Food Chemistry 245 (2018) 471–480

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Exploration of the molecular interactions between angiotensin-I-converting enzyme (ACE) and the inhibitory peptides derived from hazelnut (*Corylus heterophylla* Fisch.)

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ARTICLE INFO

Keywords:
Hazelnut protein
Angiotensin-I-converting enzyme inhibition
Purification
Molecular docking
Cation–pi interaction

ABSTRACT

The mechanism of action of food-derived angiotensin-I-converting enzyme (ACE) inhibitory peptides has not been completely elucidated. In the present study, ion-exchange chromatography, gel filtration chromatography, reverse phase-high performance liquid chromatography, and liquid chromatography-electrospray ionization–tandem mass (LC-ESI-MS/MS) were employed for purifying and identifying the ACE inhibitory peptides from hazelnut. To understand the mode of action of these peptides, ACE inhibition kinetics, *in vitro* and *in vivo* bioavailability assays, active site analysis, and interaction between the inhibitory peptides and ACE were investigated. The results identified novel ACE inhibitory peptides Ala-Val-Lys-Val-Leu (AVKVL), Tyr-Leu-Val-Arg (YLVR), and Thr-Leu-Val-Gly-Arg (TLVGR) with IC_{50} values of 73.06, 15.42, and 249.3 μM, respectively. All peptides inhibited the ACE activity via a non-competitive mode. The binding free energies of AVKVL, YLVR, and TLVGR for ACE were −3.46, −6.48, and −7.37 kcal/mol, respectively. The strong inhibition of ACE by YLVR may be attributed to the formation of cation–pi interactions.

1. Introduction

Inhibition of angiotensin-I-converting enzyme (ACE) is considered to be a useful approach for the prevention and treatment of hypertension and associated diseases. Synthetic ACE inhibitors, including captopril, enalapril, and lisinopril, are extensively used as antihypertensive drugs. However, they have been reported to cause certain adverse effects, including allergic reactions, increasing blood potassium levels, taste disturbances, and skin rashes (Balti et al., 2015). Recently, attention has been mainly focused on the ACE inhibitory peptides derived from various kinds of food items. These peptides can be produced using different enzymes or microbial fermentation (Lee & Hur, 2017). Several studies have demonstrated that these peptides are safe for consumption and may possess potent antihypertensive activities. Usually, the ACE inhibitory peptides exert their mode of action by directly binding to or indirectly inducing conformational changes in the active site of the enzyme (Jia, Wu, Yan, & Gui, 2015). One of the major challenges associated with the use of ACE inhibitory peptides for the modulation of physiological functions is understanding their inhibitory activity. The molecular interactions between purified peptides and ACE are crucial in determining the inhibitory activity. Thus, identifying natural ACE inhibitory peptides from different species and investigating their molecular interactions with ACE has become an area of immense research interest. For example, Pan, Cao, Guo, and Zhao (2011) investigated the molecular interactions between the peptides derived from whey protein hydrolysate and ACE by using a flexible molecular docking approach. Wu, Jia, Yan, Du, and Gui (2015) elucidated the molecular interactions between ACE and a purified peptide (Ala-Ser-Leu) using flexible docking simulation. These results suggested that the hydrogen bonds potentially played the most significant role in binding of the inhibitor to ACE. However, it is known that enzyme inhibition can be mediated by both covalent and noncovalent interactions. Hydrophobic and electrostatic effects were also regarded to play key roles in mediating enzyme inhibition (Qiao et al., 2016). The molecular mechanism of ACE

Abbreviations: ACE, angiotensin-I converting enzyme; AVKVL, Ala-Val-Lys-Val-Leu; BLAST, Basic Local Alignment Search Tool; BP, blood pressure; BW, body weight; CV, column volumes; DBP, diastolic blood pressure; HOMO, highest occupied molecular orbit; HPLC-MS/MS, high performance liquid chromatography/tandem mass spectrometry; HHL, hippuryl-histidyl-leucine; NCBI, National Center of Biotechnology Information; LC-ESI-MS/MS, liquid chromatography-electrospray ionization–tandem mass; LUMO, lowest unoccupied molecular orbital; QSAR, quantitative structure- activity relationship; RP-HPLC, reverse phase-high performance liquid chromatography; SHRs, spontaneous hypertensive rats; SD, standard deviation; SBP, systolic blood pressure; TLVGR, Thr-Leu-Val-Gly-Arg; YLVR, Tyr-Leu-Val-Arg; Tris, (hydroxymethyl)tromethamine

