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Full Length Research Paper

Purification and characterization of angiotensin-1 converting enzyme (ACE)-inhibitory peptide from the jellyfish, *Nemopilema nomurai*

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The *Nemopilema nomurai* hydrolysate was produced by the reaction of papain, and an angiotensin-I converting enzyme (ACE)-inhibitory peptide was purified by using the molecular cut-offs membrane filter, the gel filtration chromatography with Sephadex LH-20 and the reverse phase chromatographic method using C_{18} and C_{12} columns. Purification yield of the active peptide was estimated to be 0.2 ± 0.1%, starting from the lyophilized jellyfish. The infrared (IR), proton nuclear magnetic resonance spectroscopy (1H NMR), carbon nuclear magnetic resonance (13C NMR) and mass spectrometry (MS) spectrometer analyses elucidated that the structure of the purified peptide is tyrosine-isoleucine (Tyr-Ile). The inhibitory concentration at 50% (IC_{50}) and K_i values were calculated to be 2.0 ± 0.3 μg/ml and 3.3 ± 0.3 μM, respectively, which acts as a competitive inhibitor to ACE.

Key words: Angiotensin-I converting enzyme, Jellyfish, *Nemopilema nomurai*, Papain hydrolysate, Tyrosine-Isoleucine.

INTRODUCTION

Angiotensin-I converting enzyme (ACE, EC 3.4.15.1) is a circulating enzyme that participates in the body's rennin-angiotensin system and plays an important physiological role in regulating blood pressure. ACE acts as an exopeptidase that cleaves dipeptides from the C-terminus of various oligopeptides. It converts the inactive form of decapeptide (angiotensin-I) to potent vasoconstrictor - an octapeptide (angiotensin-II) - and inactivates the catalytic function of bradykinin, which has a depressor action (Richard et al., 2004). Recently, many ACE-inhibitory peptides have been isolated and characterized from various protein hydrolysates such as cheese (Smacchi and Gobbetti, 1998), milk protein (Gobbetti et al., 2000), egg white (Miguel et al., 2007), plant protein (Dziuba et al., 1999), meat (Jang and Lee, 2005) and marine resources (Je et al., 2005). Various ACE-inhibitory peptides have also been isolated from fish protein such as sardine (Matsui et al., 1993), tuna (Kohama et al., 1988) and cod (Kim et al., 2000). Protein hydrolysates are usually produced by gastrointestinal enzymes (for example, pepsin or pancreatin) or microbial enzymes [for example, Protamex (Novozymes)] under different conditions. Active peptides in the hydrolysates are, in general, inactive within the sequence of parent protein, but they can be released during gastrointestinal digestion or food processing. These peptides that are released from enzymatic hydrolysis exert an ACE-inhibitory activity and many physiological effects in the body (Shahidi and Zhong, 2008).

Nemopilema nomurai belongs to a giant Jellyfish species that have been blooming on the offshore areas of Korea, China and Japan in the last several years. Most of the fisheries dislike Jellyfish because it is not only difficult

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to winnow fishes from the bycaught jellyfishes but jellyfish toxins also decrease the quality of the fishes caught (Lee et al., 2007). Traditionally, Jellyfish has been eaten to treat asthma and hypertension for a long time in the East Asian countries. Salted Jellyfish has been one of the seafood delicacies owing to its unique textures for more than a thousand years. Although, Jellyfish has been traditionally used for hypertensive therapy, information on its active compound(s) is lacking.

Thus, the purpose of this study was to produce, isolate and purify the ACE-inhibitory peptides from *N. nomurai*. We hydrolyzed *N. nomurai* Jellyfish with papain, and then ACE-inhibitory peptide was purified to determine the functional properties and assess its possible application as an antihypertensive therapy. The structure of purified peptide was elucidated by the methods of the infrared (IR), proton nuclear magnetic resonance spectroscopy (1H NMR), carbon nuclear magnetic resonance (13C NMR), and mass spectrometry (MS) spectrometer analyses.

