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ARTICLE

Analysis of Novel Angiotensin I-Converting Enzyme Inhibitory Peptides from Enzymatic Hydrolysates of Cuttlefish (*Sepia officinalis*) Muscle Proteins

Rafik Balti,[†] Naima Nedjar-Arroume,[‡] Estelle Yaba Adjé,[‡] Didier Guillochon,[‡] and Moncef Nasri*,[†]

[†]Laboratoire de Génie Enzymatique et de Microbiologie—Ecole Nationale d'Ingénieurs de Sfax, BP "1173" 3038 Sfax, Tunisia, and [‡]Laboratoire de Procédés Biologiques, Génie Enzymatique et Microbien, IUT A Lille I, BP 179, 59653 Villeneuve d'Ascq Cedex, France

The angiotensin I-converting enzyme (ACE) inhibitory activities of protein hydrolysates prepared from cuttlefish (*Sepia officinalis*) proteins by treatment with various bacterial proteases were investigated. The hydrolysate generated by the crude enzyme from *Bacillus mojavensis* A21 displayed the highest ACE inhibitory activity, and the higher inhibition activity (87.11 ± 0.92% at 2 mg/mL) was obtained with hydrolysis degree of 16%. This hydrolysate was fractionated by size exclusion chromatography on a Sephadex G-25 into eight major fractions (P_1-P_8). Fraction P_6, which exhibited the highest ACE inhibitory activity, was then fractionated by reversed-phase high performance liquid chromatography (RP-HPLC). Eleven ACE inhibitory peptides were isolated, and their molecular masses and amino acids sequences were determined using ESI-MS and ESI-MS/MS, respectively. The structures of the most potent peptides were identified as Ala-His-Ser-Tyr, Gly-Asp-Ala-Pro, Ala-Gly-Ser-Pro and Asp-Phe-Gly. The first peptide displayed the highest ACE inhibitory activity with an IC_{50} of 11.6 μM. The results of this study suggest that cuttlefish protein hydrolysates are a good source of ACE inhibitory peptides.

KEYWORDS: *Sepia officinalis*; muscle protein hydrolysates; ACE inhibitory peptide; purification and identification

INTRODUCTION

High blood pressure has been considered a risk factor for developing cardiovascular diseases (arteriosclerosis, stroke and myocardial infarction) and end-stage renal disease (*1*). Angiotensin I-converting enzyme (ACE) plays an important role in the renin−angiotensin system (RAS), which regulates human blood pressure and fluid homeostasis. The main molecule of the RAS, angiotensin II, is produced through an enzymatic cascade consisting of renin, an aspartic protease that first cleaves angiotensinogen to form the decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu, 1−10), and ACE, an M2 family metallopeptidase that then further cleaves angiotensin I into the octapeptide angiotensin II (1−8) by removing the C-terminal dipeptide His-Leu (*2*). The resulting angiotensin II is a potent vasoconstrictor, which stimulates the release of aldosterone and antidiuretic hormone or vasopressin and increases the retention of sodium and water and the regeneration of rennin. In addition, ACE, also termed kininase II, inactivates the vasodilators bradykinin (Arg-Pro-Pro- Gly-Phe-Ser-Pro-Phe-Arg) and kallidin (Lys-bradykinin) in the kallikrein−kinin system by cleaving the C-terminal dipeptide Phe-Arg. These effects directly act in concert to raise blood pressure.

*Corresponding author. Tel: 216 74-274-088. Fax: 216 74-275-595. E-mail: mon_nasri@yahoo.fr; moncef.nasri@enis.rnu.tn.

Specific inhibitors of ACE have been shown to be useful as antihypertensive drugs. Three kinds of synthetic ACE inhibitors were designed; they are grouped by their ligand for the active site on ACE. Captopril, the major representative of his group, has a sulfydryl moiety, lisinopril and enalapril have a carboxyl moiety, and fosinopril has a phosphorus group (*3*). Although ACE inhibitory drugs have demonstrated their usefulness, they are not entirely without side effects, such as cough, lost of taste, renal impairment and angioneurotic edema (*4*). Therefore, research and development to find safer, innovative and economical ACE inhibitors is necessary for the prevention and remedy for hypertension.

A variety of ACE inhibitory peptides with various amino acids sequences have been purified and identified in hydrolysates from food proteins digested with different proteases such as milk protein (*5*), gelatin (*6*), wheat protein (*7*), sardinelle (*8*), sea cucumber (*9*), tuna (*10*), and tilapia (*11*). In contrast to the many ACE inhibitory peptides derived from vertebrate muscle, very few studies on ACE inhibitory peptides from invertebrate muscles have been conducted.

In this study, we investigated the ACE inhibitory activity of cuttlefish muscle protein hydrolysates obtained by various enzymatic treatments. Eleven new antihypertensive peptides were isolated from the hydrolysate obtained by treatment with crude protease extract from *Bacillus mojavensis* A21, and their amino

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acids sequences were determined. Furthermore, the inhibition pattern of the most active peptide on ACE was investigated and the stability was evaluated under simulated gastrointestinal digestion.

