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Rapid purification and characterization of angiotensin converting enzyme inhibitory peptides from lizard fish protein hydrolysates with magnetic affinity separation

Xiongdiao Lan [a], Dankui Liao [a,*], Shanguang Wu [a,b], Feng Wang [c], Jianhua Sun [a], Zhangfa Tong [a]

[a] School of Chemistry and Chemical Engineering, Guangxi University, Nanning 530004, China
[b] School of Medicine, Guangxi University of Science and Technology, Liuzhou 545006, China
[c] State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, Beijing 100029, China

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ABSTRACT

In this study, angiotensin converting enzyme (ACE) inhibitory peptides from lizard fish protein hydrolysate with neutral protease were purified through magnetic affinity separation. Magnetic agarose microsphere was prepared by reverse-phase microemulsion method, and its surface was modified with epoxy groups to immobilize ACE as a magnetic affinity medium (MAM-ACE) and then mixed with lizard fish ultrafiltration hydrolysate (<5 kDa). The MAM-ACE was recovered by a magnet. The bound peptides were released by 1 M NaCl and further purified by reverse-phase high-performance liquid chromatography. The amino acid sequence of the peptide with the highest ACE inhibitory activity was identified as Gly-Met-Lys-Cys-Ala-Phe, and its IC_{50} was 45.7 ± 1.1 μM. The result indicates that MAM-ACE is a faster and more efficient method for purifying micro-bioactive peptides from food protein complex mixtures compared with ion exchange and gel chromatography.

1. Introduction

With the rapid development of the economy and lifestyle changes, hypertension has become one of the major chronic health problems today. A previous study showed that angiotensin converting enzyme (ACE) plays an important role in the adjustment of blood pressure (Tikellis, Cooper, & Thomas, 2006). The study showed that ACE can not only passivate the vasodilator-bradykinin but also promote the conversion of angiotensin I to angiotensin II, which causes high blood pressure (Ng & Vane, 1967; Tschope, Schultheiss, & Walther, 2002). ACE inhibitors (ACEI) are a class of drugs frequently used in the treatment of hypertension. Chemically synthesized ACEI, including captopril, enalapril, fosinopril, and ramipril, are some of the drugs for lowering blood pressure currently available in the market. However, these drugs have been reported to have certain side effects, including cough, skin rashes, proteinuria, and hypotension (Thurman & Schrier, 2003; Vyssoulis, Karpanou, & Papavassiliou, 2001). Nevertheless, ACEI derived from food have displayed some potential for reducing blood pressure without obvious side effects. Thus, the food-derived ACEI could take the place of some of the drugs and provide adjuvant treatment of blood pressure in the form of functional foods (Tanzadehpanah, Asoodeh, Saberi, & Chamani, 2013).

Since Oshima, shimabukulo, and Nagasawa (1979) identified ACEI from gelatin protein hydrolysates, more than 300 types of ACE inhibitory peptides have been identified from hydrolysates and fermentations of milk protein (Miguel, Manso, Lopez-Fandino, Alonso, & Salaices, 2007; Vermeirssen, Van Camp, & Verstraete, 2005), ovalbumin (Miguel, Aleixandre, Ramos, & Lopez-Fandino, 2006; Yoshii et al., 2001), gluten (Jimsheena & Gowda, 2011; Kuba, Tana, Tawata, & Yasuda, 2005), meat (Jang & Lee, 2005; Katayama et al., 2007), and fish (Wu et al., 2008). However, purifying and characterizing target ACE inhibitory peptides can be difficult because hydrolysate and fermentation contains only trace amounts of ACE inhibitory peptides. Classical purification methods, including dialysis, membrane separation, gel chromatography, ion exchange chromatography, and reverse-phase high-performance liquid chromatography (RP-HPLC), have been combined to purify ACE inhibitory peptides (Lee, Hong, Jeon, Kim, & Byun, 2009). However, these methods have limited application because separation is based on the difference in the molecular polar interaction or weight. Thus, the samples purified with high-performance liquid chromatography (HPLC) still contain numerous non-bioactive ingredients, and the purification efficiency is normally rather low.

* Corresponding author. Tel.: +86 771 327 2702; fax: +86 771 323 3718.
E-mail address: liaodk@gxu.edu.cn (D. Liao).

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Affinity separation is a powerful protein purification method that relies on the formation of specific reversible complexes between an immobilized molecule and the ligands to be purified. Megias et al. (2006) reported the purification and partial characterization of several peptide fractions through affinity chromatography by using immobilized ACE, with the affinity medium recovered by filtration. The process took several minutes to recover the magnetic affinity medium from the mixture solution by using a magnet. Thus, recovering magnetic affinity medium was much quicker and more convenient than recovering the affinity medium through filtration.

We have reported the purification and characterization of an ACE inhibitory peptide from lizard fish protein hydrolysate with neutral protease by combining ultrafiltration, Sephadex G-15 gel chromatography, ion exchange chromatography, RP-HPLC, and MALDI-TOF–TOF (Wu et al., 2012). The results of that study showed that a variety of ACE inhibitory peptides have not yet been purified and identified.

No information regarding the purification and characterization of ACE inhibitory peptides from lizard fish protein hydrolysate through affinity chromatography has been reported. Magnetic affinity chromatography has the advantage of quicker completion of the affinity chromatography and magnetic separation processes. The aim of the present study is to prepare magnetic agarose microspheres immobilized ACE (MAM-ACE) crude extracted from pig lung and to isolate and identify an ACE inhibitory peptide from lizard fish protein hydrolysates with neutral protease by using magnetic mediums immobilized ACE, RP-HPLC, and matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry. The purified peptide was also synthesized, and its ACE inhibitory activity in vitro was confirmed to verify the potential use of the peptide.

