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In Vitro Modulation of Renin−Angiotensin System Enzymes by Amaranth (*Amaranthus hypochondriacus*) Protein-Derived Peptides: Alternative Mechanisms Different from ACE Inhibition

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ABSTRACT: Among the factors affecting the development of cardiovascular diseases, hypertension is one of the most important. Research done on amaranth proteins has demonstrated their hypotensive capacity in vivo and in vitro; nevertheless, the mechanism underlying this effect remains unclear. The aim of this study was to analyze in vitro the inhibition of peptides derived from an amaranth hydrolysate (AHH) on other RAS enzymes other than ACE. The chymase and renin activities were studied. AHH was not able to inhibit chymase activity, although a dose−response effect was found on renin activity (IC_{50} 0.6 mg/mL). To provide an approach to the renin inhibition mechanism, we analyzed AHH renin inhibition kinetics and performed a structural characterization of the peptides involved in the effect in terms of molecular size and hydrophobicity. Results suggest that amaranth peptides exhibit renin competitive inhibition behavior. Renin inhibition potency was directly related to peptide hydrophobicity. RP-HPLC separation of AHH and subsequent analysis of the peptide sequences showed 6 peptides belonging to 11S globulin (that can be grouped into 3 families) that would be responsible for renin inhibition. These results demonstrate that *Amaranthus hypochondriacus* seeds are an adequate source of peptides with renin inhibitory properties that could be used in functional food formulations.

KEYWORDS: *Amaranth hydrolysate peptides, renin, chymase, enzyme inhibition*

INTRODUCTION

According to the World Health Organization, nontransmissible diseases (NTD) are responsible for 68% of the mortality rate worldwide.[1] Within this group of diseases, cardiovascular diseases (CVD) are those that hold the highest record of fatalities. In 2012, 17.5 million of deceases from NTD were due to CVD (46% of all NTD mortality rate). Among the factors affecting the development of CVD, hypertension is one of the most important.[2] By 2025, the population suffering from hypertension will have risen to 1.5 billion.[3] Hypertension is a pathological condition characterized by an insufficient vessel relaxation and reduced blood flow.[4]

The main system involved in the regulation of blood pressure is the renin−angiotensin system (RAS) comprising a large number of different enzymes (Figure 1), regulated mainly by two of them, the angiotensin converting enzyme (ACE) and renin. The latter enzyme hydrolyzes angiotensinogen to produce angiotensin I, an inactive decapeptide, whereas ACE cleaves angiotensin I to yield angiotensin II, a vasoactive peptide which causes blood vessel contraction.[4,5]

Antihypertensive drugs developed so far are either ACE or renin inhibitors. These drugs are efficient. However, they cause side effects in some patients, such as cough fits in 5−20% of patients using ACE inhibitors[6] and gastrointestinal disorders for those employing Aliskiren, a renin inhibitor.[7] For this reason, there is increased interest in developing natural compounds that are able to coregulate blood pressure without causing side effects. Different research groups have obtained and reported several food-grade peptide inhibitors from vegetal and animal sources, so as to get a high proportion of bioactive peptides with inhibitory capacity on ACE and, more recently, on renin both in vitro and in vivo.[4,5,8,9]

Amaranth, an ancestral crop with high protein content (12−17% w/w), is considered an important source of bioactive compounds.[10,11] Bioactive peptides derived from their proteins with different biological activities have been described, including antihypertensive activity among others.[12,13]

Our research group has studied the ACE inhibiting capacity of amaranth isolates, hydrolysates, sprouts, protein fractions, and synthetic peptides and their gastrointestinal products by means of in vitro, in silico, and in vivo assays, obtaining promising results.[14−17] On the other hand, Tovar-Pérez et al.[18] analyzed the ACE inhibition capacity of albumins and globulins, while Barba de la Rosa et al.[19] characterized the amaranth trypsin-digested glutelin ability to produce nitric oxide (a

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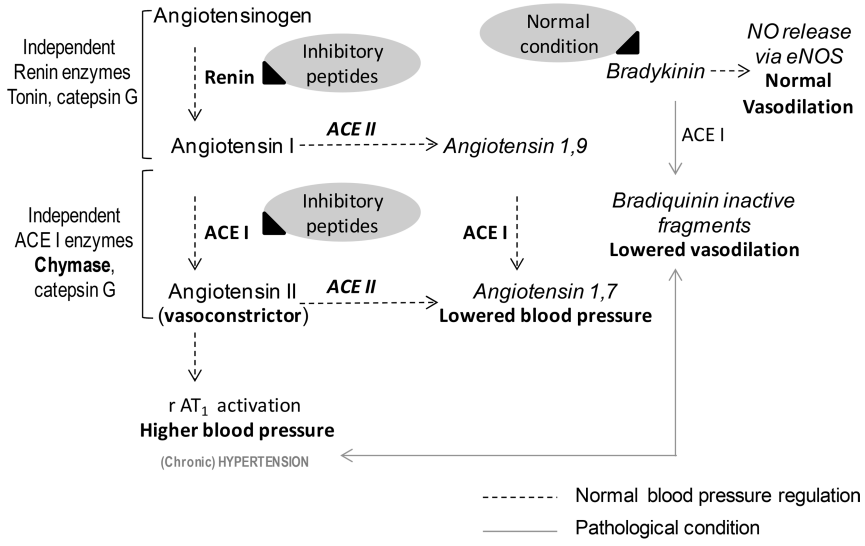

ACE I: produced in the surface of pulmonar and renal endothelium.
Renin: synthesized in granular cells of kidneys yuxtaglomerular system.
Angiotensinogen: released in liver, renin specific sustrate.
ACE II: expressed predominantly in vascular endothelial cells of heart and kidneys.
Independent ACE and renin enzymes are expressed under pathological conditions (vascular remodelation).

