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Chapter 7

Vigna Unguiculata as Source of Angiotensin-I Converting Enzyme Inhibitory and Antioxidant Peptides

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53045

1. Introduction

The frequency of lifestyle-related diseases is steadily increasing, particularly of hypertension, a risk factor for cardiovascular diseases such as coronary heart disease, peripheral arterial disease and stroke. Indeed, cardiovascular diseases are the primary cause of morbidity and mortality in Western countries, with hypertension affecting about 20% of the world's adult population [1]. Blood pressure is controlled by various regulatory factors in the body, including angiotensin I-converting enzyme (ACE-I). ACE-I (peptidyldipeptidaseA, kininase II, EC 3.4.15.1) is a zinc dipeptidylcarboxypeptidase. This membrane-bound exopeptidase is found on the plasma membranes of various cell types, including vascular endothelial cells, microvillar brush border epithelial cells and neuroepithelial cells. It is thought to be physiologically important. The primary activity of ACE-I is to cleave broad specificity free carboxyl group oligopeptides. Substrates containing Pro at the P1' position and Asp or Glu at P2' are resistant to ACE-I. However, on certain substrates ACE-I can also function as an endopeptidase or a tripeptidylcarboxypeptidase. With ACE-I, endopeptidase activity is observed on substrates having amidated carboxyl groups where the enzyme can cleave a C-terminal dipeptide amide and/or a C-terminal tripeptide amide [2]. ACE-I is responsible for converting angiotensin I (Ang I) to the powerful vasoconstrictor angiotensin II (Ang II) and inactivating the vasodilator peptide bradykinin (BK) by removal of C-terminal dipeptides [3]. In a functional sense, therefore, the enzymatic actions of ACE-I potentially cause increased vasoconstriction and decreased vasodilation. ACE-I has attracted interest for development of orally-active ACE-I inhibitors to treat hypertension due to its central role in vasoactive peptide metabolism. Inhibition of ACE-I prevents conversion of Ang I into Ang II, making it becomes one of the most effective

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methods for suppressing increases in blood pressure [4]. Since discovery of ACE-I inhibitors in snake venom, extensive research has been done on synthesizing ACE-I inhibitors such as captopril, enalapril, alacepril and lisinopril, all currently in use for treatment of hypertension and heart failure in humans. However, these synthetic drugs occasionally produce side effects such as cough, taste alterations and skin rashes. Interest has consequently increased in natural ACE-I inhibitors as safer and lower cost alternatives to synthetic ones [5].

Antioxidant deficiency also has been implicated in the occurrence of hypertension and other degenerative diseases. Reactive oxygen species (ROS) such as the superoxide anion radical (O^{-2}), hydrogen peroxide and hydroxyl radicals (●OH) are physiological metabolites formed as result of respiration in aerobic organisms. ROS are very unstable, and react rapidly with other substances including DNA, membrane lipids and proteins. Oxidative stress is produced by an imbalance between oxidizing species and natural antioxidants in the body, and has been associated with aging, cell apoptosis and severe diseases such as cancer, Parkinson, Alzheimer, and cardiovascular disorders [6]. Epidemiological studies have demonstrated an inverse association among intake of antioxidants from fruits and vegetables, and morbidity and mortality due to coronary heart diseases and cancer. In response, researchers are searching for natural antioxidants in food that may protect the body from free radicals and retard the evolution of many chronic diseases [7].

In recent years, food proteins have gained increasing value due to the rapidly expanding knowledge about physiologically active peptides. Peptides from various dietary sources have been shown to have clearly positive effects on health by functioning as antihypertensives, antioxidants, anticarcinogens, antimicrobials and anticariogenics, among others. These properties have led to their labeling as functional or biologically-active (i.e. bioactive) peptides. Bioactive peptides may be encrypted within the amino acid sequence of a larger protein. These peptides usually consist of 3-20 amino acids and are released from the original protein after degradation [8]. The most common way to produce bioactive peptides is through enzymatic hydrolysis of whole protein molecules. After enzymatic processing, amino acid sequences that were inactive in the core of the source protein are released and can exercise special properties. Many of the known bioactive peptides have been produced using gastrointestinal enzymes, usually pepsin and trypsin. Other digestive enzymes and different enzyme combinations of proteinases-including Alcalase®, chymotrypsin, pancreatin, pepsin and thermolysin have also been utilized to generate bioactive peptides from various proteins [9].

Continued population growth worldwide, consequent food resource shortages in developing countries, and the health risks associated with excessive animal protein (and saturated fats) intake has led researchers to search for new sources of proteins from non-conventional raw materials. Legumes are cultivated worldwide and constitute an excellent protein source (protein content =20-30%). Cowpea (*Vigna unguiculata*) is a major legume crop worldwide, particularly in tropical and subtropical areas such as southeast Mexico. It serves as a major dietary protein source in both human and animal diets, and its protein content makes it a good raw material for preparation of protein extracts and hydrolysates. Protein

extract has been used as substrates for production of hydrolysates with functional and/or nutritional properties better than the original extract [10]. Bioactive peptides with ACE-I inhibitory activity has been isolated from protein hydrolysates from a number of animal and vegetable sources. Vegetable origin proteins are of particular interest, and legumes are especially promising due to their high protein content and diverse physiological activities in the human organism. Extensive hydrolysis of *V. unguiculata* protein concentrates with commercial and digestives enzymes could therefore produce a number of peptides with a myriad of potential applications; for example, as natural-source therapeutic agents in medical treatments and/or as an ingredient in functional foods. Taking this into account, the aim of the present study was to modify enzymatically protein concentrates of *V. unguiculata*, evaluate the ACE-I inhibitory and antioxidant potential of the hydrolysates and relate the biological activity to their amino acid compositions.

2. Material and methods

2.1. Materials

V. unguiculata seeds were obtained from the February 2007 harvest in Yucatan state, Mexico. Reagents were of analytical grade and purchased from J.T. Baker (Phillipsburg, NJ, USA), Sigma Chemical Co. (St. Louis, MO, USA), Merck (Darmstadt, Germany) and Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Alcalase® 2.4L FG and Flavourzyme® 500MG enzymes were purchased from Novo Laboratories (Copenhagen, Denmark).

2.2. Protein isolates

A single extraction was performed with 6 kg of cowpea seeds. Impurities and damaged seeds were removed, and sound seeds milled in a Mykros impact mill (Industrial Machinery, Monterrey, Mexico) until passing through a 20-mesh screen (0.85 mm) followed by milling in a Cyclotec 1093 (Tecator, Sweden) mill until passing through a 60-mesh screen (0.24 mm). The resulting flour was processed using the wet fractionation method of Betancur-Ancona [11]. Briefly, whole flour was suspended in distilled water at a 1:6 (w/v) ratio, pH was adjusted to 11.0 with 1 M NaOH, and the dispersion was stirred for 1 h at 0.178 x *g* with a mechanical agitator (Caframo Rz-1, Heidolph Schwabach, Germany). This suspension was wet-milled with a Kitchen-Aid® food processor, and the fiber solids were separated from the starch and protein mix by straining through 80- and 150-mesh sieves and washing the residue five times with distilled water. The protein-starch suspension was allowed to sediment for 30 min at room temperature to recover the starch and protein fractions. The pH of the separated solubilized proteins was adjusted to the isoelectric point (4.5) with 1 N HCl. The suspension was then centrifuged at 1317 x *g* for 12 min (Mistral 3000i, Curtin Matheson Sci.), the supernatants were discarded, and the precipitates were freeze-dried until use.

2.3. Enzymatic hydrolysis

Hydrolysis of the protein extract was done using a totally randomized design with the treatments being the enzymatic system applied: Alcalase® 2.4L FG; Flavourzyme® 500MG; or

a sequential system using pepsin from porcine gastric mucosa (Sigma, P7000-100G) and pancreatin from porcine pancreas (Sigma, P3292-100G). The response variable was degree of hydrolysis (DH).

Hydrolysis was done under controlled conditions (temperature, pH and stirring) in a 1000 mL reaction vessel equipped with a stirrer, thermometer and a pH electrode. Hydrolysis with Alcalase® and Flavourzyme® was done according to Pedroche et al. [12]. Protein extracts were suspended in distilled water to produce a 4% (w/v) protein solution. This solution was equilibrated at optimum temperature and pH for each protease before adding the respective enzyme. Protease was then added to the solution at a ratio of 0.3 UA/g for Alcalase® and 50 UAPL/g for Flavourzyme®. Hydrolysis conditions were 90 min at 50°C for both enzymes, and pH 8.0 for Alcalase® and pH 7.0 for Flavourzyme®. The pH was kept constant by adding 1.0 M NaOH during hydrolysis. Hydrolysis with the sequential pepsin-pancreatin system was done with a pH-stat method for 90 min: pre-digestion with pepsin for 45 min followed by incubation with pancreatin for 45 min. Hydrolysis parameters were substrate concentration 4%; enzyme/substrate ratio 1:10; pH 2 for pepsin; pH 7.5 for pancreatin; and 37°C [13, 14]. In all three treatments the reaction was stopped by heating to 80°C for 20 min, followed by centrifugation at 9,880 x *g* for 20 min to remove the insoluble portion.

