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Angiotensin- I- converting enzyme (ACE) inhibitory peptides from Pacific cod skin gelatin using ultrafiltration membranes

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ABSTRACT

Angiotensin- I- converting enzyme (ACE) is crucial in the control of hypertension and the development of type- 2 diabetes and other diseases associated with metabolic syndrome. The aim of this work was to utilize Pacific cod skin to purify ACE inhibitory peptides. First, gelatin was extracted from Pacific cod skin and hydrolyzed with several enzymes (pepsin, papain, α-chymotrypsin, trypsin, neutrase, and alcalase). The pepsin hydrolysate showed the strongest ACE inhibitory effect and was further fractionated into different ranges of molecular weight (<1, 1–5, 5–10, and >10 kDa) using ultrafiltration (UF) membranes. The peptic hydrolysate below 1 kDa resulted in two potent ACE inhibitory peptides, GASSGMPG (662 Da) and LAYA (436 Da), with IC_{50} values (concentration required to decrease the ACE activity by 50%) of 6.9 and 14.5 μM, respectively. Moreover, to explore the interaction between the peptides and ACE molecule, the tertiary structure of ACE and docking simulation to the peptides were predicted using Docking Server. Pacific cod peptides can be used as functional food ingredients to prevent hypertension and its related diseases.

1. Introduction

The global burden of chronic diseases such as cardiovascular diseases (CVDs), diabetes, obesity, and cancer is increasing rapidly. High blood pressure is an independent risk factor for CVDs and is responsible for most preventable deaths worldwide [1,2]. Human angiotensin- I- converting enzyme (ACE) is crucial in the control of hypertension and electrolyte homeostasis by converting angiotensin I to angiotensin II (vasoconstrictor) and by annulling the potent vasodilator bradykinin to its inactive fragments [3,4]. Synthetic ACE inhibitors such as captopril, lisinopril, and enalapril, although used extensively, are responsible for adverse side effects such as coughing, taste disturbances, skin rashes, dizziness, headache and angioedema [5]. Therefore, it is necessary to discover ACE inhibitors from naturally available sources without side effects.

The seafood processing industry produces a large amount of by-products that usually consist of bioactive materials such as proteins, enzymes and fatty acids. Ultrafiltration (UF) membrane bioreactors can effectively produce bioactive components of desirable molecular weight (MW) such as bioactive peptides from seafood processing by-products [6,7]. Marine bioactive peptides exhibit biological activities such as antihypertensive, antioxidant, antimicrobial, anticancer, mineral-binding, antithrombotic and hypocholesterolemic effects [8–10]. ACE inhibitory peptides can prevent hypertension by binding to the ACE molecule.

In recent years, computational (*in silico*) docking has minimized the time-consuming process of molecular analyses for selecting a suitable ligand, and it has been used to predict the interaction between protein and small molecules such as bioactive peptides [11,12]. Therefore, computational approaches can be used for studying inhibitory mechanisms as an assistant tool and designing novel enzyme inhibitors. Increasingly more studies have focused on the quantitative structure- activity relationship and the mechanism of peptide binding with ACE using computational simulation, and

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current software include Discovery Studio, AutoDock and Docking Server [13,14].

In the present study, ACE inhibitory peptides were purified *via* enzymatic digestion of Pacific cod (*Gadus macrocephalus*) skin gelatin using UF membranes (10, 5, and 1 kDa), fast protein liquid chromatography (FPLC, anion-exchange column and gel filtration column), reversed-phased high-performance liquid chromatography (RP-HPLC) and quadrupole-time-of-flight (TOF) liquid chromatography (LC)/mass spectroscopy (MS)/MS mass spectrometer. Moreover, the tertiary structure of the ACE molecule and docking simulation to the peptides were predicted using Docking Server to explore the binding mechanism including estimation of the free energy of binding, estimation of the inhibition constant, Van der Waals interaction force, hydrogen bonds, polar interaction, hydrophobic interaction, electrostatic interaction force, total intermolecular energy, frequency and interaction surface between the peptides and ACE molecule.

2. Materials and methods

2.1. Materials

Pacific cod (*G. macrocephalus*) skin was collected from the Jagalchi fish market, Busan, South Korea. Captopril, papain, α-chymotrypsin, pepsin, trypsin, ACE (from rabbit lung), and hippuryl-histidyl-leucine (HHL) were provided by Sigma Chemical Co. (St. Louis, MO, USA). Alcalase and neutrase were obtained from Novozymes Co. (Bagsvaerd, Denmark). UF membranes were procured from GE Healthcare Bio-Sciences Corp. (Westborough, MA, USA). All other chemicals used in the experiments were of analytical grade.

