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Modification of ACE-inhibitory peptides from *Acaudina molpadioidea* using the plastein reaction and examination of its mechanism

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ABSTRACT

The development of peptides derived from seafood, which have potential anti-angiotensin converting enzyme (ACE) activity and other bioactivities, are of scientific interest. However, the activity and thermal stability of these peptides are relatively poor, limiting their applications. The ACE-inhibitory peptides with high activity and stability prepared from sea cucumber (*Acaudina molpadioidea*) protein were modified using the plastein reaction. The mechanism of the plastein reaction was investigated using Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and differential scanning calorimetry. FTIR data suggested that the plastein products had a new absorption peak at 1250 cm^{-1}, which suggested the occurrence of the plastein reaction. Two new diffraction peaks appeared at 7.9 and 13.6° in the XRD spectra, and they indicated that new products were obtained from the peptides. The thermal stability of peptides after the plastein reaction was enhanced, and the thermal transition temperature of the peptides increased from 120° to 134°C. Urea and SDS decreased the plastein stability, thus suggesting that non-covalent interactions, such as hydrophobic and hydrogen bonds, are important for the plastein reaction. Accordingly, the plastein reaction has the potential to enhance the activity of peptides. Therefore, it can possibly be used as an effective method for increasing the application of marine active peptides and possibly other bioactive peptides.

1. Introduction

Angiotensin converting enzyme (ACE) is critical in controlling blood pressure regulation by converting angiotensin (Ang) I into Ang II and by inactivating the vasodilator bradykinin, thus leading to the development of hypertension (Corrons, Liggieri, Trejo, & Bruno, 2017; Wu, Liao, & Udenigwe, 2017; Zhang, Roytrakul, & Sutheerawattananonda, 2017). Therefore, the inhibition of ACE is considered an effective method to prevent and manage hypertension. However, with rising concerns about food safety, the potential toxicity of pharmaceutical drugs is driving the development of food-derived ACE inhibitors, such as phenolic compounds and peptides. Among these inhibitors, peptides derived from seafood, which have many significant advantages, have been previously studied as a way to control systemic blood pressure. Although ACE-inhibitory peptides showed the required activity, further hydrolysis may cause damage to structures and activities, thus limiting their application (Sun, Li, & Zhao, 2014). Therefore, finding ways to increase the anti-ACE activity of polypeptides may be beneficial.

The plastein reaction was first observed by Danilevski and Okuneff in 1902 (Beard, 1927) and was considered the reverse reaction of protein hydrolysis (Fujimaki, Arai, & Yamashita, 1977). It is characterized by only requiring mild reaction conditions without the need for organic reagents. Aside from research on the optimum reaction conditions using different substrates, the applications of plastein reactions have been extensively studied (Eriksen & Fagerson, 1976; Sukan & Andrews, 1982). The bitterness of protein hydrolysates from bovine red blood cells was reduced using the plastein reaction with the diethyl ester of glutamic acid (Synowiecki, Jagietka, & Shahidi, 1996). Using the plastein reactions, the bioavailability and functional properties of peptides were improved (Sun & Zhao, 2012; Yamashita, Arai, Tsai, & Fujimaki, 1971; Zhao, Dan, & Li, 2010). For example, soybean peptides after being subjected to the plastein reaction showed higher scavenging activity for hydroxyl radicals and significant increases in antioxidants than the original peptides (Seberry, Xia, & Pieprzyk, 2014). The plastein reaction of a casein hydrolysate using papain with extrinsic phenylalanine or tyrosine could lead to modified hydrolysates with increased scavenging activity for DPPH or hydroxyl radicals and reducing power (Zhao, Fu, & Yue, 2014). Consequently, plastein reactions provided a

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possible way to synthesize a multifunctional peptide-based ingredient with desirable sensory, bioavailability, stability, and functional properties (Brownsell, Rjh, & Andrews, 2001). However, due to the complexity of the reaction, the mechanism of plastein reactions remains undecided and are still an intellectual curiosity (Gong, Mohan, Gibson, & Udenigwe, 2015). Some researchers argued that transpeptidation is the main mechanism (Fujimaki, Kato, Arai, & Yamashita, 2010) and that new covalent bonds would be formed during the plastein reaction (Yamashita et al., 1971). On the other hand, many researchers supported that plasteins are held together mostly by hydrophobic interaction of aggregating peptides (Andrews & Alichanidis, 1990; Stevenson, Morgan, Fenton, & Moraes, 1999; Udenigwe, Wu, Drummond, & Gong, 2013).

