In silico analysis and antihypertensive effect of ACE-inhibitory peptides from smooth-hound viscera protein hydrolysate: Enzyme-peptide interaction study using molecular docking simulation

Ola Abdelhedi[†a], Rim Nasri[a], Mourad Jridi[a], Leticia Mora[b], Miguel Eduardo Oseguera-Toledo[c], María-Concepción Aristoy[b], Ibtissem Ben Amara[a], Fidel Toldrá[b], and Moncef Nasri[a]

a. Laboratoire de Génie Enzymatique et de Microbiologie, Université de Sfax, Ecole Nationale d'Ingénieurs de Sfax, B.P. 1173-3038 Sfax, Tunisia.

b. Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Avenue Agustín Escardino, 7, 46980 Paterna, Valencia, Spain.

c. Programa de Posgrado en Alimentos del Centro de la República (PROPAC), Universidad Autónoma de Querétaro, Apdo. Postal 184, Querétaro, QRO 76010, Mexico.

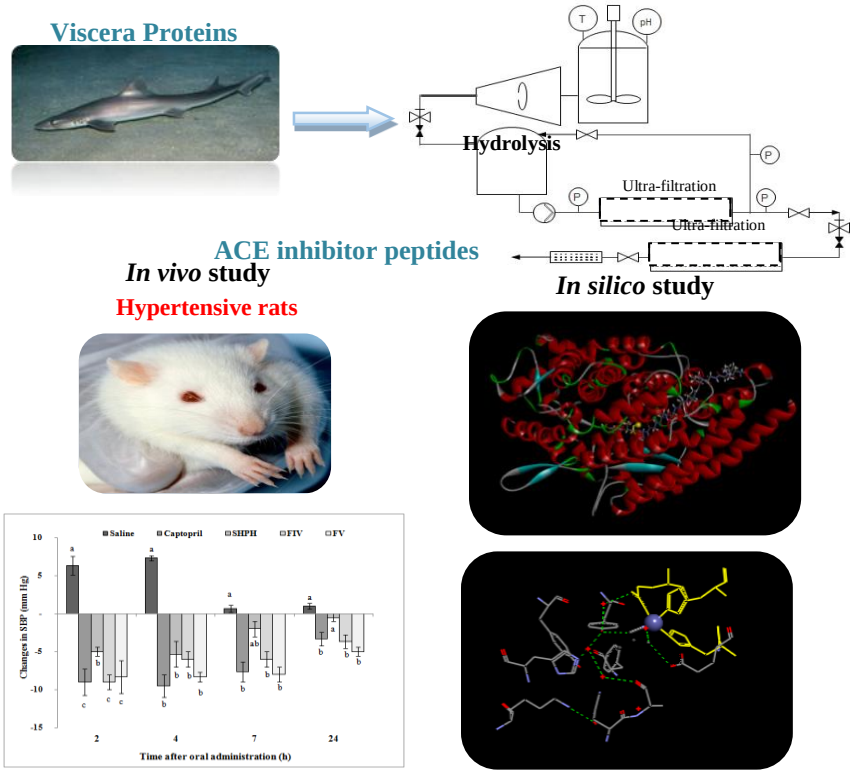
d.

† Corresponding author. Tel.: +216 23166655; Fax: +216 74275595.

Ola Abdelhedi: Laboratoire de Génie Enzymatique et de Microbiologie, Université de Sfax, Ecole Nationale d'Ingénieurs de Sfax, B.P. 1173-3038 Sfax, Tunisia.

E-mail address: abd.ola1502@gmail.com

Graphical abstract



Highlights

- Bioactive peptides from smooth hound viscera was elaborated using Esperase;
- SHPH fractions showed different characteristics and ACE-inhibitory potential;
- Peptide profiles of the promising fractions were studied through proteomic tools;
- Molecular docking study was used to reveal the ACE inhibition of peptides;
- SHPH contained peptides that could interact with the ACE active sites.

Abstract

Smooth-hound viscera proteins were enzymatically hydrolyzed, using Esperase®, and the resulting hydrolysate was fractionated by ultrafiltration through four membranes with decreasing molecular weight (MW) cut-offs. Five fractions were obtained, FI (>50 kDa), FII (5-50 kDa), FIII (3-5 kDa), FIV (3-1 kDa) and FV (<1 kDa). Their RP-HPLC peptide profile, amino acid composition, and ACE-inhibitory activity were investigated and compared to the non-fractionated hydrolysate. Data showed that fractions with low MW peptides contained the highest amounts of

hydrophobic amino acids (39.63 and 41.68% in FIV and FV, respectively). In addition, FIV and FV exhibited the strongest ACE-inhibitory activity with respective IC_{50} of 101.61 and 92.75 µg/ml. Moreover, they showed interesting blood-lowering results in hypertensive rats after 4h of oral administration (200 mg/kg body weight). Both fractions were then fractionated by RP-HPLC and eluted peptides were analyzed by nanoLC-MS/MS. The molecular docking study of IAGPPGSAGPAG, VVPFEGAV, PLPKRE, and PTVPKRPSPT showed that peptides were able to bind ACE through a complex of hydrophobic, hydrogen bonds, van der Waals and electrostatic interactions, as well as to interact with the three residues coordinating with Zn^{2+}ion. Hence, this study provides a useful bioprocess for the use of smooth-hound byproducts as a natural source of hypotensive agents.

Keywords: Smooth-hound viscera; Ultra-filtration process; ACE-inhibitory; Peptides; Molecular docking.

1. Introduction

Fish proteins exhibit an increasing market value in part due to their high nutritional value and health-promoting properties. Besides fish meat and edible parts, head, skin, and viscera can be used as raw material for the production of value-added products through the employment of hydrolysis process [1]. Enzymatic hydrolysis of fish proteins was found to be an effective way to recover potent bioactive peptides from waste materials [2]. Particularly, marine peptides have attracted the attention of the pharmaceutical, nutraceutical and medicinal industries because of their broad

spectra of bioactivities, such as antioxidant, cardioprotective (antihypertensive, anti-atherosclerotic and anticoagulant), anti-diabetic and appetite suppressing activities [3].

Among their bioactivities, the angiotensin-I converting enzyme (ACE) inhibitory effect is in continuous exploration, since the discovery of the first natural peptides in snake venom [4], until nowadays with ACE-inhibitory peptides isolated from food proteins, particularly marine sources. In fact, many investigations have been conducted on ACE-inhibitory peptides from fish proteins, such as *Stichopus horrens* [5], boarfish [6], flounder fish [7], thornback ray [8], lizard fish [9] and goby [10]. ACE inhibitors generally contain a proline residue in their C-terminal side [11]. Wu et al. [12] proposed models for ACE-inhibitory peptides with hydrophobic amino acid residues at the N-terminus, positively charged amino acids at the middle, and aromatic amino acids at the C-terminus site.

ACE, a dipeptidyl carboxypeptidase that belongs to the class of zinc proteases, plays a double action in the renin-angiotensin-aldosterone system by cleaving two amino acids from angiotensin-I, releasing angiotensin-II, a powerful vasoconstrictor, and inactivating the vasodilator bradykinin, a potent vasodilator peptide [13]. ACE inhibitors block angiotensin II conversion and cause the relaxation of blood vessels resulting in a decrease of blood pressure level. Studies on ACE-inhibitory activity also require a structure-activity relationship study through molecular docking simulation, in order to predict the 3D structure of ACE and to simulate the binding type between ACE catalytic site and the bioactive peptide.

Hypertension is a very important chronic disease affecting one third of adults worldwide and causing about half of the total mortalities, mainly due to stroke and heart problems. In fact, it accounts for 9.4 million deaths worldwide every year [14]. Studies on cardiovascular problems and particularly hypertension are based on the inhibition of ACE activity via the use of natural components, such as bioactive marine peptides [8].

Common smooth-hound (*Mustelus mustelus*) is the most abundant hound shark in Tunisian coasts. During processing, *M. mustelus* generates a relevant amount of by-products as dearth exploited wastes. Among the generated by-products, viscera can be used either as a source of digestive enzymes, or as a protein matrix for protein hydrolysates preparation with potential ACE-inhibitory activity [8]. On the other hand, the ultrafiltration (UF) membrane technology has been of great importance for the concentration and fractionation of protein mixtures and it has been one of the best available techniques for the enrichment of peptides [2].

In this study, smooth-hound viscera protein hydrolysate (SHPH) was prepared using Esperase® and later fractionated according to its MW distribution and hydrophobic character, using UF technique and reverse-phase HPLC, respectively. Then, the peptidomic identification of most active peptides, showing ACE-inhibitory activity, was carried out using tandem mass spectrometry (nLC-MS/MS). The binding interaction of ACE-inhibitory peptides within the enzymatic active site was further studied through molecular docking simulation. The short-term *in vivo* anti-hypertensive effect was also investigated in hypertensive model rats.

2. Materials and methods

2.1. Reagents

Angiotensin-converting enzyme (from rabbit lung) was purchased from Sigma (St. Louis, MO). Abz-Gly-p-nitro-phe-pro-OH trifluoroacetate salt was obtained from Bachem (Bubendorf, Switzerland). All other chemicals were of analytical grade. Solvents and chemicals used in the mass spectrometry (MS) analysis were from Sigma (St. Louis, MO) and MS grade.

2.2. Biological material

Viscera mass from Smooth-hound (*M. mustelus*) fish was obtained in the local fish market of Sfax, Tunisia. The biological material was brought to the research laboratory in polyethylene

bags, in iced conditions, within 30 minutes. Upon arrival, they were immediately well rinsed with tap water, weighed and stored in plastic bags at -20 °C until use. Before protein hydrolysate preparation, smooth-hound viscera were characterized in terms of their protein (21.60±0.75%), fat (6.20±0.84%) and ash (0.85±0.09%) contents. These results are expressed based on the wet weight matter.

2.3. Proteolytic enzyme

Esperase®, an alkaline protease from *Bacillus* sp. (pH 9.0-10.0 and temperature 50-60 °C), was purchased from Novozymes® (Bagsvaerd, Denmark). Esperase® is characterized by its excellent performance at elevated temperature and pH values. It is a serine endopeptidase that hydrolyses the internal peptide bonds. The protease activity was determined by the method of Kembhavi et al. [15] using casein as a substrate.

2.4. Protein hydrolysate preparation

Hydrolysis was carried out as previously described by Abdelhedi et al. [16]. Smooth-hound viscera were first cooked in distilled water at 95 °C for 20 min, with a solid/solvent ratio of 1:1 (w/v) to inactivate endogenous enzymes. After being homogenized, the pH of the mixture was adjusted to the optimum enzymatic activity value (pH 9.0) by adding 4 N NaOH solution. Viscera mass was then subjected to hydrolysis using Esperase® with an enzyme/protein ratio of 6/1 (U/mg of protein). During the reaction, the pH of the mixture was maintained constant at the target value by continuous addition of 4 N NaOH solution. The degree of hydrolysis (DH) was calculated as described by Adler-Nissen [17]. After the achievement of the digestion process (580 min), *i.e.* when no pH variation was detected, the reaction was stopped by heating the solution for 20 min at 95 °C to inactivate enzymes. The resulting protein hydrolysate (SHPH) was then centrifuged at 9500 g for 20 min and the soluble fraction (peptides) was freeze-dried (Bioblock Scientific Christ

ALPHA 1-2, IllKrich-Cedex, France). Undigested viscera proteins (UVPs) were treated under the same conditions, without enzyme addition, and serve as control. After freeze-drying, all powders were stored at -20 °C until use.

2.5. Ultra-filtration process

SHPH was fractionated by UF membrane technology, in a tangential filtration mode, using Millipore UF systems, a Labscale™ TFF System (USA) with membranes having molecular weight cut-offs (MWCO) of 50 kDa and 5 kDa and a stirred cell system (Amicon, Inc., MA, USA) with membranes of 3 kDa and 1 kDa cut-offs. Manometers before and after UF membranes were used to measure the inlet and the outlet pressures in order to control the trans-membrane pressures (ΔP), which were equal to 1 bar for the tangential flow filtration (TFF) and 5 bar for the frontal stirred ultrafiltration cell system. Separation was performed using a decreased molecular mass order from 50 to 1 kDa, as reported in Fig. 1A. The obtained fractions were noted FI (MW>50 kDa), FII (5<MW<50 kDa), FIII (3<MW<5 kDa), FIV (3<MW<1 kDa) and FV (MW<1 kDa) and their protein content was immediately quantified using the Lowry reagent [18]. During fractionation, the permeate flux (J, L/h.m²) was calculated as the following:

$J = V_p / (t \times S)$,

where V_p (L) is the amount of permeate collected during the period of time t (h) and the permeation surface area of membrane S (m^2).

Prior to each experiment, the stabilized water flux (J_0) of each membrane was measured in order to estimate the total fouling (TF) of each membrane. TF was calculated as follows:

$TF = (J_0 – J_e) / J_0$,

where J_e is the ending flux in the filtration of sample solution.

Finally, the collected fractions were freeze-dried, weighed to determine their proportions in the hydrolysate mixture and then assayed for their ACE inhibition activity.

2.6. Amino acids composition of SHPH and its MWCO fractions

The amino acids (AA) composition was determined according to Aristoy and Toldrá [19]. A volume of 50 µl of each sample (3 mg/ml) was mixed with 50 µl of Norleucine (100 µg/ml), used as internal standard, in vacuum-sealed glass tubes. After being dried under nitrogen-vacuum cycles, glass tubes containing protein samples were hydrolyzed in 300 µl of 6 M HCl containing 1% (v/v) phenol at 120 °C for 24 h. After hydrolysis, samples were derivatizated with phenyl isothiocyanate (PITC) and dissolved in 300 µl of sodium phosphate buffer (5 mM, pH 7.4) containing 5% (v/v) of acetonitrile. The PITC derivates were quantified by reverse phase-high performance liquid chromatography (RP-HPLC) with a 1200 Agilent liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector and using a PicoTag® column (300 mm × 3.9 mm, Waters, Ireland) set at 52 °C. The detection was carried out at 254 nm, and amino acids quantification was done compared to the amino acid standards. The amino acids content was expressed in g of amino acid per 100 g amino acids in the sample.

