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ACE-inhibitory peptides identified from the muscle protein hydrolysate of hard clam (*Meretrix lusoria*)

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ABSTRACT

The meat of hard clam was extracted using hot water. The residual meat was freeze-dried then hydrolyzed at 50 °C for 5 h by Protamex (PX). The inhibitory effects of hot water extract and hydrolysate against angiotensin I converting enzyme (ACE) were investigated. The IC_{50} value of hot water extract and hydrolysate on ACE were 1.090 and 0.036 mg/ml, respectively. The PX hydrolysate was separated into five fractions by size exclusion chromatography on a Sephadex G-25 column. The fifth fraction of the hydrolysate having molecular weight ranged 350–300 Da showed the highest inhibitory efficiency ratio (IER) being 5831%/(mg ml). The amino acid sequence of the inhibitory peptide was Tyr-Asn (IC_{50} = 51 μM). The hydrolysate showed mixed-type inhibition kinetics while Captopril, the positive control, showed competitive inhibition on ACE. Their K_i values were 0.027 mg/ml and 0.0067 μg/ml, respectively.

1. Introduction

Cardiovascular disease (CVD) is the primary cause of mortality in developed countries, and has become the leading cause of death in developing countries [1]. Hypertension is the major controllable risk factor in the development of CVD [2]. For each 5 mm Hg reduction in systolic pressure, the risk of CVD is reduced by about 16% [3,4]. Angiotensin I-converting enzyme (ACE; dipeptidyl carboxypeptidase, EC3.4.15.1) plays an important role in regulating blood pressure. In the rennin angiotensin system, ACE inactivates the vasodilator, bradykinin [5]. ACE also cleaves the dipeptide portion of angiotensin I from the C-terminal and produces a potent vasopressor angiotensin II, which induces the release of aldosterone, and causes the retention of sodium ions by kidney and elevates the blood volume, thus increases blood pressure [6]. ACE inhibitors act as vasodilators, but the most obvious potential benefit is their effect on the rennin–angiotensin–aldosterone system to reduce the levels of angiotensin II. Clinical studies have demonstrated that ACE inhibitors significantly reduce the morbidity and mortality of patients with myocardial infraction or heart failure [7,8].

ACE-inhibitory peptides have been isolated from enzymatic hydrolysates of various fish waste, such as Alaska pollack skin [9], sea bream scales [10], and yellowfin sole frame protein [11]. In addition, ACE-inhibitory peptides were also prepared from shellfish, including oyster protein digest (Leu-Phe, IC_{50} = 126 μM) [12], peptic digest of short-necked clam (Val-Glu-Val, IC_{50} = 8.7 μM) and pearl oyster (Leu-Val-Glu, IC_{50} = 14.2 μM) [13], and freshwater clam hydrolysate (Val-Lys-Pro, IC_{50} = 2.6 μM) [14]. These inhibitory peptides were found to be di- to octapeptides.

Hard clam (*Meretrix lusoria*) is important cultured shellfish in Taiwan. The changes of freshness quality, extractive components and glycogen in the edible portion of hard clam during storage were investigated [15,16]. Nowadays, the hot water extract (HWE) of clams essences have been marketed in Taiwan as nutraceuticals. Its proximate composition, free amino acids and peptides contents [17], and bioactivity on immunity [18] have been reported. The ethyl acetate extract of hard clam exhibited apoptotic effect on human cancer cells [19]. Moreover, the residual meat after hot water extraction still contributed to 90% of the total weight of fresh hard clam meat and is partially used in surimi processing. In order to make better utilization of the clam meat, the residual meat after hot water extraction was freeze-dried then hydrolyzed by Protamex, a commercial protease, to produce some bioactive peptide substances. And, the inhibitory effects on angiotensin I converting enzyme were determined to compare the efficacy of the hot water extract and the hydrolysates. The bioactive peptides of hydrolysate were fractionated using gel filtration to measure their molecular weights, purified by reverse-phase HPLC and further analyzed its amino acid sequences. Since number of ACE-inhibitory

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peptides produced from yogurt-like product [20], sour milk [21], and cheese whey [22] have shown to be effective in lowering blood pressure after oral administration by the spontaneously hypertensive rats as well as that of the mild and the moderate hypertensive subjects. It is our objective to utilize the clam essence residue to process into antihypertensive nutraceuticals

