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Analysis of novel angiotensin-I-converting enzyme inhibitory peptides from protease-hydrolyzed marine shrimp *Acetes chinensis*

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Abstract: *Acetes chinensis* is an underutilized shrimp species thriving in the Bo Hai Gulf of China. In a previous study, we had used the protease from *Bacillus* sp. SM98011 to digest this kind of shrimp and found that the oligopeptide-enriched hydrolysate possessed antioxidant activity and high angiotensin I-converting enzyme (ACE) inhibitory activity with an IC_{50} value of 0.97 mg/ml. In this paper, by ultrafiltration, gel permeation chromatography and reversed-phase high-performance liquid chromatography (RP-HPLC), five peptides with high ACE inhibitory activity were purified from the shrimp hydrolysates and their sequences were identified by amino acid composition analysis and molecular weight (MW) analysis. Three of them, FCVLRP (a), IFVPAF (f) and KPPETV (j), were novel ACE inhibitory peptides. Their IC_{50} values were 12.3 μM, 3.4 μM and 24.1 μM, respectively, and their recoveries were 30 mg/100 g (solid basis of shrimp), 19 mg/100 g and 33 mg/100 g, respectively. Lineweaver-Burk plots for the three novel peptides showed that they are all competitive inhibitors. To test the ACE inhibitory activity of peptide a, f, j after they were digested by digestive enzymes *in vivo*, 12 derived peptides from FCVLRP and IFVPAF were synthesized based on their amino acid sequences and the cleavage sites of digestive enzymes. No digestive enzyme cleavage site was found in KPPETV. The IC_{50} values of the derived peptides were determined and the result showed that except for VPAF, FC and FCVL, the ACE inhibitory activity of the other nine derived peptides did not significantly change when compared with their original peptides. Surprisingly, five peptides had lower IC_{50} values than their original peptides, particularly for RP (IC_{50} value = 0.39 μM), which is about 30 times lower than its original peptide and almost the lowest IC_{50} value for ACE inhibitory peptides reported. Therefore, the novel peptides identified from *A. chinensis* hydrolysates probably still maintain a high ACE inhibitory activity even if they are digested *in vivo*. This is the first report about novel ACE inhibitory peptides from hydrolysates of marine shrimp *A. chinensis*. The novel peptides from hydrolysate of *A. chinensis* and some of their derived peptides with high ACE inhibitory activity probably have potential in the treatment of hypertension or in clinical nutrition. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: angiotensin-I-converting enzyme (ACE); *Acetes chinensis;* ACE inhibitory peptide; hydrolysates; purification; identification

INTRODUCTION

In recent years, food scientists have paid a great deal of attention to peptides from partial enzymatic hydrolysates of food proteins or the metabolites generated during intestinal digestion. Many biological peptides with health benefits, such as immune defense, uptake of nutrients, opioid activity, antihypertensive activity, antibacterial activity, mineral-binding activity and enhancement of intestinal activity, have been classified and identified from food protein hydrolysates [1–4]. These peptides, which are inactive within the sequence of the parent protein, are liberated during enzymatic digestion or food processing. Among these bioactive peptides, a variety of angiotensin-I-converting enzyme (ACE) inhibitory peptides with various amino acid sequences have been found in hydrolysates from food proteins digested with different proteases under different hydrolysis conditions, such as milk protein [5–8], soy-protein [9], egg protein [10], muscle protein [11] and fish protein [3,12]. ACE has been classically associated with the renin–angiotensin system, which regulates peripheral blood pressure via conversion of angiotensin I to angiotensin II. Inhibition of ACE may have an antihypertensive effect as a consequence of a decrease in blood pressure. Therefore, the ACE inhibitory peptides have potential in the treatment of hypertension. It is necessary to find new peptides with high ACE inhibitory activity.