In this work, the anti-ACE activity and thermal stability of the peptide modified using the plastein reaction increased significantly. Thus, the mechanism of the plastein reaction in this case was further studied. The structural characterizations were observed using Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), and X-ray diffraction (XRD), and the types of interactions involved in the reaction were inferred.

2. Materials and methods

2.1. Materials

The sea cucumbers (*Acaudina molpadioidea*), 30 ± 3 g were purchased from a local fish market in Qingdao (Shandong province, China), kept in ice and transported to laboratory within 30 min. Then the sea cucumbers were stored at −18 °C for a maximum of 4 wk prior to further analyses. Trypsin (E.C.3.4.21.4, 4000 U/g, one unit corresponds to the amount of enzyme which increases the absorbance at 253 nm 0.001/min at pH 7.6 at 25 °C using N-benzoyl-L-arginine ethyl ester as a substrate) and papain (E.C.3.4.22.2, 800 U/mg, one unit corresponds to the amount of enzyme which increases the absorbance at 253 nm 0.001/min at pH 6.2 and 25 °C using N-benzoyl-L-arginine ethyl ester as a substrate) were purchased from Novozymes China Biotechnology Co. Ltd. (Tianjin, China). SP Sephadex G-15, ACE (E.C.3.4.15.1, 2 U/mg, one unit will produce 1.0 μmol/L of hippuric acid from hippuryl-His-Leu per min in 100 mmol/L Tris-HCl, 300 mmol/L NaCl and 10 μmol/L $ZnCl_2$ at pH 7.0 at 37 °C), and O-aminobenzoic acid-Leu-Phe-Lys-OH were obtained from Sigma-Aldrich Co. Ltd. (Shanghai, China). L-glutamic acid was supplied by BBI Life Sciences Corp. (Shanghai, China). All other reagents used were analytical-grade chemicals and purchased from Sinopharm Group Co. Ltd. (Shanghai, China).

2.2. Preparation of A. molpadioidea hydrolysates and the plastein products

*A. molpadioidea*in in distilled water 1:4 (w/w) were swelled at 120 °C for 12 h, homogenized using a high-speed homogenizer (D-500, Wiggens Corp., Straubenhardt, Germany) at 15,000 rpm for 8 min, and then extracted in hot water at 45 °C for 8 h after being centrifuged at 4 °C (7870 × *g* for 20 min) in a refrigerated centrifuge (MR-23, Jouan, Saint Nazaire, France). Hot water soluble protein was obtained and centrifuged at 4 °C at 9710 × *g* for 20 min, and the supernatant was collected and freeze-dried (Scientz-10ND, Ningbo Scientz Biotechnology Co. Ltd., Ningbo, Zhejiang, China). Then the soluble protein power was stored at −18 °C for a maximum of 4 wk prior to further analyses.

The soluble protein power was dissolved in distilled water at a concentration of 2% (w/v). Then the suspension was adjusted to pH 7.0 with 1 mol/L NaOH. Trypsin (2000 U/g of dry soluble protein) was added. After incubation at 45 °C for 6 h, the reaction mixture was boiled at 95 °C for 15 min to inactivate the trypsin. The hydrolysates were concentrated using an ultrafiltration membrane with a nominal 5000 Da cutoff (PLCC06210, Merck Millipore Corp., Beijing, China), and then freeze-dried for further studies. The trypsin hydrolysates suspension (40%, w/v) was digested with papain (2500 U/g of dry hydrolysates) at 45 °C for 1 h and then heated to 95 °C for 15 min to inactivate the papain.

