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Research Article

Identification and molecular docking of novel ACE inhibitory peptides from protein hydrolysates of shrimp waste

The effect of enzymatic hydrolysis by Savinase on the interfacial properties and antihypertensive activity of shrimp waste proteins was evaluated. The physicochemical characterization, interfacial tension, and surface characteristics of shrimp waste protein hydrolysates (SWPH) using different enzyme/substrate (E/S) (SWPH₅ (SWPH using E/S = 5), SWPH₁₅ (SWPH using E/S = 15), and SWPH₄₀ (SWPH using E/S = 40)) were also studied. SWPH₅, SWPH₁₅, and SWPH₄₀ had an isoelectric pH around 2.07, 2.17, and 2.54 respectively. SWPH₅ exhibited the lowest interfacial tension (68.96 mN/m) followed by SWPH₁₅ (69.36 mN/m) and SWPH₄₀ (70.29 mN/m). The in vitro ACE inhibitory activity of shrimp waste protein hydrolysates showed that the most active hydrolysate was obtained using an enzyme/substrate of 15 U/mg (SWPH₁₅). SWPH₁₅ had a lower IC₅₀ value (2.17 mg/mL) than that of SWPH₅ and SWPH₄₀ (3.65 and 5.7 mg/mL, respectively). This hydrolysate was then purified and characterized. Fraction F1 separated by Sephadex G25 column which presents the best ACE inhibition activity was then separated by reversed-phase high performance liquid chromatography. Four ACE inhibitory peptides were identified and their molecular masses and amino acid sequences were determined using ESI-MS and ESI-MS/MS, respectively. The structures of the most potent peptides were SSSKAKKMP, HGEGRSTHE, WLGHGGRPDHE, and WRMDIDGDMISE-QEAHQQR. The structural modeling of anti-ACE peptides from shrimp waste through docking simulations results showed that these peptides bound to ACE with high affinity.

Keywords: ACE inhibitory peptide / Mass spectrometry / Shrimp waste protein hydrolysates / Surface properties

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1 Introduction

Proteins are increasingly used to improve the functional properties in food formulations. However, near their isoelectric point (pI), the functional properties of proteins are impaired as in the case of most acidic foods. Besides, the application of enzyme technology to recover and modify fish proteins may produce several of industrial products and food ingredients for a wide range of applications. The treatment of proteins with enzyme generates various peptides and amino acids, which can modify

the functional and biological characteristics of the proteins and improve their quality [1] and offers interesting opportunities for food.

Hypertension is a major chronic disease which affects 30% of the adult population in the world. It has many deleterious effects on the body which increase the risk of coronary artery diseases, heart failure, cardiac arrhythmia, abnormal renal function and stroke and producing many other complications related to structural damage to the cardiovascular system [2].

It is well known that angiotensin I-converting enzyme (ACE) plays an important role in the fluid and salt balance in mammals, as well as the kallikrein-kinin systems and the renin-angiotensin for the regulation of blood pressure [3]. However, angiotensin converting enzyme (ACE) catalyzes the conversion of angiotensin I (an inactive decapeptide) into angiotensin II by cleaving a dipeptide from its C-terminus. Furthermore, ACE degrades bradykinin [4] which has vasodilator activity in the

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Abbreviations: ACE, angiotensin converting enzyme; pI, isoelectric point; SWPH, shrimp waste protein hydrolysate

kallikrein-kinin system and results in an increase in blood pressure [5]. Therefore, the inhibition of ACE is considered to be a useful approach in the treatment of hypertension.

Synthetic ACE inhibitors, such as captopril and enalapril and others have been used to prevent hypertension in clinical [6]. However, these synthetic ACE inhibitors can produce negative side effects such as skin rashes, allergic reactions, cough, headache and renal impairment. It is necessary to search safer, more innovative, more economical and no negative side effects ACE inhibitors in the treatment of essential hypertension and heart failure in humans [7]. Therefore, the replacement of synthetic ACE inhibitors by natural ones can have benefits due to health implications and functionality in food systems.

Recently, numerous studies are focused on the production and isolation of ACE-inhibitory peptides from different food proteins. Many ACE inhibitory peptides have been isolated from enzymatically hydrolyzed fish proteins including grass carp [8], salmon [9], cuttlefish [10] and skate [7].

As far as we know, no studies have analysed the hypotensive activity of Tunisian pink shrimp processing wastes. In this context, we describe the impact of enzymatic hydrolysis by Savinase using different E/S on the interfacial properties and antihypertensive activity of shrimp waste proteins.