MATERIALS AND METHODS

Materials

A giant Jellyfish, *N. nomurai*, was caught in the southern area of Jeju Island, Korea. Only the umbrella (mesogloea) was collected, washed with deionized water and then kept at -23°C until required. ACE from rabbit lung, a substrate hipuryl-L-histidyl-L-leucine (Hip-His-Leu), trifluoroacetic acid (TFA), acetonitrile (CH_3CN) and papain were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sephadex LH-20 (Amersham Pharmasia Biotech, Tokyo, Japan) used for gel filtration chromatography, and C_{18} and C_{12} columns used for the reverse-phase high performance liquid chromatography (HPLC) were obtained from Phenomenex Inc. (Torrance, CA, USA). Other reagents applied here are all reagent grade and used without purification.

Determination of ACE-inhibitory activity

The ACE-inhibitory activity was determined using a modified method of Cushman and Cheung (1971). The standard reaction mixture contained 5 mM Hip-His-Leu as a substrate, 0.3 M NaCl and 5 mU ACE in 50 mM sodium borate buffer (pH 8.3). For the assay, each separated sample (50 µl) was added to the enzyme solution (50 µl) and then mixed with 8.3 mM Hip-His-Leu (150 µl) containing 0.5 M NaCl to obtain the same concentration as the standard reaction mixture. After incubation at 37°C for 30 min, the reaction was stopped by addition of 1.0 N HCl (250 µl). The resulting hippuric acid was extracted by the addition of 1.5 ml ethyl acetate. After centrifugation (800 g, 15 min), 1 ml of the upper layer was transferred to a new glass tube and evaporated at 60°C for 30 min in vacuum. The extracted hippuric acid was dissolved in 3.0 ml of distilled water, and the absorbance was measured at 228 nm using a spectrophotometer (Model U-3210, Hitachi Co., Japan).

Production of Jellyfish hydrolysate

The papain hydrolysis of the Jellyfish was conducted under the following conditions. First, the Jellyfish samples were lyophilized to remove excess water and then used for hydrolysis. Enzyme reaction including 0.2% (w/v) papain was carried out for 4 h using the 10% (w/v) lyophilized Jellyfish at 60°C and pH 6.0, and then it was stopped by heat treatment at 90°C for 15 min. The resultant slurry was centrifuged at 3,000 *g* for 10 min, and the supernatant was lyophilized and then used for analysis. The degree of hydrolysis was defined as the proportion (%) of α-amino nitrogen with respect to the total-N in the samples (Taylor, 1957).

$$\text{Degree of hydrolysis (\%)} = (h/h_{tot}) \times 100$$

Where, h_{tot} is the amount of total-N of lyophilized sample, and h is α-amino nitrogen amount of the jellyfish hydrolysates.

Isolation and purification of ACE-inhibitory peptide

The papain hydrolysate showing the biggest inhibitory activity was selected and the resultant hydrolysate was fractionated through the Amicon Millipore membrane (1 kDa cut-offs; Amicon Co., Beverly, MA). The resultant fraction (F2) was lyophilized and then used for purification. The fraction (F2) was further purified using a Sephadex LH-20 (25 × 250 mm) gel filtration chromatography eluting with 30% methanol solution at a flow rate of 0.5 ml/mim. Then, a reverse-phase HPLC was conducted by eluting with a linear gradient of MeOH-H_2O [A eluent; H_2O:MeOH:TFA = 90:10:0.1 (v/v/v), B eluent; MeOH:H_2O:TFA = 90:10:0.1 (v/v/v)] at a flow rate of 2 ml/min (Phenomenex, C_{18} 5 µm Agilent's New Octadecylsilane (ODS) 3100A, 10 × 250 mm, ultraviolet (UV) detection at 214 nm). To obtain a pure peptide, a Jupiter Proteo C_{12} column (90 Å, 10 µm, 21.2 × 150 mm) was used at a flow rate of 2 ml/min with a linear gradient of CH_3CN-H_2O [A eluent; H_2O:CH_3CN:TFA = 95:5:0.1 (v/v/v), B eluent; CH_3CN:H_2O: TFA = 55:45:0.08 (v/v/v)].

