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In vitro and *in vivo* ACE inhibitory of pistachio hydrolysates and *in silico* mechanism of identified peptide binding with ACE

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ABSTRACT

The ACE inhibitory activity of pistachio (*Pistacia vera* L.) kernel's hydrolysates by gastrointestinal enzymes was studied. Results indicated that hydrolysate successively hydrolyzed by pepsin and trypsin, Pe–Tr–H, presented *in vitro* ACE inhibitory activity as IC_{50} 0.87 ± 0.04 mg/ml. The Pe–Tr–H can *in vivo* decrease around 22 mmHg in systolic blood pressure (SBP) and 16 mmHg in the diastolic blood pressure (DBP) at 4 h after the oral administration, however the pistachio kernel powder can slightly lower SBP and DBP. The Pe–Tr–H with the highest activity was then separated by ultrafiltration membrane of 3 kDa, size exclusion chromatography on Sephadex G-15 and G-10 columns and reversed phase high-performance liquid chromatography (RP-HPLC) consecutively. A novel ACE inhibitory peptide, ACKEP, with the IC_{50} value of 126 μM, was identified by MALDI–TOF/TOF system. ACKEP has the same C-terminal residue as Lisinopril and Enalapril, which plays a key role in binding with ACE. The binding mechanism was explored at a molecular basis by docking experiments, which revealed that seven residues from ACE active site (His383, His387, Glu384, Arg522, Asp358, Ala356 and Asn70) and two atoms of ACKEP (O5, H60) greatly contributed to the combinative stabilization.

1. Introduction

Hypertension is a considerable public health problem worldwide and poses a major risk factor for cardiovascular diseases, the number one cause of death in the world. The WHO estimated that by 2020, heart disease and stroke will have surpassed infectious diseases to become the leading cause of death [1]. On the other hand, health food and diet could be an aid to prevent the cardiovascular diseases, for instance, the antioxidant, vitamins, minerals and the bio-functional peptide from foods. The angiotensin I-converting enzyme (ACE) inhibitory peptide, which can be released by gastrointestinal enzyme hydrolysis and regulate blood pressure through binding to ACE [2,3]. ACE is a membrane anchored dipeptidyl peptidase that hydrolyses angiotensin I to the potent vasoconstrictor angiotensin II and by abrogating a potent vasodilator bradykinin to its inactive fragments, which play a key role in the blood pressure homeostasis. Thus, inhibition of ACE is considered as the first line of therapy for treating hypertension [4]. The first peptide inhibitors of ACE were reported from snake venom of *Bothrops jararaca* [5]. The drugs, captopril, lisinopril, enalapril for medical intervention are based on the snake venom peptide scaffold. Although these drugs show dramatically activity, however, along with various side effects such as coughing, skin rashes, and angioedema are concerned [6,7].

In recent years, ACE inhibitory peptides from food sources are promising natural bio-functional alternatives to the synthetic drugs and are currently the best known class of bioactive peptides. These specific sequences of bioactive peptides can be released through proteolysis either by food processing or by gastrointestinal digestion. Various ACE inhibitory peptides from enzymatic hydrolysates derived from different sources have been reported, such as the Atlantic salmon [8], areca nut [9], *Chlorella ellipsoidea* [10], kidney bean [11], brownstripe red snapper [12], soy bean [13], hard clam [14], wakame [15], insect protein [16], lupin [17], egg [18], peas [19], and so on.

In recent years, computational biology was used to predict the interaction between protein and small molecular such as bioactive peptides, thus, computational (*in silico*) methods can be used for inhibitory mechanism study as an assistive tool and the design of novel enzyme inhibitors [20–23]. An increasing number of

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researches are booming for the quantitative structure–activity relationship of ACE inhibitory peptide and the mechanism of peptide binding with ACE by using the molecular simulation on computer, and the common software are Discovery Studio, Autodock and Dock [24–26].

Pistachio (*Pistacia vera* L.) is a nutritional food, which is rich in protein, vitamins, minerals and oil, consuming around the world [27]. However, little study has been reported on the pistachio kernel's anti-hypertension functionality related to ACE inhibition. An attempt was made in this study to discuss the ACE inhibitory activity of gastrointestinal enzymatic hydrolysates from the pistachio kernel *in vitro* and *in vivo*. Then a novel ACE inhibitory peptide was isolated and identified from the pepsin–trypsin hydrolysate (Pe–Tr–H) with highest ACE inhibitory activity. Extend study was conducted to explore the binding mechanism including hydrogen bond, hydrophobic interaction, electrostatic interactions, Van der Waals interaction force and total energy between peptide and ACE by docking experiments.

2. Materials and methods

2.1. Materials

The pistachio (*P. vera* L.) was obtained from Paramount Farms Ltd. Co. (San QiaoKun valley, CA, USA). Pepsin, trypsin, ACE (EC 3.4.15.1) from rabbit lung and hippuryl–histidyl–leucine (HHL) as a substrate peptide of ACE were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other reagents used in this study were analytical grade chemicals.

