Research Article

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In vitro renin–angiotensin system inhibition and *in vivo* antihypertensive activity of peptide fractions from lima bean (*Phaseolus lunatus* L.)

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Abstract

BACKGROUND: The renin–angiotensin system is key in the physiopathology of arterial hypertension because it converts angiotensin I, via angiotensin I-converting enzyme (ACE), into angiotensin II. *In vitro* analyses were done of the ACE-inhibitory and renin-inhibitory activities of peptide fractions isolated by enzymatic hydrolysis of lima bean (*Phaseolus lunatus*) protein. Antihypertensive activity was confirmed *in vivo* using a rat model.

RESULTS: Lima bean protein was hydrolyzed with one of two sequential enzymatic systems (pepsin–pancreatin or Alcalase®–Flavourzyme®). Ultrafiltration of the hydrolysates produced fractions of different molecular weights. The >3 kDa fraction of the pepsin–pancreatin hydrolysate had the highest ACE-inhibitory activity (60.15%, IC_{50}: 172.62 µg mL^{-1}), while the >3 KDa fraction of the Alcalase®–Flavourzyme® hydrolysate had the highest *in vitro* renin-inhibitory activity. A weak correlation ($r = 0.44$) was found between ACE-inhibitory and renin-inhibitory activities. When tested *in vivo*, the latter fraction lowered systolic blood pressure by 64% and diastolic blood pressure by 51%.

CONCLUSION: Peptide fractions from lima bean *Phaseolus lunatus* protein hydrolysates exhibit both *in vitro* and *in vivo* antihypertensive activity. Bioactive peptides from lima bean have potential applications as ingredients in functional foods.

Keywords: *Phaseolus lunatus*; ACE; renin; hypertension; bioactive peptides

INTRODUCTION

Arterial hypertension (AH) is the number one cause of mortality worldwide, causing 7 million deaths annually. It affects one out of four adults, reducing their life expectancy by 10–15 years.[1] The renin–angiotensin system (RAS) is key to short- and long-term regulation of AH since it involves sequential transformation of different proteins. Renin first transforms angiotensinogen into angiotensin-I (Ang-I). Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) then transforms Ang-I into angiotensin-II (Ang-II), producing a vasoconstrictor response that increases peripheral vascular resistance and blood pressure (BP).[1] Pharmacotherapy is recommended for those with blood pressure (BP) higher than 140/90 mmHg.[2] Fast-acting pharmaceuticals with reversible effects are administered as competitive inhibitors to react with ACE and cause inhibition.[1] However, when taken regularly these pharmaceuticals can have adverse side effects such as cough, dry cough, irritant cough, headache, dysgeusia, hypokalemia, hyponatremia and angioedema, among others.[3] Interest is therefore increasing in production and isolation of natural ACE inhibitors derived from proteins or bioactive peptides. The potential of some promising compounds has been assessed in *in vivo* and *in vitro* studies.[4]

Several food protein-derived peptides are potent *in vitro* ACE inhibitors. The first ACE-inhibitory peptides were isolated from a gelatin hydrolysate by using collagenase. These peptides have since been isolated from diverse protein sources such as milk, fish, yeast, rice, corn, egg and bovine blood plasma.[5] Two purified rice-derived tripeptides (Val-Asp-Trp and Val-Trp-Pro) were characterized and shown to exhibit high ACE-inhibitory activity. Peptide fractions from the Southeast Asian legume *Parkia speciosa* also have proven ACE-inhibitory activity ranging from 50.6% to 80.2%.[6] Legumes are known to contain bioactive peptides, beneficial nutrients and high-quality proteins. Legume-derived proteins are important food ingredients that can improve the nutritional and technological aspects of food formulations, as well as provide potential benefits to human health.[5] Different peptides derived from several legumes (e.g. velvet bean *Mucuna pruriens*,[7] cowpea *Vigna unguiculata* and Jamapa bean *Phaseolus vulgaris*), exhibit antihypertensive activity.[8,9] Lima bean *Phaseolus lunatus* has a high nutrient content and is a potential source of bioactive

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peptides with antihypertensive, antithrombotic, anticancer and antimicrobial activities.[10]

The aim of the present study was to assess the *in vitro* ACE-inhibitory and antihypertensive activities of peptide fractions extracted from lima bean (*P. lunatus*) using two different sequential enzymatic hydrolysis systems. The results were supported by an *in vivo* trial using rats.