2.7. Evaluation of the ACE-inhibitory activity

The ACE-inhibitory activity of the SHPH, its MWCO fractions and RP-HPLC sub-fractions was measured according to Sentandreu and Toldrá [20]. This assay is based on the ability of ACE to hydrolyze the internally quenched fluorescent substrate o-aminobenzoyl-glycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe-(NO_2)-Pro). A sample solution (50 µl) was mixed with 50 µl of 150 mM Tris-base buffer (pH 8.3) containing 3 mU/ml of ACE. The reaction was initiated by the addition of 200 µl of 150 mMTris–HCl buffer (pH 8.3) containing 1.125 M NaCl and 10 mM Abz-Gly-Phe-(NO_2)-Pro. The reaction mixture was then incubated for 60 min at 37 °C. The

generation of fluorescence due to the release of o-aminobenzoyl-glycine (Abz-Gly) by the action of ACE was measured, each 15 min during 1 h, using excitation and emission wavelengths of 355 and 405 nm, respectively. Captopril was used as a positive control. ACE inhibition results are expressed as percentage and the IC_{50} (µg/ml) as the amount of sample required to inhibit 50% of ACE activity.

2.8. Blood pressure measurement

Twenty male Wistar rats, weighing between 150-200 g were purchased from the Central Pharmacy of Tunisia (SIPHAT, Tunis City, Tunisia). Animals were housed in an environmentally controlled room (temperature = 25 ± 1 °C; relative humidity = 60 ± 5 %; 12 h light/dark cycle) in the laboratory of the Faculty of Sciences of Sfax city, Tunisia, and were allowed free access to tap water and alimentation during the experimental period. Laboratory animal handling and experimental procedures were performed according to the guidelines of the Tunisian Ethical Committee for the care and use of laboratory animals. After the acclimatization period, 0.25 mg/kg of body weight (BW) of adrenaline (Adrenaline Renaudin, France) was daily injected into rats through intraperitoneal route using a 1 ml disposable syringe for one week to induce hypertension [21]. Rats were divided into five groups of 4 rats each and their daily systolic blood pressure (SBP) was evaluated, using a non invasive method, to confirm hypertension induction. After one week of hypertension induction, 1 ml of phosphate buffered saline (PBS) containing 8 g/l NaCl (pH 7.4), captopril (20 mg/kg BW), SHPH and its most *in vitro* active fractions (FIV and FV, 200 mg/kg BW), dissolved in PBS buffer, were administered to rats by oral gavage. SBP was then measured at 2, 4, 7 and 24 h. Prior to oral gavage, the baseline (time zero) SBP was determined, and the change in SBP (ΔSBP, mm Hg) was determined by subtracting the baseline data from data obtained at different time points.

2.9. RP-HPLC analysis

SHPH and its most active MWCO fraction were dissolved at a concentration of 100 mg/ml and 100 µl were injected into an Agilent liquid chromatography (1100 series Agilent Technologies, Palo Alto, CA, USA) equipped with a C18 column (250 mm × 4.6 mm, Waters) set at 30 °C. Solvent A was 0.1% trifluoroacetic acid (TFA) in double distilled water and solvent B consisted of acetonitrile (ACN) / double distilled water (60:40, v/v) containing 0.085% of TFA. Mobile phases were filtered through a 0.45 µm filter and degassed. The elution started with 100% solvent A for 2 min, followed by a linear gradient from 0 to 25% of solvent B during 30 min; thereafter, B increased to 100% at 45 min and returning to 0% at 50 min. The flow rate used was fixed at 1 ml/min and the separation was monitored at a wavelength of 214 nm. The HPLC instrument was equipped with a fraction collector to collect the eluted sample in tubes (1 ml) based on a fixed interval of time (1 min). The elution profile was monitored at 214 nm during 45 min. After evaluating their ACE-inhibitory activity, fractions showing the highest potential were freeze-dried to be further analyzed by MS/MS.

2.10. Peptide identification by tandem mass spectrometry

The identification of the peptide sequence from fractions exhibiting remarkable ACE-inhibitory activity was carried out by MS/MS analysis. The nESI-LC-MS/MS analysis was performed using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA) coupled to the quadrupole-time-of-flight (Q-ToF) TripleTOF® 5600 system from AB Sciex Instruments (Framingham, MA) that is equipped with a nanoelectrospray ionization source (nano-ESI). The selected RP-HPLC fractions were re-suspended in H_2O with 0.1% of TFA and 5 µl of the sample were injected. Diluted samples were pre-concentrated on a 0.3 × 5 mm, 3 µm, C18 trap column from LC Packings PepMap (Dionex Company, Amsterdam, The Netherlands) at a flow rate of 40

µl/min and using 0.1% TFA as mobile phase. After 5 min of pre-concentration, the trap column was automatically switched in-line onto a nanoHPLC capillary column (3µm, 75µm x 12.3 cm, C18) (NikkyoTechnos Co, Ltd. Japan). Mobile phase A contained 0.1% (v/v) formic acid in water, and solvent B, contained 0.1% (v/v) formic acid in 100% ACN. After elution, the positive TOF mass spectra were recorded on the QSTAR instrument using information dependent acquisition (IDA). TOF-MS survey scan was followed by MS/MS scans of the three most intense peaks. Typical ion spray voltage was in the range of 2.5–3.0 kV, and nitrogen was used as collision gas. Other source parameters and spray position were optimized with a tryptic digest of protein mixture digest (LC Packings; P/N 161088).

Automated spectral processing and peak list generation were performed using Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA) (http://www.matrixscience.com). The NCBInr protein database was used to identify the peptides with a significance threshold $p < 0.05$ and a FDR (False Discovery Rate) of 1.5%. The tolerance on the mass measurement was 100 ppm in MS mode and 0.3 Da in MS/MS ions. Regions of local similarity with fish protein sequence were searched using BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) database was used to search similar sequences, showing ACE-inhibitory activity, previously identified.

2.11. Computational modeling

The three-dimensional structure of native human ACE was imported from the Protein Data Bank (1O8A.pdb). Before the docking, water molecules and lisinopril were removed whereas the cofactors zinc and chloride atoms were retained in ACE model. The structure of the peptides was drawn using Accelrys Discovery Studio 4.0 Visualizer (DS 4.0, Accelrys Software Inc., San Diego, CA) and the files were saved as PDB. Peptide and ACE interaction models were predicted by

GRAMM-X protein–protein docking server (Version 12.0) [22]. Ten output conformation models were then obtained. The output file of each docking was opened with DS 4.0 and the best docking conformation was kept. These docking conformations were sent to the Rosetta FlexPepDock server to be refined from a complex between a protein receptor and an estimated conformation for a peptide, allowing full flexibility to the peptide and side-chain of the receptor. Two hundred high resolution structures were defined for each peptide run. FlexPepDock gave an output of predicted energies for each complex in Rosetta energy units which is a standard unit used by computational modeling web servers that use the Rosetta algorithm while scoring complexes [23]. The top one conformation output was saved and analyzed using DS 4.0 where peptides were added the CHARMM36 (Chemistry at HARvard Macromolecular Mechanics), a force field simulation tool, to correct any resulting mischarges before starting the protein structure–function relationships study.

2.12. Statistical analysis

Data were expressed as mean ± SD (Standard Deviation) and statistically analyzed using SPSS ver. 17.0, professional edition (SPSS Inc., Chicago, USA). A one-way analysis of variance (ANOVA) was then performed and means comparison was carried out by Duncan's multiple range test to estimate the significance among the main effects at the 5% probability level.

3. Results and discussion

3.1. Fractionation of SHPH by ultrafiltration

In this study, smooth-hound protein viscera were treated with Esperase® at pH 9.0 during 580 min. After hydrolysis, the final DH was equal to 13.7%, which considered as relatively high DH level for relatively complex fish materials (viscera).

Membrane technologies (ultra- and nano-filtration) have been widely used to fractionate by-product protein hydrolysates with the aim of enhancing their biological properties [24]. Thus, in order to improve its bioactivity, SHPH was fractionated according to its MW distribution using membranes with different cut-offs ranged from 50 to 1 kDa (Fig. 1A). In fact, SHPH was first filtered with a 50 kDa MWCO membrane to remove high MW poly-peptides, while two low MWCO membranes (3 and 1 kDa) were applied to obtain fractions enriched in low MW peptides.

During UF, the permeate flux (J) were measured with time. Fig. 1 illustrates the flux decline in terms of liters of permeate produced per unit time and membrane area (L/h m²) in the tangential flow filtration (TFF) (B) and the stirred cell (C) systems up to VCF (volume concentrate final) of 5 and 10, respectively. The initial permeates flux in the TFF system were 7.60 and 28.80 L/ h m² for 50 and 5 MWCO membranes, respectively. Thereafter, the fluxes decreased gradually over time and tended to be constant (5.01 and 28.68 L/h m², respectively). Similarly, the flux of the frontal filtration (Fig. 1C) gradually decreased to reach 21.0 and 9.76 L/h m² using 3 and 1 kDa MWCO membranes, respectively. Similar trend was previously observed for the UF of tuna protein waste hydrolysate using 4 and 1 kDa MWCO membranes [25]. The decline of flux over time could be linked to the increase of the thickness of peptide layer over the membrane surface leading to increase the resistance of the membrane against the hydrolysate solution flow and, therefore, the decline in permeate flux [26]. In fact, the total fouling values across the membranes were estimated to be 0.39 (3 kDa) and 0.48 (1 kDa). This observation is line with that reported by Zhou et al. [27] showing more flux decline accompanied with higher total fouling value during the UF of algal extracellular organic matters.

Following the UF process in successive stages, five different fractions were obtained. Small peptide fractions (FIV+FV) yielded a 27.44±1.95% of the total proteins recovered from the four UF steps. This finding suggested that studied Esperase® hydrolysis is suitable to produce a great

amount of low MW peptides, which would be related to final detected bioactivity. In fact, previous researches have shown that small sized peptides exhibited the strongest ACE-inhibitory activities [6,28]. Garcia-Moreno et al. [29] found that Alcalase and Savinase produced the most potent ACE-inhibitory hydrolysates from lentil proteins and also showed the highest yield of shorter peptide sequences from all the studied hydrolysates. ACE-inhibitory peptides generally contain short sequences (between 3 – 12 amino acids) due to the fact that the lid structure of ACE restrict the access of large and folded peptides to the active site cleft [30].

3.2. Characterization of SHPH and its derived fractions

3.2.1. Amino acid compositions

The amino acid compositions of SHPH and its MWCO fractions obtained from UF were determined and the results are illustrated in Table 1. The amino acid profiles were similar although some significant differences were detected. In this respect, the non-fractionated hydrolysate contained a significant amount of Glx and Gly, followed by Lys, Asx, Arg, Thr, Ala and Pro amino acids. However, the derived fractions, particularly FIV and FV, contained higher amounts of Ala, Arg, Tyr, Val, Met, Leu and Phe, leading to increase their total hydrophobic amino acids content (THAA), in comparison with the other fractions. These data suggested that UF improve the concentration of hydrophobic residues that reached 39.63% and 41.68%, in FIV and FV, respectively. Furthermore, among the different fractions, FIV and FV showed the highest essential amino acids contents (31.51 and 33.94%, respectively), indicating their high nutritional value and their potential use as supplements in functional food formulations. Saidi et al. [31] reported that the nanofiltration of tuna byproducts Alcalase hydrolysate a through 1 kDa membrane, gave a retentate enriched in Val, Thr, Met, Phe, Trp and Lys amino acids. The increased content in THAA content in FIV and FV fractions could account for their expected high bioactivity. In fact,

hydrophobic residues present in the three positions closest to the C-terminal site of peptides enhance their ACE-inhibitory activity [6]. These results are in line with those reported by Chi et al. [32], showing that fractions from skipjack tuna dark muscle protein hydrolysates had high levels of hydrophobic and aromatic amino acids and exhibited the best biological activities. Therefore, the presence of highest hydrophobic and aromatic amino acid contents in FIV and FV might be main reasons for the showed biological properties.

3.2.2. RP-HPLC profiles

In addition to the amino acid composition, peptide sequence and hydrophobicity represent two other key factors that affect the activity of protein hydrolysates. Successive fractionation of the starting protein hydrolysate by UF membranes allowed peptides separation according to their sizes and in turn they would exhibit distinct hydrophobic/hydrophilic properties. The RP-HPLC profiles of the SHPH fractions after UF separation are shown in Fig. 2. The obtained chromatograms showed that all the fractions exhibited different profiles according to the membrane used. A huge number of peaks was detected during the elution period. As it is shown, FI to FIII were mostly composed of peptides eluted between the 3^{rd} and 40^{th} min, whereas FIV and FV showed more simplified spectra and contained fewer and early-eluted peptides. Therefore, based on their shorter retention time, the hydrophobic properties of peptides in the last fractions might be weaker than those obtained from the first UF steps. Previous studies showed that the presence of specific amino acids may influence the interactions between peptides when the peptide layer was in contact with the UF membrane [24], leading to different peptide profiles in the resulting fractions.

