Purification and the secondary structure of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from the alcalase hydrolysate of seahorse protein

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Abstract Bioactive peptides with blood pressure-lowering functions have received increasing attention. In recent years, many ACE-inhibiting peptides have been widely purified from various food-derived proteins and have received considerable interest owing to their potential role in cardiovascular diseases and in the reduction of side effects. In this study, we hydrolyzed a three-spot seahorse (*Hippocampus trimaculatus* Leach) protein by alcalase to obtain a hydrolysate containing angiotensin I-converting enzyme (ACE) inhibitory peptide. Then, the hydrolysate was fractionated by dialysis, Sephadex G-25 gel filtration chromatography, and reverse-phase high performance liquid chromatography. After consecutive purification, a potent ACE-inhibiting peptide composed of 8 amino acids (Pro-Ala-Gly-Pro-Arg-Gly-Pro-Ala; MW: 721.39 Da; IC₅₀ value: 7.90 μM) was successfully isolated from three-spot seahorse protein. For the first time, a novel ACE-inhibiting peptide (PAGPRGPA) was isolated from the seahorse. Circular dichroism (CD) analyses suggested that the secondary structure of the purified peptide was mainly composed of random coil. Therefore, the peptide from seahorse protein may be used as a favorable ingredient in nutraceuticals, medicines, and functional foods against antihypertensive and related diseases.

Keywords Three-spot seahorse protein hydrolysate · ACE inhibitory peptide · Alcalase · Protein secondary structure

Introduction

In recent years, hypertension has been a serious chronic disease that can cause high-risk diseases, such as arteriosclerosis, stroke, and myocardial congestion. Hypertensive patients also show a trend of younger age (Balti et al. 2015). Hence, development of an effective treatment for hypertension is urgently needed. Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is a zinc metal peptidase that regulates blood pressure via the renin–angiotensin system and the kallikrein kinin system (Ko et al. 2012). Based on the principle of ACE action, several synthetic ACE inhibitors have been developed, including captopril, lisinopril, enalapril, and alacepril. They exert significant effects on the treatment of hypertension but are also accompanied by obvious drug-associated side effects, such as cough, taste disorder, rash, and angioedema (Ahn et al. 2012). Therefore, development of non-toxic, economical, safe, and effective ACE inhibitors is important.

Bioactive peptides refer to specific portions of protein having a number of amino acids of 2–20 and having biological activities which are effective for human health, such as blood pressure-lowering, anti-inflammatory, lipid-lowering, cholesterol-lowering, anti-oxidation, immunomodulation, and anti-thrombosis (de Castro and Sato 2015). Bioactive peptides can be released by digestion with gastrointestinal proteases in vivo and can also be obtained by microbial fermentation and enzymatic hydrolysis in vitro (Mohanty et al. 2016). In particular, the development of bioactive peptides research has allowed obtaining blood pressure-lowering peptides from natural

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resources (Boschin et al. 2014; Zhang et al. 2017). Compared with synthetic ACE inhibitors, ACE inhibitory peptides derived from natural resources are safer, healthier, and have less side effects (Mohanty et al. 2016). Given these advantages, bioactive peptides with ACE inhibition have greatly stimulated the interest of scientific researchers and gradually become a research hotspot.

Seahorse is an extremely unusual marine bony fish belonging to the Syngnathidae family, and it is mainly distributed in subtropical and tropical shallow sea areas (Lourie et al. 1999). Hainan Island, a characteristic of tropical marine life, is close to the South China Sea and has a unique geographical environment and abundant seahorse resource. With their complex and diverse living environment, marine organisms have become a major source for extracting biologically active components with novel structures and multiple biological activities, and these bioactive ingredients play a significant role in human health (Girgih et al. 2013). Recent studies have shown that seahorse is rich in active ingredients, such as steroids, proteins, fatty acids, and phospholipids, especially protein content as high as 67.90–73.56% (dry weight) (Qin et al. 2014; Zhang et al. 2016). As a traditional marine Chinese herbal medicine in Asia, seahorse shows physiological effects, such as anti-fatigue, anti-tumor, anti-inflammatory, and anti-aging (Zhang et al. 2016; Qin et al. 2014), and thus has broad development and application prospects. Existing studies focused on seahorse population distribution (Lourie et al. 1999), seahorse aquaculture (Koldewey and Martin-Smith 2010), fat-soluble component analysis (Finkel and Holbrook 2000), and glycolipid (Chen et al. 2015). Few studies on seahorse protein extraction have been reported. Scientists have recently isolated ACE-inhibiting peptides from various marine biological proteins, such as salmon (Ahn et al. 2012), lizard fish (Wu et al. 2015a, b), and Pacific salmon skin gelatin (Himaya et al. 2012). These studies indicated that marine biological proteins can be an important source for extracting ACE inhibitory peptides. However, the ACE-inhibiting rate of seahorse has not been reported.