2.2. Enzymatic hydrolysis

Pistachios were shelled to obtain the kernel, and then were milled into powder in a food processor (JYL-B031, Joyoung, Jinan, China) to obtain pistachio kernel powder (PKP). PKP was mixed with distilled water (10%, w/v) and digested with pepsin (5000 U/g powder, pH 1.8) at 37 °C for 4 h. The hydrolysis reaction was terminated by heating in a boiling water bath for 10 min, and then the pH was adjusted to 7.8. The mixture was further digested with trypsin (5000 U/g powder) at the same condition for another 6 h. After heating in a boiling water bath for 10 min, the hydrolyzed solution was then centrifuged at 9000*g* at 4 °C for 25 min. The supernatant was collected and lyophilized as the pepsin–trypsin hydrolysate (Pe–Tr–H). The pepsin hydrolysate (Pe–H) and trypsin hydrolysate (Tr–H) were obtained at the same condition.

2.3. ACE inhibition measurement

The ACE inhibitory activity was measured according to the method of Cushman and Cheung [28] with slight modifications. Ten milligram sample was dissolved in 1 ml distilled water and then diluted to seven different concentrations for ACE inhibitory measurements. Fifteen microliter of sample solution in certain concentration added with 15 μl substrate HHL (8.3 mM Hip–His–Leu in 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3) was pre-incubated at 37 °C for 5 min, and the reaction was initiated by adding 5 μl of ACE solution (310 mU/ml) and incubated for 60 min at the same temperature. The reaction was terminated by the addition of 1.0 M HCl (200 μl). Ten microliters of the reaction solution were injected directly onto a Thermo BDS-C18 column (3.0 mm × 250 mm, 5 μm, Thermo Scientific Co. Ltd., USA). The mobile phase was 10% acetonitrile and 90% water with 0.1% trifluoroacetic acid (TFA). The flow rate was 0.7 ml/min and monitored at 228 nm to evaluate the ACE inhibition activity of hydrolysates. All determination was carried out at least in triplicate. The inhibition activity was calculated using the following equation:

$$\text{ACE inhibition}\,(\%) = \left[1\left(A_{\text{inhibitor}}/A_{\text{control}}\right)\right] \times 100$$

where $A_{\text{inhibitor}}$ and A_{control} are, respectively, the relative areas of the hippuric acid (HA) peak of the assay with inhibitor and of the control sample without inhibitor.

The IC_{50} value was defined as the concentration of inhibitor that could inhibit 50% of the ACE activity.

2.4. SHRs and measurement of blood pressure

In vivo testing was conducted by the methods of Matsui [3] with some modifications. The Spontaneously hypertensive rats (SHRs, 10-week-old, male, specific pathogen-free, 250–320 g body weight) with tail systolic blood pressure over 180 mmHg were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). SHRs were then housed individually in steel cages (6 SHRs per group) in a room kept at 22 ± 2 °C with a 12 h light–dark cycle, and fed a standard laboratory diet. Tap water was freely available. The SHRs experiment was conducted by Shanghai Second Military Medical University (Shanghai, China). The study was approved by the Shanghai Second Military Medical University Animal Care and Use Ethics Committee, and the animals were cared for in accordance with the institutional ethical guidelines.

PKP and gastrointestinal enzymatic hydrolysate powders were mixed in saline and were orally injected into SHR at a dose of 1 g per kg body weight, Control rats were administrated with the same volume of saline solution. After oral administration, SHRs were warming up in a chamber maintained at 37 °C for 10 min. The systolic blood pressure (SBP) and the diastolic blood pressure (DBP) were measured by tail-cuff method with a Softron BP system (Softron BP-98A, Tokyo, Japan) and all the results were measured three times.

2.5. Purification and identification of ACE inhibitory peptide

The Pe–Tr–H powder was first separated by UF membrane with 3 kDa molecular weight cut-off (Millipore Co., Beverly, USA). Two fractions with molecular weights of <3 kDa, and >3 kDa were collected to assay the ACE inhibition *in vitro* and then lyophilized for further use. The lyophilized powder was dissolved in distilled water and was applied to a Sephadex G-15 followed by G-10 column (1.0 cm × 80 cm; Pharmacia, Sweden), eluted with ultrapure water at a flow rate of 48 ml/h and were monitored at 215 nm by way of fast protein liquid chromatography (FPLC) (AKTA P-900; GE, USA). Fractions were collected automatically and the highest active fraction obtained was pooled and further purified using RP-HPLC on an Ultimate 3000 C18 semi-prep column (10 mm × 150 mm; DIONEX, USA). The mobile phase was 0.1% TFA in water (v/v) as eluent A and acetonitrile containing 0.1% TFA as eluent B. A linear gradient was conducted by the eluent B (5–50% in 60 min) at a flow rate of 1.5 ml/min and monitored at 215 nm. Fractions were collected based on the profile of the eluted peptides, and then lyophilized.

Molecular weight of purified peptide from pistachio kernel's hydrolysates was determined by electrospray ion trap mass spectrometry (LCQ Deca Model 5890, HP, USA), and the amino acid sequence was determined in positive ion mode by de novo sequencing method of MALDI–TOF/TOF system. The sample was dissolved in the water and acetonitrile (1:1), and Cyano-4-hydroxycinnamic acid (CHCA) was used as substrate. MALDI–TOF MS spectra were acquired with an AB Sciex 4800plus, MALDI–TOF/TOF MS equipped with a Nd:YAG laser (emitting at 355 nm, operated at 200 Hz). The spectra were recorded in the reflectron positive ion mode and externally calibrated with "TOF/TOF calibration" standard solution.