2 Materials and methods

2.1 Reagents

Common solvents and chemicals of analytical grade used in this study were obtained from different commercial sources. The ACE synthetic substrate hippuryl-L-histidyl-L-leucine (HHL) and the ACE enzyme from rabbit lung were purchased from Sigma Chemicals Co (St Louis, MO, USA). Acetonitrile was of HPLC grade. Sephadex G-25 was purchased from Pharmacia (Uppsala, Sweden). All solutions were freshly prepared in distilled water. All other reagents and chemicals used were of analytical grade.

2.2 Enzyme

Savinase® (EC 3.4.21.62) (16 KNPU/g) was obtained from Novozymes® (Bagsvaerd, Denmark). Protease activity was calculated according to the method of Kembhavi et al [11] using casein as a substrate. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μ g of tyrosine per minute under the experimental conditions used.

2.3 Preparation of raw material

The deep-water pink shrimp (*Parapenaeus longirostris*) wastes included heads, shells, cephalothorax, and appendix, which were obtained in fresh condition from a shrimp processing plant located in Sfax, Tunisia. The sample was packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w), and transported to the research laboratory within 30 min.

Upon arrival, the shrimp wastes were washed with distilled water. Then, the wastes were stored at -20°C until further analysis.

2.4 Preparation of hydrolysates from pink shrimp waste proteins

Two hundred grams of shrimp waste were homogenized in 200 mL distilled water by using a homogenizer (MAMMONLEX, R.O.C, model no JW. 1001, Taiwan), then cooked at 90°C for 5 min to inactivate endogenous enzymes. Afterward, the cooked sample was homogenised for 5 min. The mixture was adjusted to pH 10 and temperature 55°C for optimal Savinase® activity. The hydrolysis reaction was initiated by the addition of the enzyme with three different enzyme/substrate ratio ($E/S = 5$; $E/S = 15$ and $E/S = 40$) (U/mg). The reaction was conducted for 3 h. During the reaction, the pH of the mixture was maintained at pH 10 by continuous addition of NaOH (4 N). After 3 h of hydrolysis, the reaction was stopped by heating the solutions for 15 min at 80°C to inactivate the enzyme. Then, the protein hydrolysates were centrifuged at $5000 \times g$ for 20 min using a refrigerated centrifuge (Hettich Zentrifugen, ROTINA 380R, Germany). Finally, the three different soluble fractions, referred to as shrimp waste protein hydrolysates (SWPH5, SWPH15, and SWPH40) according to $E/S = 5$, $E/S = 15$ and $E/S = 40$, were freeze-dried at -50°C and 121 mbar using a freeze dryer (CHRIST, ALPHA 1–2 LD plus, Germany).

2.5 Chemical composition

The moisture and ash content were determined according to the AOAC standard methods as 930.15 and 942.05, respectively. Total nitrogen content was determined by using the Dumas Elemental Rapid N cube 161 15054 (Donaustrasse, Germany) which based on the quantitative combustion digestion of the sample at approximately 900°C in excess oxygen [12]. Crude fat was determined gravimetrically after Soxhlet extraction of dried samples with hexane. All measurements were performed in triplicate.

2.6 Determination of color

The color of the different hydrolysates was measured by a tristimulus colorimeter (CHROMA METER CR-400/410. KONICA MINOLTA, Japan) using the CIE Lab scale ($C/2^{\circ}$), where L^* , b^* and a^* refer to the parameters measuring lightness, yellowness, and redness, respectively. The results were the average of three measurements taken at ambient temperature at different points on the samples.

2.7 Surface charge (zeta potential) measurement

The surface charges for SWPH5, SWPH15, and SWPH40 were determined by using a Delsa Nano C Instrument (Malvern Instruments, Westborough, MA) at pH 7, ambient temperature and a concentration of 0.25% (w/w).

2.8 Surface tension measurements

The surface tension measurements were realized at pH 7 and a concentration of 0.25% (w/w) from a suspension of different shrimp waste protein hydrolysates (SWPH). The automated drop volume tensiometer TVT1 (Lauda, Germany) was used in the dynamic mode for measuring the surface tension. This method is based on a continuous formation of drops at the capillary tip with a definite diameter. The drop falls when a critical volume is reached and then a new one is formed.

Thus, it is possible to establish surface tension as a function of the drop time curves [$y = f(t)$]. A capillary tip of 1.055 mm internal radius was connected to a Lauda syringe (2.5 mL). The drop-forming time was from 0.07 to 0.8 s/ μ L.

