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Purification and Identification of an ACE Inhibitory Peptide from Walnut Protein

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ABSTRACT: In the present study, a novel angiotensin I-converting enzyme (ACE)-inhibitory peptide, P-2a2, was purified to homogeneity from walnut protein hydrolysate by ultrafiltration, consecutive column chromatography, and high-performance liquid chromatography. The purified peptide was characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrophotometry and a liquid-phase peptide sequencer. The molecular mass of P-2a2 was tested to be 1033.42 D. Its amino acid sequence was determined to be Trp-Pro-Glu-Arg-Pro-Pro-Gln-Ile-Pro. The potent ACE-inhibitory peptide is an enneapeptide and shows a high ACE-inhibitory activity, with an IC_{50} value of 25.67 μg/mL.

KEYWORDS: *walnut, peptide, ACE-inhibitory activity, purification, characterization*

INTRODUCTION

Angiotensin I-converting enzyme (ACE, EC3.4.15.1) is a dipeptidyl carboxypeptidase associated with the regulation of blood pressure as well as cardiovascular function.[1] Consequently, ACE-inhibitory substances are used to decrease the blood pressure of hypertensive patients.[2] Potent synthetic ACE inhibitors such as captopril, enalapril, and lisinopril are widely used in the clinical treatment of hypertension in humans.[3] However, these synthetic drugs have several side effects including coughing and skin rashes. Food-derived ACE inhibitors have safety advantages over the synthetic compounds.[4] At present, many ACE-inhibitory peptides have been isolated from food proteins, such as soybean, fermented soybean, cheese whey, casein, and corn gluten.[5−9] Some have been found to be effective for antihypertensive agents *in vivo* without causing side effects in spontaneously hypertensive rats (SHR) and hypertensive humans.[10]

Walnuts have been investigated as sources of food with health-promoting functions, such as antioxidant,[11] antitumor,[12] neuronal signaling increasing and stress signals decreasing,[13] and antihypertensive properties.[14] Many studies showed that the bioactive peptides as a result of enzymatic hydrolysis of nut proteins have better functions than proteins.[15] In resent years, to produce peptides with better functions, researchers focused on the optimization of enzymatic hydrolysis processing conditions. However, not all of the hydrolysis fractions but those peptides with special amino acid sequences have certain bioactivity, which prompt researchers to purify and identify these functional peptides. Until now, there is no report related to the purification and identification of ACE-inhibitory peptides from walnut proteins.

In the present study, a novel ACE-inhibitory peptide was isolated from walnut protein hydrolysate, and its molecular mass and amino acid sequence were identified.

MATERIALS AND METHODS

Materials and Chemicals. Defatted walnut residues were purchased from Beijing Lvhu Industrial & Trade Co., Ltd. (Beijing, China). Neutral proteinase As1.398 (6.0×10^4 U/g) was purchased from Novozymes China (Beijing, China). Angiotensin converting enzyme (ACE) and hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Sephadex G-15 and HiTrap Mono Q resins were purchased from GE Healthcare (Beijing, China).

Preparation of Crude Peptides. The crude proteins were isolated from the defatted walnut residues and then hydrolyzed by neutral proteinase As1.398 for 5 h at 45 °C, pH 7.0. The ratio of enzyme to substrate is 3:100, and the concentration of protein is 50 mg/mL.[16] The antihypertensive peptide will be purified from the walnut protein hydrolysate (WPH). The protein concentration was determined by the modified Lowry method with bovine serum albumin as standard.[17]

Purification of ACE-Inhibitory Peptides. The WPH was fractionated using dead-end ultrafiltration (8050, Millipore, Beijing, China) with 3K molecular weight cutoff UF membranes (Millipore, Beijing, China) for lab scale at 4 °C. The fraction with molecular weights lower than 3K was gathered and lyophilized in a freezing drier (ALPHA 1−4, Christ, Osterode, Germany). The fraction was loaded in a Sephadex G-15 size exclusion column (16 mm × 100 cm; GE Healthcare, Beijing, China) pre-equilibrated with deionized water and eluted at a flow rate of 5 mL/min. The highest inhibitory fraction was further isolated using a HiTrap Mono Q (1 mL) strong anion exchange chromatography column (GE Healthcare, Beijing, China) pre-equilibrated with Tris-HCl buffer (pH 8.0). The elution was performed using 1 mol/L NaCl and 0.05 M Tris-HCl (pH 8.0) buffer as a mobile phase with a flow rate of 1 mL/min and a 30 mL of NaCl linear salt gradient (0−100%). The elution was operated at 4 °C and monitored at 280 and 220 nm.

