Bioresource Technology 100 (2009) 5255–5259

Contents lists available at ScienceDirect

Bioresource Technology

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

Purification and characterization of angiotensin I converting enzyme inhibitory peptides from the rotifer, *Brachionus rotundiformis*

Jung Kwon Lee [a], Suhee Hong [a], Joong-Kyun Jeon [a], Se-Kwon Kim [b], Hee-Guk Byun [a,*]

[a] Faculty of Marine Bioscience and Technology, Kangnung-Wonju National University, 120 Gangneung Daehakro, Gangneung 210-720, Republic of Korea
[b] Department of Chemistry, Pukyong National University, Busan 608-737, Republic of Korea

ARTICLE INFO

Article history:
Received 23 February 2009
Received in revised form 22 May 2009
Accepted 22 May 2009
Available online 18 June 2009

Keywords:
Angiotensin I converting enzyme
Marine rotifer
Peptide
Alcalase
Hydrolysates

ABSTRACT

Angiotensin I converting enzyme (ACE) inhibitory peptide was isolated from the marine rotifer, *Brachionus rotundiformis*. ACE inhibitory peptides were separated from rotifer hydrolysate prepared by Alcalase, α-chymotrypsin, Neutrase, papain, and trypsin. The Alcalase hydrolysate had the highest ACE inhibitory activity compared to the other hydrolysates. The IC_{50} value of Alcalase hydrolysate for ACE inhibitory activity was 0.63 mg/ml. We attempted to isolate ACE inhibitory peptides from Alcalase prepared rotifer hydrolysate using gel filtration on a Sephadex G-25 column and high performance liquid chromatography on an ODS column. The IC_{50} value of purified ACE inhibitory peptide was 9.64 μM, and Lineweaver–Burk plots suggest that the peptide purified from rotifer protein acts as a competitive inhibitor against ACE. Amino acid sequence of the peptide was identified as Asp-Asp-Thr-Gly-His-Asp-Phe-Glu-Asp-Thr-Gly-Glu-Ala-Met, with a molecular weight 1538 Da. The results of this study suggest that peptides derived from rotifers may be beneficial as anti-hypertension compounds in functional foods resource.

1. Introduction

Hypertension is a major health issue, estimated to be affecting about 20% of the world's adult population (Alper et al., 2001). Among processes related to hypertension, angiotensin I converting enzyme (ACE) plays an important role in the regulation of blood pressure. Angiotensin I converting enzyme (ACE) is a dipeptidyl carboxypeptidase (EC. 3.4.15.1.) that not only converts the decapeptide (angiotensin I) to the potent vasoconstring octapeptide (angiotensin II), but also inactivates the antihypertensive vasodilator bradykinin, a process that increases blood pressure (Skeggs et al., 1957). Inhibition of ACE activity leads to a decrease in the concentration of angiotensin II and consequently reduces blood pressure (Skeggs et al., 1957). Since the discovery of ACE inhibitors in snake venom, effort has been focused on synthesizing ACE inhibitors such as captopril, enalapril, alacepril and lisinopril, which have been used extensively in the treatment of hypertension and heart failure in humans (Ondetti et al., 1977). However, the aforementioned ACE inhibitors are believed to have a number of side-effects, including the inducement of coughing, taste disturbances and skin rashes (Atkinson and Robertson, 1979). As a result, recent investigations have focused on ACE inhibitors derived from other organisms.

Bioactive peptides can be obtained from organisms proteins by enzymatic hydrolysis of proteins (Je et al., 2005). Functional peptides can be induced from enzymatic hydrolysis of various proteins and may act as potential physiological modulators of metabolism during intestinal digestion of nutrients. Bioactive peptides are liberated depending on their structural, compositional and amino acid sequence. These peptides exhibited various bioactivities such as antioxidative (Rajapakse et al., 2005), antihypertensive (Byun and Kim, 2001) and antimicrobial (Kim et al., 2001a,b). ACE inhibitory peptides have been isolated from enzymatic hydrolysates of various fish waste, such as Alaska pollack skin (Byun and Kim, 2001), sea bream scales (Fahmi et al., 2004), yellowfin sole frame protein (Jung et al., 2006), oyster proteins (Wang et al., 2008) and shark meat (Wu et al., 2008).