Figure 1. Simplified scheme of blood pressure regulation by the renin−angiotensin system in physiological and pathological conditions. It shows the action points of the enzymes in study renin and chymase.

potent vasodilator) by serine phosphorylation in coronary endothelial cells. Besides, Medina-Godoy et al.[20] obtained an engineered amaranth 11S globulin (AMC 3) expressed in *E. coli* and subjected to gastrointestinal digestion. The effect of this preparation was analyzed on spontaneously hypertensive rats (SHR), and good results were obtained. Although these results are interesting and promising, the regulatory mechanisms of blood pressure exerted by amaranth peptides are not completely known.

The aim of this study was to analyze in vitro the inhibition of amaranth peptides on other RAS enzymes other than ACE. In particular, the inhibitory capacity of AHH on chymase and renin activities was studied. In order to provide an approach for the renin inhibition mechanism, we analyzed AHH renin inhibition kinetics and performed a structural characterization of the peptides involved in the effect in terms of molecular size and hydrophobicity.

MATERIALS AND METHODS

Chemicals. All chemicals and solvents were of analytical grade. Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemical (Ann Arbor, Michigan, USA). Chymase Kit and Alcalase solution (protease from *Bacillus licheniformis*, >2.4 U/g, Anson Units) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

One-Anson Unit is defined as the amount of enzyme which, under specified conditions, digests urea-denatured hemoglobin at an initial rate such that there is liberated an amount of TCA-soluble product per minute which gives the same color with Folin-Ciocalteu Phenol reagent as one milliequivalent of tyrosine at 25 °C at pH 7.50.

Materials. *Amaranthus hypochondriacus* cv. *Antorcha* samples were harvested at Instituto de Agrobiotecnología Rosario (INDEAR), Rosario, Santa Fe, Argentina.

Sample Preparation. The ground flour obtained from whole seeds was obtained from an UDY Cyclone Sample Mill 0.5 mm mesh and defatted with hexane during 24 h at room temperature.

Protein Isolate. The *A. hypochondriacus* protein isolate (AHI) was prepared from defatted flour. The flour was suspended in water (10% w/v) and adjusted to pH 9 by adding 2 N NaOH. Proteins were extracted with continuous stirring for 1 h at room temperature and then centrifuged at 9000*g* for 20 min. The supernatants were adjusted to pH 5 with 2 N HCl. The suspension was centrifuged at 9000*g* for 20 min at 4 °C. The precipitates were suspended in water, neutralized with 0.1 N NaOH, and freeze dried.

Protein Hydrolysate. AHI was suspended in water in a proportion of 1 g/0.1 L at pH 10 with continuous stirring for 1 h at 37 °C in a thermomixer (Eppendorf, Hamburg, Germany). Then alcalase solution was added (0.16 μL alcalase/mg AHI), and the mixture was incubated for 5 h. The pH value was controlled and adjusted every 15 min during the first hour and every 60 min from then on. The enzymatic reaction was stopped by thermal treatment at 90 °C for 10 min. The Amaranth protein hydrolysate was frozen and freeze dried (AHH).

The protein content of AHI and AHH was determined by Kjeldhal's method (protein/nitrogen coefficient = 5.85), 82 ± 1% w/w and 80.5 ± 2.2% w/w (dry base), respectively.

Degree of Hydrolysis. The degree of hydrolysis (DH) was measured by the reaction of released amino groups with *o*-phthaldialdehyde (OPA)[21] and calculated with the following formula

$$\%DH = \frac{[-NH_2]_h - [-NH_2]_0}{[-NH_2]_\infty - [-NH_2]_0} \times 100$$

where $[-NH_2]_0$ is the concentration of free amino groups in the nonhydrolyzed sample (mol/L) and $[-NH_2]_h$ is the concentration of free amino groups in the hydrolyzed samples (mol/L).

The parameter $[-NH_2]_\infty$ (mol/L) was estimated according to

$$[-NH_2]_\infty = \frac{1}{M_{aa}} \times (1 + f_{Lys}) \times C_{prot}$$

where M_{aa} is the average molecular weight of amino acids present in amaranth proteins (130 g/mol), f_{Lys} is the proportion of lysine in these proteins (1/20),[10] and C_{prot} is the protein concentration in g/L.

From the degree of hydrolysis, the average peptide length was estimated according to

$$L = \frac{100}{\%DH}$$

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where L is the average peptide length and % DH is the degree of hydrolysis.

Protein Content Determination. The nitrogen content of AHI, AHH, size exclusion fractions, and RP-HPLC fractions was determined according to Nkonge and Ballance (1982).[22] Briefly, samples, control solutions ($(NH_4)_2SO_4$, $(NH_2)_2CO$), and standards (albumin) were digested in a 2040 digestor (Foss Tecator, Hillerød, Denmark) in 5 mL of concentrated H_2SO_4 and 1 g of catalyst mixture ($NaSO_4$ and $CuSO_4$, 10:1). After acid digestion, cooled samples were diluted to 50 mL with distilled water. Blanks were prepared in an identical manner using only the catalyst and the acid.

The nitrogen content was derived from a calibration curve and expressed in milligrams. Finally, to convert nitrogen content into protein mass, the protein/nitrogen coefficient (5.85 mg protein/mg N) was employed.

