Phytopharmacology

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Contribution of non-peptide substances to inhibition of angiotensin I-converting enzyme (ACE) by aqueous extract of brown seaweed *Undaria pinnatifida*

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Received: 2 March 2012, **Revised**: 2 April 2013, **Accepted**: 9 April 2013

Abstract

Brown seaweeds are thought to be beneficial to the human health, but little is known about their functional abilities to manage the blood pressure. Then, the effects of the extracts prepared from sporophyll, stem and leaf of brown seaweed *Undaria pinnatifida* on angiotensin I-converting enzyme (ACE) were examined to assess their antihypertensive activities. The extracts inhibited the enzyme activity in a concentration-dependent manner, and the 50% inhibition was obtained by approximately 15 μl of the extract. Further studies showed that sporophyll extract inhibited the enzyme in a competitive manner, and the inhibitory activity was recovered in the non-peptide fraction from the separation processes using both spin column and HPLC systems. Therefore, brown seaweed, particularly its sporophyll, was considered to contain non-peptide substances to inhibit ACE activity, thereby improving the hypertensive conditions by reducing the production of active hypertensive peptide.

Keywords: Brown seaweed, Sporophyll extract, Non-peptide substance, Functional food, Antihypertensive effect, High blood pressure

Introduction

Hypertension is one of the risk factors for cardiovascular diseases, and the implication of the renin-angiotensin-aldosterone system in the regulation of blood pressure has been established. In particular, angiotensin-II is generally recognized as an active peptide playing

a pathogenic role in the onset of hypertension, and angiotensin I-converting enzyme (ACE), the enzyme catalyzing the conversion of inactive angiotensin-I to the active hypertensive peptide, has therefore come to draw a lot of attention. Moreover, the inhibition of this enzyme is considered to reduce the blood pressure, thereby improving the conditions of hypertensive patients. Based on this concept, much work has previously been done to find out potential active substances inhibiting the production of this hypertensive peptide in the vascular system, and a variety of natural food materials, such as plant foods including wheat, rice, peas, corn, fruits and vegetables have been enthusiastically evaluated by determining their inhibitory effects on ACE activity *in vitro*. Consequently, various peptides derived from dietary sources have been reported to inhibit ACE activity, thus proposing their potential abilities for the improvement of hypertension (Marti'nez-Maqueda, et al., 2012). As several specific examples, the active peptides inhibiting ACE activity have been identified in the extracts prepared from cowpea and Korean rice wine (Kang, et al., 2012; Segura Campos, et al., 2010). Moreover, the synthetic peptides containing selenocysteine and cysteine residues have also been reported to cause the inhibitory effect on ACE activity (Bhuyan and Mugesh, 2012). Therefore, it seemed interesting and significant to investigate the potential inhibitory effects of peptide-rich food materials on ACE activity *in vitro*.

Brown seaweed *Undaria pinnatifida* is one of the popular marine products, and widely used in East Asian region as a food stuff. Because of low-calorie and low-fat content, brown seaweed is favorable as an ingredient of diet food in dietetic treatment for adiposis and obesity. Recently, seaweeds have been shown to cause the anti-cancer and anti-proliferative actions (Furusawa and Furusawa, 1989; Lins, et al., 2009; Maruyama, et al., 2003; Riou, et al., 1996; Yuan and Walsh, 2006), and reported to have various biological activities (Smit, 2004) including the anti-bacterial and anti-viral actions (Bansemir, et al., 2004; Damonte, et al., 2004; Kolanjinathan, et al., 2009; Thompson and Dragar, 2004). On the other hand, the alcoholic extract, but not aqueous extract, prepared from the sporophyll of brown seaweed has been reported to cause the toxic damage to human colorectal cancer cells (Nishibori, et al., 2012a), but the aqueous extract of the sporophyll has been shown to be oppositely protective against iron-induced oxidative damage to PC12 cells, thus suggesting that brown seaweed sporophyll may contain a variety of active substances, which may be differentially extracted by different methods. Therefore, it seems interesting and worth examining the biological activities of various extracts prepared from brown seaweed by different preparation methods.