This study aimed to purify the ACE inhibitory peptide from the alcalase hydrolysate of seahorse protein and study its primary and secondary structure. To our knowledge, this study is the first to isolate and purify ACE inhibitory peptides from seahorse protein. Therefore, this work could contribute to the development and utilization of seahorse protein in Hainan and provide a theoretical basis for the development of functional, nutritional, and medicinal foods for the treatment of hypertension.

Materials and methods

Materials and reagents

The three-spot seahorse dried product (10–13 cm) was purchased from Hainan Longsheng Bio-tech Development Co., Ltd (Haikou, China) and stored in a desiccator at room temperature. Cellulose dialysis bag with a molecular weight cut off of 500–1000 Da was purchased from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China). ACE (from rabbit lung; ≥ 2.0 units protein) and N-Hippuryl-His-Leu hydrate (HHL) were obtained from Sigam-Aldrich (St. Louis, MO, USA). Alcalase was provided by Beijing Solarbio Science and Technology Co., Ltd (Beijing, China). Acetonitrile (HPLC grade) was purchased from Fisher Scientific Inc (Fair Lawn, NJ, USA). Trifluoroacetic acid (TFA) was provided by Shanghai Maclean Biochemical Technology Co., Ltd (Shanghai, China). All other reagents and chemicals were of HPLC or analytical grade.

Preparation of protein hydrolysate from three-spot seahorse

The three-spot seahorse powder was defatted with ethyl acetate at a material-liquid ratio of 1:6 (g/mL). Alcalase was used to prepare peptides by the enzymatic hydrolysis of seahorse protein under optimum reaction conditions (pH 9.5, 60 °C). The substrate-to-enzyme ratio was 100:1. In brief, 20 g of defatted three-spot seahorse powder, 300 mL of distilled water, and 0.2 g of enzyme were mixed and reacted for 6 h. Once the enzymatic reaction was completed, the hydrolysate was adjusted to pH 7.0 and boiled at 100 °C for 10 min to inactivate the enzyme. The impurities were removed via centrifugation at 5000 rpm for 20 min at 4 °C. All the hydrolysates were lyophilized and stored at -20 °C.

Isolation and purification of ACE-inhibiting peptides

After dialysis for 72 h, the resulting solutions were lyophilized and the IC_{50} values were measured. The dialysis product (100 mg) was again filtered through a needle-type 0.45 μ m PES filter membrane and loaded into a Sephadex G-25 column (1.6 cm $\times$ 90 cm) previously equilibrated with ultrapure water. After loading, it was eluted with ultrapure water at a flow rate of 0.3 mL/min. Fractions detected at 280 nm were collected at 10 min intervals using an autocollector. The fraction with the highest ACE-inhibiting activity were lyophilized. The lyophilized fraction was filtered through a needle-type 0.22 μ m PES filter

membrane and further purified with RP-HPLC on a Hypersil™ ODS C₁₈ column (10 mm × 250 mm, 5 μm). The mobile phase consisted of eluent A of ultrapure water containing 0.1% trifluoroacetic acid (TFA) (v/v) and eluent B of acetonitrile containing 0.1% TFA (v/v). Elution was carried out at a flow rate of 3.0 mL/min. An isocratic step was carried out with 10% acetonitrile for 10 min, and a subsequent linear gradient of 10–42% acetonitrile was performed for 40 min. The UV absorbance of each elution peak was monitored at 215 nm. Potent peaks were collected and concentrated using a rotary evaporator. The collection procedure was repeated until sufficient samples were collected for evaluating the ACE-inhibiting activity and identifying the primary structure.