2.2. Gelatin extraction and hydrolysis

Gelatin was extracted as described in Ref. [15]. The collected gelatin was separately hydrolyzed with pepsin (pH 2, 37 °C), papain (pH 6, 37 °C), α-chymotrypsin (pH 8, 37 °C), trypsin (pH 8, 37 °C), neutrase (pH 8, 50 °C), and alcalase (pH 7, 50 °C) [16]. For each enzyme, the enzyme/substrate ratio was 1/100. The resulting mixture was stirred for 4 h and then heated at 100 °C for 10 min to inactivate the enzyme. The pepsin hydrolysate was desalted and separated into four ranges of MW (>10, 5–10, 1–5, and <1 kDa) using UF membranes of 10, 5, and 1 kDa, respectively (GE Healthcare Bio-Sciences Corp, Westborough, MA, USA). All fractions were desalted and lyophilized in a freeze dry system. The bioactive peptide was isolated from the peptic hydrolysate below 1 kDa *via* FPLC (AKTA, Amersham Bioscience Co., Uppsala, Sweden) on a HiPrep 16/10 diethylaminoethyl fast-flow (DEAE FF) anion-exchange column (16 × 100 mm, Amersham Biosciences, Piscataway, NJ, USA) and a GE Healthcare Superdex^{TM} Peptide 10/300 GL gel filtration column (10 × 300 mm). The purified peptide was desalted and then subjected to amino acid sequencing.

2.3. Measurement of ACE inhibitory activity

The ACE inhibitory effect was measured by measuring the release of hippuric acid from the substrate HHL using the method proposed by Jimsheena and Gowda [15,17] with slight modifications.

2.4. Computational docking

The PDB files of human ACE metalloprotease (1O8A) and captopril were downloaded from RCSB (www.rcsb.org). In addition, the peptides were drawn and converted to PDB file format using the ChemBioOffice 2010 tool. The docking of the target protein and the ligands was simulated using Docking Server (http://www.dockingserver.com/web) [18].

Essential hydrogen atoms, Kollman united atom-type charges, and solvation parameters were added with the aid of AutoDock tools. Affinity (grid) maps of XXÅ grid points and 0.375 Å spacing were generated using the Autogrid program [19].

Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis and Wets local search method [20]. The initial position, orientation, and torsions of the ligand molecules were set randomly. All rotatable torsions were released during docking. Each docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250,000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of five were applied.

2.5. Statistical analysis

The results of the analyses were presented as mean ± standard deviation from three independent experiments. Statistical comparisons between different treatments were done using Student's *t*-test. Differences with a value of $p < 0.05$ were considered significant.

3. Results and discussion

3.1. ACE inhibitory activity of enzymatic hydrolysates using UF membranes

To release ACE inhibitory peptides, gelatin was separately hydrolyzed using different enzymes such as pepsin, α-chymotrypsin, trypsin, papain, alcalase, and neutrase. Among them, at 1 mg/ml concentration, the pepsin- hydrolysate showed the strongest ACE inhibitory effect of about 91% (Fig. 1A). The ACE inhibitory activity of the hydrolysate varied with the MW distribution, and the MW distribution of the desired functional peptide can be controlled by using a UF membrane bioreactor system [21,22]. Therefore, the peptic hydrolysate was further fractionated into different MWs using UF membranes of 10, 5, and 1 kDa. Four fractions with different MWs of >10, 5–10, 1–5, and <1 kDa were obtained. As shown in Fig. 1B, VW, a commercial drug, was used as the positive control, which showed the strongest ACE inhibitory activity. Meanwhile, the peptic hydrolysate <1 kDa showed the strongest ACE inhibitory effect of 70% at 500 μg/ml (Fig. 1B). This result is consistent with previous studies of ACE inhibitory peptides, in which the low-MW peptides had more potent ACE inhibitory activity than the high-MW peptides. Ko et al. [23] found that the marine *ellipsoidea* protein hydrolysate can be fractionated into three fractions (>10, 5–10, and <5 kDa) by UF according to MWs, and the fraction below 5 kDa showed the strongest ACE inhibitory activity. Ngo et al. [24] separated skate (*Okamejei kenojei*) skin gelatin hydrolysate using an UF membrane (1 kDa), and the fraction below 1 kDa exhibited the highest ACE inhibitory activity. Thus, the peptic hydrolysate below 1 kDa was selected for further purification.

3.2. Purification of ACE inhibitory peptides

To obtain active peptides with ACE inhibitory activity, the peptic hydrolysate below 1 kDa was further separated into three fractions using the FPLC technique (Fig. 1C), and the resultant fractions were also analyzed for their ability to inhibit ACE. At 250 μg/ml concentration, fraction 1 exhibited a strong effect on the ACE inhibitory property of about 81% (Fig. 1C). Therefore, fraction 1 was further purified.