Recent studies have reported that several oligopeptides isolated from the enzymatically hydrolyzed brown seaweed can inhibit ACE activity *in vitro*, resulting in the reduction of blood pressure in spontaneously hypertensive rats, thereby proposing the possibility that brown seaweed may be able to reduce the blood pressure in patients with hypertension (Sato, et al., 2002; Suetsuna, et al., 2004). However, these active peptides are virtually produced by the enzymatic digestion of brown seaweed, and therefore seems possible that these peptides may be artificial products derived from the proteinous components of the seaweed. Then, to answer the question whether brown seaweed *Undaria pinnatifida* may intrinsically contain natural substances which can be essentially active as a potential inhibitor of ACE activity, we prepared the aqueous extracts from different parts of seaweed, such as sporophyll, leaf and stem, and examined the direct effects of these extracts on the enzyme activity *in vitr*o. Consequently, the extract of sporophyll was shown to cause the most potent inhibition of ACE

activity as compared to the extracts of leaf and stem, and therefore the present study is possibly considered to provide evidence for suggesting that the extract of brown seaweed sporophyll may be able to effectively reduce the blood pressure in the hypertensive patients.

Materials and Methods

Chemicals

Rabbit lung angiotensin I-converting enzyme was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hippuryl-L-histidyl-L-leucine (HHL) tetrahydrate was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals used here were commercially available reagent grade. Brown seaweed *Undaria pinnatifida* was raised and kindly provided by Tokushima Prefectural Senior High School of Science and Technology (Tokushima, Japan).

*Preparation of brown seaweed extract*s

Frozen sporophyll, leaf and stem of *Undaria pinnatifida* (Harvey) Suringar were chopped with a food processor, and the chopped seaweed (about 40 g wet weight) was suspended in 100 ml of distilled water. The suspension was autoclaved at 121°C for 30 min, and then kept in an autoclave chamber for overnight to allow it cool down. The autoclaved suspension was filtered through a Whatman No. 1 filter paper, and the obtained filtrate was then centrifuged at 5,000 x g for 20 min to precipitate the dregs. The supernatant fraction was moreover centrifuged at 12,000 x g for 20 min to clarify the solution, and the extract was sterilized by filtering through a 0.2 µm syringe-top disk filter. The obtained extract was aliquoted, and stored at -20°C until use.

Determination of ACE activity

The enzyme activity was determined using a synthetic peptide HHL as a substrate by the method described previously (Mallikarjun-Gouda, et al., 2006). Briefly, the reaction mixture containing 0.1 M Borate buffer (pH 8.3), 1 M NaCl, 1 mM HHL, 0.5 mU of the enzyme and 100 µl of the test samples in 150 µl of total volume was made up on ice, and then incubated at 37°C for 60 min. The reaction was terminated by adding 10 µl of 5 M HCl, and the mixture was kept on ice for 30 min or longer, then filtered through a syringe-top filter (0.45 µm-pore size). The amount of hippuric acid formed enzymatically during the reaction period was determined using a HPLC system as follows.

The formation of hippuric acid from HHL was determined using a reverse-phase HPLC system with a UV-VIS detector. Briefly, hippuric acid contained in 20 µl of the reaction mixture was separated on a CAPCELL PAK C18 MG2 column (2 mm diameter x 150 mm length) with a mobile phase containing 20 % methanol and 0.1 % TFA for 15 min at a flow rate of 0.1 ml/min, and detected by measuring the absorbance at 228 nm. The column was refreshed by washing with 100 % methanol-0.1 % TFA in the intervals of assays.

Isolation of small peptides

Small oligopeptides contained in the sporophyll extract were isolated from non-peptide substances by the method reported previously (Aito-Inoue, et al., 2006). Briefly, the aqueous extract (10 ml) was mixed with 30 ml of ethanol, and centrifuged at 12,000 x g for 10 min. The supernatant fraction was evaporated under the vacuum conditions, and the residue was then dissolved in 5 ml of 50% methanol containing 10 mM HCl, and kept in a refrigerator until use. The sample solution (350 µl) was loaded onto a spin column AB1150 (Atto Corp., Tokyo, Japan) packed with AG50W-X8 (5 mm length x 5 mm internal diam-eter), and the column was washed twice with 200 µl of 50% methanol containing 10 mM HCl. The solutions passing through the column and washing the resin were combined and dried up under vacuum, and the residue was then dissolved in 350 µl of distilled water (Non-peptide fraction). Following to the washing process, the column was rinses with 200 µl of 50% methanol containing 2 M ammonium hydroxide, and eluted 7-times with 200 µl of 50% methanol containing 7.5 M ammonium hydroxide. All solutions were combined and dried up under vacuum, and the residue was then dissolved in 350 µl of distilled water (Peptide fraction). The approximate concentrations of peptides in these two fractions were estimated by measuring the absorbance at 230 nm, and then adjusted with distilled water to compare their inhibitory potencies.

