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ORIGINAL PAPER

Broken Rice as a Potential Functional Ingredient with Inhibitory Activity of Renin and Angiotensin-Converting Enzyme(ACE)

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Abstract

The aim of this work was to evaluate the ability of broken rice, an underutilized industrial by-product, as a potential functional and health promoting ingredient. With this purpose, the ability to inhibit the angiotensin converting enzyme and renin of a rice protein hydrolyzate (RPH) obtained from a high-protein variety of broken rice (var. Nutriar FCAyF) was analyzed (IC_{50} = 0.87 and 2.7 mg/mL, respectively). RPH was separated by gel permeation chromatography and in a second purification step by RP-HPLC. The sequence of antihypertensive peptides presented in two RP-HPLC fractions was analyzed. Peptides capable of interacting with the active sites of both enzymes were identified. In this study, we demonstrate that the hydrolysis treatment improves functional and biological properties of rice proteins. Protein preparations obtained from a by-product of rice industry, such as broken rice, are a promising ingredient with potentially good biological properties.

Keywords Broken rice · ACE inhibitory peptides · Renin inhibitory peptides · Molecular docking analysis

Introduction

Rice (*Oryza sativa* L.) is one of the leading food crops in the world, with a global annual production estimated at about 480 million metric tons (milled rice basis) [1] cultivated today in more than 100 countries. Even though, Asiatic continent is the main rice producer and consumer followed by Africa and United States, nowadays rice production in South America and Caribbean region is increasing due to regional soil characteristics and the abundant reserves of water (www.agroindustria.gob.ar, October 2017). In Argentina in particular, a great part of rice annual production (1.5 million tons) is exported, being a minority the one used in the domestic market [2, 3].

As part of the Rice Program research framework within Facultad de Ciencias Agrarias y Forestales (UNLP) and local rice producers from Provincia de Entre Ríos, Argentina, a high-protein variety of rice, Nutriar (with a 30% more protein than common varieties), was breeded in the Julio Hirschhörn Experimental Station, La Plata [4]. This registered variety is the result of a genetic crossing between rich-glutelin content Philippine rice variety and a local one adapted to regional agronomic conditions. The variety registration was made in the year 2003 in Ministerio de Agroindustrias, Argentina.

Although rice is not a protein rich crop and its proteins have poor digestibility and solubility [5] at neutral pH, in recent years the nutritional and health properties of rice proteins have been widely recognized. Nowadays rice protein-containing products have become commercially available. The increasing interest of industry and the academic research communities in these ingredients enabled the development of processes for the extraction, enrichment, purification and functionalization of rice proteins. A possible course to improve rice functionality is to subject it to controlled hydrolysis treatments. Other low cost and rapid process used widely is *in vitro* gastrointestinal digestion [6, 7]. These hydrolysis processes allow improving the functional properties of these proteins as well as

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generating beneficial bioactive peptides for health. In particular, it has been described peptides with antioxidative activity [6], anticancer property [8], and ability to disrupt cholesterol micelles [9]. Besides that, different peptide sequences have been identified in rice bran protein hydrolysates, particularly di and tripeptides, with relevant inhibitory capacity of ACE *in vitro* [10] (additional data are given in on line resource, literature cited, 3–5). Most of the described peptides were obtained from rice bran. However, an important by-product of rice production is broken rice, which has a low commercial value and is mainly used for animal feed and vegetable oil industry [11]. This by-product could become a source of bioactive peptides.

In recent years, bioinformatic tools have played a decisive role in the discovery of bioactive peptides [12]. One of the most used tools are molecular docking techniques. This method can be used to identify ligands that binds to a specific receptor binding site and to determine its preferred, more energy-efficient binding posture [13]. This technique allows estimating the binding energy of the peptides with the enzyme studied and, in this way, this tool can contribute to select one or several probable active sequences from a list of identified peptides.

The main objective of this work was to evaluate the ability of broken rice, an underutilized industrial product, as a potential health-promoting ingredient. In order to achieve this purpose, antihypertensive ability of rice peptides was examined by studying enzymatic inhibition of renin and angiotensin converting enzyme (ACE), the main enzymes that control blood pressure. Subsequently, peptides identified in active fractions were selected to analyze, through docking techniques, a possible relationship between potent enzyme inhibitors and their structural interactions with the enzymes, ACE and renin.