Structural Characterization. *SDS-Tris/Tricine-Polyacrylamide Gel Electrophoresis (Tricine-SDS-PAGE).* Electophoresis was performed in minislabs (BioRad Mini Protean II Model, Hercules, USA) using 160, 100, and 40 g/L of acrylamide for separating, spacing, and stacking gels, respectively. Protein samples (20 mg/mL) were dissolved in tricine sample buffer centrifuged at 13 000g for 15 min at room temperature. The supernatants were separated, and a volume of 10 μL was loaded onto the gel.[23]

The Low Molecular Weight Calibration Kit (St_1) (MW 14.1−97 kDa) (GE Healthcare, Uppsala-Sweden) and polypeptide SDS-PAGE standards (St_2) (MW 1.4−26.6 kDa) (BioRad) were used as molecular markers. Gels were fixed and stained with Coomasie brilliant Blue.

Gel Filtration Liquid Chromatography. The AHH profile was analyzed at room temperature in a Superdex Peptide 10/300 GL column using a FPLC, ÄKTA Purifier (GE-Healthcare). Briefly, the AHH (20 mg) was dissolved in 1 mL of buffer (100 mmol/L NH_4HCO_3, pH 8), centrifuged at 13 000g for 15 min, at room temperature. The supernatants were separated, filtered, loaded onto the column, and eluted with the same buffer at a flow rate of 0.2 mL/min. Fractions of 2 mL were collected and the elution profile (miliabsorbance at 214 nm) was obtained. Column calibration was made with aprotinin (Sigma-Aldrich 6.500 kDa), B_{12} vitamin (Sigma-Aldrich 1.355 kDa), and hippuric acid (Sigma-Aldrich 0.179 kDa). Curves were processed, and data were evaluated using the Unicorn Software. Fractions obtained from six experiments were collected and freeze dried.

RP-HPLC. *Preparative RP-HPLC.* A Sun Fire prep C8, 5 μm ST 10/250 (Waters Corp., Milford, Massachusetts, USA) column was used to separate the peptides present in the AHH soluble fractions on a Waters System HPLC (Waters) equipped with a diode array detector. Prior to analysis, the AHH was suspended in buffer (50 mmol/L Tris-HCl, 10 mmol/L NaCl, pH 8) at a ratio of 25 mg/mL, centrifuged at 13 000g for 20 min at 25 °C, and filtered (0.45 μm mesh). Samples (2 mL) were injected and eluted with a linear gradient from 0 to 100% solvent B in 55 min, employing a flow rate of 5.2 mL/min. Solvent A was made up of water and acetonitrile (98:2) with trifluoroacetic acid (TFA, 650 μL/L), and solvent B was made up of water and acetonitrile (35:65) with TFA (650 μL/L). The separation was done at 40 °C, and the detection was performed at 210 nm. Six fractions were collected, pooled, and concentrated in a Concentrator Plus (Eppendorf) in order to eliminate acetonitrile and then freeze dried.

Analytical RP-HPLC. An analytical Microsorb-MV C18, 100 Å-5 μm, 4.6/250 (Agilent Technology, Santa Clara, USA) column was used to separate the peptides obtained in the gel filtration liquid chromatography (fractions f4−f10) using a Waters System HPLC (Waters) equipped with a diode array detector. Prior to analysis, freeze-dried fractions collected from gel filtration liquid chromatography were suspended in 500 μL of buffer (50 mmol/L Tris-HCl, 10 mmol/L NaCl, pH 8), centrifuged at 13 000g, and filtered (0.45 μm). Samples (50 μL) were injected and eluted with a linear gradient from 0 to 100% solvent B in 55 min at a flow rate of 1.1 mL/min. Solvent A consisted of water and acetonitrile (98:2) with trifluoroacetic acid TFA (650 μL/L), and solvent B consisted of water and acetonitrile (35:65) with TFA (650 μL/L). The separation was done at 40 °C, and the detection was performed at 210 nm.

MALDI-TOF-MS-Analysis. Two microliters (2 μL) of RP-HPLC fraction sample was injected into a nano HPLC (EASY-nLC 1000-RP-HPLC, Thermo Scientific, Waltham, MA, USA). The mobile phase was composed of solution A (formic acid/water 0.1/100 v/v) and solution B (acetonitrile/formic acid 100/0.1 v/v). A mass spectrometer (Thermo Scientific model Q- Exactive) was coupled to this nano HPLC with a high-collision dissociation cell and an Orbitrap analyzer. This platform allows peptide identification and chromatographic separation simultaneously. Sample ionization was performed by electrospray (Thermo Scientific, EASY-SPRAY) using a spray voltage of 3.5 kV. Protein identification was achieved by comparing mass data against the UniProt databank restricted to the Amaranthus taxonomy using Proteome Discover 1.4 software (Thermo Scientific). The parameters used for database searches included miscleavage 2, variable oxidation of methionins, statics carbamidomethylation of cysteines, and tolerance of the ions at 10 ppm for parents and 0.05 Da for fragments. Peptides were considered as valid when they present high confidence levels, which is provided by the program in searches according to the score thresholds computed by Proteome Discover.

