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Screening for Angiotensin 1-Converting Enzyme Inhibitory Activity of *Ecklonia cava*

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Abstract

Seven brown algal species (*Ecklonia cava, Ishige okamurae, Sargassum fulvellum, Sargassum horneri, Sargassum coreanum, Sargassum thunbergii* and *Scytosiphon lomentaria*) were hydrolyzed using five proteases (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase) and screened for angiotensin 1-converting enzyme (ACE) inhibitory activities. Most algal species examined showed good ACE inhibitory activities after the enzymatic hydrolysis. However, *E. cava* was the most potent ACE inhibitor of the seven species. Flavourzyme digest of *E. cava* exhibited an IC_{50} of around 0.3 μg/mL for ACE; captopril has an IC_{50} of ∼0.05 μg/mL. The Flavourzyme digest was separated to three fractions by an ultrafiltration membrane (5, 10, 30 kDa MWCO) system according to the molecular weights. The active components were mainly concentrated in >30 kD fraction which are composed of the highest protein content (27%) and phenolic content (261 mg/100 mL) compared to the other two smaller molecular weight fractions. Therefore, the active compounds appear to be relatively high molecular weight complex molecules associated with protein (glycoprotein) and polyphenols. Therefore, *E. cave* is a potential source of antihypertensive compound.

Key words: brown algae, *Ecklonia cava*, ACE inhibition, enzymatic hydrolysis, Flavourzyme

INTRODUCTION

ACE plays an important physiological role in the regulation of blood pressure and electrolyte homeostasis. It cleaves angiotensin I to angiotensin II, which is a powerful vasoconstrictor and salt-retaining octapeptide. Moreover, it catalyzes the inactivation of bradykinin, which is a vasodilator and natriuretic nonapeptide (1-3). More than a dozen ACE inhibitors have been used extensively in the treatment of essential hypertension and heart failure in humans; these include alacepril, benazepril, captopril, cilazapril, enalapril, fosinopril, lisinopril, moexipril, perindopril, quinapril, ramipril, tandolapril, and zofenopril (4-6). However, these synthetic drugs are believed to have certain side effects, such as cough, taste disturbances and skin rashes (7). Therefore, a search for ACE inhibitors from foods has become a major area of research.

Bioactive peptides can be released by enzymatic proteolysis of food proteins and may act as potential physiological modulators of metabolism during the intestinal digestion of the diet. The possible regulatory effects of peptides related to nutrient uptake include immune defense, opioid and antihypertensive activities (8). The ACE-inhibitory activity of foods has been studied, and it was found that some ACE-inhibitory peptides are produced by enzymatic digestion of various food proteins, including casein (9,10), zein (11,12), soybean protein (13), dried-salted fish (14), ovalbumin (15), fish sauce (16), and fish water-soluble protein (17).

Recently, seaweeds have been received attention, because they are a nutritious food with health-stimulating properties and medicinal effects. Dietary ingestion of seaweeds has been shown to decrease blood pressure in humans (18). Also, in spontaneously hypertensive rats, oral dosage of peptides isolated from algae caused a significant, sustained reduction in blood pressure up to 24 hr after administration (19).

Hence, scientists have focused special attention on marine algae in order to identify and evaluate natural antihypertensive compounds. Even if seaweeds contain a low amount of protein, the amino acid heterogeneity of those proteins is very high among seaweeds. Therefore, it is believed that the evaluation of algal species is promising way to find new anti-hypertensive compounds.

In this study, seven brown algal species (*E. cava, I. okamurae, S. fulvellum, S. horneri, S. coreanum, S. thunbergii* and *S. lomentaria*) were hydrolyzed using five commercially available proteases: Protamex, Kojizyme, Neu-

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trase, Flavourzyme and Alcalase, and their hydrolysates were screened to evaluate their antihypertensive activities.

