



Antioxidative and angiotensin I-converting enzyme inhibitory activities of Eastern eel (*Anguilla japonica*)

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민물장어의 항산화 활성과 안지오텐신 I 전환효소 저해 효과

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Abstract

This study was designed to investigate the antioxidative and angiotensin I-converting enzyme (ACE) inhibitory activities of Eastern eel (*Anguilla japonica*). Hot water extracts were prepared over a period of 25 h at 100°C from the meat and whole body of the Eastern eel. Protein hydrolysates of Eastern eel were prepared over a period of 10 h using different hydrolytic enzymes, such as Alcalase[®], Protamex[®], and Alcalase[®] + Protamex[®]. The antioxidative and ACE inhibitory activities of the eel hydrolysates were higher than those of the extracts, and showed similar trends for hydrolysis time on each assay system due to similar enzyme specificity. The hydrolysates produced using Alcalase[®] exhibited the highest DPPH scavenging activity (IC₅₀=3.02 mg/mL), while the Protamex[®] hydrolysates exhibited the highest ABTS (IC₅₀=1.13 mg/mL), hydroxyl radical (IC₅₀=1.30 mg/mL), and hydrogen peroxide (IC₅₀=1.11 mg/mL) scavenging activities and ACE inhibitory activity (IC₅₀=110.37 µg/mL). These results revealed that the Eastern eel hydrolysates exhibited strong radicals (DPPH, ABTS, superoxide, hydroxyl, and hydrogen peroxide) scavenging properties and ACE inhibitory activity, indicating that they can be potentially used as functional food ingredients with potential to prevent oxidation and reduce blood pressure.

Key words : Eastern eel (*Anguilla japonica*), hydrolysate, extract, antioxidative activity, ACE inhibitory activity

Introduction

The Eastern eel (*Anguilla japonica*) is one of 19 species within the freshwater eel family Anguillidae. They are common across the world, especially in East and South East Asian countries, predominantly in South Korea, China, Japan,

Thailand, and Philippines (Seo et al., 2013). They are used in many different ways including consuming as food and using in traditional Chinese medicine to cure skin diseases (e.g., atrophoderma) and hemorrhoids (Ekanayake et al., 2005). It is well known that fish and fishery products represent a valuable source of nutrients of fundamental

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importance for diverse and healthy diets. Fish provides high-value protein, polyunsaturated omega-3 fatty acids (docosahexaenoic acid and eicosapentaenoic acid), and a wide range of essential micronutrients, including various vitamins (D, A, and B), minerals (e.g., calcium, iodine, zinc, iron, and selenium) (FAO, 2012). In addition to their high nutritious value, studies have been identified fish meat as a rich source of bioactive peptides with potentially valuable nutraceutical and pharmaceutical properties (Elavarasan et al., 2014; Halim et al., 2016). Bioactive peptides could be released from inactive original protein by enzymatic hydrolysis. These fish protein-derived bioactive peptides exhibit various biological activities such as antioxidative, anti-diabetic activities, anti-hypertensive, anti-coagulant, anti-proliferative, anti-obesity, and calcium-binding (Ngo et al., 2012). Moreover, it has been demonstrated that protein hydrolysates exhibiting antioxidative potential may contain peptides with other biological activities such as angiotensin I-converting enzyme (ACE) inhibitory activity (Samaranayaka et al., 2010).

Antioxidants are very important to human health. They can inhibit the oxidation reaction that cause cell damage or death in human body by removing free radical intermediates. Therefore, it is believed that antioxidants can decrease the risk of developing more than 100 diseases, including diabetes, heart disease, cancer, HIV (AIDS), stroke, and atherosclerosis, and then can slow the aging process (Ekanayake et al., 2005). Many natural antioxidants originating from plant and fish sources have been identified as free radical active scavengers (Clarke et al., 2013; Elavarasan et al., 2014; Mandal et al., 2009; Zhou et al., 2012). Hypertension, a long-term medical condition in which the blood pressure in the arteries is persistently elevated, is a major risk factor for cardiovascular disease. ACE plays a vital physiological role in the regulation of blood pressure. ACE cleaves angiotensin I to the potent vasoconstrictor angiotensin II and inactivates the vasodilator bradykinin (Ghanbari et al., 2015). These functions of ACE increase blood pressure and finally lead to hypertension. In addition, angiotensin II produced by ACE increases oxidative stress, inducing the production of reactive oxygen species (ROS) under high blood pressure conditions (Schiffirin and Touyz, 2004). Therefore, the inhibition of ACE activity or the blockade of angiotensin II formation may enhance the

antioxidative system.

A number of studies have reported the presence of bioactivities in enzymatically hydrolyzed fish proteins from salmon (*Salmo salar*) (Darewicz et al., 2014), rainbow trout (*Oncorhynchus mykiss*) (Kim and Byun, 2012), bigeye tuna (*Thunnus obesus*), dark muscle (Qian et al., 2007), and chum salmon (*Oncorhynchus keta*) (Ono et al., 2003). Recently, there are several published studies reporting the presence of bioactivities in enzymatically hydrolyzed Asian swamp eel (*Monopterus* sp.) proteins (Azemi et al., 2017; Baharuddin et al., 2016; Halim et al., 2017; Halim et al., 2018; Jamil et al., 2016). They report that Asian swamp eel protein hydrolysate using alcalase have the antioxidant activity (Azemi et al., 2017), ACE inhibitory activity (Azemi et al., 2017; Baharuddin et al., 2016) and anticancer activity (Halim et al., 2018). However, there was little known about the potential for multiple bioactivities of protein derived from Eastern eel (*A. japonica*). The main objective of the present study was to evaluate and compare the antioxidative potential and ACE inhibitory activity of enzymatic hydrolysates and hot water extracts of Eastern eel (*A. japonica*).