Biological Characterization. *Chymase Inhibition Assay.* The in vitro inhibition of the chymase activity was realized according to the instructions of the Chymase Kit (Sigma- Aldrich). A chymotrypsin-like substrate (*N*-succinic-Ala-Ala-Pro-Phe-*p*-nitroanilide) is cleaved, and a product is obtained whose absorbance is read at 405 nm. The experiment was performed in a microplate format using a microplate of 96 wells. The following reagents were added in each well: water, assay buffer, substrate, and DMSO for the activity reaction or inhibitor reaction. The plate was mixed and equilibrated in the plate chamber at 37 °C for 2 min. The reaction was initiated by the addition of a chymase solution on the different wells except in the backgrounds. The absorbance increment at 405 nm was recorded during the first 10 min of the reaction every 20 s in a microplate reader (BiotekSyenrgy HT, Winooski, VT, USA). To estimate chymase inhibition reaction speed, the slope for each curve was calculated. An inhibition of 50% or more in chymase activity is considered as a satisfactory result.

Renin Inhibition Assay. The in vitro inhibition activity on human recombinant renin was performed with the Renin Inhibitor Screening Assay Kit (Cayman Chemical) according to their instructions. First, substrate and assay buffer were added to the following wells: (a) background, (b) 100% initial activity, (c) background peptide inhibitor sample, and (d) peptide inhibitor. The enzymatic reaction was initiated by the addition of renin to b and d treatment wells. The microplate was shaken for 10 s for blending and incubated at 37 °C for 15 min, and fluorescence intensity (FI) was then recorded using an excitation wavelength of 340 nm and an emission wavelength of 490 nm in a microplate reader (Biotek Synergy HT).

The IC_{50} value was the concentration of protein hydrolysate that inhibited 50% of renin activity.

AHH Renin Kinetics Parameters. Renin inhibition kinetics studies were performed using 1.25, 2.5, 3.5, 5, 7.5, and 10 μmol/L substrate in the absence and presence of AHH (0.32 and 0.64 mg protein/mL). The renin inhibition behavior was determined from Lineweaver−Burk plots, and inhibition parameters (V_{max} and K_M) were calculated as the Y- and X-axis intercepts, respectively. K_i was calculated as the X-axis intercept of the lines obtained from a plot of Lineweaver−Burk slopes versus peptide concentration.

Statistical Analyses. Samples were assayed in triplicate. Results were expressed as the mean ± SD. One-way analysis of variance (ANOVA) was performed with the Origin Lab software. Fisher's protected LSD ($p < 0.01$) values were calculated for the appropriate data.

RESULTS AND DISCUSSION

Hydrolysis of *A. hypochondriacus* Protein Isolates (AHI). Structural Characterization. To obtain renin and chymase inhibitor peptides from AHI, we used alcalase under conditions pre-established in our laboratory.[14]

The hydrolysis degree (% DH) allowed us to measure the extent of this process which was found to be % DH = 21 ± 4,

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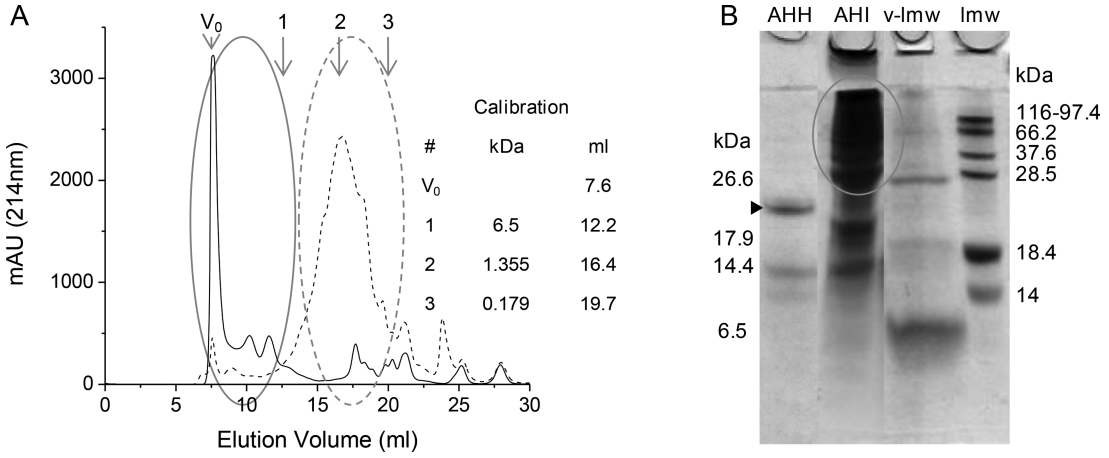

Figure 2. (A) Superdex peptide 10/300 GL gel filtration profile. AHI: *A. hypochondriacus* isolate (continuous line). AHH: *A. hypochondriacus* hydrolysate (dashed line). Gray arrows indicate the elution volumes of standard proteins. V0: Void volume; 1: Aprotinin (6.500 kDa); 2: Vitamin B12 (1.355 kDa); 3: Hippuric acid (0.179 kDa). (B) Tricine-SDS-PAGE (reducing conditions). St_1: standard proteins (Ge-Healthcare). St2: standard proteins (BioRad). On the left are the standard molecular masses (kDa) of St_2. On the right are the standard molecular masses of St_1 (kDa).

