Peptides xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Peptides

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

A novel angiotensin-I converting enzyme (ACE) inhibitory peptide from gastrointestinal protease hydrolysate of silkworm pupa (*Bombyx mori*) protein: Biochemical characterization and molecular docking study

Qiongying Wu[a,b,*,1], Junqiang Jia[a,b,**,1], Hui Yan[a,b], Jinjuan Du[a], Zhongzheng Gui[a,b]

[a] School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, Jiangsu Province 212018, PR China
[b] Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, Jiangsu Province 212018, PR China

ARTICLE INFO

Article history:
Received 30 June 2014
Received in revised form 30 July 2014
Accepted 30 July 2014
Available online xxx

Keywords:
Silkworm pupa (*Bombyx mori*)
ACE inhibitory peptide
Gastrointestinal protease
Inhibition pattern
Molecular docking

ABSTRACT

Silkworm pupa (*Bombyx mori*) protein was hydrolyzed using gastrointestinal endopeptidases (pepsin, trypsin and α-chymotrypsin). Then, the hydrolysate was purified sequentially by ultrafiltration, gel filtration chromatography and RP-HPLC. A novel ACE inhibitory peptide, Ala-Ser-Leu, with the IC_{50} value of 102.15 μM, was identified by IT-MS/MS. This is the first report of Ala-Ser-Leu from natural protein. Lineweaver–Burk plots suggest that the peptide is a competitive inhibitor against ACE. The molecular docking studies revealed that the ACE inhibition of Ala-Ser-Leu is mainly attributed to forming very strong hydrogen bonds with the S1 pocket (Ala354) and the S2 pocket (Gln281 and His353). The results indicate that silkworm pupa (*B. mori*) protein or its gastrointestinal protease hydrolysate could be used as a functional ingredient in auxiliary therapeutic foods against hypertension.

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Introduction

Angiotensin-I converting enzyme (ACE; dipeptidyl carboxypeptidase, EC3.4.15.1) plays an important role in regulating blood pressure. This enzyme can hydrolyse angiotensin I to the potent vasoconstrictor angiotensin II and inactivate the potent vasodilator bradykinin [23]. Inhibition of ACE activity is thus considered to be a pivotal therapeutic approach for treating hypertension.

Many synthetic ACE inhibitors including captopril, enalapril and lasinopril and others have been used to prevent hypertension in clinical [8]. However, these synthetic ACE inhibitors have been suspected to threaten health by causing cough, taste disturbances and renal impairment [3]. There, thus, is a growing interest in finding ACE inhibitors from natural products. Numerous studies are focused on the production and isolation of ACE-inhibitory peptides from different food proteins, such as grass carp protein [7], Haruan (*Channa striatus*) myofibrillar protein [8], whey protein [18], porcine skeletal muscle protein [4], salmon protein [1] and soybean protein [9]. These peptides usually were found to be di- to tridecapeptide, and their activities are dependent on their amino acid composition, structure and hydrophobicity [16,24].

Silkworm pupa (*Bombyx mori*), an edible insect, is traditionally used as food material and traditional medicine in some countries, such as China, Japan, Korea, India and Thailand [28]. It is known for its nutritional value due to the presence of high protein content (around 48–60% crude protein) and high fat content (about 30% of dry pupa weight) [26]. Several protease hydrolysates with ACE-inhibitory activities, such as neutrase hydrolysate, acidic protease hydrolysate, flavourzyme hydrolysate and trypsin hydrolysate, have been reported for silkworm pupa protein [25]. Although the production of ACE-inhibitory peptides by various proteases has been extensively studied, peptides produced through gastrointestinal protease are rarely reported. In addition, the molecular docking studies of the peptides at the ACE active site have not yet been reported.

Abbreviations: ACE, angiotensin-I converting enzyme; HHL, hippuryl-histidine-leucine; SPP, silkworm pupa protein; SPPH, silkworm pupa protein hydrolysate; MW, molecular weight; RP-HPLC, reversed-phased high performance liquid chromatography; IT-MS, ion trap-mass spectrometry; IT-MS/MS, ion trap-mass spectrometry/mass spectrometry; ESI, electro-spray ionization.