Materials and methods

Chemicals

Hydrogen peroxide (H₂O₂), 1,1'-diphenyl-2-picrylhydrazyl (DPPH), thiobarbaturic acid (TBA) and angiotensin I-converting enzyme (ACE) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylene diamine tetra-acetic acid (EDTA), 2,2'-azino-bis (3-ethylbenzthiazolin)-6-sulfonic acid (ABTS), iron sulphate (FeSO₄ · 7H₂O), peroxidase, 2-deoxyribose, monopotassium phosphate (H₂HPO₄), potassium diphosphate (KH₂PO₄), hydrochloric acid (HCl), vitamin C and pyrogallol were purchased from Fluka Co. (St. Louis, MO, USA). All the other reagents used were either extra pure or analytical grade. Alcalase[®] (Alcalase 2.4L) and Promatex[®] were obtained from Novozymes (Copenhagen, Denmark).

Fish protein extract and hydrolysate preparation

Eastern eel (*Anguilla japonica*) were collected from local reservoirs (Chilbo-susan, Jeongeup, Korea), frozen immediately with the help of fishermen, and thawed rapidly below 35°C for analysis. The fish meat and the fish whole body

of eel including the frame, dark muscle, cut offs, viscera, skin, scales, bones and fins, were collected for the hot water extraction of eel. These were minced and mixed with water in the ratio of 1:10 (fish mince: water). The extraction was done on 100°C with reflux, where samples were collected on different hours (0, 5, 10, 15, 20 and 25 h). For the preparation of enzymatic hydrolysates, the fish meat was minced, mixed with the pre-warmed 0.1 M sodium phosphate buffer (pH 7) in the ratio of 1:10 (fish mince: sodium phosphate buffer) and each protease was added as the final conc. of 1.15%. The hydrolysis reaction was performed at 50°C for 10 h. The hydrolysates were collected at 2 hours' interval (0, 2, 4, 6, 8 and 10 h). The collected hydrolysates were inactivated in boiling water for 10 min. After cooling at room temperature, each sample was clarified by centrifugation at 3,000 rpm for 20 min to remove any residue and stored at -20°C. Hydrolysates were designated as ME-ALP (Alcalase[®]), ME- PRH (Promatex[®]) and ME-APH (Alcalase[®] + Promatex[®]) based on the enzyme used for hydrolysis. Hot water extracts based on the source for extraction, were expressed as ME (meat extract) and WBE (whole body extract).

Chemical analysis

Moisture and ash contents of Eastern eel (*Anguilla japonica*) were determined according to the AOAC (2005), standard methods 930.15 and 942.05, respectively. Total nitrogen content was determined by using the Kjeldahl method according to AOAC (2005) method number 984.13. Crude protein was estimated by multiplying total nitrogen content by the factor 6.25. Crude fat was determined gravimetrically after Soxhlet extraction of samples with hexane. All measurements were performed in triplicate.

Protein concentration determination

The protein contents of the hot water extracts and hydrolysates were estimated by the Lowry assay (Lowry et al., 1951). According to this procedure, the samples were first pretreated with copper ions in an alkali solution. The aromatic amino acids in the treated samples reduced the phosphomolybdic-phosphotungstic acid present in the Folin reagent. Since the endpoint of the reaction has a blue color, the amount of protein in the sample were estimated by reading the absorbance using a spectrophotometer (Optizen

3220 UV-VIS spectrophotometer, Daejeon, Korea) at 750 nm. BSA was used as standard.

Determination of DPPH radicals scavenging activity

Determination of DPPH radicals scavenging activity was estimated with the modification of the methods used by Khan et al. (2012). A 100 µL of 0.4 mM DPPH solution and 100 µL of the sample were added, mixed well in 96 microwell plates for 10-20 seconds and incubated for 30 min at room temperature in the dark. After incubation, the absorbance was measured at 540 nm against the corresponding blank solution (ethanol without the sample). The control was prepared by taking 100 µL DPPH and 100 µL of distilled water. Percentage inhibition of free radical DPPH was calculated based on following equation.

$$\text{DPPH scavenging (\%)} = [(Ac - As) / (Ac - Ab)] \times 100.$$

Where Ac is the absorbance of control; As is the absorbance of mixture containing sample; Ab is the absorbance of blank.

Determination of ABTS radical-scavenging activity

ABTS radical-scavenging activity was determined by ABTS assay as described by Binsan et al. (2008). The working solution was prepared by mixing the two stock solutions (7 mM ABTS solution and 2.45 mM potassium persulfate) in equal quantities and allowing them to react for 12-16 h at room temperature in the dark. ABTS solution was then diluted by mixing distilled water to obtain an absorbance of 0.7-0.8 at 734 nm using a spectrophotometer (Optizen 3220 UV-VIS Spectrophotometer, Gyeonggi-do, Korea). 180 µL ABTS solution and 20 µL of the sample were mixed, left at room temperature for 2 min in the dark and read at 734 nm. ABTS radical-scavenging activity was calculated by the following equation.

$$\begin{aligned} \text{ABTS radical scavenging (\%)} \\ = [(Ac - As) / (Ac - Ab)] \times 100 \end{aligned}$$

Where Ac is the absorbance of control (ABTS and distilled water); As is the absorbance of mixture containing sample; Ab is the absorbance of blank (distilled water).

Superoxide (O_2^-) scavenging assay

Ability of the samples to inhibit the autoxidation of pyrogallol was done by modifying Marklund and Marklund (1974). Briefly, 0.3 mL of the sample and 2.65 mL of 50 mM phosphate buffer (pH 7.4 at 37°C) containing 1 mM Na_2EDTA were mixed in a cuvette. Freshly prepared 50 μ L of 60 mM pyrogallol in 1 mM HCl was added and the inhibition of pyrogallol autoxidation was measured at 325 nm using spectrophotometer. Absorbance of sample was recorded at 0 min and after 5 min and the increment of absorbance was calculated by the difference.

$$O_2^- \text{ Scavenging activity (\%)} = [(\Delta Ac - \Delta As) / \Delta Ac] \times 100$$

Where ΔAs is the absorbance increment of the mixture containing the sample and ΔAc is absorbance increment of the mixture without the sample.