Rotifers are the most commonly used marine zooplankton as live feed for fish larvae cultures (Rønnestad et al., 2003). Rotifers are small size, rich nutrients, and an ideal feed source for large quantity fish cultivation (Helland et al., 2003). Generally, larval fish was demand high dietary protein. Hence, their feed required high content protein and rapidly growth rate. Rotifer has a lot of amino acid pool for product high content protein (Rønnestad et al., 2003). To date, bioactive materials have not been reported from marine zooplankton, such as rotifers. In addition, ACE inhibitory effects of marine zooplankton are yet to be reported.

The purpose of this study was purification and characterization of an ACE inhibitory peptides derived from enzymatic hydrolysates of rotifer protein.

* Corresponding author. Tel.: +82 33 640 2854; fax: +82 33 640 2340.
E-mail address: hgbyun@nukw.ac.kr (H.-G. Byun).

0960-8524/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2009.05.057

2. Methods

2.1. Materials

S-type rotifer, *Brachionus rotundiformis,* was purchased from Aquanet Co. Ltd. (Tong-young, Korea), and lyophilized at −50 °C using a freeze dryer. Lyophilized rotifer powder was stored at −80 °C until use. Alcalase and Neutrase were purchased from Novozyme Co. (Bagsvaerd, Denmark), α-chymotrypsin, trypsin, papain, ACE (lung acetone powder from rabbit), substrate (Hip-His-Leu) and Sephadex G-25 were purchased from the Sigma Chemical Co. (St. Louis, MO). Pepsin was purchased from Junsei (Japan). All other reagents were of the highest grade commercially available.

2.2. Proximate composition and amino acid composition assay

Proximate compositions of *B. rotundiformis* were assayed as described by AOAC Official Methods (AOAC, 2000). Moisture was determined by oven-drying method at 105 ± 1 °C. Crude lipid was measured in a Soxhlet system by extraction with diethyl ether solvent. Total nitrogen content was analyzed by the Kjeldahl procedure (Kjeltec1035, Foss, Sweden). Crude protein content was calculated using a conversion factor of 6.25. Ash content was determined by incineration of samples at 550 °C in a muffle furnace (F6000, Barnstead Thermolyne Co., USA). For total amino acids analysis, rotifer was hydrolyzed in 6 N HCl for 24 h at 110 °C. Amino acids were analyzed by using the Agilent 1100 HPLC system (Santa Clara, California, USA) after pre-derivatization with OPA and β-mercaptoethanol. Separations were performed with a C18 column (5 μm, 4.6 × 250 mm, Waters, Massachusetts, USA). The amino acid concentrations of samples were calculated from calibration curves made with amino acid standard solutions (Sigma–Aldrich Co., St. Louis, USA).

2.3. Preparation of enzymatic hydrolysates

For the production of ACE inhibitory activity peptide from *B. rotundiformis*, enzymatic hydrolysis was performed using various enzymes (Alcalase, α-chymotrypsin, Neutrase, papain, pepsin, and trypsin) at their optimal conditions. At enzyme/substrate ratio of 1/10 (w/w), substrate and enzyme were mixed in a 100 ml flask with buffer, temperature and pH control devices (Table 1). The mixture was incubated for 12 h at each optimal temperature with stirring, and then heated in a boiling water bath for 10 min to inactivate the enzyme. Hydrolysis yields were measured as weight of the rotifer hydrolysates.