Sporophyll extract was fractionated using a reverse-phase HPLC system. The extract (100 µl) was loaded onto an Inertsil ODS3 column (4.6 mm diameter x 150 mm length), and washed with 5% methanol containing 0.1% TFA at a flow rate of 0.5 ml/min for 5 min, and then eluted with a mobile phase containing the increasing concentrations of methanol from 5% to 100% and 0.1 % TFA at a flow rate of 0.5 ml/min for 50 min. The absorbance of the effluent was measured at 230 nm to monitor the elution time. The fractionation was repeated several times, and the obtained fractions were combined, and dried up under vacuum. The residue was dissolved in the same volume of distilled water to that of the original fraction.

Statistical analysis

Results were presented as the mean ± SEM, and the statistical analyses were carried out using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (GraphPad Prism 4 Software). The difference between two values with $p < 0.05$ was regarded as indicating a statistically significant.

Results and discussion

The conversion of angiotensin I to angiotensin II is the process producing the active-form of hypertensive peptide, and recognized to result in the elevation of blood pressure. Therefore, the inhibition of this pathway is considered to be one of the effective measures to reduce the blood pressure in hypertensive patients. Recently, the peptides produced by digesting brown seaweed have been reported to inhibit ACE activity, thus resulting in the reduction of blood pressure in the hypertensive animal model. In the present study, the aqueous extracts were prepared from different parts of seaweed to confirm the effects of natural substances contained intrinsically in brown seaweed. Consequently, the extracts of sporophyll,

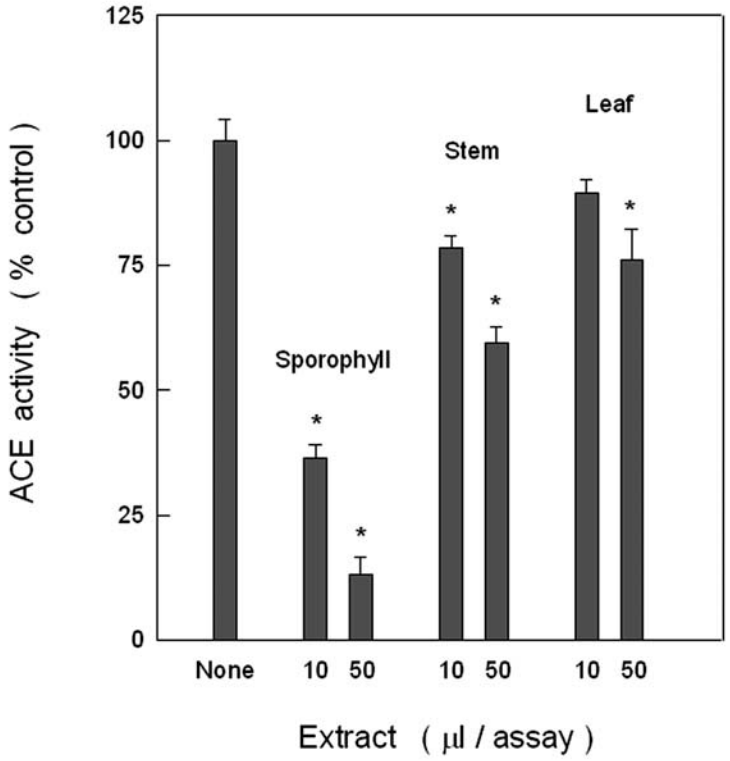

Figure 1. Inhibitory effects of brown seaweed extracts on ACE activity. The enzyme was incubi-ted at 37°C for 60 min in the mixture containing the extracts from different parts of seaweed, and the enzyme activity was determined as described in the text. Results were expressed as a percent of the control. Values are the mean ± SEM (* $p < 0.05$ vs. no extract, $n = 6$).