MATERIALS AND METHODS

Reagents. Angiotensin I-converting enzyme from rabbit lung and the ACE synthetic substrate hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma Chemicals Co (St. Louis, MO). Acetonitrile was of HPLC grade. Sephadex G-25 was purchased from Pharmacia (Uppsala, Sweden). Water was obtained from a Culligan system; the resistivity was approximately 18 MΩ. All other chemicals and reagents used were of analytical grade.

Materials. Cuttlefish (*Sepia officinalis*), in the size range of 8−10 cuttlefish/kg, was purchased from the fish market of Sfax city, Tunisia. The samples were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w) and transported to the research laboratory within 30 min. The mantle was cleaned, deskinned and eviscerated and then stored in sealed plastic bags at −20 °C until used.

Proteolytic Enzymes. Alcalase 2.4L serine-protease from *Bacillus licheniformis* was supplied by Novozymes (Bagsvaerd, Denmark). Pepsin (10 U, from porcine stomach mucosa), chymotrypsin (5 U, from bovine pancreas) and trypsin (10 U, from bovine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO). Crude enzyme preparations from *Aspergillus clavatus* ES1 (*12*), *Bacillus licheniformis* NH1 (*13*) and *Bacillus mojavensis* A21 (*14*) were prepared in our laboratory.

Preparation of Cuttlefish Muscle Protein Hydrolysates (CMPHs) Using Various Proteases. Cuttlefish (*S. officinalis*) muscle (500 g), in 1000 mL of distilled water, was minced, using a grinder (Moulinex Charlotte HV3, France), and then cooked at 90 °C for 20 min to inactivate endogenous enzymes. The cooked muscle sample was then homogenized in a Moulinex blender for about 2 min. The samples were adjusted to optimal pH and temperature for each enzyme: Alcalase (pH 8.0; 50 °C), crude enzymes from *B. licheniformis* NH1 and *B. mojavensis* A21 (pH 10.0; 50 °C) and crude enzyme from *A. clavatus* ES1 (pH 8.0; 40 °C). Then, the substrate proteins were digested with enzymes at a 3:1 (U/mg) enzyme/protein ratio for 4 h. Enzymes were used at the same activity levels to compare hydrolytic efficiencies. During the reaction, the pH of the mixture was maintained at the desired value by continuous addition of 4 N NaOH solution. The enzymatic hydrolysis was stopped by heating the solutions at 80 °C during 20 min. Protein hydrolysates were then centrifuged at 5000*g* for 20 min to separate soluble and insoluble fractions. Finally, the soluble fractions were freeze-dried using freeze-dryer (Bioblock Scientific Christ ALPHA 1-2, Illkirch-Cedex, France) and stored at −20 °C for further use.

Determination of the Degree of Hydrolysis. The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds broken (h) to the total number of peptide bonds per unit weight (h_{tot}), in each case, was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis (*15*) as given below.

$$\mathrm{DH}\ (\%) = \frac{h}{h_{\mathrm{tot}}} \times 100 = \frac{B \times \mathrm{Nb}}{\mathrm{MP}} \times \frac{1}{\alpha} \times \frac{1}{h_{\mathrm{tot}}} \times 100$$

where B is the amount of NaOH consumed (mL) to keep the pH constant during the reaction. Nb is the normality of the base, MP is the mass (g) of protein ($N \times 6.25$), and α is the average degree of dissociation of the α-NH_2 groups released during hydrolysis expressed as

$$\alpha = \frac{10^{\mathrm{pH}-\mathrm{pK}}}{1 + 10^{\mathrm{pH}-\mathrm{pK}}}$$

where pH and pK are the values at which the proteolysis was conducted. The total number of peptide bonds (h_{tot}) in a fish protein concentrate was assumed to be 8.6 mequiv/g (*15*).

Chemical Analysis. The moisture and ash content were determined according to the AOAC (*16*) standard methods 930.15 and 942.05, respectively. Total nitrogen content of cuttlefish protein hydrolysates was determined by using the Kjeldahl method. Crude protein was estimated by multiplying total nitrogen content by the factor of 6.25. Lipids were determined gravimetrically after Soxhlet extraction of dried samples with hexane. All measurements were performed in triplicate.

Determination of ACE Inhibition Activity. The ACE inhibition activity was measured as reported by Nakamura et al. (*17*). A sample solution (80 μL) containing different concentrations of protein hydrolysates was added to 200 μL containing 5 mM hippuryl-L-histidyl-L-leucine (HHL), and then preincubated for 3 min at 37 °C. CMPHs and HHL were prepared in 100 mM borate buffer, pH 8.3, containing 300 mM NaCl. The reactions were then initiated by adding 20 μL of 0.1 U/mL ACE from rabbit lung prepared in the same buffer and incubated for 30 min at 37 °C. The enzyme reaction was terminated by the addition of 250 μL of 1.0 M HCl. The released hippuric acid (HA) was quantified by RP-HPLC on a Vydac C_{18} column connected to a system composed of a Waters TM 600 automated gradient controller pump module, a WaterWisp 717 automatic sampling device and a Waters 996 photodiode array detector. The sample was then eluted using an acetonitrile gradient from 0 to 28% and from 28 to 47% in 0.1% trifluoroacetic acid (TFA) (v/v) for 50 and 20 min, respectively. The eluate was followed at 228 nm. Spectral and chromatographic data were stored on a NEC image 446 computer. Millennium software was used to acquire, analyze and plot chromatographic data. The average value from three determinations at each concentration was used to calculate the ACE inhibition rate as follows:

$$\text{ACE inhibition}\ (\%) = \left[\frac{B-A}{B-C}\right] \times 100$$

where A is the absorbance of HA generated in the presence of ACE inhibitor component, B the absorbance of HA generated without ACE inhibitors and C is the absorbance of HA generated without ACE (corresponding to HHL autolysis in the course of enzymatic assay).