MATERIALS AND METHODS

Materials

Lima bean seeds (2014 harvest) were acquired from a local market in Yucatan, Mexico. After removal of impurities, the seeds were milled in a roller mill (Cemotec 1990, Tecator, Sweden) and then in an impact mill (Cyclotec, Tecator). The resulting flour was passed through a 200-mesh sieve producing a powder of 500 μm particle size.[11] Inputs were acquired from Sigma-Aldrich: porcine-origin pepsin (EC 3.4.23.1) (9001-75-6); porcine-origin pancreatin (8049-47-6); Flavourzyme® (9014-01-1) from *Aspergillus oryzae*; Alcalase® (EC 3.4.21.62) (9014-01-1) from *Bacillus licheniformis*; subtilisin A; *ortho*-phthalaldehyde (OPA) (643-79-8); hippuryl-L-histidyl-L-leucine (HHL) (207386-83-2); 2 U ACE (905-82-1); and Captopril® (62571-86-2). The Renin Inhibitor Screening Assay Kit was acquired from Cayman Chemical (Ann Arbor, MI, USA). Teklad Global Rodent Diets® were supplied by Harlan Laboratories.

Protein concentrate

A single protein extraction was done of the lima bean flour using an established wet fractionation method.[12]

Enzymatic hydrolysis

Enzymatic hydrolysis was done with either an Alcalase®–Flavourzyme® (AF) enzymatic system or a pepsin–pancreatin (PP) enzymatic system.[13] Conditions in the AF system were 1:10 (v/v) enzyme-to-substrate ratio, 50 °C, 90 min, 0.3 UA g^{-1} enzymatic activity for Alcalase® at pH 8, and 50 LAPU g^{-1} enzymatic activity for Flavourzyme® at pH 7. Conditions in the PP system were 1:10 (v/v) enzyme-to-substrate ratio, 90 min, 37 °C, pH 2 for pepsin and pH 7.5 for pancreatin. A 40 g kg^{-1} protein content solution was used as substrate. After hydrolysis, the samples were centrifuged (Thermo Scientific RC6+ Sorvall) at 11951.9 × *g* for 20 min. The soluble fractions were frozen for later ultrafiltering. Degree of hydrolysis (DH) was quantified following Nielsel *et al.*[14]

Fractionation by ultrafiltration

Following Cho *et al.*,[15] the soluble fractions of the enzymatic hydrolysates were fractionated by ultrafiltration with 1, 3, 5 and 10 kDa membranes (Millipore 2000 ultrafiltration unit, Marlborough, MA, USA). This produced five peptide fractions of different molecular weights (MW) from each hydrolysate: >10 kDa; 5–10 kDa; 3–5 kDa; 1–3 kDa; and <1 kDa. These fractions were frozen at −4 °C and later lyophilized at −47 °C and 13×10^{-3} mbar (Labconco). The protein content in each fraction was measured following Lowry *et al.*[16] The ACE- and renin-inhibitory activities were quantified for the hydrolysates and the ultrafiltered peptide fractions.