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http://dx.doi.org/10.1016/j.foodchem.2017.10.095
Received 24 May 2017; Received in revised form 13 September 2017; Accepted 18 October 2017
Available online 20 October 2017

inhibition has not been completely elucidated.

Changbai mountain hazelnut (*Corylus heterophylla* Fisch.) is a well-known wild hazelnut species, which belongs to the family Betulaceae. The area under *Corylus heterophylla* plantation has increased up to a million hectare in northeast China. Hazelnuts are considered to be a rich source of several compounds that are useful for human health. Hazelnut dregs (containing around 58.8 ± 3.2% protein) are the by-products of edible oil production which have not been fully exploited. Recently, the use of hazelnut protein hydrolysates has been the focus of several studies. This is because of their biological activities, such as antiatherogenic, anti-inflammatory, and antimutagenic activities (Masthoff et al., 2013; Nitride et al., 2013). Our previous study revealed that the proteins isolated from hazelnut have a well-balanced amino acid composition, and are particularly rich in hydrophobic amino acids. Moreover, it has been previously demonstrated that peptides that are rich in hydrophobic residues demonstrated high ACE inhibition and the proteins that contained abundant hydrophobic amino acids produced more ACE inhibitory peptides (He et al., 2012). Thus, the production of novel value-added protein hydrolysates from Changbai mountain hazelnut may provide an alternative source for obtaining ACE inhibitory peptides for preventing hypertension. However, up till now, ACE inhibitory peptides from Changbai mountain hazelnut proteins or protein hydrolysates have not been reported. Additionally, information about the structural characteristics of these peptides is also not available.

For understanding the ACE inhibitory activities of protein hydrolysates from wild hazelnuts, the role of individual peptides in the hydrolysate should be examined. To the best of our knowledge, the molecular interactions of these inhibitory peptides with ACE have not been reported. Therefore, the aim of the present study was to isolate and identify the ACE inhibitory peptides from hazelnut using ion-exchange chromatography, gel filtration chromatography and high performance liquid chromatography/tandem mass spectrometry (HPLC–MS/MS). In addition, bioavailability and antihypertensive effects of the hazelnut protein hydrolysates with high ACE inhibitory activity were evaluated using the spontaneous hypertensive rat (SHR) model. Recently, molecular docking has been reported to be a useful tool for analyzing the interactions between ACE and its inhibitors with a high degree of accuracy and versatility. Previous studies have used molecular docking to elucidate the interactions between small molecules and proteins with high accuracy. Accordingly, molecular interactions between the purified peptides and ACE were elucidated. Thus, this study can provide the theoretical basis and technological support for industrial production of ACE inhibitory peptides from Changbai mountain hazelnut.

2. Materials and methods

2.1. Materials

Hazelnut protein was obtained from the College of Food Science and Engineering, Jilin Agricultural University (Changchun, China). ACE from rabbit lungs, hippuryl-histidyl-leucine (HHL), Alcalase® 2.4 L food grade, pepsin from porcine stomach mucosa, trypsin from porcine pancreas, and chymotrypsin were purchased from Sigma Chemical Co. (St Louis, MO, USA). HPLC-grade acetonitrile and trifluoroacetic acid were obtained from Fischer Scientific Co. (Waltham, MA, USA). All other reagents and chemicals used were of analytical grade and procured from Sigma Chemical Co. (St Louis, MO, USA).