There is a great variety and abundance of marine organisms and most of them are a good source of human food and drugs. However, a lot of these organisms have been underutilized. *Acetes chinensis* is an underutilized shrimp species thriving in the Bo Hai Gulf of China. Every year, about 300 000 tons of this shrimp is dried or made into sauce, which have a low commercial value. We have found that the oligopeptide-enriched hydrolysates from the marine *A. chinensis* digested with a protease produced

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by *Bacillus* sp. SM98011 possessed both antioxidant activity and high ACE inhibitory activity [13]. In the present study, the oligopeptides with high ACE inhibitory activity were isolated from hydrolysates of *A. chinensis* and were identified and characterized.

MATERIALS AND METHODS

Materials

A. chinensis, freshly harvested in the spring in China, were purchased from Chinese Dayudao Group. They were packed in polyethylene bags and stored at −20 °C before use. The protease SM98011 was extracted from the culture of *Bacillus* sp. SM98011 preserved in our lab.

Protease Preparation and Activity Assay

Bacillus sp. SM98011 was cultivated according to the previously reported method for 36 h [13]. After cultivation, the culture was centrifuged at 10 000*g* for 20 min at 4 °C. The supernatant was collected and was diluted by distilled water to 4000 U/ml before it was used for protein hydrolysis. The protease activity in the supernatant was determined by the previously reported method [14].

Acetes chinensis Hydrolysis

A. chinensis was hydrolyzed using the previously reported method [13]. Whole shrimps were minced by a mincer before hydrolysis. Fifty grams of minced *A. chinensis* and 10 ml of diluted protease solution (the activity is 4000 U/ml) were combined in a 250 ml Erlenmeyer flask and adjusted to pH 7.0. The reactor vessel was placed in a thermostatically controlled water-bath with constant agitation (160 rpm) at 50 °C for 5 h. The reaction was stopped by incubating the solution at 90 °C for 15 min and the resulting slurry was centrifuged at 9000 g for 20 min at 4 °C. The supernatant was freeze-dried and kept at 4 °C.

ACE Inhibitory Activity Assay

ACE inhibitory activity was assayed using the method described in Cushman and Cheung, modified by Nakamura *et al.* [15,16]. Each 300 μl assay mixture contained the following components: 0.02 mM sodium borate buffer (pH 8.3), 0.06 M NaCl, 1 μM Hip-His-Leu (Sigma), 35 μl of peptide solution and 2 mU ACE (Sigma). After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 20 μl 0.1% trifluoroacetic acid (TFA). Released hippuric acid was quantified by reversed-phase high-performance liquid chromatography (RP-HPLC) onto a Symmetry (Waters, Milford, MA, USA) C18 column (21 × 150 mm). Detection was performed at 228 nm [6]. The amount of hippuric acid liberated from Hip-His-Leu under test conditions without an inhibitor was defined as 100% ACE activity. The IC_{50} value of ACE inhibitor was expressed as the amount of hydrolysates or peptide fraction needed to inhibit 50% of the original ACE activity.

Purification of Peptides from the Hydrolysates of *Acetes chinensis*

Peptides in the hydrolysates of *A. chinensis* were purified with Arihara's method with some modification [11]. Peptides with molecular weight (MW) more than 3000 Da in *A. chinensis* hydrolysates supernatants were removed by ultra filtration with an Amicon PM-3 membrane. (Millipore Co.Bediord, USA). The filtrate was then separated on a Sephadex G-15 column (2.5 × 150 cm, medium Pharmacia) eluted with deionized water at a flow rate of 30 ml/h and fractions of 5 ml were collected. Each gel chromatographic profile was obtained by monitoring the absorbance at 220 nm. The active fractions were collected and prepared for further purification.

The active fractions were fractionated by RP-HPLC on a Shim–Pack VP-ODS column (150 × 4.6 mm). Elution was performed with a linear gradient system from solvent A (0.1% TFA in distilled water) to solvent B (0.1% TFA in methanol) for 60 min at a flow rate of 1 ml/min, and absorbance was detected at 214 nm. The active fraction was lyophilized, dissolved in distilled water, and rechromatographed under the same conditions as described earlier.