2. Material and methods

2.1. Materials

Hippuryl-L-histidyl-L-leucine (HHL), rabbit lung ACE was purchased from Sigma. Lizard fish was purchased from a local market in Nanning, China. Pig lung was obtained from a local market. The ACE extract was prepared as described: pig lung (100 g) was homogenized in 500 mL of 0.1 mol/L borate buffer (BBS) containing 0.25 mol/L sucrose at pH 8.3, and the homogenate was centrifuged at 6000 rpm for 20 min; solid ammonium sulfate was added to provide fractional precipitation from 1.6 mol/L to 2.6 mol/L. The secondary precipitates were collected for ACE immobilization. The other chemicals and reagents used were of analytical grade.

2.2. Preparation of magnetic agarose microspheres and immobilized ACE

Magnetic agarose microspheres were prepared using the water/oil (W/O) microemulsion technique (Gustavsson & Larsson, 1996). The oil phase was prepared using liquid paraffin (25 mL) and Span 80 (1.0 g). Agarose (0.125 g) was dissolved in 4 mL of water and boiled to dissolve completely and then mixed with 1 mL of Fe_3O_4 magnetic fluid. The solution was added drop wise into the oil phase under constant stirring (800 rpm) at 80 °C for 15 min. The microspheres were washed with petroleum ether and distilled water.

Approximately 0.7 mL of epichlorohydrin was added to 1 mL of NaOH solution (0.9 M, containing 2 mg/mL $NaBH_4$) with 0.2 g of magnetic microspheres and shaken for 4 h at 40 °C. The magnetic functionalized microspheres were separated by magnet and washed with distilled water to remove the residues.

Subsequently, 2 mL of 8 mg/mL ACE extract solution (0.1 M BBS, pH 8.3, containing 0.3 M NaCl) were mixed with the functionalized magnetic microspheres and gently stirred for 2 h at 50 °C. The obtained magnetic affinity medium immobilized ACE were washed several times with water and stored at −22 °C in the BBS.

2.3. Preparation of lizard fish muscle protein hydrolysate

Lizard fish muscle protein was dissolved in ultrapure water with a ratio of 2:100 (w/v) and placed in boiling water for 10 min to ensure deactivation of endogenous enzymes. The proteins were hydrolyzed with neutral protease at an enzyme/protein substrate ratio of 10,000 u/g at 48 °C and pH 7.0. During the reaction, the hydrolysis pH was maintained at 7.0 through the continuous addition of 0.1 M NaOH. The hydrolysate was subsequently heated at 95 °C for 10 min to terminate the reaction for 2 h under continuous stirring. Reactions were terminated by heating the solution in a boiling water bath for 15 min to ensure enzyme deactivation. After the hydrolysate was centrifuged at 8000 g for 20 min (4 °C), the supernatant was collected and passed through a molecular weight (WM) membrane (5000 Da) by using Labscale TFF System (Millipore Co., Billerica, MA, USA). The portion with MW less than 5000 Da was lyophilized and stored at −80 °C for further use.

2.4. Purification of ACE inhibitory peptides

The hydrolysates were dissolved in 0.1 M BBS (pH 8.3, containing 0.3 M NaCl), and 0.05 g of magnetic affinity medium was mixed with different concentrations of hydrolysates at a ratio of 1:10 (w/v) and stirred at 30 °C at different times. After incubation with the hydrolysates, the magnetically immobilized extract was recovered through a magnet and washed several times with the same buffer until absorbance of the rinsed buffer at 280 nm reached a baseline. The adsorption capacities were calculated indirectly. The adsorbed peptides were released through incubation with 1 M NaCl and separated from the immobilized extract by magnet as above.

The eluent was concentrated and freeze-dried and then applied to a preparative HPLC reverse-phase column (Zorbax SB-C18, 4.6 mm × 250 mm, Agilent, Santa Clara, CA, USA) to separate the peptides into different fractions. A linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid (0–30% in 35 min) at a flow rate of 1 mL/min at 30 °C was used. Elution was monitored at 280 nm. The fractions collected from HPLC were lyophilized for further assay of ACE inhibitory activity. The lyophilized fraction with the highest ACE-inhibitory activity was purified for the second step RP-HPLC. The linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid (0–50% in 35 min) at a flow rate of 1 mL/min at 30 °C was used, and elution was monitored at 280 nm. These purification procedures were repeated until sufficient samples were collected to perform an assay of the ACE inhibitory activity and sequence identification.

2.5. Assay of ACE activity

ACE activity is usually evaluated by determining the amount of hippuric acid (HA) generated. In this work, ACE activity was measured using the method as described previously (Wu et al., 2012) with slight modifications. First, ACE and substrate HHL were dissolved in 0.1 mol/L BBS (pH 8.3, containing 0.3 mol/L NaCl), and then 130 μL of BBS and 30 μL of ACE solution were mixed and pre-incubated at 37 °C for 10 min. Next, 40 μL of HHL were added to start the reaction. The reaction was terminated by adding 150 μL of 1 M HCl after 30 min of incubation at 37 °C. The HA content of the mixture was determined through RP-HPLC (Agilent 1260) with Zorbax SB C18 column (4.6 mm × 150 mm, 5 μm, Agilent, Santa