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Angiotensin-I converting enzyme (ACE) inhibitory activity of aqueous extract prepared from fermented brown rice: A potential functional food for management of hypertension

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Abstract

Angiotensin-II is a hypertensive peptide formed from angiotensin-I by the action of angiotensin-I converting enzyme (ACE), thereby being implicated in the pathogenic process of hypertension, and hence the substances inhibiting this enzyme can be expected to reduce the blood pressure in hypertensive patients. On the other hand, previous studies have shown that brown rice fermented with *Aspergillus oryzae*, designated as FBRA, is a dietary fiber-rich food and possibly beneficial to human health. Then, the effect of FBRA extract on ACE activity was examined to evaluate its potential activity to ameliorate hypertension. Consequently, the extract was shown to inhibit the enzyme activity in a competitive manner, and this inhibitory effect was furthermore suggested to be attributed to small peptides in the extract. Thus, FBRA is clearly shown to cause the reduction of blood pressure by inhibiting the production of hypertensive peptide.

Keywords: FBRA extract; ACE activity; Competitive inhibition; Small peptides; Antihypertensive effect

Introduction

Angiotensin I-converting enzyme (ACE) is generally known to play a pathogenic role in the onset of hypertension through its enzymatic activity to catalyze the conversion of angiotensin-I to angiotensin-II, which is an active peptide inducing the elevation of blood pressure, and hence the inhibition of this enzyme is considered to reduce the blood pressure, there-

by improving the physical conditions of hypertensive patients. Based on this concept, much work has previously been done to find out potentially active substances to inhibit the formation of this hypertensive peptide, and a variety of natural food materials, such as plant foods including wheat, rice, peas, corn, fruits and vegetables have been evaluated by determining their inhibitory effects on ACE activity *in vitro*, and many peptides derived from dietary sources have previously been reported to inhibit ACE activity, 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 been reported to cause the inhibitory effect on ACE activity (Bhuyan and Mugesh, 2012). Therefore, it seemed significant to investigate the potential inhibitory effects of peptide-rich food materials on ACE activity *in vitro*.

Brown rice is commonly known to be more nutritious and healthy as compared with polished rice, based upon its germ part and husks containing large amounts of vitamins and minerals, and hence the mixture of these ingredients was processed by fermenting with *Aspergillus oryzae* to produce a fiber-rich, low-calorie food "Fermented Brown Rice with *Aspergillus oryzae*" without spoiling their prebiotic properties. The effect of this processed food, designated as "FBRA", on the inflammatory bowel diseases has been investigated in the animal model, and the feeding of FBRA-containing diet has been suggested to suppress the induction of acute colitis by dextran sulfate sodium probably through the modification of colonic microbiota (Kataoka, et al., 2007; Kataoka, et al., 2008). Furthermore, FBRA-containing diet has been reported to indicate the tendency to prevent the development of hereditary hepatitis in Long-Evans Cinnamon rats (Shibata, et al., 2006). On the other hand, the effect of FBRA on chemical carcinogenesis has been extensively studied, and FBRA-containing diet has been shown to prevent the production of cancer induced by the administration of several chemical agents in rats and mice (Katayama, et al., 2003; Katayama, et al., 2002; Phutthaphadoong, et al., 2009; Tomita, et al., 2008). Recently, FBRA extract has been shown to cause the apoptotic death of human colorectal tumor HCT116 cells, proposing the possibility that the daily intake of FBRA may be beneficial to the prevention of carcinogenesis and/or suppressing the tumor growth in the digestive tract (Itoh, et al., 2012). However, despite these previous studies, the pharmacological as well as the chemical properties of FBRA are not sufficiently characterized, and hence necessary to be further investigated.

FBRA is made from the mixture of brown rice, rice bran and fungi, and therefore presumed to contain large amounts of proteins and peptides, which are considered to have a potential activity to inhibit ACE activity. Then, FBRA was suspended in distilled water, and autoclaved to extract the potentially active ingredients including various small peptides, and the effect of this aqueous extract on ACE activity was examined *in vitro* to assess its potential activity as a functional food effective in the improvement of hypertensive symptom.