2.4. Degree of hydrolysis

DH was calculated by calculating free amino groups with o-phthaldialdehyde [15]: $DH = h/h_{tot} * 100$, where h_{tot} is the total number of peptide bonds per protein equivalent, and h is the number of hydrolyzed bonds. The h_{tot} factor is dependent on raw material amino acid composition.

2.5. Hydrolysate fractionation by ultrafiltration

Following Cho et al. [16], the hydrolysate was fractionated by ultrafiltration using a high performance ultrafiltration cell (Model 2000, Millipore, Inc., Marlborough, MA, USA). Five fractions were prepared using four molecular weight cut-off membranes: 1 kDa, 3 kDa, 5 kDa and 10 kDa. Soluble fractions prepared by centrifugation were passed through the membranes stating with the largest Molecular Weight Cut off (MWCO) membrane cartridge (10 kDa). The retentate and permeate were collected separately, and the retentate recirculated into the feed until maximum permeate yield was reached, as indicated by a decreased permeate flow rate. The permeate from the 10 kDa membrane was then filtered through the 5 kDa membrane with recirculation until maximum permeate yield was reached. The 5 kDa permeate was then processed with the 3 kDa membrane and the 3 kDa permeate with the 1 kDa membrane. This process minimized contamination of the larger molecular weight fractions with smaller molecular weight fractions while producing enough retentates and permeates for the following analyses. The five ultrafiltered peptide fractions were designated as >10 kDa (10 kDa retentate); 5-10 kDa (10 kDa permeate-5 kDa retentate); 3-5 kDa (5 kDa permeate- 3 kDa retentate); 1-3 kDa (3 kDa permeate-1 kDa retentate); and <1 kDa (1 kDa permeate).

2.6. ACE-I inhibitory activity

ACE-I inhibitory activity in the hydrolysate and its purified peptide fractions was analyzed following Hayakari et al. [17]. ACE-I hydrolyzes hippuryl-L-histidyl-L-leucine (HHL) to yield hippuric acid and His-Leu. This method relies on the colorimetric reaction of hippuric acid with 2,4,6-trichloro-s-triazine (TT) in a 0.5 mL incubation mixture containing 40 μmol potassium phosphate buffer (pH 8.3), 300 μmol sodium chloride, 40 μmol 3% HHL in potassium phosphate buffer (pH 8.3) and 100 mU/mL ACE-I. The mixture was incubated at 37ºC for 45 min and then reaction terminated by addition of TT (3% v/v) in dioxane and 3 ml of 0.2 M potassium phosphate buffer (pH 8.3). After centrifuging the reaction mixture at 10,000 x *g* for 10 min, enzymatic activity was determined in the supernatant by measuring absorbance at 382 nm. All runs were performed in triplicate. ACE-I inhibitory activity was quantified by a regression analysis of ACE-I inhibitory activity (%) versus peptide concentration and defined as an IC_{50} value, that is, the peptide concentration in (μg protein/mL) required to produce 50% ACE-I inhibition under the described conditions.

2.7. Antioxidant activity by ABTS assay

2,2'azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS with potassium persulfate following Pukalskas et al. [18]. To prepare the stock solution, ABTS was dissolved at a 2 mM concentration in 50 mL phosphate-buffered saline (PBS) prepared from 4.0908 g NaCl, 0.1347 g KH_2PO_4, 0.7098 g Na_2HPO_4, and 0.0749 g KCl dissolved in 500 mL ultrapure water. If pH was lower than 7.4, it was adjusted with NaOH. A 70 mM $K_2S_4O_8$ solution in ultrapure water was prepared. $ABTS^{\bullet+}$ radical was produced by reacting 10 mL of ABTS stock solution with 40 μL $K_2S_4O_8$ solution and allowing the mixture to stand in darkness at room temperature for 16-17 h before use. The radical was stable in this form for more than 2 days when stored in darkness at room temperature.

Antioxidant compound content in the hydrolysates and their UF peptide fractions was analyzed by diluting the $ABTS^{\bullet+}$ solution with PBS to an absorbance of 0.800 ± 0.030 AU at 734 nm. After adding 990 μL of diluted $ABTS^{\bullet+}$ solution ($A_{734\ nm}$= 0.800 ± 0.030) to 10 μL antioxidant compound or Trolox standard (final concentration 0.5-3.5 mM) in PBS, absorbance was read at ambient temperature exactly 6 min after initial mixing. All analyses were run in triplicate. The percentage decrease in absorbance at 734 nm was calculated and plotted as a function of the concentration of Trolox for the standard reference data. The radical scavenging activity of the tasted samples, expressed as inhibition percentage, was calculated by the following formula:

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100$$

Where A_B was the absorbance of the blank sample (t=0), and A_A was the absorbance of sample with antioxidant after 6 min.

Trolox equivalent antioxidant coefficient (TEAC) was quantified by a regression analysis of % Inhibition versus Trolox concentration using the following formula:

$$TEAC = (\%I_M - b)/m$$

Where b was the intersection and m was the slope.

2.8. G-50 gel filtration chromatography

After **f**iltration through 10, 5, 3 and 1 kDa membranes in a high performance ultrafiltration cell, 10 mL of the fraction with highest ACE-I inhibitory activity was injected into a Sephadex G-50 gel filtration column (3 cm x 79 cm) at a flow rate of 25 mL/h of 50 mM ammonium bicarbonate (pH 9.1). The resulting fractions were collected for to assay ACE-I inhibitory activity. Peptide molecular masses were determined by reference to a calibration curve created by running molecular mass markers on the Sephadex G-50 under conditions identical to those used for the test samples. Molecular mass standards were thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), equine myoglobin (17 kDa), vitamin B12 (1.35 kDa) and Thr-Gln (0.25 kDa). Fractions selected for further purification of peptides were pooled and lyophilized before RP-HPLC.

2.9. HPLC C18 chromatography

The fractions isolated with the Sephadex G-50 column were redissolved in deionized water and injected into a preparative HPLC (Agilent, Model 1110, Agilent Technologies, Inc. Santa Clara, CA, USA) reverse-phasecolumn (C18 Hi-Pore RP-318, 250 mm x10 mm, Bio-Rad). The injection volume was 100 μL, and the sample concentration was 20 mg/mL. Elution was achieved by a linear gradient of acetonitrile in water (0-30% in 50 min) containing 0.1% trifluoroacetic acid at a flow rate of 4 mL/min and 30°C [13]. Elution was monitored at 215 nm, and the resulting fractions were collected for assay of ACE-I inhibitory activity as described above.

2.10. Amino acid composition

Protein amino acid composition was determined for the hydrolysate, and the peptides were purified by ultrafiltration, gel filtration chromatography and HPLC [19]. Samples (2-4 mg protein) were treated with 4 mL of 6 mol equi/L HCl, placed in hydrolysis tubes and gassed with nitrogen at 110°C for 24 h. They were then dried in a rotavapor and suspended in 1 mol/L sodium borate buffer at pH 9.0. Amino acid derivatization was performed at 50°C using diethyl ethoxymethylenemalonate. Amino acids were separated using HPLC with a reversed-phase column (300 x 3.9 mm, Nova Pack C18, 4 mm; Waters), and a binary gradient system with 25 mmol/L sodium acetate containing (A) 0.02 g/L sodium azide at pH 6.0, and (B) acetonitrile as solvent. The flow rate was 0.9 mL/min, and the elution gradient was: time 0.0–3.0 min, linear gradient A:B (91:9) to A-B (86:14); time 3.0–13.0 min, elution with A-B (86–14); time 13.0–30.0 min, linear gradient A-B (86:14) to A-B (69:31); time 30.0–35.0 min, elution with A-B (69:31).

2.11. Statistical analysis

All results were analyzed in triplicate using descriptive statistics with a central tendency and dispersion measures. One-way ANOVAs were performed to evaluate protein isolate

hydrolysis data and *in vitro* ACE-I inhibitory activity. A Duncan's multiple range test was used to determine differences between treatments. All analyses were performed according to Montgomery [20] and processed using the Statgraphics Plus version 5.1 software.

3. Results and discussion

3.1. Protein extract hydrolysis

Alcalase®, Flavourzyme® and pepsin-pancreatin were used to produce extensively hydrolyzed *V. unguiculata* protein extracts. Degree of hydrolysis (DH) differed ($P<0.05$) between the enzymatic systems with values of 53.0%, 58.8%, and 35.7% for Alcalase® hydrolysate (AH), Flavourzyme® hydrolysate (FH) and Pepsin-pancreatin hydrolysate (PPH), respectively.