Hydroxyl radical ($\cdot OH$) scavenging assay

The $\cdot OH$ scavenging activity was determined by modified methods of Chung et al. (1997). A 100 μ L sample and 500 μ L of 0.1 M phosphate buffer (pH 7.4) were mixed with 100 μ L of 10 mM $FeSO_4$, 100 μ L of 10 mM EDTA and 100 μ L of 10 mM 2-deoxyribose in order to make a total volume of 900 μ L, then 100 μ L of 10 mM of H_2O_2 were added, and incubated at 37°C for 4 h. After incubation, 0.5 mL of 2.8% TCA and 0.5 mL of 0.1% TBA dissolved in water were added to the reaction mixture and kept in a boiling water bath for 10 min. The absorbance was measured at 532 nm.

$$\text{Hydroxyl radical scavenging (\%)} = [(Ac - As) / Ac] \times 100$$

Where Ac is the absorbance of mixture without sample; As is the absorbance of mixture containing sample.

Hydrogen peroxide (H_2O_2) scavenging assay

The H_2O_2 scavenging activity was determined according to the method of Muller (1985). The 20 μ L of sample was dissolved in 100 μ L of 0.1 M phosphate-buffered saline (pH 5.0), mixed with 20 μ L of 10 mM H_2O_2 , and incubated at 37°C for 5 min. Then, 30 μ L of 1.25 mM ABTS and 30 μ L of peroxidase (1 unit/mL) were added to the mixture and re-incubated at 37°C for 10 min. The incubation of ABTS

with peroxidase resulted in the production of the radical $ABTS^+$, which is blue green and absorbance of each sample was measured at 405 nm.

Hydrogen peroxide scavenging (%)

$$= [(Ac - As) / Ac] \times 100$$

Angiotensin I-converting enzyme (ACE) inhibitory activity

ACE inhibitory activity was measured according to the method described by Cushman and Cheung (1971) with some modification. This method was based on liberation of hippuric acid (HA) from hippuryl-L-histidyl-L-leucine (Hip-His-Leu, HHL) catalyzed by the ACE. The sample (25 μ L) was pre-incubated with 50 μ L substrate solution (8.3 mM HHL in 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3) at 37°C for 10 min. The reaction started by adding 50 μ L of pre-heated ACE (8 mU) solution in sodium borate and the mixture was incubated for 30 min at 37°C. The reaction was stopped by addition of 125 μ L 1 N HCl. The formed HA was extracted with 750 μ L ethyl acetate and centrifuged (3,000 rpm, 15 min). The organic phase (500 μ L) was evaporated at 50°C for 4 h. The dried residue was dissolved in 750 μ L of pre-heated water (60°C), vortexed for 5 min and the absorbance was read at 228 nm in a spectrophotometer (Optizen 3220 UV-VIS spectrophotometer, Korea). The extent of ACE inhibition was calculated using the following formula:

$$\text{ACE Inhibition (\%)} = [(Ac - As) / (Ac - Ab)] \times 100$$

Where Ac is the absorbance of control (HHL and ACE), As is mixture containing sample, and Ab is blank (distilled water). The antihypertensive agent, captopril was used as a standard.

Amino acid composition analysis

The sample preparation was carried out by hydrolysis with 6 M HCl at 110°C for 24 h and derivated by using phenyl isothiocyanate prior to HPLC analysis. The total amino acids compositions were analyzed by an automated amino acid analyzer (L-8900; Hitachi High-Technologies Corp., Tokyo, Japan).

Statistical analysis

All tests were conducted in triplicates. All results were expressed as mean \pm SD. The data was statistically analyzed by one-way ANOVA followed by Duncan's multiple range tests using SPSS package program (Version 26, IBM SPSS statistics, Armonk, NY, USA). p-value less than 0.05 were considered statistically significant.

Results

Chemical composition of Eastern eel

According to the chemical composition results, Eastern eel contains 61.8% moisture, 14.8% protein, 20.7% fat, 1.5% ash, and 1.2% carbohydrate. In particular, the fat and protein contents are very high in Eastern eel. Excluding the moisture content, the ratios of fat and protein were 54.2% and 38.7% on a dry basis.

Production of Eastern eel extracts and protein hydrolysates

To monitor the degree of protein extraction, the protein concentrations of ME and WBE were determined at intervals of 5 h over an extraction time of 25 h (Fig. 1A). The initial concentrations in the ME and WBE were 0.364 and 0.116 mg/ml, respectively. The final protein concentrations in the ME and WBE were 5.08 and 6.87 mg/mL, respectively, corresponding to 14.1- and 59.1-fold increases from the initial protein concentration, indicating that the protein yield of the WBE was higher than that of the ME. The protein concentration curves of the hydrolysates tended

to have an initial rapid phase during the first 2 h followed by a phase of slow increase after the core proteins had been hydrolyzed (Fig. 1B). The initial average protein concentration of eel meat was 0.147 mg/mL. The final protein concentrations in the ME-ALP, ME-PRH, and ME-APH were 10.6, 11.22, and 12.1 mg/mL, respectively, corresponding to 71.9-, 76.3-, and 82.2-fold increases from the initial protein concentration for ME-ALP, ME-PRH, and ME-APH, respectively. All hydrolysates showed higher protein yields than the eel extracts. At the same concentration, the ME-APH showed greater proteolytic activity.

DPPH radical scavenging activity

While the ME and WBE at 25 h did not show DPPH radical scavenging activity, the ME-APH was found to have greater scavenging ability (56.6%) followed by the ME-ALP (47.0%) and ME-PRH (30.1%) at 10 h (Fig. 2A; $p < 0.05$). The changes in DPPH radical scavenging activity were monitored during the hydrolysis process, as shown in Fig. 2B. The highest level of activity in the ME-ALP was 57.0% at 2 h; that in the ME-APH and ME-PRH was 59.9% and 48.1% after 8 h of hydrolysis, respectively.

