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Dipeptide (Tyr-Ile) Acting as an Inhibitor of Angiotensin -I-Converting Enzyme (ACE) from the Hydrolysate of Jellyfish *Nemopilema nomurai*

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Abstract

The jellyfish *Nemopilema nomurai* was hydrolyzed with papain and a novel dipeptide purified via ultrafiltration, gel filtration chromatography with Sephadex LH-20, and reverse phase chromatography using C_{18} and C_{12} columns. The IR, 1H NMR, 13C NMR, and MS spectrometer analyses showed that the dipeptide comprised tyrosine-isoleucine (Tyr-Ile). The IC_{50} and K_i values were 6.56 ± 1.12 and 3.10 ± 0.28 μM, respectively, indicating competitive inhibition of angiotensin-I-converting enzyme (ACE). As a novel ACE-inhibitory active peptide, Tyr-Ile may have potential for use in antihypertensive therapy.

Key words: ACE, Jellyfish, *Nemopilema nomurai*, Papain, Tyrosine-isoleucine

Introduction

Functional foods may contain bioactive peptides since they exert a physiological effect in the body. These peptides are released from dietary protein by enzymatic hydrolysis or during processing. Bioactive peptides that regulate blood pressure are believed to be inhibitors of angiotensin-I-converting enzyme (ACE). Those peptides are, in general, inactive within the sequence of the parent protein, but can be released during gastrointestinal digestion or food processing (Shahidi and Zhong, 2008). 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; Wu et al., 2008), meat (Jang and Lee, 2005), and marine resources (Je et al., 2005; He et al., 2006). Various ACE-inhibitory peptides have also been isolated from fish protein, *e.g*., sardines (Matsui et al., 1993), tuna (Kohama et al., 1988), and cod (Kim et al., 2000). Protein hydrolysates are usually produced by gastrointestinal enzymes (*e.g*., pepsin or pancreatin) or microbial enzymes (*e.g*., Protamex; Novozymes, Bagsvaerd, Denmark) under varying conditions.

ACE (EC 3.4.15.1) is a circulating enzyme that participates in the 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 a potent octapeptide vasoconstrictor (angiotensin II), and inactivates the catalytic function of bradykinin, which has a depressor action (Ondetii et al., 1977; FitzGerald et al., 2004).

Nemopilema nomurai is a giant jellyfish species that has been blooming offshore of Korea, China, and Japan in the last several years. Jellyfish have traditionally been consumed to treat asthma and hypertension in East Asian countries. Despite this, knowledge of the active compound(s) is lacking. Thus, the aim of this study was to determine the functional prop-

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erties of the active compounds of *N. nomurai* and assess its possible application in antihypertensive therapy. We hydrolyzed *N. nomurai* jellyfish with papain, and then purified and elucidated the structure and the inhibition pattern of an ACE-inhibitory peptide.

Materials and Methods

Materials

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

Enzymatic hydrolysis

Papain hydrolysis of the jellyfish was conducted under the following conditions. First, jellyfish samples were lyophilized to remove excess water. Enzymatic hydrolysis (100 mL) was carried out for 4 h with 10% (w/v) lyophilized jellyfish at 60°C and pH 6.0. The ratio of enzyme to reaction volume was varied from 0 to 2% (w/v). Each enzyme reaction 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 total-N of lyophilized samples, and h is the quantity of α-amino nitrogen.

Determination of ACE-inhibitory activity

ACE-inhibitory activity was determined using the 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). A sample (50 μL) was added to 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 the addition of 1.0 N HCl (250 μL). The resulting hippuric acid was extracted by 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 room temperature for 2 h in a vacuum. The extracted hippuric acid was dissolved in 3.0 mL distilled water, and the absorbance at 228 nm was measured using a spectrophotometer (model U-3210; Hitachi Co., Tokyo, Japan).

Fractionation of jellyfish hydrolysates

The papain hydrolysate showing the greatest inhibitory activity was selected and the resultant hydrolysate was fractionated through an Amicon Millipore membrane (1 kDa cutoffs; Amicon, Beverly, MA, USA). The resultant fraction (FII) was lyophilized and then stored at -82°C until required.