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Finally, to further purify the ACE inhibitor, the fraction showing the strongest activity in anion exchange chromatography was collected, lyophilized, and further applied to a GPC-HPLC system (Ultrahydrogel 250 column, 7.8 mm × 300 mm; Waters, MA, USA). Elution was carried out using a mixture liquor of acetonitrile (20%) and 0.1 M NaH_2PO_4–Na_2HPO_4–NaCl (pH = 7) (80%) at a flow rate of 0.5 mL/min at 30 ± 5 °C and 360 psi. The effluent was monitored by a UV/visible detector at 215 nm, and the fractions were collected for assay of ACE-inhibitory activity.

ACE-Inhibitory Activity Assay. ACE-inhibitory activity was measured by a spectrophotometric method[18] with minor modifications as follows. The reaction mixture contained 50 μL of 20 mmol/L HHL as a substrate, 50 μL of ACE powder (0.02 unit) in 150 mmol/L sodium borate buffer (pH 8.3), and 50 μL of the sample solution. The reaction was carried out at 37 °C for 30 min and terminated by adding 100 μL of 1.75 mol/L HCl and 1 mL of ethyl acetate. After centrifugation, 1 mL of the supernatant was transferred into a test tube and evaporated at room temperature for 2 h in a vacuum. The hippuric acid was redissolved in 1 mL of distilled water, and the absorbance was measured at 228 nm. IC_{50} was defined as the concentration of the peptide that inhibits 50% of the ACE activity and determined from the ACE-inhibitory activity and peptide contents of each sample after regression analysis.

Identification of ACE-Inhibitory Peptide. The molecular mass and the amino acid sequence of the purified peptide were determined using a matrix-assisted laser desorption ionization time-of-flight mass spectrophotometer (MALDI-TOF-MS, Voyager-DE STR, PerSeptive Biosystem Inc., Framingham, MA, USA) and a liquid-phase peptide sequencer (476A-01-120, Applied Biosystems Co., Foster, CA, USA),[17] respectively.

RESULTS AND DISCUSSION

ACE Inhibitory Activity of Fractions of WPH with Different Molecular Mass. To isolate the active peptide, the WPH was separated by an ultrafiltration membrane with a cutoff of 3K. The ACE-inhibitory activity of the permeate is 88.6% when the protein concentration is 1.0 mg/mL, which is higher than that of the retentate (47.3%) under the same concentration. Moreover, the amount of permeate protein (82.4%) is higher than the amount of retentate proteins (17.6%). Therefore, the permeate fraction was selected for further study.

Isolation of ACE-Inhibitor Peptide by Sephadex G-15 and Mono Q. The ACE-inhibition peptide was first purified by a Sephadex G-15 chromatography column. As shown in Figure 1, there are two remarkable fractions, P-1 and P-2. The ACE-inhibition activity of P-1 and P-2 is 53.5% and 74.8%, respectively, at a concentration of 500 μg/mL. P-2 was collected and concentrated by an ultrafiltration membrane (cutoff 500D) and then loaded on the Mono Q, which was used for the subsequent chromatography column. The elution results (Figure 2) showed six apparent fractions, P-2a, P-2b, P-2c, P-2d, P-2e, and P-2f. Their ACE-inhibition activities were 72.17%, 63.87%, 19.61%, 34.65%, 10.28%, and 48.52%, respectively, at the same concentration of 50 μg/mL. Both the yield and the ACE-inhibition activity of P-2a are the highest among the six fractions from Mono Q chromatography.

Purification of ACE Inhibitor Peptide by GPC-HPLC. P-2a was collected and concentrated by ultrafiltration. Then, GPC-HPLC was carried out for further separation and purification. As shown in Figure 3, the eluate was divided into three major fractions (P-2a1, P-2a2, and P-2a3) from the Ultrahydrogel 250 column. Fraction P-2a2 was prepared by preparative liquid chromatography. The purity of P-2a2 was also confirmed by Ultrahydrogel 250 column analysis (Figure 4). A single peak of P-2a2 in the elution of GPC-HPLC indicated its high purity. The yield of P-2a2 in each purification step is shown in Table 1. The final yield of this new peptide is 10.5 mg/g protein.