ABTS radical scavenging activity

As shown in Fig. 2A, the ABTS radical scavenging activities of the ME and WBE were 55.5% and 57.5% at 25 h, but they were not significantly different ($p = 0.169$). All hydrolysates showed an ABTS radical scavenging power of $> 98\%$. ABTS radical scavenging activities higher than 50% were recorded after 15 h of extraction (54.5% at 15 h for

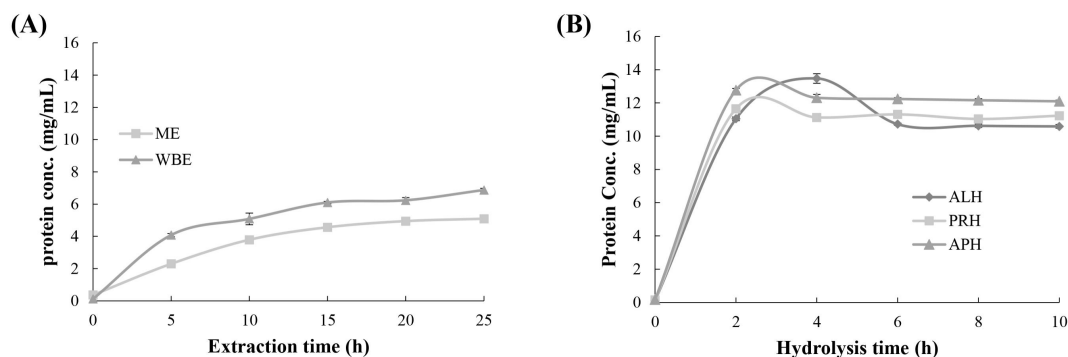


Fig. 1. Protein concentrations of hot water extracts (A) and enzyme hydrolysates (B) prepared from Eastern eel at different times.

ME, meat extracts; WBE, whole body extracts; ALP, Alcalase[®]-treated hydrolysates (ME-ALP); PRH, Promatex[®]-treated hydrolysates (ME-PRH); APH, the mixture of Alcalase[®] and Promatex[®]-treated hydrolysates (ME-APH).

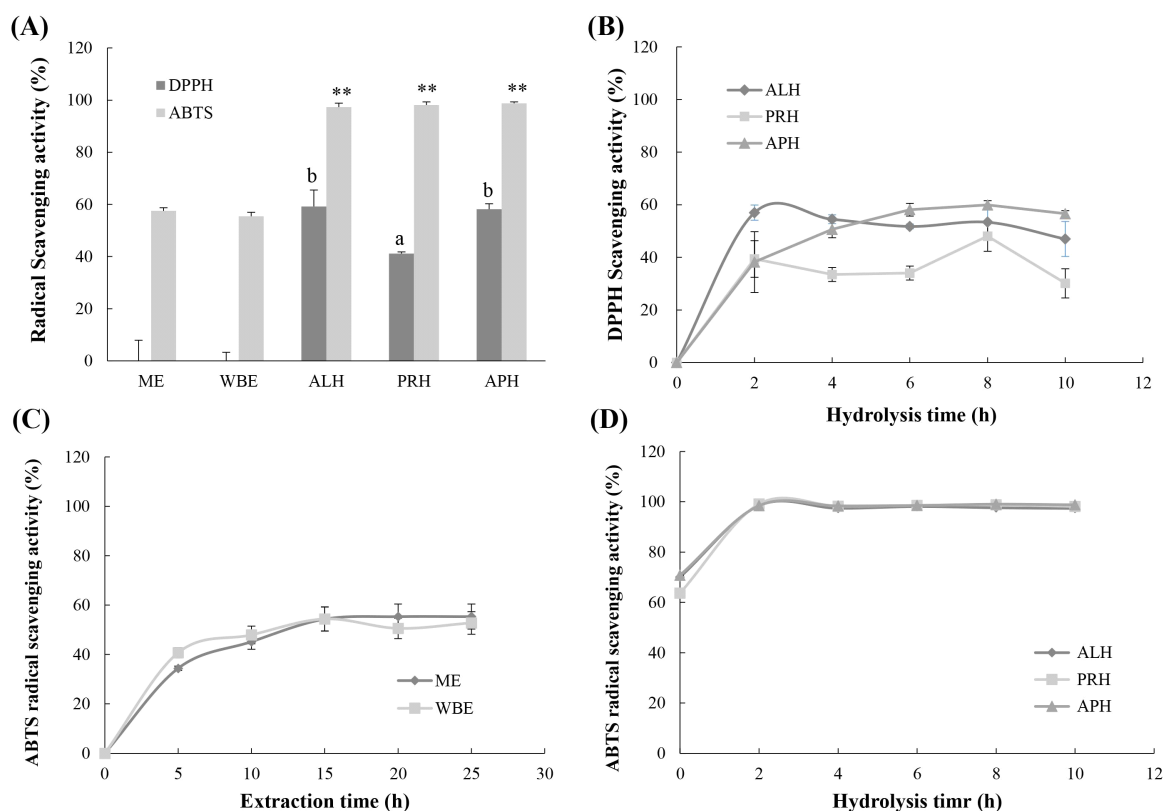


Fig. 2. DPPH and ABTS radical scavenging activities in hot water extracts and hydrolysates prepared from Eastern eel.

A, comparison of DPPH and ABTS radical scavenging activities at final extraction (25 h) and hydrolysis (10 h) times; B, DPPH radical scavenging activities of Eastern eel hydrolysates for 10 h hydrolysis times; C, ABTS radical scavenging activities of hot water extracts for 25 h extraction times; D, ABTS radical scavenging activities of Eastern eel hydrolysates for 10 h hydrolysis times.

ME, meat extracts; WBE, whole body extracts; ALP, Alcalase[®]-treated hydrolysates (ME-ALP); PRH, Promatex[®]-treated hydrolysates (ME-PRH); APH, the mixture of Alcalase[®] and Promatex[®]-treated hydrolysates (ME-APH).

** and the different letters in (A) mean significant difference ($p < 0.01$).

the ME and 55.4% at 20 h for the WBE; Fig. 2C), while all hydrolysates reached > 98% ABTS radical scavenging power at 2 h. The ME-PRH (99.1%) at 2 h had the highest level of activity, followed by the ME-APH (99.03%) at 8 h and ME-ALP (98.50%) at 2 h (Fig. 2D).

