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High throughput and rapid screening of marine protein hydrolysates enriched in peptides with angiotensin-I-converting enzyme inhibitory activity by capillary electrophoresis

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Abstract

Twelve kinds of marine protein materials, including fish, shrimp, seashell, algae and seafood wastes were selected for the hydrolysis using four different proteases. The IC_{50} values for angiotensin-converting enzyme (ACE) inhibitory activity of 48 hydrolysates were rapidly determined by capillary electrophoresis (CE). The values ranged from 0.17 to 501.7 mg/ml, and were affected by both the marine protein resources and the selected proteases. Hydrolysates of the lowest IC_{50} values were from shrimp (*Acetes chinensis*), shark meat, mackerel bone, *Polysiphonia urceolata* and *Spirulina platensis*, indicating these five kinds of marine food proteins contained beneficial materials for the production of ACE inhibitory peptides by proteolysis. The hydrolysates obtained using proteases Protamex and SM98011 had lower IC_{50} values, showing these two proteases were superior to others. The CE method achieved the same sensitivity as the high performance liquid chromatography (HPLC) method. However, the CE method was faster and, as a result, more economical. Therefore, CE had potential for rapid screening of marine protein hydrolysates enriched in ACE inhibitory peptides.

Keywords: Angiotensin-converting enzyme (ACE); ACE inhibitory activity; Capillary electrophoresis (CE); Hydrolysate; Marine proteins

1. Introduction

Angiotensin-converting enzyme (ACE) is a dipeptidyl carboxypeptidase that catalyzes the conversion of angiotensin I to angiotensin II, a process that increases blood pressure. Inhibition of ACE activity leads to a decrease in the concentration of angiotensin II and consequently reduces blood pressure (Skeggs et al., 1957). Although synthetic ACE inhibitors, including captopril, enalapril and listinopril, are remarkably effective as anti-hypertensive drugs, they inevitably cause adverse side effects (Atkinson and Robertson, 1979). Therefore, it is necessary to find innovative, safer, and more economical ACE inhibitors for the prevention and remedy of hypertension.

To date, more than 200 kinds of ACE inhibitory peptides have been reported in hydrolysates from diverse food proteins digested with different proteases (He et al., 2004). Most of these peptides were identified from hydrolytic products of land proteins, such as milk protein (Meisel, 1998; Nakamura et al., 1995; Pihlanto-Leppala, 2001), soy protein (Wu and Ding, 2002), egg protein (Yoshii et al., 2001), muscle protein (Arihara et al., 2001) and other proteins (Nakagomi et al., 2000; Yust et al., 2003). A wide variety and amount of marine protein resources exist in the ocean, including fish, shrimp, seashells, algae and seafood wastes generated from the processing of marine organisms. The composition and primary sequences of

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amino acids of these marine proteins are different from those of land proteins; therefore, marine proteins may become important protein resources for the selection of novel ACE inhibitory peptides by enzymatic hydrolysis. A few novel ACE inhibitory peptides have already been identified from the enzymatic products of marine fish protein and algae (Hordur and Barbara, 2000; Fujita and Yoshikawa, 1999; Suetsuna and Chen, 2001), but more extensive studies are needed to further characterize these marine proteins. It is important to determine whether hydrolysates have ACE inhibitory activity before attempting to purify the putative inhibitory peptides. Thus, it is imperative to establish a reliable method for high throughput and rapid screening of marine protein hydrolysates with potent ACE inhibitory activity.

High performance liquid chromatography (HPLC) is frequently used to quantitatively determine the amount of inhibitor needed to inhibit 50% of the original ACE activity (IC_{50}) (He et al., 2006). However, the HPLC method requires repeating a large number of ACE inhibitory reactions, which is time-consuming and inefficient. Therefore, it is difficult to achieve high throughput and rapid screening of the ACE inhibitory activities of large numbers of hydrolysate samples with the HPLC method. Recently, CE was successfully used to determine the activity of ACE inhibitors (Hillaert and Van den Bossche, 2001; Zhang et al., 2000) in lesser time than the HPLC method (Kemp, 1998). In this study, the CE method was used for the first time to throughput and rapidly screen marine protein hydrolysates enriched in peptides with angiotensin-I-converting enzyme inhibitory activity.