Materials and Methods

Materials

The Nutriar FCAyF rice (*Oryza sativa* L.) cultivar, was supplied by Programa Arroz, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Argentina.

Chemicals were obtained from commercial sources: trizma base, sodium dodecylsulphate (SDS), tricine, bovine serum albumin, *o*-phtalaldehyde (OPA), hippuryl-histidyl-leucine (HHL), Captopril [(2-methyl 3-sulfanylpropanoyl) pyrrolidine-2-carboxylic acid] and cyanuric chloride (2,4,6-Trichloro-1,3,5-triazine) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pepsin (E.C. 3.4.23.1) 5X NF was from MP Biomedicals LLC (Solon, OH, USA) and Renin Inhibitor Screening Assay Kit from Cayman Chemical (Ann Arbor, MI, USA).

Preparation of Protein Samples

Broken Rice Flour (BRF) Husk and bran layers were removed by means of an experimental mill (Guidetti and Artioli universal type, Renazzo, Italy). An Udy mill of 1 mm mesh (UDY Corporation and Alpha Plastic and Design, Fort Collins, CO, USA) was used to obtain ground rice flour and sieved through a 150 mesh. Ground and sieved flour was defatted with hexane (10 g flour/100 mL solvent) for 24 h at room temperature.

Rice Protein Isolated (RPI) BRF was suspended in water (10 g flour/100 mL solvent) and pH was adjusted to 12 with 2 mol/L NaOH. The suspension was stirred for 60 min and centrifuged for 30 min at 9000 *g* at 25 °C. The obtained supernatant was adjusted to pH 5.5 (2 mol/L HCl) and centrifuged at 9000 *g* for 20 min at 4 °C. Precipitate was suspended in water, neutralized and freeze-dried.

Rice Protein Hydrolyzate (RPH) RPI was suspended in water (10 g RPI/100 mL solvent). The pH was adjusted to 2 and hydrolyzed with pepsin using 0.1 g pepsin/g protein (1:15000 5X NF units, MPBiomedicals, Santa Ana, CA, USA). The suspension was stirred for 60 min at 37 °C and pH value was controlled and adjusted every 15 min using 0.1 mol/L NaOH. The enzymatic activity was stopped by thermal treatment at 90 °C for 10 min. RPH was neutralized and freeze-dried.

Structural Characterization

Determination of the Protein Content The protein content of BRF and sample preparations was determined by the micro-Kjeldhal method using a protein-nitrogen factor of 5.95 [14]. The Lowry method was used for the determination of solubility and protein concentration adjustment for ACE and renin.

Determination of the Hydrolysis Degree The hydrolysis degree (DH) was measured by means of the reaction of free amino groups with OPA [15]. The percentage of hydrolysis was calculated according to Quiroga *et al.* [16].

Tricine-Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis RPI and RPH were analyzed by tricine-SDS-PAGE under reducing conditions according to Schägger [17]. Briefly, an equivalent protein mass (20 mg) was loaded onto each lane and runs were carried out in a Mini Protean II Dual Slab Cell (BIO-RAD, Hercules, USA). Polypeptide SDS-PAGE standards (MW 1.4–26.6 kDa) (BIO-RAD) were used as molecular markers.

Gel Filtration Liquid Chromatography RPI and RPH soluble fractions were dissolved in 0.2 mol/L borate buffer (pH 8.3)

0.4 mol/L NaCl (exclusion buffer) and analyzed in an FPLC ÄKTA purifier (GE-Healthcare, Uppsala, Sweden) with a Superdex 75 10/30 HR column. Mass molecular calibration markers were run in exclusion buffer (conalbumin 75 kDa, ovoalbumin 43 kDa, ribonulease A 13.7 kDa and aprotinin 6.5 kDa). Protein samples (4 mg) were dissolved with the exclusion buffer, solubilized for 1 h with agitation, and centrifuged at room temperature. Polypeptides and peptides were detected at 214 nm. Data were processed and evaluated using the Unicorn Software (GE-Healthcare, Uppsala, Sweden).