The AH had a 53.0% DH, which is lower than reported by Vioque et al. [21] for rapeseed protein hydrolysates (60% DH) produced with a mixture of Alcalase® and Flavourzyme® during 180 min. However, this DH was higher than that reported for mung bean protein hydrolysates (20.0% DH) produced with Alcalase® for 10 h[22] and for *V. unguiculata* hydrolysates (32.3% DH) produced with Alcalase® for 60 min [10].The variation in DH observed here is probably the result of protease specificity since Alcalase® is an industrial alkaline protease produced from *Bacillus licheniformis*, the main enzyme component of which (serine endopeptidase subtilisin Carlsberg) presents broad specificity and hydrolyzes most peptide bonds, with a preference for those containing aromatic amino acid residues [23]. ACE-I prefers substrates or competitive inhibitors containing hydrophobic amino acid (aromatic or branched lateral chain) residues (Hong et al., 2005). Alcalase® is therefore very suitable for production of bioactive peptides, such as those with ACE-I inhibitory activity. According to Pedroche et al. [24] the controlled liberation of biologically active peptides from protein by enzymatic hydrolysis is one of the most promising trends concerning medical applications of the protein hydrolysates with DH higher than 10% while hydrolysates with a low DH (lower than 10%) are used for the improvement of functional properties of flours or protein isolates. Therefore, the results suggest (DH=53.0%) that *V. unguiculata* protein is an appropriate substrate for producing these bioactive peptides when hydrolyzed with Alcalase®.

Hydrolysis with Flavourzyme® produced a *V. unguiculata* hydrolysate with 58.8% DH, somewhat higher than obtained with the Alcalase® system. A similar discrepancy has been reported for chickpea protein hydrolysates (27.0% DH) produced with Flavourzyme® for 180 min [25]. DH was higher with Flavourzyme® since it is a protease complex produced by *Aspergillus orizae,* which contains endoproteinases and exopeptidases. The fungal protease complex Flavourzyme® has a broader specificity; thus, when combined with its exopeptidase activity high DH values can be achieved, perhaps as much as 50% giving mostly dipeptides in the hydrolysate.

Sequential hydrolysis with pepsin-pancreatin produced cowpea protein hydrolysates with the lowest DH (35.74%) of the three studied enzymatic systems. This DH was similar to

37.0% at 360 min reported for sunflower protein hydrolysates obtained with the same system [13], but higher than the reported values for soy protein hydrolysates produced with pancreatin for 60 (11.0%) and 180 min (17.0%) [26]. The *V. unguiculata* PPH represents a pool of peptides resembling those generated during digestion of *V. unguiculata* proteins in the organism. This coincides with the behavior of extensively hydrolyzed sunflower protein reported by Megías et al. [13].Pepsin is the main proteolytic enzyme generated in the stomach during food digestion, while pancreatin includes proteases such as trypsin, chymotrypsin and elastase, which are released by the pancreas in the small intestine. The resulting peptides are therefore resistant to pepsin and pancreatin, suggesting that they might be absorbed by digestive epithelial cells in the small intestine, probably might be bioavailable and exercise their biological activity.

3.2. ACE-I inhibitory activity

ACE-I inhibitory activity of AH, FH and PPH was measured and calculated as IC_{50}. Biological activity was highest in the PPH, as indicated by a lower IC_{50} value (1397.9 μg/ml) compared to the AH (2564.7 μg/ml) and FH (2634.4 μg/ml). In other words, more ACE-I inhibitory active peptides were produced using the pepsin-pancreatin treatment.

ACE-I inhibitory activity has been reported for enzymatic hydrolysates from different protein sources with IC_{50} values ranging from 0.2 to 246.7 μg/mL [22]. Many of these hydrolysates have been shown to have antihypertensive activities in spontaneously hypertensive rats (SHR). In the present study, the IC_{50} value for the hydrolysate prepared with pepsin-pancreatin at 90 min incubation is within the concentration range likely to mediate an antihypertensive effect. Therefore, it is to be expected that *V. unguiculata* protein derived ACE-I inhibitory peptides would have antihypertensive activity. However, further investigations are necessary to examine whether the peptide mixture may exert antihypertensive activity *in vivo* because the inhibitory potencies of the peptides on ACE-I activity do not always correlate with their antihypertensive activities found in SHR[22].

Ultrafiltration of AH, FH and PPH produced peptide fractions with increased biological activity (Fig. 1). ACE-I inhibitory activity of peptide fractions ranged from 24.3-123 μg/ml in the AH, from 0.04 to 170.6 μg/ml in the FH and from 44.7 to 112 μg/ml in the PPH. ACE-I inhibitory activity was significantly (P<0.05) dependent on peptide fraction molecular weight, with the lowest activity being in the >10kDa fractions and the highest in the <1kDa fractions for all hydrolysates. Similar ACE-I inhibitory activity behavior was reported by Je et al.[5]for five peptide fractions from pepsin-hydrolyzed Alaska pollack frame protein run through an ultrafiltration membrane-bioreactor system with MWCOs of 30, 10, 5, 3 and 1 kDa. Higher ACE-I inhibitory activity (%) in lower molecular weight fractions was also reported by Xue-Ying et al. [27] for yak casein hydrolysate fractions separated using 6 and 10 kDa MWCOs: >10kDa (23.1%), 6-10kDa (29.2%) and <6 kDa (85.4%).

Of the three hydrolysates tested in the present study, FH, which had the highest DH, also exhibited the highest ACE-I inhibitory activity in the <1kDa fraction. The biological activity of this fraction provides it potential commercial applications as a 'health-enhancing ingredient' in functional food production.

In terms of ACE-I inhibitory activity, the pepsin-pancreatin system produced hydrolysate with the highest activity, while the Flavourzyme® system produced peptide fractions with the highest activity. This also confirms that *V. unguiculata* is a good protein source for bioactive peptide extraction by gastrointestinal or commercial proteases.

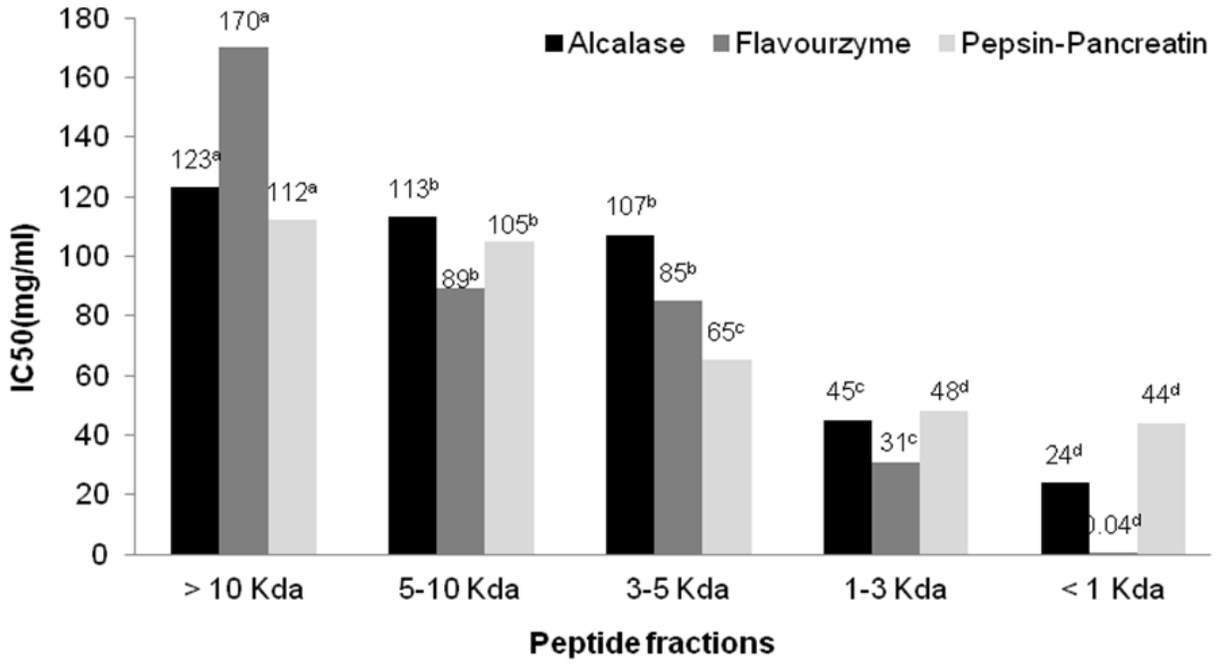


Figure 1. IC_{50} values of peptide fractions obtained by ultrafiltration from *V. unguiculata* protein hydrolysates.

3.3. Antioxidant activity by ABTS assay

Antioxidant activity of the protein hydrolysates and their corresponding UF peptide fractions was quantified and calculated as TEAC values (mM/mg protein). Antioxidant activity was not significantly different ($P>0.05$) between the three hydrolysis systems (14.7 for AH, 14.5 for FH and 14.3 mM/mg protein for PPH). Ultrafiltration improved antioxidant activity, which was dependent ($P<0.05$) on fraction molecular weight (Fig. 2).TEAC values were 303.2-1457 mM/mg protein for the AH, 357.4-10211 mM/mg protein for the FH, and 267.1-2830.4 mM/mg protein for the PPH. These values, and consequently the fraction's antioxidant activities, were higher ($P<0.05$) as fraction molecular weight decreased. According to Dávalos et al. [28] this behavior among the *V. unguiculata* peptide fractions may reflect the enhanced accessibility of small peptides to the redox reaction system, for the prescence of critical amino acid residues.