2.9 Angiotensin-I-converting enzyme inhibitory activity

The angiotensin I-converting enzyme (ACE) inhibition activity was determined according to the method reported by Nakamura et al [13]. Sample solutions containing different concentrations of different hydrolysates obtained from shrimp waste proteins by enzymatic treatment, 2.17 mM hippuric-L-histidyl-L-leucine (HHL) and 55 mU/mL ACE solutions were prepared in borate buffer (100 mM) pH 8.3 containing 300 mM NaCl. The reaction was performed in a 96-well microplate. Then, the reactions were started by mixing 30 μ L of ACE and 30 μ L of hydrolysates solutions. The negative control is measured by replacing the 30 μ L of hydrolysates solutions with 30 μ L borate buffer. Then, the mixture was incubated for 5 min at 37°C. Afterward, 100 μ L of HHL solution were added to negative control, different hydrolysates solutions and blank (60 μ L borate buffer) and the microplate was further incubated 30 min at 37°C in a Thermo Cell Mixing Block under constant mild agitation (Bioer, Binjiang, China). The enzyme reaction was then inactivated by adding 100 μ L HCl (1.0 M). The released hippuric acid (HA) was quantified by RP-HPLC on a C₁₈ 150 $\times$ 4.60 mm 100 Å Kinetex column (Phenomenex Inc., Torrance, USA) connected to a system composed of a Waters TM 600 automated gradient controller pump module, a Water-Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. The sample was then eluted at 1 mL/min using a 25% acetonitrile, 0.1% trifluoroacetic acid (TFA) (v/v) solvent for 5 min. The eluate was followed at 228 nm and HA was released at a retention time of 1.2 min. Spectral and chromatographic data were stored on an NEC image 446 computer. Millennium software was used to acquire, analyze and plot chromatographic data. The peak area values average from three determinations at each concentration was used to calculate the ACE inhibition rate as follows:

$$\text{ACE activity inhibition (\%)} = 1 - \frac{A_{\text{sample}} - B}{A_{\text{negative control}} - B} \times 100$$

Where A_{sample} is the peak area of HA generated in the presence of ACE inhibitor component, $A_{\text{negative control}}$ is the peak area of HA generated without ACE inhibitors (negative control) and B the peak area of HA generated without ACE (HHL autolysis).

The IC₅₀ value was defined as the concentration of hydrolysate (mg/mL) required to reduce the hippuric acid peak by 50% (indicating 50% inhibition of ACE).

2.10 Purification of ACE inhibitory peptides

2.10.1 Fractionation of protein hydrolysate with Sephadex G-25 gel filtration

The lyophilized SWPH₁₅ (1 g) was dissolved in 10 mL of distilled water, then separated onto a Sephadex G-25 gel filtration column (5.2 $\times$ 56 cm) pre-equilibrated and eluted with distilled water. Fractions (6 mL) were collected separately at a flow rate of 25 mL/h and absorbance was measured at 280 nm to determine the elution profile of hydrolysate. Fractions that showed ACE inhibition activity were pooled and lyophilized.

2.10.2 Reversed-phase high pressure liquid chromatography

The most active fraction was suspended in Milli Qwater, filtered through 0.22 μ m filters, and then separated by Reverse-phase high-pressure liquid chromatography (RP-HPLC) on a Waters C₁₈ column (4.6 mm $\times$ 250 mm) (XBridge™, Ireland). Peptides were first eluted with eluent A (water containing 0.1% trifluoroacetic acid (TFA)) for 5 min, then with a linear gradient of acetonitrile (5–45% for 85 min) containing 0.1% TFA at a flow rate of 1 mL/min and absorbance scans were measured between 200 and 300 nm at a rate of one spectrum per second with a resolution of 1.2 nm. Chromatographic analyses were completed with Millennium software. Each fraction was manually collected and then the solvent was evaporated using a speed-vac. The ACE-inhibition activities of the eluted peaks were determined. The liquid chromatographic system consisted of a Waters 600E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on an NEC Image 466 computer. Millennium software was used to plot, acquire and analyze chromatographic data.