The IC_{50} value was defined as the concentration of hydrolysate (mg/mL) required to reduce the hippuric acid peak by 50% (indicating 50% inhibition of ACE).

Purification and Characterization of ACE-Inhibitory Peptides. The freeze-dried hydrolysate (1 g), with a DH of 16% obtained by treatment with alkaline proteases from *B. mojavensis* A21, was suspended in 5 mL of distilled water, and then loaded onto a Sephadex G-25 gel filtration column (5 cm × 57 cm), pre-equilibrated and eluted with distilled water. Fractions (4 mL each) were collected at a flow rate of 30 mL/h, and the absorbance was measured at 226 nm. Fractions associated with each peak showing ACE inhibitory activity were pooled and freeze-dried. Peptides in fraction P_6 from Sephadex G-25, which exhibited the highest ACE inhibitory activity, were dissolved in distiller water, filtered through 0.22 μm, and then separated by RP-HPLC on a Vydac C_{18} column (10 mm × 250 mm) (Grace-Vydac, USA). Peptides were eluted with eluent A (water containing 0.1% trifluoric acetic acid (TFA) for 20 min), then with a linear gradient of acetonitrile (0−50% in 35 min) containing 0.1% TFA at a flow rate of 1 mL/min. Online UV absorbance scans were performed between 200 and 300 nm at a rate of one spectrum per second with a resolution of 1.2 nm. Chromatographic analyses were completed with Millennium software. The ACE inhibitory activities of the eluted peaks were determined. The liquid chromatographic system consisted of a Waters 600E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 computer. Millennium software was used to plot, acquire and analyze chromatographic data.

Identification of the Amino Acid Sequence of ACE Inhibitory Peptides. The molecular mass and peptide sequencing were done on positive ion mode using electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS), respectively. ESI mass spectrometry was performed using a triple quadrupole instrument Applied Biosystems API 3000 (PE Sciex, Toronto, Canada) equipped with an electrospray ion source. The system is controlled by the Analyst Software 1.4, allowing the control of the spectrometer, the analysis and the processing data. Interpretations of MS/MS spectra were made with Bioanalyst software. The freeze-dried samples from RP-HPLC were dissolved in acetonitrile/water (20/80; v/v) containing 0.1% formic acid for the positive mode. The solution was injected (nebulized) uninterrupted, by a pump (model 22, Harvard Apparatus, South Natick, MA) with a flow rate of 5 μL/min. The potential of ionization was of 5000 V in positive

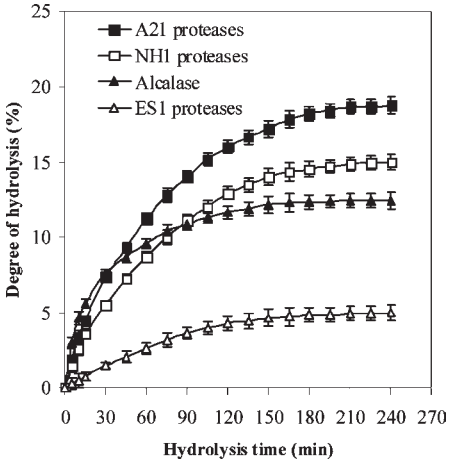

Figure 1. Hydrolysis curves of cuttlefish muscle proteins treated with different enzymes.

mode. At the time of the recording of the spectrum, 30 scans on average were added (MCA mode) for each spectrum. The gases used (nitrogen and air) were pure (up to 99%) and produced by a compressor Jun-Air 4000−40 M and a nitrogen generator Whatman model 75-72 (Whatman Inc., Haverhill, MA). Polypropylene glycol (PPG) was used for the calibration and the optimization of the machine. The peptide sequence was determined from the CID spectrum of the protonated analyze $[M + H]^+$ by MS/MS experiments. Peptide sequences were done using the bioanalyst software (Applied Biosystems, USA).

Stability of Ala-His-Ser-Tyr against Gastrointestinal Proteases. *In vitro* digestion of the potent purified ACE-inhibitory peptide (Ala-His-Ser-Tyr) was carried out according to the method of Kuba et al. (*18*). Peptide (0.2 mL, 1 mg/mL) was incubated with 0.05% pepsin (pH 2.0), chymotrypsin or trypsin (pH 8.0) for 3 h at 37 °C. In successive digestion test, the peptide was first incubated with pepsin for 3 h, evaporated in a centrifugal concentrator and then incubated for 3 h at 37 °C with both 0.025% (w/v) chymotrypsin and 0.025% (w/v) trypsin. The reactions were then heated for 5 min in boiling water to inactivate the enzyme. After enzymatic treatment, each sample was centrifuged and the supernatant was adjusted to pH = 8.3 and used for the ACE inhibitory activity determination.