MATERIALS AND METHODS

Materials

Marine brown algal species were collected close to the shores of Jeju Island in Korea during March and October 2004. Salt, sand and epiphytes were removed using tap water. Finally, seaweed samples were rinsed carefully with fresh water and freeze-dried at -20°C for further experimentation. Proteases such as Protamex (hydrolysis of food proteins), Kojizyme 500MG (boosting of the Soya sauce fermentation), Neutrase 0.8L (an endoprotease), Flavourzyme 500MG (containing both endopeptidase and exopeptidase activities), Alcalase 2.4L FG (an endoprotease) were obtained from Navo Co. (Novozyme Nordisk, Bagsvaed, Denmark). Hippuryl-L-histydyl-L-leucine (HHL) and angiotensin 1-converting enzyme (ACE) were obtained from Sigma Chemicals Co. (St. Louis, MO). All other chemicals used in this study had 90% or more purity.

Digestion of algal species

Dried algae sample was ground (MFC SI mill, Janke and Kunkel Ika-Wreck, Staufen, Germany) and sifted through a 50 mesh standard testing sieve. One hundred gram alga samples were homogenized with water (2 L), and mixed with 1 g or 1 mL enzyme. The enzymatic hydrolytic reactions were performed for 12 h to achieve optimum degree of the hydrolysis. Before digestion, the pHs of the homogenates were adjusted to be within the optimal range for the respective enzymes. Following digestion, the digests were boiled for 10 min at 100°C to inactivate the enzymes. Each sample was clarified by centrifugation (3,000 rpm, for 20 min at 4°C) to remove the residue. All samples were adjusted to pH 7.0 and stored at -20°C for further experiments.

Water extraction

Three grams of the ground algal powder was mixed with 50 mL of water and placed in shaking incubator for 12 hr at 25°C. The mixtures were then centrifuged at 10,000 rpm for 20 min at 4°C and filtered with Whatman filter paper. Finally, each supernatant was then assayed for ACE inhibition activity.

Determination of protein content

The amount of crude protein was determined by the Lowry method, absorbance at 540 nm using brovine serum albumin as the calibration standard (20).

ACE inhibitory activity

ACE inhibitory activity was assayed by the method of Cushman and Chung (21) with slight modifications. HHL was dissolved in 100 mM sodium borate buffer, pH 8.3, containing 300 mM NaCl. 200 μL of a 5 mM HHL solution was mixed with 80 μL of a captopril solution, and then preincubated for 3 min at 37°C. The reaction was initiated by adding 20 μL of an ACE solution in distilled water (100 mU/mL), and the mixture was incubated for 30 min at 37°C. The reaction was stopped by addition 250 μL of 1 M HCl. The hippuric acid liberated by the ACE reaction was extracted with 1.7 mL ethyl acetate, and the solvent was removed by evaporation in an oven (120°C). The residue was dissolved in 1 mL distilled water and its UV spectra density was measured at 228 nm.

The extent of inhibition was calculated as follows:

$$\text{Inhibition }\% = Ac - (As - Ab)/Ac$$

Ac=Absorbance of control sample

As=Absorbance of test solution

Ab=Absorbance of blank solution

Molecular weight fractionation of algal extracts

Algal extracts were passed through microfiltration membranes (5, 10 and 30 kD) using Millipore's Labscale TFF system (Millipore Corporation, Bedford, Massachusetts, USA) to obtain different molecular weight fractions. Finally, fractions (>30, 30~10, 10~5 and <5 kD) were separately processed to evaluate ACE inhibition activity.

Determination of total polyhenolic content

Total polyphenolic compounds were determined according to a protocol similar to that of Chandler and Dodds (22). *E. cava* extract (1 mL) was mixed with 1 mL of 95% ethanol, 5 mL of distilled water and 0.5 mL of 50% Folin-Ciocalteu reagent. The mixture was allowed to react for 5 min and 1 mL of 5% Na_2CO_3 was added. Finally, each sample was mixed thoroughly and placed in the dark for 1 h and absorbance was measured at 725 nm with a UV-vis spectrophotometer. A gallic acid standard curve was obtained for the calculation of polyphenolic content.