Structure elucidation of the purified peptide

The IR and UV spectra were obtained using a Bruker IFS88 and a Thermo Electron 9423B UV/Vis spectrophotometer. The NMR spectrum was recorded on a JEOL JNM ECP-400 (400 MHz for 1H, 100 MHz for 13C) spectrometer (JEOL Ltd., Akishima, Tokyo, Japan). Chemical shift (*δ*) values were expressed in ppm and were referenced to the residual solvent signals with resonances at *δ*H/*δ*C, 7.26/77.0 ($CDCl_3$). To calculate the molecular weight (Mw), the purified sample was processed on an Agilent 1100 LC/MSD spectrometer (Agilent Tech., CA, USA) with direct injection onto an electrospray interface in the positive or negative mode.

Kinetic analysis

The inhibitory concentration at 50% (IC_{50}) value of the purified peptide was determined by the standard method, and it was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity. For Lineweaver-Burk polt, 0 to 6 µg/ml of the purified peptide was added to the reaction solution as an inhibitor, and the inhibition pattern and K_i were estimated using the program of Grafit 5.0 (Surry, RH6 9YJ, UK).

RESULTS AND DISCUSSION

Production and isolation of the active fraction

After 4 h digestion with 0.2% (w/v) papain, the hydrolysis rates of jellyfish mesogloea reached 12.2 ± 1.6%. The ACE-inhibitory activity of the hydrolysate was notably

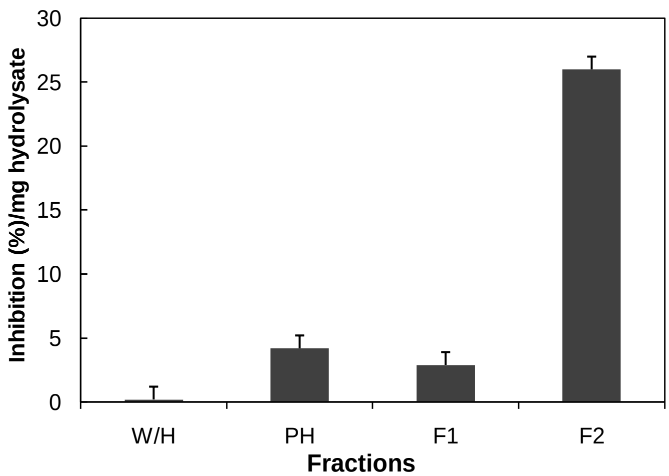

Figure 1. Angiotensin-I converting enzyme (ACE)-inhibitory activity of the fractionated Jellyfish hydrolysate. The resultant hydrolysate was fractionated using amicon millipore membrane filters (1 kDa cut-offs), then the inhibitory activity was determined by the standard method with the sample concentration of 1 mg hydrolysate/ml. W/H, without hydrolysis; F1, >1 kDa; F2, <1 kDa.

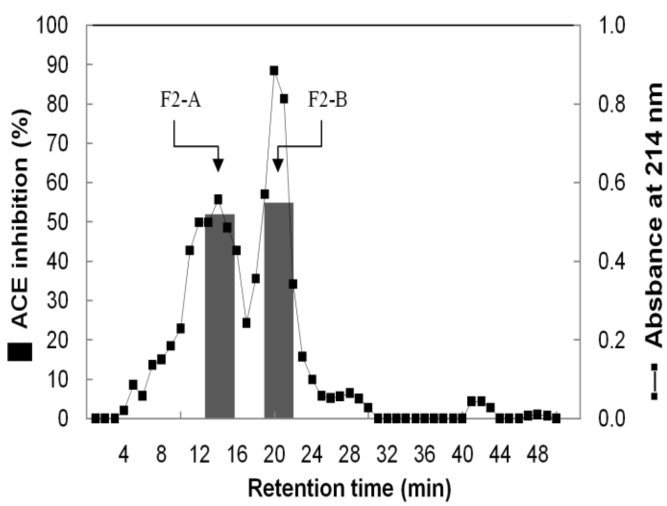

Figure 2. Gel filtration chromatography of the fraction F2 on Sephadex LH-20. Separation was performed with 30% aqueous methanol solution at a flow rate of 0.5 ml/min and collected with fraction volume of 5 ml. The fraction corresponding to F2-B was collected and the inhibitory activities was determined using the sample concentration of 200 µg/ml. ■─■, absorbance at 214 nm; ▌, ACE inhibition (%).