Determination of ACE Inhibition Pattern. To clarify the inhibitory mechanism of the most potent peptide (Ala-His-Ser-Tyr) on ACE, different concentrations of the ACE inhibitory peptide were added to each reaction mixture according to the method of Wu and Ding (*19*), with slight modifications. The enzyme activities were measured with different concentrations of the substrate (HHL). ACE inhibitory pattern in the presence of the inhibitor was determined with Lineweaver−Burk plot (*20*).

Statistical Analysis. Statistical analyses were performed with Statgraphics ver. 5.1, professional edition (Manugistics Corp., USA) using ANOVA analysis. Differences were considered significant at $p < 0.05$. Correlation coefficient between degree of hydrolysis (DH) and ACE-inhibitory activity was performed by SPSS software using Pearson's correlation coefficient.

RESULTS AND DISCUSSION

Preparation of Cuttlefish Protein Hydrolysates Using Various Proteases. The specificity of the enzyme used for the proteolysis and process conditions greatly influenced the molecular weight and amino acid residue composition of cuttlefish protein hydrolysates and thus their ACE inhibitory activities (*21*). In the present study, to produce ACE inhibitory peptides, cuttlefish muscle was hydrolyzed with various enzymes: Alcalase, crude enzyme preparations from *B. mojavensis* A21, *B. licheniformis* NH1 and *A. clavatus* ES1. The proximate composition of the cuttlefish muscle showed that it had high protein content (80.67 ± 0.26%). The ash and lipid contents of the muscle were 9.91 ± 0.04 and 3.4 ± 0.1%, respectively.

Table 1. Degrees of Hydrolysis of Cuttlefish Muscle Proteins, ACE Inhibitory Activities and IC_{50} Values of CMPH Obtained with Various Protease Treatments

enzyme	DH (%)	ACE inhibn (%)	IC_{50} (mg/mL)
A21 proteases	18.7	70.00 ± 1.00	1.12 ± 0.07
NH1 proteases	15.0	49.32 ± 0.62	2.01 ± 0.21
Alcalase	12.5	51.47 ± 1.50	1.84 ± 0.11
ES1 proteases	5.0	22.57 ± 1.30	4.43 ± 0.36

The extent of protein degradation by proteolytic enzymes was estimated by assessing the DH. The hydrolysis curves of cuttlefish proteins with the different enzymes after 4 h of incubation are shown in **Figure 1**. The hydrolysis of cuttlefish proteins was characterized by a high rate of hydrolysis for the first 1 h. The rate of enzymatic hydrolysis was subsequently decreased, and then the enzymatic reactions reached the steady-state phase when no apparent hydrolysis took place.

The crude protease from *B. mojavensis* A21 was the most efficient (DH = 18.7%), followed by the crude enzyme from *B. licheniformis* NH1 (DH = 15%), while that of *A. clavatus* ES1 was the least efficient (DH = 5%). High hydrolysis degree obtained with crude proteases from NH1 and A21 strains could be due to the fact that these extracts contain multiple proteases which offer the ability to achieve higher degree of hydrolysis of cuttlefish proteins (*14*, *22*). However, in the case of *A. clavatus* ES1, Hajji et al. (*23*) reported the production of only one protease by this strain.

The shape of hydrolysis curves is similar to those previously published for hydrolysates from smooth hound (*24*), Atlantic salmon (*25*), silver carp (*26*) and sardinelle (*8*).

ACE Inhibitory Activity of Cuttlefish Muscle Protein Hydrolysates. ACE inhibitory activity of the four CMPHs is presented in **Table 1**. ACE inhibitory activity of hydrolysates at 2 mg/mL varied greatly from 22.6% to 70.0%. The difference between ACE inhibitory activities might be attributed to the different molecular weights and different amino acid composition of ACE inhibitory peptides present in the CMPHs.

The protein hydrolysate generated by the crude enzyme preparation from *A. clavatus* ES1 showed the weakest ACE inhibitory activity with an IC_{50} value of 4.43 ± 0.36 mg/mL, while that obtained by the crude enzyme preparation from *B. mojavensis* A21 exhibited the highest ACE inhibitory activity with an IC_{50} value of 1.12 ± 0.07 mg/mL. The obtained results clearly show that high ACE inhibitory activity was observed only with hydrolysates with high DH, demonstrating that low molecular weight peptides had more ACE inhibitory activity than high molecular weight peptides. This is in line with previous works reporting that low molecular weight peptides exhibited higher activity than high molecular weight peptides (*9*, *27*). In addition, the obtained results suggest that protein hydrolysate prepared by treatment with crude protease from A21 strain possibly contained more potent ACE inhibitory activity peptides.