Protein Solubility Protein suspension 1 g/100 mL were prepared either in water or in one of the following 0.2 mol/L sodium salt buffers: **a)** 0.17 mol/L $C_6H_8O_7$and 0.03 mol/L $C_6H_7O_7^{1-}$ (pH 2); **b)** 0.08 mol/L and 0.12 mol/L$C_6H_7O_7^{1-}$ (pH 3); **c)** 0.015 mol/L $C_6H_8O_7$, 0.15 M $C_6H_7O_7^{1-}$, 0.035 M $C_6H_6O_7^{2-}$ (pH 4); **d)** 0.06 mol/L $C_6H_7O_7^{1-}$, 0.14 mol/L $C_6H_6O_7^{2-}$ (pH 5); **e)** 0.18 mol/L $H_2PO_4^{1-}$, 0.02 mol/L HPO_4^{2-} (pH 6); **f)** 0.12 mol/L $H_2PO_4^{1-}$, 0.08 mol/L HPO_4^{2-} (pH 7); **g)** 0.132 mol/L $H_2BO_3^{1-}$, 0.068 mol/L HBO_3^{2-} (pH 8) and **h)** 0.046 mol/L $H_2BO_3^{1-}$, 0.154 mol/L HBO_3^{2-} (pH 9). To maintain the ionic strength at 0.54 NaCl was added when needed. Samples were prepared by agitation for 1 h at 25 °C, 500 rpm (Thermomixer, Eppendorf, Hamburg, Germany) and centrifuged 10 min at 10,000 *g* at 25 °C. Supernatants were analyzed and soluble protein concentration determined by the Lowry method.

Antihypertensive Properties

ACE Inhibitory Assay The ACE inhibitory activity assay was performed according to Hurts and Lovell-Smith [18]. Briefly, a protein solution (5 mg/mL, 25 μL) were added to a 0.2 M borate buffer (pH 8.3) + 2 M NaCl, and 5 mmol/L hyppuryl-histidyl-leucine (HHL) and ACE (Sigma-Aldrich) were incubated at 37 °C for 30 min at 500 rpm in a shaker (Thermomixer, Eppendorf, Hamburg, Germany). The enzymatic reaction was stopped by heating at 90 °C for 15 min. Then, 600 μL of 0.2 mol/L KH_2PO_4 buffer, pH 8.3 were then added and followed by the addition of 515 μL of 3% *w/v* cyanuric chloride in dioxane. The hippurate released was measured at 382 nm in a Beckman DU 650 spectrophotometer.

To select the fractions that would be sequenced later, *in vitro* ACE inhibitory activity of the fractions obtained by gel filtration chromatography and RP-HPLC, were assessed using 25 μl of an ACE crude extract (ACEce) obtained from rabbit lungs instead of 25 μl of commercial ACE (additional data are given in on line resource).

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, Winooskii, USA).

The IC_{50} values were calculated by dose–response curves. The logarithmic values of four different sample concentrations (mg/mL) against the inhibitory activity (%) were plotted.

The IC_{50} values were calculated by dose-response curves using nonlinear regression sigmoidal curve fit functions in GraphPad prism 6.01 (Graphpad Software Inc., San Diego, CA, USA).

RPH Fractionation and Characterization of Inhibitory Peptides

Gel Filtration Liquid Chromatography The RPH profile was analyzed at room temperature in a Superdex Peptide 10/300 GL column using a FPLC ÄKTA Purifier (GE-Healthcare, Uppsala, Sweden). Briefly, the RPH (20 mg) was dissolved in 1 mL of buffer 100 mmol/L NH_4HCO_3 (pH 8), centrifuged at 13,000 *g* 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 (absorbance at 214 nm) was obtained. Column calibration was made with aprotinin (Sigma-Aldrich 6.5 kDa), B12 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 (GE-Healthcare, Uppsala, Sweden). Fractions obtained from three experiments were collected and freeze-dried.