Amino Acid Sequence Analysis and Peptide Synthesis

The amino acid sequences of the ACE inhibitory peptides were determined by Gobbetti's method [5]. Peptides were hydrolyzed in 6 N HCl at 110 °C for 22 h. The amino acid analysis was carried out using the automatic aminoanalyser, HITACHI 835. The molecular formulas of peptides obtained by chromatography were confirmed by a triple quadrupole mass spectrometer (PE SCIEX API 3000 LC/MS-MS systems).

Peptides were synthesized using a solid-phase method on a CS536 peptide synthesizer (CS Bio Company, USA) by CL.(XIAN)Bio-Scientfic.Co., LTD. The purity of the synthesized peptides was greater than 95% as determined by HPLC analysis.

RESULTS

Purification of ACE Inhibitory Peptides from the Hydrolysates of *Acetes chinensis*

Our previous study showed that the untreated *A. chinensis* extract exhibited low ACE inhibitory activity (IC_{50} = 103 mg/ml). When *A. chinensis* was digested by protease SM98011, the ACE inhibitory activity of the hydrolysates (IC_{50} = 0.97 mg/ml) was markedly increased and the total recovery of peptides in the hydrolysates was 41.7% (w/w solid basis of shrimp). These results indicated that ACE inhibitory peptides might be rich in the hydrolysates of *A. chinensis* digested by the protease SM98011 [13]. According to these results, the hydrolysates of *A. chinensis* were made using the same method as in our previous study and were used to isolate peptides with high ACE inhibitory activity. Since most of the ACE inhibitory peptides reported have low MW [17], *A. chinensis* hydrolysates were ultrafiltered through an

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ultrafiltration membrane with a MW cut-off of 3000 Da to enrich specific peptide fractions. The total recovery of peptides in the ultrafiltrate was 10.2% (w/w solid basis of shrimp) for the whole procedure based on protein. The ACE inhibitory activity of the ultrafiltrate ($IC_{50} = 0.22$ mg/ml) was much higher than that of the hydrolysates, indicating that the MW of most of the ACE inhibitory peptides in the hydrolysates should be less than 3000 Da.

The ultrafiltrate was further separated by gel permeation chromatography on a Sephadex G-15 column (2.5 × 150 cm, medium Pharmacia) and nine peaks were eluted. The fractions were designated as peak I–IX (Figure 1). Each peak was pooled and concentrated by lyophilization and its ACE inhibitory activity was assayed. The ACE inhibitory activities of peak II, peak III and peak IV (IC_{50}value = 0.11, 0.059, 0.096 mg/ml, respectively) were markedly higher than that of other fractions (Figure 2). Therefore, they were further purified by RP-HPLC. The fractions of peak II, peak III and peak IV were pooled, and concentrated by lyophilization. The elution profiles of the peaks are shown in Figure 3. Sixteen peaks (a ~ p + q) were obtained and the ACE inhibitory activity of each fraction was determined at the same primary content (Figure 3). The peptides in peak a, f, j, m and n produced an ACE inhibition greater than 70% and the ACE inhibitory activities of the peptides in the other peaks were all lower than 30% (Figure 4). Therefore, the peptides in peak a, f, j, m and n were identified and characterized.

Identification of ACE Inhibitory Peptides

The purified peptide a, f, j, m and n were hydrolyzed in 6 N HCl and their amino acid composition was analyzed. The ion peak of each inhibitor appeared at m/z of the theoretical value in the ESI-MS/MS. The structures of peptides were identified using protein sequencing (Table 1). Thus, the amino acid sequences of peptide a, f, j, m and n are Phe-Cys-Val-Leu-Arg-Pro (a), Ile-Phe-Val-Pro-Ala-Phe (f), Lys-Pro-Pro-Glu-Thr-Val (j), Tyr-Leu-Leu-Phe (m) and Ala-Phe-Leu (n), respectively. Hexapeptide a, f, and j are three novel peptides with ACE inhibitory activity that had never been reported. Tripeptide n and tetrapeptide m had been separated from the hydrolysates of microalgae and milk, respectively [18,19].

