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In silico analysis and molecular docking study of angiotensin I-converting enzyme inhibitory peptides from smooth-hound viscera protein hydrolysates fractionated by ultrafiltration

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ABSTRACT

Smooth-hound viscera hydrolysates (SHVHs) were prepared by treatment with Neutrase (SHVH-N) and Purafect (SHVH-P). Hydrolysates were then separated according to their molecular weight, using the ultra-filtration membrane system, into 5 fractions (≥50, 50–5, 5–3, 3–1 and ≤1 kDa). Fractions showed different amino acid compositions and angiotensin I-converting enzyme (ACE) inhibitory potentials. The SHVH-P-FV (≤1 kDa) and SHVH-N-FIV (3–1 kDa) fractions showed the best ACE-inhibitory activities with IC_{50} values of 53.31 and 75.05 μg/ml, respectively. According to their high ACE-inhibitory potential, FIV and FV were fractionated by RP-HPLC and then analyzed by LC–MS/MS to identify peptide sequences. A systematic peptidomic study resulted in the identification of numerous novel sequences. Furthermore, *in silico* data, based on the molecular docking simulation, showed that GPAGPRGPAG, AVVPPSDKM, TTMYPGIA, and VKPLPQSG could bind ACE active site with low interaction scores. Indeed, they share hydrogen bonds and Van der Waals and electrostatic interactions with ACE catalytic pockets.

1. Introduction

Depending on their origin, fish protein hydrolysates are characterized by different nutritional properties related to their amino acids content. They are rich in potent bioactive peptides, which are encrypted within the primary structure of proteins. Generally, these peptides are initially latent in the protein of origin but they remain active upon release by proteases action (Korhonen, 2009). Bioactive peptides usually contain between 2 and 20 residues. Their bioactivity depends on the amino acid composition, sequence and length, which, in turn, are affected by processing conditions and enzyme specificity.

Marine-derived bioactive peptides are recently emerging nutraceutical field as supplements in health functional food formulations due to their diverse health-promoting benefits including antioxidation and blood pressure lowering (angiotensin I-converting enzyme (ACE) inhibitory) effect (Lassoued et al., 2015), anti-tumor activity (Hsu, Lin, Tzen, & Chen, 2011), anti-inflammation (Ahn, Cho, & Je, 2015), appetite suppressing ability (Cudennec et al., 2012), etc. Stabilium® is a fish protein hydrolysate obtained from enzymatic autolysis of blue ling viscera. It is accounted for reducing anxiety in humans and improving memory and concentration (Le Poncin, 1996; Le Poncin & Lamproglou, 1996). Actually, casein peptides are commercialized as a therapeutic natural medicine used for the treatment of high blood pressure, high cholesterol, intestinal disorders, cancer prevention and stress reduction. However, people with milk allergy are usually allergic to fragments derived from milk proteins, including casein peptides. Maine peptides, however, with ACE-inhibitory activity, have been consumed as antihypertensive drugs, without undesirable side effects (Cheung, Ng, & Wong, 2015).

Interestingly, bioactive peptides are able to inhibit the ACE, involved in the regulation of human blood pressure and fluid homeostasis via the renin-angiotensin system. In fact, the ACE is responsible to convert the angiotensin I into angiotensin II that constricts the arteries and, as a consequence, increases the blood pressure. Further, it is involved in the inactivation of the bradykinin, which is a known vasodilator.

The identification of ACE-inhibitory bioactive peptide sequences requires sensible fractionation and isolation techniques, by using mass spectrometry (MS) analysis coupled to different separation techniques. A combination of chromatography analysis is usually done to simplify the complex mixture of peptides. Furthermore, a structure-activity relationship study actually required, molecular docking simulation, to study the interaction between

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the ACE and peptides in order to evaluate the binding interaction between peptide (N-terminus or C-terminus) and ACE catalytic site. Short sequences (with 2–3 amino acids) are widely prevalent in the literature and the most popular commercial functional foods claiming antihypertensive effects contain the tripeptides VPP and IPP (García-Mora et al., 2017). However, functional foods containing larger peptides are less studied. Thus, their identification and the study of their structure-activity relationship would be interesting.

Among sharks, common smooth-hound (*Mustelus mustelus*) represents one of the most consumed fishes in the Mediterranean countries, including Tunisia. Regarding the huge quantities of viscera mass generated in the local freshwater market and in order to obtain new products from these wastes, the recent scientific tendencies are focalized on their conversion into more interesting products, by the production of protein hydrolysates using enzymatic treatment (Abdelhedi et al., 2016) and the screening of ACE-inhibitory peptides (Abdelhedi et al., 2017).