Preparative RP-HPLC A SunFire C8 column (5 μm, ST 10/250) (Waters Corp., Milford, USA) column was used to separate the peptides present in the RPH soluble fractions on a Waters System HPLC (Waters Corp., Milford, USA) equipped with a diode array detector. Prior to analysis, the RPH was suspended in buffer 50 mmol/L Tris-HCl, 10 mmol/L NaCl pH 8 at a ratio of 25 mg/mL, centrifuged at 13,000 *g* for 20 min at 25 °C, and filtered (nylon filter 0.45 μm pore size). Samples (2 mL) were injected and eluted with a linear gradient from 0 to 100% solvent B in 55 min (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. In order to eliminate acetonitrile six RP-HPLC fractions collected, pooled and concentrated in a

Concentrator Plus (Eppendorf, Hamburg, Germany) and then freeze-dried.

LC-MS/MS Analysis The amino acid sequence in active fractions was determined using a nano-HPLC coupled to a mass spectrometer with a high-collision dissociation cell and an Orbitrap analyser (LC-MS/MS). This platform allows peptide identification and chromatographic separation simultaneously. Two microliters (2 μL) of RP-HPLC fraction sample was injected into a nano-HPLC (EASY-nLC, Thermo Scientific, Waltham, USA), equipped with a C18 column (EASY-Spray Accucore ES801: 2.6 μm, 75 μm × 150 mm) at 35 °C. 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). Samples (2 μl) were injected and eluted with a gradient (5% B to 35% B in 110 min; 35% to 95% B in 1 min and remained in 95% during 9 min). Flow rate of 300 nL/min.

Sample ionization was performed by electrospray (Q Exactive Hybrid Quadrupole System, Thermo Scientific, Waltham, USA) using a spray voltage of 3.5 kV. Protein identification was achieved by comparing mass data against the UniProtKB databank (https://www.uniprot.org) restricted to the *Oryzia sativa* taxonomy (February 2017) using Proteome Discover 1.4 software (Thermo Scientific, Waltham, USA). 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.

Molecular Docking

The molecular structures of peptides were generated with PepFold3 (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/) [19]. The crystal structure of human ACE (PDB ID 4APH) and renin (PDB ID 2V0Z) were obtained from Protein Data Bank (www.rcsb.org) and docking analysis was performed using AutoDock Tools 4.2.6 and AutoDock 4.0 (Script Rearch Institute, La Jolla, CA, USA). Before the docking, water molecules and other molecules (angiotensin II for ACE and aliskiren for renin) were removed whereas the cofactors zinc and chloride atoms were retained in ACE model. The polar hydrogens were then added. For ACE the docking runs were carried out with coordinates x: 13.499, y: 6.014 and z: −18.880 and with a radius of 9.375 Å. To renin a binding site with coordinates x: 34.414, y: 13.1777, z: 87.996 and a radius of 9.375 Å was created around the ligand. The best ranked docking pose of peptides in the active site of ACE and renin was obtained according to the scores and binding-energy value.

Statistical Analysis

A multifactorial variance analysis (ANOVA) was performed using the Statgraphics Corp. software, (Rockville, USA). LSD's multirange test ($p \leq 0.05$) was used to compare different variables means. Results were expressed as the mean of at least three measurements.

Results and Discussion

Structural and Functional Characterization

The BRF protein content was 11.2 ± 0.5%. RPI and RPH protein contents were 97.7 ± 0.4 and 88 ± 2% (*w*/w; wet basis) respectively. These results are in line with the ones using rice bran subjected to enzymatic and/or physical and enzymatic treatments.

The protein samples hydrolysis degree (DH) was measured. RPI was considered as hydrolysis degree reference. The value for DH in RPH sample was 5.46 ± 0.07%. This result is in coincidence with Adebiyi *et al.* [20], who found similar values for hydrolysis with pepsin.