Antioxidant activity in the *V. unguiculata* protein hydrolysates and their UF peptide fractions was measured with an ABTS assay, which quantifies an antioxidant's (i.e. hydrogen or electron donor) suppression of the radical cation $ABTS^{\bullet+}$ based on single-electron reduction of the relatively stable radical cation $ABTS^{\bullet+}$ formed previously by an oxidation reaction. When added to PBS medium (pH 7.2) containing $ABTS^{\bullet+}$, the proteins in the hydrolysates and peptide fractions very probably acted as electron donors, transforming

this radical cation (maximum absorbance at 734nm) into the non-radical ABTS. The higher antioxidant activity of the UF peptide fractions versus their source hydrolysates is related to unique properties provided by their amino acid composition. The fractions' increased ability to decrease free radical reactivity is linked to the greater exposure of their amino acids, which leads to increased peptide/free radical reactions.

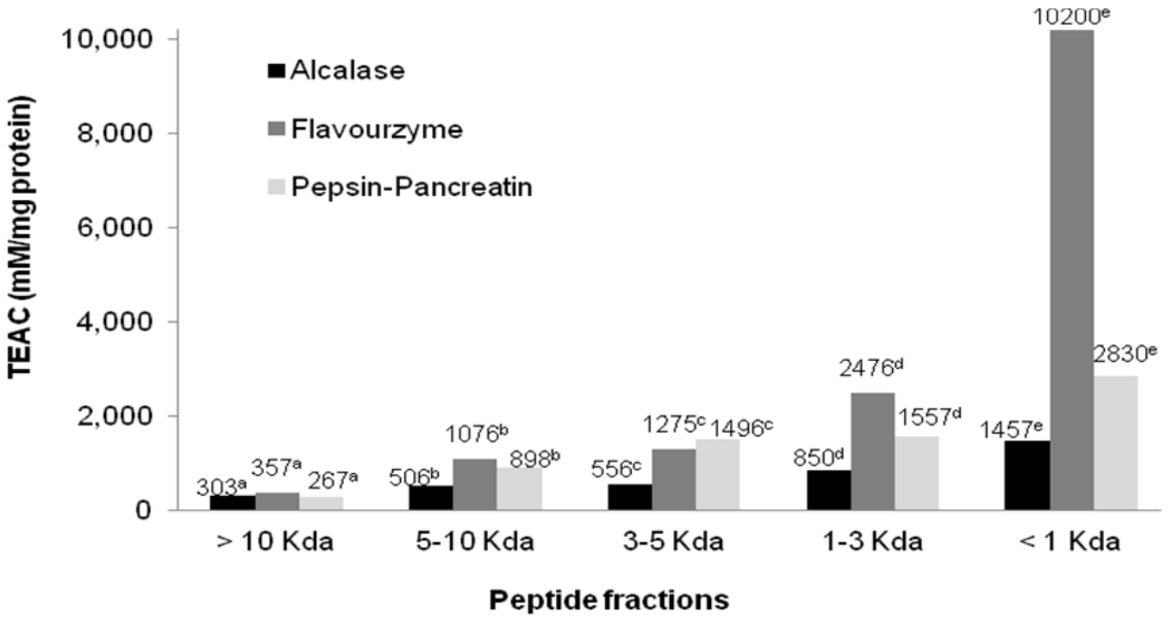


Figure 2. Antioxidant activity of peptide fractions obtained by ultrafiltration from *V. unguiculata* protein hydrolysates.

Overall, the <1 kDa peptide fraction from the FH had the highest TEAC values and was shown to undergo single-electron transfer reactions in the $ABTS^{\bullet+}$ reduction assay, demonstrating its antioxidant capacity. This is an extremely attractive property since oxidants are known to be involved in many human diseases and aging processes. Oxidants are associated with the chronic damage of ageing, and destructive oxidants and oxygen-free radicals can be extremely toxic to tissues by promoting tissue necrosis and cell damage. Some authors claim that proteins possess antioxidant properties; for instance, [29] reported that protein insufficiency aggravates enhanced lipid peroxidation and reduces antioxidative enzyme activities in rats, while Larson et al. [30] observed that proteins affect lipid metabolism in laboratory animals. The biological effect exhibited by the FH<1kDa fraction apparently reinforces the claim that proteins possess antioxidant properties. This makes the FH<1kDa fraction a potential "antioxidant" ingredient in functional food production.

3.4. Gel filtration chromatography

Because it exhibited the highest ACE-I inhibitory (IC_{50} value of 0.04 μg/mL) and antioxidant activity (TEAC value of 10211 mM/mg protein), the <1 kDa fraction from FH was selected for further fractionation. Gel filtration chromatography was used to generate a molecular weight profile of this fraction (Fig. 3).

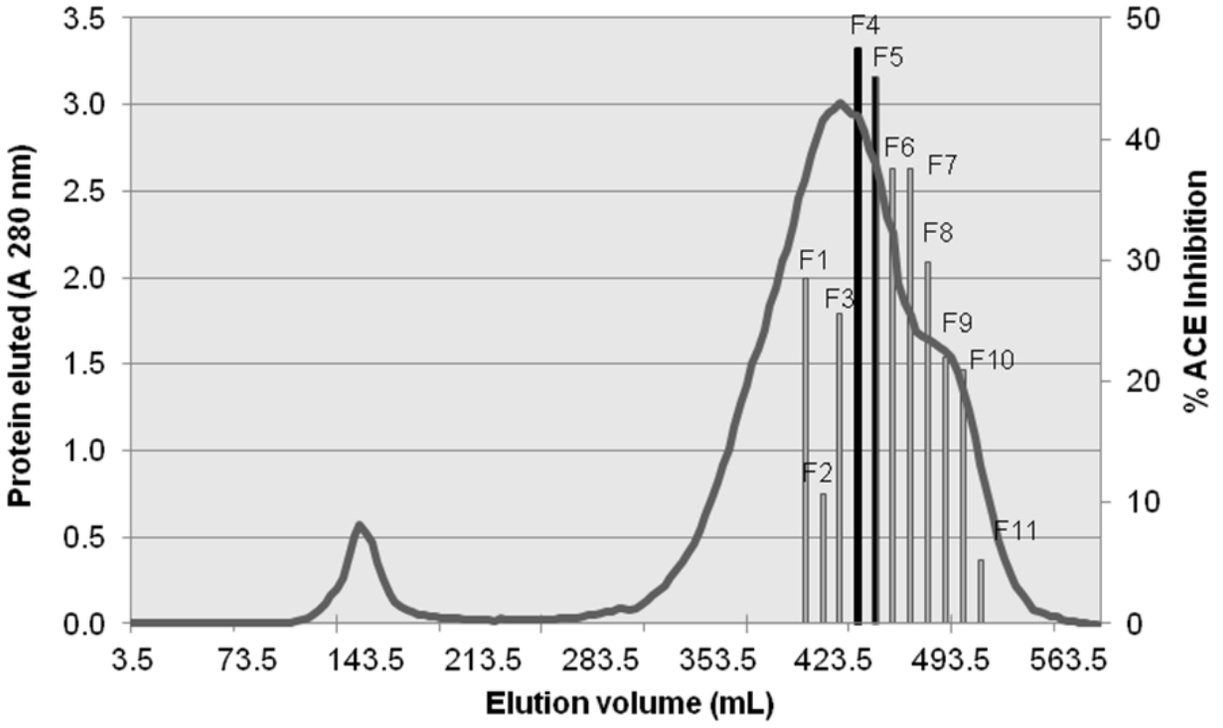


Figure 3. Elution profile of the <1 kDa ultrafiltration fraction of the cowpea *V. unguiculata* protein hydrolysate with Flavorzyme® purified in a Sephadex G-50 gel filtration column.