The IC_{50} value of A21 cuttlefish protein hydrolysate is lower than those of hydrolysates from oyster, scallop, codfish skin, and codfish bone, which presented an IC_{50} greater than 10 mg/mL (*28*), whereas it is higher than those of β-conglycinin and glycinin hydrolysates (0.126 and 0.148 mg/mL, respectively) (*29*).

Effect of the Degree of Hydrolysis on ACE Inhibitory Activity. Since the extent of hydrolysis highly influences the recovery and the functionality of the proteins, it is imperative to control the hydrolysis of proteins. Thus, cuttlefish muscle proteins were hydrolyzed by the crude enzyme from *B. mojavensis* A21, and the degree of hydrolysis and the ACE inhibition activity were measured. As shown in **Figure 2**, no ACE-inhibitory activity was detected with the undigested cuttlefish muscle proteins, while

ACE inhibitory activity of hydrolysates increased with extended incubation time. Higher ACE inhibition ($p < 0.05$) activity (87.11 ± 0.92% at 2 mg/mL) was obtained with a DH of 16% (about 2 h) and further digestion resulted in a decrease in the inhibition level (**Figure 2**).

The degree of hydrolysis (DH) is an important indicator used to describe a protein hydrolysis reaction (*15*). Many studies have suggested the importance of DH relating to ACE inhibitory activity. In the present study, we found that ACE-inhibitory activity was positively correlated with DH value ($r = 0.8333$, $p < 0.001$) based on Pearson's correlation analysis (**Figure 3**), suggesting that, for cuttlefish muscle proteins, it is essential to reach a certain level of degree of hydrolysis to allow maximum release of active peptides from inactive protein precursors.

The above results indicated that the hydrolysate obtained by treatment with crude enzyme preparation from *B. mojavensis* A21

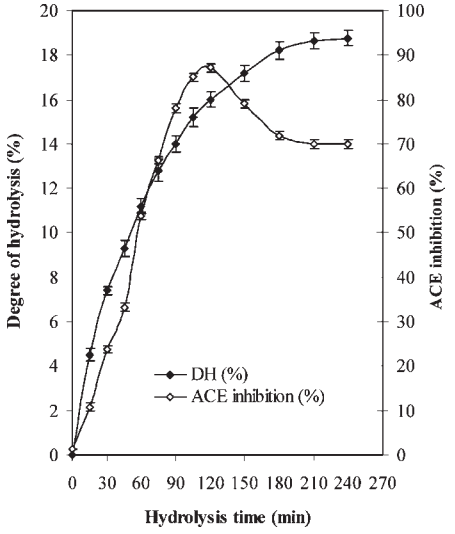

Figure 2. Comparison of ACE inhibitory activity and degree of hydrolysis of CMPH as a function of hydrolysis time.

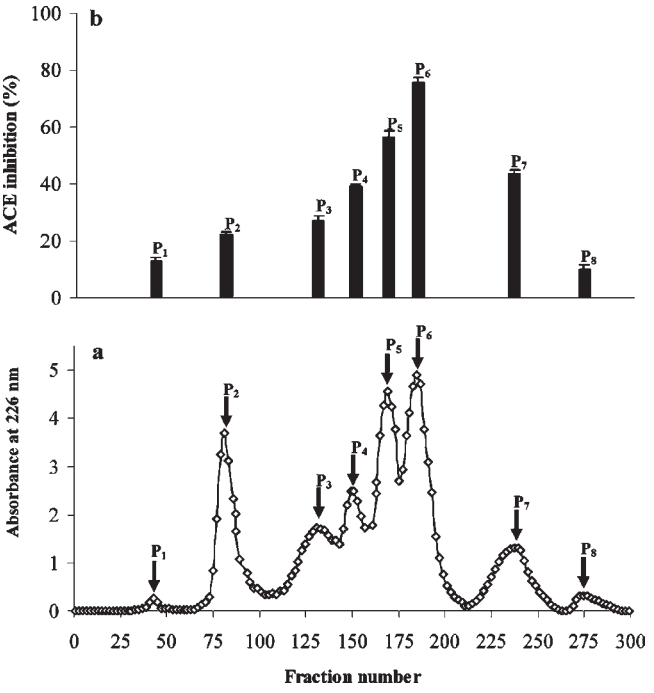

Figure 4. Elution profile of CMPH obtained with crude enzyme from *B. mojavensis* A21 separated by size exclusion chromatography on Sephadex G-25 (lower panel) and the ACE inhibitory activities of the separated fractions (P_1−P_8) (upper panel). Values presented are the means of triplicate analyses.