RESULTS AND DISCUSSION

In this study, water extracts of seven brown algal species were screened for their potential ACE inhibitory activities. ACE inhibitory activities of water extracts from seven brown algal species (*E. cava*, *I. okamurae*, *S. fulvellum*, *S. horneri*, *S. coreanum*, *S. thunbergii* and *S. lomentaria*) are shown in Table 1. The water extract of *E. cava* had the highest ACE inhibitory activity (36%). Water extracts of *S. fulvellum* and *I. okamurae* also exhibited moderate ACE inhibitory activities of 24 and 22%, respectively, whereas *S. horneri* and *S. thun-*

Table 1. ACE inhibitory activities of several brown algal water extracts

Species	ACE inhibitory activity (%)
Ecklonia cava	36.1±2.1[1)]
Ishige okamurae	22.9±1.3
Sargassum fulvellum	24.3±1.5
Sargassum horneri	18.4±1.6
Sargassum coreanum	11.2±0.8
Sargassum thunbergii	17.3±0.8
Scytosiphon lomentaria	5.1±1.2

[1)]Values are mean±SD of two determinations, and the sample concentration is 1 mg/mL.

bergii water extracts exhibited weak activities. Compared to other algal species, the water extract of *S. lomentaria* showed the least ACE inhibitory activity (5%). According to our results, the water extracts of all algal species exhibited moderate ACE inhibition. It has been reported that the anti-hypertensive compounds in extracts from 10 fruit bodies of edible mushrooms have been screened for the inhibitory activity against angiotensin 1-converting enzyme. The most potent ACE inhibitory activity (58.7%) has been obtained from cold-water extract of *Grifola frondosa* with an IC_{50} of 0.95 mg (23).

Since all algal water extracts contained ACE inhibitory compounds, those samples were then subjected to enzymatic hydrolysis using five proteases (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase). After enzymatic hydrolysis of those algal species, they were then again evaluated for ACE inhibitory assay (Table 2). Most algal species in this study showed superior potential ACE inhibition after enzymatic hydrolysis. As it has been reported from previous studies, the enzymatic extraction of algae enhances ACE inhibition making them superior to water extracts (18). As shown in Table 2, all enzymatic extracts of *E. cava* showed more than 60% ACE inhibition activities at 1 mg/mL concentration, especially Flavourzyme and Neutrase enzymatic hydrolysates showed higher activities (80 and 76%), respectively than their counterparts. *I. okamurae* hydrolysates also had considerable ACE inhibition activity. Except for the Flavourzyme extract of this species, other four counterparts showed over 50% ACE inhibitory activities. The highest activity (85%) of this species was recorded from the Alcalase extract. Kojizyme and Protamax extracts of *S. fullvelum* exhibited very high ACE inhibitory activities (97 and 74%), in contrast, the other hydrolysates of this species did not exhibit significant ACE inhibition. According to the results, *S. horneri* hydrolysates also had good ACE inhibitory activities. Especially, Kojizyme and Neutrase digests of *S. horneri* showed good ACE inhibitory activities (91 and 86%) respectively. However, Alcalase hydrolysate did not show ACE inhibitory activity. All enzymatic extracts of *S. coreanum* showed moderate ACE inhibitory activities. However, Protamax, Neutrase and Flavourzyme extracts had better activities than the other extracts of this species. The extracts of *S. thunbergii* and *S. lomentaria* exhibited relatively lower activities, but Kojizyme and Neutrase extracts of *S. lomentaria* showed considerable activities (62 and 60%), respectively.