increased as much as 4.7 ± 0.5%/mg hydrolysate although a negligible activity (<0.2%/mg hydrolysate) was detected in the intact Jellyfish (Figure 1). The papain hydrolysate was fractionated using an ultrafiltrating membrane of 1 kDa molecular cut-offs, then the activity was determined. The inhibitory activity of the resulting fraction (F2, <1 kDa) showed the activity of 26 ± 1.3%/mg hydrolysate, whereas the other fraction (F1, >1 kDa) showed an activity as low as 2.9 ± 0.4%/mg hydrolysate (Figure 1). These findings led us to assume that the most active compounds were contained in the fraction F2, and that those activities were newly exposed by the papain treatment, because no recognizable inhibitory activity was detected in the plain water extract. Molecular weights (Mws) of fraction F2 was supposed to be less than 1 kDa, as the ultrafiltrating membrane filter (1 kDa cut-offs) was used to obtain the fraction.

To carry out the ACE-inhibitory activity *in vivo*, peptides have to reach to the blood system intact. Many different barriers in the human body might limit or enhance the activity of the peptides *in vivo*. When proteins or peptides are ingested, the first barriers are the stomach and small intestines where proteins and peptides are broken down by enzymes like as pepsin, trypsin and α-chymotrypsin. Then, the resulting oligopeptides and free amino acids are absorbed into the blood. During the absorption, peptides are further hydrolyzed by brush border peptidases and peptidases in enterocytes (Vermeirssen et al., 2004). So, the smaller size of peptide would have the better inhibitory activity *in vivo*. Byun and Kim (2001) observed the correlation between the Mw of hydrolysate and the specificity of the ACE-inhibitory activity, and revealed that the activities were markedly increased with the decrease of Mw. However, we were unable to find the correlation between the Mw and the ACE-inhibitory activity.

Purification of ACE-inhibitory peptide

To purify the ACE-inhibitory peptide, fraction F2 was subjected to a Sephadex LH-20 and two major fractions were obtained: F2-A and F2-B. The ACE-inhibitory activities of F2-A and F2-B showed 54.01 and 55.08%, respectively (Figure 2). Of these two fractions, F2-B was applied for further purification using a reverse-phase column (ODS C_{18}) by HPLC. The inhibitory activities were detected in a wide range of eluted fractions (F2-B(I) - F2-B(VII)), indicating that many inhibitory active peptides were exposed by the papain treatment (Figure 3). For further purification of the fraction, F2-B(IV) that exhibited the highest ACE-inhibitory activity, another reverse-phase column (ODS C_{12}) was subjected and a pure active peptide, F2-B(IV)7 was obtained. As shown in Table 1, the purification yield and IC_{50} value of the F2-B(IV)7 were 0.2 ± 0.1% and 2.0 ± 0.3 µg/ml, respectively.

Lee et al. (2005) purified the ACE-inhibitory peptides from the goat's milk casein hydrolysates of pepsin, demonstrating those sequences to be Ala-Tyr-Phe-Tye and Pro-Tyr-Tyr. Gao et al. (2010) have also produced the ACE-inhibitory peptides from papain-involved cottonseed hydroysate and calculated the IC_{50} values to range from 0.159 to 0.792 mg/ml, even though they did not elucidate the structure.