Purification of ACE-inhibitory peptide

The active fraction (FII) was further purified by Sephadex LH-20 (25 × 250 mm) gel filtration chromatography, eluting with 30% methanol at a flow rate of 0.5 mL/min, and reverse-phase HPLC 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 (C_{18} 5 μM ODS 3100A, 10 × 250 mm, UV detection at 214 nm; Phenomenex). To obtain pure peptide, a Jupiter Proteo C_{12} column (90 Å, 10 μm, 21.2 × 150 mm; Phenomenex) was used at a flow rate of 2 mL/min with a linear CH_3CN-H_2O gradient (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 of the purified peptide

The IR spectra were recorded on a Fourier transform (FT)-IR model IFS-88 spectrometer (Bruker, Billerica, MA, USA). NMR spectra were recorded on a JEOL JNM ECP-400 (400 MHz for 1H, 100 MHz for 13C) spectrometer (JEOL Ltd., Tokyo, Japan). Chemical shift (δ) values were expressed in ppm and were referenced to the residual solvent signals with resonances at δH/δC, 7.26/77.0 ($CDCl_3$). To calculate molecular weights, purified samples were processed on an 1100 LC/MSD spectrometer (Agilent Technologies, Santa Clara, CA, USA) with direct injection onto an electrospray interface in the positive or negative mode. The structure of the purified peptide was conformed using Chemdraw 11 (CambridgeSoft, Cambridge, MA, USA).

IC_{50} values and kinetic analysis

The IC_{50} value of the purified peptide was determined by a standard method, and was defined as the concentration of inhibitor required to inhibit 50% of ACE activity. For a Lineweaver-Burk plot, 0-4 μg/mL of purified peptide was added to the reaction solution as an inhibitor, and the inhibition pattern

and K_i were estimated using GraFit 5.0 (GraFit Software, Surrey, UK).

Results and Discussion

Papain digestion of jellyfish

The hydrolysis rates of jellyfish mesogloea ranged from 2 to 17%, depending on the papain concentration used. Examination of the ACE-inhibitory activity of each hydrolysate revealed that the activity increased significantly by as much as 62% after 4 h digestion, although negligible activity was detected in intact jellyfish. The greatest inhibitory activity was obtained with an enzyme concentration of 0.2% (w/v), and it then leveled off even in a prolonged reaction (Fig. 1). Papain can digest most protein substrates and exhibits a broad substrate specificity, cleaving the peptide bonds of basic amino acids, leucine, or glycine. Because of this broad substrate specificity, many peptides and amino acids have been produced, leading to higher ACE-inhibitory activity. Lee et al. (2001) and Kim et al. (1996) reported that no relationship existed between the degree of hydrolysis of laver and freshwater fish and ACE-inhibitory activity. In the case of laver hydrolysate, both neutrase and papain achieved a similar degree of hydrolysis (50.7% and 51.3%, respectively) and ACE-inhibitory activity (34.3% and 34.9%, respectively), whereas the hydrolysis rate and ACE-inhibitory activity of jellyfish differed markedly. This indicates that different substrates will show clear differences in reaction pattern.

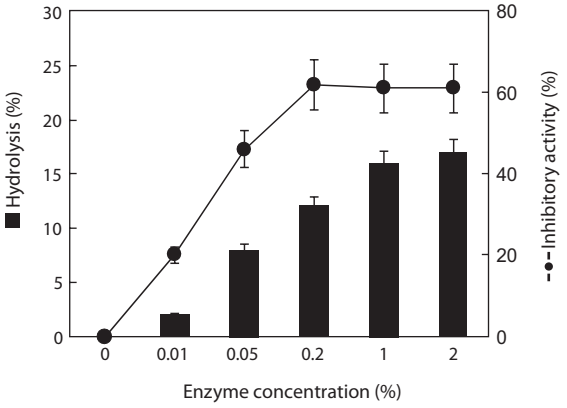

Fig. 1. Degree of hydrolysis and angiotensin-I-converting enzyme (ACE)-inhibitory activity of jellyfish hydrolysate. Papain was used to hydrolyze *Nemopilema nomurai* and the inhibitory activity of the resulting hydrolysate was determined by the method mentioned in Materials and Methods. ■, hydrolysis (%); -●-, ACE inhibition (%).