The profile was typical of a protein hydrolysate formed by a pool of peptides, with gradually decreasing molecular masses. Elution volumes between 406 and 518 mL included free amino acids and peptides with molecular masses ranging from 3.6 to 0.4 kDa. This range was fractionated into eleven fractions (1 to 11) and ACE-I inhibitory activity determined for each. Fractions with elution volumes smaller than 406 mL and greater than 518 mL were not analyzed because they largely included peptides with high molecular weights, as well as free amino acids. ACE-I inhibitory activity (%) in the eleven fractions ranged from 5.29 to 47.43% and differed (P<0.05) between fractions (Table 1). The highest ACE-I inhibitory activity was observed in fractions F4 (47.43%; 437.5-444.5 mL elution volume) and F5 (45.14%, 448-455 mL elution volume), which were not statistically different (P<0.05). Their molecular masses were approximately 1.8 kDa (indicative of 7 amino acid residues) and 1.5 kDa (indicative of 10 amino acid residues), respectively. The IC_{50} value for F4 (14.19 μg/mL) was similar than those of *Fagopirum esculentum* peptide fractions purified by Sephadex LH-20 gel filtration (15.1 μg/mL) [31], but lower than those of gel filtration (Sephadex G-25) peptide fractions from tuna broth hydrolysate (210 to 25,260 μg/mL) [32], from *Fagopyrum esculentum* Moench (Sephadex C-25 = 25,715.1 μg/mL; Sephadex G-10 = 21,315.1 μg/mL), and from the peptic hydrolysate of *Acetes chinensis* (Sephadex C-15 = 770 – 1590 μg/mL) [33]. A similar behavior pattern was observed with Konjac peptides purified by series connection of Sephadex G-25 and Sephadex G-15 columns that resulted in purified peptides with molecular weights of 1500 and 1000 Da and IC_{50} values of 120 μg/mL and 88 μg/mL, respectively [34]. Ji-Eun et al. [35] reported similar results to separate peptide fractions below 3 kDa through size exclusion chromatography (IC_{50}= 500 μg/mL) from

textured and fermented vegetable protein (IC_{50} = 2190 μg/mL). They purified peptide fraction with a molecular weight range of 500-999 Da with IC_{50} values of 94 μg/mL and that represented peptides of approximately 7 amino acids residues.

Fraction	ACE-I inhibitory activity (%)	Fraction	ACE-I inhibitory activity (%)
F1	28.47^{C}	F7	37.61^{d}
F2	10.67^{a}	F8	29.9^{c}
F3	25.61^{bc}	F9	22.01^{b}
F4	47.43^{e}	F10	20.92^{b}
F5	45.14^{e}	F11	5.29^{a}
F6	37.58^{d}		

Table 1. ACE-I inhibition percentage of peptide fractions purified in a Sephadex G-50 gel filtration column.[a-e]Different superscripts letters indicate statistical difference (P <0.05). Data are the mean of three replicates.

3.5. Reverse-phase HPLC chromatography of pooled fractions

Fractions F4 and F5 from the gel filtration chromatography treatment were pooled and analyzed using RP-HPLC to produce a chromatographic profile from mass-transfer between stationary and mobile phases. Mixture components were separated by dissolving fractions F4 and F5 in acetonitrile and forcing them through a chromatographic column under high pressure. The mixture resolved into its components in the column, separating F4 and F5 based on differences in hydrophobicity. In this process, the components of both fractions passed over stationary-phase particles containing pores large enough for them to enter, and in which interactions with the hydrophobic surface removed them from the flowing mobile-phase stream. The strength and nature of the interaction between the sample particles and stationary phase depended on hydrophobic and polar interactions. As the eluent organic solvent concentration increased, it reached a critical value for each analyte and desorbed it from the hydrophobic stationary-phase surface to allowing it to elute from the column into the flowing mobile phase. Because this elution depended on the precise distribution of hydrophobic residues in each specie, each analyte eluted from the column at a characteristic time and the resulting peaks were used to qualitatively analyze both fractions' components. Within each gel filtration fraction, the eluates were divided into four major fractions: F4-1, F4-2, F4-3, F4-4; F5-1, F5-2, F5-3, F5-4. The peptides were relatively pure, although a small shoulder still appeared behind the peaks in the chromatogram.

Enough material from each fraction was collected in successive analyses to determine ACE-I inhibitory activity (Fig. 4). Fraction F4 had a larger (P<0.05) ACE-I inhibitory activity range (33.83 to 75.42%) than F5 (32.31 to 49.71%). Overall, F4-2 (75.42%) had the highest ACE-I inhibitory activity (IC_{50} = 0.4704 μg/mL). This value is lower than the 6.3 μg/ml reported for a peptide from *F. esculentum* Moench purified by RP-HPLC [31] and within the 8.1 to 91.6 μg/mL range reported for fractions from caprine kefir water-soluble extract purified by preparative RP-HPLC [36]. The same behavior was observed when comparing the results of

RP-HPLC fractions obtained of *Mustelus mustelus* intestines with alkaline proteases (130- 783 μg/ml) [37] and with peptide fractions obtained from the peptic hydrolysate of the freshwater rotifer *Brachionus calyciflonus* (40.01 μg/ml) [38].

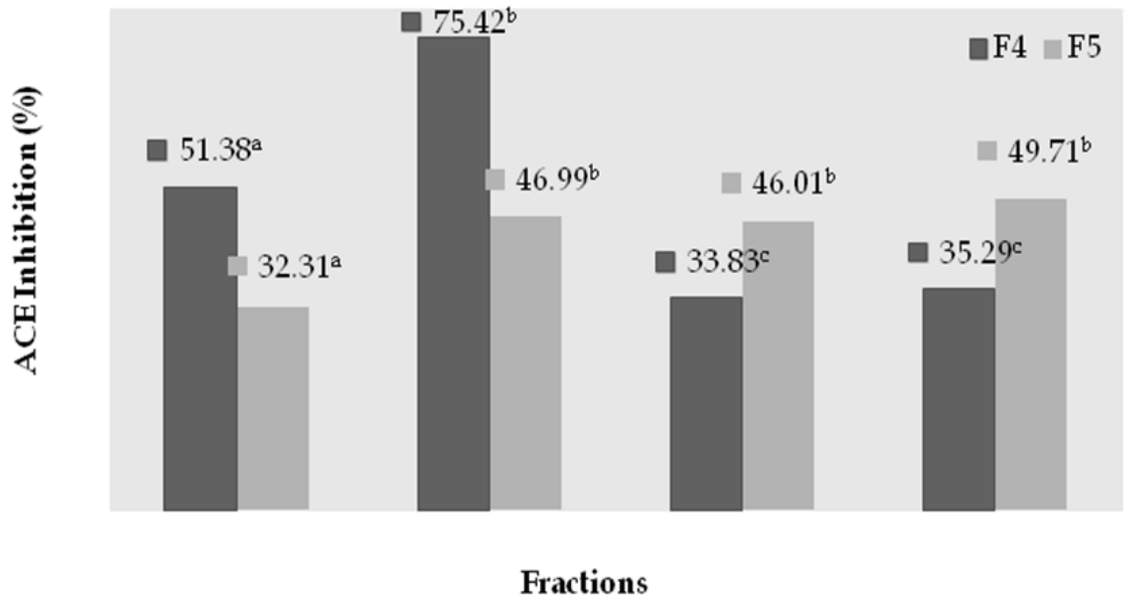


Figure 4. ACE inhibition percentage of RP-HPLC fractions obtained from fractions F4 and F5 produced after hydrolysis of *V. unguiculata* with Flavourzyme®.[a-c]Different letters in the same gel filtration chromatography fraction indicate statistical difference (P<0.05). Data are the mean of three replicates.

3.6. Amino acid composition

Fractions with the highest ACE-I inhibitory activity were analyzed to produce an amino acid profile. During hydrolysis, asparagine and glutamine partially converted to aspartic acid and glutamic acid, respectively; the data for asparagine and/or aspartic acid were therefore reported as Asx while those for glutamine and/or glutamic acid were reported as Glx. The higher ACE-I inhibitory activity exhibited by the <1 kDa fraction (IC_{50}=0.04 μg/mL) compared to FH (IC_{50}=2634.4 μg/mL) was probably due to its higher concentration of neutral amino acids, such as Ser (3.03%) and Thr (11.36%), hydrophilics such as His (12.5%) or hydrophobics such as Ala (7.84%), Pro (23.80%), Val (10%), Met (66.66%), Ile (10%), Leu (21.6%), Phe (18.6%) and Trp (16.6%) (Table 2).

Compared to the <1 kDa fraction amino acid profile, the G-50 gel filtration chromatography fractions had higher Asx, Glx and Arg concentrations. Hydrophobic amino acid content decreased by 20.27% in F4 (34.56g/100g) and 21.19% in F5 (34.16g/100g) compared to the <1 kDa fraction (43.35 g/100g), while hydrophilic residues increased by 19.25% (48 g/100g) in F4 and 22.48% (50 g/100g) in F5. The ACE-I inhibitory activity observed in F4 (47.43%) and F5 (45.14%) could therefore be the result of their higher Arg, Asx or Asp concentrations. These residues are known to play an important role in the antihypertensive activity of peptides from white and red wines and from French flor-sherry wine [38]. The F4 and F5 fractions may also have the added benefit of low bitterness. Many of the ACE-I-inhibitory peptides

isolated from food sources are composed of multiple food components and hydrophobic and/or aromatic amino acid residues. However, practical use of these food protein hydrolysates is complicated by formation of peptides, which impart a bitter taste, the result of the formation of low molecular weight peptides containing mostly hydrophobic amino acids. To address this problem, Kim et al. [40] recommended use of Flavourzyme®, a fungal endoprotease and exo-protease complex that produces hydrolysates or peptides with ACE-I inhibitory activity and low bitterness. Fractions F4 and F5 are promising prospects for use in new product development because they had clear ACE-I inhibitory activity and low hydrophobic amino acid content, which may ensure that they have low bitterness.