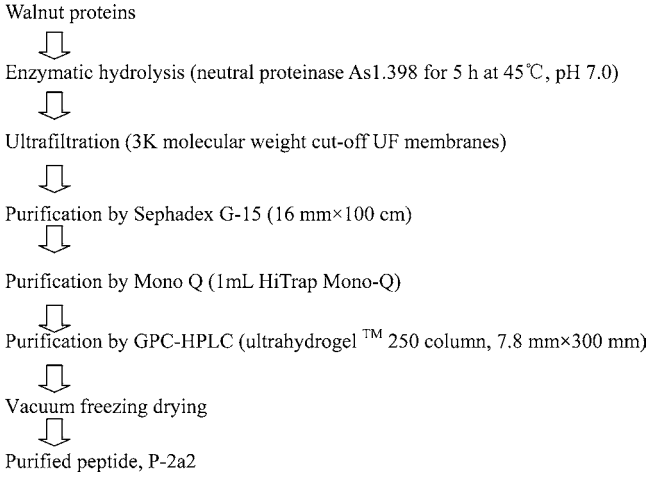

Figure 1. Flowchart of purification of the ACE-inhibitory peptide. The purification procedures of the ACE-inhibitory peptide are laid out step by step, and some key parameters for each procedure are shown.

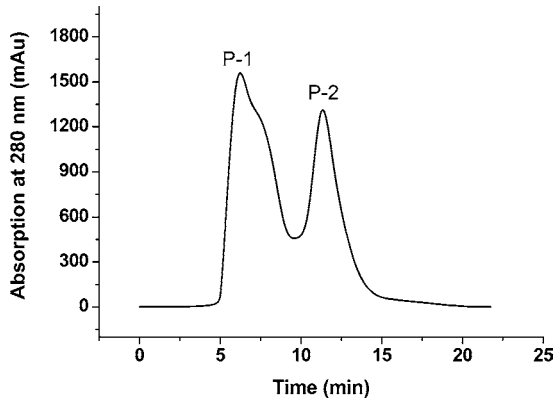

Figure 2. Elution diagram of Sephadex G-15. The filtrate with molecular weight lower than 3K was loaded on a Sephadex G-15 column (16 mm × 100 cm) pre-equilibrated with deionized water and eluted at a flow rate of 5 mL/min.

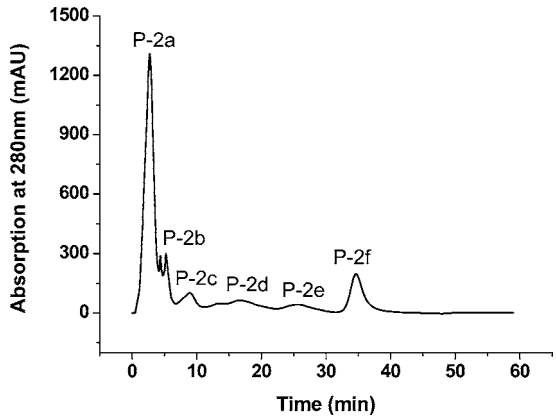

Figure 3. Elution diagram of the HiTrap Mono Q column (1 mL) (GE Healthcare, Beijing, China) pre-equilibrated with Tris-HCl buffer (pH 8.0). The elution was performed using 1 mol/L NaCl and 0.05 M Tris-HCl (pH 8.0) buffer as a mobile phase with a flow rate of 1 mL/min and a 30 mL of NaCl linear salt gradient (0–100%).