* Corresponding author at: School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, Jiangsu Province 212018, PR China.
Tel.: +86 511 85616716; fax: +86 511 85616716.
** Corresponding author at: School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, Jiangsu Province 212018, PR China.
Tel.: +86 511 85639697; fax: +86 511 85635850.
E-mail addresses: wuqy1@163.com (Q. Wu), jiajq@just.edu.cn (J. Jia).
[1] These authors contributed equally to this study.

http://dx.doi.org/10.1016/j.peptides.2014.07.026
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Please cite this article in press as: Wu Q, et al. A novel angiotensin-I converting enzyme (ACE) inhibitory peptide from gastrointestinal protease hydrolysate of silkworm pupa (*Bombyx mori*) protein: Biochemical characterization and molecular docking study. Peptides (2014), http://dx.doi.org/10.1016/j.peptides.2014.07.026

In the present study, gastrointestinal proteases were used to hydrolyze silkworm pupa (*B. mori*) protein. The ultrafiltration, gel filtration chromatography and reversed-phased high performance liquid chromatography (RP-HPLC) were used to purify the ACE inhibitory peptide. The sequence of purified peptide was identified using Ion Trap-Mass Spectrometry/Mass Spectrometry (IT-MS/MS) and its inhibition pattern on ACE was investigated with Lineweaver–Burk plots. Furthermore, binding interaction of purified peptide within the active site of ACE was also determined by using Discovery Studio 2.1 software.

Materials and methods

Materials and chemicals

Silkworm pupa (*B. mori*) powder was provided by Sericultural Research Institute, Chinese Academy of Agricultural Sciences (Zhenjiang, China). ACE (from rabbit lung) and Hippuryl-His-Leu (HHL) were purchased from Sigma–Aldrich Trading Co. (Shanghai, China). Pepsin, trypsin and α-chymotrypsin were purchased from Sangon Biotechnology Co. (Shanghai, China). Sephadex G-10 and G-25 were purchased from Auyoo Biotechnology Co. (Shanghai, China). All other chemicals and solvents were of analytical grade.

Production of silkworm pupa protein (SPP)

Silkworm pupa powder was defatted with petroleum ether and dried at 50 °C using a drying oven. The defatted flour was dispersed in deionized water at ratio 1:20 (w/v). The suspension pH was adjusted to 9.5 by using 1 M NaOH. After stirring for 1 h, the suspension was centrifuged at 3000 × *g* for 15 min. The supernatant was adjusted to pH 4.5 using 1 M HCl to precipitate the proteins, centrifuged again at 3000 × *g* for 15 min. The precipitates were lyophilized using a freeze-dryer (EYELA FDU-1100, Shanghai Ailang Instruments Co., Shanghai, China) and stored in polyethylene bag at −20 °C until used.

Preparation of silkworm pupa protein hydrolysate (SPPH)

The hydrolysis of SPPH was designed to simulate the human gastrointestinal digestion process. The hydrolysis process was carried out according to the method of Himaya et al. [10] with some modifications. Silkworm pupae protein was dissolved in distilled water at a concentration of 20 mg/ml and hydrolyzed for 1.5 h using pepsin (enzyme–substrate ratio, 1000 U/mg) at pH 2.0 and 37 °C with stirring. Then the reaction solution was further digested by trypsin and α-chymotrypsin (enzyme–substrate ratio of 1000 U/mg for each enzyme) for 2.5 h at pH 6.5 and 37 °C with stirring. The reaction was stopped by heating the mixture in a boiling water bath for 15 min. Then, the solution were centrifuged at 5000 × *g* for 10 min and the supernatant was lyophilized and stored at −20 °C until used.