2. Materials and methods

2.1. Materials

The hard Clam (*Meretrix lusoria*) was cultivated in Chia-Yi County, Taiwan. Protamex (from *Bacillus*, activity labeled 1.5 AU/g) was purchased from Novo Nordisk A/S Co. (Denmark). Hippuryl-L-histidyl-L-leucine (Hip-His-Leu), angiotensin-converting enzyme of rabbit lung, captopril and other chemicals of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of hot water extract and hydrolysates

Whole hard clam was added with tap water in 1:1 (w/w) ratio and boiled for 40 min according to the commercial procedure. This liquid part was filtered through no. 2 filter paper (Toyo Roshi Kaisha, Tokyo Japan) and lyophilized to be defined as hot water extract (HWE). Then, the residual meat was freeze-dried. The powder of residual meat was homogenized with 10 times of deionized water for 2 min and boiled for 10 min then cooled to room temperature. Protamex (PX) was added at 1% (the ratio of enzyme to meat protein was 1:100, w/w) to the hard clam homogenate and was incubated at 50 °C for 5 h and heated at 98 °C for 10 min to inactivate the protease. The insoluble material was removed by centrifugation at 12,000 × *g* (Hitachi SCR 20BA, Tokyo, Japan) for 20 min. The supernatant was filtered through no. 2 filter paper. The filtrate was used as hydrolysate (PX5), which was lyophilized to a powdered form ready for analysis.

2.3. Measurement of soluble protein content

The soluble protein content of the hot water extract and hydrolysate was determined by the Folin-Lowry method [23,24]. One milliliter of the sample was mixed with 1 ml of an alkaline-copper reagent and 3 ml of the Folin-Ciocalteu's phenol reagent (Merck, Germany) at 10-fold dilution with deionized water. After the solution was allowed to stand for 30 min, the absorbance at 540 nm was measured with a spectrophotometer (Model UV-160A, Shimadzu, Kyoto, Japan). The soluble protein content was quantified using bovine serum albumin as standard.

2.4. Measurement of peptide content

The peptide content of the hot water extract and hydrolysate was measured according to the method of Church et al. [25] with some modifications. The sample solution (50 mg/ml) was filtered by 0.2 μm membrane. The filtrate passed through an ultrafiltration membrane with molecular weight (MW) cut-offs of 5000 Da (Millipore, Bedford, MA, USA). This permeate was defined as small peptides (MW < 5000 Da). Fifty milliliters of reagent was prepared by mixing 25 ml of 100 mM borax, 2.5 ml of 20% (w/w) sodium dodecyl sulfate, 40 mg of *o*-phthaldialdehyde solution (dissolved in 1 ml methanol) and 100 μl of β-mercaptoethanol. The volume was adjusted to 50 ml with deionized water. Fifty microliters of this permeate was mixed with 2 ml of reagent. The reaction mixture was incubated for 2 min at ambient temperature, and the absorbance at 340 nm was measured with spectrophotometer (Model UV-160A, Shimadzu, Kyoto, Japan). The peptide content was quantified using Leu-Gly (Sigma Chemical Co., St. Louis, MO, USA) as standard.

2.5. In vitro assay for ACE-inhibitory activity

The inhibitory activity of angiotensin-converting enzyme was performed on reversed-phase high performance liquid chromatography (RP-HPLC) and assayed with a modified spectrophotometric method [26,27]. Fifteen millimolar of hippuryl-L-histidyl-L-leucine (HHL) was dissolved in 100 mM Na-borate buffer (pH 8.3) containing 300 mM NaCl. Rabbit lung ACE was dissolved in the same buffer at a concentration of 8 mU/ml. A mixture containing 75 μl of ACE solution and 75 μl of sample with 5000 Da molecular weight cut-off (Millipore, Bedford, MA, USA) was incubated at 37 °C for 10 min, then added with 75 μl of HHL solution and incubated for 30 min. The reaction was stopped by adding 250 μl of 1N HCl and 10 μl of the assay solution injected directly onto a Luna C_{18} column (4.6 mm × 250 mm, particle size, 5 μm; Phenomenex, Torrance, CA, USA) to separate the product and hippuric acid (HA) from HHL. The column was eluted with 50% methanol in water (v/v) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.8 ml/min using a pump (Model L-7100, Hitachi Co. Ltd., Tokyo, Japan). The detector was monitored at 228 nm by a UV spectrophotometer (UV/Visible detector 118, Gilson Medical Electronics, Villiers-le-Bel, France). The inhibition activity was calculated using the following equation:

$$\text{Inhibition activity } (\%) = \left[\frac{E_c - E_s}{E_c - E_b}\right] \times 100 \quad (1)$$

where E_c is the absorbance of the buffer (control), E_s is the absorbance of the reaction mixture (sample), E_b is the absorbance when the stop solution was added before the reaction occurred (blank). The IC_{50} value was defined as the concentration of peptide in mg/ml required to reduce 50% of ACE activity, which was determined by regression analysis of ACE inhibition (%) versus peptide concentration.