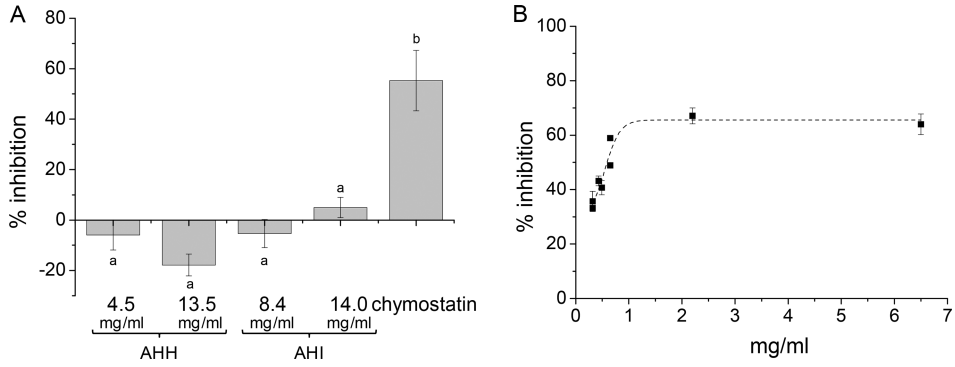

Figure 3. (A) Percentage of chymase inhibition caused by different concentrations of AHH (AHH1, 4.5 mg/mL; AHH2, 13.5 mg/mL) and AHI (AHI1, 8.4 mg/mL; AHI2, 14 mg/mL). Chymostatin: commercial chymase inhibitor. Bars (mean ± standard deviation) with different letters have mean values that are significantly different ($p < 0.05$). (B) Percentage of renin inhibition caused by different concentrations of AHH.

values that are in accordance with those found for pea (*Pisum sativum*), cowpea (*Vigna unguiculata*), and rapeseed (*Brassicus napus*) protein hydrolysates.[24−26]

According to the calculated *L* parameter after enzymatic hydrolysis, the peptides mean size obtained was between 4 and 6 amino acids. This peptide length is optimum to cross the intestinal epithelium and to be absorbed without further hydrolysis.[27,28]

In order to characterize AHH, gel filtration chromatography and tricine-SDS-PAGE were performed. Figure 2A shows the main components of the AHI (Figure 2A continuous line) and its hydrolyzed products after 6 h of enzymatic treatment (AHH), which rendered several low molecular weight peptides (Figure 2A, dashed line). The AHH principal components were species with molecular masses between 1.355 and 0.179 kDa (Figure 2A) that correspond to peptides of 12 amino acids to free amino acids. This result is in agreement with the *L* average value calculated for this sample.

AHI and AHH tricine-SDS-PAGE protein profiles are shown in Figure 2B. Results are coincident with the gel filtration profile, which shows the reduction in molecular size of the major components of AHI upon enzymatic treatment (Figure 2B, AHI, gray circle). However, after 6 h of hydrolysis, a band of approximately 20 kDa remained, which corresponded to globulin basic subunit that is resistant to enzymatic treatment (Figure 2B, AHH, black arrow). These results are in agreement with those reported by Fritz et al.[14]

AHH Enzyme Inhibitory Activities. Results obtained for chymase and renin inhibition are presented in Figure 3A and 3B, respectively. Neither AHI nor AHH was able to inhibit chymase. This finding is not surprising because even though some chymase inhibitors are peptides,[29] most of the inhibitors described so far are known to be of a nonpeptidic nature.[30] Furthermore, there is no literature data on the chymase inhibition by peptides derived from food proteins.

Nevertheless, AHH inhibited renin in a dose−response manner. The IC_{50} value (0.6 mg/mL) was calculated from a nonlinear fitting. This result is in agreement with those informed by other authors working with different protein sources. Girgih et al.[31] found an IC_{50} = 0.81 mg/mL for hemp (*Cannabis sativa*) protein hydrolysate. Fitzgerald et al.[32] also reported an IC_{50} value higher than 1 mg/mL for palmate (*Macroalga palmaria*) protein hydrolysate, and Girgih et al.[3] found an IC_{50} = 0.26 mg/mL for salmon (*Salmo salar*) protein hydrolysate. He et al.[33,34] reported a renin inhibition of 80% at 1 mg/mL for rapeseed (*Brassica napus*) protein hydrolysate. When we compared the renin inhibition activity before and after the hydrolysis treatment, the AHH proved to be a stronger

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inhibitor than the AHI. To achieve a value similar to AHH, an AHI protein concentration 22 times higher (data not shown) was needed. This finding indicated that the enzymatic treatment released encrypted peptides from AHI that were able to inhibit the activity of renin.

Unlike ACE, there are scarce in vitro reports on food protein-derived peptides with renin inhibitory properties or on the existence of a double ACE and renin inhibitory capacity. He et al.[34] reported an inhibition capacity on ACE of 76% when a complete rapeseed protein hydrolysate was used. Instead, Girgih et al.,[3] who worked with salmon proteins, obtained an IC $_{50}$ (ACE) = 0.10 mg/mL, and Onuh et al.,[35] who worked with chicken skin protein hydrolysates, reported an IC_{50} (ACE) = 0.64 mg/mL. In general, the ACE IC_{50} inhibition values for hydrolysates are lower than those found for renin, which is in line with our results, where the IC_{50} value for renin (0.6 mg/mL) is higher than the IC_{50} value for ACE (0.12 mg/mL) obtained in a previous work.[14,16]

Aluko[5] suggested that the peptides inhibitory mechanism for ACE and renin are different. The differences between both enzymes originate in the tertiary structure, with the active site of ACE being more accessible than that of renin.[28,31]

AHH Renin Inhibition Kinetics Parameters. The study of enzymatic mechanisms and the analysis of the structural consequences derived from enzyme−inhibitor interactions is a powerful tool to enhance the design of new and potent blood pressure-reducing peptides. In a first approach, we determined the AHH renin inhibition kinetics parameters by means of Lineweaver−Burk plots (Figure 4).