Superoxide (O_2^-) scavenging activity

The highest O_2^- scavenging activities of the eel hot water extracts were recorded as 41.0% at 5 h for the ME and 28.7% at 20 h for the WBE (Fig. 3A), respectively. The O_2^- scavenging activity of the eel hydrolysates increased rapidly to 40% within 2 h of hydrolysis and slowly settled to approximately 65% at 10 h. The O_2^- scavenging activities of the hydrolysates at the end of hydrolysis (10 h) were recorded as 59.9% for the ME-ALP, 67.0% for the ME-PRH, and 64.5% for the ME-APH. The highest level of

O_2^- scavenging activity was recorded at 10 h for the ME-PRH (67.00%) (Fig. 3B).

Hydroxyl radical ($\cdot OH$) scavenging activity

The greatest $\cdot OH$ scavenging activities observed in the hot water extracts were 22.4% at 25 h and 21.5% at 15 h for the ME and WBE (Fig. 3C), respectively; those in the hydrolysates were 61.8% and 63.9% at 4 h for the ME-ALP and ME-PRH, and 68.0% at 6 h for the ME-APH (Fig. 3D).

Hydrogen peroxide (H_2O_2) scavenging activity

The highest H_2O_2 scavenging activities were 47.4% at 10 h for the ME and 51.1% at 10 h for the WBE (Fig. 3E). The highest H_2O_2 scavenging activities in the hydrolysates were 52.6% after 4 h of hydrolysis for the ME-ALP, and 55.4% and 53.8% at 8 h for the ME-PRH and ME-APH (Fig. 3F).

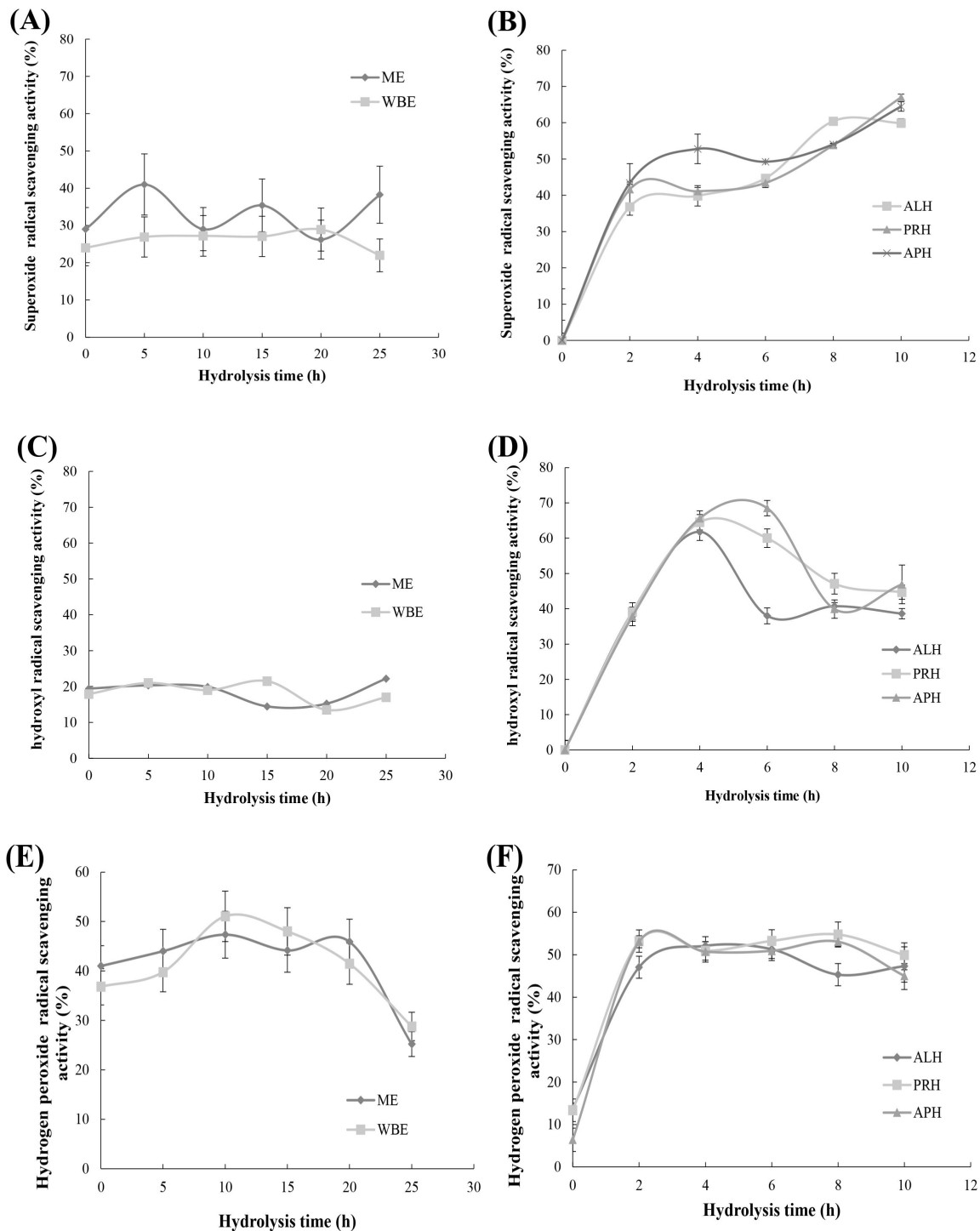


Fig. 3. Superoxide (O_2^-), hydroxyl radical ($\cdot OH$), and hydrogen peroxide (H_2O_2) scavenging activities in hot water extracts and hydrolysates prepared from Eastern eel at different hydrolysis times.

A, superoxide (O_2^-) scavenging activities of hot water extracts; B, superoxide (O_2^-) scavenging activities of hydrolysates; C, hydroxyl radical ($\cdot OH$) radical scavenging activities of hot water extracts; D, hydroxyl radical ($\cdot OH$) radical scavenging activities of hydrolysates; E, hydrogen peroxide (H_2O_2) scavenging activities of hot water extracts; F, hydroxyl radical ($\cdot OH$) radical scavenging activities of hydrolysates.