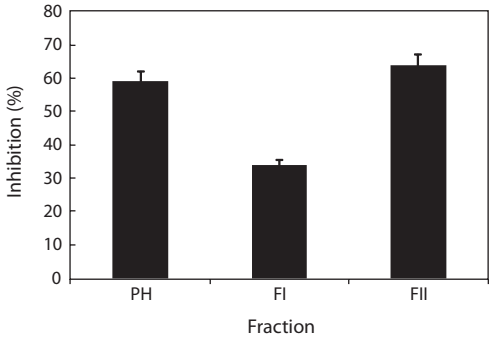

Fig. 2. Angiotensin-I-converting enzyme (ACE)-inhibitory activity of the fractionated jellyfish hydrolysate. The resultant hydrolysate was fractionated using amicon millipore membrane filters (1kDa cut offs), then the inhibitory activity was determined by the standard method. PH, papain hydrolysate before fractionation; FI, >1 kDa; FII, <1 kDa.

Fractionation and characterization of papain hydrolysate

The papain hydrolysate was fractionated by ultrafiltration (1 kDa MWCO) and the activity determined. The resulting fraction (FII, <1 kDa) possessed 61.1% of the total activity, whereas the retained fraction (FI, >1 kDa) possessed 35.0% (Fig. 2). These findings suggested that the most active compounds were present in the FII fraction and that this activity was newly exposed by papain treatment because no recognizable inhibitory activity was detected before papain hydrolysis. Molecular weights (Mw) of fraction FII were less than 1 kDa, since a 1 kDa MWCO membrane was used.

To inhibit ACE *in vivo*, peptides must reach the blood intact. Many barriers in the 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 intestine, where proteins and peptides are broken down by enzymes such as pepsin, trypsin, and α-chymotrypsin. The resulting oligopeptides and free amino acids are absorbed into the blood. During absorption, peptides are further hydrolyzed by brush border peptidases and peptidases in enterocytes (Vermeirssen et al., 2004). Byun and Kim (2001) observed a correlation between the molecular weight of hydrolysate and the specificity of the ACE-inhibitory activity, and revealed that activities increased with decreasing molecular weight. However, we were unable to detect a correlation between molecular weight and ACE-inhibitory activity.

Purification of the ACE-inhibitory peptide

To purify the ACE-inhibitory peptide, fraction FII was subjected to a Sephadex LH-20 column, and two major fractions were obtained: FII-A and FII-B (Fig. 3). The ACE-inhibitory activities of FII-A and FII-B represented 54.01% and 55.08%, respectively. FII-B was further purified by HPLC using a reverse-phase column (ODS C_{18}). Inhibitory activity was detected in a wide range of eluted fractions (FII-B(I)-FII-B(VII)), indicating that many inhibitory peptides were exposed by papain treatment. For further purification, FII-B(IV), which

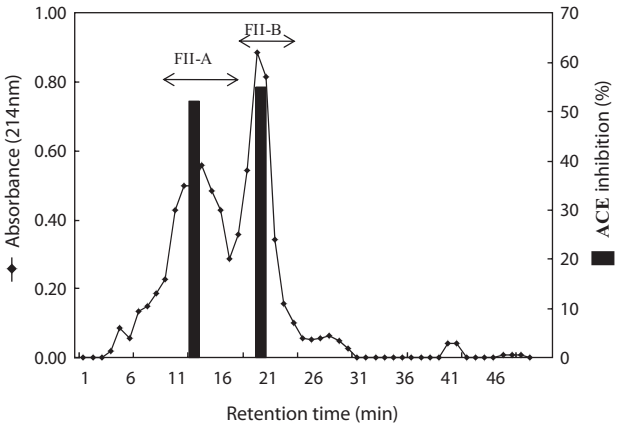

Fig. 3. Gel filtration chromatography of the fraction FII on Sephadex LH-20. Separation was performed with 30% aqueous methanol solution at a flow rate of 0.5 mL/min and collected with fraction volume of 5 mL. The fraction corresponding to FII-B was collected and determined the inhibitory activities. ✦, Absorbance at 214 nm; ▮, ACE inhibition (%).