Assay of ACE-inhibitory activity

The ACE inhibitory activity was measured according to our previously described method [27]. The 10 μl of sample solution (sample in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) with 45 μl Hippuryl-His-Leu (HHL) solution (6.5 mM HHL in 0.1 M borate buffer containing 0.3 mol/L NaCl, pH 8.3) was pre-incubated at 37 °C for 5 min, and then incubated with 10 μl ACE (ACE in 0.1 M borate buffers containing the 0.3 M NaCl, pH 8.3) at 37 °C for 30 min. The reaction was stopped by adding 85 μl of 1 M HCl to the samples except for the blank (85 μl of 1 M HCl were added before the pre-incubation). The hippuric acid formed was extracted with 1000 μl of ethyl acetate. Then 800 μl of the ethyl acetate layer was collected and evaporated in a dry-oven at 100 °C. The residue was dissolved in 800 μl distilled water and its absorbance was measured at 228 nm. The inhibition activity was calculated using the following equation:

$$\text{ACE-inhibition activity}\,(\%) = \frac{C - S}{C - B} \times 100 \quad (1)$$

where *C* is the absorbance without sample (buffer for samples), and *S* is the absorbance in the presence of both ACE and sample. *B* is the absorbance of blank (hydrochloric acid was added before ACE). The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

Purification of ACE-inhibitory peptide

Ultrafiltration

For purification of ACE-inhibitory peptide, 3 L of SPPH solution (0.6%, w/v) was filtered sequentially using an ultrafiltration unit (Pellicon XL, Millipore, USA) through two ultrafiltration membranes with molecular weight (MW) cut-off of 10 and 5 kDa, respectively. Three fractions with MWs <5 kDa, 5–10 kDa and >10 kDa were obtained. The fraction with the highest ACE-inhibitory activity was lyophilized and stored at −20 °C until use.

Gel filtration chromatography

The fraction with the highest ACE-inhibitory activity after ultrafiltration separation was re-dissolved in distilled water, then further purified using a Sephadex G-25 column (2.5 cm × 70 cm) which was eluted with distilled water at a flow rate of 1.0 ml/min. The fractions were collected at 3 min intervals with a fraction collector, and measured at 220 nm. The fraction exhibiting the greatest ACE inhibitory activity was pooled and further separated using Sephadex G-10 column (1.5 cm × 50 cm) which was eluted with distilled water at a flow rate of 0.5 ml/min. The fractions were collected and monitored under the same conditions as that used for the Sephadex G-25 column. Each fraction was assayed for ACE inhibitory activity.

Reversed-phased high performance liquid chromatography (RP-HPLC)

The faction exhibiting the highest ACE inhibitory activity after gel filtration chromatography was further purified by RP-HPLC (L-7100, Hitachi High-Technologies Co., Japan) on ODS C_{18} column (4.6 mm × 250 mm, 5 μm, Shimadzu, Tokyo, Japan). The column was eluted by a linear gradient of acetonitrile (0–50%) containing 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. Absorbance of the eluent was monitored at 220 nm. Fractions were collected according to the elution peaks and lyophilized immediately. The lyophilized fraction with the highest ACE-inhibitory activity was dissolved at 7 mg/ml and purified for the second step RP-HPLC using the same column as that used for the first step RP-HPLC. The elution was conducted at a flow rate of 0.5 ml/min using a linear gradient of acetonitrile (5–50%) containing 0.1% trifluoroacetic acid. The eluted peaks were detected at 220 nm, and they were collected and lyophilized immediately. The fraction exhibiting the highest ACE inhibitory activity was followed by identification of the amino acid sequence.

Amino acid sequence of the purified peptide

Amino acid sequence of the purified peptide from silkworm pupa protein hydrolysate (SPPH) was determined using an Ion Trap-Mass Spectrometry (IT-MS; Thermo LXQ, Massachusetts, USA) with an electro-spray ionization (ESI) source. Sample dissolved in methanol/water (1:1, v/v) was infused into the ESI source. The capillary temperature was set at 400 °C. Spray voltage was 4000 V. Nitrogen was maintained at 40 psi for nebulization and 9 L/min. Mass spectra were acquired over the range from 50 *m/z* to

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