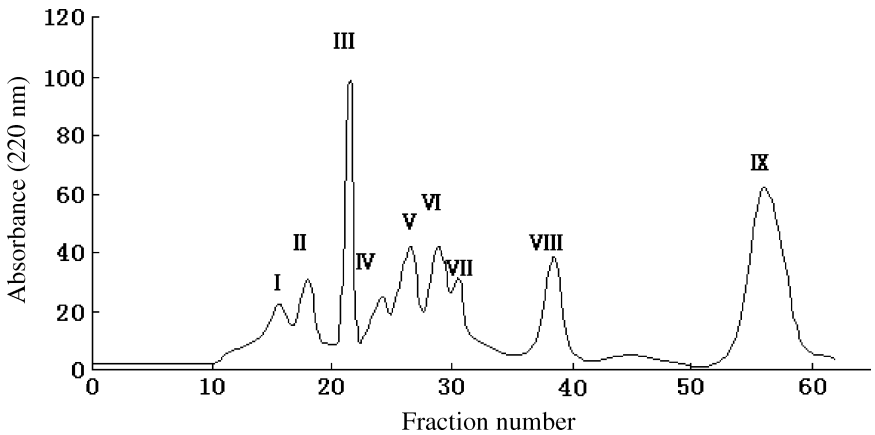

Figure 1 Separation of the peptide fractions of the ultrafiltrate on a Sephadex G-15 column. The fraction was eluted with distilled water at a flow rate of 30 ml/h. The data are represented and expressed as the mean value of three experiments.

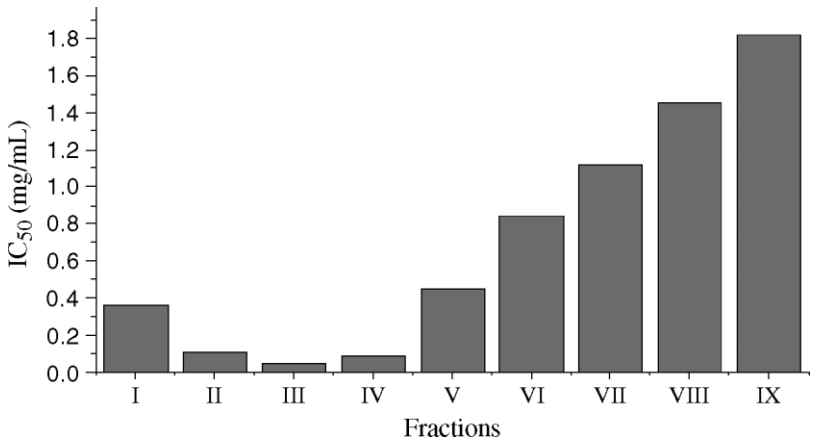

Figure 2 The IC_{50} values of fraction peaks I–IX. Each panel represents the mean value of three experiments.