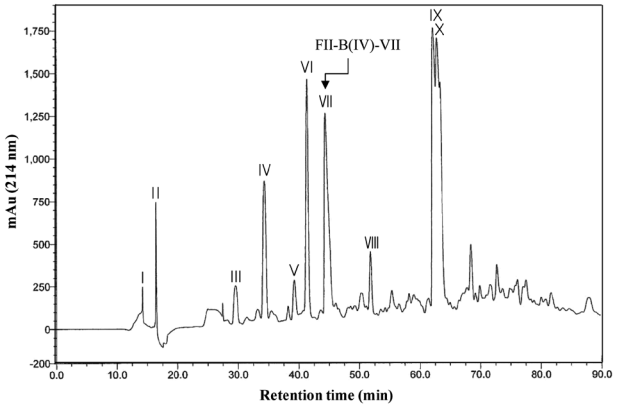

Fig. 4. Separation chromatogram of the FII-B(IV) by using C_{12} reverse-phase column. The resultant active fraction (FII-B(IV)) after C_{18} column chromatography was loaded on a Jupiter Proteo C_{12} column (90Å, 10 μm, 21.2 × 150 mm) and eluted with a linear gradient of CH_3CN-H_2O [A eluent; H_2O:CH_3CN:TFA = 95:5:0.1 (v/v/v), B eluent; CH_3CN:H_2O:TFA = 55:45:0.08 (v/v/v)] at a flow rate of 2 mL/min. The inhibitory activity of each elute from I to X was determined and the resultant active fraction, FII-B(IV)-VII was obtained.

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

Fraction	IC_{50} (μg/mL)	Purification yield (%)
PH	12.0 ± 1.21 (×10^3)	100
FII	1.90 ± 0.27 (×10^3)	9.40 ± 1.71
FII-B	0.20 ± 0.03 (×10^3)	2.01 ± 0.27
FII-B(IV)	12.01 ± 0.91	0.51 ± 0.08
FII-B(IV)-VII	1.93 ± 0.33*	0.08 ± 0.02

The fraction was obtained after each separation step. Purification yield (%) was calculated with the amount of total-N obtained. Values were expressed as mean ± SD. ACE, angiotensin-I-converting enzyme; PH, Papain hydrolysate; FII, ultrafiltrate (<1 kDa); FII-B, Sephadex LH-20; FII-BIV, ODS C_{18}; FII-BIV-VII, ODS C_{12}. *Based on the molecular weight (294.16 Da) of FII-B(IV)-VII, the IC_{50} value was calculated to be 6.56±1.12 μM.

exhibited the highest ACE-inhibitory activity, was applied to another reverse-phase column (ODS C_{12}), and a pure active peptide, FII-B(IV)-VII, was obtained (Fig. 4). The purification yield and IC_{50} value of FII-B(IV)-VII were 0.08 ± 0.02% and 1.93 ± 0.33 μg/mL, respectively (Table 1). Based on the molecular weight (294.16 Da) determined by an Agilent 1100 LC/MSD spectrometer, the IC_{50} value was 6.56 ± 1.12 μM.

Lee et al. (2005) purified ACE-inhibitory peptides from goat's milk casein hydrolysates of pepsin, and showed their sequences to be Ala-Tyr-Phe-Tye and Pro-Tyr-Tyr. Gao et al. (2010) also produced ACE-inhibitory peptides from papain-digested cottonseed hydrolysate, 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 purified an ACE-inhibitory peptide (FII-B(IV)-VII) from the hydrolysate. These findings led us to postulate that many proteolytic enzymes, including papain, may be applied to produce ACE-inhibitory peptides of varying molecular weights from several jellyfish species.

Structure of the purified peptide

To elucidate the structure of FII-B(IV)-VII, several techniques were employed. The IR spectrum showed an NH/OH vibrational frequency at 3,398 cm^{-1} and peptide bond amine peak (CN) at 1,203 cm^{-1}, whereas the carbonyl and broad peaks of the cyclic ring appeared at 1,672 and 1,468 cm^{-1}, respectively. These IR data indicated that the peptide contains a phenolic ring.

The 1H, 13C NMR, and DEPT data allowed assignment of seven methine, two methylene, and two methyl groups. The remaining quaternary centers consisted of a carbonyl (172.4 ppm), hydroxyl (155.2 ppm), and double-bonded (163.3 ppm) oxygenated carbon signals. The 1H NMR spectrum showed that the peaks at δ 1.14, 1.32, 2.95, and 3.08 ppm were related to the methylene protons of the ester group (Table 2). These data convinced us that FII-B(IV)-VII contains tyrosine and isoleucine. The methine protons of isoleucine appeared at δ 1.87 and 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 a doublet. The 13C NMR spectrum showed two carbonyl peaks at δ 172.4 (C=O ester) and δ 163.3 ppm (C=O amide), δ 116.1-155.2 ppm (benzilic carbons), δ 35.1, 58.3, and 62.6 ppm (methine carbons), δ 11.1 and 14.4 ppm (methyl carbons), which correlated with the structure of tyrosine-isoleucine (Tyr-Ile) (Table 2). These data strongly suggest that the purified peptide, FII-B(IV)-VII, is a Tyr-Ile dipeptide, 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 5.9, respectively, using the ChemBioDraw 11 program, corresponding to the LC/MS data (294.16 Da).