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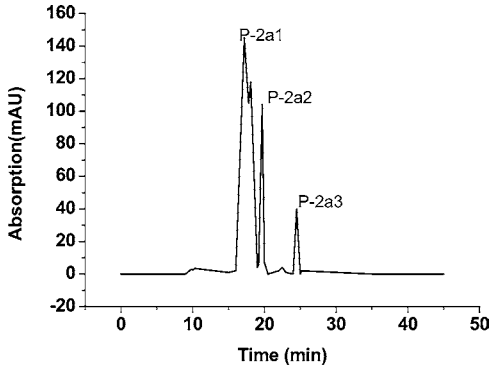

Figure 4. GPC-HPLC of P-2a from Mono Q. The sample was applied to a GPC-HPLC system (Ultrahydrogel 250 column, 7.8 mm × 300 mm; Waters, MA, USA). Elution was carried out using a mixture liquor of acetonitrile (20%) and 0.1 M NaH_2PO_4–Na_2HPO_4–NaCl (pH = 7) (80%) at a flow rate of 0.5 mL/min at 30 ± 5 °C and 360 psi.

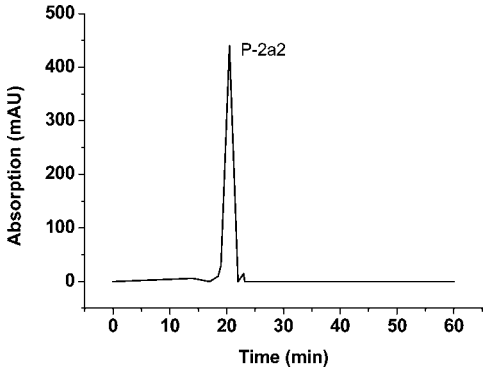

Figure 5. GPC-HPLC of purified peptide P-2a2. The GPC-HPLC system was equipped with an Ultrahydrogel 250 column (7.8 mm × 300 mm) (Waters, MA, USA). Elution was carried out using a mixture liquor of acetonitrile (20%) and 0.1 M NaH_2PO_4–Na_2HPO_4–NaCl (pH = 7) (80%) at a flow rate of 0.5 mL/min at 30 ± 5 °C and 360 psi.

Table 1. Yield of Each Purification Procedure of P-2a2

procedure	yield (mg/g protein)
after enzymatic hydrolysis	806.1
after ultrafiltration	626.2
after purification by Sephadex G-15	212.9
after purification by Mono Q	68.8
after purification by GPC-HPLC	10.5

Identification of ACE Inhibitor Peptide. The molecular mass of the purified peptide was 1033.42 D according to the results of MALDI-TOF-MS. It was analyzed using a peptide sequencer and was identified to be an enneapeptide, Trp-Pro-Glu-Arg-Pro-Pro-Gln-Ile-Pro. Hydrophobic amino acids in the N-terminal region of the active peptide play important roles in binding the ACE active site.[19−23] The most favorable sequence consists of hydrophobic amino acid residues at the N-terminus, positively charged amino acids at the middle position, and aromatic amino acids at the C-terminus and is the proposed typical model for ACE-inhibitory peptides through computational analysis.[24] The purified peptide identified here shares the entire representative structural motif. The N-terminus has two hydrophobic amino acids, Trp and Pro, which might contribute to ACE binding. It has both positively charged amino acids in the middle and aromatic amino acid residues at the C-terminus.

An assay of ACE-inhibitory activity of purified P-2a2 revealed that its activity is concentration dependent (Table 2). The IC_{50} value of P-2a2 is 25.67 μg/mL, which is significantly higher than those of fermentation soybean extracts (1.46 mg/mL), miso paste (1.27 mg/mL), natto (0.16 mg/mL), and tofuyo (1.77 mg/mL).[9,25]

Table 2. ACE Inhibition Rate of the Purified Peptide

number	protein concentration (μg/mL)	ACE inhibition rate (%)
1	15	38.5
2	30	52.1
3	45	61.9
4	60	70.8
5	75	77.6
6	90	82.8

CONCLUSION

In the present study, a novel ACE-inhibitory peptide, P-2a2, was consecutively purified from the walnut protein hydrolysate by Sephadex G-15 size exclusion chromatography, HiTrap Mono Q anion exchange chromatography, and an Ultrahydrogel 250 column in GPC-HPLC, combined with ultrafiltration and lyophilization. P-2a2 was an enneapeptide, Trp-Pro-Glu-Arg-Pro-Pro-Gln-Ile-Pro, and showed a high ACE-inhibitory activity with an IC_{50} of 25.67 μg/mL, which indicates that it can be used as a functional ingredient in food with antihypertensive properties.

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Notes

The authors declare no competing financial interest.

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