2.11 Mass spectrometry analysis

The molecular mass and peptide sequencing were performed on positive ion mode using electrospray ionization-mass spectrometry (ESI-MS) and the tandem mass spectrometry (MS/MS). ESI mass spectrometry was realized using a triple quadrupole instrument Applied Biosystems API 3000 (PE Sciex, Toronto, Canada) equipped with an electrospray ion source. The system is controlled by the Analyst Software 1.4, allowing the control of the spectrometer, the analysis and the processing data. Interpretations of spectra (MS/MS) were made with the BioAnalyst software. The lyophilized samples from RP-HPLC were dissolved in acetonitrile/water (20/80; v/v) containing 0.1% formic acid for the positive mode.

Afterward, the solution was injected continuously, by a pump at a flow rate of 5 μ L/min. The potential of ionization was of 5000 V in positive mode. At the time of the recording of the spectrum, 30 scans on average were added (MCA mode) for

each spectrum. The gases used (nitrogen and air) were pure (up to 99%) and produced by a compressor Jun-Air 4000–40M and a nitrogen generator Whatman model 75-72 (Whatman Inc., Haverhill, MA, USA). The polypropylene glycol (PPG) was used for the calibration and the optimization of the machine. The peptide sequence was determined from the CID spectrum of the protonated analyte $[M+H]^+$ by MS/MS experiments. Peptide sequences were determined using the BioAnalyst software (Applied Biosystems, USA).

2.12 Molecular docking of the purified peptides in the ACE binding site

The three-dimensional structure of Angiotensin-I converting enzyme (ACE) from rabbit lung ACE in complex with lisinopril (1O8A.pdb) was derived from the RCSB PDB Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>). Before the docking, water molecules and the inhibitor lisinopril were removed whereas the cofactors zinc and chloride atoms were retained in ACE model. The polar hydrogens were then added to the ACE structure. Peptides structures were generated by automated peptide modeling program PEP-FOLD [14, 15]. The molecular docking of the peptides at the ACE-binding site was performed using autodock Vina software [16]. The docking runs were carried out with a radius of 126 Å³ with coordinates x: 40.6559, y: 37.3827, and z: 43.3401. The best ranked docking pose of each peptide in complex with ACE was obtained according to the scores and binding-energy value.

2.13 Statistical analysis

Results were expressed as mean values $\pm$ standard error of three independent determinations. Statistical analyses were determined using a statistical software program (SPSS for Windows version 11.0). The data were subjected to analysis of variance using the general linear model to determine significant differences between samples ($p < 0.05$).

3 Results and discussion

3.1 Preparation and characterization of shrimp waste protein hydrolysates (SWPHs)

In recent years, being aware of the importance of health, people begin to pay more attention to their diet. Functional foods, which have nutritional value and exert a physiological effect on the body are favored by people and have a marked effect on the food sector [17]. The bioactive peptides have an affinity to regulate blood pressure and generally believed to be inhibitors of the angiotensin I converting enzyme. In addition to their therapeutic quality, bioactive peptides should possess specific functional properties to facilitate processing. The changes in functional properties of native proteins are related to peptides and free amino acids produced by enzymatic hydrolysis. In fact, the controlled enzymatic hydrolysis of protein produces smaller polypeptides that modify and even enhance functional characteristics for a wide range of applications [18]. It is well known that biological properties of

protein hydrolysates depend on the degree of hydrolysis, the protein substrate, the conditions used during hydrolysis (pH, temperature and enzyme/substrate ratio) and the specificity of the enzyme used for the hydrolysis [18]. In the present work, bioactive peptides were produced from shrimp waste with Savinase[®] using three different enzyme/substrate ratios. Savinase[®] contains both endoprotease and exoprotease activities, which offer the ability to achieve higher deproteinization degree. Garcia-Mora et al [19] reported that Alcalase[®] and Savinase[®] digestion at atmospheric pressure gave rise to higher values of ACE inhibition (57 and 46%, respectively) than Protamex and Corolase 7086 (36 and 34%, respectively). In the same context, Garcia-Mora et al [20] investigated the effectiveness of Alcalase[®] and Savinase[®] for the simultaneous release of bioactive peptides and phenolics with antioxidant, angiotensin I converting enzyme (ACE) inhibitory and anti-inflammatory activities. Garcia-Mora et al [21] showed that hydrolysis of pinto bean protein concentrate by Savinase[®] generated the best ACE-inhibitory and radical scavenging activities and was considered the most effective treatment to increase small peptides (<3 kDa). In another study Garcia-Mora et al [22] reported that savinase is the most suitable enzyme for releasing peptides from lentil (*Lens culinaris* var. Castellana) protein concentrates with multifunctional properties.

