Purification and Molecular Docking Study of Angiotensin-I Converting Enzyme (ACE) Inhibitory Peptide from Alcalase Hydrolysate of Hazelnut (*Corylus heterophylla* Fisch) Protein

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**Abstract**

Although a number of bioactive peptides are capable of angiotensin I-converting enzyme (ACE) inhibitory effects, little is known regarding the mechanism of hazelnut peptides using molecular simulation. In the present study, 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, the interaction between the inhibitory peptides and ACE was investigated. The results identified novel ACE inhibitory peptides Asp-Asp-Glu-Leu-Arg-Gln-Ala (DDELRQA), Asp-Asp-Glu-Leu-Arg-Ala-Ala (DDELRAA), and Asp-Gly-Glu-Leu-Arg-Glu (DGELRE). The binding free energies of DDELRQA, DDELRAA, and DGELRE for ACE were −10.2, −9.0, and −8.8 kcal/mol, respectively. This study proves the high stability of ACE inhibitory peptides derived from hazelnut against different temperature and pH of processing.

**Keywords**

Hazelnut Protein, Angiotensin-I-Converting Enzyme Inhibition, Purification, Molecular Docking

1. Introduction

Hypertension is a major chronic disease affecting 30% of the adult population in the world [1]. Although there are many causes of hypertension, it is well recog-
nized that angiotensin I-converting enzyme (ACE) plays an important role in the fluid and salt balance in mammals, as well as the rennin-angiotensin and the kallikrein-kinin systems for the regulation of blood pressure [2] [3]. Many synthetic ACE inhibitors including captopril, enalapril and lisinopril and others have been used to prevent hypertension in clinical [4]. However, these synthetic ACE inhibitors have been suspected to threaten health by causing cough, taste disturbances and renal impairment [5]. There, thus, is a growing interest in finding ACE inhibitors from natural products [6]. Food-derived peptides with antihypertensive properties have received great interest during the past 30 years [7]. Various food proteins, derived from plant protein and animal protein had ACE inhibitory activity in vitro [8].

The hazel species *Corylus. heterophylla* Fisch is a nut tree that belongs to the Betulaceae family, which is the most well-known species of wild hazelnuts. Moreover, the wild hazelnuts (species *Corylus. heterophylla* Fisch) are considered a rich source of many important compounds (polyphenols, protein, fat acid, carbohydrates, dietary fibre, aminophenols and microelements) useful for human diet health, which mainly distributed in Changbai Mountain, Jilin Province, in northeast of China, for centuries; its nuts have been harvested for oil extraction and as a food source. It is an economically important species, and its production accounts for nearly three quarters of the total output of the Chinese domestic market. The hazelnut dregs (containing around 58% of protein) are by-products of edible oil production were lost.

In our previous work, we have analyzed the immunomodulatory effects of hazelnut hydrolysed peptides isolated and identified in the hazelnut dregs, by-products (35% - 45% of waste production) from the hazelnut industries [9]. Recently, the use of hazelnut protein hydrolysates has been the subject of several research works, because of their biological activities, such as antioxidant, anti-inflammatory and antimutagenic properties [10]. For understanding the ACE inhibitory activities of protein hydrolysates from wild hazelnuts, the role of individual peptides in the hydrolysate should be examined. Therefore, the aim of the present study was to isolate and identify the ACE inhibitory peptides from hazelnut using gel filtration chromatography and high performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS). 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.

2. Materials and Methods

2.1. Materials

Hazelnut protein was obtained from the College of Food Science and Engineer-
ing, 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. (Wal-tham, 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 Dayong Ren (2016) with some modification. Hazelnut protein and distilled water were mixed to obtain a solution with the concentration of 2.0% and then incubated in a water bath at 90°C for 15 min to denature protein. After the mixture was cooled, Alcalase 2.4 L (dosage: 10,000 U/g) was added, and the solution was been constant stirred at a constant temperature of 54°C and pH of 8.0 in a heated water bath for 2.5 h. Following hydrolysis, the solution was incubation at 100°C for 10 min to inactivate the enzyme, and the pH was adjusted to neutral. The mixture was centrifuged at 3910×g for 15 min. The supernatant was collected, freeze-dried, and stored at −20°C for future use. The purity of obtained hydrolysates was 80.5% measured by folin-phenol protein quantitative assay.