Materials and Methods

Chemicals

Rabbit lung-derived 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 were commercially available reagent grade. FBRA powder was produced by fermenting the mixture of brown rice and rice bran with *Aspergillus oryzae*, and provided by Genmaikoso Co. Ltd. (Sapporo, Japan).

Preparation of FBRA extract

FBRA powder was suspended in distilled water at the ratio of 20 g to 100 ml, and autoclaved at $121°C$ for 30 min, and the suspension was then kept in an autoclave chamber for overnight to allow it cooling down. The mixture was centrifuged at 5,000 x g for 20 min to remove the dregs, and the supernatant fraction was filtered through a Whatman No. 1 filter paper. The filtrate was sterilized by filtering through a syringe-top disk filter (0.2 μm-pore size). Aliquots of the sterile extract were stored in a freezer until use.

Determination of ACE activity

The enzyme activity was determined using a synthetic peptide HHL as a substrate according to the method described previously (Mallikarjun-Gouda, et al., 2006; Nishibori, et al., 2012). Briefly, the reaction mixture containing 0.1 M Borate buffer (pH 8.3), 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 disk 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 enzymatic formation of hippuric acid from HHL was determined using a reverse-phase HPLC system with a UV-VIS detector (Nishibori, et al., 2012). Briefly, hippuric acid contained in 20 μl of the reaction mixture was separated on a CAPCELL PAK C18 MG2 column (2 x 150 mm) 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 oligopeptides

FBRA extract was fractionated using a reverse-phase HPLC system. The extract (100 μl) was loaded onto an Inertsil ODS3 column (4.6 x 150 mm), 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 eluate at 230 nm was measured 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.

Small peptides contained in FBRA 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 supern-

atant 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, Centrifugal Filter Unit (Millipore Corp., Billerica, MA, USA) packed with AG50W-X8 (5 mm length x 5 mm internal diameter), 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 distil-led water (Non-peptide portion). 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 portion). 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.

Statistical analysis

The enzyme activity was calculated as a percent of the control. The results were presented as the mean ± SEM. The statistical analyses were carried out using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The difference between two values with $p < 0.01$ was regarded as indicating a statistically significant.