In order to characterize the samples, gel filtration chromatography and tricine-SDS-PAGE were performed. Figure 1a shows the main components of the RPI (continuous line) and RPH (dashed line). The RPI main components were species poorly resolved by the column with molecular masses greater than 75 kDa corresponding to glutelins and to a lesser extent, some loose polypeptides with molecular masses less than 16 kDa. On the other hand, RPH shows protein profile with less molecular size than the species present in RPI because of the hydrolysis treatment. RPH principal components were species with molecular masses between 30 and 2.1 kDa, respectively. RPI and RPH tricine-SDS-PAGE protein profiles are shown in Fig. 1b. Results are coincident with the gel filtration profile, which shows the reduction in molecular size of the major components of RPI upon enzymatic treatment (Fig. 1b**,** RPI, red circle). The majority of rice grain proteins are in the glutelin fraction. These proteins are extensively aggregated, disulfide bonded, and glycosylated and difficult to solubilize [21, 22]. Rice glutelins are composed of high-molecular-weight proteins ranging from 45 to 150 kDa and they are formed by several polypeptides of 57, 34–37, 25, 21–23, 16, and 14 kDa [23, 24].

On the other hand, the RPH sample showed two well-defined hydrolyzed polypeptide groups, one group ranging between 14.2–6.5 kDa and another group comprising polypeptides smaller than 6.5 kDa. A polypeptide with a molecular mass of 20 kDa was present at a very low proportion. In other species, this polypeptide is known to be highly resistant to hydrolysis [21].

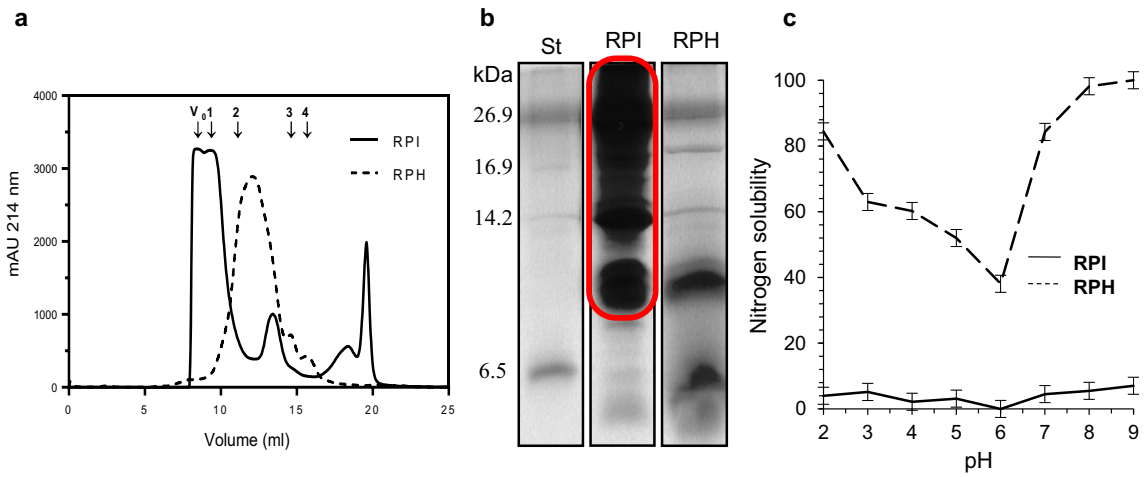


Fig. 1 **a** Gel filtration chromatography (Superdex 75 10/30 HR column). RPI: rice protein isolate, RPH: rice protein hydrolysate. Arrows indicate the elution volumes of standard proteins. V0: Void volume; 1: Albumin (66 kDa); 2: Carbonic anhydrase (29 kDa); 3: Cytochrome C (12.4 kDa); 4: Aprotinin (6.5 kDa). **b** Tricine SDS-Page. St: standard low molecular weight marker. RPI: rice protein isolate. RPH: rice protein hydrolysate. **c** Protein solubility at different pHs of RPI and RPH

To analyze the change in the functionality of rice proteins, we analyzed protein solubility before and after the hydrolysis treatment. Figure 1c shows solubility along a pH gradient ranging from 2 to 9. RPI presented the lowest solubility, since glutelins are the main fraction (about 80%) [14], soluble in dilute acid and alkali solutions. Previous works performed on this variety [4] have reported that Nutriar variety exhibit different protein structural characteristics between them the possibility that it may possess two glutelin subfamilies with different degrees of polymerization compared to a standard cultivar rice isolate as El Paso variety. On the other hand, RPH exhibited the highest ($P < 0.05$) solubility at all the assayed pHs due to the reduction in the molecular weights caused by hydrolysis. This fact indicates the generation of an increased number of smaller, more hydrophilic and solvated peptides units after enzymatic hydrolysis. The increase of the protein solubility in the whole range of pH analyzed, allows extending its application in formulated food systems, since a good solubility is crucial for an attractive appearance and a softer mouthfeel of the final product [7].