In vitro ACE-inhibitory activity

Preparation of ACE was done according to Hayakari *et al.*[17] Rabbit lungs were used as starting material and the resulting aliquots were stored at −20 °C. This method is based on the colorimetric reaction of hippuric acid with 2,4,6-trichloro-*s*-triazine (TT). An HHL substrate was used at a 3 g L^{-1} concentration in 0.1 mol L^{-1} phosphate-based buffers (pH 8.3) and a 5 mol L^{-1} NaCl solution. Once assay time was complete, the reaction was stopped with TT in dioxane (30 mg mL^{-1}) and 5 min later the samples were centrifuged at 15 250 × *g* for 10 min at 4 °C. Absorbance was read at 382 nm. All runs were done in triplicate. The IC_{50} value (the peptide concentration required to produce 50% ACE inhibition) was quantified by a regression analysis of ACE-inhibitory activity (%) *versus* peptide concentration (μg protein mL^{-1}).

Renin-inhibitory activity

Hydrolysate and peptide fraction renin-inhibitory activities were measured with the Renin Inhibitor Screening Assay Kit (Cayman Chemical), according to Li and Aluko.[18] The control microplate was filled with 20 μL substrate, 160 μL assay buffer and 10 μL high-performance liquid chromatography (HPLC)-grade water. The maximum activity (100%) microplate was filled with 20 μL substrate, 150 μL assay buffer and 10 μL HPLC-grade water. The inhibition cell microplate was filled with 20 μL substrate, 150 μL assay buffer, and 10 μL sample (inhibitor). Fluorescence intensity (FI) was measured with a Thermo Scientific Appliskan and the Skanit 2.3 software, using an excitation wavelength of 340 nm and an emission wavelength of 500 nm. A regression analysis was run to establish the potential correlation between ACE-inhibitory and renin-inhibitory activities.

Antihypertensive activity in Wistar rats

The ultrafiltered peptide fraction exhibiting the highest *in vitro* RAS inhibitory activity was assessed *in vivo* in 25 male Wistar rats (15 days of age, average weight 279 ± 18 g). Blood pressure in the rats' tails was measured using a non-invasive plethysmograph (Kent Scientific CODA Standard model, Científica Senna, Mexico). All animal trials were conducted in accordance with the guidelines for care and use of experimental animals of the European Communities Council Directive 86/ 609/EEC. The animals were housed in accordance with the official standard 'Norma Oficial Mexicana NOM-062-ZOO-1999'. Laboratory animal care and use practices followed the ethical standards of the Autonomous University of Morelos.

Hypertension was induced following Zicha *et al.*,[19] with some modifications. For 3 weeks, the rats were orally administered the nitric oxide synthase inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) dissolved in drinking water at 60 mg kg^{-1} body weight. Before the treatments were begun, the L-NAME dose was reduced to 20 mg kg^{-1} for all groups. This was done to ensure that any decline in BP during treatment was due to the antihypertensive agent and not elimination of L-NAME. The negative control group consisted of five rats administered no L-NAME before treatment. The rats were kept in plastic cages with stainless steel tops, under a natural light–dark cycle, 25 °C temperature and with water freely available. Treatments were randomly assigned to one cage containing five experimental units (i.e. animals). Antihypertensive activity in the *in vivo* experiment was evaluated with a 2 × 2 × 4 factorial design to measure the effect of the factors (antihypertensive agent, concentration and time) on the response variable (BP). Three factors were analyzed: [1] antihypertensive agent, PP (PP >3 kDa) or Captopril® (Capto); [2] agent concentration, 5 (C5) or 15 (C15) mg kg^{-1}; and [3] time period (1, 2, 3 or 4 weeks). Animals were weighed weekly to adjust agent concentration (5 or 15 mg

kg^{-1}) for the peptide fractions and positive control pharmaceuticals (Captopril® and L-NAME). All treatments were orally administered daily for 4 weeks using 500 µL volume direct cannulation to the stomach.[20] The effect of time period was assessed by weekly measurement of BP in rats employing the tail-cuff method in a non-invasive CODA device. Results were expressed as average variation in individual rat BP. Three replicates were done per peptide fraction, measuring BP with the same electrophysiological system. Antihypertensive effect, expressed as a percentage reduction in BP, was calculated from the formula

$$\text{Antihypertensive effect } (\%) = 100 \times \left(BP_{exp}\right) / \left(BP_{ctrl}\right)$$

where BP_{exp} is systolic and diastolic blood pressure observed after administration of hydrolysates, ultrafiltered peptide fractions and captopril; and BP_{ctrl} is basal blood pressure observed in week 0 (after L-NAME-induced hypertension).