3.2.3. Evaluation of the ACE-inhibitory activity

ACE-inhibitory activity of the resulting fractions was evaluated (Table 2). Data indicated that the SHPH exhibited a strong ability with an IC_{50} of 164.56 µg/ml, proving the effective role of Esperase® on the release of ACE-inhibitory peptides from the smooth-hound viscera. The FIV and FV fractions, showed lower IC_{50} values (101.61 and 92.75 µg/ml, respectively), compared to the SHPH and the other fractions. This observation is in good agreement with previous studies reporting greater *in vitro* activity of low MW peptides rather than those with high MW. However, both fractions were less potent than Captopril (IC_{50} = 19.31 µg/ml). Hayes et al. [6] have shown that after fractionation, the 3-kDa boarfish protein hydrolysate generated using protease AP inhibited the ACE by 85.8 % at 1 mg/ml, while the hydrolysate showed an activity of 72.08%. On the other hand, Silvestre et al. [11] have reported that the UF process may cause the retention of peptides with structures (aromatic amino acids or proline at the C-terminal position) that favor ACE-inhibitory activity. However, in some cases, UF showed no effect on the enhancement of the ACE-inhibitory activity. In this context, Mullally et al. [33] demonstrated that no change in ACE-inhibitory activity was observed when a 10 kDa cut-off membrane was used to fractionate trypsin-hydrolyzed whey proteins. In fact, it may occur a synergistic action between peptides found in the whole hydrolysate. He et al. [34] found, similarly, a synergistic effect between peptides in the rapeseed protein hydrolysate which exhibited stronger ACE-inhibitory activity (IC_{50} = 93.2 µg/ml), than fractions obtained from electrodialysis with ultrafiltration membrane.

3.2.4. In vivo evaluation of the antihypertensive activity

In order to confirm *in vitro* findings, SHPH and the most active MWCO fractions (FIV and FV) were assayed for their antihypertensive effect *in vivo*. The results reported in Fig. 3 represent the short-term changes in SBP of hypertensive rats evaluated during a 24 h period. Rats received

by oral gavage 200 mg SHPH, FIV or FV per kg of BW, 20 mg capropril per kg BW (positive control group), and 1 ml of saline solution (negative control group). Differences in SBP (mm Hg) were analyzed after 2, 4, 7 and 24 h of saline administration compared to t = 0 min (before oral gavage). Saline solution administration increased significantly SBP during the first 4 h, while the SHPH and its MWCO fractions showed an antagonist affect. The maximum SBP decreases were early obtained (after 4 h). SHPH was the least active treatment, showing the highest SBP values among the treated groups, at a dose of 200 mg/kg BW; then, this effect was almost lost after 24 h. Similarly, Lafarga et al. [35] study showed no significant differences between saline-treated rats and those receiving bovine serum albumin hydrolysate (200 mg/kg BW) after 24 h. However, used at the same dose, FIV and FV gave the best blood-lowering results and the SBP values decreased to -9.0±1.0 and -8.33±2.19 (after 2 h), respectively. At 4, 7 and 24 h post-administration, FV was the most effective as an SBP-lowering agent, with respective SBP values of -8.3±0.67, -8.0±1.0 and -5.0±0.58 mmHg. All over the treatment times, FV showed a similar trend to that of captopril in lowering the SBP. Similar to our results, Girgih et al. [36] have shown that a dose of 200 mg/kg BW of salmon protein hydrolysate and 30 mg/kg BW of its most *in vitro* active RP-HPLC peptide fraction were able to reduce the SBP after 24 h of saline administration. In addition, the after oral administration of hemp seed hydrolysate fractions (<1 and 1-3 kDa) at 100 mg/kg BW to hypertensive rats produced the maximum systolic blood pressure reduction (-15 mmHg) after six hours [37].

Overall results showed that fractions containing the smallest peptides (FIV and FV), were the strongest ACE-inhibitor and antihypertensive agents, thus they were chosen to be further fractionated for bioactive peptides identification.

3.3. RP-HPLC fractionation of ACE-inhibitory peptides in FIV and FV

FIV and FV fractions obtained from the SHPH were separated according to their hydrophobicity using RP-HPLC. Fig. 4 shows the elution profile of both samples and their ACE-inhibitory activity. Several peaks of activity, in both FIV and FV fractions were detected during the elution period. Four major sub-fractions (SFs) in FIV, eluted at 15, 19, 33 and 37-39 min, showed high ACE-inhibitory potential, whereas the best ACE-inhibitory activity peaks in FV were eluted at 3, 15, 20 and 33 min. Based on their activities, two sub-fractions, SF1 (37-39 min) and SF2 (20 min) were collected and freeze-dried to be analyzed by tandem mass spectrometry (nano ESI-LC-MS/MS), with the aim to characterize their peptide content.

3.4. Identification of bioactive peptides by tandem-mass spectrometry

After nLC-MS/MS and Mascot analysis, a total number of 475 and 221 peptides were sequenced in SF1 and SF2, respectively, with a percentage of confidence ≥ 95%. Interestingly, all peptides were found novelty identified when compared to previously reported ACE-inhibitory peptides available in the literature.

Fig. 5 shows the distribution in percentages of the identified peptides according to their origin proteins. Main identified peptides are derived from collagen (30 and 49% in SF1 and SF2, respectively). Collagen-derived proteins have been reported to give rise to biological active peptides with high ACE-inhibitory capacity [8,38]. Furthermore, actin-derived peptides were found at a percentage of 12 and 10% in SF1 (Fig. 5A) and SF2 (Fig. 5B), respectively. Actin is an important protein in skeletal muscle responsible for cell movement and muscle contraction. Previous study have shown that ACE-inhibitory peptides from thornback ray muscle hydrolysates were mainly derived from actin (more than 47%) [8].

Moreover, identified peptides were characterized in terms of their observed mass/charge (*m/z*), expected and calculated molecular masses. Table 3 reported the sequences of some peptides, selected according to their length (between 6 and 12 amino acids). In fact, Shimizu et al. [39] demonstrated that oligo-peptides with 4 to 9 amino acids in length could be able to diffuse passively through the membranes' wall. In addition, Euston et al. [40] reported that protein hydrolysates with hydrophobic amino acids may be easily absorbed, which facilitate their *in vivo* biological properties. As shown in Table 3, peptides identified in SF1 and SF2 were mainly hydrophobic, and therefore they may exert their effect in the organism. However, a major part of SF2 peptides shared identical amino acids (Pro, Gly, Ala and Leu) in their sequences, which were present as repeating tri-peptides in the different positions (Table 3b). For instance, the sequences GPA, GPP, LPG, GLP, and PLP, exhibiting potent ACE-inhibitory abilities with respective IC_{50} of 405, 23.10, 5.73, 1.62, and 430 µM (Biopep database), were frequently detected in these peptides. In addition, the motif GPX (GPA, GPH, GPQ, GPR, GPG, etc.) was observed in several peptides such as PRGPAGPHGPP and GPAGPRGPA. In this context, Gu and Wu [41] and Lassoued et al. [42] studies have shown that peptides, with the repeated motifs PX and GPX, tend to be satisfactory ACE inhibitors.

Peptides of sub-fractions SF1 and SF2 were analyzed using BIOPEP data base to be compared to previously reported bioactive sequences. Interestingly, almost peptides share a partial homology, particularly in their N-terminal and C-terminal sites, with ACE-inhibitory, antioxidant and anti-thrombotic peptides (Table 4).

Extended comparison revealed that the sequences WDDMEK and IWHHT were frequently repeated, partially or totally, among numerous SF1 peptides. In fact, N<u>WDDMEK</u>**<u>IWH</u>**<u>,</u> <u>WDDMEK</u>**IWHH**, <u>DDMEK</u>**IWHH**, <u>MEK</u>**IWHHT** were composed of two parts, the N-ter one

sowed a partial or a total homology with the antioxidant peptide, WDDMEK [43], while their C-terminus contained the sequence IWHHT, which is an ACE inhibitor peptide (IC_{50} = 5.80 µM) [44]. In addition, the peptide **MYPGIA**DRM contained the sequence **MYPGIA** in its C-ter position, which was previously reported by Escudero et al. [45] as ACE inhibitor, with an IC_{50} value of 641.02 µM. Similarly, VFPS (IC_{50} = 0.46 µM) represent the C-ter part of GDDAPRA**VFPS**, suggesting that this peptide could display an ACE-inhibitory potential.

EIFDKARQ**AAP** contained interesting C-terminus tri-peptide, AAP previously isolated from casein and known for its ACE-inhibitory potential [46]. Furthermore, the AAP tri-peptide was recorded, in different positions of ACE inhibitors isolated from ham meat (KAA**AAP**, KPV**AAP** and **AAP**LAP) [47], and in the C-ter of C**AAP**, an antioxidant peptide isolated from flounder fish (*Paralichthys olivaceus*) using digestive proteases. In addition, **EKSYELP**DGQVI showed high similarity (7 identical residues) with **EKSYELP**, a potential antihypertensive and ACE-inhibitory peptide identified in the protein hydrolysate of cuttlefish (*Sepia officinalis*) muscle protein hydrolysate [48]. Thus, this peptide could display an antihypertensive effect.

When analyzing SF2 sequences, the collagen-derived peptide **GPA**GPR**GPA** shares the sequence GPA, with the ACE inhibitor peptide **GPA**GAPGAA. In addition, PRGPAGPH**GPP** contains the sequence GPP, previously identified as ACE inhibitor and antioxidant peptide [49,50]. In addition, **IAG**PPGSAGPAG shares the sequence IAG with the previously described ACE-inhibitory peptide **IAG**RP (IC_{50} = 25.94 µM), which was identified from Spanish dry-cured ham [47]. The N-terminal sequence VVP found in **VVP**FEGAV was observed in the ACE-inhibitory peptide **VVP**P, identified in the milk casein (IC_{50} = 258.21 µM) [51]. Thus, **VVP**FEGAV could be ACE inhibitor. Moreover, the peptide **PLP**KREE contained the ACE inhibitor tri-peptide PLP (IC_{50} = 430 µM) [52] and the anti-thrombotic tetra-peptide KREE [53]. DSFEG**LQQ** shows the

sequence LQQ in its C-terminus, which is an ACE-inhibitor peptide with an IC_{50} value of 100 µM [54].

Thus, the presence of these interesting N-ter and C-ter sequences in SHPH-derived peptides could be probably responsible for their expected potent ACE-inhibitory activity. In this respect, three peptides from SF1 (MYPGIADRM, MEKIWHHT, and GDDAPRAVFPS) and eight peptides from SF2 (GPAGPRGPA, IAGPPGSAGPAG, PRGPAGPHGPP, VVPFEGAV, PLPKREE, DSFEGLQQ, PTVPKRPSPT, and EGLQQLR) were selected to study their interaction with human ACE by computational modeling, in order to establish the relationship between their structure and activity.

3.5. Molecular docking simulation

The ACE-inhibitory activity has been increasingly studied over the last three decades. In fact, ACE is a meltallo-protease involved in the regulation of blood pressure homeostasis. Pina and Roque [55] have reported that ACE contained three active pockets, S1, S2 and S1'. S1 included Ala354, Glu384 and Tyr523 residues, S2 included Gln281, His353, Lys511, His513 and Tyr520 residues, while S1' contained Glu162 residue. Further, as a metalloenzyme, ACE had a zinc ion (Zn^{2+}) in its active site that coordinates with His383, His387 and Glu411.

Eleven peptides from the SHPH sub-fractions were analyzed by automated docking to study the structure-activity relationships between these peptides and ACE active site. The total energy score, the interaction score, and the numbers of predicted hydrogen bonds and ACE residues in interaction with the peptide were determined (Table 5).

Results showed that all peptides were able to inhibit the ACE activity, but with different extents, as demonstrated by the total energy and the interaction scores. Particularly, the lowest interaction energy scores were recorded in IGPPGSAGPAG (-21.076), followed by PLPKREE (-

21.143) and PTVPKRPSPT (-21.679), indicating that these peptides could interact efficiently with ACE. Furthermore, PLPKREE and PTVPKRPSPT could interact with all the ACE key residues, through noncovalent interactions particularly Van der Waals and hydrogen bonds. Also, a number of 11 ACE key amino acids were in interaction with VVPFEGAV peptide. The highest number of H-H bonds was recorded in GPAGPRGPA and IGPPGSAGPAG, which could establish 9 hydrogen bonds with ACE residues.

Therefore, the molecular docking results of IGPPGSAGPAG, VVPFEGAV, PLPKREE, and PTVPKRPSPT were further detailed. The list of ACE key residues (S1, S2 and S1' pockets and those in coordination with Zn^{2+}) involved in the interaction with selected peptides, are illustrated in Table 6. Additionally, the best pose of each peptide is shown in Fig. 6 (2D diagrams) and Fig. 7 (3D diagrams). Our results revealed that the binding site of all peptides were located at the catalytic region of the enzyme. In addition, almost key residues of the ACE active site contribute to the peptide binding and stabilizing, through the formation of hydrogen bonds and hydrophobic and electrostatic (Pi and van der Waals) interactions. Further, the four peptides were able to interact with S1 and S2 pockets, while the S1' (Glu162) was not usually accessible.

Surprisingly, the molecular docking of IGPPGSAGPAG on the ACE binding site revealed that it could make contact with ACE residues via two strong positions, either by its C-terminus tri-peptide PAG (Fig. 6Ai) or its N-terminal sequence IAGP (Fig. 6Aii). Thus, the present peptide may affect the enzyme activity through two different inhibition mechanisms. Interestingly, in the first position, 9 H-bonds could be made between the peptide and ACE, in which three bonds were established with His383, Glu384, and His513. Van der Waals interactions were also made with Gln281, Ala354, Ala356, and His410, and electrostatic interactions with Lys511. Other unfavorable bumps with His353, His387, Glu411 and Tyr523 contributed also to the peptide pose stabilization. In addition, the C-terminal di-peptide of VVPFEGAV was responsible of the

interaction with almost ACE key residues, while the 2nd Pro residue was more involved in the inhibitory effect of PLPKREE by interacting with Ala354, Glu384, His513, Tyr520, His 353, and Tyr523 via hydrogen and electrostatic bonds (Fig. 6C). This peptide could also emit interactions either with Glu162, Gln281, His383, and Lys511 residues via its N-terminal di-peptide (PL) or with His387 and Glu411 via its C-ter sequence, suggesting that PLPKREE peptide could effectively interacted with the ACE active pockets, and thus contributed to its stronger inhibition activity. Similarly, Val-Pro residues of PTVPKRPSPT were found to be the major residues involved in the interaction with ACE active site. These results are in line with previous studies showing that Pro is the most residue involved in the ACE inhibition [11].