The average compositions of shrimp waste protein hydrolysates (SWPH₅, SWPH₁₅ and SWPH₄₀) were determined and compared to shrimp waste. As shown in Table 1, the SWPH₄₀ had higher protein content (78.7% $\pm$ 0.5) than that of SWPH₅ and SWPH₁₅ (71% $\pm$ 0.1 and 75.98% $\pm$ 0.9, respectively). The high protein content can be explained by the solubilization of protein during hydrolysis, the elimination of insoluble undigested non-protein substances and the partial removal of lipid after proteolysis [23]. Similar compositions were reported for barbel protein hydrolysates [24].

Color influences the overall acceptability of food products. During hydrolysis, SWPH₅, SWPH₁₅ and SWPH₄₀ turned a brownish colour. In a previous work [25] reported that shrimp (*P. longirostris*) waste was the lightest ($L^* = 80.7$) and more red ($a^* = 17.5$). Whereas, protein hydrolysates were darker and less red. No significant differences ($P > 0.05$) were found between the yellow color of waste shrimp and protein hydrolysates. Indeed, enzymatic browning reactions are assumed to have contributed to the reduction in the luminosity. The reduction of the red colour of fish protein hydrolysate was probably a result of oxidation of the melanin pigment and the myoglobin of the raw material [23]. Furthermore, astaxanthin doesn't affect the color of the sample because the astaxanthin is denatured at high temperature. Indeed, the preparation of the protein hydrolysates requires a high temperature pretreatment to denature the endogenous enzymes, an enzymatic treatment and finally a denaturation of the enzyme at high temperature. So after this treatment, the astaxanthin will be denatured. In this context, Sila et al [25] demonstrated that the color of carotenoprotein hydrolyzate is influenced by enzymatic treatment.

3.2 Surface charge (zeta potential) measurements

Zeta potential provides a measure of the net surface charge and potential charge distribution at the interface [26]. Net surface

Table 1. Physicochemical characterization of shrimp waste and its protein hydrolysates (SWPHs).

Parameters	Shrimp waste	SWPH ₅	SWPH ₁₅	SWPH ₄₀
Moisture (%)	66 ± 0.1 ^c	7.94 ± 0.05 ^a	7.63 ± 0.1 ^a	9.8 ± 0.06 ^b
Protein (%)	31.3 ± 0.6 ^a	71 ± 0.1 ^b	75.98 ± 0.9 ^c	78.7 ± 0.5 ^d
Ash (%)	28.6 ± 0.7 ^d	16.84 ± 0.2 ^c	12.66 ± 0.08 ^b	9.46 ± 0.07 ^a
Fat (%)	3.97 ± 0.4 ^b	3.91 ± 0.1 ^b	2.11 ± 0.05 ^a	1.96 ± 0.1 ^a
Chitin (%)	35.9 ± 1.6	-	-	-
Colour				
<i>L</i> *	80.7 ± 1.0 ^c	51.98 ± 0.3 ^b	50.28 ± 0.01 ^b	43.22 ± 0.6 ^a
<i>a</i> *	17.5 ± 0.4 ^c	8.29 ± 0.2 ^b	5.6 ^a	5.4 ± 0.4 ^a
<i>b</i> *	22.4 ± 0.2 ^a	22.21 ± 0.5 ^a	22.6 ± 0.01 ^a	22.65 ± 0.05 ^a

Physicochemical composition was calculated based on the dry mater. Values represent averages ± standard deviations for triplicate experiments. Different superscripts in the same row indicate the significant differences ($p < 0.05$).

Figure 1. (A) Zeta potential for shrimp waste protein hydrolysates as function of pH. (B) Evolution of the interfacial tension of shrimp waste protein hydrolysates (SWPHs).

charge was determined by potentiometric titration, the results are shown in Fig. 1. Shrimp protein hydrolysates displayed both positive and negative charge, depending on the pH. SWPH₅, SWPH₁₅ and SWPH₄₀ have an isoelectric pH at around 2 (2.07, 2.17 and 2.54 respectively). The surface charge of all samples, as indicated by their zeta potentials, has shown a net negative charge at pH 7, which is readily explained by the fact that this pH value was well above the isoelectric point of shrimp protein hydrolysates. The pH plays an important role for the change of charge on the weakly acidic and basic side chain groups and generally, hydrolysates present low solubility at their isoelectric points which limits their use in highly acidic foods. The variation of solubility may be attributed to the net charge of peptides, which increases with the number of the exposed ionisable

carboxyl and amino groups that improve intra and intermolecular electrostatic repulsion and foster the unfolding of protein, reducing protein–protein aggregation and increasing protein–water interactions [27].