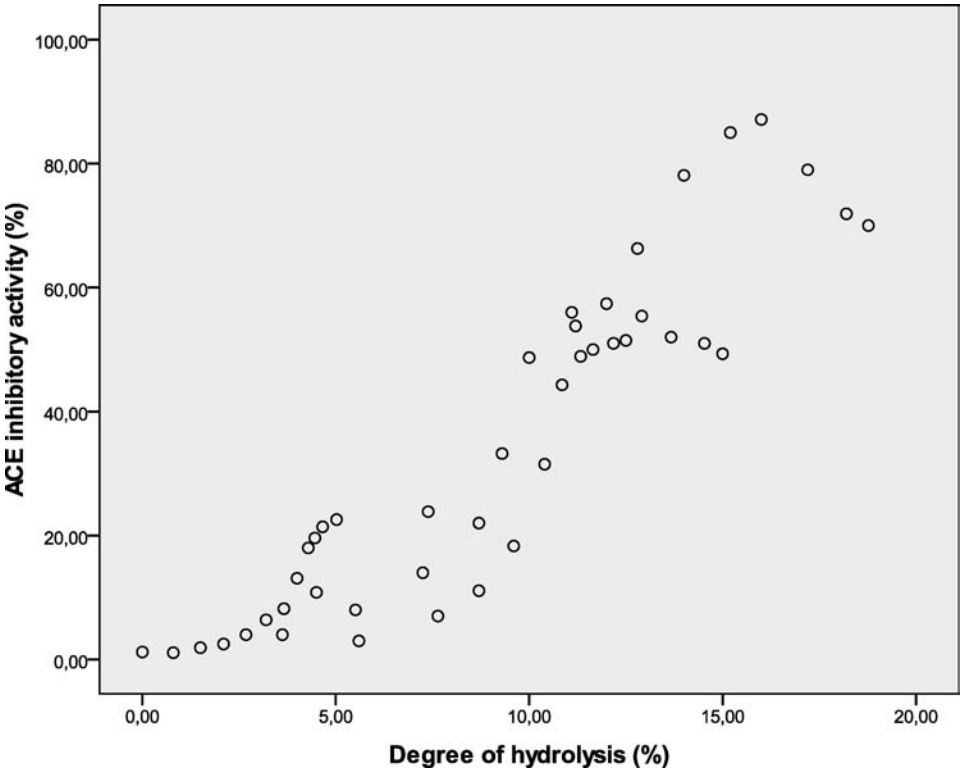

Figure 3. Relationship between degree of hydrolysis (DH) of cuttlefish muscle proteins treated with varied proteases and ACE-inhibitory activity based on Pearson's correlation analysis ($r = 0.8333$, $p < 0.001$).

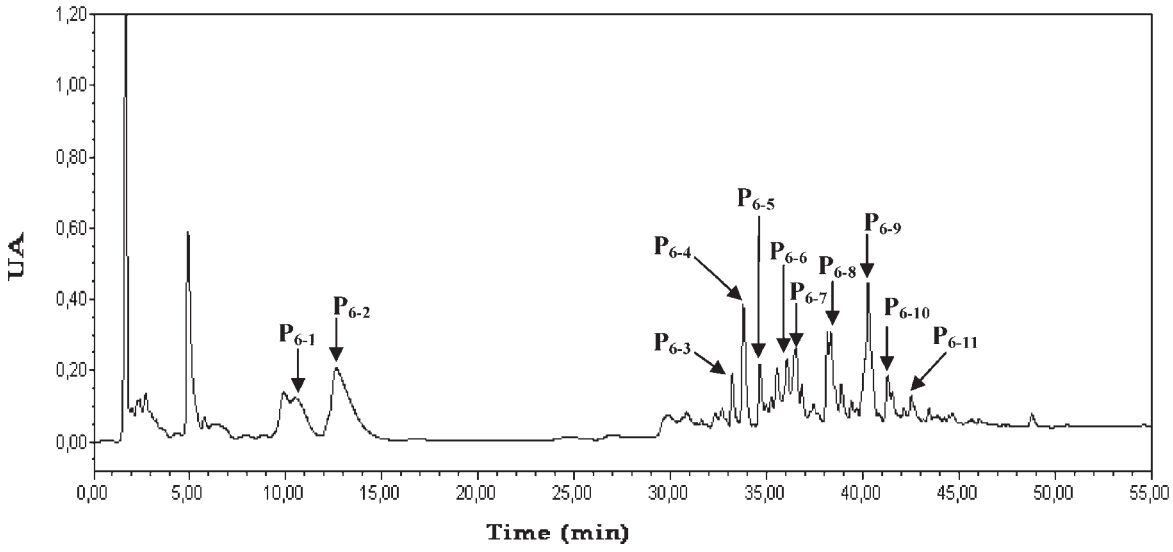

Figure 5. Purification of ACE inhibitory peptides in peak P_6 by reversed-phase HPLC chromatography on a Vydac C_{18} column (10 mm × 250 mm).

Table 2. Structure, Molecular Weight and IC_{50} Values of Peptides in Fraction P_6 from Gel Filtration Separated by RP-HPLC

fractions	mass $(M + H)^+$ in Da	sequence	IC_{50} value[a] (μM)
P_{6-1}	270.3	Gly-His-Gly	122.0
P_{6-2}	359.1	Gly-Asp-Ala-Pro	22.5
P_{6-3}	234.1	Ala-Gly-Ser	527.9
P_{6-4}	321.0	Ala-Gly-Ser-Ser	672.1
P_{6-5}	477.1	Ala-His-Ser-Tyr	11.6
P_{6-6}	331.2	Ala-Gly-Ser-Pro	37.2
P_{6-7}	520.1	Gly-Val-His-His-Ala	71.8
P_{6-8}	338.0	Asp-Phe-Gly	44.7
P_{6-9}	280.0	Phe-Gly-Gly	82.5
P_{6-10}	288.2	Ala-Val-Val	66.6
P_{6-11}	302.1	Ile-Ala-Val	153.4

[a] The IC_{50} value of each peptide is the mean of three experimental repeats.

possessed potent ACE inhibitory peptides. Therefore, this hydrolysate was selected for further study.