Biological Activities: ACE and Renin Inhibition Capacity

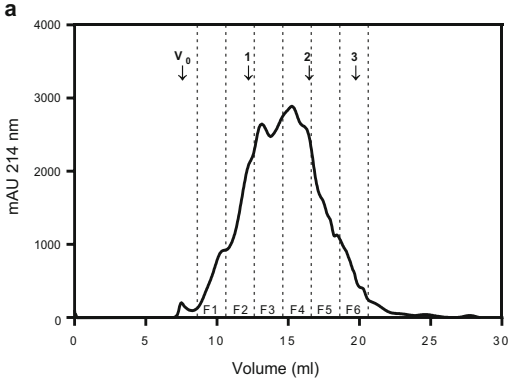
As expected RPH presented higher inhibition capacity than RPI sample for both enzymes. RPH inhibit ACE and renin in a dose-response manner (IC_{50} = 0.87 and 2.7 mg/mL, respectively). In general, the ACE IC_{50} inhibition values for hydrolysates are lower than the ones found for renin, which is in line with our results [16, 22, 25].

RPH present a IC_{50} value for renin higher than that reported for different protein sources [16, 26, 27] and this work is the first one to report the presence of renin inhibitory peptides from rice. Previously, other authors [28] reported inhibitory activity of renin in a methanol extract obtained from rice, but these authors identified oleic and linoleic acid as responsible for the inhibition. On the other hand, ACE IC_{50} value is comparable with values reported by other authors for rice protein hydrolysates although the majority of them uses bran rice proteins [10] (additional data are given in on line resource, LITERATURE CITED, 3–5).

Figure 2 shows RPH fractionation by gel filtration chromatography (a) and ACE and renin inhibitory capacity of each fraction (b). RPH was fractionated into six fractions (F1 − F6). The obtained results show that, statistically, all the fractions inhibit ACE activity in equal percentage, while F5 and F6 show the highest inhibitory activity with respect to renin which suggests that these fractions would be enriched in the more active renin inhibitory peptides.

According to bibliographic data, it is known that the peptides with the highest inhibitory potency over ACE are of small size (di, tri or tetrapeptides) [26] (additional data are given in on line resource, LITERATURE CITED, 2). On the other hand, although several sequences of food-derived peptides with renin inhibitory activity have been identified, little is known about the relationship activity-structure. Although some authors [25, 26, 29] have explored the characteristics of peptides capable of inhibiting renin, such as its molecular size and hydrophobicity, the results provided do not go in the same direction. It has been suggested that the presence of N-terminal aliphatic and C-terminal bulky amino acid residues contributes to higher renin inhibitory activity at least in dipeptides [30]. Considering these results we selected F5 and F6 for further fractionation by RP-HPLC because they were the most active fractions for renin inhibition and they were enriched in small peptides that has been described as the most active inhibitory peptides to ACE.

a



b

Fraction	% ACE inhibition	% Renin inhibition
F1	16 ± 4 [ab]	ND
F2	ND	17 ± 9 [a]
F3	19 ± 3 [b]	17 ± 4 [a]
F4	16 ± 4 [ab]	11 ± 8 [a]
F5	10 ± 4 [a]	42 ± 5 [b]
F6	10 ± 2 [a]	31 ± 3 [b]

Fig. 2 **a** RPH fractionation in a Superdex peptide 10/300 GL. Arrows indicate the elution volumes of standard proteins. V_0: Void volume; 1: Aprotinin (6.5 kDa); 2: Vitamin B12 (1.355 kDa); 3: Hippuric acid (0.179 kDa). **b** ACE and renin inhibitory capacity of each fraction at concentration of 1 and 0.3 mg/mL of protein soluble, respectively. Different letters in the same column mean significant differences at $p \leq 0.05$ according to LSD's test. ND: Non detected

Figure 3 shows the chromatogram obtained using RP-HPLC of F5 (a) and the inhibitory capacity of some fractions on ACE and renin (b). F6 (data not shown) was solved in a smaller number of peaks of which three were selected to measure its activity. The results obtained were significantly lower than those found for F5; therefore, we chose the F5-IV and F5-V fractions to be sequenced.