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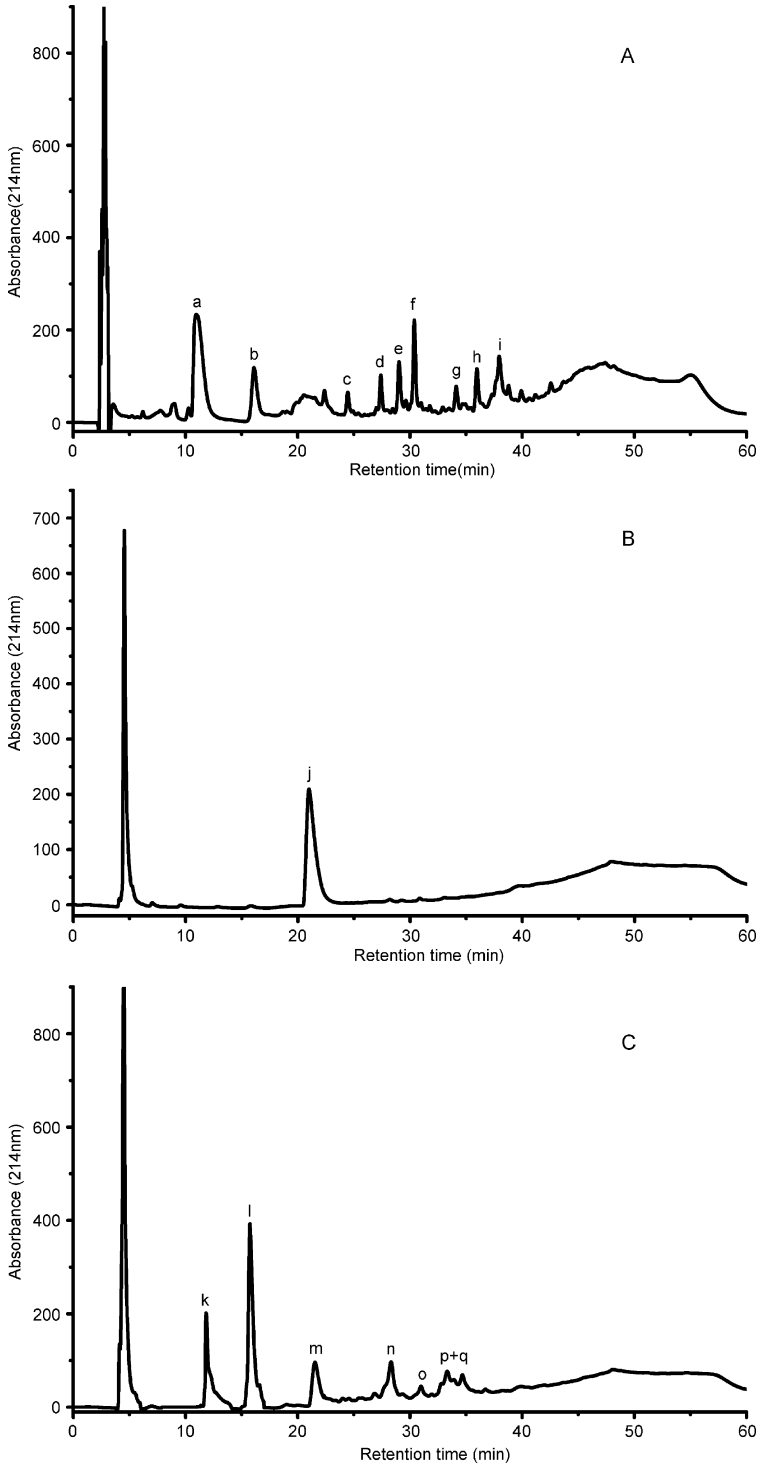

Figure 3 The purification of ACE inhibitory peptides from the elution of peak II (A), peak III (B) and peak IV (C). Reversed-phase HPLC chromatography of the elution peak II, peak III and peak IV on a ODS column (4.6 × 150 mm) using a gradient elution from 0to 60% methanol in 0.1% trifluoroacetic acid (TFA) for 60 min at a flow rate of 1.0 ml/min and the elution was monitored at 214 nm.

ACE Inhibitory Activity and Inhibition Mechanism of Three Novel Peptides

The IC_{50} values of the novel peptides were determined after logarithmic linearization (Figure 5). The ACE inhibitory activities of peptide a, f and j were found to be dose-dependent with an IC_{50} value of 12.3 μM, 3.4 μM and 24.1 μM, respectively (Table 1), indicating that peptide f had a higher ACE inhibitory activity than the others.