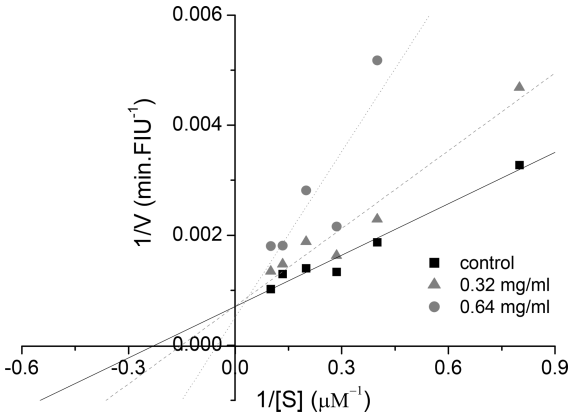

Figure 4. Lineweaver−Burk plot of human recombinant renin inhibition at different concentrations of AHH.

The kinetics parameters determined were V_{max} and K_M and are presented in Table 1. Results indicated that the AHH exhibits a renin competitive inhibition behavior. This type of enzymatic inhibition is characterized by an increase of K_M values, while V_{max} remains unchanged, thus indicating that the AHH protein inhibitor may interact with renin but not with the renin−substrate complex.

Table 1. Catalytic Parameters Obtained from Lineweaver−Burk Plots for Renin Inhibition at Different AHH Concentrations

	peptide concentration (mg/mL)		
catalytic parameter	0	0.32	0.64
K_M or K_M' (μM)	4.5 ± 0.8	6 ± 3	13 ± 5
V_{max} or V_{max}' (FI/min)	1429 ± 225	1272 ± 582	1305 ± 460
K_i (mg/mL)	0.25	0.25	0.25

The K_M value (4.4 μM) obtained without the addition of the inhibitor was similar to the value reported by other authors.[3,28,31,36] Slight differences in the results may be due to the different renin sources used in each study.

The K_i value (0.25 mg/mL) is lower than that obtained for hemp seed hydrolysate,[31] which implies that a lower amount of AHH is required for renin inhibition.

The renin inhibition kinetics has been studied for a variety of peptides from different plant and animal protein sources. Different types of renin inhibition mechanisms (competitive, mixed, noncompetitive, and uncompetitive) were found.[3,28,31,35,37,38]

Although some competitive inhibitors have been described, most peptides reported in the literature are noncompetitive or mixed inhibitors, suggesting the existence of other sites on the enzyme that could interact with the inhibitory peptides and induce changes in the protein three-dimensional structure, thereby modifying the active site of the enzyme.

Characterization of Renin Inhibitory Peptides. *AHH Fractionation by Size Exclusion Chromatography and Determination of Renin Inhibition.* The AHH gel filtration fractionation and the renin inhibitory capacity of some fractions are presented in Figure 5A and 5B, respectively). Fractions f4, f5, and f6 showed high renin inhibition activity (29 ± 2%, 49 ± 5%, and 25 ± 1%, respectively); however, these fractions had undetectable protein levels by the micro-Kjeldahl method. These results could be attributed to the presence of very small amounts of proteins/peptides in the samples and/or to the existence of compounds of nonprotein nature with renin inhibitory activity. However, the latter option is more remote because, unlike ACE,[39] there is no available information about nonprotein molecules with biological activity (such as flavonoids or polyphenols) capable of inhibiting renin. Concerning the presence of nonprotein compounds, we cannot reject neither that hypothesis nor the possibility of a highly potent peptide/s present in a low protein concentration

In fractions f7−f10, variable amounts of peptides were detected. Figure 5B shows the percentage of inhibition per milligram of peptide in every fraction.

These results suggest that the AHH has several renin inhibitory peptides that act synergistically, since 1 mg peptide/mL of AHH inhibited about 65% of the enzymatic activity. Size fractionation of such peptides led to an activity loss (f7−f10 did not exceed 50% of inhibition/mg peptide). This may be because the peptides that act synergistically in the AHH are in different fractions, as reported by other authors.[31,35]

No clear evidence is available for renin inhibitor peptides. Most of the renin inhibitory peptides found in the literature are small, positively charged, and containing hydrophobic amino acids.[8] However, Fitzgerald et al.[32] reported a tridecapeptide obtained from the macroalga *Palmaria palmata* with an IC_{50} value of 3.344 mM for renin inhibition.

Fractions f7−f10 were analyzed by RP-HPLC using an analytical C18 column (Figure 5C). These results suggest that several renin inhibitory peptides with a wide range of hydrophobicity (related with elution time) are present in each fraction.