ME, meat extracts; WBE, whole body extracts; ALP, Alcalase[®]-treated hydrolysates (ME-ALP); PRH, Promatex[®]-treated hydrolysates (ME-PRH); APH, the mixture of Alcalase[®] and Promatex[®]-treated hydrolysates (ME-APH).

IC₅₀ values showing antioxidative activities of eel protein hydrolysates

The hydrolysates showed similar trends in the DPPH radical, ABTS radical, O₂⁻, •OH, and H₂O₂ scavenging assays in terms of their biological activities. Thus, the IC₅₀ values (concentration required to scavenge 50% of radical, anion, or H₂O₂) of the hydrolysates (10 h) were compared in each assay. Ascorbic acid and α-tocopherol were used as positive controls. As shown in Table 1, the lowest IC₅₀ value of DPPH was 2.73 mg/mL in the ME-ALP; that of α-tocopherol was 0.36 mg/mL. However, the lowest IC₅₀ values from the ABTS scavenging activity assay was found in the ME-PRH with 1.13 mg/mL. The lowest IC₅₀ values from the •OH and H₂O₂ scavenging activity assays were also found in the ME-PRH, at 1.11, and 1.3 mg/mL, respectively. The IC₅₀ values of O₂⁻ scavenging activity were 3.01, 3.07, and 3.14 mg for the ME-ALP, ME-PRH, and ME-APH, respectively, which are not significantly different. The IC₅₀ value of ascorbic acid was 0.22 mg/mL for ABTS radical scavenging activity, 0.31 mg/mL for H₂O₂ radical scavenging activity, and 0.42 mg/mL for •OH scavenging activity. The ME-PRH exhibited the highest ABTS, •OH, and H₂O₂ scavenging activities, while the ME-ALP exhibited the highest DPPH scavenging activity.

ACE inhibitory activity of eel extracts and hydrolysates

The ACE inhibitory activities of the hot water extracts at 25 h were 8.6% for the ME and 0% for the WBE, while those of the hydrolysates at 10 h were approximately 75% for all hydrolysates (72.6% for the ME-ALP, 75.7% for the

ME-PRH, and 75% for the ME-APH; Fig. 4A). The ACE inhibitory activities of the hydrolysates at 10 h of hydrolysis were not significantly different (p=0.108). The ACE inhibitory activities of the hydrolysates showed an initial rapid increase during the first 2 h of hydrolysis and then maintained a similar activity for all hydrolysates. The highest activity levels were 74.79% (8 h) for the ME-ALP, 78.30% (8 h) for the ME-PRH, and 80.42% (2 h) for the ME-APH (Fig. 4B). To characterize the ACE inhibitory activity of the hydrolysates further, IC₅₀ values for ACE inhibition of the hydrolysates were determined as shown in Table 1. The IC₅₀ values for the ME-ALP, ME-PRH, and ME-APH were 132.91, 110.37, and 115.17 μg/mL, respectively (p=0.164). The IC₅₀ value for captopril, a synthetic ACE inhibitor (positive control), was 0.0567 μg/mL. Similar to the results for antioxidative activity, the greatest ACE inhibitory activity was found in the ME-PRH, although the values were not significantly different among the hydrolysates.

Amino acid composition of eel extracts and hydrolysates

The amino acid composition of eel meat extract and hydrolysates were compared in Table 2. The contents of glycine and alanine were to approximately 37% and the ratio of hydrophobic amino acids to total amino acids was 0.2081 on ME. On three hydrolysates of Eastern eel, the contents of leucine, valine and tyrosine, which were hydrophobic amino acids, were increased more than 2 times and glycine and alanine were decreased to 8.46 and 8.56%, respectively. The ratios of hydrophobic amino acids to total

Table 1. IC₅₀ values of Eastern eel hydrolysates on various antioxidative and ACE inhibitory assay systems

Hydrolysates	DPPH (mg/mL)	ABTs (mg/mL)	Superoxide (mg/mL)	Hydrogen peroxide (mg/mL)	Hydroxyl (mg/mL)	ACE inhibitory (μg/mL)
ME-ALP	2.73±0.02 ^{a1)}	1.28±0.08 ^c	3.01±0.05	1.83±0.01 ^{7b}	1.37±0.00 ^b	132.91±17.31
ME-PRH	3.02±0.03 ^b	1.13±0.02 ^a	3.07±0.05	1.11±0.00 ^a	1.30±0.00 ^a	110.37±12.85
ME-APH	3.21±0.01 ^c	1.17±0.00 ^b	3.14±0.03	1.85±0.00 ^b	1.61±0.00 ^c	115.17±4.48
Ascorbic acid	- ²⁾	0.22±0.03	-	0.31±0.03	0.42±0.04	-
α-Tocopherol	0.36±0.01	-	0.41±0.08	-	-	-
Captopril						0.06±0.01

¹⁾Mean±SD (n=3) within each column followed by different letters are significantly different (p<0.05).

²⁾Not tested.

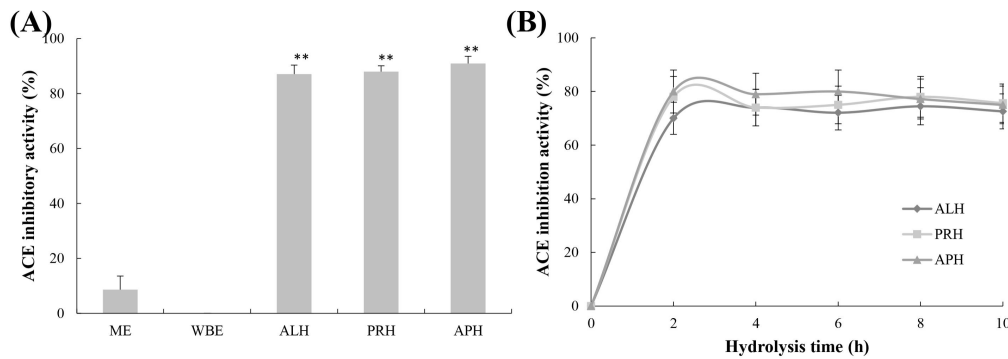


Fig. 4. Comparison of ACE inhibitory activities of Eastern eel extracts and hydrolysates.