2.6. In vitro gastrointestinal digestion

In vitro digestion was carried out in triplicate according to the method of Wu and Ding [27]. A sample solution of 3.5% (w/v) in 0.1 M KCl–HCl (pH 2.0) buffer was added with pepsin (4% w/w). The mixture was reacted for 4 h at 37 °C then stopped by boiling in water bath for 10 min and neutralized to pH 7.0 by addition of 2N NaOH solution. Neutralized suspension (50 ml) was centrifuged (10,000 × *g*, 30 min) and the supernatant was assayed for ACE-inhibitory activity. The remaining neutralized suspension was further digested by reacting with 4% (w/w) Pancreatin at 37 °C for 4 h. The enzyme was inactivated by boiling for 10 min and followed by centrifugation (10,000 × *g*, 30 min). The supernatant was ready for determination of ACE-inhibitory activity.

2.7. Kinetic evaluation of ACE-inhibitory hydrolysate

A Lineweaver–Burk plot was drawn to estimate the ACE-inhibitory types of the hydrolysate. The quantity of ACE used was maintained constant at 50 mU/ml. The ACE-inhibitory activities of the hydrolysate at a concentration ranged from 0.0194 to 0.0774 mg/ml and captopril (from 0.0011 to 0.0044 μg/ml) were measured in substrate (HHL) concentrations being 5, 2.5, 1.25 and 0.625 mM. The catalysis rate obtained from the double-reciprocal plot of ACE (μmol/min) was used to analyze the inhibition types of hydrolysate, the peptide inhibitor, against ACE. The inhibition constant (K_i) of the peptide inhibitor was obtained from the Lineweaver–Burk plot. The intercept on the horizontal axis is $1/K_i$.

2.8. Size exclusion chromatography

The lyophilized hydrolysate (50 mg) was dissolved with 2 ml of deionized water. The resulting solution was passed through a 5 kDa molecular weight cut-off membrane (MWCO) and fractionated by gel filtration on Sephadex G-25 column (1.6 cm × 90 cm; Amersham Pharmacia Biotech AB, Sweden), and eluted with deionized water. Fractions in 5 ml each were collected at a flow rate of 0.5 ml/min, and the absorbance was measured at 280 nm. Insulin B chain (MW = 3495.9 Da), Gastin I (MW = 2126 Da), Bacitracin (MW = 1422.0 Da), penta-L-phenylalanine (MW = 753.9 Da), and tryptophan (MW = 204.2 Da) were used as molecular weight standards.

2.9. Purification of ACE-inhibitory peptide

The hydrolysate (25 mg/ml) was applied on a Sephadex G-25 column (1.6 cm × 90 cm) and eluted with deionized water. The fraction with the highest ACE-inhibitory activity was collected, lyophilized and further separated by reversed-phase HPLC (Model L-7100, Hitachi, Tokyo, Japan), using a Synergi 4u Hydro-RP 80A C_{18} column (10 mm × 250 mm; particle size, 4 μm; Phenomenex, Torrance, CA, USA). Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in deionized water, and solvent B was 0.1% (v/v) TFA in 100% (v/v) acetonitrile (CH_3CN) solution. The separation was performed with a linear gradient from 0 to 12% of solvent B within 60 min at a flow rate of 1.5 ml/min at room temperature. The absorbance of the eluate was monitored at 220 nm with a UV spectrophotometer (UV/Visible detector 118, Gilson Medical Electronics, Villiers-le-Bel, France) linked to a data station (715 system controller, Gilson Medical Electronics, Villiers-le-Bel, France). These peaks were collected separately through repeated chromatography and each were confirmed as single component by reversed-phase HPLC column (ODS C_{12} Joupiter 4 μm Proteo 90 A, 250 mm × 4.6 mm, Phenomenex, Torrance, CA, USA) with linear gradients of acetonitrile solution from 0 to 40%. The peak with ACE-inhibitory activity was collected, lyophilized and its amino acid sequence was identified.

2.10. Sequence analysis

The sequence of each peptide was identified by automated Edman degradation using a protein sequencer 492 Procise (PerkinElmer, Applied Biosystem Inc., Foster City, CA, USA).

2.11. Statistical analysis

Analysis of variances of results was carried out using the general linear model procedure of SAS [28]. Multiple comparisons of means were carried out by Duncan's multiple range tests.