Enzymatic hydrolysis of proteins allows the preparation of bioactive peptides to more complex food source proteins having a potential use as nutraceuticals. Bioactive peptides can be obtained by *in-vitro* hydrolysis of protein substrates using appropriate enzymes (24). The physico-chemical conditions of the reaction media, such as temperature and pH of the protein solution, must then be adjusted in order to optimize the activity of the enzyme used. Any given enzymatic hydrolysate cannot fulfill many properties or be universal in its use. Enzymatic hydrolysates are therefore specific and tailor-made for each application (25). Consistent with those previous observations, this study also obtained different activities after enzymatic hydrolyses. This fact may be due to different physiochemical conditions of the reaction mixture. When we evaluate all results of this study, it is clear that all enzymatic extracts of *E. cava* exhibited relatively higher ACE active compounds. There-

Table 2. Angiotensin 1-converting enzyme inhibitory activity (%) of brown algae hydrolyzed with proteases

Algal species	Enzymes treated[1)]				
	1	2	3	4	5
Ecklonia cava	67.8±1.4[2)]	65.1±1.5	76.9±2.3	80.0±2.1	60.0±1.1
Ishige okamurae	66.4±2.1	59.7±2.3	64.1±1.6	39.5±2.2	85.8±1.7
Sargassum fulvellum	74.0±1.5	97.0±0.8	0	0	0
Sargassum horneri	57.3±0.3	91.6±1.3	86.7±1.5	21.6±2.3	0
Sargassum coreanum	69.9±1.8	45.4±0.	65.0±1.9	65.0±2.7	44.0±0.6
Sargassum thunbergii	37.0±1.8	25.0±1.7	52.0±1.7	21.0±1.6	34.0±0.9
Scytosiphon lomentaria	35.0±1.4	62.0±1.2	60.0±2.1	22.0±1.3	39.0±1.8

[1)]1, Protamex; 2, Kojizyme; 3, Neutrase; 4, Flavourzyme; 5, Alcalase.
[2)]Values are mean±SD of two determinations and the sample concentration is 1 mg/mL.