Amino acid	Composition (g/100g)*				
	PC	**FH**	**F<1 kDa**	**F4**	**F5**
Asx	10.8 ± 0.003	9.6 ± 0.387	9.4 ± 0.448	14 ± 0.274	16.7 ± 0.152
Glx	19 ± 0.016	18.6 ± 0.013	14.9 ± 0.171	17.9 ± 0.182	18.3 ± 0.17
Ser	6.5 ± 0.041	6.4 ± 0.016	6.6 ± 0.046	5.8 ± 0.302	5.0 ± 0.126
His	2.9 ± 0.028	2.8 ± 0.001	3.2 ± 0.024	2.9 ± 0.008	2.7 ± 0.061
Gly	4.4 ± 0.005	4.5 ± 0.006	4.1 ± 0.035	4.3 ± 0.011	3.0 ± 0.177
Thr	4.3 ± 0.009	3.9 ± 0.009	4.4 ± 0.032	4.0 ± 0.040	3.4 ± 0.036
Arg	7.8 ± 0.067	7.9 ± 0.025	6.0 ± 0.073	7.5 ± 0.026	6.9 ± 0.039
Ala	4.4 ± 0.008	4.7 ± 0.002	5.1 ± 0.106	4.3 ± 0.016	3.6 ± 0.625
Pro	2.7 ± 0.078	1.6 ± 0.038	2.1 ± 0.01	0 ± 0	0 ± 0
Tyr	2.6 ± 0.098	2.7 ± 0.006	2.6 ± 0.026	2.2 ± 0.018	1.2 ± 0.078
Val	5.4 ± 0.052	6.3 ± 0.015	7.0 ± 0.029	5.3 ± 0.004	4.2 ± 0.211
Met	0.1 ± 0.109	0.3 ± 0.001	0.9 ± 0.013	0.7 ± 0.006	2.2 ± 0.292
Cys	0.4 ± 0.002	0.4 ± 0.006	0.2 ± 0.002	1.2 ± 0.01	3.2 ± 0.1
Ile	4.4 ± 0.019	5.4 ± 0.207	6.0 ± 0.073	5.2 ± 0.015	4.7 ± 0.236
Leu	9.2 ± 0.115	9.8 ± 0.175	12.5 ± 0.029	11.6 ± 0.092	12.6 ± 0.229
Phe	6.9 ± 0.008	7.0 ± 0.004	8.6 ± 0.056	6.7 ± 0.046	5.8 ± 0.043
Lys	7.3 ± 0.089	7.1 ± 0	5.3 ± 0.072	5.7 ± 0.042	5.3 ± 0.263
Trp	0.7 ± 0.064	1.0 ± 0.038	1.2 ± 0.041	0.8 ± 0.241	1.0 ± 0.535

*Data are the mean of three replicates

Table 2. Amino acid contents of the cowpea *V. unguiculata* protein concentrate (PC), Flavourzyme hydrolysate (FH), <1 kDa ultrafiltered fraction and F4 and F5 gel filtration chromatography fractions.

Comparison of amino acid composition and properties between the cowpea *V. unguiculata* hydrolysate and its peptides isolated by ultrafiltration and G-50 gel chromatography showed the most active fraction to be the <1 kDa fraction, which had abundant aromatic (e.g., Phe) and cyclic amino acids (e.g., Pro). Amino acids such as Phe, with large bulky chains and hydrophobic side chains, are preferred in both positions of a dipeptide for their high steric properties and low lipophilicity, while amino acids such as Pro are preferred in the carboxyl terminus of active tripeptides for their low lipophilicity, and high steric and electronic properties [41]. Aromatic and basic amino acids are important to the ACE-I

inhibitory activity of peptides. For instance, similar observations have been made for an orientase hydrolysate with notable ACE-I inhibition attributed to its high basic and aromatic amino acids contents [33]. Cheung et al. [43] reported a peptide with strong, competitive ACE-I inhibition in which aromatic amino acid residues at its C-terminal and basic or hydrophobic ones at its N-terminal played an essential role. Based on the above, the higher ACE-I inhibitory potential of the <1 kDa fraction from the cowpea hydrolysate can be attributed to the steric properties of its aromatic amino acids and the lipophilicity and electronic properties of its cyclic amino acids.

The most active fraction among those purified from F4 by preparative RP-HPLC was F4-2, which had much higher neutral amino acid content (80.6 g/100g) than F4-1 (46.6 g/100g), F4-3(6.1 g/100g) and F4-4 (28.6 g/100g) (Table 3). Of the neutral amino acids, Tyr was higher in F4-2 (71.3 g/100g) compared to F4-3 (1.5 g/100g), F4-4 (3.9 g/100g) and F4-1 (3.9 g/100g). This supports the importance of aromatic residues in a peptides' biological potential, probably due to their high steric properties and low lipophilicity. The Tyr amino acid has been reported in peptides from milk (e.g., Tyr-Pro-Tyr-Tyr) isolated by a combination of lactic acid bacteria fermentation and Flavourzyme® hydrolysis. These peptides are bioavailable and exhibit *in vitro* (90.9 μM) and *in vivo* ACE-I inhibitory activity, the latter in the form of reduced hypertension in SHR (15.9 mmHg reduction in systolic blood pressure) [43].

F4-2 was also unique in containing Met (0.5 g/100g) and Trp (0.2 g/100g), as well as higher concentrations of Ile (3.2 g/100g) and Leu (10.2 g/100g) than the other fractions eluted from F4, all of which could have significantly increased its relative ACE-I inhibition activity. This would coincide with previous reports of inhibitory activity in peptides with these amino acids. The residues Val, Leu and Ile are preferred in the amino terminal position in active tripeptides for their low lipophilicity and steric properties or side chain bulk/ molecular size [40]. Clearly, the structure of ACE-I inhibitory peptides influences their activity, as shown by Cheung et al. [42], who reported that peptides with Tyr at the C-terminus and Ile at the N-terminus exhibit highly potent inhibitory activity. In a randomized, double-blind, placebo-controlled human study, a significant depressor effect was observed in mild essential hypertensive volunteers and Val-Tyr was shown to be one of the predominant ACE-I inhibitory peptides involved in this effect [44]. In addition to its high Tyr content, F4-2 may also be absorbed intact into the human circulatory system and induce a reduction in blood pressure. For instance, intravenous and oral administration of Val-Tyr in SHR have shown that this di-peptide caused a long-lasting depressor effect. Val-Tyr is known to be absorbed intact into the human circulatory system, and studies using cross-mated transgenic mice carrying the human renin gene and the human angiotensinogen gene have shown that, as a natural ACE-I inhibitory dipeptide, Val-Tyr regulates the enhanced human renin-angiotensin system and induces a prolonged reduction in blood pressure [45].

Of the fractions purified from F5 by preparative RP-HPLC chromatography (Table 3), F5-2, F5-3 and F5-4 exhibited ACE-I inhibition that was not different among them (P<0.05), but was greater than that of F5-1. The amino acids Arg, Tyr, Met, Ile, Leu, Phe and Lys very probably played a key role in providing greater activity to the first three fractions. As mentioned above, residues such as Leu and Ile are preferred at the amino terminus of

tripeptides with ACE-I inhibitory activity due to their low lipophilicity and steric properties or side chain bulk/molecular size values. In addition, Lys and Arg are expected in positions adjacent to the amino terminus due to their low electronic properties and high lipophilicity and steric property values, while residues such as Phe are preferred at the carboxyl terminus due to their low lipophilicity and high steric and electronic property values [41].

Although the precise substrate specificity is not fully understood, ACE-I appears to prefer substrates containing hydrophobic amino acid residues at the three C-terminal positions, suggesting that the higher hydrophobic amino acid content in F5-2 (9.1 g/100g), F5-3 (89.8 g/100g) and F5-4 (34.2 g/100g) versus F5-1 (1.1 g/100g) probably made a substantial contribution to their inhibitory potency. This agrees with Wu et al. [40], who state that aromatic, positively-charged and hydrophobic amino acids are preferred in active tripeptides. Due to substrate specificity differences between the two ACE-I catalytic sites, ACE-I inhibitors may inhibit only one site. Moskowitz [46] proposed a model explaining the clinical superiority of hydrophobic ACE-I inhibitory drugs relative to hydrophilic ones: all ACE-I inhibitors bind to the C-terminal catalytic site, but only hydrophobic ones bind to the occluded N-terminal catalytic site and are therefore better at blocking Ang II production. This would also explain why hydrophobic ACE-I inhibitors have specific local benefits such as organ damage prevention, in addition to reducing blood pressure [46]. The high hydrophobic amino acid (particularly aromatic side-chains) content in the F5 may therefore make a substantial contribution to its fractions' ACE inhibitory activity by blocking Ang II production.

