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Purification and characterization of a novel angiotensin-I converting enzyme (ACE) inhibitory peptide derived from enzymatic hydrolysate of grass carp protein

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ABSTRACT

Peptides inhibiting angiotensin-I converting enzyme (ACE, EC. 3.4.15.1) are possible cures of hypertension. Food-derived ACE-inhibitory peptides are particularly attractive because of reduced side effects. Previously, we reported ACE-inhibitory activity of grass carp protein hydrolysates. In this work, we report steps for purifying the ACE-inhibitory peptide from the hydrolysate and its biochemical properties. Following steps of ultrafiltration, macroporous adsorption resin, and two steps of reversed phase high performance liquid chromatography (RE-HPLC), a single Val-Ala-Pro (VAP) tripeptide was identified. The tripeptide with excellent ACE-inhibitory activity (IC_{50} value of 0.00534 mg/mL) was a competitive ACE inhibitor and stable against both ACE and gastrointestinal enzymes of pepsin and chymotrypsin. This is the first report of food-derived VAP. The identified unique biochemical properties of VAP may enable the application of grass carp protein hydrolysates as a functional food for treatments of hypertension. The developed purification conditions also allow the production of VAP for pharmaceutical applications.

1. Introduction

The population of adults affected by hypertension was estimated to be 972 million worldwide in 2000 and was predicted to reach 1.56 billion by 2025 [19]. This is a severe concern because hypertension is frequently linked with high risks of cardiovascular and renal diseases [6]. While there are many causes of hypertension, ACE is well recognized for its important physiological roles in the regulation of blood pressure [6]. Biochemically, ACE converts angiotensin-I, a decapeptide that is inactive, to angiotensin-II, an octapeptide that is a potent vasoconstrictor [29]. ACE also inactivates bradykinin, a vasodilator [29]. These established biochemical mechanisms have led to the synthesis of ACE inhibitors such as captopril, lisinopril, enalapril and fosinopril for treatment of hypertension [5,7]. Although effective, these synthetic ACE inhibitors cause side effects such as coughing, taste disturbances, skin rashes and angioneurotic edema, and their long term administration may cause aldosterone escape phenomenon and reduce the efficacy of ACE inhibitors [2,11,36]. Therefore, much interest has focused on ACEIP derived from food materials to reduce side effects while maintaining physiological functions like body weight reduction and immune regulation.

Oshima [30] first reported ACEIP derived from food natural sources. Since then, a large number of ACEIP have been isolated and identified from proteins or their hydrolysates originating from milk [4], egg [27], rapseed [25], soybean [32,42], peanut [16], rice [22], corn [20], wheat [15], beef [14], porcine [18], chicken [12,33], among others. ACEIP derived from seafood proteins have also been reported. Kohama et al. [21] observed that an octapeptide in an acid extract from tuna muscle, with an amino acid sequence of GD at the amino terminus, showed strong anti-hypotensive activity. More recently, many ACEIP have been isolated from bonito [13,40],

Abbreviations: ACE, angiotensin-I converting enzyme; ACEIP, angiotensin-I converting enzyme inhibitory peptides; RE-HPLC, reversed phase high performance liquid chromatography; HPLC, high performance liquid chromatography; HHL, hippuryl-histidine-leucine; HA, hippuric acid; TFA, trifluoroacetic acid; DH, degree of hydrolysis; BV, bed volume; PTH, phenylthiohydantoin.

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cuttlefish [31], pipefish [37], sardine [26], squid [3], and yellowfin [17], and the "Katsuo-bushi oligopeptide" is now sold in Japan as a functional food product claiming anti-hypertension benefits [12,13].

We are interested in farm-raised freshwater fish as protein sources of ACEIP, particularly grass carp because its production ranks the first in China, about 3.7 million tons annually [24], and second in the world. Grass carp proteins are easy to extract and their functional and nutritional properties can be improved by methods such as enzymatic hydrolysis. Hydrolysates with unique properties add significant values to aquaculture. Previously, we hydrolyzed grass carp protein using alcalase and observed significant ACE-inhibitory activities in the derived peptides [8,9]. Because high-purity ACEIP are required for applications such as admission by hypertension patients, the major objective of this work was to purify ACEIP from grass carp protein hydrolysates using RP-HPLC. Our secondary objectives were to evaluate the ACE-inhibitory functions of the purified ACEIP and characterize its stability during simulated digestions by gastrointestinal enzymes.

2. Materials and methods

2.1. Materials

Grass carp was purchased from the supermarket of Wushang Wholesale Chain Company (Wuhan, China). Alcalase, pepsin and chymotrypsin were purchased from Novozymes Biotechnology Company (Shanghai, China). ACE from rabbit lungs, HHL and HA were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile and TFA were HPLC grade, and other reagents were analytical grade, purchased from Sinopharm Chemical Reagent Company (Shanghai, China).