A, ACE inhibitory activities of Eastern eel at final extraction (25 h) and hydrolysis (10 h) time; B, ACE inhibitory activities of Eastern eel hydrolysates prepared by different enzymes for 10 h hydrolysis times.

ME, meat extracts; WBE, whole body extracts; ALP, Alcalase®-treated hydrolysates (ME-ALP); PRH, Promatex®-treated hydrolysates (ME-PRH); APH, the mixture of Alcalase® and Promatex®-treated hydrolysates (ME-APH).
** means significant difference ($p < 0.01$).

Table 2. The amino acid composition of eel extracts and hydrolysates

Amino acid	ME	ME-ALP	ME-PRH	ME-APH
Histidine	4.69	3.94	4.45	4.15
Arginine	4.41	5.01	4.98	5.24
Threonine	6.75	5.76	5.64	5.95
Methionine	1.88	4.28	3.73	4.05
Isoleucine ¹⁾	2.27	6.75	6.52	6.53
Leucine ¹⁾	4.79	11.02	11.02	10.68
Phenylalanine ¹⁾	1.26	4.98	5.21	4.92
Tryptophan ¹⁾	0.00	0.00	0.00	0.00
Lysine	6.21	8.92	10.22	8.52
Valine ¹⁾	2.75	7.73	7.37	7.67
Cysteine / cystine	0.00	0.00	0.00	0.00
Glutamine / glutamic acid	9.44	8.28	8.45	8.74
Aspartic acid / asparagine	6.08	3.12	3.10	3.03
Serine	2.93	3.28	3.03	3.40
Glycine	23.98	8.46	8.55	8.70
Alanine	12.83	8.56	7.93	8.19
Proline ¹⁾	8.81	6.52	6.60	6.75
Tyrosine ¹⁾	0.93	3.40	3.20	3.49
Total amino acid	100.00	100.00	100.00	100.00
Sum of hydrophobic amino acids	20.81	40.40	39.92	40.04

¹⁾Hydrophobic amino acids.

amino acids for the ME-ALP, ME-PRH, and ME-APH were 0.404, 0.3992, and 0.4004, respectively, indicating the hydrophobic amino acids on three hydrolysates of Eastern eel were released via enzymatic hydrolysis.

Discussion

The eels were considered as a nutritious and tasty species, and as an oriental medicine (Khanh and Ngan, 2010). Especially in Korea and Japan, eels were thought to boost stamina in hot weather, and therefore they have been consumed as the various forms including hot water extracts. Recently, fish protein hydrolysates are widely focused as the main source of bioactive peptides for various health benefits. In this study, to evaluate the health benefits of Eastern eel extracts and hydrolysate, the antioxidative and ACE inhibiting effects of them were compared.

An efficient way of assessing antioxidative activity of eel extracts and hydrolysates is to examine the ability to trap the free radicals produced in various multiple reactions. The radical system used for evaluation of antioxidative activity may influence the experimental result, because of differences in the mechanism of antioxidative action being measured or the reaction conditions used. Therefore, two or more radical systems are required to investigate radical scavenging capacities (Yu et al., 2002). A range of assays, including DPPH, ABTS, O_2^- , $\bullet OH$, and H_2O_2 scavenging activities, were performed to assess the antioxidative activities of the

eel extracts and hydrolysates. These trapping techniques measure the radical scavenging activities of samples against free radicals such as DPPH, ABTS, O_2^- , $\bullet OH$, and H_2O_2 .

DPPH is a stable free radical molecule that changes color from deep violet to colorless or pale yellow on reduction by either hydrogen or electron donation. Substances that can donate hydrogen or electrons are considered to have anti-oxidative activity. The blue/green $ABTS^{+}$ radical cation produced through the reaction between ABTS and potassium persulfate is converted to a colorless neutral form by a reaction with antioxidants (Carocho and Ferreira, 2013). O_2^- is normally formed during the first step of a cellular oxidation reaction, and its effect can be magnified as it produces other kinds of cell-damaging free radicals and oxidizing agents (Liu and Ng, 2000). $\bullet OH$ is the neutral form of the hydroxide ion (OH^-) and the most ROS in biological systems. This radical can damage all types of macromolecules, including carbohydrates, nucleic acids, lipids, and amino acids, and it has the strongest cell-damaging action among free radicals (Chung et al., 1997; Liu and Ng, 2000). In addition, this radical combine with the nucleotides in DNA and cause strand breakage, leading to carcinogenesis, mutagenesis, and cytotoxicity (Khan et al., 2012). H_2O_2 is a non-reactive but unstable molecule. Sometimes it is converted to free OH^- radicals, which react with the proteins, membrane lipids, and DNA in living cells and cause tissue damage and cell death (Khan et al., 2012).

In our present study, the antioxidative activities of the eel hydrolysates were higher than those of extracts in all types of antioxidative assay systems. These results are in line with Slizyte et al.'s report (2016), showing that the fish protein hydrolysates from defatted salmon backbones obtained with commercial enzymes increased the antioxidative DPPH radical scavenging ability by 10-25% compared to those without hydrolysis. Among eel hydrolysates, especially the eel hydrolysate treated with Protamex (ME-PRH) exhibited the highest ABTS, $\bullet OH$, and H_2O_2 scavenging activities, indicating ME-PRH could be potential anti-oxidant against free radicals with the strong cell-damaging action.