Results and discussion

The inhibition of ACE activity is generally recognized to contribute to the reduction of blood pressure in hypertension, thereby being considered to be one of the effective measures to improve the physical conditions of patients. Therefore, we have been making efforts to find out potentially active substances to inhibit the formation of this hypertensive peptide, and trying to isolate them from a variety of plant food materials, such as wheat, rice, peas, corn, fruits and vegetables. As a result of this attempt, the aqueous extract of lotus root, particularly its non-editable joint part, has previously been found to cause the inhibition of ACE activity *in vitro*, and the inhibitory effect of lotus root extract has furthermore been suggested to be possibly due to polyphenolic compounds contained in the extract (Nishibori, et al., 2012). In the present study, we prepared the aqueous extract from FBRA, and examined the effect of this extract on the enzyme activity. As shown in Fig. 1, FBRA extract caused the inhibition of ACE activity in a concentration-dependent manner. A significant inhibition of the enzyme activity (approximately 30% reduction of the activity) was observed at the concentration of 0.2 mg/assay, and the maximum inhibitory effect was obtained at approximately 1.6 mg/assay. Then, the kinetic property of the enzyme inhibition was analyzed using a Lineweaver-Burk plot, and FBRA extract was shown to cause the competitive inhibition of ACE activity under the assay conditions used here (Fig. 2). Furthermore, FBRA extract was dialyzed against distilled water, and the inhibitory effect of the dialyzed extract on ACE activity was examined to roughly estimate the size of potential active substances contained in the extract. As shown in Fig. 3, the dialyzed extract failed to cause any significant inhibition of the enzyme activity under the conditions in which the inhibitory effect of the original extract was evidently observed, thus suggesting that the putative inhibitory substances conta-inned in the extract might be mostly removed during the dialysis. Thus, these findings seemed
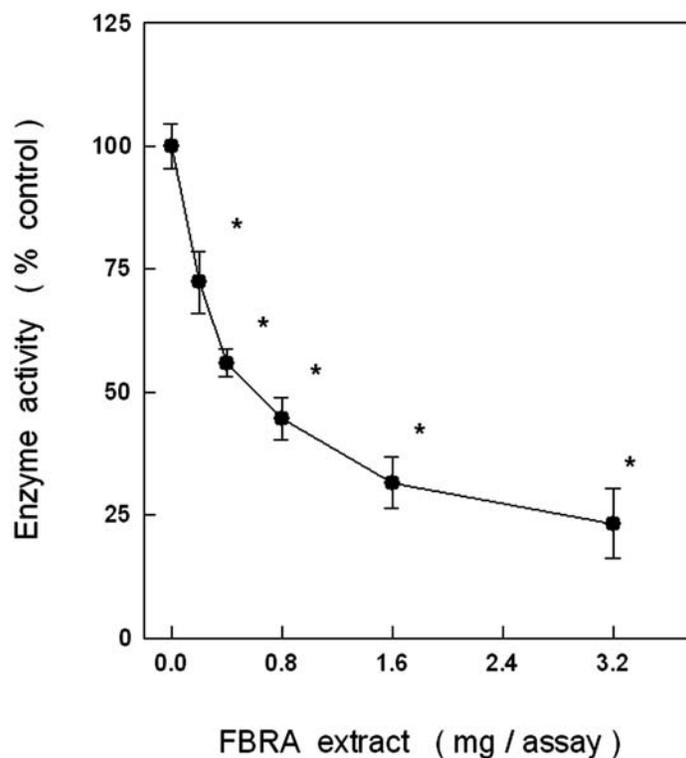
Figure 1. Inhibitory effect of FBRA extract on ACE activity. ACE was incubated in the reaction mixture conta-ining various concentrations of FBRA extract, and the enzyme activity was then determined as described in the text. Results were expressed as a percent of the control. Values are the mean ± SEM (* *p* < 0.01 *vs*. no extract, n = 6).
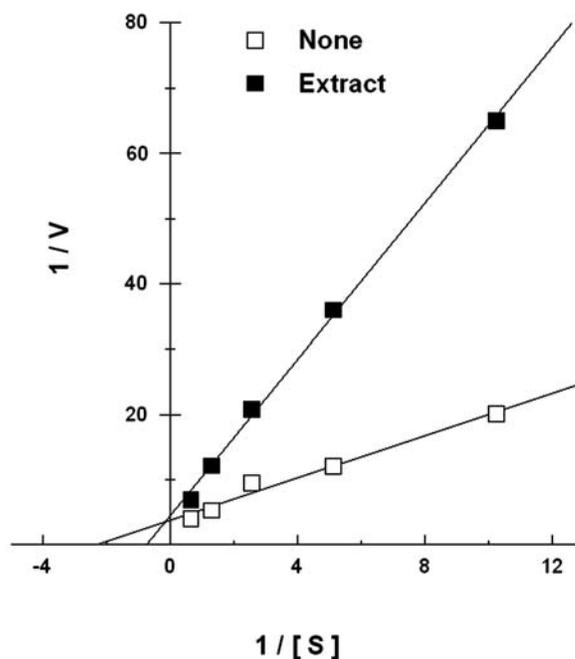