In this work, we hydrolyzed Jellyfish with papain and

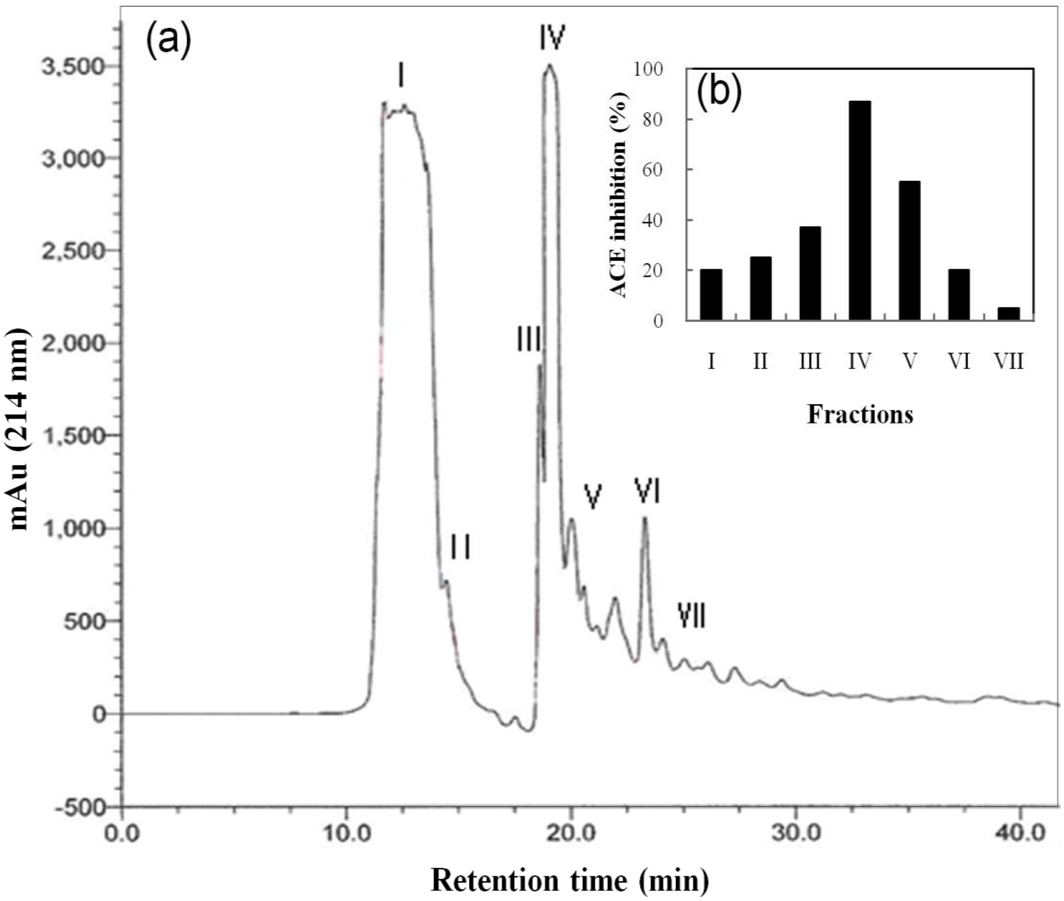

Figure 3. The C_{18} column chromatography (a) of F2-B and the inhibitory activities (b) of the elutes. The elution was performed with linear gradient of MeOH-H_2O [A eluent; H_2O:MeOH:TFA = 90:10:0.1 (v/v/v), B eluent; MeOH:H_2O: TFA=90:10:0.1 (v/v/v)] at a flow rate of 2 ml/min. Each elute was collected based on the whole peak area and numbered from I to VII. The inhibitory activity was determined with the sample concentration of 20 μg/ml.

purified an ACE-inhibitory peptide (F2-B(IV)7) from the hydrolysate. These findings led us to postulate that many proteolytic enzymes including papain may be applied in the production of ACE-inhibitory peptides different in Mws from several jellyfish species.