Statistical analysis

All results were analyzed in triplicate using descriptive statistics with a central tendency and distributions. A one-way ANOVA was run to evaluate *in vitro* ACE-inhibitory and renin-inhibitory activities. Duncan's multiple range test was applied to identify differences between treatments. All analyses were processed using the Statgraphics Plus version 5.1 software.

RESULTS AND DISCUSSION

Degree of hydrolysis

The AF system produced a hydrolysate with a higher ($P < 0.05$) DH (77.30%) than that of the PP system (32.33%). The higher DH with the AF system may be attributed to the dual endo- and exopeptide nature of its enzymes, which allows them to attack most of the protein's peptide bonds. The lower DH in the PP system was probably due to both enzymes having only endopeptidase activity, with potentially lower catalytic action on the protein concentrate. Pepsin mainly attacks peptide bonds containing aromatic amino acids, methionine or leucine, whereas pancreatin has a higher specificity for the C-terminal bonds of methionine and leucine residues. However, *in vitro* application of a sequential PP enzymatic system can prevent peptide hydrolysis during gastric digestion.[21] Sequential use of proteases does generate extensive hydrolysates (DH > 10%), and peptides derived from these hydrolysates may exhibit biological activity (i.e. the capacity to affect physiological processes). The extensive DH values obtained with both the AF and PP systems suggest the lima bean protein hydrolysates analyzed here are potential sources of bioactive peptides.[10]

ACE-inhibitory activity

Ultrafiltration of the protein hydrolysates resulted in ten peptide fractions differentiated by hydrolysis system and MW: F1, AF > 10 kDa; F2, AF > 5 kDa; F3, AF > 3 kDa; F4, AF > 1 kDa; F5, AF < 1 kDa; F6, PP > 10 kDa; F7, PP > 5 kDa; F8, PP > 3 kDa; F9, PP > 1 kDa; and F10, PP < 1 kDa (Fig. 1). All the evaluated protein hydrolysates and peptide fractions exhibited ACE-inhibitory activity (Fig. 1), although the two highest activities were observed in PP > 3 kDa (60.15%) and PP > 1 kDa (50.37%). Both these values are lower than the maximum ACE inhibition percentages reported for pepsin-based hydrolysates from different legumes: chickpea (86%); Jamapa bean (77%); lentil (79%); lupine (*L. albus*) (80%); lupine (*L. angustifolius*) (89%); pea (71%); and soy (88%).[4] Differences between the present results and those of previous studies are probably due to protein sources, enzymatic systems and operating conditions. The AF fractions' ACE inhibition values probably differed from those of the PP fractions and of previous reports for much the same reasons.

The fraction with the highest ACE-inhibitory activity (60.15%) was PP > 3 kDa. Its IC_{50} was 172.62 µg mL^{-1}, notably higher than previously reported values for peptide fractions produced under different hydrolysis conditions (e.g. 560 µg mL^{-1}; 370.5 µg mL^{-1}).[3,8]

Renin-inhibitory activity

The AF hydrolysates and peptide fractions had renin-inhibitory activity values ranging from 15.82% to 31.73%, the AF > 3 kDa fraction having the highest degree of inhibition (Fig. 2). Renin-inhibitory activity in the PP system hydrolysates and fractions ranged from 5.36% to 30.05%.