To clarify the inhibitory mechanism kinetically, Lineweaver–Burk plots were determined for the three

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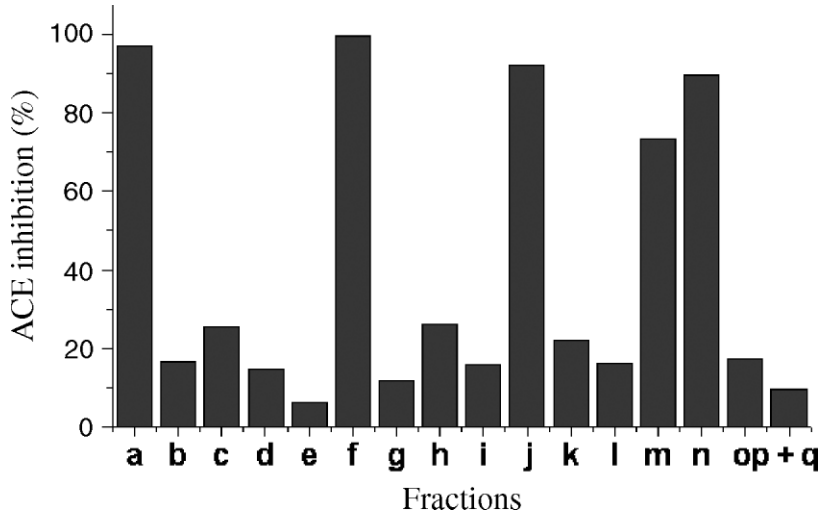

Figure 4 ACE inhibitory activity of fractions separated by HPLC. Each panel represents the mean value of three experiments.

Table 1 Analytical data of peptides isolated from hydrolysates and their ACE inhibitor activity

Peptide	Sequence	Amino acid ratio in HCl hydrolysates (n M)	Mass/ charge	IC_{50} (μM)	Recovery (mg/100 g {solid shrimp})
a	Phe-Cys-Val-Leu-Arg-Pro	Phe1.03, Cys1.12, Val1.08, Leu 0.96, Arg 1.01, Pro 1.14	734	12.3	30
f	Ile-Phe-Val-Pro-Ala-Phe	Ile 1.05, Phe 1.98, Val 1.11, Pro 0.97, Ala 1.04	693	3.4	19
j	Lys-Pro-Pro-Glu-Thr-Val	Lys 1.14, Pro2.12, Glu 1.02, Thr0.96, Val 1.01	671	24.1	33
m	Tyr-Leu-Leu-Phe	Tyr 1.03, Leu2.07, Phe0.94	473	172	14
n	Ala-Phe-Leu	Ala1.08, Phe1.14, Leu0.92	349	65.2	12

novel peptides. These plots demonstrated that they were competitive inhibitors, with an intercept on the 1/S axis (Figure 5). These plots indicated that the novel peptides bind to the catalytic site of ACE and they could be hydrolyzed by ACE.

ACE Inhibitory Activity of Synthetically Derived Peptides

When peptides are absorbed from the intestinal tract *in vivo*, digestive enzymes, such as trypsin, pepsin and chymotrypsin might break them down. To test the ACE inhibitory activity of peptide a, f, and j after they were digested by digestive enzymes *in vivo*, seven fragment-peptides derived from peptide a and five fragment-peptides derived from peptide f were synthesized based on their amino acid sequences and the cleavage sites of digestive enzymes, respectively. No peptide was derived and synthesized from peptide j because no digestive enzyme cleavage site was found in peptide j. The IC_{50} values of the synthetic peptides were measured and compared with the original peptide of a and f, respectively (Table 2). Except for VPAF, FC and FCVL, the ACE inhibitory activity of the other nine derived peptides did not significantly change when compared with their original peptides. Five derived peptides had lower IC_{50} values than their parental peptides, particularly for VLRP and RP (IC_{50}value = 0.89 μM and 0.39 μM) whose IC_{50} values were about 13 times and 30 times lower than that of their original peptide, respectively. These results indicated that the novel peptides identified from *A. chinensis* hydrolysates even if they were digested *in vivo*, probably still possessed high ACE inhibitory activity.