2.3. Determination of ACE Inhibitory Activity

The ACE inhibitory activity of peptides was evaluated using the method described by da Cruz et al. [11]. 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:

\[
\text{ACE-inhibition activity (\%) = } \frac{A_b - A_{bc}}{A_b - A_c} \times 100
\]

here \( A_b \) is the absorbance without the addition of sample solution (buffer solution added instead of sample) and \( A_s \) 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. Gel Filtration Chromatography
The ACE inhibitory peptides were purified according to the method described by Sangsawad et al. [12], with slight modifications. 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.

2.4.2. RP-HPLC
Following gel filtration chromatography, the fraction exhibiting the highest ACE inhibitory activity was further purified by RP-HPLC using the Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) with a Diamonsil C18 HPLC column (4.6 × 250 mm, 5 µm; loading quantity, 80 µL). The column was eluted using a linear gradient of acetonitrile (5% - 60%) supplemented with 0.1% trifluoroacetic acid, at a flow rate of 0.5 mL/min. The fractions, corresponding to the elution peaks, were collected and lyophilized immediately for determining their ACE inhibitory activities. Next, fraction with highest activity was pooled using Waters 2545 QGM semi-preparative HPLC (Waters Corporation, Milford Massachusetts, USA) with a SunFire Prep C18 column (10 × 250 mm, 5 µm), which was eluted using a linear gradient of acetonitrile (5% - 60%; supplemented with 0.1% trifluoroacetic acid) at a flow rate of 5 mL/min and a loading quantity of 2 mL. Absorbance of the eluent was measured at 215 nm. Next, the amino acid sequence of the peptide fraction exhibiting the highest ACE inhibitory activity was determined.

2.5. Identification of Hazelnut Peptide
Based on the previous experiments, the amino acid sequence of the peptides in the fraction showing the highest ACE inhibitory activity was identified as described previously [1], with slight modifications. The Agilent 1200 HPLC system coupled with an Agilent 6520 Q-TOF MS was used. Peptides were separated using the Agilent Eclipse Plus C18 column (2.1 × 150 mm, 3.5 µm) with Eluent A (an aqueous solution of 0.1% formic acid) and Eluent B (acetonitrile in water supplemented with 0.1% formic acid). Elution was performed using a linear gradient of Eluent B (5% - 30%) under the following conditions: flow rate, 0.4 mL/min; time, 30 min; column temperature, 35°C; sample volume, 5 µL. Positive-ion mode electrospray ionization (ESI+) mass spectrometry was performed. The scan range was m/z 100 - 2000. Dry gas flow velocity (N₂) was 9 L/min and the drying temperature was 300°C. The other parameters were as follows: atomizing voltage, 35 psig; capillary voltage, 3.5 kV; cataclastic voltage, 175 V; ca-
pillary tube voltage, 65 V; radio frequency voltage, 250 V; and the secondary mass spectrometry collision voltage, 12 - 15 eV. The de novo amino acid sequence was determined using Peaks 7.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada). The identified peptides were subjected to in silico ACE inhibitory activity analysis using the “profile of potential biological activity” analysis tool of the BIOPEP database (http://www.uwm.edu.pl/biochemia) (da Cruz, Pimenta, de Melo, & Nascimento, 2016). The BLAST program was used for performing sequence homology searches for the obtained sequences against the non-redundant protein sequence database from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6. Molecular Docking

The three-dimensional structure of human ACE-lisinopril complex (1O8A.pdb) was derived from the RCSB PDB Protein DataBank (http://www.rcsb.org/pdb/home/home.do). Before the docking, water molecules and the inhibitor lisinopril were removed whereas the cofactors zinc and chloride atoms were retained in ACE model. The polar hydrogens were then added to the ACE model. Structure of the purified peptide was generated using ChemOffice2004 software (Cambridge Soft Co., USA) and its energy was minimized with the CHARMM program. The molecular docking of the peptide and ACE was studied according to a method described by Tao et al. [13] with some modifications. The flexible docking tool of Discovery Studio 2.1 software was used to perform the molecular docking of the peptide at ACE-binding site. The docking runs were carried out with a radius of 9˚A, with coordinates x: 40.6559, y: 37.3827 and z: 43.3401. The best ranked docking pose of the peptide in the active site of ACE was obtained according to the scores and binding-energy value.