In a previous study of renin-inhibitory activity, peptides isolated from *M. pruriens* using a pepsin/pancreatin sequential system had IC_{50} values ranging from 99.94 to 252.97 µg mL^{-1}.[22] Even

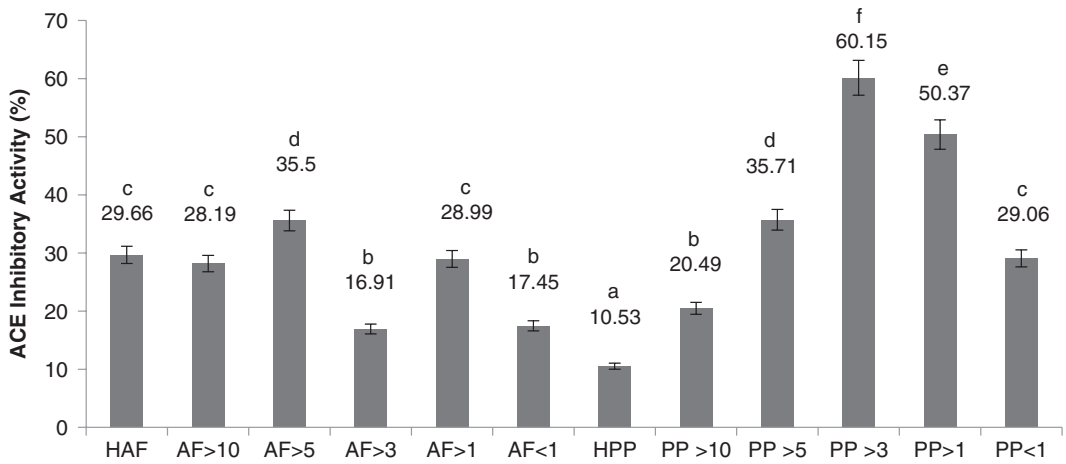

Figure 1. ACE-inhibitory activity of hydrolysates and peptide fractions from lima bean (*P. lunatus*). Each value is the mean ± standard deviation ($n = 3$). Different lower-case letters indicate significant difference ($P < 0.05$). AF = Alcalase®–Flavourzyme® enzymatic system; HAF, AF hydrolysate; PP, pepsin–pancreatin enzymatic system; HPP, PP hydrolysate.

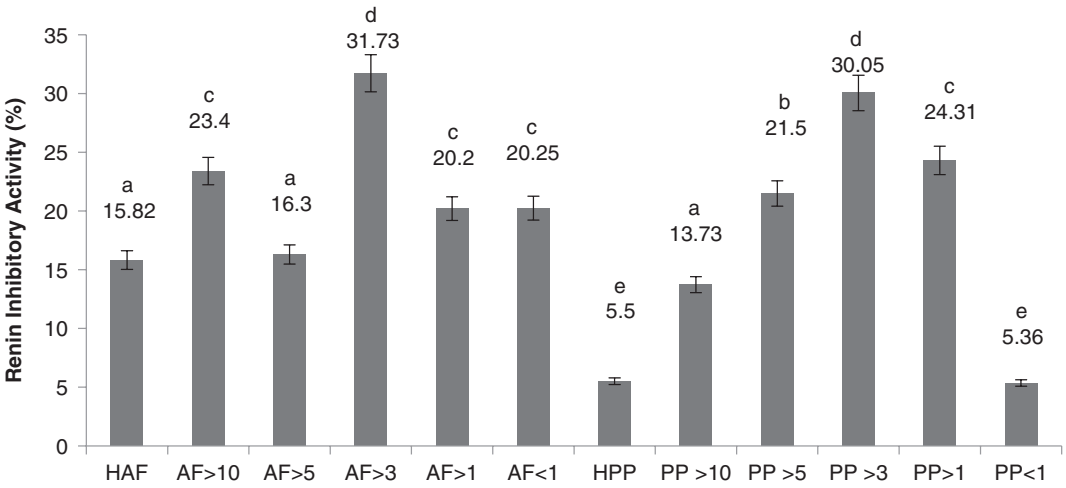

Figure 2. Renin-inhibitory activity of hydrolysates and peptide fractions from lima bean (*P. lunatus*). Each value is the mean ± standard deviation ($n = 5$). Different lower-case letters indicate significant difference ($P < 0.05$). AF, Alcalase®–Flavourzyme® enzymatic system; HAF, AF hydrolysate; PP, pepsin–pancreatin enzymatic system; HPP, PP hydrolysate.