The peptidic hydrophobicity behavior was compared for fractions f7−f10, splitting the chromatogram into two arbitrary regions. Peak areas were calculated on a range of eluted

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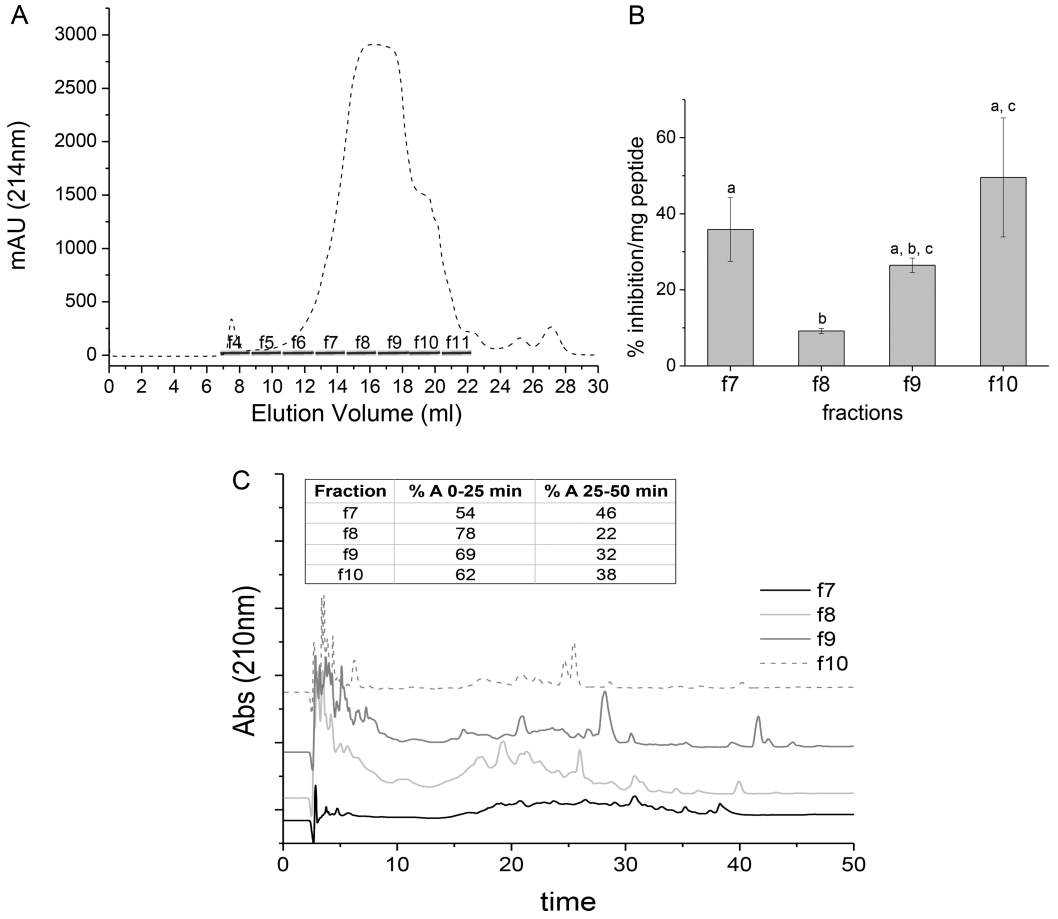

Figure 5. (A) Superdex peptide 10/300 GL gel filtration profile of AHH. At the bottom fractions f4−f11 are indicated. (B) Renin inhibitory activity of AHH fractions from gel filtration. Bars (mean ± standard deviation) with different letters have mean values that are significantly different ($p < 0.05$). (C) Analytical RP-HPLC profile of AHH fractions from gel filtration (f7−f10). (C, insert) Percent area of the hydrophilic (% *A* 0−25 min) and hydrophobic (% *A* 25−50 min) species present in the analyzed samples were indicated.

fractions: hydrophilic zone, between 0 and 25 min time elution; hydrophobic zone, between 25 and 50 min time elution (insert Figure 5C). The area of the more hydrophobic region was lower for f8 (22%), whereas the corresponding areas for f7, f9, and f10 were greater (46%, 32%, and 38%, respectively). These results suggest that the potency of renin inhibition was directly related to the hydrophobicity level. This finding is in agreement with the results reported by Aluko,[8] who found that the potency of renin inhibitory peptides increases with the presence of branched, positively charged, aromatic and hydrophobic amino acids.

AHH Fractionation by RP-HPLC Chromatography and Determination of Renin Inhibition. Identification of Possible Renin Inhibitor Peptides. Figure 6 shows the results of RP-HPLC AHH fractionation and the renin inhibitory activity of each fraction. This amaranth hydrolysate profile was separated into six fractions (FI−FVI) according to their hydrophobicity. These results confirmed the above-mentioned relationship between hydrophobicity and potency for renin inhibition. Similar results were reported by other authors who found that renin inhibitory peptides contain high levels of hydrophobic amino acids.[3,5,32,40]

Although fractions FV and FVI are capable of inhibiting renin (approximately 39% and 27%, respectively), the amount of nitrogen detected in these fractions is very low (approximately 0.2 $\mu g/\mu L$). Nevertheless, we cannot dismiss the presence of either a small amount of highly active peptide/s or an active nonprotein component. This last option is more remote since there are no reports in the literature that account for renin inhibitors associated with secondary metabolites present in the seeds, and their relative abundance in AHH is quite scarce.

FIV showed an inhibition of 67 ± 5%/mg of peptide. These results were equivalent to the AHH inhibition, and for this reason the peptide sequences present in FIV were analyzed by liquid chromatography coupled to tandem mass spectrometry.