Figure. 2. Lineweaver-Burk analysis of inhibitory effect of FBRA extract on ACE activity. ACE was incubated with or without FBRA extract (1.6 mg) in the reaction mixture containing different concentrations of the substr-ate, and the enzyme activity was then determined as described in the text. The enzyme kinetics was graphically represented as the double-reciprocal plot.
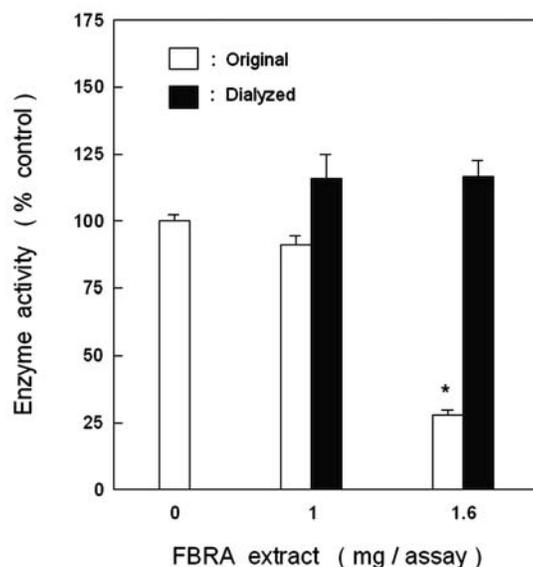
Figure 3. Inhibitory effect of dialyzed FBRA extract on ACE activity. FBRA extract was dialyzed against distilled water (50-volume) at 4°C for overnight (exchanged 3-times), and the inhibitory effects of the dialyzed and non-dialyzed extracts on ACE activity were determined as described in the text. Results were expressed as a percent of the control. Values are the mean ± SEM (* $p < 0.01$ *vs*. no extract, n = 6).
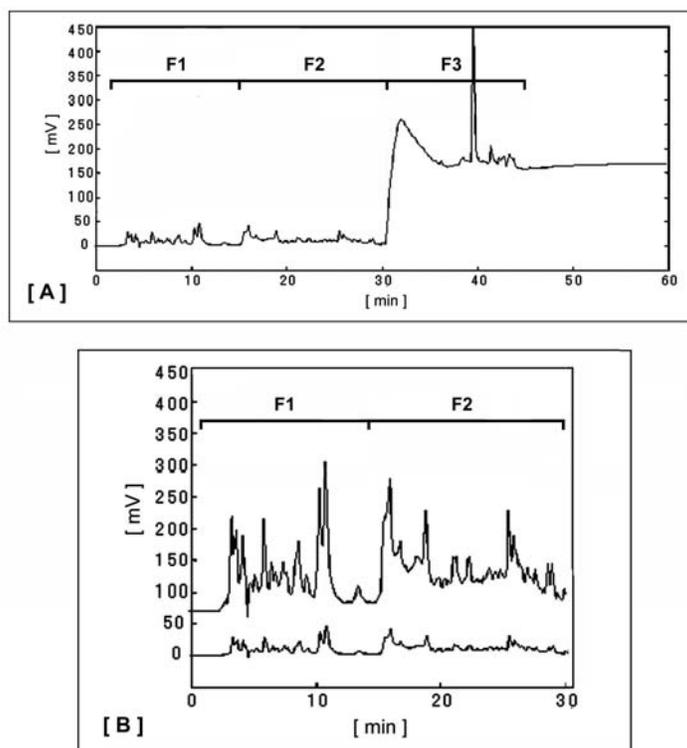

Figure 4. Reverse-phase HPLC analysis of oligpeptides in FBRA extract. The extract was subjected to a reverse-phase HPLC separation system, and the elution profile was monitored by measuring the absorbance at 230 nm [A]. The elution profile between 0-time and 30 min was enlarged by a data-analyzing device of HPLC system [B]. The eluate collected for 0 - 15, 15 - 30 and 30 - 45 min were designated as F1, F2 and F3, respectively.
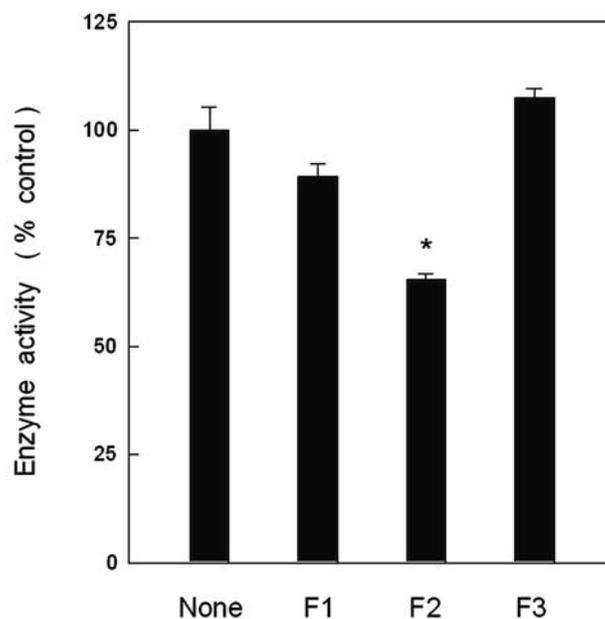
Figure 5. Effects of HPLC fractions on ACE activity. ACE was incubated in the reaction mixture containing the fractions (10 μl/assay) obtained from HPLC separation process, and the enzyme activity was then determined as described in the text. Results were expressed as a percent of the control. Values are the mean ± SEM (* $p < 0.01$ *vs*. no extract, n = 6).