Structure elucidation of the purified peptide

To elucidate the structure of F2-B(IV)7, several analyzing techniques were employed. The UV spectrum found that the λmax (log ε) of the purified peptide was at 214 nm, and the Mw was estimated to be 294.16 Da by Agilent 1100 LC/MSD spectrometer. The IR spectrum showed the NH/OH vibrational frequency at 3398 cm^{-1} and amine peak (CN) of peptide bond at 1203 cm^{-1} whereas the carbonyl peak and broad peak for cyclic ring appeared at 1672 and 1468 cm^{-1}, respectively. These specific IR absorptions inform us that the peptide includes a phenolic ring in the peptide. The 1H, 13C NMR and DEPT experiments allowed the assignment of seven methine, two methylene and two methyl groups. The remaining quaternary centers consisted of a carbonyl (172.4 ppm), a hydroxyl (155.2 ppm) and a double-bonded (163.3 ppm) oxygenated carbon signals. The 1H NMR spectrum showed that the peaks at δ 1.14, δ1.32, δ2.95 and δ 3.08 ppm are related to the methylene protons of the ester group. These data from the several analyzing techniques convinced us that the F2-B(IV)7 contains tyrosine and isoleucine in the molecule. The methine protons of isoleucine appeared at δ 1.87 and δ 3.73 ppm, and those of tyrosine appeared at δ 3.97 ppm. The methine protons attached to the cyclic side chain appeared at δ 6.71 and 7.01 ppm as doublet. The 13C NMR spectrum showed two carbonyl peaks at δ 172.4 (C=O ester) and δ 163.3 (C=O amide), δ116.1 - δ 155.2 ppm (benzilic carbons), δ35.1, δ58.3 and δ 62.6 ppm (methine carbons), δ11.1 and δ 14.4 ppm (methyl carbons)

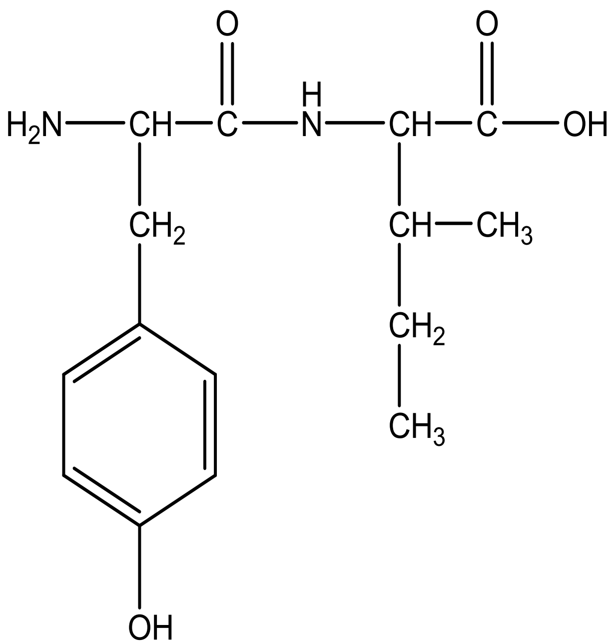

Figure 4. The proposed structure of the purified dipeptide, F2-B(IV)7. The structure was elucidated to be Tyr-Ile.