Moreover, the 3D interactions of the best docking poses of the studied peptides showed that the predicted interaction modes between peptides and ACE were different (Fig. 7). Despite peptides did not coordinate directly with the active site Zn(II), they were able to be positioned to coordinate with His383, His387 and Glu411. Together with the three residues (His383, Glu411 and His 387) coordinating with Zn^{2+}, peptides around the zinc ion formed a distorted geometry, as compared to the native position (Fig. 8). All these changes may help to stabilize the complex peptide-enzyme and therefore contributing to its better inhibition strength. Similarly, the captopril, a synthetic antihypertensive drug, was able to interact with His383, His387, and Glu411 via basic and acidic interactions. In the same context, Wu et al. [56] have reported that TLS displayed a high ACE-inhibitory activity with no direct interaction between the peptide and the Zn(II) atom. In addition, Jimsheena and Gowda [57] described that arachin ACE-inhibitory peptides did not directly coordinate with the Zn(II) atom, but, their interaction with His383, His387 and Glu411 promoted their inhibitory activity. In contrast, Ko et al. [7] studies have shown that MEVFVP, a competitive ACE inhibitor peptide, could bind the enzymatic active site through a metal ion interaction (Zn701), H-bond interactions including Glu411, Arg522, Asn66, and a Pi interaction bond via the Tyr523. Non-competitive ACE inhibitors able to interact to ACE leading to its conformational change, have been frequently reported [56,58]. However, competitive inhibitors have been found rarely described. Therefore, this study is very important finding for identifying potential

hypertensive agents that can be used as potential candidates to ACE inhibitor commercial medicines.

4. Conclusion

Novel peptides from smooth-hound viscera protein hydrolysate, produced by Esperase®, were isolated by two successive purification steps, ultrafiltration and reverse phase-HPLC. The use of mass spectrometry in tandem analysis, allows the identification of peptides released during proteolysis. After structure-activity relationship study, results from the docking simulation suggested that peptide sequences were able to bind the ACE through interactions with at least two active pockets, besides their interaction with residues that coordinate with Zn^{2+} ion. In further research, these peptides will be tested after the gastrointestinal digestion and *in vivo* (long-term treatment), in order to confirm their bioavailability under physiological conditions. Smooth-hound viscera proteins could serve, therefore, as a source for the generation of hydrolysates containing ACE-inhibitory peptides, which might be applied as ingredients in functional foods, dietary supplements or in pharmaceutical field.

Acknowledgements

This work was funded by the Ministry of Higher Education and Scientific Research, Tunisia. Emerging Research Group Grant from Generalitat Valenciana in Spain (GV/2015/138) and Juan de la Cierva de Incorporación postdoctoral contract of L.M. are acknowledged. The proteomic analysis was carried out by the SCSIE University of Valencia Proteomics Unit (Spain), a member of ISCIII ProteoRed Proteomics Platform. Authors want to acknowledge also the Laboratory of Animal Ecophysiology (University of Sfax, Faculty of Sciences of Sfax) for rats care and treatment.

References

[1] Slizyte R., Mozuraityte R., Martinez-Alvarez O., Falch E., Fouchereau-Peron M., Rustad T., Functional, bioactive and antioxidative properties of hydrolysates obtained from cod (*Gadus morhua*) backbones, Process Biochem. 44 (2009) 668–677.

[2] Chalamaiah M., Dinesh Kumar B., Hemalatha R., Jyothirmayi T., Fish protein hydrolysates: proximate composition, amino acid composition, antioxidant activities and applications: a review, Food Chem. 135 (2012) 3020-3038.

[3] Cheung R.C.F., Ng T.B., Wong J.H., Marine Peptides: Bioactivities and Applications, Mar. drugs. 13 (2015) 4006-4043.

[4] Ferreira S. H., Bartelt D.C., Greene L.J., Isolation of bradykinin-potentiating peptides from *Bothrops jararaca* venom. Biochemistry. 9 (1970) 2583–2593.

[5] Forghani B., Zarei M., Ebrahimpour A., Philip R., Bakar J., Abdul Hamid A., Saari N., Purification and characterization of angiotensin converting enzyme-inhibitory peptides derived from *Stichopus horrens*: Stability study against the ACE and inhibition kinetics, J. Funct. Foods. 20 (2016) 276–290.

[6] Hayes M., Mora L., Hussey K., Aluko R.E., Boarfish protein recovery using the pH-shift process and generation of protein hydrolysates with ACE-inhibitory and antihypertensive bioactivities in spontaneously hypertensive rats, Innov. Food Sci. Emerg. Technol. 37 (2016) 253-260.

[7] Ko J.-Y., Kang N., Lee J.-H., Kim J.-S., Kim W.-S., Park S.-J., Kim Y.-T., Jeon Y.-J., Angiotensin I-converting enzyme inhibitory peptides from an enzymatic hydrolysate of flounder fish (*Paralichthys olivaceus*) muscle as a potent anti-hypertensive agent, Process Biochem. 51(2016) 535–541.

[8] Lassoued I., Mora L., Barkia A., Aristoy M.-C., Nasri M., Toldrá F., Angiotensin I-converting enzyme inhibitory peptides FQPSF and LKYPI identified in *Bacillus subtilis* A26 hydrolysate of thornback ray muscle, Int. J. Food Sci. Food Tech. 51 (2016) 1604-1609.

[9] Wu S., Feng X., Lan X., Xu Y., Liao D., Purification and identification of Angiotensin-I Converting Enzyme (ACE) inhibitory peptide from lizard fish (*Saurida elongata*) hydrolysate, J. Funct. Foods. 13 (2015) 295–299.

[10] Nasri R., Chataigné G., Bougatef A., Karra-Chaâbouni M., Dhulster P., Nasri M., Nedjar-Arroume N., Novel angiotensin I-converting enzyme inhibitory peptides from enzymatic hydrolysates of Goby (*Zosterisessor ophiocephalus*) muscle proteins, J. Proteomics. 91 (2013) 444-452.

[11] Silvestre M.P.C., Silva M.R., Silva V.D.M., Souza M.W.S., Junior C.O.L., Afonso W.O., Analysis of whey protein hydrolysates: peptide profile and ACE-inhibitory activity, Braz. J. Pharm. Sci. 48 (2012) 747-757.

[12] Wu J., Aluko R.E., Nakai S., Structural requirements of angiotensin I-converting enzyme inhibitory peptides: quantitative structure-activity relationship study of di- and tripeptides, J. Agr. Food Chem. 54 (2006) 732–738.

[13] Erdös E.G., Tan F., Skidgel R.A., Angiotensin I-converting enzyme inhibitors are allosteric enhancers of kinin B1 and B2 receptor function, Hypertension. 55 (2010) 214-20.

[14] WHO, A global brief on hypertension: Silent killer, global public health crisis (2013).

[15] Kembhavi A., Kulkarni A., Pant A., Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM No. 64, Appl. Biochem. Biotechnol. 38 (1993) 83–92.

[16] Abdelhedi O., Jridi M., Jemil I., Mora L., Toldrá F., Aristoy M.-C., Boualga A., Nasri M., Nasri R., Combined biocatalytic conversion of smooth hound viscera: Protein hydrolysates elaboration and assessment of their antioxidant, anti-ACE and antibacterial activities, Food. Res. Int. 86 (2016) 9-23.

[17] Adler-Nissen J., Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid, J. Agr. Food. Chem. 27 (1979). 1256-1262.

[18] Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J., Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951). 265-275.

[19] Aristoy M.-C., Toldrá F., Deproteinization techniques for HPLC amino acids analysis in fresh pork muscle and dry cured ham, J. Agr. Food. Chem. 39 (1991) 1792-1795.

[20] Sentandreu M.A., Toldrá F., A rapid, simple and sensitive fluorescence method for the assay of angiotensin-I converting enzyme, Food. Chem. 97 (2006) 546–554.

[21] James O., Friday E.A., Unekwuojo E.G., Antihypertensive effect of methanol extract of *Napoleona imperialis* (p. beauv) in adrenaline induced hypertensive Albino rats, Int. J. Biochem. Res. Rev. 1 (2011) 47-57.

[22] Tovchigrechko A., Vakser I.A., GRAMM-X public web server for protein–protein docking, Nucleic. Acids. Res. 34 (Web Server Issue) (2006) W310–W314.

[23] Oseguera-Toledo M.E., de Mejia E.G., Amaya-Llano S.L., Hard-to-cook bean (*Phaseolus vulgaris* L.) proteins hydrolyzed by alcalase and bromelain produced bioactive peptide fractions that inhibit targets of type-2 diabetes and oxidative stress, Food. Res. Int. 76 (2015) 839-851.

[24] Picot L., Ravallec R., Fouchereau-Péron M., Vandanjon L., Jaouen P., Chaplain-Derouiniot M., Gurerard F., Chabeaud A., LeGal Y., Alvarez O.M., Berg´ e J.-P., Piot J.-M., Batista I., Pires C., Thorkelsson G., Delannoy C., Jakobsen G., Johansson I., Bourseau P., Impact of ultrafiltration and nanofiltration of an industrial fish protein hydrolysate on its bioactive properties, J. Sci. Food Agric. 90 (2010) 1819–1826.

[25] Saidi S., Ben Amar R., Valorisation of tuna processing waste biomass for recovery of functional and antioxidant peptides using enzymatic hydrolysis and membrane fractionation process, Environ. Sci. Pollut. Res. 23 (2016) 21070-21085.

[26] Khemakhem I., Gargouri O.D., Dhouib A., Attia H., Ayadi M.A., Bouaziz M., Oleuropein rich extract from olive leaves by combining microfiltration, ultrafiltration and nanofiltration, Sep. Purif. Technol. 172 (2017) 310-317.

[27] Zhou S., Shao Y., Gao N., Li L., Deng J., Tan C., Zhu M., Influence of hydrophobic/hydrophilic fractions of extracellular organic matters of *Microcystis aeruginosa* on ultrafiltration membrane fouling, Sci. Total Environ. 470-471 (2014) 201-207.

[28] Nasri R., Jridi M., Lassoued I., Jemil I., Ben Slama-Ben Salem R., Nasri M., Karra-Châabouni M., The Influence of the extent of enzymatic hydrolysis on antioxidative properties and

ACE-inhibitorynhibitory activities of protein hydrolysates from goby (*Zosterisessor ophiocephalus*) muscle, Appl. Biochem. Biotechnol. 173 (2014) 1121-1134.

[29] García-Moreno P.J., Espejo-Carpio F.J., Guadix A., Guadix E.M., Production and identification of angiotensin I-converting enzyme (ACE) inhibitory peptides from Mediterranean fish discards, J. Funct. Foods. 18 (2015) 95-105.

[30] Sirtori C.R. Galli C., Anderson J.W, Arnoldi A., Nutritional and nutraceutical approaches to dyslipidemia and atherosclerosis prevention: focus on dietary proteins, Atherosclerosis. 203 (2009) 8‑17.

[31] Saidi S., Deratani A., Belleville M.-P., Amar R.B., Production and fractionation of tuna by-product protein hydrolysate by Ultrafiltration and Nanofiltration: Impact on interesting peptides fractions and nutritional properties, Food Res. Int. 65 (2014) 453-461.

[32] Chi C.-F., Hu F.-Y., Wang B., Li Z.-R., Luo H.-Y., Influence of amino acid compositions and peptide profiles on antioxidant capacities of two protein hydrolysates from Skipjack Tuna (*Katsuwonus pelamis*) dark muscle, Mar. Drugs. 13 (2015) 2580–2601.

[33] Mullally M.M., Meisel H., FitzGerald R.J., Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins, Int. Dairy J. 7 (1997) 299-303.

[34] He R., Girgih A.T., Rozoy E., Bazinet L., Ju X.-R., Aluko R.E., Selective separation and concentration of antihypertensive peptides from rapeseed protein hydrolysate by electrodialysis with ultrafiltration membranes, Food Chem. 197 (2016) 1008-1014.

[35] Lafarga T., Aluko R.E., Rai D.K., O'Connor P., Hayes M., Identification of bioactive peptides from a papain hydrolysate of bovine serum albumin and assessment of an antihypertensive effect in spontaneously hypertensive rats, Food Res. Int. 81 (2016) 91-99.

[36] Girgih A.T., Nwachukwu I.D., Hasan F.M., Fagbemi T.N., Malomo S.A., Gill T.A., Aluko R.E., Kinetics of *in vitro* enzyme inhibition and blood pressure-lowering effects of salmon (*Salmo salar*) protein hydrolysates in spontaneously hypertensive rats, J. Funct. Foods. 20 (2016) 43-53.

[37] Girgih A.T., Udenigwe C.C., Li H., Adebiyi A.P., Aluko R.E., Kinetics of enzyme inhibition and antihypertensive effects of hemp seed (*Cannabis sativa* L.) protein hydrolysates, J. Am. Oil Chem. Soc. 88 (2011) 1767-1774.

[38] Dave L.A., Hayes M., Montoya C.A., Rutherfurd S.M., Moughan P.J., Human gut endogenous proteins as a potential source of angiotensin-I-converting enzyme (ACE-I)-, renin inhibitory and antioxidant peptides, Peptides, 76 (2016) 30–44.

