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Short communication

Antihypertensive effect of an angiotensin I-converting enzyme inhibitory peptide from bullfrog (*Rana catesbeiana* Shaw) muscle protein in spontaneously hypertensive rats

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Abstract

To investigate biomedical and nutraceutical benefits of bullfrog (*Rana catesbeiana* Shaw) muscle protein, we examined an angiotensin I-converting enzyme (ACE I) inhibitory activity of various enzymatic hydrolystes of *R. catesbeiana* muscle protein in the present study. Among the enzymatic hydrolysates prepared using various commercial enzymes such as Alcalase, neutrase, pepsin, papain, α-chymotrypsin, and trypsin, Alcalase-proteolytic hydrolysates showed the highest ACE I inhibitory activity. During consecutive purification using a Hiprep 16/10 DEAE FF anion exchange and an octadecylsilane (ODS) C18 reversed phase liquid chromatographic techniques, a potent ACE I inhibitory peptide composed of 12 amino acids, Gly-Ala-Ala-Glu-Leu-Pro-Cys-Ser-Ala-Asp-Trp-Trp (M_w: 1.3 kDa) was isolated from *R. catesbeiana* muscle hydrolysates degraded by Alcalase. The purified peptide from *R. catesbeiana* muscle (RCMP-alca) has IC_{50} value of 0.95 μM, and Lineweaver–Burk plots suggest that RCMP-alca play act as a non-competitive inhibitor against ACE I. Antihypertensive effect in spontaneously hypertensive rats (SHR) also revealed that oral administration of RCMP-alca can decrease systolic blood pressure significantly ($P < 0.05$). In addition, MTT assay showed no cytotoxicity on human embryonic lung fibroblasts cell line (MRC-5). The result of this study suggests that the ACE inhibitory peptide derived from *R. catesbeiana* muscle (RCMP-alca) could be potential candidates to develop nutraceuticals and pharmaceuticals.

Keywords: Bullfrog (*Rana catesbeiana* Shaw) muscle; Alcalase; Hydrolysates; ACE I inhibitory peptide; Antihypertensive effect

1. Introduction

Angiotensin I-converting enzyme (ACE I; dipeptidyl carboxy peptidase; EC 3.4.15.1) is a multifunctional zinc-containing enzyme, located in different tissues. This enzyme plays a key physiological role in the control of blood pressure, by virtue of the rennin–angiotensin system [1]. It acts as an exopeptidase that cleaves dipeptides from the C terminus of various oligopeptides [2]. ACE I converts an inactive form of decapeptide (angiotensin I) to octapeptide (angiotensin II), which is a potent vasoconstrictor, and inactivates bradykinin. Many studies have been attempted in the synthesis of ACE inhibitors such as captopril, enalapril, alacepril, and lisinopril, which are currently used in the treatment of essential hypertension and heart failure in humans [3] since the discovery of ACE inhibitors in snake venom [4]. However, these synthetic drugs are believed to have certain side effects such as cough, taste disturbances, and skin rashes. Therefore, the search for natural and non-toxic ACE inhibitors derived from proteins of livestock, grain, fish, etc., as alternatives to synthetic ones is of great interest among researchers for safe and economical use. Bioactive peptides, which are inactive within the sequence of the parent protein, could be released due to enzymatic hydrolysis. Among these, peptides with specific C-terminal sequences exposed by endo- or exo-proteolysis could interact with ACE I catalytic or binding site. The

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relationships between the structure and activity level of various ACE inhibitory peptides indicate that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate [5].

To investigate biomedical and nutraceutical benefits of bullfrog (*Rana catesbeiana* Shaw) muscle, the bullfrog muscle protein was hydrolyzed by various commercial enzymes. The market for anurans, mainly frogs, has grown substantially in response to rising demand from frog products in high purchasing power markets, both in the food industry and as live laboratory material. Bullfrog is the most commercially important anuran species distributed group of amphibians, have proved to be a particularly rich source of peptides. However, studies on the biomedical utilization of *R. catesbeiana* muscle as a natural source of ACE I inhibitor are scarce. In the present study, the objective was to isolate and characterize an ACE inhibitory peptide derived from enzymatic hydrolysates of *R. catesbeiana* muscle protein. Moreover, we have also investigated antihypertensive action of the purified peptide by oral administration in spontaneously hypertensive rats (SHR).

2. Materials and methods

2.1. Materials

Bullfrog (*R. catesbeiana* Shaw) was purchase from a local market in Yangsan and Busan, S. Korea. *R. catesbeiana* muscle was rapidly separated and rinsed with deionized water to eliminate contaminants under −4 °C, and then stored at −20 °C until use. ACE (from rabbit lung) and a substrate peptide (Hip-His-Leu), α-chymotrypsin, papain, pepsin and trypsin were purchased from Sigma Chemical Co., USA. Alcalase and Neutrase, were purchased from Novozymes Co. (Bagsvaerd, Denmark). All other reagents were of the highest grade available commercially.