Isolation and Purification of ACE Inhibitory Peptides. The ACE inhibitory peptides, in cuttlefish muscle protein hydrolysates with a DH of 16%, treated with the *B. mojavensis* A21 proteases, were separated by gel filtration chromatography on a Sephadex G-25 column. As reported in **Figure 4a**, eight fractions were separated and designated as P_1−P_8. Each fraction was pooled and freeze-dried, and its ACE inhibitory activity was determined. All fractions (at 1 mg/mL) displayed ACE inhibitory activity as shown in **Figure 4b**. Of the eight fractions collected, fraction P_6 exhibited the highest level of ACE inhibitory activity (IC_{50} = 0.52 ± 0.03 mg/mL), followed by fraction P_5 (IC_{50} = 0.93 ± 0.07 mg/mL).

Fraction P_6 was further separated by RP-HPLC on a C_{18} column using a linear gradient of acetonitrile. As can be seen from the chromatographic profile at 214 nm, given in **Figure 5**, at least eleven subfractions designated $P_{6\text{-}1}$−$P_{6\text{-}11}$ were present in fraction P_6. These fractions were collected, and their ACE inhibitory activities were determined. Although ACE inhibitory activity was observed in all subfractions, fraction $P_{6\text{-}5}$ exhibited the highest inhibitory activity (IC_{50} value 0.0055 mg/mL) followed by $P_{6\text{-}2}$, $P_{6\text{-}6}$ and $P_{6\text{-}8}$ with IC_{50} values of 0.008, 0.0123, and 0.015 mg/mL, respectively. The other fractions showed moderate or very low ACE inhibitory activity.

Identification of the ACE Inhibitory Peptides. Peptides in subfractions $P_{6\text{-}1}$−$P_{6\text{-}11}$ were analyzed by ESI-MS for molecular mass determination and by ESI-MS/MS for amino acid sequences. Eleven peptides were identified, and their IC_{50} values were determined (**Table 2**). These inhibitors are novel peptides with ACE inhibitory activity that had never been reported. The amino acid sequences of the three potent ACE inhibitors were Ala-His-Ser-Tyr, Gly-Asp-Ala-Pro and Ala-Gly-Ser-Pro, and their IC_{50} values were 11.6, 22.5, and 37.2 μM, respectively.

According to previous reports on the structure−activity relationships between different peptide inhibitors of ACE (*30*), binding to ACE is strongly influenced by the C-terminal amino acid residue. Gobbetti et al. (*31*) reported that peptides with Trp, Tyr, Phe, Pro or hydrophobic amino acids at the C-terminal were effective for ACE inhibitory activity. Several identified ACE inhibitory peptides have a proline residue in the C-terminal position, but this is neither sufficient nor essential to confer activity. Further, Cheung et al. (*32*) indicated that ACE prefers competitive inhibitors that contain hydrophobic amino acid residues such as Pro, Phe, and Tyr at the three positions from the C-terminal. In this study, two of the three potent ACE inhibitory activity peptides identified had Pro at C-terminal and the most potent inhibitory peptide had Tyr at the C-terminal position. On the other hand, several studies reported that hydrophobic amino acid residues were preferred at the N-terminal position. Among the eleven identified peptides, nine peptides had Ala, Gly or Ile at the N-terminal.

Interestingly, Ala-His-Ser-Tyr peptide with Tyr residue at C-terminal was found to possess the strong ACE-inhibitory activity with an IC_{50} value of 11.6 μM. The ACE inhibitory activity of the most potent inhibitor was higher than Ileu-Trp-Glu (IC_{50}, 315.3 μM) and Ileu-Ala-Glu (IC_{50}, 34.7 μM) peptides from the sea cucumber (*Acaudia molpadiodea*) hydrolysate (*9*), and Leu-Val-Gln-Gly-Ser (IC_{50}, 43.7 μM) from fermented soybean extract (*33*).

The peptides with moderate ACE inhibitory activity had a high content of branched and/or aromatic amino acids (Pro, Glu, Val, Phe and Tyr) in their peptide sequences such as Asp-Phe-Gly and Gly-Val-His-His-Ala peptides with IC_{50} values of 44.7 and 71.8 μM, respectively. Ala-Gly-Ser and Ala-Gly-Ser-Ser showed only weak ACE inhibitory activity. The presumed reason for this low level of activity inhibition was that these peptides were not sufficiently hydrophobic for ACE inhibition and contained Ser, a hydrophilic amino acid, at the C-terminal.