Overall, the highest *in vitro* ACE-I inhibitory activity (IC_{50}) among the cowpea hydrolysate and its derivative fractions was present in the ultrafiltered <1 kDa fraction (0.04 μg/mL), followed by the RP-HPLC F4-2 fraction (0.4704 μg/mL), the gel filtration chromatography fraction F4 (14.195 μg/mL) and finally the hydrolysate (2634.4 μg/mL). Although the IC_{50} values for the hydrolysate, F4 and F4-2 fractions were substantially higher than that of the F<1 kDa fraction, they are still in the same order of magnitude as values reported for many other natural ACE-I inhibitory peptides. Nevertheless, the IC_{50} values for all the studied *V. unguiculata* derivatives are far higher than that of the synthetic ACE-I inhibitor Captopril® (0.0013 μg/mL) [32]. The biological potential in the peptides purified from *V. unguiculata*, and the high ACE-I inhibitory activity in the F<1 kDa fraction, reinforce the need for ACE-I inhibitory peptides to be rich in hydrophobic amino acids (aromatic or branched chains) and peptides rich in Pro. Pro is well-documented as the most favorable amino acid for ACE-I binding (most commercial inhibitors include this residue), but it was not present in the peptides with potential biological activity that had been purified by G-50 gel filtration chromatography and RP-HPLC.

The studied cowpea *V. unguiculata* protein hydrolysate has potential applications in the development of physiologically functional foods aimed at preventing and/or treating hypertension. An added benefit is the balanced amino acid profile of the protein hydrolysate and its peptide fractions, which makes them an appropriate protein source in human nutrition. It should be considered, however, that the *in vitro* ACE-I inhibitory potencies of peptides do not always correlate with their *in vivo* antihypertensive activities as quantified in SHR. This is because they must be absorbed and transported intact from the

intestine to the blood stream (in the case of oral administration) and resist plasma peptidase degradation (in the case of oral and intravenous administration) to reach their target sites and exert an antihypertensive effect *in vivo*. Therefore, *in vivo* research is needed to determine to what extent any of the studied ACE-I inhibitory peptides can exercise their antihypertensive activity *in vivo*.

Amino acid	Content (g/100g)*							
	F4-1	F4-2	F4-3	F4-4	F5-1	F5-2	F5-3	F5-4
Asx	9.9 ± 0.063	1.2 ± 0.009	0.8 ± 0.001	12.5 ± 0.242	2.1 ± 0.492	1.2 ± 0.005	2 ± 0.004	10.6 ± 0.731
Glx	5.1 ± 0.214	1.4 ± 0.048	1.1 ± 0.006	12.6 ± 0.329	0 ± 0	1.5 ± 0.046	2 ± 0.071	14.2 ± 0.762
Ser	22.7 ± 0.291	1 ± 0.001	1.2 ± 0.021	8.4 ± 0.177	30.5 ± 0.834	0.9 ± 0.014	1 ± 0.014	8.4 ± 0.009
His	8.2 ± 0.677	0.4 ± 0.015	0.3 ± 0.009	4.1 ± 0.133	16.3 ± 0.194	0.3 ± 0.049	0.4 ± 0.017	2.7 ± 0.957
Gly	19.6 ± 0.983	1.4 ± 0.008	1.7 ± 0.031	9.2 ± 0.226	13.3 ± 0.159	2.9 ± 0.017	2 ± 0.016	10 ± 0.057
Thr	4.3 ± 0.987	0.3 ± 0.005	0.4 ± 0.018	3.5 ± 0.066	5.8 ± 0.069	0.3 ± 0.048	0.4 ± 0.024	2.7 ± 0.957
Arg	3.2 ± 0.065	0.8 ± 0.002	0.8 ± 0.01	7.1 ± 0.151	0 ± 0	0.2 ± 0.236	0.6 ± 0.028	3 ± 0.139
Ala	14.3 ± 0.620	0.4 ± 0.001	0.3 ± 0.005	3.8 ± 0.046	1.1 ± 1.536	0.3 ± 0.048	0.5 ± 0.012	3.4 ± 0.089
Pro	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Tyr	0 ± 0	71.3 ± 0.012	1.5 ± 0.026	3.9 ± 0.109	0 ± 0	74.7 ± 0.280	0.7 ± 0.109	4.2 ± 0.289
Val	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Met	0 ± 0	0.5 ± 0.013	0 ± 0	0 ± 0	0 ± 0	0.6 ± 0.071	0.1 ± 0.096	0.5 ± 0.039
Cys	0 ± 0	6.6 ± 0.001	1.3 ± 0.085	3.6 ± 0.288	31 ± 0.369	8.8 ± 0.098	0.9 ± 0.019	6.8 ± 0.070
Ile	0.4 ± 0.555	3.2 ± 0.041	0.2 ± 0.020	2.8 ± 0.059	0 ± 0	1.6 ± 0.035	0.5 ± 0.026	1.8 ± 0.032
Leu	9.3 ± 0.217	10.2 ± 0.006	0.3 ± 0.008	4.7 ± 0.024	0 ± 0	3.1 ± 0.066	0.8 ± 0.093	6.1 ± 0.896
Phe	0 ± 0	0.8 ± 0.003	89.6 ± 0.230	20 ± 0.778	0 ± 0	3.5 ± 0.765	87.9 ± 0.234	22.4 ± 0.304
Lys	3± 0.532	0.3 ± 0.007	0.3 ± 0.008	3.8 ± 0.068	0 ± 0	0.2 ± 0.009	0.3 ± 0.002	2.4 ± 0.073
Trp	0 ± 0	0.2 ± 0.040	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 3. Amino acid contents of fractions from the F4 and F5 gel filtration chromatography fraction purified by preparative RP-HPLC C-18 chromatography.

4. Conclusions

After modification by Alcalase®, Flavourzyme® and pepsin-pancreatin cowpea *V. unguiculata* proteins proved to be a source of bioactive peptides with ACE-I inhibitory and antioxidant activity. Fractionation of *V. unguiculata* enzymatic hydrolysates by ultrafiltration enhanced their ACE-I inhibitory and antioxidant activity in all the resulting peptides, although the <1 kDa fraction of the Flavourzyme hydrolysate had the highest overall biological activity. Further purification of this fraction by gel filtration chromatography and RP-HPLC produced fractions with different activities, all of which were much higher than the source hydrolysate. Separation of protein hydrolysates by molecular weight and hydrophobicity clearly enhanced peptide ACE-I inhibitory activity, particularly purification by ultrafiltration and chromatography. The highest biological potential among the purified peptides was observed in the ultrafiltered <1 kDa fraction, which supports the importance of high hydrophobic amino acid and proline content in ACE-I inhibitory peptides. These results highlight the promise of controlled protein hydrolysis with a fungal protease complex to isolate bioactive peptides from cowpea *V. unguiculata* proteins, which can then be further purified and/or used as an ingredient in functional foods designed for specific diets.

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Acknowledgement

This research was supported by the Consejo Nacional de Ciencia y Tecnología (CONACYT) through doctoral scholarship 164186. This research forms part of Project 153012 "Actividad biológica de fracciones peptídicas derivadas de la hidrólisis enzimática de proteínas de frijoles lima (*Phaseolus lunatus*) y caupí (*Vigna unguiculata*)", financed by the CONACYT.

5. References

[1] Miguel M, Alonso M, Salaices M, Aleixandre A, López-Fandiño R. Antihypertensive, ACE inhibitory and vasodilator properties of an egg white hydrolysate: Effect of a simulated intestinal digestion. Food Chemistry 2011; 104: 163-168.
[2] Hooper NM, Turner AJ. An ACE structure. Nature Structural & Molecular Biology 2003; 10: 155-157.

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[3] Tzakos AG, Galanis AS, Spyroulias GA, Cordopatis P, Manessi-Zoupa E, Gerothanassis IP. Structure-function discrimination of the N and C-catalytic domains of human angiotensin-converting enzyme: implications for Cl-activation and peptide hydrolysis mechanisms. Protein Engineering 2003; 16: 993-1003.

[4] Saiga A, Tanabe S, Nishimura T. Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. Journal of Agricultural and Food Chemistry 2006; 51: 3661-3667.

[5] Je J Y, Park PJ, Kwon, J Y, Kim AK. A novel angiotensin I converting enzyme inhibitory peptide from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. Journal of Agricultural and Food Chemistry 2004; 52: 7842-7845.

[6] Giasson BI, Ischiropoulos H, Lee VMY, Trojanowski JQ. The relationship between oxidative/nitrosative stress and pathological inclusions in Alzheimer's and Parkinson's diseases.Free Radical Biology & Medicine 2002; 32: 1264-1275.

[7] Pellegrini N, Del Rio D, Colombi B, Bianchi M, Brighenti F. Application of the 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation assay to a flow injection system for the evaluation of antioxidant activity of some pure compounds and beverages. Journal of Agricultural and Food Chemistry 2003; 51: 260-264.

[8] Moller NP, Scholz-Ahrens KE, Schrezenmeir NR. Bioactive peptides and protein from foods: indication for health effects. European Journal of Nutrition 2008; 47: 171-182.

[9] Korhonen H, Pihlanto A. Bioactive peptides: Production and functionality. International Dairy Journal 2006; 16: 945-960.