2.7. Stability of ACE Inhibitory Peptides

The purified peptides (1 mg/mL) were incubated separately at 20, 40, 60, 80, and 100˚C for 2 h. Before the ACE inhibitory activity was determined, the samples were allowed to cool to room temperature (25˚C) and the pH was adjusted to 8.3. Then, the purified peptides (1 mg/mL) were also incubated at 40˚C at pH values of 2, 4, 6, 8 and 10 for 2 h. Subsequently, pH values were adjusted to 8.3 before the ACE inhibitory activity was determined. Subsequently, ACE inhibitory peptide solutions (1 mg/mL) were treated at different metal ions Cu²⁺, Mg²⁺, Zn²⁺, Ca²⁺, and K⁺ (at a concentration of 100 μg/mL) and different concentrations of NaCl solution for 1 h.

The stability of the ACE inhibitory peptides when challenged with in vitro gastrointestinal digestion was assessed by mixing a peptide solution (10 mg/mL) in 0.1 M KCl-HCl (pH 2.0) buffer with 1% (w/w) pepsin. The mixture was incubated in a 37˚C water bath for 4 h and then neutralized to pH 7.0 with addition of 2 M NaOH solution. The neutralized suspension (1 mL) was centrifuged at 10,000× g for 20 min and the supernatant was assayed for ACE inhibitory activity. The remaining neutralized suspension was further digested by 1% (w/w)
chymotrypsin at 37˚C for 4 h. The pancreatic digestion was terminated by boiling the reaction mixture for 15 min followed by centrifugation at 10,000× g for 20 min. The supernatant was used for ACE inhibitory activity assays. Controls were prepared by mixing the inactivated pepsin and chymotrypsin (by boiling enzymes for 15 min) with the peptide solution.

2.8. Statistical Analysis

Analysis of variance was performed using the SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA). All experiments were performed in triplicates. Data are represented as the mean ± standard deviation. The results were considered to be statistically significant at P < 0.05.

3. Results and Discussion

3.1. Purification of Hazelnut Peptides

High degree of hydrolysis can be used for obtaining low-molecular-weight peptides. Thus, alcalase was used for the preparation of ACE inhibitory peptides due to its broad substrate specificity and high degree of hydrolysis. After hydrolysis with alcalase, the hydrolysate was subjected to gel filtration chromatography, and RP-HPLC, which separated the ACE inhibitory peptides (from the bioactive fractions) based on differences in molecular weight, and hydrophobicity. As shown in Figure 1(a), following Sephadex G-15 chromatography, two major fractions were obtained. The fractions were labeled A1 and A2. Next, they were pooled, lyophilized, and subjected to the ACE inhibition assay. The highest ACE inhibitory activity (72.31% ± 2.47%, P < 0.05) was observed for fraction A1. Since the Sephadex G-15 column was used, these results indicated that the majority of peptides present in fraction A1 were high-molecular-mass peptides. These results were similar to those reported by Connolly, O’Keeffe, Piggott, Nongonierma and FitzGerald (2015), who demonstrated that the low-molecular-mass peptides were not always associated with a high ACE inhibitory activity. Therefore, the results of the present study suggested that molecular mass may not be the only physicochemical characteristic associated with ACE inhibition. It has also been reported that certain high-molecular-mass peptides may act as substrates for ACE, thereby interfering with the hydrolysis of the synthetic substrate (HHL) used in the assay.