2. Methods

2.1. Materials

ACE, Hip-His-Leu (HHL), hippuric acid (HA) and captopril were obtained from Sigma Chemical Co. All other reagents used in this study were reagent grade chemicals. Twelve kinds of marine organisms or seafood wastes were selected for hydrolysis. Shrimp (*Acetes chinensis*) with low commercial value, scallops, oysters, shark meat, six kinds of seafood wastes (codfish bone, codfish skin, mackerel head, mackerel bone, crucian skin, crucian bone) were obtained from Shandong Dayudao Group Co. Ltd. (Yantai, China). Marine red alga (*Polysiphonia urceolata*) was collected from Huiquan Bay, Qingdao, P. R. China. Blue–green alga (*Spirulina platensis*) were cultured and collected in our lab using previously described methods (Zhang et al., 1999). Samples were packed in polyethylene bags and stored at −20 °C prior to hydrolysis experiments.

Four kinds of proteases were selected as enzymes for hydrolysis. Alcalase, Flavourzyme and Protamex were obtained from Novo Nordisk (Bagsvaerd, Denmark); Protease SM98011 was produced by the strain *Bacillus* sp. SM98011 preserved in our lab. All enzyme preparations were food-grade.

2.2. Proteases activity assay and preparation of hydrolysates

Protease activity was determined using a previously described method (He et al., 2004). Prior to enzymatic hydrolysis, the fresh marine proteins obtained from the above organisms were minced to a uniform consistency. Then 50 g samples of the minced marine proteins were digested in 50 ml of a diluted protease solution containing 4000 U/ml Alcalase, Flavuorzyme, Protamex or SM98011. The reactor vessel was placed in a thermostatically constant agitation (160 rpm) to allow adequate digestion of the marine proteins by the proteases. The digestion was continued until no increasing ACE inhibitory activity was detected. And then, it was terminated by incubation at 90 °C for 15 min. Tubes were centrifuged at 9000*g* for 20 min at 4 °C, and the supernatants were lyophilized and stored at 4 °C. An appropriate amount of the powder was dissolved into 5 ml of 0.02 M sodium borate buffer (pH 8.3).

2.3. Determination of ACE inhibitory activity by CE

The CE method was based on the liberation of HA from HHL catalyzed by ACE (Cushman and Cheung, 1971). For direct CE analysis, the total reaction volume was 30 μl, including 10 μl of 1 mM HHL, 10 μl of 0.8 mU ACE and 10 μl of different concentrations of Captopril or marine hydrolysates. All reactions were prepared in 100 mM sodium borate buffer (pH 8.3) containing 300 mM NaCl. The reactions were allowed to incubate at 37 °C for 30 min and then terminated with the addition of 0.1% TFA (10 μl). The amount of HA liberated from HHL per minute in the absence of ACE inhibitor was defined as 100% ACE activity. The IC_{50} value was defined as the amount of inhibitor required to inhibit 50% of the baseline uninhibited ACE activity. External standard HA samples were prepared and used for calculation of the concentration of HA.

Validation of the method and the experiments was accomplished using CE performed on a Beckman Coulter P/ACE MDQ instrument (Fullerton, CA) equipped with a photodiode array detector. For data collection, data analysis, and system control, P/ACE MDQ software from Beckman Coulter (Fullerton, CA) was used. ACE inhibitory reaction mixtures were directly introduced into the capillary by pressure injection (30 mbar) for 6 s. A constant voltage of 20 kV was applied and UV absorbance at 228 nm was employed for detection. The running buffer consisted of 20 mM boric acid–borate buffer (pH 9.18).

3. Results

3.1. Analysis of inhibitory activity of ACE inhibitor by CE

The CE method was used to directly determine the ACE inhibitory reactions. The total volume of the reaction mixtures was 30 μl, and complete baseline separation of HA and HHL was achieved in 5 min. The most anionic HA and