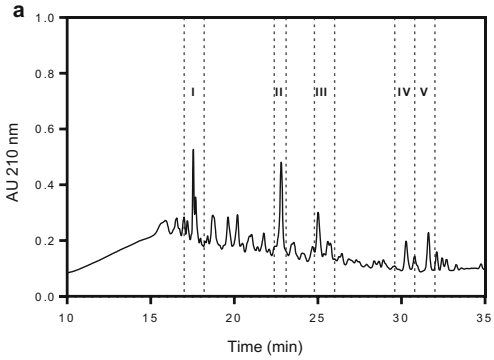
Peptide Identification and Molecular Docking Analysis

Most abundant peptides in active mixture F5-IV and F5-V were identified by liquid chromatography coupled to tandem mass spectrometry. Both fractions presented a complex mix of peptides (molecular masses between 800 and 1400 Da) from different rice proteins mainly glutelins and prolamins, the main storage proteins of this cereal. Table 1 summarizes the most relevant sequences identified. The peptides with masses 1215.616, 1195.576 and 948.457 were present in both F5-IV and F5-V and they were also identified as the same peptides, FNVPSRYGIY, PWHNPRQGGF and SPFWNINA, respectively. This is a common phenomenon when two adjacent fractions from chromatography are analyzed [31].

The sequences found were compared with bibliography data and databases. The ACE inhibitory peptides previously reported were explored by searching in BIOPEP-UWM (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep, June 2018) and the published literature. Renin is a much less studied enzyme as a target in food science and this is reflected because its inhibition does not exist as an activity in BIOPEP-UWM. In this case, the comparison was made with sequences reported in literature. Table 1 shows that several of the identified peptides contain encrypted small sequences reported as ACE inhibitors; therefore, those peptides must be part of the active factors that made the fraction exhibit high ACE inhibitory activity. The octapeptide SPFWNINA present in F5-IV and F5-V has been previously reported with a different activity: as a prolyl endopeptidase (EC 3.4.21.26) inhibitor. This cytosolic enzyme is involved in the maturation and degradation of peptide hormones and neuropeptides. Saito *et al.* [32] identify this sequence from sake, a traditional alcoholic beverage obtain from rice in Japan.

The modeling of the structure of a protein-ligand complex is important to understand the binding interactions between a potential ligand and its target. Even though this modeling usually helps to evaluate the thermodynamic stability of the complex, protein–peptide docking is generally more challenging than docking of other type of molecules. Short peptides

a



b

Fraction	% ACE inhibition	% Renin inhibition
F5-I	9 ± 2 [a]	27 ± 2 [a]
F5-II	11 ± 2 [ab]	23 ± 9 [a]
F5-III	15 ± 2 [b]	26 ± 2 [a]
F5-IV	27 ± 3 [c]	23 ± 2 [a]
F5-V	26 ± 2 [c]	49 ± 9 [b]

Fig. 3 **a** F5 fractionation in a preparative C8 by RP-HPLC. Fractions I − V are indicated. **b** ACE and renin inhibitory capacity of each fraction. Different letters in the same column mean significant differences at $p \leq 0.05$ according to LSD's test. ND: Non detected

Table 1 Peptide profile identified by LC-MS/MS in HPLC fraction of rice hydrolysate