Measurement of ACE-inhibiting activity

The ACE-inhibiting activity was measured using the method of Ko et al. (2016) with a slight modification. The peptide sample solution (50 μL) was pre-incubated with 150 μL HHL solution (8.3 mM HHL in 100 mM sodium borate buffer containing 0.3 M NaCl, pH 8.3) for 5 min at 37 °C. At the same temperature, 50 μL ACE solution (25 mU/mL) was added to the mixture for 60 min. The reaction was stopped by the addition of 250 μL of 1.0 M HCl. The released hippuric acid (HA) was extracted with 1.5 mL of ethyl acetate. After centrifugation (3000 rpm, 10 min, 25 °C), 1.0 mL of the upper layer was transferred to a centrifuge tube and evaporated under vacuum at 60 °C for 3 h. The residue was reconstituted by adding 3.0 mL of distilled water, and the absorbance was detected at 228 nm. The inhibitor concentration that inhibits 50% of ACE-inhibiting activity is defined as the IC₅₀ value. The ACE inhibition rate was calculated according to the following equation:

$$\text{ACE-inhibiting activity (\%)} = \frac{A_b - A_s}{A_b - A_c} \times 100,$$

where A_b , A_s , and A_c represent the absorbance values of without inhibitor (blank), reaction mixture (sample), and buffer (control), respectively.

Identification of the amino acid sequence of the purified peptide

The accurate molecular mass and amino acid sequence of the RP-HPLC-purified peptide that exhibited high ACE-inhibiting activity were obtained using a Linear Trap Quadrupole Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, USA) in positive mode. Buspirone was used to calibrate and optimize the machine. The lyophilized sample was dissolved in 0.1% formic acid aqueous solution and then loaded onto an autosampler. ESI

served as a source of ions and polarity. The ion transport capillary temperature was set to 250 °C, and the spray voltage was 1800 V. The MS scanning method was adopted for information-dependent analysis, and the fragmentation method was used to achieve collision-induced dissociation. Mass spectrum were obtained over a range of 50–750 *m/z*. Mascot 2.3 software (Matrix Science, Boston, MA, USA) was used for peptide identification. The peptide mass was identified by searching the masses from the seahorse database, and MS/MS spectrum matched to the sequence was obtained according to the mass.

Circular dichroism spectra analysis

The secondary structure of the purified peptide was evaluated by using circular dichroism (CD) spectroscopy. Briefly, peptide sample was prepared in 20 mM phosphate buffer solution (pH 7.2) to 0.2 mg/mL. The Chirascan qCD recorded the far-ultraviolet (190–260 nm) spectrum of the peptide sample solution. The path length of the quartz cell (1 mm long) was used at room temperature. The scan rate was 120 nm/min, and the bandwidth was 1.0 nm. A buffer was used as a blank. The final CD spectra were obtained by averaging three consecutive scans. The secondary structure of the peptide sample was fitted using CDNN software (Applied Photophysics Ltd., Surrey, UK). In the Milli-Degress mode, the contents of α -helix, β -sheet (anti-parallel and parallel), β -turn, and random coil in different wavelength intervals were calculated.

Statistical analysis

Final data were expressed as the mean $\pm$ standard deviation ($\bar{X} \pm s$). ANOVA with Duncan's multiple range test was employed to assess the differences among groups. Statistical analysis was performed using SPSS 21.0. Significant differences were denoted by $P < 0.05$.

Results and discussion

Purification of ACE-inhibiting peptides from three-spot seahorse protein hydrolysate

A common method for obtaining bioactive peptides from food-derived proteins is to hydrolyze proteins by proteases, of which alcalase is more commonly used (Mohanty et al. 2016). Enzymatic hydrolysis is simple and easy to control the digestion conditions. Many studies have reported that alcalase hydrolysates from food-derived proteins exhibit functional properties, such as emulsifying properties (Jamdar et al. 2010) and potential biological activities,

particularly antioxidant (Alemán et al. 2011; Sarbon et al. 2018) and ACE-inhibiting activities (Chen et al. 2012; Pan et al. 2016). The endo-peptidase properties of alcalase account for the biological activity of various bioactive peptides produced by alcalase. Some of the biological activities, including ACE-inhibiting activity, are explained by the terminal amino acid sequences generated by alcalase (Wijesekara et al. 2011; Ko et al. 2012).

