Chem* **132**:468–475 (2012).
32 Jamdar S, Rajalakshmi V, Pednekar M, Juan F, Yardi V and Sharma A, Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. *Food Chem* **121**:178–184 (2010).
33 Zhu Z, Qiu N and Yi J, Production and characterization of angiotensin converting enzyme (ACE) inhibitory peptides from apricot (*Prunus armeniaca* L.) kernel protein hydrolysate. *Eur Food Res Technol* **231**:13–19 (2010).
34 Segura Campos MR, Chel Guerrero LA and Betancur Ancona DA, Angiotensin-I converting enzyme inhibitory and antioxidant activities of peptide fractions extracted by ultrafiltration of cowpea *Vigna unguiculata* hydrolysates. *J Sci Food Agric* **90**:2512–2518 (2010).
35 Valdez-Ortiz A, Fuentes-Gutiérrez CI, Germán-Báez LJ, Gutiérrez-Dorado R and Medina-Godoy S, Protein hydrolysates obtained from Azufrado (*sulphur yellow*) beans (*Phaseolus vulgaris*): Nutritional, ACE-inhibitory and antioxidative characterization. *LWT – Food Sci Technol* **46**:91–96 (2012).
36 Mäkinen S, Johannson T, Gerd EV, Pihlava JM and Pihlanto A, Angiotensin I-converting enzyme inhibitory and antioxidant properties of rapeseed hydrolysates. *J Funct Foods* **4**:575–583 (2012).
37 Udenigwe CC, Lin Y-S, Hou W-C and Aluko RE, Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. *J Funct Foods* **1**:199–207 (2009).
38 Harnedy PA and FitzGerald RJ, In vitro assessment of the cardioprotective, anti-diabetic and antioxidant potential of *Palmaria palmata* protein hydrolysates. *J Appl Phycol* **25**:1793–1803 (2013).
39 Chen Gt, Zhao L, Zhao Ly, Cong T and Bao SF, In vitro study on antioxidant activities of peanut protein hydrolysate. *J Sci Food Agric* **87**:357–362 (2007).
40 He R, Girgih AT, Malomo SA, Ju X and Aluko RE, Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. *J Funct Foods* **5**:219–227 (2013).
41 Preza AM, Jaramillo ME, Puebla AM, Mateos JC, Hernández R and Lugo E, Antitumor activity against murine lymphoma L5178Y model of proteins from cacao (*Theobroma cacao L.*) seeds in relation with in vitro antioxidant activity. *BMC Complem Altern Med* **10**:61 (2010).
42 Tironi VA and Añón MC, Amaranth proteins as a source of antioxidant peptides: effect of proteolysis. *Food Res Int* **43**:315–322 (2010).