Using gel filtration chromatography, the A1 fraction, which showed the highest ACE inhibitory activity was further purified, and subjected to RP-HPLC. RP-HPLC is widely used as the final step in peptide purification and possesses several advantages such as high sensitivity, resolution, and column efficiency. Eight major fractions, designated B1-B8, were obtained and their ACE inhibitory activities were measured (Figure 1(d)). Fraction B5 showed highest ACE inhibition (47.07% ± 1.08%, P < 0.05). Subsequently, the purity of B5 was analyzed using RP-HPLC (data not shown). The results indicated high purity for B5, and thus, it was further subjected to amino acid sequencing.
Figure 1. Chromatograms and ACE inhibitory activity at various purification steps. (a) Purification of the ACE inhibitory peptide from hazelnut protein hydrolysates by Sephadex G-15 chromatography; (b) ACE inhibition of fraction A1 and A2; (c) Purification from the fraction B1 by RP-HPLC chromatography; (d) ACE inhibition of fraction B1-B8.

3.2. Identification of Hazelnut Peptides

The B5 fraction, which exhibited the highest ACE inhibitory activity, was analyzed using LC-ESI-MS/MS, in order to identify the constituent peptides that may be potentially responsible for ACE inhibition. A search of potential ACE inhibitory activity that can be derived from the identified peptides was carried out with the BIOPEP database in which the identified peptides were compared to previously reported sequences. As shown in Figure 2, three novel peptides were screened de novo from fraction B5, namely Asp-Asp-Glu-Leu-Arg-Gln-Ala (DDELRQA), Asp-Asp-Glu-Leu-Arg-Ala-Ala (DDELRAA), and Asp-Gly-Glu-Leu-Arg-Glu (DGELRE).
According to previous reports on the relationship between structure and activity of ACE inhibitory peptides, the C-terminal end of the peptide is a major contributor to the activity, and the nature of the C1-C4 amino acids seems to control the inhibitory potential [7]. Many ACE inhibitory peptides exhibit some common structural properties, including abundant hydrophobic amino acids,
and present hydrophobic amino acids at the N-terminal position [12]. Sornwatanakul et al. reported Chebulin binds to ACE by the hydrogen bond, hydrophobic and ionic interactions via the interactions of C-terminal Phe (Phe-6), and N-terminal residues (Asp-1 and Glu-2) with the amino acid residues on noncatalytic sites of the ACE [14]. In this study, DDELRQA and DDELRAA contained a large number of hydrophobic amino acids (Leu, Ala) and N-terminal Asp contributed to their inhibitory activity.

However, the structure-activity relationship of ACE inhibitory peptides has not been fully established. Some longer peptides possessed high ACE inhibitory activity and some peptides with hydrophilic amino acid residues in their sequences possessed low inhibitory activities (Ashok & Aparna, 2017; Li et al., 2017). This may be due to the presence of hydrophilic amino acid residues, which affect the ACE inhibitory activity by restricting the entry of the peptide to the enzyme active site. Hence, the structure-activity relationship of ACE inhibitory peptides needs to be further explored. Recently, the quantitative structure-activity relationship (QSAR) models for ACE inhibitory peptides have been studied to analyze structure information. However, significant time and resources will be required to build models.

3.3. Inhibition Mechanism of Hazelnut Peptides

Understanding the molecular interactions between ACE and the ACE inhibitory peptides will help in designing and synthesizing more efficient peptide inhibitors. To elucidate the molecular interactions between the purified hazelnut peptides and ACE, molecular docking studies were performed. The results predicted the preferred orientations of the three peptides required for the formation of a stable complex with ACE.

The molecular docking results of synthetic peptides and ACE were shown in Figure 3. It was observed that the active pocket of ACE was surrounded by hazelnut peptide. The affinity force of DDELRQA, DDELRAA and TLVGR with ACE were −10.2, −9.0, and −8.8 kcal/mol, respectively (Table 1). This indicated that DDELRQA could bind tightly to ACE, and thus, the DDELRQA-ACE complex was stable. Although previous studies suggested that the interaction between most active peptides and ACE was dynamic, but molecular docking results showed that some sites could interaction. For example, hydrogen-bonding formed between DDELRQA and the amino acid residues Lys511, Gln281, Asp453, and Glu376 at the ACE active center. Since the hydrogen bond was stable during the binding of the inhibitor to the enzyme, the number of hydrogen bonds also determined the affinity of the peptide. Therefore, DDELRQA and ACE had a good affinity, forming 11 hydrogen bonds. Compared with DDELRQA, less hydrogen bonds and van der Waals forces were formed between DDELRAA and ACE, which means that DDELRAA with weaker ACE inhibitory activity. Pan et al. studied the molecular docking of whey protein-derived ACE inhibitory peptide LL and ACE, which also indicated that the amino acid residues Tyr523,
Figure 3. The general overview docking pose showing interactions between the peptides of (a) DDELRQA, (b) DDELRAA, and (c) DGELRE with ACE (PDB: 1O8Å).