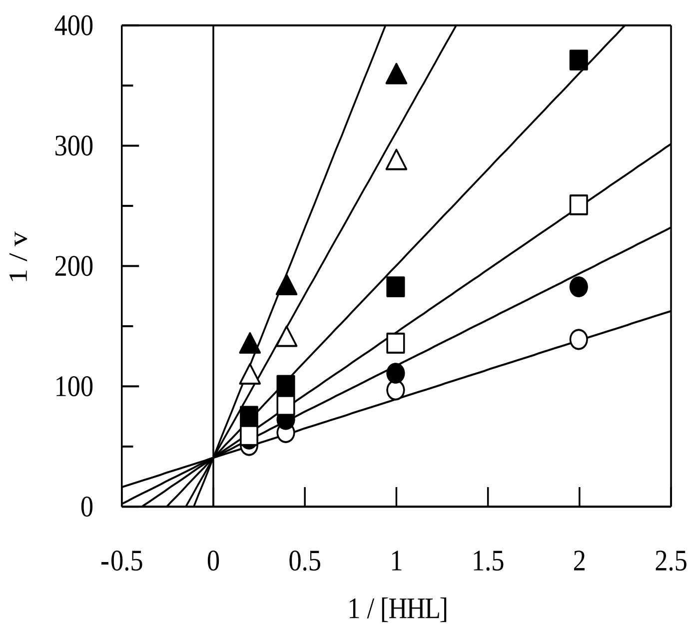

Figure 5. Lineweaver-Burk plot of F2-B(IV)7 on ACE reaction. A series of F2-B(IV)7 (0, 0.5, 1.0, 2.0, 4.0 and 6.0 μg/ml) was included in the reaction solution and the K_i was calculated by using the Grafit 5.0 program. ○, 0 μg/ml; ●, 0.5 μg/mL; □, 1.0 μg/ml; ■, 2.0 μg/ml; △, 4.0 μg/ml; ▲, 6.0 μg/ml; HHL, hipuryl-L-histidyl-L-leucine.

which are correlated with the structure of tyrosine-isoleucine (Tyr-Ile) (Figure 4). These data elucidate that the purified peptide, F2-B(IV)7, is the dipeptide of Tyr-Ile, which is a novel dipeptide reported for the first time in this work. The molecular weight and Isoelectric point (PI) of Tyr-Ile were calculated to be 294.35 Da and PI 5.9 by the ChemBioDraw 11 program, respectively, corresponding to the LC/MS data (294.16 Da).

Kawasaki et al. (2000) reported that the deptide (Val-Tyr) purified from the sardine muscle hydrolysate has a significant antihypertensive effect on mild hypertensive subjects *via* ACE inhibition. Erdmann et al. (2006) reported on other sardine muscle hydrolysate, stating that the dipeptide (Met-Tyr) has ACE-inhibitory activity, while it was also capable of diminishing free radical formation in human endothelial cells.

Kinetic analysis

The ACE inhibition pattern of the purified peptide was investigated by applying the Lineweaver-Burk plot. It was found that the Tyr-Ile acts as a competitive inhibitor on ACE, suggesting that the Tyr-Ile from *N. nomurai* competes with the substrate at the active site of ACE. The IC_{50} and K_i values of the Tyr-Ile were 2.0 ± 0.3 μg/ml and 3.3 ± 0.3 μM, respectively (Figure 5). The inhibition pattern of the Tyr-Ile was similar to those of fibrinogen pentapeptides, casein fragment, porcine plasma tripeptides and tuna muscle octapeptide (Astawan et al., 1995). Cheung et al. (1980) reported that tryptophan, tyrosine, proline or phenylalanine at the carboxy-terminal and branched-chain aliphatic amino acids at the amino-terminal are suitable for a peptide binding to ACE as a competitive inhibitor. Fujita and Yoshikawa (1999) reported that LKPNM is a pro-drug type of ACE-inhibitory peptide because LKPNM was hydrolyzed by ACE to produce LKP, which had an ACE-inhibitory activity 8-fold higher than LKPNM. After oral administration in spontaneously hypertensive rats (SHR), the antihypertensive effect of LKPNM showed a maximal effect after 6 h, while LKP showed a maximal effect at 4 h.

Some antihypertensive drugs are known to produce side effects, such as an abnormal elevation of the blood pressure after administration. However, Jellyfish is a favourite seafood in Southeast and East Asian countries. Thus, the Jellyfish may be a useful functional food for maintenance of blood pressure within the normal range. Our results also suggest that an ACE inhibitor derived from *N. nomurai* may be utilized in developing physiologically functional foods.

ACKNOWLEDGEMENT

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Table 1. Purification yield and ACE-inhibitory activity of each step.

Fraction	Purification step	Purification yield (%)	IC_{50} (μg/ml)
W/H	Papain hydrolysis	100	12,000 ± 1,210
F2	Ultrafiltrate (<1 kDa)	9.4 ± 1.7	1,900 ± 270
F2-B	Sephadex LH-20	2.0 ± 0.3	200 ± 30
F2-B(IV)	ODS C_{18}	0.5 ± 0.1	12.0 ± 0.9
F2-B(IV)7	ODS C_{12}	0.2 ± 0.1	2.0 ± 0.3

The fractions were obtained after each separation step. Purification yield (%) was calculated with the amount of total-N obtained. Values were expressed as mean ± SD.

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