3.3 Aqueous suspensions properties: surface tension measurements

The ability of shrimp waste protein hydrolysates (0.25%, w/w; pH 7.0) to lower the interfacial tension between two phases was evaluated (Fig. 1.B). Among all shrimp waste protein hydrolysates, SWPH₅ exhibited the lowest interfacial tension (68.96 mN/m) followed by SWPH₁₅ (69.36 mN/m) and SWPH₄₀ (70.29 mN/m). Indeed, SWPH₅ and SWPH₁₅ were able to decrease interfacial tension relative to water (72 mN/m) (Data not shown), indicating that SWPH₅ and SWPH₁₅ were surface-active. However, the increase of enzyme/substrate ratio led to a reduction in the surface activity by a decrease in the first drop value. This can be explained by the fact that hydrolysis leads to the release of small peptides into solution. These small peptides, with the same charge, presented antagonist behaviour and can compete to reach the air–water interface, which ultimately results in the slow diffusion into interface. This competition effectively reduces the diffusion to the interface. Therefore, the rate of diffusion depends on the molecular size of proteins and on the modified conformation of the protein with the condition used during hydrolysis and the degree of hydrolysis due to the action of the enzyme on the native protein. Besides, Morales et al [28] demonstrated that sardine muscle hydrolysates at degree of hydrolysis DH = 5% with different enzymes (Trypsine and Alcalase®) have a significant effect on the interfacial tension. Furthermore, Mokni Ghribi et al [29] confirmed that the increase in the DH value led to a reduction in the surface activity by a decrease in the first drop value. Felmeister [30] demonstrated that biomolecules exhibited the ability to accumulate at a monolayer/water or air/water interface.

3.4 ACE inhibitory activity of shrimp protein hydrolysates

Research and development to find innovative, safer, and economical ACE inhibitors are necessary for the prevention and

Table 2. IC₅₀ values of the ACE inhibition activity of shrimp waste protein hydrolysates and of sub-fractions separated by RP-HPLC on a Waters C₁₈ column

ACE inhibition activity	Shrimp waste protein hydrolysates (mg/mL)			Captopril® (μg/mL)	RP-HPLC sub-fractions (mg/mL)	
	SWPH ₅	SWPH ₁₅	SWPH ₄₀		P7	P8
IC ₅₀ value	3.65 ^{a)} ± 0.23	2.17 ^{b)} ± 0.16	5.7 ^{c)} ± 0.07	0.00177 ^{d)} ± 0.00003	7.593 [#]	0.083 [□]

Values represent averages ± standard deviations for triplicate experiments. All samples were compared to Captopril. The letters refer to the difference between Shrimp waste protein hydrolysates and Captopril ($p < 0.05$). The symbols refer to the difference between the sub-fractions and Captopril ($p < 0.05$).

remedy of hypertension [10]. Bioactive peptides with antihypertensive activity are receiving much interest because of their beneficial effects in cardiovascular diseases.

The angiotensin I converting enzyme inhibitory activity of shrimp protein hydrolysates (SWPH₅, SWPH₁₅ and SWPH₄₀) was determined at different concentrations (1–10 mg/mL). Results indicated that hydrolysates exhibited concentration-dependent ACE inhibitory activities. As shown in Table 2, SWPH₁₅ had a lower IC₅₀ value (2.17 mg/mL) than that of SWPH₅ and SWPH₄₀ (3.65 and 5.7 mg/mL, respectively). These results demonstrate that SWPH₁₅ exhibited the highest ACE inhibition activity. In this context, Lee et al [9] reported IC₅₀ values of 1.08, 1.23 and 1.40 mg/mL for peptides generated from Chum salmon (*Oncorhynchus keta*) by trypsin, neutrase and α -chymotrypsin, respectively. Furthermore, Gu et al [31] reported IC₅₀ values of 1.165 ± 0.087 mg/mL for peptides isolated from Atlantic salmon (*Salmo salar* L.) skin by Alcalase®. The IC₅₀ value of shrimp protein hydrolysates were lower than those of hydrolysates from oyster, scallop, codfish bone, and codfish skin whose presented an IC₅₀ higher than 10 mg/mL [32].