leaf and stem of seaweed caused the significant inhibition of ACE activity, and the most potent effect was obtained by the extract of brown seaweed sporophyll under the assay conditions used here (Fig. 1). We then focused on the extract of brown seaweed sporophyll in the following experiments. Further studies were carried out to characterize the inhibitory action of this extract on ACE activity, and clearly showed that the extract caused the inhibition of ACE activity in a manner dependent on its concentration (Fig. 2). Then, the inhibitory potency of sporophyll extract was compared with that of captopril, a typical ACE inhibitor used commonly for the treatment of hypertension, and the inhibitory effect of the seaweed extract observed at the concentration of 10 μl was shown to correspond to that caused by 1 nM captopril (data not shown). Furthermore, the kinetic analysis showed that, just similar to captopril, sporophyll extract increased the Km value without any noticeable change in the Vmax, thus considering that the extract could cause the competitive inhibition of ACE activity under the experimental conditions used in this study (Fig. 3). Therefore, it seems possible to speculate that the extract of brown seaweed sporophyll may contain considerably large amounts of the active substances inhibiting the ACE activity, and can be potent enough to lower the blood pressure in hypertensive subjects.

In general, most of the ACE inhibitory substances derived from a variety of foods, plants and other natural sources have been reported to inhibit ACE activity in a competitive manner. Here, the extract of brown seaweed sporophyll was also shown to cause the competitive inhibition of the enzyme, and therefore it seems possible to speculate that the inhibito-

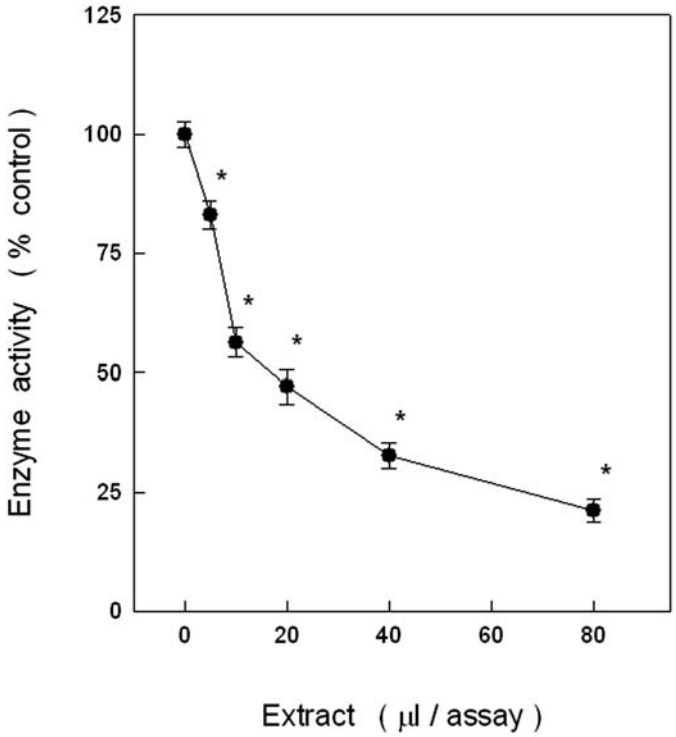

Figure 2. Concentration-dependent effect of sporophyll extract on ACE activity. The enzyme was incubated at 37°C for 60 min in the mixture containing different amounts of sporophyll extract, and the enzyme activity was determined as described in the text. Results were expressed as a percent of the control. Values are the mean ± SEM (* $p < 0.05$ vs. no extract, $n = 6$).

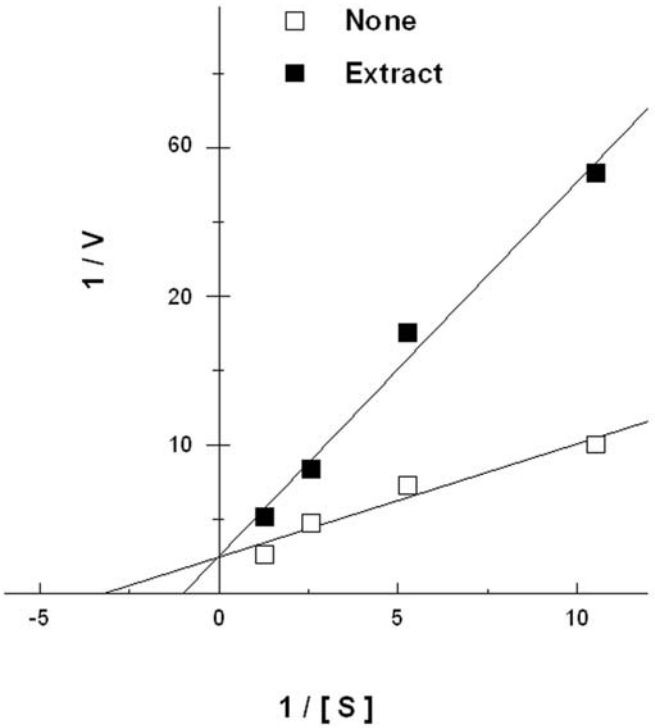

Figure 3. Kinetic study on inhibitory effect of sporophyll extract on ACE activity. The enzyme was incubated at 37°C for 60 min with or without sporophyll extract (20 µl) in the mixture containing different concentrations of the substrate, and the enzyme activity was determined as described in the text. The enzyme kinetics was graphically represented as the double-reciprocal plot.