Taking into consideration that AHH comes from AHI and this was composed by seed storage proteins, we search for peptides belonging to 11S globulin (the only amaranth seed storage protein that is sequenced until now). Six peptides belonging to this globulin were identified and grouped into 3 families (Table 2), which correspond to sequences that colocalize in the same region of 11S globulin sequence. The sequenced peptides contain about 43% hydrophobic residues of which 27% were aliphatic (A, V, I, L) and 17% aromatic amino acids (F, W, Y). The hydrophilic residues represent the 47% of which 17% were acidic (D, E), 20% polar uncharged (S, T, N, Q), and 10% basic amino acid (K, R, H). The remaining 10% of the total amino acids in the three families identified was P and G amino acid. According to the analysis of information made in specialized databases such as MEROPS (http://merops.sanger.ac.uk/cgi-bin/pepsum?id=A01.007) and BRENDA (http://

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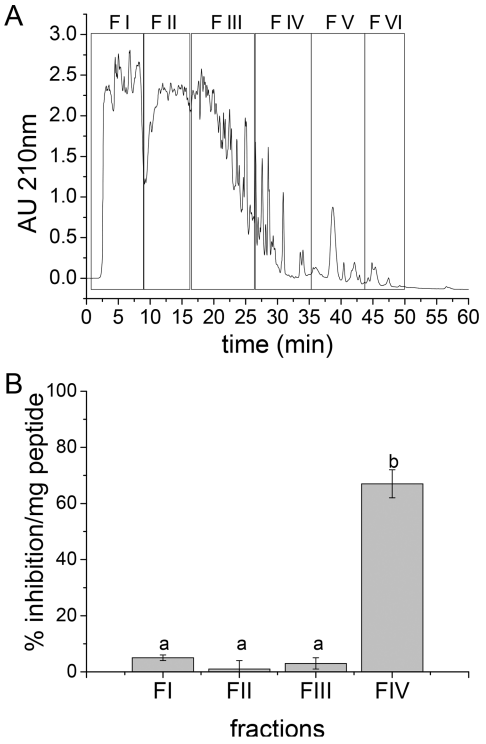

Figure 6. (A) Preparative RP-HPLC. AHH fractionation. (Top) Fractions FI–FVI are indicated. (B) Renin inhibitory activity of AHH fractions from RP-HPLC fractionation. Bars (mean ± standard deviation) with different letters have mean values that are significantly different ($p < 0.05$).

Table 2. Peptides Identified by MALDI-TOF/MS in FIV RPHPLC Fractionation of AHH

family	sequence	Mr	number of aa
1	QAFEDGFEWVSFK	1589,7271	13
	-AFEDGFEWVSFK	1461,6685	12
2	SFNLPILR	959,5673	8
	-FNLPILR	872,5352	7
	SFNLPIL–	872,5352	7
3	VNVDDPSKA	944,4683	9

www.brenda-enzymes.org/enzyme.php?ecno=3.4.24.11) known renin inhibitors are synthetic molecules and peptidomimetics. On the other hand, in the scarce references available for peptidic renin inhibitors, we found that in the great majority these are di- or tetrapeptides, with little information on its structure–inhibitory relationship. Udenigwe et al.[41] predicted that the most active dipeptides according to a QSAR model would be those containing aromatic and voluminous amino acids (W, Y, F) at the C-terminus with less voluminous hydrophobic residues (V, L, I, A) at the N-terminus. In the case of the tetrapeptides, Girgih et al.[40] reported a group of sequences which in some cases suggest competitive and nonspecific inhibition mechanisms due to the binding of peptides to different sites than the active site. In the case of WYT, SVYT and IPAGV found that they act in a noncompetitive way.[42] He et al.,[36] based on docking studies, indicated that LY and RALP inhibit renin activity is due to the active site distortion, resulting in a loss of effectiveness of the interaction between the catalytic residues and the substrate. These authors suggest that the presence of a positively charged amino acid such as R may improve formation of multiple interactions and contribute to improved affinity of inhibitory peptides for renin. Moreover, a structure-based design in silico assay reported small renin inhibitory peptides, including 2–3 and even 4 amino acids.[43] On the other hand, Fitzgerald et al.[32] reported the existence of a tridecapeptide of *Palmaria palmata* macroalgae with in vitro renin inhibitory activity in agreement with peptide length found in this work.

Amaranth-identified sequences are not homologous to any of the reported peptide renin inhibitors. It is not a valid approach to infer the sequence responsible for the activity detected in the active fraction exclusively from its amino acid composition, especially considering the limited number of renin inhibitory peptides available in the literature. On the other hand, some of the peptides identified in amaranth contain 12–13 amino acid residues and could therefore adopt some three-dimensional conformation that could affect their activity. Further studies are needed to confirm these results, and it cannot be ruled out that the renin inhibitory activity is due or not to the combination of more than one peptide. Future assays will be done in order to study complementarity between enzyme and substrate (docking) with these peptides and small peptides derived from them, simultaneously with synthesis of peptides to assess their activity and validate in silico analysis and determine their kinetic parameters.

Bearing in mind that the renin in the RAS system specifically acts on one kind of substrate (angiotensinogen), inhibition of this enzyme is a key step in blood pressure regulation. The results presented herein have shown that amaranth seed proteins are a good source of peptides able to decrease renin activity. Besides, some authors have described ACE inhibitor peptides in amaranth proteins.[14−18,44,45] This fact turns amaranth seeds into an interesting source of bioactive peptides acting on the two major enzymes that regulate the RAS. Thus, amaranth proteins are good candidates to be used in the formulation of functional foods. The highest renin inhibitory activity determined for the whole AHH is also an advantage to lower additional costs of obtaining purified fractions.

This work is a first approach to the study of the amaranth renin inhibitory activity, and further experimental work should be performed to prove the in vitro action of sequenced peptides found in fraction FIV and to determine the in vivo effects of these preparations. However, this work contributes to increasing potential uses of amaranth as a renewable source of proteins and provides an opportunity for development of a cardioprotective ingredient for functional foods.

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Notes

The authors declare no competing financial interest.

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