2.4. Measurement of ACE inhibitory activity

The ACE inhibitory activity assay was performed according to the methods of Cushman and Cheung (1971) with slight modification. A hydrolysate solution (50 μl) with 50 μl of ACE solution (25 munits/ml) was pre-incubated at 37 °C for 5 min, and the mixture was subsequently incubated with 150 μl of substrate (8.3 mM Hip-His-Leu in 50 mM sodium borate buffer) for 60 min at the same temperature. The reaction was terminated with addition of 250 μl 1 M HCl. The resulting hippuric acid was extracted with 0.5 ml of ethylacetate. After centrifugation (3000 rpm, 15 min), 0.2 ml of the upper layer was transferred into a test tube, and dried at 80 °C for 1 h. The hippuric acid was dissolved in 0.5 ml of distilled water and absorbance was measured at 228 nm using an UV-spectrophotometer (Jasco, Japan). The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of ACE inhibitory activity.

Table 1
Optimum conditions of enzymatic hydrolysis for various enzymes.

Enzyme	Buffer	pH	Temp. (°C)
Alcalase	50 mM sodium phosphate	7.0	50
α-Chymotrypsin	50 mM sodium phosphate	8.0	37
Neutrase	50 mM sodium phosphate	8.0	50
Papain	50 mM sodium phosphate	6.0	37
Pepsin	20 mM glycine–HCl	2.0	37
Trypsin	50 mM sodium phosphate	8.0	37

2.5. Purification of ACE inhibitory peptide

Rotifer hydrolysate showing ACE inhibitory activity was dissolved in distilled water and loaded onto a Sephadex G-25 gel filtration column (2.5 × 70 cm), equilibrated with distilled water. The column was eluted with distilled water at a flow rate of 1.5 ml/min, and fractions showing ACE inhibitory activity were pooled and lyophilized. The fraction with the highest ACE inhibitory activity was dissolved in distilled water, peptides were separated by reversed-phase HPLC on a Grom-sil 120 ODS-5 ST column (5 μm, 10.0 × 250 mm) using a linear gradient of acetonitrile (0–50% v/v, 50 min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 ml/min. Elution peaks were monitored at 215 nm. Finally, the amino acid sequence of purified peptide from Alcalase prepared hydrolysate was analyzed.

2.6. Determination of molecular weight and amino acid sequence

Accurate molecular weights of the Alcalase hydrolysate were determined with a Q-TOF mass spectrometer (Micromass, Manchester, UK) coupled with electrospray ionization (ESI) source. The peptide solution was desalted using Capcell Pak C_{18} UG120 V (5 μm, 1.5 × 250 mm, Shiseido, Tokyo, Japan). Approximately 50 μl of the sample was placed into a metal-coated glass capillary (Protana Co., Odense, Denmark). Applied voltage to produce electrospray was 2950 eV, and cone voltage was 30 eV. MS/MS spectra were acquired in data dependant MS/MS mode of collision cell, where voltage was rapidly switched between low (10 V) and high (30 V) to obtain spectra of intact and fragmented peptides, respectively. The instrument was calibrated externally, and no post-acquisition recalibration of MS/MS spectra was performed. Sequencing of ACE inhibitory activity peptide was obtained over the m/z range 50–2000 and sequenced using the PepSeq de nove sequencing program (Micromass Co., Manchester, UK).

2.7. Determination of ACE inhibition pattern

Different concentrations of ACE inhibitory peptide were added to each reaction mixture according to the method of Kim et al. (2001a,b). Enzyme activity was measured with different concentrations of substrate. ACE inhibitory pattern in presence of the inhibitor was determined with Lineweaver–Burk plots.

2.8. Statistical analysis

Data were expressed as mean ± standard deviation of three determinations.

3. Results and discussion

3.1. Rotifer composition

The proximate analysis of the rotifer is shown in Table 2. Crude protein content was high at 63.53%, while lipid, carbohydrates, and ash were 17.17%, 6.54% and 4.72%, respectively. Crude protein