Table 1. Affinity force of peptide and ACE.

<table>
<thead>
<tr>
<th>Number</th>
<th>Peptide sequence</th>
<th>Affinity (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DDELRQA</td>
<td>−10.2</td>
</tr>
<tr>
<td>2</td>
<td>DDELRAA</td>
<td>−9.0</td>
</tr>
<tr>
<td>3</td>
<td>DGELRE</td>
<td>−8.8</td>
</tr>
</tbody>
</table>

Ala354 and Glu384 of ACE active center played an important role in the ACE active center, which forming hydrogen bonds with inhibitor [15]. It is beneficial to increase the affinity of the peptide with the ACE molecule to higher inhibitory activity. In addition, Zn$^{2+}$ is an important ligand ion of ACE active center, and electrostatic interaction with Zn$^{2+}$ may directly lead to inactivation of ACE molecules [16]. In future, flexible docking may be adopted in calculating the change of distance and energy between the residue and Zn$^{2+}$.

3.4. Stability of ACE Inhibitory Peptides against Heat Treatment and in Vitro Digestion

ACE inhibitory peptides could exert in vivo antihypertensive effect if they reach...
the blood stream in an active form [17]. So, after oral ingestion, peptides need to resist complete hydrolysis by gastrointestinal enzymes and brush border peptidases, and be able to pass through the intestinal wall with preserving their biological activity [18]. Peptides can be degraded in this process and their biological activity can be activated or inactivated. As Figure 4(a) showed ACE peptide retained its inhibitory activity even after severe thermal treatments at 100°C. In addition, inhibitory activity remained constant over a wide pH range of 2 - 10 (Figure 4(b)), indicating that peptides were both thermo and pH stable. In addition, activity of hazelnut peptide decreased when treated with Cu²⁺, Ca²⁺ and high concentration of NaCl, but other metal ion could not affect their inhibition.

Figure 4. Stability of hazelnut-derived ACE inhibitory peptides after: (a) 2 h incubation at various temperatures, (b) incubation at different pH, (b) incubation at different metal ion, (b) incubation at different concentration of NaCl and (b) incubation at different digestive enzymes. Both case compared to a control (c). Bars represent means ± SD. Bars with different letters are significantly different at P < 0.05.
Moreover, the ACE inhibitory peptides from hazelnut could not show resistance to in vitro gastrointestinal digestion, revealing that the peptides may be partially degraded into smaller peptides. Previous studies have reported that small peptides still presented ACE inhibitory activity after digestion [19] [20]. These results indicated that orally administered ACE inhibitory peptides may not keep their sequence integrity in the stomach, but break into new smaller bioactive peptides that could reach the blood stream.

4. Conclusion

Novel ACE inhibitory peptides from hazelnut protein hydrolysates, namely Asp-Asp-Glu-Leu-Arg-Gln-Ala (DDELRQA), Asp-Asp-Glu-Leu-Arg-Ala-Ala (DDELRAA), and Asp-Gly-Glu-Leu-Arg-Glu (DGELRE) were successfully purified and identified using gel filtration chromatography, RP-HPLC, and LC-ESI-MS/MS. This screening method may be used as a valuable tool for identifying novel food-derived ACE inhibitory peptides. Furthermore, molecular docking results indicated that the interaction of peptides with certain amino acids in the ACE active site markedly contributed towards stabilizing the docking complex. The strong inhibition of ACE exhibited by DDELRQA may be attributed to the formation of hydrogen bond interactions. The above results provided novel insights and helped in improving our current understanding of the interaction between ACE and its inhibitors.

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Conflicts of Interest

There are no conflicts to declare.

References