In this study, the hydrolysate obtained by hydrolysis of three-spot seahorse protein with alcalase showed 51.85% ACE-inhibiting activity at 1.0 mg/mL and had an IC_{50} value of 0.81 mg/mL. Many studies revealed the probability of developing natural ACE inhibitors from fish protein-derived hydrolysates (He et al. 2013a, b). The hydrolysate showed ACE-inhibiting activity confirming this view and also demonstrated alcalase activity. The dialysis product showed higher ACE-inhibiting activity than the hydrolysate (Table 1). The above results indicated that ACE inhibiting peptides could be enriched from three-spot seahorse protein hydrolysate.

The 0.45 μ m PES membrane-treated dialysis product was separated into three adsorption fractions via Sephadex G-25 gel filtration chromatography (Fig. 1), in which the second fraction (F2) achieved the strongest ACE inhibitory activity with an IC_{50} of 0.11 mg/mL (Table 1). The 0.22 μ m PES filter-treated lyophilized fraction F2 was then

Fig. 1 Elution profile of the dialysis product by Sephadex G-25 gel filtration chromatography

further separated via RP-HPLC. As shown in Fig. 2, the RP-HPLC profile of F2 presents many peaks, indicating that a large amount of peptides emerged during elution. The hydrophilic salt NaCl present in the three-spot seahorse protein hydrolysate possibly affected the separation effect. The fractions were collected by peaks, and the ACE inhibitory activity of each fraction was determined for the selection of the active fraction. However, only six peptide fractions showed ACE-inhibiting activities, and the IC_{50} values of F2 (1–6) were 39.6 μ g/mL, 17.6 μ g/mL, 246.9 μ g/mL, 5.7 μ g/mL, 35.4 μ g/mL, and 44.5 μ g/mL, respectively. Considering the IC_{50} values, we selected F2-4 in subsequent studies.

Table 1 displays the purification procedure of a novel ACE-inhibiting peptide derived from the three-spot seahorse protein hydrolysate. After three steps of purification, a potent ACE inhibitor was purified by 142.11 fold.

Sequence and characterization of the ACE-inhibiting peptide

The amino acid sequence of F2-4 was identified with ESI-MS/MS. The peptide sequence was Pro-Ala-Gly-Pro-Arg-Gly-Pro-Ala (PAGPRGPA; MW = 721.39 Da) (Fig. 3). F2-4 exhibited a high activity (IC_{50} of 7.90 μ M). The result is consistent with the view that marine protein-derived ACE-inhibiting peptides are typically composed of 3–12 amino acid residues (He et al. 2013a, b). To the best of our knowledge, the present study is the first to isolate an ACE-inhibiting peptide (PAGPRGPA) from the seahorse.

Bioactive peptides typically consist of 3–20 amino acids, and the biological activities of low-molecular-weight peptides may be stronger than those of high-molecular-weight peptides (García-Moreno et al. 2015). Obviously,

Table 1 IC_{50} values and purification folds of a novel ACE-inhibiting peptide

Purification step	IC_{50} value (mg/mL) ^a	Purification fold ^b
Hydrolysate	0.81 ± 0.12 ^B	1.00
Dialysis	0.44 ± 0.26 ^B	1.84
G-25 gel filtration chromatography		
F1	0.34 ± 0.35 ^B	
F2	0.11 ± 0.08 ^C	7.36
F3	3.76 ± 0.19 ^A	
RP-HPLC		
F2-1	0.0396 ± 0.0003 ^D	
F2-2	0.0176 ± 0.0010 ^E	
F2-3	0.2469 ± 0.0013 ^B	
F2-4	0.0057 ± 0.0009 ^F	142.11
F2-5	0.0354 ± 0.0005 ^D	
F2-6	0.0445 ± 0.0007 ^D	

The fractions with the strongest ACE inhibitory activity were shown in bold face

^aThe IC_{50} values are shown as mean ± standard deviation of the triplicates

^bThe IC_{50} of the hydrolysate was divided by the IC_{50} of the different steps as the purification fold

A, B, C, D, E, F Different superscript letters in different fractions indicate significant differences ($p < 0.05$)

Fig. 2 Elution profile of fraction F2 by RP-HPLC on a HypersilTM ODS C₁₈ column (10 mm × 250 mm, 5 μm)

121145-NO #1808 RT: 10.00 AV: 1 NL: 3.88E4
T: ITMS + c NSI r d w Full ms2 361.70@cid35.00 [85.00-735.