to provide evidence for suggesting that the inhibitory substances of FBRA extract might be small molecules and have the chemical structures similar to the enzyme substrate, thereby allowing us to speculate that small oligopeptides might be one of the most likely candidates for putative ACE inhibitory substances contained in the extract.
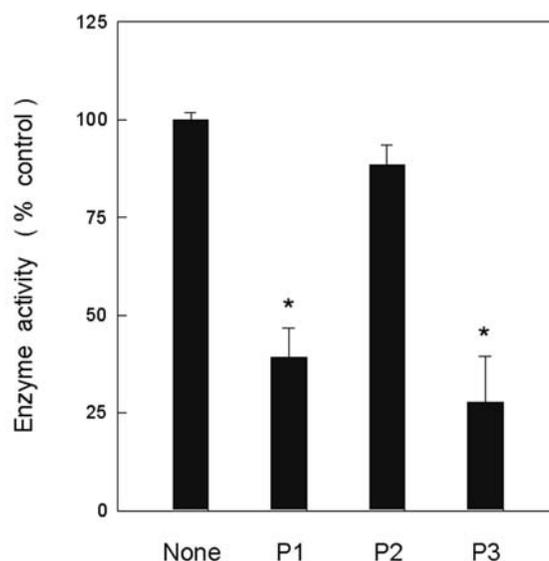

Figure 6. Effects of spin-column portions on ACE activity. ACE was incubated in the reaction mixture containing 10 μl of the original extract (P1), the non-adsorbed non-peptide portion (P2) and the adsorbed peptide portion (P3), and the enzyme activity was determined as described in the text. Results were expressed as a percent of the control. Values are the mean±SEM (* $p < 0.01$ *vs*. no extract, n = 6).

To further investigate the inhibitory substances contained in FBRA extract, the extract was subjected to the separation process targeting on small oligopeptides using a reverse-phase HPLC system, and the column eluate was monitored by measuring the absorbance at 230 nm, which is considered to reflect the concentrations of oligopeptides, and three fractions, F1 (0 - 15 min), F2 (15 - 30 min) and F3 (30 - 45 min), were obtained as shown in Fig. 4. Then, the inhibitory effects of these fractions on ACE activity were examined, and only the fraction F2 was shown to cause the significant inhibition of the enzyme activity under the *in vitro* conditions used here (Fig. 5).

Furthermore, the isolation of small peptides from the extract was also carried out using a spin column according to the method reported previously (Aito-Inoue, et al., 2006), and the inhibitory effects of the original extract (P1), the non-adsorbed non-peptide portion (P2) and the adsorbed peptide portion (P3) on ACE activity were then examined. The peptide portion P3 was shown to cause the inhibitory effect on the enzyme activity, almost similar in extent to the original portion P1 (Fig. 6). Therefore, these results seemed to provide evidence for suggesting that FBRA extract could inhibit ACE activity in a competitive manner, and the inhibitory effect might be due to small peptides contained in the extract. However, these peptides were not yet identified, and their amino acid composition still remained entirely unknown.

In conclusion, a variety of natural materials, such as fruits, vegetables and other plants, have been investigated to identify the potentially active substances inhibiting ACE activity, suggesting considerable number of the inhibitory substances contained in natural materials to be beneficial to the improvement of hypertensive conditions. Particularly, polyphenolic compounds contained in plant materials have been considered to be possibly responsible for the inhibitory effects of these materials on ACE activity as well as their radical scavenging and antioxidant activities. On the other hand, small oligopeptides derived from natural and processed food materials have been expected to cause their inhibitory effects on ACE activity probably through the competition with the enzyme substrate, thus resulting in the reduction of blood pressure in hypertensive patients. In the present study, FBRA extract was shown to inhibit ACE activity, and this inhibitory effect was speculated to be possibly attributed to oligopeptides contained in the extracts. Thus, FBRA is demonstrated to be not only an excellent low-calorie foodstuff but also one of the prominent functional foods. However, the properties of the inhibitory substances still remain unknown, and hence should be more precisely investigated to identify the bioactive substances contained in this material.

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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