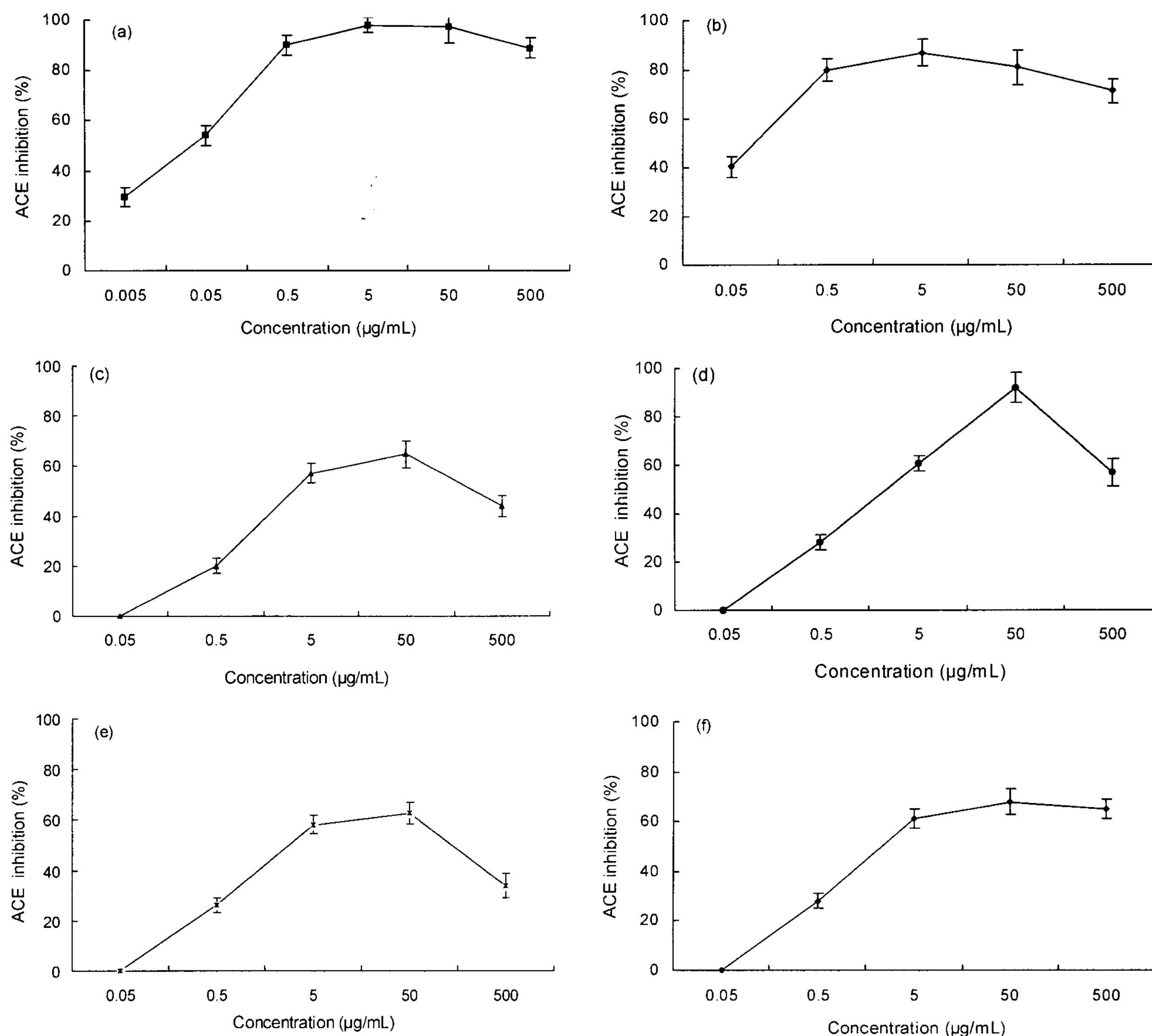

Fig. 1. ACE inhibition by enzymatic hydrolysate of *E. cava* and captopril. (a) captopril, (b) Flavourzyme digest, (c) Neutrase digest, (d) Alcalase digest, (e) Protamex digest, (f) Kojizyme digest.

fore, dose-dependent activity of protein hydrolysates from *E. cava* was evaluated in order to examine the exact IC_{50} value of each sample, and the IC_{50} values of the extracts were compared with that of captopril.

The dose-dependant ACE inhibitory activity of captopril is shown in Fig. 1a. As we expected, ACE inhibitory activity of captopril dramatically increased with increasing concentration. The activity reached a maximal level (98%) at 5 μg/mL and the IC_{50} value for captopril was approximately 0.05 μg/mL (Table 3). Watanabe et al. (26) reported that the IC_{50} of captopril is 0.08 μg/mL.

The dose-dependant ACE inhibitory activity of *E. cava* is shown in Fig. 1b. Flavourzyme hydrolysate of *E. cava* inhibited ACE activity in a concentration-dependant manner from 0.05 to 500 μg/mL and reached maximum at 5 μg/mL, and then decreased as sample concentration was increased above 50 μg/mL. Previously it has been reported that a good ACE inhibitory activity was obtained from *Porphyra yezoensis* (18). When *P. yezoensis* was hydrolyzed with pepsin, protease P, Denazyme AP and Bioprase PN4, the inhibitory activity of pepsin digest was the most potent with an IC_{50} of just 1.52 mg/mL of reaction mixture. In this study, thus, Flavourzyme

Table 3. ACE inhibition by enzymatic hydrolysate of *E. cava*

Protease	IC_{50} (μg/mL)
Flavourzyme	0.30 ± 0.5[1)]
Neutrase	4.47 ± 1.3
Alcalase	4.10 ± 0.8
Protamex	4.12 ± 0.6
Kojizyme	4.10 ± 1.2
Captopril	0.05 ± 0.8

[1)]Values are mean ± SD of three determinations.

hydrolysate of *E. cava* exhibited a promising ACE inhibitory activity with an IC_{50} value of 0.3 μg/mL.