higher IC_{50} values have been reported for unhydrolyzed *P. vulgaris* (270–420 μg mL^{-1}) and *V. unguiculata* (360 μg mL^{-1}).[23] The renin enzyme limits Ang-II synthesis, and renin inhibitors may produce more complex blocking of the RAS, highlighting the promise of the *P. lunatus* peptide fractions in the present study. The correlation coefficient (*r*) between ACE inhibition and renin inhibition was 0.4482, indicating a relatively weak relationship between inhibitory activities ($P < 0.05$).

Antihypertensive activity in Wistar rats

Before induction of hypertension with L-NAME, the rats were in a normotensive state: mean DBP = 96 ± 8.34; mean SBP = 132 ± 9.89. These values are slightly higher than the 80–90 mmHg DBP and 100–120 mmHg SBP reported elsewhere for normotensive rats. [24] After 3 weeks of L-NAME induction the rats were considered hypertensive, exhibiting increases of 47.45% in DBP (142 ± 8.53 mmHg) and 37.51% in SBP (182 ± 8.26 mmHg). Blood pressure values were time dependent in all the L-NAME-induced hypertensive groups. This coincides with a study of rats administered 60 mg kg^{-1} body weight L-NAME in which DBP values increased to 130 mmHg beginning at day 7 and SBP values increased to 150–170 mmHg after day 12.[19] These increases were not homogeneous in all rats in either study. In another study, administration of 60 mg kg^{-1} L-NAME for 3 weeks raised BP in rats by 25 mmHg.[20] Administering L-NAME in rats clearly raises systemic pressure levels above 140 mmHg, creating a hypertension model induced by nitric oxide (NO) deficiency.

As an irreversible inhibitor of the enzyme nitric oxide synthase (NOS), L-NAME is responsible for production of NO in the endothelium from L-arginine. Vascular tone and endothelial function, and consequently BP, depend on NO. Inhibition of NOS decreases NO production, raising BP.[25] This constitutes endothelial dysfunction, a pathological condition associated with hypertension, hypercholesterolemia, diabetes and oxidative stress. Loss of endothelium-dependent vasodilation in response to declines in vascular wall NO activity is produced by reduced or aberrant NO production or increased NO degradation. Prevention of cardiovascular disease is therefore fundamentally aimed at preventing endothelial dysfunction and associated factors.

Decreasing NO levels also favors vasoconstriction, leading to increased renin release and hypertension. Reduction in the glomerular filtration rate, a characteristic described in primary or essential hypertension in humans, has also been observed in this rat model. The model used closely mimics systemic arterial hypertension in humans since it increases BP as well as damaging target organs. It has been widely used in studies of compounds and plants with hypotensive and ACE-inhibitory activities.[26]

Administration of the PP >3 kDa fraction and Captopril® produced an antihypertensive effect in the rats with L-NAME-induced hypertension, despite their receiving a constant dose of 20 mg kg^{-1} L-NAME. Both time period and agent concentration affected ($P < 0.05$) SBP (Fig. 3a) and DBP (Fig. 3b). The highest reductions in BP (51% systolic and 64% diastolic) were produced with the PP > 3 kDa peptide fraction at 15 mg kg^{-1} three times a week. However, the overall efficacies of the peptide fractions and Captopril® differed ($P < 0.05$) only slightly beginning at week three since all lowered BP values. Clearly, time period had the most notable effect on BP. The greatest reductions were observed beginning in week 3, probably the time required by the animals to adapt to the treatments. The effect of agent concentration was less consistent, although all the peptide fraction treatments had systolic and diastolic BP values higher than in the 15 mg kg^{-1} Captopril® treatment. No differences were identified between most of the groups, and the least significant difference test ($P < 0.05$) found differences only between the mean values of some treatments.