Kawasaki et al. (2000) reported that a dipeptide (Val-Tyr)

Table 2. 1H and 13C NMR chemical shifts for the FII-B(IV)-VII

Position	13C NMR	1H NMR
Tyr 1		
CO	163.3	
Cα/Hα	58.3	3.97
Cβ/Hβ	35.9	2.95, 3.08
C1	126.2	
C2,6/H2,6	130.9	7.01
C3,5/H3,5	116.1	6.71
C4/OH	155.2	4.72
Ile 2		
COO	172.4	
Cα/Hα	62.6	3.73
Cβ/Hβ	35.1	1.87
Cγ(CH3)	14.4	0.85
Cγ(CH2)	24.8	1.14, 1.32
Cδ/Hδ	11.1	0.77

The chemical shift (δ) values were expressed in ppm and were referenced to the residual solvent signals with resonances at δH/δC, 7.26/77.0 ($CDCl_3$).

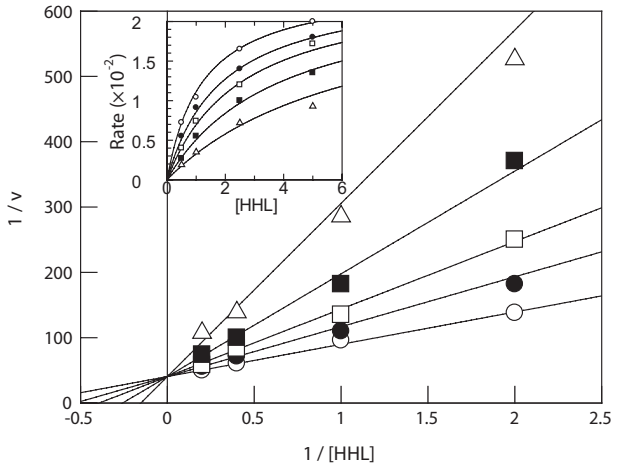

Fig. 5. Lineweaver-Burk plot of FII-B(IV)-VII on ACE reaction. A series of FII-B(IV)-VII (0, 0.5, 1.0, 2.0, 4.0 μg/mL) was included in the reaction solution and the K_i was calculated by using the Grafit 5.0 program. HHL, hipuryl-L-histidyl-L-leucine; ○, 0 μg/mL; ●, 0.5 μg/mL; □, 1.0 μg/mL; ■, 2.0 μg/mL;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

purified from sardine muscle hydrolysate had a significant antihypertensive effect on mildly hypertensive subjects via ACE inhibition. Erdmann et al. (2006) reported that sardine muscle hydrolysate contained a Met-Tyr dipeptide that showed ACE-inhibitory activity and was capable of diminishing free radical formation in human endothelial cells.

IC_{50} value and kinetic analysis

The ACE inhibition pattern of the purified peptide was investigated by applying a Lineweaver-Burk plot. Tyr-Ile acts as a competitive inhibitor of ACE, suggesting that Tyr-Ile from *N. nomurai* competes with the substrate at the active site of ACE. The IC_{50} and K_i values of Tyr-Ile were 6.56 ± 1.12 μM (1.93 ± 0.33 μg/mL) and 3.10 ± 0.28 μM, respectively (Fig. 5). The inhibition pattern of Tyr-Ile was similar to those of fibrinogen pentapeptides, casein fragments, porcine plasma tripeptides, and tuna muscle octapeptide (Sugiyama et al., 1991; 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 competitive inhibition of ACE. Fujita and Yoshikawa (1999) reported that LKPNM is a prodrug type of ACE-inhibitory peptide because LKPNM was hydrolyzed by ACE to produce LKP, which exhibited an ACE-inhibitory activity eightfold higher than LKPNM. After oral administration in SHR, the antihypertensive effect of LKPNM was maximal after 6 h and that of LKP at 4 h.

Some antihypertensive drugs are known to produce side effects, such as an abnormal elevation of blood pressure. However, jellyfish is a favorite seafood in Southeast and East Asian nations. Thus, 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* can be utilized in the development of physiologically functional foods.

Acknowledgments

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