2.2. Preparation of grass carp protein hydrolysate

Grass carp protein hydrolysates were prepared according to our previously described method [9]. The reactor (MUT-Tschamber, Roedermark, Germany) was equipped with a stirrer, a thermostatic water-circulator bath (Huayi, Gongyi, China) for temperature control, and a pH-adjustment system (Mettler Toledo, Shanghai, China) enabling a constant pH during hydrolysis. The solution with 5.5% (w/w) grass carp protein was homogenized at 0 °C for 30 min and pre-incubated at 50 °C for 5 min. The hydrolysis was performed using alcalase at a level of 48 AU per kg protein at a constant temperature of 50 °C and pH 9. At a DH of 17.25%, the hydrolysis was terminated by adjusting pH to 4.5, followed by centrifugation at 10,000 × *g* for 10 min. The supernatant was transferred and lyophilized after adjusting pH to neutral. The final powder was collected and stored at −20 °C until further experiments.

2.3. Measurement of ACE-inhibitory activity

The ACE inhibitory activity was measured by the method of Wu et al. [38] with slight modifications. The 30 μL of a 2.5 mM HHL solution was mixed with 10 μL of an inhibitor solution, followed by incubation for 5 min at 37 °C. Afterwards, 20 μL of a 0.1 U/mL ACE solution (prepared in a 50 mM borate buffer adjusted to pH 8.3, containing 0.3 M NaCl) was added, followed by incubation at 37 °C for 60 min. The reaction was terminated by adding 70 μL of 1 M HCl before the following assays. A blank sample was prepared by replacing the inhibitor solution with the 50 mM borate buffer. Samples were filtered through a 0.45 μm nylon syringe filter and then separated by a C_{18} column (4.6 mm × 150 mm, 5 μm). HA and HHL were detected at 228 nm. The column was eluted at a flow rate of 0.8 mL/min with two solutions – (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile – using a gradient of 10–60% B in 10 min and another 60–10% B in 2 min. Peak areas were used to quantify HA. The degree of ACE inhibition (in percentages) was calculated according to the following equation:

$$\text{ACE inhibition } (\%) = \left[1 - \left(\frac{A_{\text{inhibitor}}}{A_{\text{blank}}}\right)\right] \times 100 \quad (1)$$

where $A_{\text{inhibitor}}$ and A_{blank} are peak areas corresponding to HA for an inhibitor sample and the blank sample, respectively.

The IC_{50} value was defined as the concentration of inhibitor required to reduce the HA peak area by 50% (indicating 50% inhibition of ACE).

2.4. Purification of ACE inhibitory peptides from hydrolysates

The lyophilized hydrolysate was dissolved at 50 mg/mL in deionized water. The solution was filtered using two ultrafiltration membranes with 3 and 10 kDa molecular-weight-cut-off to obtain three fractions corresponding to MW below 3 kDa, between 3 and 10 kDa, and above 10 kDa. Each fraction was assayed for ACE-inhibitory activity. The fraction with the highest ACE-inhibitory activity was lyophilized, and the powder obtained was prepared at 10 mg/mL and loaded to a DA 201-C macroporous resin column at a flow rate of 1 BV/h. After loading, the column was washed with deionized water at a flow rate of 1 BV/h until the eluent had the same conductivity as deionized water. Then, four fractions were obtained from step-elution using 25%, 50%, 75% and 90% aqueous ethanol solution at a flow rate of 3 BV/h. The fraction with the highest ACE-inhibitory activity was lyophilized and further purified in two RP-HPLC (model 1525-2998, Waters Corp., Torrance, USA) steps. A Jupiter C_{18} column (5 μm, 10 mm × 250 mm, Phenomenex Co., Torrance, CA, USA) was adopted in the first step. After equilibrating the column with 5% acetonitrile, 2 mL sample, prepared with the lyophilized powder at 10 mg/mL, was injected. A linear gradient of 5–60% acetonitrile (in deionized water, containing 0.1% TFA) was carried out in 60 min at a flow rate of 2 mL/min, with the absorbance of eluent 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 10 mg/mL and separated for the second step RP-HPLC using a Luna C_{18} column (5 μm, 4.6 mm × 250 mm, Phenomenex Co., Torrance, CA, USA). After injecting 250 μL sample, the elution was conducted at a flow rate of 0.8 mL/min using an isocratic step with 10% acetonitrile for 10 min and a subsequent linear gradient of 10–25% acetonitrile in 45 min. The detection of eluent was also based on the absorbance at 220 nm. Similar to the first step, fractions were collected and lyophilized immediately for further use. Another RP-HPLC analysis was performed for the fraction showing the highest ACE-inhibitory activity from the second step to evaluate the purity of peptide. The separation was achieved with a Jupiter C_{18} column (5 μm, 4.6 mm × 250 mm, Phenomenex Co., Torrance, CA, USA) and a sample injection volume of 20 μL. To elude the adsorbed peptide, an isocratic step with 10% acetonitrile for 5 min and a subsequent linear gradient of 10–100% acetonitrile in 25 min were used at a flow rate of 0.8 mL/min. The absorbance at 220 nm was monitored during elution.

2.5. Amino acid sequencing of the purified peptide

The purified peptide was dissolved in 1% TFA (in deionized water) solution and then transferred onto a polyvinylidene fluoride membrane. After fixing by polybrene, the amino acid sequence of the peptide was characterized using an automated protein/peptide sequencer (model PPSQ-33A, Shimadzu, Tokyo, Japan) equipped with an online detection system for PTH-amino acids.