[39] Shimizu M., Tsunogai M., Arai S., Transepithelial transport of oligopeptides in the human intestinal cell, Caco-2, Peptides. 18 (1997) 681-687.

[40] Euston S.R., Finnigan S.R., Hirst R.L., Heat-induced destabilization of oil-in water emulsions formed from hydrolyzed whey protein, J. Agric. Food Chem. 49 (2001) 5576-5583.

[41] Gu Y., Wu J., LC-MS/MS coupled with QSAR modeling in characterising of angiotensin I-converting enzyme inhibitory peptides from soybean proteins, Food Chem. 141 (2013) 2682–2690.

[42] Lassoued I., Mora L., Barkia A., Aristoy M.-A., Nasri M., Toldrá F., Bioactive peptides identified in thornback ray skin's gelatin hydrolysates by proteases from *Bacillus subtilis* and *Bacillus amyloliquefaciens*. J. Proteomics. 128 (2015) 8-17.

[43] Liu R., Zheng W., Li J., Wang L., Wu H., Wang X., Shi L., Rapid identification of bioactive peptides with antioxidant activity from the enzymatic hydrolysate of *Mactra veneriformis* by UHPLC–Q-TOF mass spectrometry, Food Chem. 167 (2015) 484-489.

[44] Li G.-H., Le G.-W., Shi Y.-H., Shrestha S., Angiotensin I–converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects, Nutr. Res. 24 (2004) 469-486.

[45] Escudero E., Sentandreu M.A., Arihara K., Toldrá F., Angiotensin I-converting enzyme inhibitory peptides generated from *in vitro* gastrointestinal digestion of pork meat, J. Agric. Food Chem. 58 (2010) 2895-2901.

[46] Meisel H., Casokinins as bioactive peptides in the primary structure of casein, in: K.D. Schwenken, R. Mothes, Weinheim VCh (Eds.), Food proteins, structure and functionality, Publishing Inc., New York-Basel-Cambridge-Tokyo, 1993, pp. 67-75.

[47] Escudero E., Mora L., Toldrá F., Stability of ACE inhibitory ham peptides against heat treatment and *in vitro* digestion, Food Chem. 161 (2014) 305-311.

[48] Balti R., Bougatef A., Sila A., Guillochon D., Dhulster P., Nedjar-Arroume N., Nine novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (*Sepia officinalis*) muscle protein hydrolysates and antihypertensive effect of the potent active peptide in spontaneously hypertensive rats, Food Chem. 170 (2015) 519-525.

[49] Murray B.A., FitzGerald R.J., Angiotensin converting enzyme inhibitory peptides derived from food proteins: biochemistry, bioactivity and production, Curr. Pharm. Des. 13 (2007) 773-791.

[50] Zou T-B., He T-P., Li H-B., Tang H-W., Xia E-Q., The structure-activity relationship of the antioxidant peptides from natural proteins, Molecules. 21 (2016) 1-14.

[51] Wang Z., Zhang S., Wang W., Feng F., Shan W., A novel angiotensin I converting enzyme inhibitory peptide from the milk casein: virtual screening and docking studies, Agric. Sci. China, 10 (2011) 463-467.

[52] Kohmura M., Nio N., Kubo K., Minoshima Y., Munekata E., Ariyoshi Y., Inhibition of angiotensin-converting enzyme by synthetic peptides of human β-casein, Agric. Biol. Chem. 53 (1989) 2107-2114.

[53] Meddeb S., Demaret J., Ballini J.P., Fiat A.-M., Jolles P., Ptak M., Vigny P., A conformational study of Lys-Arg-Asp-Ser and analogs, a series of potent antithrombotic peptide. An approach based on simulated annealing and 1H-NMR, J. Biomol. Struct. Dyn. 11 (1994) 959-981.

[54] Yano S., Suzuki K., Funatsu G., Isolation from alpha-zein of thermolysin peptides with angiotensin I-converting enzyme inhibitory activity, Biosci. Biotech. Biochem. 60 (1996) 661-663.

[55] Pina A.S., Roque A.C.A., Studies on the molecular recognition between bioactive peptides and angiotensin-converting enzyme, J. Mol. Recognit. 22 (2009) 162-168.

[56] Wu Q., Du J., Jia J., Kuang C., Production of ACE inhibitory peptides from sweet sorghum grain protein using alcalase: Hydrolysis kinetic, purification and molecular docking study, Food Chem. 199 (2016) 140-149.

[57] Jimsheena V.K., Gowda L.R., Arachin derived peptides as selective angiotensin I-converting enzyme (ACE) inhibitors: Structure–activity relationship, Peptides. 31 (2010) 1165-1176.

[58] Fu Y., Young J.F., Rasmussen M.K., Dalsgaard T.K., Lametsch R., Aluko R.E., Therkildsen M., Angiotensin I–converting enzyme–inhibitory peptides from bovine collagen: insights into inhibitory mechanism and transepithelial transport, Food Res. Int. 89 (2016) 373-381.

Figure captions:

Figure 1: Experimental set-up of ultrafiltration process after enzymatic protein hydrolysis (A). The permeate flow as a function of time of the tangential flow filtration (B) and the stirred cell filtration (C) systems.

Figure 2: Reversed-phase chromatographic profiles of smooth-hound protein hydrolysate fractions obtained from UF process.

Figure 3: Short-term (24 h) changes in systolic blood pressure (SBP) of hypertensive rats after oral administration of saline, captopril (20 mg/kg BW), smooth-hound protein hydrolysate (SHPH) and its most *in vitro* active MWCO fractions (FIV and FV) used at 200 mg/kg BW. Data are expressed as mean ± standard error mean (SEM) (n=4). Different letters in each time represent significant differences between treatments ($p < 0.05$).

Figure 4: ACE-inhibitory of FIV **(A)** and FV **(B)** fractions following their fractionation with RP-HPLC; Selected sub-fractions were noted SF and collected to be subjected to the peptides sequence analysis by nESI-LC-MS/MS.

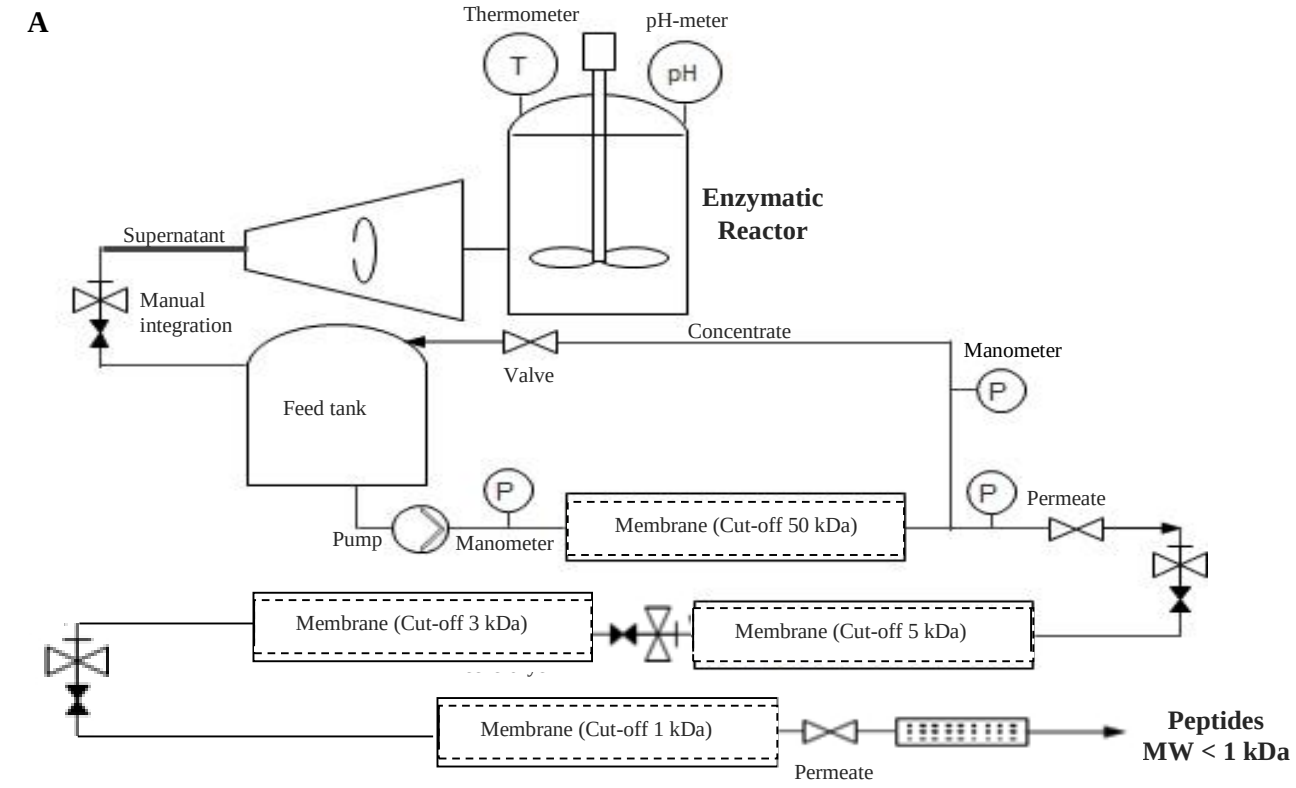
Figure 5: Distribution in percentages of the ACE-inhibitory peptides identified in the FIV **(A)** and FV **(B)** fractions according to the protein of origin.

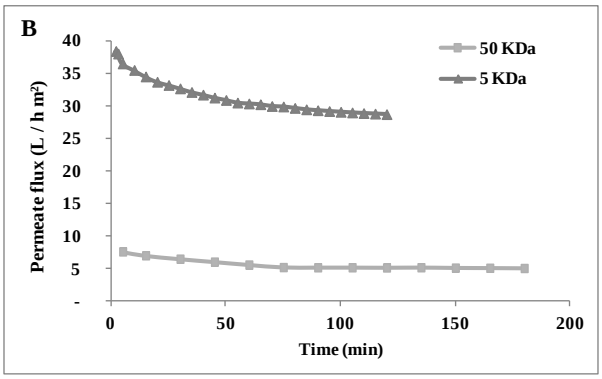
Figure 6: Bi-dimensional (2D) diagrams of predicted interactions between peptides and ACE amino acid residues. Images of the best poses of IAGPPGSAGPAG **(Ai**, **Aii)**, VVPFEGAV **(B)**, PLPKREE **(C)**, and PTVPKRPSPT **(D)** were obtained with Accelrys DS Visualiser software.

Figure 7: General (a) and local (b) overview poses of the best interaction poses after automated docking of peptide-ACE active site; **(A)** IAGPPGSAGPAG, **(B)** VVPFEGAV, **(C)** PLPKREE, and **(D)** PTVPKRPSPT. Peptide residues are represented in blue and grey, while zinc atom in yellow. (c) Schematic interaction of peptide (green)/zinc ion (purple) at the ACE active site after docking. ACE residues (His383, His387 and Glu411) that coordinate with zinc are presented in yellow; hydrogen bonds are presented in green dashed lines and zinc coordination bonds in grey lines. Images were obtained with Accelrys DS Visualiser software.

Figure 8: Details of the zinc ion (purple) tetrahedrally-coordinated with His383, His387 and Glu411 ACE residues (yellow) before docking. Other presented ACE residues are shown in grey, hydrogen bonds are presented with green dashed lines and zinc coordination bonds with grey bold lines. Image obtained with Accelrys DS Visualiser software.