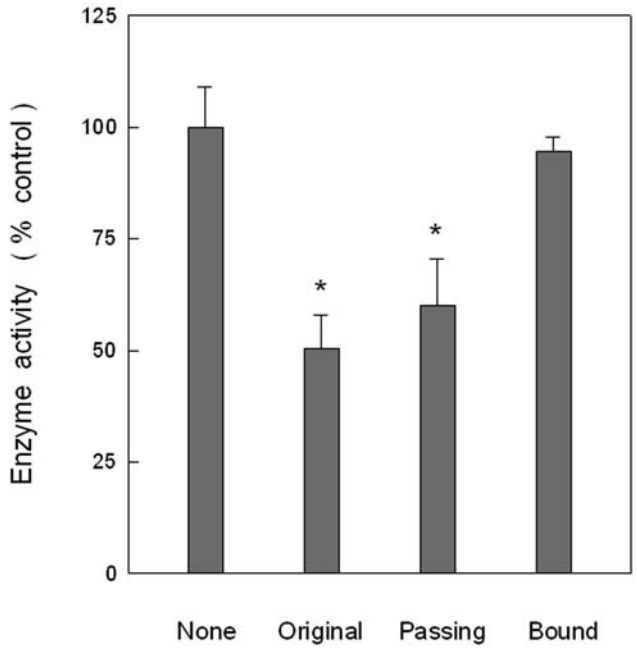

Figure 4. Effects of spin-column fractions on ACE activity. The enzyme was incubated at 37oC for 60 min in the mixture containing 10 µl of the original extract, the non-adsorbed non-peptide fraction (passing) and the adsorbed peptide fraction (bound), and the enzyme activity was determined as described in the text. Results were expressed as a percent of the control. Values are the mean ± SEM (* $p < 0.05$ vs. no extract, $n = 6$).

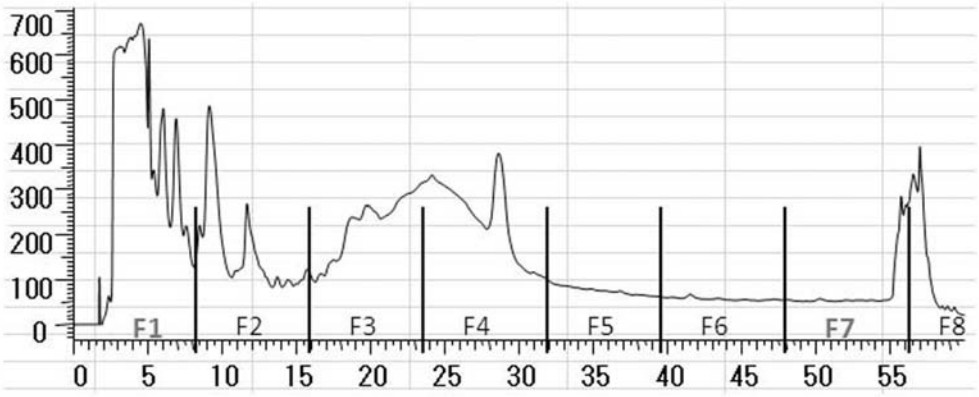

Figure 5. Elution pattern of reverse-phase HPLC separation of sporophyll extract. The extract was subjected to a reverse-phase HPLC system as described in the text, and the elution pattern was monitored by measuring the absorbance at 230 nm. The eluate was collected for every 8 min throughout the period of analysis.