[10] Araujo-González F, Chel-Guerrero L, Betancur-Ancona D. Functional properties of hydrolysates from cowpea (*V. unguiculata*) seeds. Advances in Food Science and Food Biotechnology in Developing Countries 2008; 24: 173-183.

[11] Betancur-Ancona D, Gallegos-Tintoré S, Chel-Guerrero L. Wet fractionation of *Phaseolus lunatus* seeds: partial characterization of starch and protein. Journal of the Science of Food and Agriculture 2004; 84: 1193-1201.

[12] Pedroche J, Yust MM, Girón-Calle J, Alaiz M, Millán F, Vioque J. Utilisation of chickpea protein isolates for production of peptides with angiotension I-converting enzyme (ACE) inhibitory activity. Journal of the Science of Food and Agriculture 2002; 82(1): 960-965.

[13] Megías C, Yust MM, Pedroche J, Lquari H, Girón-Calle J, Alaiz M, Millán F, Vioque J. Purification of an ACE inhibitory peptide after hydrolysis of sunflower (*Helianthus annuus* L.) protein isolates. Journal of Agricultural and Food Chemistry 2004; 52: 1928-1932.

[14] Yang Y, Marczak DE, Yokoo M, Usui H, Yoshikawa M. Isolation and antihypertensive effect of Angiotensin I-Converting Enzyme (ACE) inhibitory peptides from spinach rubisco. Journal of Agricultural and Food Chemistry 2003; 51: 4897-4902.

[15] Nielsen P, Petersen D, Dammann C. Improved method for determining food protein degree of hydrolysis. Journal of Food Science 2001; 66(5): 642-646.

[16] Cho MJ, Unklesbay N, Hsieh F, Clarke AD. Hydrophobicity of bitter peptides from soy protein hydrolysate.Journal of Agricultural and Food Chemistry 2004; 52 (19): 5895-5901.

[17] Hayakari M, Kondo Y, Izumi H.A rapid and simple spectrophotometric assay of angiotensin-converting enzyme. Analytical Biochemistry 1978; 84 (1): 361-369.
[18] Pukalskas A, Van Beek T, Venskutonis R, Linssen J, Van Veldhuizen A, Groot A. Identification of radical scavengers in sweet grass (*Hierochloe odorata*), Journal of Agricultural and Food Chemistry 2002; 50: 2914-2919.
[19] Alaiz M, Navarro JL, Giron J, Vioque E. Amino acid analysis by high-performance liquid chromatography after derivatization with diethylethoxymethylenemalonate. Journal of Chromatography 1992; 591: 181-186.
[20] Montgomery D. Diseño y análisis de experimentos (2th Ed.). Mexico: Limusa-Wiley 2004; 686 p.
[21] Vioque J, Sánchez-Vioque R, Clemente A, Pedroche J, Bautista J, Millan F. Production and characterization of an extensive rapeseed protein hydrolysate. Journal of the American Oil Chemists Society 1999; 76(7): 819-823.
[22] Hong LG, Wei L, Liu H, Hui SY. Mung-bean protein hydrolysates obtained with Alcalase® exhibit angiotensin I-converting enzyme inhibitory activity. Food Science and Technology International 2005; 11(4): 281-287.
[23] Doucet D, Otter DE, Gauthier SF, Foegeding EA. Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: Peptide identification and determination of enzyme specificity Journal of Agricultural and Food Chemistry 2003; 51: 6300-6308.
[24] Pedroche J, Yust M, Girón-Calle J, Vioque J, Alaiz M, Millán F. Plant protein hydrolysates and tailor-made foods. Electronic Journal of Environmental, Agricultural and. Food Chemistry 2003; 2(1): 233-235.
[25] Clemente A, Vioque J, Sánchez-Vioque R, Pedroche J Millán F. Production of extensive chickpea (*Cicer arietinum* L.) Protein hydrolysates with reduced antigenic activity. Journal of Agricultural and Food Chemistry 1999; 47: 3776-3781.
[26] Qi M, Hettiarachychy N, Kalapathy U. Solubility and emulsifying properties of soy protein isolates modified by pancreatin.Journal of Food Science 1997; 62(6): 1110-1115.
[27] Xue-Ying M, Jin-Ren N, Wei-Ling S, Peng-Peng H, Li F. Value-added utilization of yak milk casein for the production of angiotensin-I-converting enzyme inhibitory peptides. Food Chemistry 2007; 103: 1282-1287.
[28] Dávalos A, Miguel M, Bartolomé B, López-Fandiño R. Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. Journal of Food Protection 2004; 67(9): 1939-1944.
[29] Hui A, Furong W, Chaoliang L. Antioxidant activities of protein-enriched fraction from the larvae of housefly, *Musca domestica*. Natural Product Research 2008; 22(6): 507-515.
[30] Larson M, Donovan SM, Potter SM. Effects of dietary protein source on cholesterol metabolism in neonatal pigs. Nutrition Research 1996; 16(9): 1563-1574.
[31] Ma MS, Bae IY, Lee HG, Yang CB. Purification and identification of angiotensin I-converting enzyme inhibitory peptide from buckwheat (*Fagopyrum esculentum* Moench). Food Chemistry 2006; 96: 36-42.

[32] Hwang JS, Ko WC. Angiotensin I-converting enzyme inhibitory activity of protein hydrolysates from tuna broth. Journal of Food and Drug Analysis 2004; 12(3): 232-237.

[33] Cao W, Zhang C, Hong P, Ji H, Hao J. Purification and identification of an ACE inhibitory peptide from the peptic hydrolysates of *Acetes chinensis* and its antihypertensive effects in spontaneously hypertensive rats. International Journal of Food Science and Technology 2010; 45(1): 959-965.

[34] Wang L, Xu H, He X, Wang X. Studies on the preparation of ACE inhibitory peptides from konjac fly powder by alkaline protease enzymolysis. Journal of Chinese Institute of Food Science and Technology 2010; 1: 42-47.

[35] Ji-Eun K, Ki H, Sam-Pin L. ACE inhibitory and hydrolytic enzyme activities in textured vegetable protein in relation to the solid state fermentation period using *Bacillus subtilis* HA. Food Science and Biotechnology 2010; 19(2): 487-495.

[36] Quirós A, Hernández-Ledesma B, Ramos M, Amigo L, Recio I. Angiotensin- converting enzyme inhibitory activity of peptides derived from caprine kefir. Journal of Dairy Science 2005; 88: 3480-3487.

[37] Bougatef A, Balti R, Nedjar-Arroume N, Ravallec R, Adje EY, Souissi N, Lassoued I, Guillochon D, Nasri M. Evaluation of angiotensin I-converting enzyme (ACE) inhibitory activities of smooth hound (*Mustelus mustelus*) muscle protein hydrolysates generated by gastrointestinal proteases: identification of the most potent active peptide. European Food Research and Technology 2010; 231(1): 127-135.

[38] Lee JK, Lee MS, Park HG, Kim S, Byun HG. Angiotensin I converting enzyme inhibitory peptide extracted from freshwater zooplankton. Journal of Medicinal Food 2010; 13(2): 357-363.

[39] Pozo-Bayón MA, Alcaíde JM, Polo MC, Pueyo E. Angiotensin I converting enzyme inhibitory compounds in white and red wines. Food Chemistry 2007; 100: 43-47.

[40] Kim JM, Whang JH, Suh HJ. Enhancement of angiotensin I converting enzyme inhibitory activity and improvement of the emulsifying and foaming properties of corn gluten hydrolysate using ultrafiltration membranes. European Food Research and Technology 2004; 218: 133-138.

[41] Wu J, Aluko RE, Nakai S. Structural requirements of angiotensin I-converting Enzyme inhibitory peptides: Quantitative structure-activity relationship study of di- and tripeptides. Journal of Agricultural and Food Chemistry 2006; 54: 732-738.

[42] Cheung HS, Wang FL, Ondetti MA, Sabo EF Cushman DW. Binding of peptide substrates and inhibitors of Angiotensin-converting enzyme. Journal of Biological Chemistry 1980; 255(2): 401-407.

[43] Jenn-Shou T, Tai-Jung C, Bonnie-Sun P, Shein-Da G, Mei-Yuh C. Antihypertensive effect of bioactive peptides produced by preotease-facilitated lactic acid fermentation of milk. Food Chemistry 2008; 106(2): 552-558.

[44] Matsufuji H, Matsui T, Seki E, Osajima K, Nakashima M, Osajima Y. Angiotensin I-converting enzyme inhibitory peptides in an alkaline protease hydrolysate derived from sardine muscle. Bioscience, Biotechnology and Biochemistry 1994; 58: 2244–2245.

[45] Matsui T, Hayashi A, Tamaya K, Matsumoto K, Kawasaki T, Murakami K, Kimoto K. Depressor effect induced by dipeptide, Val-Tyr, in part, to the suppression of human circulating renin-angiotensin system. Clinical and Experimental Pharmacology 2003; 30: 262-265.
[46] Moskowitz DW. Is 'somatic' angiotensin I-converting enzyme a mechanosensor? Diabetes Technology and Therapeutics 2003; 4: 841-858.

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