DISCUSSION

There have been many studies on the ACE inhibitory activity of peptides derived from food proteins by enzymatic hydrolysis. Most of them are from terrestrial protein hydrolysates and information on the ACE inhibitory activities and hypertensive activities of marine proteins is relatively less. *A. chinensis* is an underutilized shrimp species thriving in the Bo Hai Gulf of China. When it was digested by the crude protease from *Bacillus* sp. SM98011, the hydrolysates of *A. chinensis* shrimp, with high concentration of peptides, had high ACE inhibitory activity with an IC_{50} value of 0.97 mg/ml, which was relatively low when compared

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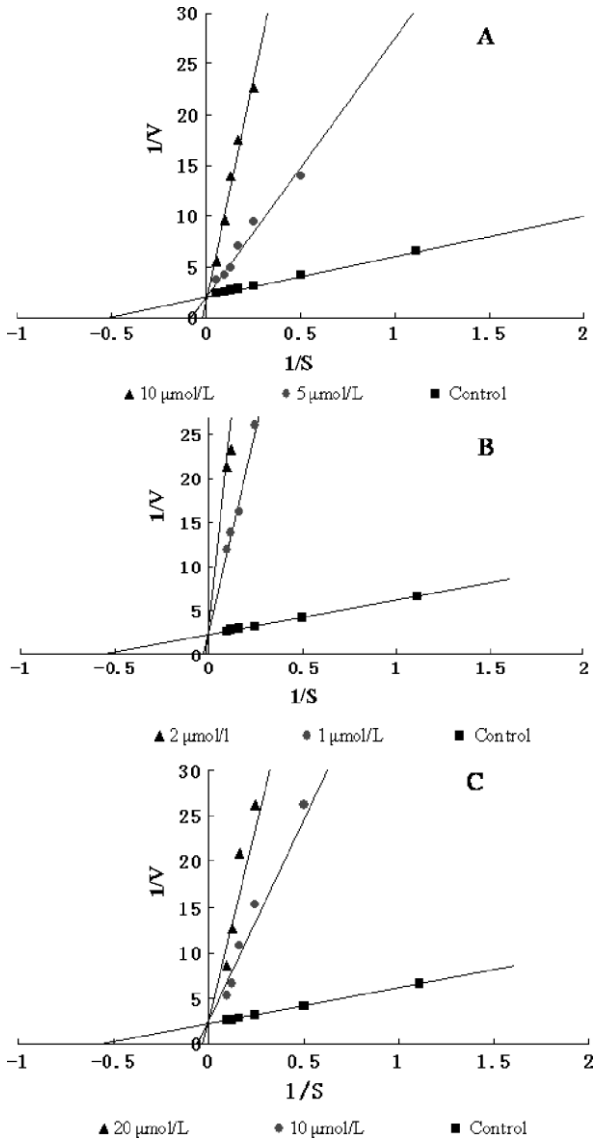

Figure 5 Lineweaver–Burk plots of the inhibition of ACE by peptide a (A), f (B) and j (C). Each point represents the mean value of three experiments.

with the IC_{50} values of proteolytic hydrolysates in the range 0.18 ~ 246.7 mg/ml in other reports [20,21]. Thus, in the present study, this protease SM98011 was used to digest *A. chinensis* to produce ACE inhibitory peptides, and the peptides with high ACE inhibitory activity in the hydrolysates were purified and identified.