In this study, hydrolysates produced from smooth-hound viscera (SHVHs) using 2 microbial enzymes, Neutrase and Purafect, were fractionated according to their molecular weight (MW) and then their hydrophobicity, using the ultra-filtration (UF) process and reverse-phase liquid chromatography (RP-HPLC), successively. Then, proteomic identification of potential biopeptides (anti-ACE) has been done by using tandem mass spectrometry (nLC-MS/MS). The most potent peptides were selected based on *in silico* screening and their binding interaction within the active site of ACE was determined through molecular docking simulation.

2. Materials and methods

2.1. Reagents and proteolytic enzymes

Angiotensin-converting enzyme (from rabbit lung) has been purchased from Sigma (St. Louis, MO). Abz-Gly-p-nitro-Phe-pPro-OH trifluoroacetate salt was obtained from Bachem (Bubendorf, Switzerland). All other chemicals were of analytical grade. Solvents and chemicals used in the mass spectrometry analysis were from Sigma (St. Louis, MO) and MS grade. Neutrase® and Purafect® were purchased from Novozymes® (Bagsvaerd, Denmark). The enzymatic activity was evaluated as previously described by Kembhavi, Kulkarni, and Pant (1993), using casein as a substrate.

2.2. Biological raw material preparation

Visceral mass (stomach and intestine) were obtained following the processing of fresh filleted Smooth-hound (*M. mustelus*) fish available in the local fish market of Sfax City, Tunisia. The biological material was brought to the research laboratory in polyethylene bags, in iced conditions, within 30 min. Upon arrival, they were immediately rinsed with tap water to remove contaminants, then, stored in plastic bags at −20 °C until they were used for protein hydrolysates production, less than 1 week later.

2.3. Protein hydrolysates preparation

Smooth-hound viscera were hydrolyzed as previously described by Abdelhedi et al. (2016). Viscera were firstly minced into small pieces and then cooked in distilled water at 95 °C for 15 min, with a solid/solvent ratio of 1:1 (w/v) to inactivate endogenous enzymes. After being well homogenized, the pH of the mixture was adjusted to the optimum activity value of the used enzyme by adding NaOH solution (4 N). Thereafter, viscera treated mass was subjected to enzymatic hydrolysis, using Neutrase or Purafect® at pH 7.0 and 10.0, respectively, with an enzyme/protein ratio of 6/1 (U/mg of protein). The obtained protein hydrolysates prepared after treatment with Neutrase and Purafect were named SHVH-N and SHVH-P, respectively. During the reaction, the pH of the mixture was maintained constant at the desired value by continuous addition of 4 N NaOH. The degree of hydrolysis (DH) was calculated based on the volume of NaOH added during the reaction, as described by Adler-Nissen (1986). After the achievement of the digestion process, the reactions were stopped by heating for 20 min at 95 °C to inactivate enzymes. Protein hydrolysates were then centrifuged at 9500*g* for 20 min to separate soluble (peptides) and insoluble (non hydrolyzed proteins) fractions. Finally, the soluble fractions were freeze-dried using freeze-dryer (Bioblock Scientific Christ ALPHA 1–2, IllKrich-Cedex, France) and the resulting powders were stored at −20 °C for further use. Hydrolysate powders were recovered with respective yields of 6.5% and 8.4% for SHVH-N and SHVH-P (Abdelhedi et al., 2016).

2.4. Ultra-filtration

Both SHVHs were fractionated by UF membrane technology, in a tangential filtration mode, using Millipore UF systems. First, a Labscale™ TFF System (USA) with molecular weight cut-off (MWCO) membranes of 50 and 5 kDa was applied to separate high and medium MW peptides. Thereafter, the permeate, recovered from the 5 kDa membrane, was subjected to a frontal ultrafiltration using a stirred cell system (Amicon, Inc., MA, USA) with MWCO membranes of 3 and 1 kDa. Separation was done using a decreased molecular mass order from 50 to 1 kDa. The obtained fractions were noted FI (MW > 50 kDa), FII (5 < MW < 50 kDa), FIII (3 < MW < 5 kDa), FIV (1 < MW < 3 kDa) and FV (MW < 1 kDa). The collected fractions were freeze-dried, weighed to determine their proportions in the hydrolysate mixture and then assayed for their ACE-inhibitory activity.

2.5. Reversed-phase high performance liquid chromatography (RP-HPLC)

Samples 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 Symmetry Prep™ C18 column (7.8 mm × 300 mm, Milford, Waters) set at 30 °C. Solvent A was 0.1% trifluoroacetic acid (TFA) in double distilled water and solvent B consisted of acetonitrile/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 was equipped with a collector which automatically turned each one minute. Collected fractions (1 ml) were monitored at 214 nm during 45 min and assayed for their ACE-inhibitory activity. Fractions showing remarkable inhibition were freeze dried to be further analyzed.

2.6. Evaluation of the anti-ACE activity

The ACE-inhibitory activities of SHVHs and their fractions were measured according to Sentandreu and Toldrá (2006). 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)