2.2. Preparation of enzymatic hydrolysates

To extract ACE inhibitory peptide from *R. catesbeiana* muscle protein, enzymatic hydrolysis was performed using various commercial enzymes (Alcalase, α-chymotrypsin, Neutrase, papain, pepsin, and trypsin) with each optimal condition. At enzyme/substrate ratio of 1/100 (w/w), 1% substrate and enzyme were mixed. The mixture was incubated for 8 h at each optimal temperature with stirring and then heated in a boiling water bath for 10 min to inactivate the enzyme. Degree of hydrolysis was determined by measuring the nitrogen content soluble in 10% (v/v) trichloroacetic acid as discussed by Kim et al. [6] and lyophilized hydrolysates were stored under −80 °C until used.

2.3. Measurement of ACE inhibitory activity

ACE inhibitory activity assay was performed using a modified version of the method of Cushman and Cheung [7] with slight modifications. The reaction mixture contained 5 mM Hip-His-Leu as a substrate, 0.3 M NaCl and 5 μl ACE in 50 mM sodium borate buffer (pH 8.3). A sample (50 μl) was added to the above reaction mixture (50 μl) and mixed with 8.3 mM Hip-His-Leu (150 μl) containing 0.5 M NaCl. After incubation at 37 °C for 60 min, the further reaction was stopped by the addition of 0.1N HCl (250 μl). The resulting hippuric acid was extracted by the addition of 1.5 ml ethyl acetate. After centrifugation (800 × *g*, 15 min), 1 ml of the upper layer was transferred into a glass tube and evaporated at room temperature for 2 h in vacuum. The hippuric acid was redissolved in 3.0 ml of distilled water, and absorbance was measured at 228 nm using spectrophotometer (Model U-3210, Hitachi Co., Tokyo, Japan). The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE inhibitory activity.

2.4. Purification of ACE inhibitory peptide

2.4.1. Ion exchange chromatography

ACE inhibitory peptide was purified from enzymatic hydrolysates using fast protein liquid chromatography (FPLC ÄKTA, Amersham Bioscience Co., Uppsala, Sweden) on a HiPrep 16/10 CM FF ion-exchange column. The hydrolysate was loaded onto a HiPrep 16/10 CM FF ion-exchange column equilibrated with 20 mM sodium acetate buffer (pH 4.0), and eluted with a linear gradient of NaCl (0–2 M) in the same buffer at a flow rate of 62 ml h^{-1}. Each fraction was monitored at 215 nm, collected at a volume of 4 ml and concentrated using a rotary evaporator; ACE inhibitory activity was also investigated. A strong ACE inhibitory active fraction was lyophilized, and chromatography was used as the next step.

2.4.2. High performance liquid chromatography (HPLC)

The fraction exhibiting ACE inhibitory activity was further purified using reversed-phase high performance liquid chromatography (RP-HPLC, Dionex Korea Ltd., USA) on a Primesphere 10 C_{18} (10 mm × 250 mm, Phenomenex, Cheshire, England) column with a linear gradient of acetonitrile (0–35% in 35 min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.2 ml min^{-1}. Elution peaks were detected at 215 nm, and active peak was concentrated using a rotary evaporator. Potent peaks were collected, evaluated ACE inhibitory activity, and then lyophilized. The active fraction from analytical column was further applied onto a SynChropak RP-P-100 column (4.6 mm × 250 mm, SynChrom, Inc., Indiana, USA) with a linear gradient of acetonitrile (15%, v/v, in 20 min) containing 0.1% TFA at flow rate of 1.2 ml min^{-1}. Finally, the purified peptide from Alcalase-digests of *R. catesbeiana* muscle (RCMP-alca) was analyzed amino acid sequence.

2.5. Determination of molecular mass and amino acid sequence

Accurate molecular mass of the RCMP-alca were determined with a Q-TOF mass spectrometer (Micromass, Altrincham, UK) coupled with electrospray ionization (ESI) source. Amino acid sequence of RCMP-alca was analyzed using an automated protein sequencer (Perkin-Elmer, Model 491, Branchburg, NJ, USA), and the phenylthiohydantoin derivatives were identified using an automatic analysis system.

2.6. Determination of the inhibition pattern on ACE

Different concentrations of ACE inhibitory peptide were added to each reaction mixture according to the method of Kim et al. [6] with some modifications. The enzyme activity was measured with different concentrations of the substrate. ACE I inhibitory pattern in the presence of the inhibitor was determined by the Lineweaver–Burk plots.

2.7. Cytotoxicity assay in vitro

For the cytotoxicity assay, the colorimetric MTT assay was performed [8]. Cell were seeded at 1.3×10^4 cell/well in 96-well microliter plates in complete medium, DMEM with 10% FBS for human lung (MRC-5) cell. After 24 h incubation in a humidified 5% (v/v) CO_2/air environment at 37 °C, 20 μl of MTT dye solution was added to each well. After 4 h incubation, 200 μl of solubilization/stop solution was added to dissolve the formazan crystals and incubated the mixture at 37 °C overnight. The absorbance was read using Genious Multifunction microplate reader (Tecan, UK) at 540 nm.

2.8. Experimental animals and measurement of systolic blood pressure

Spontaneously hypertensive rats (10-week-old, male, SHR/Hos, SPF, 180–240 g BW) with tail systolic blood pressure (SBP) over 180 mmHg were obtained from Korea Research Institute of Bioscience and Biotechnology (DaeJeon, Korea). Spontaneously hypertensive rats (SHRs) were housed individually in steel cages in a room kept at 24 °C with a 12-h light/12-h dark cycle, and fed a standard laboratory diet. Tap water was freely available.