As shown in Fig. 1c, the Neutrase digest of *E. cava* inhibited ACE in a dose-dependant manner. However, no ACE inhibition was observed at the lowest concentration tested in this study. This hydrolysate reached its maximum inhibitory activity (64%) at 50 μg/mL. Addition of this extract to the mixture rendered an IC_{50} value of 4.47 μg/mL (Table 3). In Fig. 1d, *E. cava* Alcalase digest results for the ACE inhibition are presented. ACE inhibition activity of this sample increased linearly with the increasing sample concentrations, however at a concentration of 50 μg/mL, the activity started to decrease. The highest inhibition activity (91%) of this sample was at a 50 μg/mL concentration and the IC_{50} was 4.1 μg/mL. ACE inhibitory activity of Protamex hydrolysate (Fig. 1e) and Kojizyme extract (Fig. 1f) of *E. cava* exhibited a similar trend to that of Neurase hydrolysate, and both extracts reached a maximum (approximately 68%) at 50 μg/mL.

Enzymatic hydrolysis is a process based on the use of proteases for the modification (breakdown) of proteins. The degree of hydrolysis is the extent of peptide bond breakage by the enzymatic hydrolysis reaction. A fifty percent degree of hydrolysis measurement may not mean that fifty percent of the protein is hydrolyzed. Rather, it may mean that a particular enzyme has hydrolyzed fifty percent of the available bonds for that specific enzyme (24,25). According to our results, Flavourzyme is able to enhance the digestion of specific peptide bonds of algal mass to increase ACE activity. Therefore, it is the enzyme of choice for further experiments to digest specific peptide bonds present in the intact protein of *E. cava*. In order to characterize the molecular weight of the active fraction, the Flavourzyme digest of *E. cava* was passed through ultrafiltration membranes (5, 10 and 30 kD) and relevant molecular weight cut-off fractions were separated and evaluated for ACE inhibition. The molecular weight of the hydrolysate is one of the most important factors in producing protein hydrolysates with the desired functional properties to use as functional materials (27). The most potent activity (76%) was obtained from the >30 kD fraction with high protein concentration. Below 5 and 5~10 kD molecular weight cut-off fractions had 18 and 12% ACE inhibition activities, respectively, however no activity was observed in the 10~30 kD fraction.

As has been mentioned in previous experiments, Flavourzyme has been used to prepare hydrolysates from corn glutein (27) and ACE inhibitory active fractions have been obtained followed by ultrafiltration membranes. The results of that study indicated that separation of hydrolysates based on molecular weight distinctly enhanced the ACE inhibitory activity. Also, there are reports of the presence of high molecular weight protein (22~24 kD) called "oligomers" with potential ACE inhibitory activity. Furthermore, Paulis and Bietz (28) have suggested oligomer formation in zein, an extract from gluten with potential ACE inhibitory activity.

In this study, the >30 kD fraction contains the highest amount of protein and phenolic compounds compared to the other two smaller molecular weight fractions (Table 4). Based on the above results, the active compounds appear to be high molecular weight complex forms with protein (or glycoprotein) and phenolic compounds. Recently, it has been found several interesting polyphenolic compounds with potential ACE inhibitory activity (29-32). Polyphenolic compounds inhibit ACE activity through sequestration of the enzyme metal factor (Zn^{2+}) (29). Furthermore, recently several important tannin compounds, which are involved in polyphenolic compounds, have been isolated that inhibited ACE activity (IC_{50}) at concentrations less than 200 μM (29). Therefore, it can be assumed that polyphenolic compounds present in *E. cava* might form some type of complex associated with proteins or glycoproteins and have ACE inhibitory activity. Therefore, further experiments are needed to evaluate the exact bioactive compound.

Although the ACE inhibiter derived from *E. cava* was weaker than captopril, further purification may improve its activity, even as a crude extract it has a great potential in ACE inhibition. Therefore, separation of pure active compounds from the Flavorzyme hydrolysate of *E. cava* is required for further studies.

Table 4. ACE inhibition activities of molecular weight fractionations of Flavourazyme hydrolyzed *E. cava* and their protein contents

Fraction	ACE inhibition	Protein (%)	Total phenolic compounds (mg/100 mL)
Above 30 kD fraction	76.6±2.1	27	261
30~10 kD fraction	-	13	112
10~5 kD fraction	12.1±2.0	13	124
Below 5 kD fraction	18.3±1.3	13	61

Results are means of two determinations and the sample concentration is 80 μg/mL.

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