Treatment time-dependent antihypertensive activity has been previously reported for legume-derived bioactive peptides. Cú-Cañetas *et al.*[20] performed an *in vivo* assessment in rats of peptide fractions (<1 kDa) from *V. unguiculata* produced with Flavourzyme® and PP. The fractions were orally administered during 4 weeks at a 10 mg kg^{-1} concentration. The Flavourzyme® peptide fractions resulted in a 4.92% drop in diastolic BP and a 10.27% drop in systolic BP. In contrast, the PP peptide fractions resulted in 17.12% and 15.98% reductions, respectively, which were higher than those obtained with the Captopril® control treatment.

In another study, Chel-Guerrero *et al.*[7] assessed *M. pruriens*-derived hydrolysates produced separately with the Alcalase® and Flavourzyme® enzymes at different hydrolysis

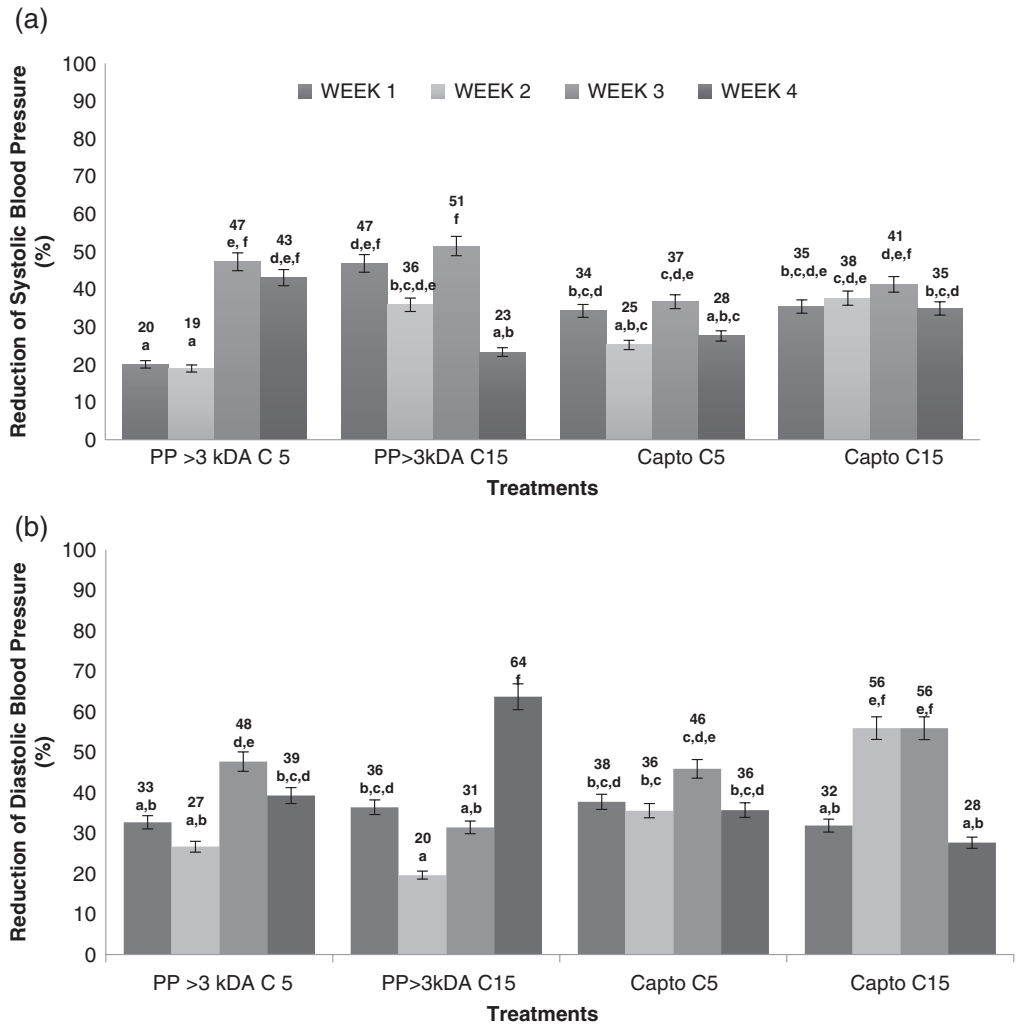

Figure 3. Antihypertensive effect of orally administered *P. lunatus* PP > 3 kDa peptide fraction isolated from the pepsin–pancreatin (PP) hydrolysate and Captopril® (Capto) at two concentrations in rats with L-NAME-induced hypertension: (a) systolic blood pressure; (b) diastolic blood pressure. Each value is the mean ± standard deviation ($n = 5$). Different lower-case letters indicate significant difference between means ($P < 0.05$). C5 = 5 mg kg^{-1} concentration; C15 = 15 mg kg^{-1} concentration.

times. At a 5 mg kg^{-1} concentration, the Alcalase® hydrolysates produced at 90 min reduced BP by 25.53 ± 5.46%, although this did not differ ($P > 0.05$) from the Flavourzyme® hydrolysates produced at 90 min (29.37 ± 13.03%) or the Captopril® control (28.56 ± 5.23%). Even at a 10 mg kg^{-1} concentration, the Flavourzyme® hydrolysates produced at 5 min exhibited the lowest antihypertensive effect (14.96 ± 4.32%).