ACE is a dipeptidyl carboxypeptidase (EC. 3.4.15.1) that was originally isolated from horse blood. It exists as a membrane-bound enzyme as well as a circulatory or globular enzyme (Ortiz-Salmeron et al., 1998). In the renin-angiotensin system, renin promotes the hydrolysis of angiotensin

to the decapeptide angiotensin I and ACE hydrolyzes the dipeptide His-Leu at the C-terminus of angiotensin I to produce active angiotensin II, which has a vasoconstricting action. In addition, ACE hydrolyzes and inactivates the vasodilator bradykinin (Das and Soffer, 1975). Therefore, the inhibition of ACE will prevent the production of angiotensin II and inhibit the hydrolysis of bradykinin, which will be useful for controlling high blood pressure and hypertension. In fact, ACE inhibitors are used to treat myocardial infraction, hypertension, and other cardio-related diseases. However, most ACE inhibitors are synthetic, and these drugs have certain adverse side effects (Raghavan and Kristinsson, 2009). Therefore, a number of studies have been conducted to find ACE inhibitors from natural resources, including food protein hydrolysates; the most useful natural ACE inhibitors were found to be peptides from protein hydrolysates (Iwaniak et al., 2014). Sila et al. (2013) recorded 87% ACE inhibition activity for an Alcalase[®] hydrolysate from the muscle of Barbel (*Barbuscallensis*), and Athukorala and Jeon (2005) recorded 85% for Alcalase[®] and 74% for Promatex[®] hydrolysates of different algal species. In our results, the measurements were very high (> 70%). The IC_{50} values for the ME-ALP, ME-PRH, and ME-APH were 132.91, 110.37, and 115.17 $\mu g/mL$, respectively. In Coscueta et al. (2016)'s reports, the potential antihypertensive peptides from milk and soy derived protein hydrolysates showed the IC_{50} values of 33-930 $\mu g/mL$, corresponding to the range of eel hydrolysates. In addition, Karaki et al. (1990) reported the anti-hypertensive effect in hypertensive rats using a casein hydrolysate with IC_{50} value of 166 $\mu g/mL$. The standard ACE inhibition peptides, Val-Trp and Ala-Tyr, were reported to have the ACE inhibition activity with IC_{50} value of 200-900 $\mu g/mL$ (Sato et al., 2002, Slizyte et al., 2016). Baharuddin et al. (2016) reported the ACE inhibitory activity of eel (*Monopterus* sp.) protein hydrolysate with IC_{50} value of 2.128 mg/mL at DH 36%. These results were rather lower than ACE inhibition activities of Eastern eel hydrolysates treated with Alcalase[®], Protamex[®], and their mixture in this study, confirming that enzymatic hydrolysates of Eastern eel (*A. japonica*) could be excellent natural source for ACE inhibitor candidates.

Generally, it has been demonstrated that the biological activities of proteins can be increased via hydrolysis with various enzymes (Chen et al., 1995). During hydrolysis,

native proteins are cleaved by enzymes, and hidden hydrophobic sites inside the proteins are exposed. These exposed hydrophobic amino acid residues in the peptides obtained confer various biological activities, especially antioxidative and ACE inhibitory activities (Faithong et al., 2010). Additionally, the biological activities of protein hydrolysates depend on the protein substrate, the specificity of the enzyme, the conditions used during proteolysis, and the degree of hydrolysis (Sila et al., 2013). Elavarasan et al. (2014) reported that protein hydrolysates differ in their antioxidant properties based on the nature of the proteases used as the sequence of released peptides are different chiefly based on the type of enzyme used for cleavage. Protein hydrolysates produced by different enzymes have different-sized peptides and amino acid sequences, which can have significantly different biological activities (Centenaro et al., 2011). In this study, the observed antioxidative and ACE inhibitory activities may be related to the enzyme used and each hydrolysate's composition. Alcalase[®] is a serine alkaline endoprotease with broad specificity; it hydrolyzes most peptide bonds, but preferentially those containing aromatic amino acid residues (Doucet et al., 2003). Promatex[®] is also an endopeptidase with broad specificity for hydrophobic amino acids (Ward, 1983). Therefore, each hydrolysate produced by these enzymes may produce various hydrophobic peptides, which might stabilize radicals and inhibit ACE. Actually, Table 2 shows that hydrophobic amino acid contents on three hydrolysates of Eastern eel were higher than on meat extract, indicating the release of hydrophobic peptides via enzymatic hydrolysis. However, additional investigations are required to obtain more information about the amino acid sequences of the active peptides.

Collectively, the enzymatic hydrolysates of Eastern eel (*A. japonica*) by Alcalase[®] and Promatex[®] could be excellent natural source for antioxidant and ACE inhibitor candidates.

요약

본 연구는 민물장어의 생리활성을 확인하기 위해 민물장어 열수추출물과 효소가수분해물을 제조하여 항산화 활성과 ACE 저해 활성을 비교하였다. 항산화 활성은 다양한 형태의 라디칼인 DPPH, ABTS, O₂⁻, •OH, and H₂O₂ 등에 대한 소거능으로 비교하였다. 연구결과, 민물장어 추출물은 단백질 수

율 및 항산화 활성 및 ACE 저해활성이 효소가수분해물에 비해 대체적으로 낮았다. Alcalase[®] 가수분해물은 DPPH 라디칼 소거능이 가장 뛰어났으며, IC₅₀값은 3.02 mg/mL였다. 반면 ABTS, •OH, and H₂O₂ 등에 대한 소거능은 Protamex[®] 가수분해물이 가장 높게 나타났으며, 그 IC₅₀값은 각각 1.13 mg/mL, 1.30 mg/mL, 1.11 mg/mL이었다. 민물장어 가수분해물의 ACE 저해활성도 대단히 높게 나타났으며, Protamex[®] 가수분해물의 경우 IC₅₀값이 110.37 µg/mL였으며, 기존에 보고된 다양한 가수분해물의 저해활성에 비해 매우 높은 ACE 저해 활성을 보였다. 이상의 결과에서 민물장어 효소가수분해물은 항산화제와 ACE 저해제로 노화를 방지하고 혈압을 감소시킬 수 있는 생리기능성을 가진 식품 소재로서의 가능성을 보였다.

Conflict of interests

The authors declare no potential conflict of interest.

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