Table 3. *In vitro* Stability of Ala-His-Ser-Tyr to Digestive Proteases

enzyme	ACE inhibn (%)
control	100
pepsin	100
trypsin	99.2
chymotrypsin	98.9
pepsin + trypsin + chymotrypsin	98.1

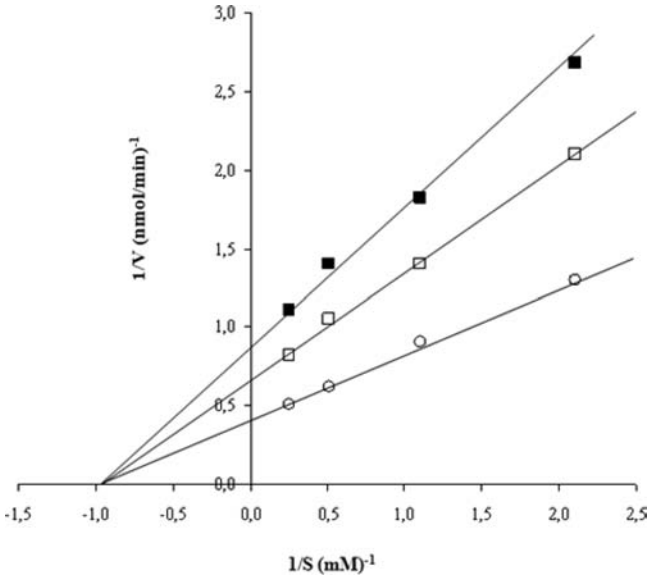

Figure 6. Lineweaver−Burk plot of the inhibition of ACE by the most potent purified peptide (Ala-His-Ser-Tyr) from cuttlefish muscle protein. ACE activity was measured in the absence or presence of inhibitory peptide (Ala-His-Ser-Tyr) as described in the text using HHL as the enzyme substrate (○, control; □, 25 μM; ■, 50 μM). 1/*V* and 1/*S* represent the reciprocal of velocity and substrate, respectively. Each point represents the mean value of three experiments.

Stability of Ala-His-Ser-Tyr against Gastrointestinal Protease. In order to exert an antihypertensive effect *in vivo*, the ACE inhibitory peptides must be absorbed in their intact form from intestine and further be resistant to plasma peptidase degradation to reach their target sites. Thus, to predict the antihypertensive effect *in vivo*, the stability of the most potent ACE inhibitory peptide, Ala-His-Ser-Tyr, against gastrointestinal proteases *in vitro*, was evaluated. As reported in **Table 3**, the peptide is stable, since incubation with gastrointestinal proteases did not change its activity ($p > 0.05$) (**Table 3**). Due to its digestion-resisting properties and long-lasting antihypertensive activity, this peptide may potentially be used as a functional food useful in prevention and/or treatment of hypertension.

Determination of ACE Inhibition Pattern of Ala-His-Ser-Tyr. The ACE inhibition pattern of the most potent ACE inhibitory peptide, Ala-His-Ser-Tyr, was investigated by Lineweaver−Burk plot (**Figure 6**). The kinetic study revealed that ACE inhibitor Ala-His-Ser-Tyr acts as a noncompetitive inhibitor. This indicates that the novel peptide cannot bind to the catalytic site of ACE and thus could not be hydrolyzed by the enzyme.

Although most of the reported peptides acted as competitive inhibitors for ACE, a few peptides inhibiting ACE activity in a noncompetitive manner have been reported, including Val-Gly-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe from algae protein waste (*34*), Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe from oyster (*Crassostrea talienwhanensis Crosse*) proteins (*35*), Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala from Alaska Pollack (*Theragra chalcogramma*) frame protein (*36*) and Ile-Phe-Leu and Trp-Leu from fermented soybean food (*18*). The inhibition site of the noncompetitive inhibitor on ACE was not specified, and the precise inhibition mechanism of ACE inhibitory peptide is also not yet clear.

Conclusion. ACE inhibitory peptides were selected from hydrolysates of continental proteins, and there were only a few reports on the identification of peptides from marine protein hydrolysates and in particular from invertebrate muscle. In the present study, cuttlefish muscle protein hydrolysates obtained by treatment with various proteases were found to possess ACE inhibitory activity. Among the four proteolytic preparations, crude enzyme from *B. mojavensis* A21, which contained multiple proteases, was the most suitable for the production of the hydrolysate with the highest ACE inhibitory activity.

By gel permeation chromatography on a Sephadex G-25, and reversed-phase HPLC, eleven ACE inhibitory peptides were purified from the muscle of cuttlefish hydrolysates with a DH of 16% and their sequences were identified by ESI-MS/MS and molecular weight analysis. The peptide Ala-His-Ser-Tyr displayed the highest ACE inhibitory activity with an IC_{50} of 11.6 μM. Further, this purified peptide maintained inhibitory activity even after incubation *in vitro* digestion with gastrointestinal proteases. The kinetic study revealed that the ACE inhibitor of the potent peptide acts as a noncompetitive inhibitor.

The obtained results show that cuttlefish muscle is a promising protein source for the production of ACE inhibitory peptides that could be utilized to develop functional foods for prevention of hypertension. Further work should be done to purify and identify ACE inhibitory peptides in the other fractions collected by gel filtration. In addition, potent ACE inhibitory peptides should be tested on spontaneously hypertensive rats to examine their properties and functions as antihypertensive compounds *in vivo*.

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