ry substance contained in this extract may be analogs of the enzyme substrates, thereby focusing on small oligopeptides as the most likely candidate for the ACE inhibitory substance contained in the extract. Then, the extract was applied to a spin column packed with a cation-exchange resin to separate small-size oligopeptides as described previously (Aito-Inoue, et al., 2006), and the inhibitory effects of the obtained fractions on ACE activity were examined. Consequently, the non-adsorbed non-peptide fraction was shown to cause the inhibitory effect on the enzyme activity, almost similar in extent to the original extract, but the adsorbed fraction expected to contain oligopeptides failed to cause any significant inhibition of the enzyme activity (Fig. 4). Furthermore, the extract was subjected to a reverse-phase HPLC separation system with a UV detector, and the effluent was monitored by measuring the absorbance at 230 nm (Fig. 5), and collected for every 8 min, and the inhibitory effect of each fr-

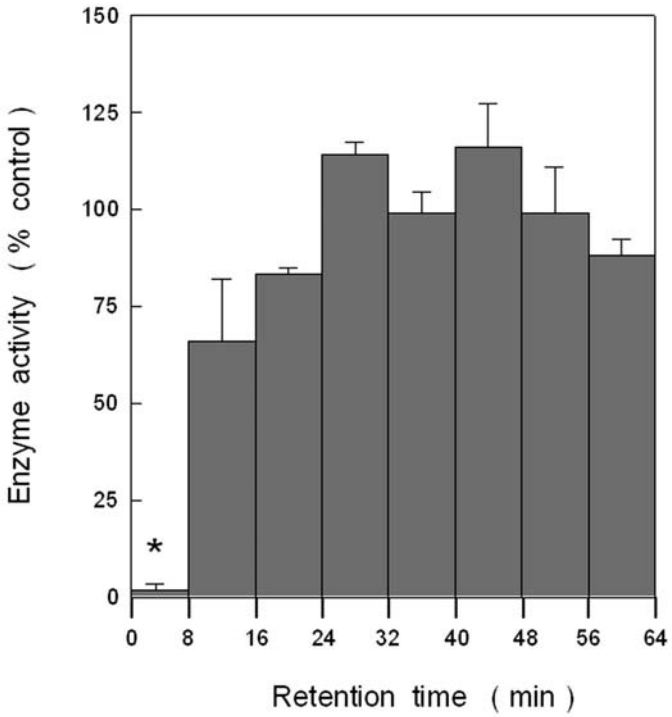

Figure. 6. Effects of HPLC effluent fractions on ACE activity. The enzyme was incubated in the mixture containing the fractions (10 μl/assay) obtained from HPLC separation process, and the enzyme activity was determined as described in the text. Results were expressed as a percent of the control. Values are the mean ± SEM (* $p < 0.05$ *vs.* no extract, n = 6).

action on the enzyme activity was then examined. Contrary to expectation, the inhibitory activity was detected only in the first fraction collected from 0 min to 8 min, which might contain all substances passing freely through the column (Fig. 6). Therefore, these results seemed to provide evidence for suggesting that the inhibitory substances contained in the extract of brown seaweed sporophyll might be non-peptide small molecules, but putative inhibitory substances were not yet identified, and intrinsic active factors still remained entirely unknown.

In conclusion, brown seaweed *Undaria pinnatifida*, particularly its sporophyll, was shown to contain potential active substances inhibiting ACE activity in vitro, thereby being expected to cause the reduction of blood pressure in hypertensive patients. Recently, the digestion of brown seaweed with proteolytic enzymes has been shown to produce several oligopeptides, which have the inhibitory effect on ACE activity in vitro, and also shown to cause the antihypertensive effect in hypertensive animals (Sato, et al., 2002; Suetsuna, et al., 2004). In contrast to these previous studies, we tried to find out potential active substances inhibiting ACE activity in unprocessed brown seaweed, and then prepared the aqueous extracts from different parts of brown seaweed without proteolytic digestion. In the present study, we suggest that brown seaweed, particularly its sporophyll, may originally contain novel non-peptide substances which have the abilities to inhibit ACE activity in vitro, thus proposing the possibility that a sporophyll of brown seaweed *Undaria pinnatifida* may be able to cause the reduction of blood pressure, thereby being useful and beneficial as a functionnal food to patients with hypertension. However, the characteristics of these novel active

substances still remain entirely unknown, and therefore it seems necessary to further investingate these bioactive substances contained in this material.

Acknowledgment

The authors are grateful to students at Tokushima Prefectural Senior High School of Science and Technology (Tokushima, Japan) to raise brown seaweed and kindly provided it for us. This project was financially supported in part by the fund provided from Kohken Co. Inc. (Sapporo, Japan).

Conflict of Interest statement

There is no conflict of interest associated with the authors of this paper, and the fund sponsors did not cause any inappropriate influence on this work.

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