Fig. 1





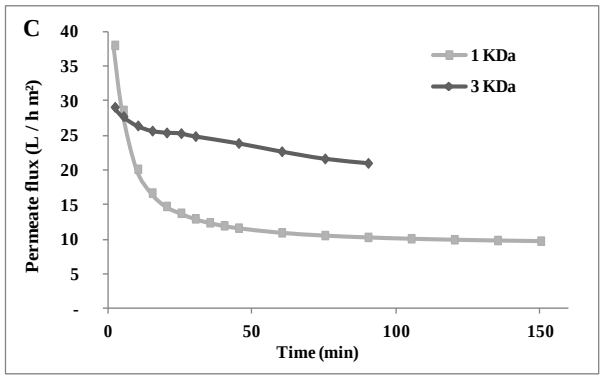


Fig. 2

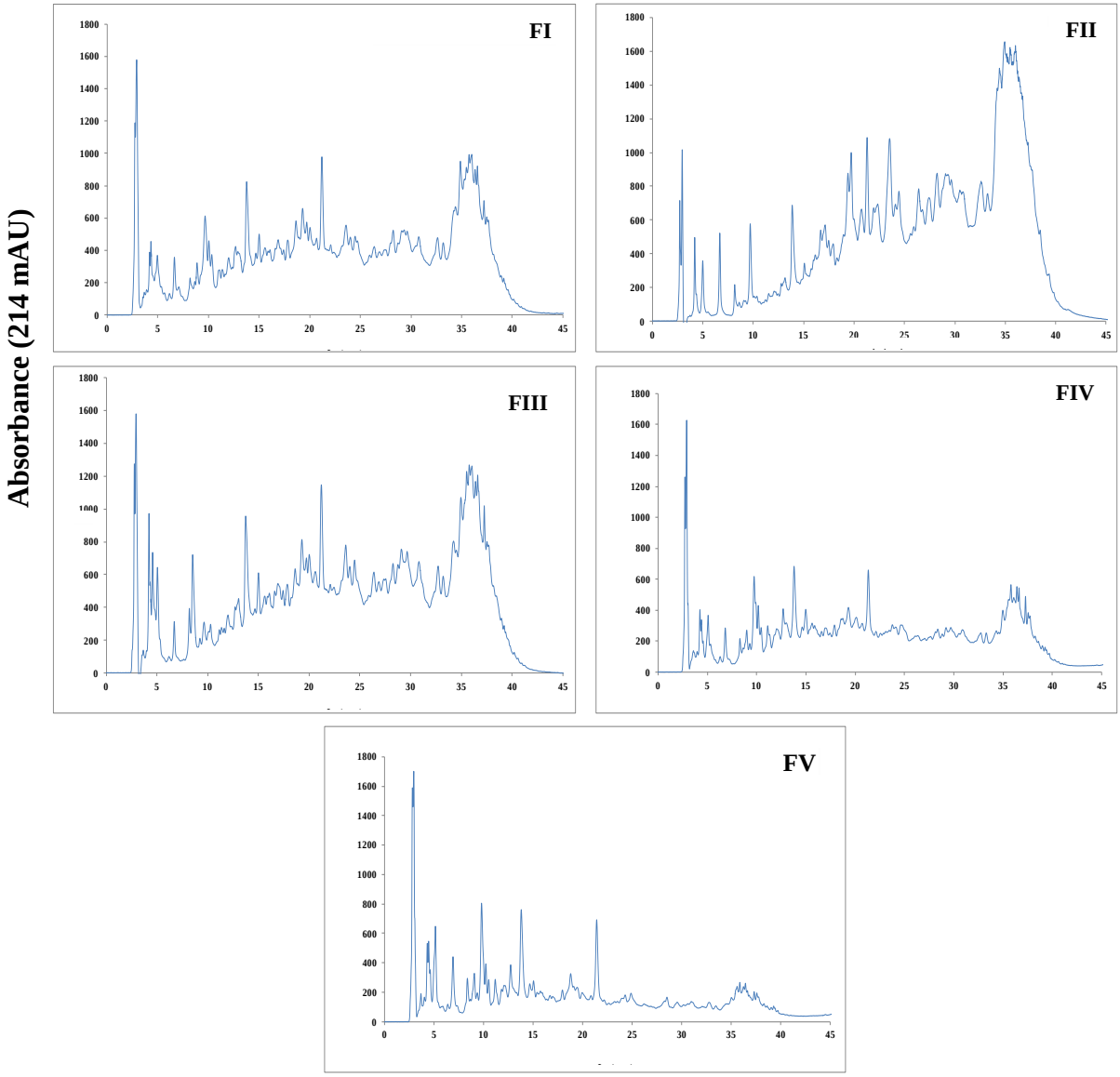


Fig. 3

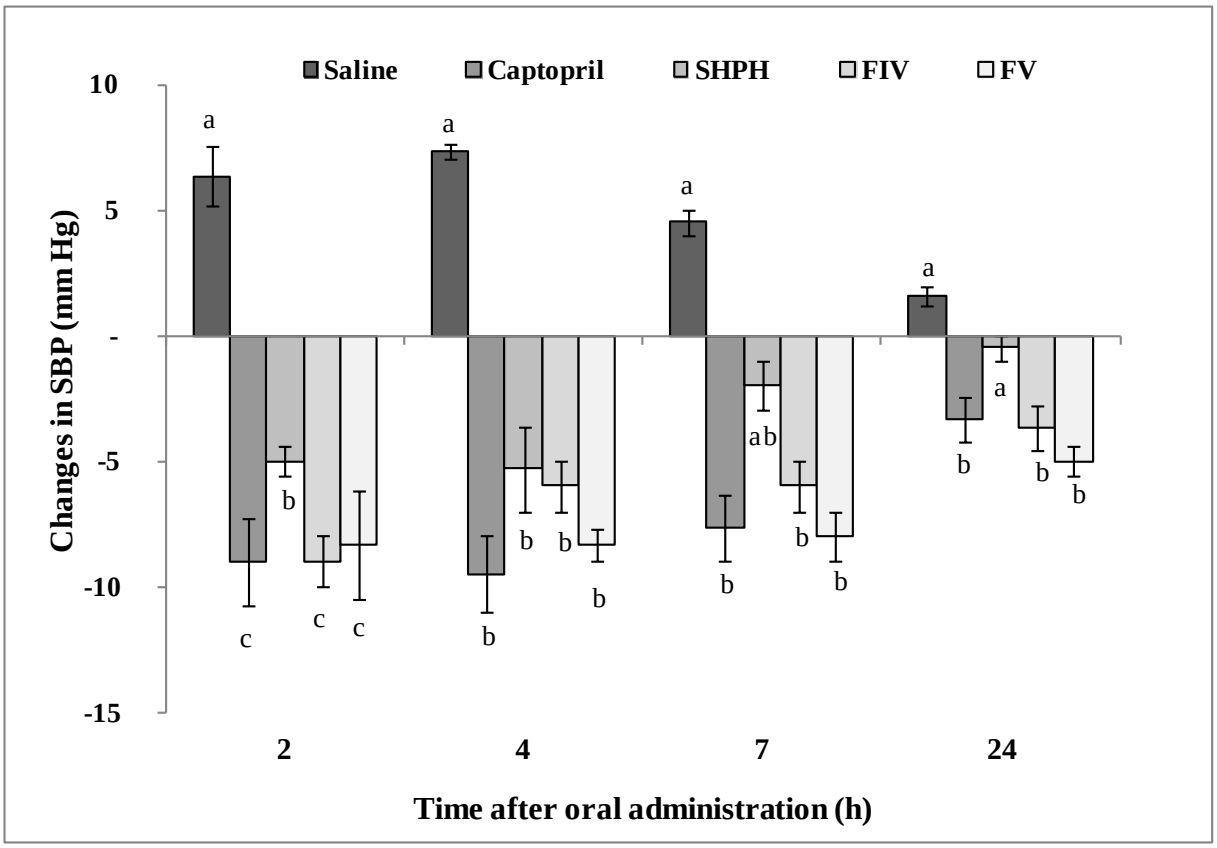


Fig. 4

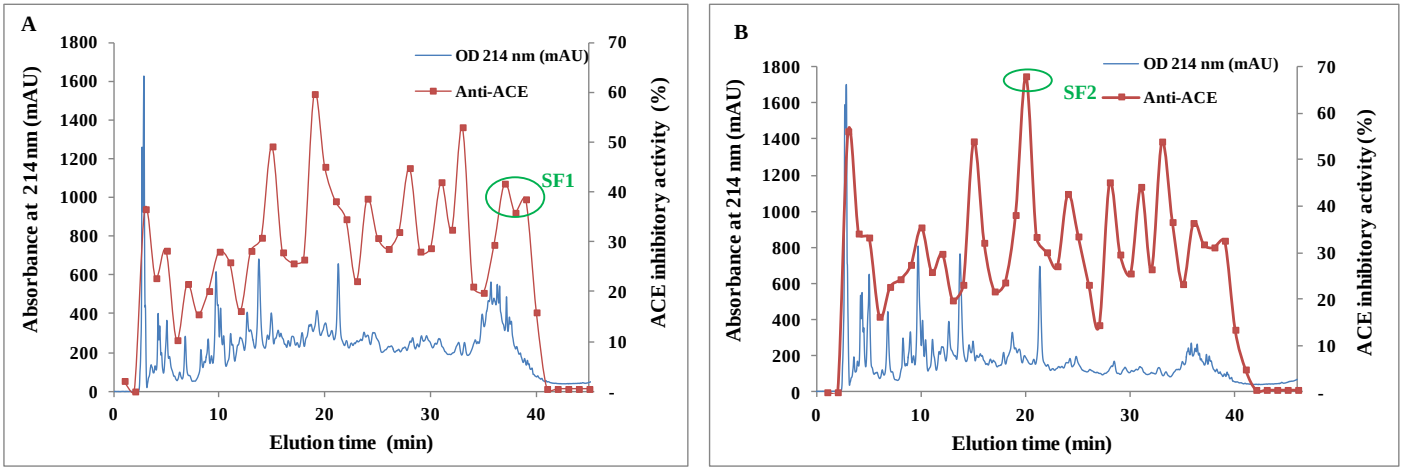


Fig. 5

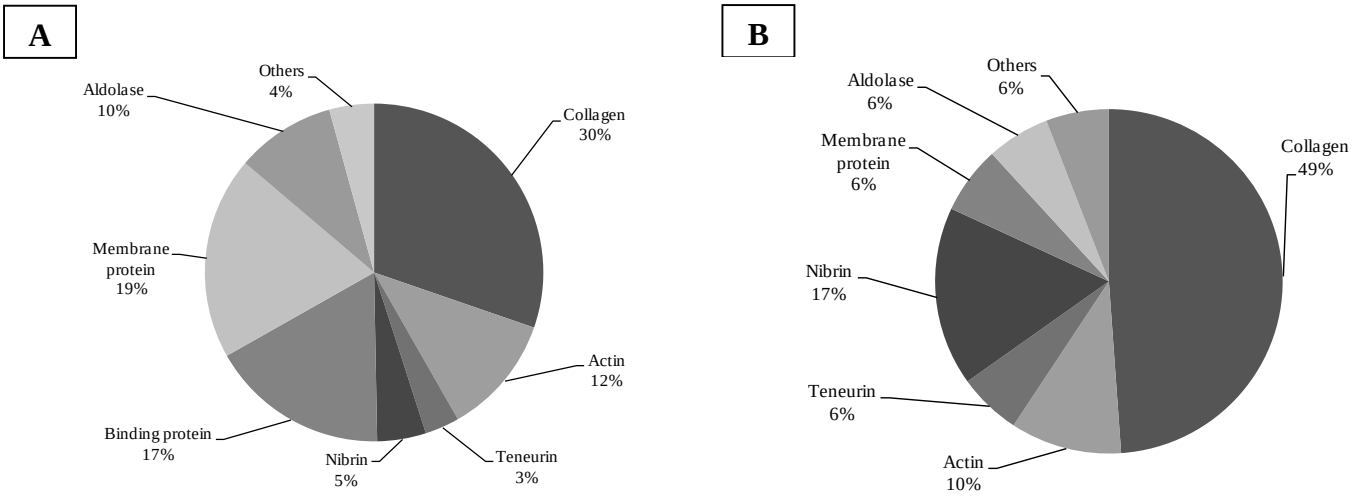
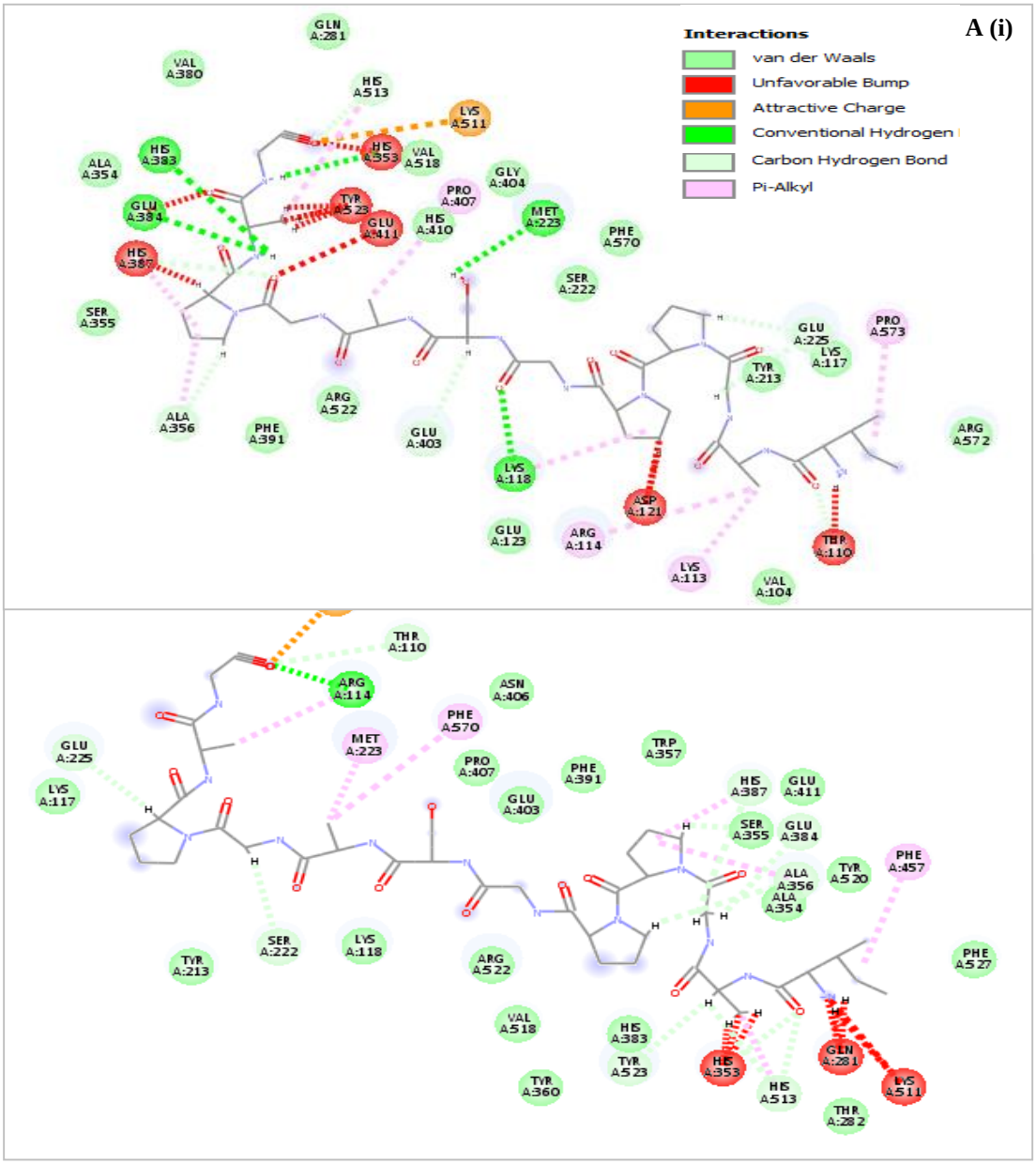
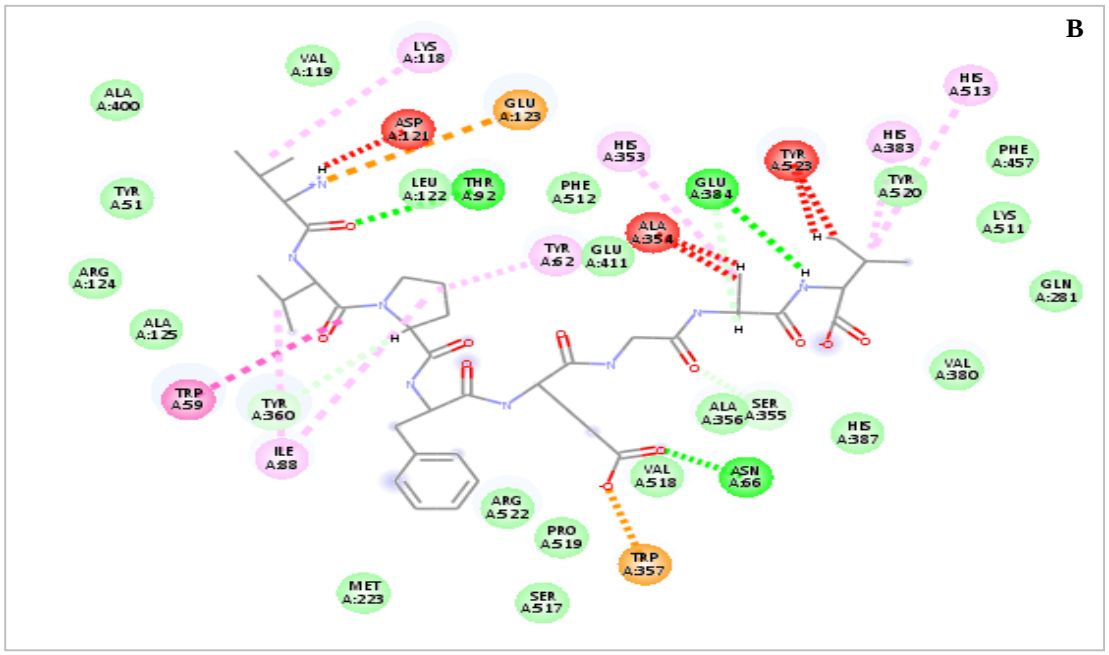
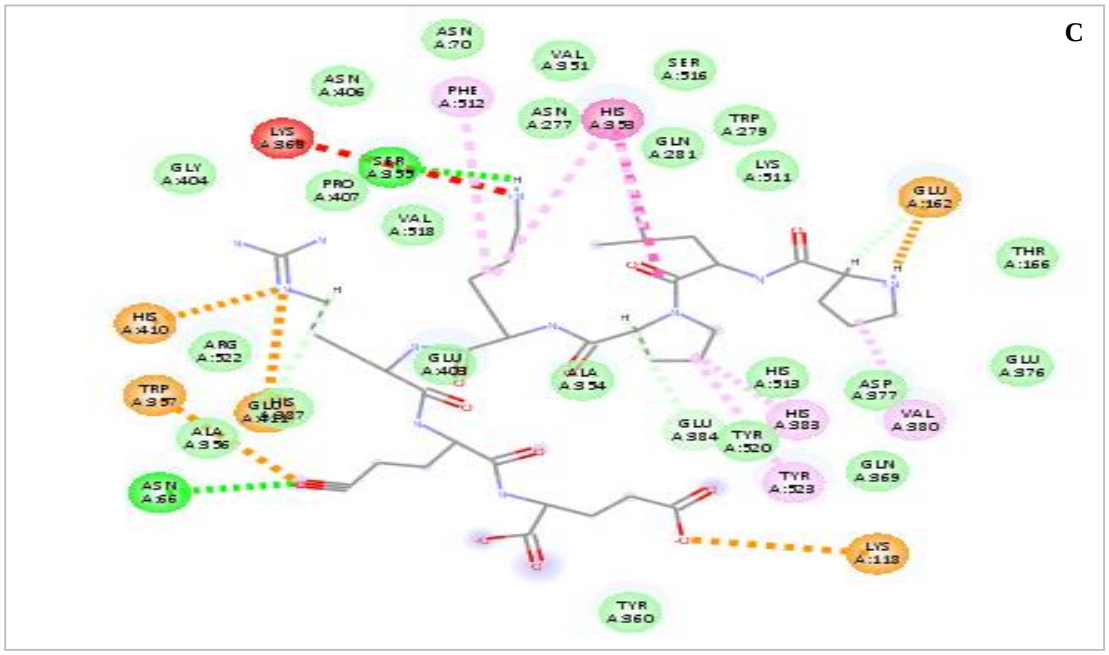