3.5 Purification of ACE inhibitory peptide from SWPH₁₅

To purify ACE inhibitory peptides, SWPH₁₅ which exhibited the highest ACE inhibition activity, was initially separated on a Sephadex G-25 gel filtration chromatography. As shown in Fig. 2A, there are five major absorbance peaks at 280 nm, designated as F1, F2, F3, F4 and F5. Fractions associated with each peak were collected separately, freeze-dried and its ACE inhibitory activity was assayed.

All fractions displayed ACE inhibitory activity unless F5. At 2 mg/mL, angiotensin-I converting enzyme inhibitory activity of F1, F2, F3 and F4 were 88.9, 53.64, 18.4 and 25.12%, respectively. As reported in Fig. 2B, these four bioactive fractions were found to exhibit ACE inhibitory activity with IC₅₀ values of 0.85, 1.8, 7.9 and 3.5 mg/mL, respectively. Among the four active fractions, F1 displayed the highest ACE-inhibitory activity.

F1 was further separated by reversed-phase HPLC and fractionated into eight major sub-fractions (P1–P8) with different elution times (Fig. 3. A). Sub-fractions were collected separately through repeated chromatographic fractionation using an RP-HPLC column, freeze-dried and their ACE inhibitory activities were measured. At the same concentrations, angiotensin-I converting enzyme inhibitory activity was widely observed only in P7 and P8 and was absent in the other sub-fractions. The highest

ACE inhibition was obtained for sub-fraction P8, with IC₅₀ values of 83 μg/mL (Table 2). The purity of this sub-fraction was further analysed by HPLC. Results presented in Fig. 3.B showed that one major peak was identified.

3.6 Identification of the ACE inhibitory peptides

Peptides in sub-fraction P8, were analysed by ESI/MS for molecular mass determination and by ESI-MS/MS for amino acid sequences (Data not shown). The relationship between peptide structure and ACE inhibitory activity is not well established, although some general features have been already identified [33].

As shown in Table 3, four ACE inhibitory peptides were identified in the sub-fraction P8: SSSKAKKMP, HGEGRSTHE, WLGHGGRPDHE and WRMDIDGDIMISEQEAHQ. Their molecular mass values were 963.49, 1065, 1253.71 and 2330.9 Da, respectively. Generally, ACE-inhibitory peptides have a proline or aromatic residue at their C-terminal and a hydrophobic amino acid residue at the N-terminal [34]. In the case of WLGHGGRPDHE and WRMDIDGDIMISEQEAHQ, the presence of Try, a hydrophobic amino acid residue at the N-terminal.

Generally, ACE inhibitors contain one or more molecular functionalities such as zinc binding ligands (usually either a phosphate or carboxylate oxygen, or thiol sulfur), a hydrogen-bond donor and carboxyl-terminal group [35].

Furthermore, among the N-terminal amino acids of the highly active short-chain peptides, the branched-chain aliphatic amino acids I and V are predominant. Interestingly, the most potent ACE inhibitory peptide derived from shrimp waste protein contain aliphatic amino acids such as A, L, I, G and P. Glycine, aspartic acid, proline and alanine were all shown in other ACE inhibitory peptides from food proteins, such as ACE inhibitory peptides from jellyfish [36] from chum salmon [9] and from lizard fish [37].

In the case of ACE inhibitory peptides, the most potent and specific peptide inhibitors have similar structures and ACE activity is strongly influenced by the C-terminal tripeptide sequence. The tripeptides with W, T, F, P and a hydrophobic amino acid at the C-terminal were effective for ACE inhibitory activity because of the interaction between three subsites at the active site of ACE [38].

Binding of inhibitors or the natural substrate to ACE takes place predominantly via the carboxyl terminal tripeptide residues. In this study, novel ACE inhibitory peptides from shrimp waste protein hydrolysates contained hydrophobic amino acids at the N-terminal which may contribute to the

Figure 2. (A) Gel filtration chromatography of shrimp waste protein hydrolysate (SWPH₁₅) on a Sephadex G-25. (B) Angiotensin I-converting enzyme inhibitory activities of Sephadex G-25 fractions.

Figure 3. (A) Reverse-phase HPLC profile of fraction F1 on a Waters C18 column. (B) Reverse-phase HPLC profile of fraction P8 on a Waters C18 column.