Fraction	Peptide	MW (Da)	Protein
F5-IV	QLFNPS*TNP*WH	1340.640	Glutelin
	PWHNPRQGGF*	1195.576	(UniProtKB AC Q0E2D2; A1YQG5; A1YQH4; D6BV14; A2Z708; A2X2Z1; Q6K7K6; Q6T725)
	YQNAL*LSP*F	1052.540	
	SP*FWN*INA**	948.457	
	I*LSPFWN*VNAH	1297.670	
	VIRRVIQPQGL	1278.800	
	IAVPAGVAHWL	1133.647	
	FNVPSRYGIY***	1215.616	Prolamin
	LAFNVPSRY	1066.568	(UniProtKB AC Q9SAY8; Q5EFA3; Q5W755; A1YQF0; Q8GVK5)
	Y*IAP*RSIPTVGG	1230.684	
F5-V	PWHNPRQGGF*	1195.576	Glutelin
	QLFGPNVNPWHN	1422.691	(UniProtKB AC D6BV14; A1YQG5)
	SP*FWN*INA**	948.457	
	*VALP*AGVAHW	1020.563	
	FNVPSRYGIY***	1215.616	Prolamin (UniProtKB AC A0A0N7KKJ8)
	WEDIGTIK	961.499	UniProtKB AC B9FWD3

With asterisks, the identical peptides identified in both fractions are indicated. Italic letters indicate ACE inhibitor peptides reported in BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep)

are often highly flexible, with few rigid parts, such as double bonds or ring systems and this is the most important limitation of this type of analysis.

To select the peptides for the molecular docking studies, the size of the peptide and of the active sites of ACE and renin must be considered. In this sense, the ACE site imposed the greatest restrictions due to its location in the bottom of a ~ 20 × 30 Å cavity [33], while the active renin site is located in a less deep groove on the surface of its structure [34].

Binding energy was estimated using the Lamarckian genetic algorithm of AutoDock [35]. For each ligand, 50 runs were performed with a population size of 150 and a maximum of 2,500,000 energy evaluations *per* generation ("Materials and Methods" section). As a control the same protocols was used for co-crystallized inhibitors of both enzymes: brankidine (ACE) and aliskiren (renin). In addition to ACE, angiotensin II was used, a natural peptidic product of this enzyme that acts as a competitive inhibitor thereof. The binding energy found was −13.10, −10.82 and − 2.01 Kcal/mol, respectively.

Peptides present in the majority rice proteins capable to interact with the sites of both enzymes were identified (Table 2). The energy of interaction was in the order of that estimated for the controls (competitive inhibitors, bradykinin (ACE) and aliskiren (renin)). SPFWNINA has the greatest potential to inhibit both enzymes. It is interesting to note that the prolyl endopeptidase (EC 3.4.21.26) is able to hydrolyze brankidine (RPPGFSPFR) giving RPPGLYF, SP and FR. On the other hand, the activity of inhibitory peptides of ACE has been correlated with the presence of proline by several authors. This, together with the results observed in molecular docking, suggests that the presence of a voluminous residue such as W following the SPF motif inhibits the catalytic activity of these enzymes.

Conclusions

The results presented in this work have shown that rice proteins are a source of potentially antihypertensive peptides. These peptides were able to inhibit ACE and renin activity *in vitro*, the two major enzymes that regulate the RAS system. Moreover, this is the first work that reports the bioactivity of

Table 2 Binding energy estimated with Lamarckian genetic algorithm of AutoDock

Peptide	MW (Da)	Binding energy (Kcal/mol) ACE	Binding energy (Kcal/mol) Renin
SPFWNINA	948.457	−8.01	−7.34
IAVPAGVAHWL	1133.647	−5.03	+5.33
VALPAGVAHW	1020.563	−5.67	−3.04

peptides derived from rice proteins capable of inhibit *in vitro* renin enzyme. Docking studies carried out indicated that the octapeptide SPFWNINA has the greatest potential to inhibit both enzymes. This peptide was previously reported as an *in vitro* prolyl endopeptidase (EC 3.4.21.26) inhibitor. Therefore, rice proteins from broken rice are potential candidates as a source of multifunctional peptides that can be used in the formulation of bio-functional foods adding value to this by-product. However, it remains unknown the bioavailability of these peptides and their *in vivo* activity in order to fully consider this by-product as a functional ingredient.

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Compliance with Ethical Standards

Conflict of Interest The author declare that they have no conflict of interest.

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