Finally, Betancur-Ancona *et al.*[8] performed an *in vivo* biological assessment of Alcalase®-produced peptide fractions (<1 kDa) from *P. lunatus* and *P. vulgaris* in a (female) Wistar rat model. Decreases in BP were greatest at the lowest peptide fraction concentration, with the highest reduction (54.74) in SBP observed at 5 mg kg^{-1}, followed by the 15 mg kg^{-1} concentration (39.46%) and the 10 mg kg^{-1} concentration (7.33%). The greatest decrease in DBP (98.9%) was also observed at the 5 mg kg^{-1}. These fractions had high contents of essential (Thr, Trp, Val, Phe, Ile, Leu, Lys and Met) and hydrophobic amino acids. These may lower BP, suggesting they could contribute to these peptide fractions' high ACE-inhibitory activity.[8] This in turn could explain the good *in vitro* and *in vivo* ACE-inhibitory activities observed in the present study for the PP > 3 kDa fraction from *P. lunatus*.

It was decided to work with male rats in the present study because the molecular mechanisms involved in regulating blood pressure are different and more complex in females than in males.[27] In particular, nuclear receptors (ERα and ERβ) and G protein-coupled estrogen receptors (GPER) are more abundant in female endothelia. Other studies also report sexual dimorphism in the regulation of vascular tone, where perivascular adipose tissue regulates vascular tone differentially between the sexes. These results demonstrate a significant difference in adiponectin vascular tone regulation in females.[27,28] Studies of the antihypertensive effect of *P. lunatus* protein hydrolysates and peptide fractions on female rat models are needed to generate data to support differential response by sex.

CONCLUSIONS

Peptide fractions produced by sequential hydrolysis of lima bean proteins followed by ultrafiltration exhibited biological *in vitro* RAS activity, ACE-inhibitory activity and renin-inhibitory activity. They also reduced BP in rats in an *in vivo* experimental model. Hydrolyzed proteins from lima beans can clearly contribute to lowering blood pressure, thus preventing many cardiopathies.

They could have applications as a functional ingredient in specialized health foods.

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