Table 3. Molecular weight and structure characteristics of ACE inhibitory peptides in RP-HPLC sub-fraction “P8”

Mass (M+H) ⁺ in Da	Sequence	Residue number	Hydrophobic amino acids (%)	Charge state	Secondary structure
963.49	SSSKAKKMP	9	22	+3	100% Helice α
1065	HGEGGRSTHE	10	0	−1	Random coil
1253.71	WLGHGGRPDHE	11	18	−1	Random coil
2330.90	WRMDIDGDIMISEQEAHQ	19	36	−3	Random coil

ACE inhibitory activity. This is the case of WLGHGGRPDHE and WRMDIDGDIMISEQEAHQ. Byun et al [39] reported that the aromatic amino acids at the C-terminal and aliphatic amino acids at the N-terminal were a designated peptide binding site for ACE as a competitive inhibitor. However, Contreras et al [40] reported that the casein hydrolysate had a hydrophobic character and low molecular weight. More specifically, they were rich in the amino acid P, which contributes to the correct location of the peptide in the active site of ACE, most likely due to the rigid structure of this residue. In addition, their study suggested that the Pro at the C-terminus may contribute to ACE inhibiting activity. Based on the results of this research, it appears that the novel ACE inhibitory peptides in subfraction P₈, may have antihypertensive function.

3.7 Docking of peptides in the ACE active site

The pose for each peptide corresponds to the best score. Peptide 1 (SSSKAKKMP) has a helical structure. The best pose corresponds to a peptide binding to a cavity on the molecule surface that is located away from the catalytic cavity (Fig. 4). Peptides 2 (HGEGGRSTHE) and 3 (WLGHGGRPDHE) display an extended structure. The poses corresponding to the best binding scores for peptide 2 and peptide 3, were embedded within the active site which satisfactorily explain their inhibiting activity (Fig. 4A). Peptide 4 (WRMDIDGDIMISEQEAHQ) is a long extended structure. Its best binding pose corresponds to a peptide blocking the active site entrance. Its N-terminal amino acids are deeply embedded in the catalytic cavity (Fig. 4A). Except peptide 1, all the other peptides were found to bind to the catalytic cavity of the enzyme. In particular, peptides 2 and 3 are completely embedded within the catalytic cavity whereas P4 is laying on the substrate recognition surface and is obstructing its entry (Fig. 4B). These peptides can strongly compete with the substrate which explains their inhibitory capacity. The similar behavior of peptides 2 and 3 can be attributed to their sequence similarity since they share an amino acid identity of about 60%, in addition to their similar length (10 and 11 residues). The inhibitory effect of peptide 1 might be due to solvent accessible cavity that can be important for the substrate recognition and binding before being processed in the catalytic site. Upon binding, the peptide might also exert constraints on the protein core preventing it from undergoing little conformational changes that are necessary for the enzyme activity.

4 Concluding remarks

In the present study, the effect of enzymatic hydrolysis by Savinase on the functional properties and antihypertensive activity of shrimp waste protein hydrolysates was studied. The results showed that SWPH₁₅ exhibited the highest ACE inhibition activity. Fractions separated by G25 which present the best ACE inhibition activity were analysed by RP-HPLC and by mass spectrometry in tandem (ESI-MS/MS) to identify the peptide sequences. A total of four peptides in sub-fraction P₈ were separated and the amino acid sequences were determined. The results suggested that SWPH₁₅ is a good source of bioactive peptide which

Figure 4. Representation of the Angiotensin-I converting enzyme (ACE) from rabbit lung as cartoon (A) or surface (B) representations, in complex with peptides P1 (SSSKAKKMP; green sticks), P2 (HGEGGRSTHE; blue sticks), P3 (WLGHGGRPDHE; red sticks) and P4 (WRMDIDGDIMISEQEAHQ; orange sticks). All peptides poses corresponding to the best scores. The inhibitor lisinopril bound to the catalytic cavity is shown in wheat. Zinc ion is shown as a blue sphere. In the surface representation, oxygen atoms are red, nitrogens are in blue whereas carbon and hydrogen atoms are in cyan.

could be useful as functional foods and antihypertensive agents. However, further research is needed to test the antihypertensive activity of SWPH₁₅ on spontaneously hypertensive rats.

Practical application

Marine proteins and their hydrolysates offer huge potential as novel sources of natural bioactive peptides with angiotensin I-converting enzyme (ACE) inhibitory activity.

Four ACE inhibitory peptides were identified and their molecular masses and amino acid sequences were determined using ESI-MS and ESI-MS/MS, respectively.

The structures of the most potent peptides were SSSKAKKMP, HGEGGRSTHE, WLGHGGRPDHE, and WRMDIDGDIMISEQEAHQ. These peptides may have antihypertensive effect as natural and safe nutraceutical/functional ingredients.

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