Fig. 6







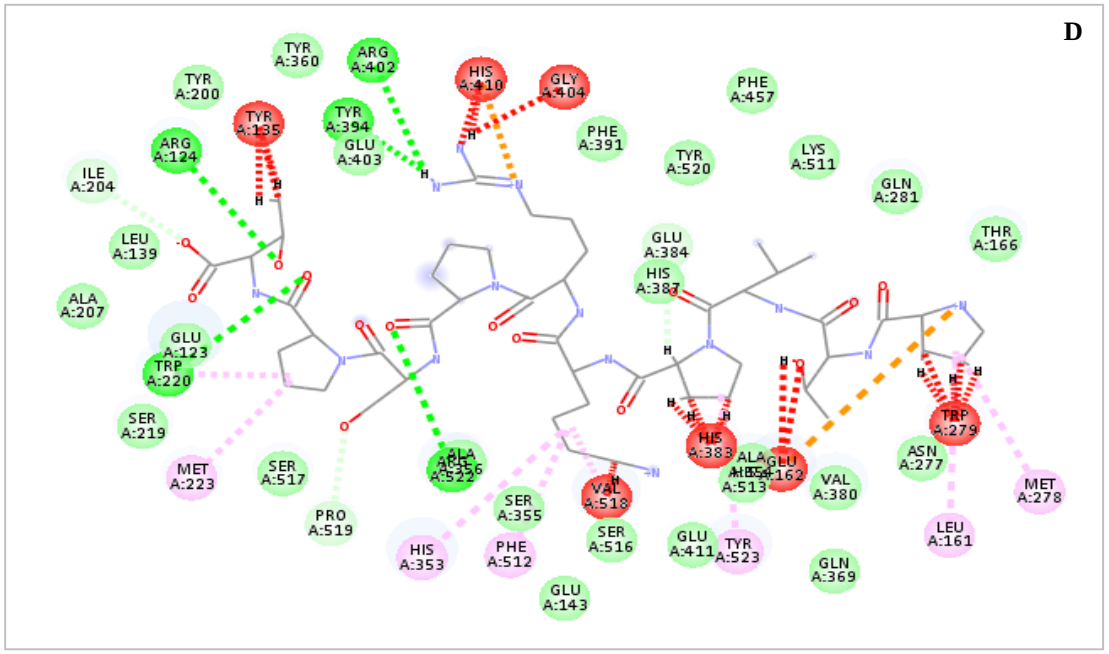


Fig. 7

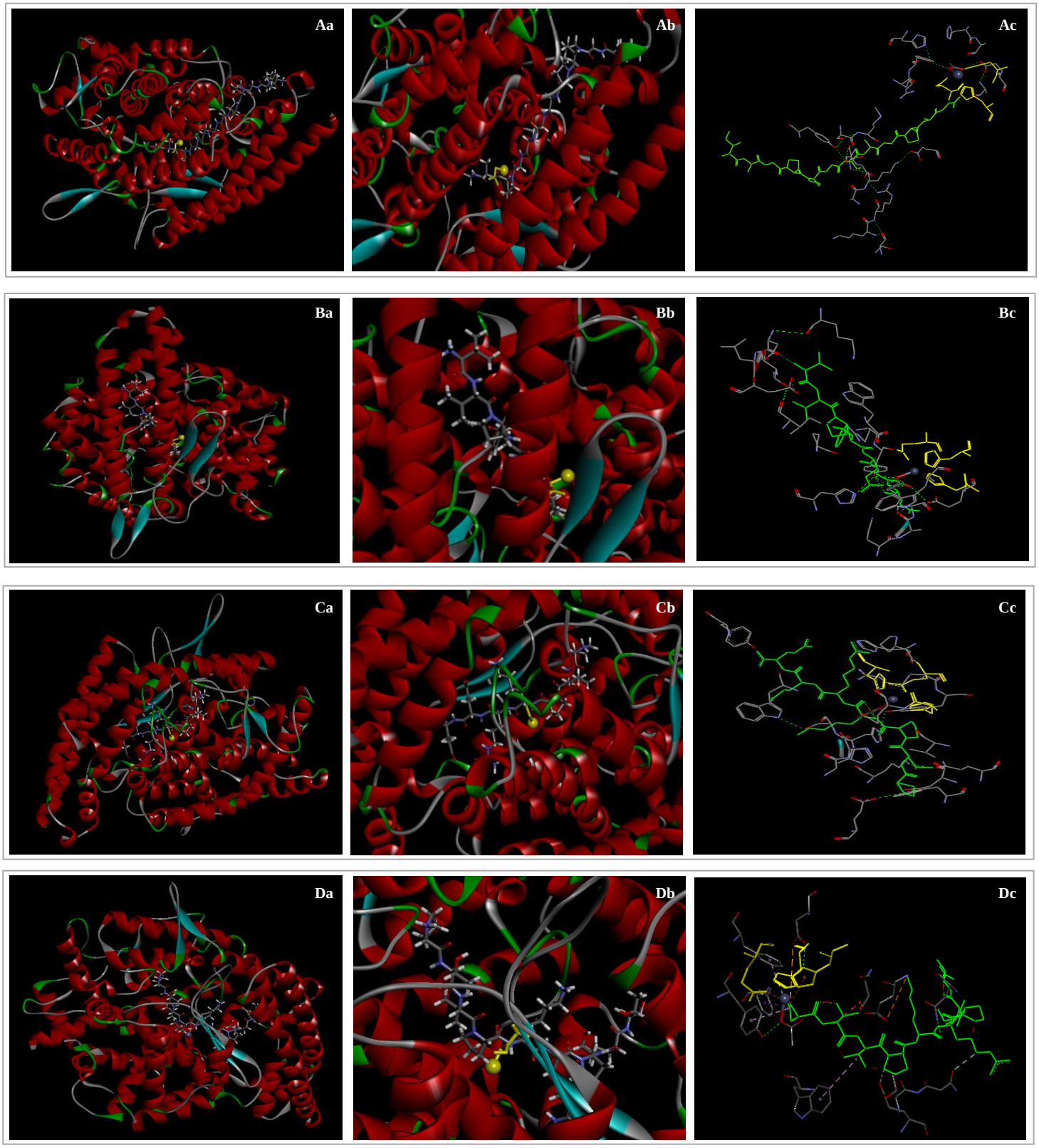


Fig. 8

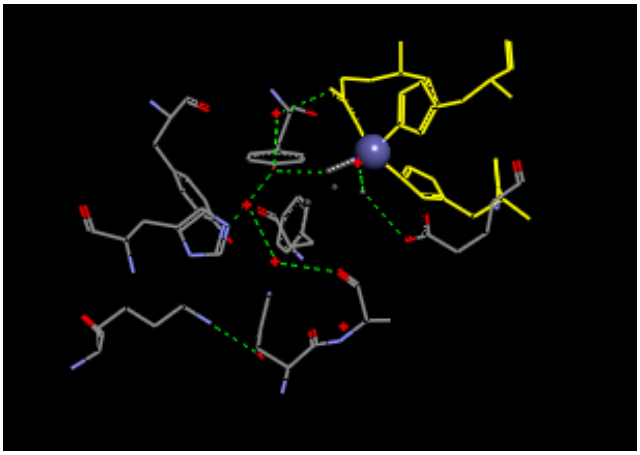


Table 1: Amino acid compositions of the smooth-hound protein hydrolysate and its fractions