Five peptides with high ACE inhibitory activity were purified from the *A. chinensis* hydrolysates and identified. FCVLRP (a), IFVPAF (f) and KPPETV (j) were novel ACE inhibitory peptides that had never been reported, whereas Tyr-Leu-Leu-Phe and Ala-Phe-Leu had been previously separated from the hydrolysates of microalgae and milk, respectively [18,19]. According to previous reports, proline residues are abundant in ACE inhibitory peptides and are found in antepenultimate or *C*-terminal positions, such as YPER, FALPQY, LKPNM, ALPHA, FFVAP and VPP with IC_{50} values of 132, 4.3, 17, 10, 6 and 9 μM, respectively [6,12,22]. The three novel ACE inhibitory peptides identified in this study contained at least one proline residue. The proline residues were known to increase resistance to proteolysis and the proline residue in peptides might protect them from degradation *in vivo* [23]. It was expected that these proline residue-containing peptides with high ACE inhibitory activity probably had sufficient antihypertensive properties *in vivo*. In addition, peptides FCVLRP, IFVPAF and KPPETV had hydrophobic amino acids at the *N*- and *C*-terminals. Thus, these hexapeptides might have potential value as ACE inhibitory peptides. The ACE inhibitors identified in the hydrolysates have an IC_{50} in the micromolar level, whereas traditional inhibitors such as Captopril have an IC_{50} value of 22 n M [24]. Although synthetic ACE inhibitors, including Captopril, Enalapril and Listinopril are remarkably effective as antihypertensive drugs, however, they inevitably cause adverse side effects [25]. In contrast, the ACE inhibitory peptides from protein hydrolysates have little side effects in spite of their IC_{50} in the micromolar level. In this study, ACE inhibitory peptides was found in hydrolysates of food protein sources like *A. chinensis*. They can be used as a food supplement to control hypertension.

Table 2 The IC_{50} values of the synthetic fragment-peptides

Peptides	Molecular weight (Da)	IC_{50} value (μM)
Phe-Cys-Val-Leu-Arg-Pro	734	12.3
Cys-Val-Leu-Arg-Pro	587	6.46
Val-Leu-Arg-Pro	483.7	0.89
Phe-Cys-Val-Leu	481	19.8
Cys-Val-Leu	334	6.8
Arg-Pro	271	0.38
Val-Leu	230	3.9
Phe-Cys	268	478.6
Ile-Phe-Val-Pro-Ala-Phe	693	3.4
Ile-Phe-Val-Pro-Ala	545	3.9
Phe-Val-Pro-Ala-Phe	580	4.5
Val-Pro-Ala-Phe	433	575.4
Val-Pro-Ala	285	4.4
Ile-Phe	278	4.2
Lys-Pro-Pro-Glu-Thr-Val	671	24.1

To test the ACE inhibitory activity of the three novel peptides after they were digested by digestive enzymes *in vivo*, 12 fragment-peptides containing part of the original peptide sequences were synthesized according to the cleavage sites of digestive enzymes *in vivo*. It was surprising that except for VPAF, FC and FCVL, cleavage of part of the peptide sequence did not significantly alter their inhibitory activities. More surprisingly, five derived peptides had very low IC_{50} values, particularly dipeptide RP with an IC_{50} value of 0.39 μM (almost the lowest IC_{50} value of ACE inhibitory peptides), which was about

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30 times lower than its original peptide. Thus, it was expected that these fragment-peptides would have effect on hypertension *in vivo*. The results from the present study indicated that even if the hexapeptides were cleaved into small fragments by enzymatic digestion *in vivo*, they still possessed high ACE inhibitory activity.

ACE inhibitory peptides were selected mainly from hydrolytic products of continental proteins [26–32], and there were only a few reports on the selection of peptides from enzymatic products of marine proteins [33]. There is a wide variety of proteins in the ocean, including those from fish, shrimp, seashells, algae and biological waste from seafood. The compositions and primary sequences of amino acids in marine proteins are much different from continental proteins and thus, the efficient value-added utilization of marine protein resources is a significant topic in marine biotechnology. *A. chinensis* shrimp is an edible seafood in China, but with a low commercial value. This study demonstrated that many kinds of ACE inhibitory peptides could be generated from *A. chinensis* hydrolysates digested with the protease produced by *Bacillus* sp. SM98011. This might be due to the fact that ACE inhibitory peptides generated from *A.chinensis* is complex of many kinds of proteins. Most of the reported bioactive peptides were isolated from the enzymatic hydrolysates of one protein. Here, three novel ACE inhibitory hexapeptides were purified from *A. chinensis* hydrolysates and their ACE inhibitory activity and inhibition mechanism were characterized. The roles of these ACE inhibitory peptides in hypertension treatment or in clinical nutrition should be further investigated.

Acknowledgements

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