2.2. Preparation of hazelnut protein hydrolysates

Hazelnut protein hydrolysates were prepared according to the method described by Lin, Yang, Cheng, Wang, and Qin (2017), with slight modifications. Distilled water was added to hazelnut proteins to obtain a 2% protein solution. To denature the proteins, the solution was incubated in a water bath at 100 °C for 15 min. After cooling, the solution was incubated in a water bath maintained at 54 °C. The pH was adjusted to 8.5 and Alcalase (10,000 U/g) was added to the solution. After the optimum temperature and pH were adjusted, the reaction mixture was constantly stirred for 2.5 h. To inactivate the enzyme, the reaction mixture was incubated for 10 min in a water bath at 100 °C, and subsequently the pH was adjusted to neutral. Next, the mixture was centrifuged at 3910*g* for 15 min. The purity of the hazelnut peptide extract was determined to be 80.50% using the Folin phenol protein quantitative assay. The peptides concentrated under vacuum, freeze-dried, and stored at −20 °C until future use.

2.3. ACE inhibitory activity

ACE inhibition was evaluated using the method described by Wu et al. (2015). Briefly, 10 μL sample solution (containing the sample in 0.1 M borate buffer supplemented with 0.3 M NaCl, pH 8.3) with 45 μL HHL solution (6.5 mM HHL in 0.1 M borate buffer supplemented with 0.3 M NaCl, pH 8.3) was preincubated at 37 °C for 5 min, and subsequently incubated with 10 μL ACE (0.1 U/mL) in 0.1 M borate buffer supplemented with 0.3 M NaCl, pH 8.3) at 37 °C for 30 min. The reaction was terminated by adding 85 μL of 1 M HCl to all samples, except for the blank control (prior to preincubation, 85 μL of 1 M HCl was added). The hippuric acid formed was extracted using 1 mL ethyl acetate. Subsequently, 800 μL of the ethyl acetate layer was collected and evaporated for 30 min in a drying oven at 100 °C. The residue was dissolved in 800 μL distilled water and absorbance of the solution was measured at 228 nm. The ACE inhibitory activity was determined using the following equation:

$$\text{ACE-inhibition activity } (\%) = \frac{A_b - A_a}{A_b - A_c} \times 100$$

where A_b is the absorbance without the addition of sample solution (buffer solution added instead of sample) and A_a is the absorbance in the presence of ACE and the sample solution. A_c is the absorbance of the blank (HCl was added prior to the addition of ACE). The half-maximal inhibitory concentration (IC_{50}) was defined as the concentration of inhibitor required to inhibit 50% of ACE activity.

2.4. Purification of the ACE inhibitory peptides

2.4.1. Ion-exchange chromatography

The ACE inhibitory peptides were purified according to the method described by Kleekayai et al. (2015), with slight modifications. The HP HiTrap Q preloaded column (1.6 cm × 2.5 cm; GE Healthcare, Buckinghamshire, UK) coupled with an ÄKTA purifier system was used. Briefly, the hazelnut protein hydrolysates were resuspended at a concentration of 1 mg protein/mL in buffer A (20 mM Tris-HCl, pH 9) and filtered using a 0.22-μm membrane. Next, 2 mL sample was loaded onto the column that had been previously equilibrated with 5 column volumes (CVs) of buffer A. The flow rate was maintained at 0.5 mL/min. The unbound fraction was removed by washing the column with 3 CVs of buffer A. Subsequently, the bound fractions were sequentially eluted using a gradient (0–100%) of 3 CVs of buffer B (1 M NaCl, pH 8.5). The chromatograms were evaluated at 220 nm. Fractions corresponding to each major peak were pooled and freeze-dried prior to the analysis of their ACE inhibitory activities.

2.4.2. Gel filtration chromatography

The ACE inhibitory peptides were purified according to the method described by Wu, Du, Jia, and Kuang (2016), with slight modifications. Following ion-exchange chromatography, the fraction showing the highest ACE inhibitory activity was redissolved in distilled water at a concentration of 30 mg/mL and subjected to further purification using the Sephadex G-15 column (1 × 50 cm), which was eluted using distilled water at a flow rate of 1 mL/min. The absorbance of the fractions was measured at 220 nm. The fractions exhibiting the highest ACE inhibitory activity were pooled. Each pooled fraction was analyzed for the ACE inhibitory activity.