Fig. 3 ESI-MS/MS spectrum of the fraction F2-4 (m/z 721.39)

bioactive peptides that are effective in preventing and treating hypertension play a significant role in the health of hypertensive patients, reducing the demands for antihypertensive drugs with strong side effects. Indeed, ACE inhibitors consist of at least one molecular functional group, such as a hydrogen bond donor, a zinc binding ligand, and a carboxy terminal group (Andrews et al. 1985). Most reports suggested that ACE-inhibiting peptides have aromatic amino acids, such as Pro, Tyr, and Phe,

at the C-terminus and aliphatic amino acids, such as Ile and Val, at the N-terminal, thus exhibiting highly potent inhibiting activity (Cheung et al. 1980; Kapel et al. 2006). Proline is stable to digestive enzymes and can enter the blood circulation from capillaries in the form of short peptide molecules (Wijesekara et al. 2011). In addition, ACE-inhibiting peptides consisting of hydrophobic amino acids at the N-terminus, positively charged amino acids in the middle, and aromatic amino acids at the C-terminus

show strong ACE-inhibiting activity (Wu et al. 2015a, b). Most importantly, the relationship between peptide structure and ACE inhibitory activity remains the hottest and one of the research directions worthy of further research to understand structure–activity relationship. In the present study, a novel ACE-inhibiting peptide (PAGPRGPA) was isolated from three-spot seahorse protein hydrolysate. In the peptide sequence, three proline and two alanine (hydrophobic amino acids) accounted for 37.5% and 25.0%, respectively, and repeated in the sequence. These residues may contribute to the ACE-inhibiting activity of the peptide.

Circular dichroism spectra analysis

As a simple, effective, and valuable spectroscopic technique, circular dichroism has been widely used to study the secondary structure, kinetics, and folding of proteins and peptides (Whitmore et al. 2010). The basic elements of the secondary structure, such as α -helix and β -turn, show dichroic activity over the wavelength range of 190–260 nm (far ultraviolet) (Kelly and Price 1997). When the α -helix content of the protein is high, it is characterized by a positive ellipticity at 193 nm and a negative ellipticity at 208 and 222 nm. When the content of β -sheets in the protein is high, it is characterized by a broad negative band around 218 nm and a large positive band around 195 nm. The negative bands near 195 nm and the low ellipticity above 210 nm are characteristic of disordered proteins (Greenfield 2006).

The far UV CD spectrum of the purified peptide was displayed in Fig. 4. The CD spectrum showed a negative

peak around 198 nm and a negative shoulder in the range of 215–225 nm, indicating the absence of the typical signatures of α -helix and β -sheet structures. The CDNN software was used to fit the five secondary structures of the purified peptide, namely α -helix, anti-parallel, parallel, β -turn, and random coil. The CD results showed that the purified peptide consisted of 6.0% α -helix, 26.6% anti-parallel, 4.9% parallel, 31.4% β -turn, and 35.6% random coils. The number and position of amino acids in the peptide chain affect its secondary structure, hydrophobicity, and other activities (de Souza et al. 2015). These results were consistent with those of a previous report that investigated recombinant proteins and peptides through circular dichroism spectroscopy at 25 °C (Creasey et al. 2012).

Conclusion

Using consecutive chromatographic methods, we isolated and characterized a novel ACE-inhibiting peptide (PAGPRGPA) from the hydrolysate of three-spot seahorse protein with alcalase. The peptide exhibited potent ACE-inhibiting activity, with an IC_{50} value of 7.90 μ M. Circular dichroic analysis showed that the random coil was the main secondary structure in the purified peptide. In conclusion, our research helps increase the value of seahorse protein utilization and provides a theoretical basis for the development of related nutraceuticals, pharmaceuticals, and functional foods.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Fig. 4 Far ultraviolet circular dichroism spectrum of the purified peptide

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