2.3. Change in the molecular weight distribution profile

The molecular weight distribution of *A. molpadioidea* hydrolysates was obtained using a high-performance size-exclusion chromatograph equipped with a ultraviolet detector (LC-20AT, Shimadzu Corp., Tokyo, Japan) system with a TSK-Gel 2000 SWXL column (7.8 × 300 mm, Agilent Technologies, Santa Clara, CA, USA). A 20 μL sample was injected into the column. The mobile phases were acetonitrile, Milli-Q water (Milli-Q integral, Merck Millipore Corp.), and trifluoroacetic acid (v/v/v: 50/50/1). The flow rate was 0.5 mL/min for 30 min, with the column temperature set to 30 °C and monitored at 220 nm using the ultraviolet detector. The protein standards (Sigma-Aldrich Co. Ltd.) used for the calibration were tyrosine (182 Da), glutathione (307 Da), bacitracin (1423 Da), insulin (5733 Da), and cytochrome C (12590 Da). The equation of the standard curve was as follows:

$$\mathrm{Lg\ MW} = -0.1844\, t + 6.322 (R^2 = 0.9968), \quad (1)$$

where MW is the molecular weight of the samples, and t is the retention time of the samples.

2.4. Fourier transform infrared (FTIR) spectra

The FTIR spectra of the *A. molpadioidea* hydrolysates and the plastein products were measured using an FTIR spectrophotometer (Nicolet iS10; Thermo Scientific Corp., Madison, WI, USA). The spectra were collected at ambient conditions in the transmittance mode from an accumulation of 64 scans with a 4 cm^{-1} resolution over the 4000–400 cm^{-1} region.

2.5. Changes in the free amino acids in the plastein reaction system

The o-phthaldialdehyde method was used to detect the free amino acids (Antoine et al., 2010). Free amino acids were determined using a reversed phase high-performance liquid chromatography (LC-20AT, Shimadzu Corp.) equipped with a fluorescence detector (RF-10AXL, Shimadzu Corp.) using a Hypersil ODS C18 column (4.0 × 125 mm, 5-micron particles, Dalian Elite Analytical Instruments Co., Ltd., Dalian, China). The fluorescence detector was used with the excitation monochrometer set at 338 nm and the emission monochrometer at 430 nm. The column temperature was 40 °C and the flow rate was 1.0 mL/min (mobile phase A was 80:19:1 of 50 mmol/L, pH 5.5 sodium phosphate buffer, methanol, and tetrahydrofuran. Mobile phase B was composed of 80:20 of methanol and 50 mmol/L, pH 5.5 sodium phosphate buffer. The gradient program used was as follows: 0–15% B in 5 min, 15–50% B in 5 min, and 50–100% B in 24 min) Free amino acids and small peptides in the samples were extracted using trichloroacetic acid (TCA). Five mL of 5% TCA was added to 1 g of sample, and the mixture was shaken for 5 min, then TCA (5%, w/v) was added to a final volume of 25 mL and kept at 4 °C for 2 h. The supernatant was filtered through the ultrafiltration membrane. One mL filtrate was centrifuged at 9710 × *g* for 10 min. Then amino acids and small peptides were derivitized using o-phthalaldehyde, 100 μL of amino acid standard (BBI Life Sciences Corp., Shanghai, China) or diluted sample supernatant and 400 μL of o-phthalaldehyde were added, followed by thorough mixing for 2 min using a Vortex (XW-80A, Shanghai Huxi Co. Ltd., Shanghai, China). The sample was injected onto the column of the HPLC system and the gradient run started. The concentrations of only the different free amino acids were measured from the standard curves of the L-glutamic acid (Sigma-Aldrich Co. Ltd.) prepared and derivatised simultaneously with the samples and were run under identical conditions. The equation of the standard curve was as follows:

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