AA	SHPH	FI	FII	FIII	FIV	FV
Asx	9.73±0.95^{a}	8.10±0.16^{a}	4.35±0.07^{c}	8.49±0.18^{a}	8.51±0.16^{a}	7.46±0.15^{b}
Glx	13.84±0.35^{a}	12.30±0.25^{b}	12.48±0.21^{b}	13.42±0.29^{a}	13.07±0.25^{a}	13.63±0.27^{a}
Hpx	2.87±0.13^{e}	5.67±0.11^{c}	10.36±0.17^{a}	6.44±0.14^{b}	3.72±0.07^{c}	3.18±0.02^{d}
Ser	5.10±0.00^{a}	4.50±0.09^{c}	4.14±0.07^{d}	4.32±0.09^{d}	4.63±0.09^{b}	4.85±0.10^{b}
Gly	10.70±0.21^{e}	15.61±0.31^{c}	20.92±0.35^{a}	16.73±0.36^{b}	13.75±0.26^{d}	10.82±0.22^{e}
Tau	4.18±0.11^{b}	2.07±0.04^{c}	0.29±0.00^{e}	1.64±0.04^{d}	2.47±0.05^{c}	5.50±0.11^{a}
His	1.25±0.03^{a}	1.04±0.02^{b}	0.96±0.02^{c}	1.12±0.02^{b}	1.17±0.02^{b}	1.17±0.00^{b}
Thr	5.97±0.15^{a}	3.73±0.08^{e}	4.25±0.07^{d}	5.04±0.11^{c}	5.52±0.11^{b}	5.56±0.11^{b}
Ala	5.96±0.15^{b}	7.39±0.15^{a}	5.62±0.09^{c}	5.77±0.13^{cb}	6.13±0.12^{b}	7.85±0.16^{a}
Arg	7.57±0.19^{c}	7.41±0.15^{c}	8.85±0.15^{a}	8.00±0.17^{b}	7.53±0.14^{c}	6.16±0.12^{d}
Pro	5.90±0.15^{d}	8.08±0.16^{b}	9.88±0.17^{a}	7.93±0.17^{b}	7.00±0.13^{c}	5.14±0.10^{e}
Tyr	1.24±0.03^{c}	1.42±0.03^{b}	0.91±0.02^{d}	0.80±0.02^{e}	1.67±0.03^{a}	1.48±0.03^{b}
Val	3.73±0.10^{b}	2.92±0.06^{c}	2.16±0.04^{d}	2.80±0.06^{c}	3.57±0.07^{b}	4.19±0.08^{a}
Met	1.51±0.04^{d}	1.72±0.03^{c}	0.90±0.02^{f}	1.29±0.03^{e}	2.08±0.04^{b}	2.49±0.05^{a}
Ile	2.84±0.07^{a}	2.16±0.04^{c}	1.80±0.03^{e}	1.98±0.04^{d}	2.59±0.05^{b}	2.69±0.05^{ab}
Leu	4.43±0.11^{b}	3.82±0.08^{c}	2.22±0.04^{f}	2.76±0.06^{e}	3.70±0.07^{d}	4.84±0.10^{a}
Phe	3.23±0.08^{b}	2.59±0.05^{d}	1.82±0.03^{e}	2.41±0.05^{d}	2.87±0.06^{c}	3.56±0.07^{a}
Lys	9.95±0.25^{a}	9.49±0.19^{b}	8.10±0.14^{d}	9.08±0.20^{c}	10.01±0.19^{a}	9.44±0.19^{b}
HAA	28.84	39.58	33.41	34.80	39.63	41.68
EAA	32.90	27.46	22.20	26.47	31.51	33.94
Total	100	100	100	100	100	100

Results are expressed in % (g per 100 g of amino acids); SHPH represents the smooth-hound protein hydrolysate prepared using Esperase; FI, FII, FIII, FIV and FV represent fractions with molecular weight > 50, 50-5, 5-3, 3-1 and < 1 kDa, respectively.

Asx: Asp + Asn ; Glx: Glu + Gln;

HAA: Hydrophobic amino acids (Ala, Pro, Tyr, Val, Met, Ile, Leu, Phe) ;

EAA: Essential amino acids (His, Thr, Val, Met, Ile, Leu, Phe, Lys);

Different letters in the same line indicate significant difference ($p < 0.05$).

Table 2: ACE-inhibitory activity of the undigested viscera proteins, smooth-hound protein hydrolysate and its fractions obtained from UF

	IC_{50} (µg/ml)
Captopril	19.31±3.36
UVPs	731.22±0.98 [a]
SHPH	164.56±2.65 [e]
FI	571.39±4.64 [b]
FII	174.24±4.02 [d]
FIII	183.58±1.22 [c]
FIV	101.61±7.56 [e]
FV	92.75±1.31 [f]

UVPs and SHPH represent the undigested viscera proteins and the smooth-hound protein hydrolysate prepared using Esperase®, respectively; FI, FII, FIII, FIV and FV represent fractions with molecular weight > 50, 50-5, 5-3, 3-1 and < 1 kDa, respectively.

Different letters indicate significant difference ($p < 0.05$).

Table 3: Peptide sequences identified in SF1 (a) and SF2 (b) sub-fractions

a ved m/z [a]	Expected m/z [b]	Charge [c]	Calculated m/z [d]	P0	Peptide sequence	Pf
517.2642	1032.5138	2	1032.5128	S	YELPDGQVI	T
527.2475	1052.4804	2	1052.4783	T	MYPGIADRM	Q
541.2671	1080.5196	2	1080.5175	D	MEKIWHHT	F
561.7575	1121.5005	2	1121.4852	N	WDDMEKIW	H
515.2855	1028.5565	2	1028.5502	L	GEDIDLIVR	C
566.277	1130.5393	2	1130.5356	A	GDDAPRAVFPS	I
401.2238	1200.6494	3	1200.6462	T	TAEREIVRDI	K
601.7944	1201.5743	2	1201.5728	F	AGDDAPRAVFPS	I
605.7705	1209.5264	2	1209.5237	W	DDMEKIWHH	T
618.777	1235.5394	2	1235.5281	T	NWDDMEKIW	H
652.8438	1303.673	2	1303.666	S	YELPDGQVITIG	N
687.306	1372.5975	2	1372.587	T	NWDDMEKIWH	H
689.3503	1376.686	2	1376.6823	L	EKSYELPDGQVI	T
696.3587	1390.7028	2	1390.698	K	SYELPDGQVITIG	N
466.2071	1395.5995	3	1395.603	N	WDDMEKIWHH	T
623.3193	1244.624	2	1244.6513	R	EIFDKARQAAP	C
535.8091	1069.6037	2	1069.6131	Y	LSPEVLRKE	P
673.3311	1344.6475	2	1344.7554	V	IQPGRGFVLYPV	K
670.3356	1338.6566	2	1338.7143	D	LKPNNLLLDENG	V
568.2962	1134.5778	2	1134.5808	F	IEEDELKLF	L
593.2879	1184.5612	2	1184.6336	M	STHKAALCKVQ	I
653.2993	1304.584	2	1304.6976	S	QVSLLSVEGNLF	E
693.8473	1385.6801	2	1385.8031	F	QYVKPLLAAEVR	R
564.292	1126.5694	2	1126.5216	P	MLIEDGYSVT	Q
552.7954	1103.5762	2	1103.5975	I	DTVSRVAFPL	V
628.3161	1254.6177	2	1254.6166	V	LSAFCTELTGIT	Q
632.3014	1262.5883	2	1262.6295	V	LAPNWKYPSST	A
571.8315	1141.6485	2	1141.7183	N	KKGKSLLQQL	E
422.5527	1264.6362	3	1264.5976	Q	EPVPTSVPNYY	K
675.326	1348.6375	2	1348.6775	L	ERNGLYPFSGLP	G
673.3311	1344.6475	2	1344.7554	V	IQPGRGFVLYPV	K
592.326	1182.6374	2	1182.6649	Y	KSLPIDPAVPF	D

b

Observed m/z [a]	Expected m/z [b]	Charge [c]	Calculated m/z [d]	P0	Peptide sequence	Pf
376.6817	751.3489	2	751.3501	P	GPSGPPGPS	G
390.2106	778.4066	2	778.4086	T	GPAGPRGPA	G
391.1973	780.38	2	780.3766	I	AGPPGSAGPA	G
405.2169	808.4193	2	808.4443	A	AGLPGVAGAP	G
405.7011	809.3876	2	809.4144	R	GRAGPAGPAG	A
407.2004	812.3863	2	812.3665	P	GPDGGKGEP	G
412.2491	822.4836	2	822.4236	G	PAGIAGPPGS	A
540.3311	1078.6477	2	1078.6135	K	PTVPKRPSPT	N
476.2487	950.4829	2	950.4821	G	IAGPPGSAGPAG	K
491.2415	980.4685	2	980.5039	V	GEPGRLGPAGA	S
421.2487	840.4828	2	840.4818	S	GLTGARGLP	G
434.7314	867.4481	2	867.4087	P	GDQGLPGPAG	V
436.2261	870.4377	2	870.3906	G	MPGDQGLPG	P
440.7304	879.4462	2	879.445	P	GPAGIAGPPGS	A
384.7018	767.389	2	767.3813	S	NYISKGS	T
389.2425	776.4705	2	776.3929	P	HHPSAKT	S
383.7088	765.4031	2	765.4497	G	ARGPVGPI	G
520.2714	1038.5283	2	1038.5359	G	PRGPAGPHGPP	G
376.6817	751.3489	2	751.3501	P	GPPGSPGSP	G
391.2159	780.4173	2	780.4242	C	VHRDLAA	R
383.7178	765.4211	2	765.4749	A	PKPTVPK	R
409.2191	816.4236	2	816.4382	G	VVPFEGAV	C
413.2137	824.4128	2	824.4392	V	IGLPGPQGS	F
423.2316	844.4486	2	844.4443	R	GPPGLPGPPG	I
469.7595	937.5045	2	937.4869	G	LPGPPGEKGS	T
441.2423	880.4701	2	880.4039	G	GPGPQGPQGS	T
434.7118	867.409	2	867.4814	I	PLPKREE	R
409.2104	816.4063	2	816.4745	C	LNPVLYV	C
422.2056	842.3966	2	842.461	F	EGLQQLR	H
462.2391	922.4637	2	922.4032	E	DSFEGLQQ	L
357.2413	712.468	2	712.4119	G	ILQVPGS	E

a Relation of mass/charge observed in the nLC–MS/MS spectrophotometer expressed in m/z

b Expected mass calculated from the observed mass according to the charge state of the ion

c Charge state of the ion

d Calculated relative molecular mass of the matched peptide in Da.

P_0 and P_f represent the amino acid residue preceding and following the peptide sequence, respectively.

Table 4: Selected peptide sequences identified in SHPH sub-fractions in comparison with previously described bioactive peptides, based on the BIOPEP data base.

Sub-fraction name	Peptide sequence	Previously identified bioactive peptides	Activities
SF1	**MYPGIA**DRM	**MYPGIA**	ACE inhibitor
	MEK**IWHHT**	**IWHHT**	ACE inhibitor
	NWDDMEK**IWH**	**IWH**	ACE inhibitor
	EKSYELPDGQVI	**EKSYELP**	ACE inhibitor
	GDDAPRA**VFPS**	**VFPS**	ACE inhibitor
	YLAAVLEYLT	**YLA**GNQ	ACE inhibitor
	EIFDKARQ**AAP**	**AAP** ; KAA**AAP** ; KPV**AAP**	ACE inhibitors
	LSPEVLRKE	**LSP**	ACE inhibitor
	LAPNWKYPSST	**LAP**PG	ACE and DPP-IV inhibitor
	GEDIDL**IVR**	**IVR**	ACE inhibitor
	PPPRLISMQAL	**PPP**VHL	ACE inhibitor
	LKPNNLLLDENG	**LKP** ; **LKP**MN	ACE inhibitors
	IQPGRGFVL**YPV**	**IQP** ; VLP**YPV**	ACE inhibitors
	STHKAALCKVQ	**STH**GVY	ACE inhibitor
SF2	**GPA**GPR**GPA**	**GPA** GPRGPA	ACE inhibitor ACE-inhibitor and anti-thrombotic
	GPDGGK**GEP**	**GEP**	ACE inhibitor
	GLTGAR**GLP**	**GLP**	ACE inhibitor
	MPGDQG**LPG**	**LPG;** QE**LPG** ; KV**LPG**	ACE inhibitors
	GPGPQGP**QGS**	Y**QGS**	ACE inhibitor
	IAGPPGSAGPAG	**IAG**RP	ACE inhibitor
	GEPGRLG**PAGA**	L**PAGA** ; **GEP**	ACE inhibitors
	AGPPGSA**GPA**	**AGP**VLL ; **GPA**	ACE inhibitors
	PRGPAGPH**GPP**	**GPP**	ACE inhibitor and antioxidant
	VHRD**LAA**	**LAA**	ACE inhibitor
	ARGPV**GPI**	YPFP**GPI**	ACE inhibitor
	PKPT**VPK**	**VPK**	ACE inhibitor
	VVPFEGAV	**VVP**P	ACE inhibitor
	PLPKREE	**PLP** **KREE**	ACE inhibitor Anti-thrombotic
	LNPVLYV	**LNP**	ACE inhibitor
	PTVPKRP**SPT**	VPL**SPT ;** VGPL**SPT ;** MG**SPT**	Antioxidant
	DSFEG**LQQ ;** EG**LQQ**LR	**LQQ**	ACE inhibitors

Table 5: Computational modeling energy scores and interaction results of the top ranked poses of docked peptides identified in SF1 and SF2 sub-fractions, and ACE (PDB: 1O8A)

Sub-fraction name	Peptide sequence	Total energy score (REU)	Interaction score (REU)	Number of predicted hydrogen bonds	Number of ACE residues*
Lisinopril	-	-	-	10	8
SF1	MYPGIADRM	-217.68	-23.96	3	7
	MEKIWHHT	-219.553	-22.31	3	10
	GDDAPRAVFPS	-216.523	-23.701	4	9
SF2	GPAGPRGPA	-215.957	-22.637	9	8
	IAGPPGSAGPAG	-217.023	-21.076	9	10
	PRGPAGPHGPP	-218.008	-21.774	4	10
	VVPFEGAV	-219.327	-22.591	5	11
	PLPKREE	-218.334	-21.143	4	12
	PTVPKRPSPT	-217.579	-21.679	8	12
	DSFEGLQQ	-217.974	-22.31	1	10
	EGLQQLR	-219.704	-23.991	5	9

* indicates the number of amino acids in the ACE active site in interaction with the peptide.

REU: Rosetta energy units are a standard unit used by computational modeling to score complexes.

Table 6: ACE residues in coordination with Zn^{2+} and amino acids of S1, S2 and S1' active pockets involved in interaction with selected peptides after molecular docking simulation

ACE residue	**Lisinopril**	**IAGPPGSAGPAG**	**VVPFEGAV**	**PLPKREE**	**PTVPKRPSPT**
His383	√	√	√	√	√
His387	√	√	√	√	√
Glu411	√	√	√	√	√
Ala354	√	√	√	√	√
Tyr523	√	√	√	√	√
Glu384	√	√	√	√	√
Gln281	-	√	√	√	√
His353	-	√	√	√	√
Lys511	-	√	√	√	√
His513	√	√	√	√	√
Tyr520	√	-	√	√	√
Glu162	-	-	-	√	√

(√) indicates the presence of the residue

.