J. Food Sci. Nutr.
Vol. 5, No. 2, p. 75~80 (2000)

Purification and Characterization of Angiotensin I-Converting Enzyme Inhibitors from *Sinapis alba* L.

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Abstract

To separate ACE inhibitors from edible plants, spices, and herbs, 285 extracts of 95 sources were screened for ACE inhibitory activity. The extract of *Sinapis alba* L. had the most potent ACE inhibitory activity. Mustard seeds were crushed homogeneously and extracted with hexane and water successively. Lyophilized water extract was fractionated with H_2O: butanol (1:1). The ACE inhibitor was purified from butanol fraction by methanol precipitation, gel filtration, HPLC, and FPLC with Superdex peptide HQ 10/30 column. The active fraction has been purified to homogeneity, which was proven by gel filtration using FPLC system. The yield was 0.02%. The compound has a molecular weight of about 640. The compound competitively inhibited ACE activity and the IC_{50} value was 79 μg/ml. The purified compound showed uterus contraction activity in isolated rat uterus.

Key words: angiotensin converting enzyme, inhibitor, *Sinapis alba*, uterus contraction activity

INTRODUCTION

High blood pressure is one of the leading risk factors for heart attacks and strokes. Hypertension could be caused by hardening and narrowing of the arteries that carry blood to the kidney. Reduced blood flow to the kidney leads to an excessive release of renin, a potent hormone that increases blood pressure. Angiotensin I-converting enzyme (ACE), a zinc-containing enzyme, catalyzes the formation of the potent vasopressor angiotensin II from angiotensin I and inactivates bradykinin which has a vasodialating action (1,2). ACE is also known as kininase II which is involved in the breakdown of kinins, potent vasodilators (3).

The first reported ACE inhibitor was a natural peptide extracted from a Brazilian snake (4-6). Using this peptide sequence, researchers have tried to synthesize ACE inhibitors that show more favorable pharmacokinetics. Captopril and other ACE inhibitors (Enalapril, Fosinopril) act by binding the zinc ion on ACE (7-9). They reduce blood pressure through vasodilation and reduction of blood volume.

Recently, two main kinds of ACE inhibitors have been screened from natural sources. One is peptides derived from casein, fish muscle, and other food proteins, and the other is flavonoids derived from plant materials such as herbs and spices. The constituents have been shown to be effective in lowering blood pressure of spontaneously hypertensive rats (SHR) after oral administration although the inhibitory activities of these food-derived peptides and flavonoids were weaker than those of synthetic drugs such as captopril and enalapril. However, these synthetic drugs are thought to contribute to side effects such as cough, taste disturbances, and skin rash (10).

The reported ACE inhibitors isolated from food materials are as follows; peptides from dried bonito digested by thermolysin (11), *Bacillus licheniformis* alkaline protease hydrolysates of sardine muscle (12), a peptide isolated from an acid extract of tuna (13), peptides from soy sauce (14), muracein muramyl peptides produced by *Nocardia orientalis* (15), the extracts from miso (16), synthetic peptide fragments of human κ-casein (17), various flavonoids isolated from leaves of persimmon (18,19), and sake and its by-products (20). Some of these peptides, however, have a bitter taste and those from fish protein have a fishy odor (21).

In order to find a new active compound that has a potent inhibitory effect on ACE from natural sources, we screened many kinds of edible plants, spices, and herbs. In this report, we describe an anti-ACE activity from the extract of *Sinapis alba*.

MATERIALS AND METHODS

Materials

Herb materials used in this experiment were purchased from specialized spice shops or Kyung-Dong market in Seoul, Korea. Sephadex LH-20 and Superdex peptide HQ 10/30 column were purchased from Phamacia Co. (Uppsala, Sweden) and μBondapak C_{18} column from Waters Co. (Milford, MA, USA). Dynamax silica column was from Rainin Co. (Woburn, MA, USA). ACE (rabbit lung acetone powder), Hippuric acid-Histidine-Leucine (Hip-His-Leu), Bradykinin (BK), and Epigallocatechin (EGCG) were purchased from Sigma Co. (St. Louis, MO, USA). Enalapril was purchased from Chong Kun

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Dang (Seoul, Korea). The other chemicals were analytical grade reagents and obtained from commercial sources.

Preparation of the extracts

Edible plants, spices, and herbs were autoclaved for 5 min to get rid of enzyme activities that might be possessed in the materials. Then the materials (5 g as dry weight) were completely homogenized with distilled water (100 ml) and centrifuged at 10,000×g for 30 min. Fraction I was prepared by lyophilization of the supernatant. The residues were continuously refluxed with 100 ml of hexane, acetone, methanol, and distilled water for 2 hr, respectively. Each fraction was centrifuged and filtrated with Whatman paper No. 541. Fraction II, III, IV, and V were prepared by evaporation or lyophilization of each solvent supernatant. Each fraction was used as a sample for ACE inhibitor screening.

Determination of optimum extraction conditions

To select the optimal extraction solvent, milled mustard (5 g) was extracted with 100 ml of hexane, 70% acetone, 70% methanol, and hot water for 2 hr using reflux extractor, respectively. After determining the optimal extraction solvent, the determination of the optimum extraction temperature was performed at 20°C, 60°C, and 100°C water for 2 hr. The water extract was continuously fractionated with hexane, diethyl ether, ethyl acetate, and butanol with water by the ratio of 1:1, respectively, using a separating funnel. After extraction the residue was separated by filtration with Whatman filter paper No. 541.

Preparation of the enzyme

Crude ACE solution was prepared as follows; one gram of the rabbit lung acetone powder was blended in 10 ml of 0.2 M borate-NaCl buffer, pH 8.3, followed by centrifugation for 30 min at 10,000×g. The clear supernatant contains a highly active ACE and the activity is stable for months at 5°C.

Angiotensin-converting enzyme assay

The method of Cheung and Cushman (22) was modified in this experiment. Each assay mixture contains the following components; 0.05 ml of extracted sample, 0.1 ml of 12.5 mM Hip-His-Leu (HHL), and 0.15 ml of the enzyme solution. The enzyme solution was added last and the reaction mixtures were incubated at 37°C for 1 hr in a water bath. The reactions were terminated by the addition of 0.25 ml of 0.5 N HCl. For the zero-time control assay, 0.25 ml of 0.5 N HCl was added to the reaction mixture before the enzyme was added. The hippuric acid formed by the action of the angiotensin-converting enzyme on HHL was extracted with 1.5 ml of ethyl acetate by vortex mixing for 15 sec. After centrifugation at 2,800 rpm for 7 min, 0.5 ml of each ethyl acetate layer was transferred to a clean tube by means of an automatic pipette. The ethyl acetate was evaporated by heating at 140°C for 30 min in an oil bath. The hippuric acid was redissolved in 3.0 ml of 1.0 M NaCl solution and the amount of hippuric acid was determined from the absorbance at 228 nm. The activity was expressed by the amount of hippuric acid produced per mg of protein for 1 min. The ACE inhibition ratio was determined by using the equation described below:

$$\text{Inhibition ratio (\%)} = (Ec-Es) / (Ec-Eb) \times 100$$

where Ec is the absorbance of the sample without the extract, Es is the absorbance of the sample containing the extract, and Eb is the absorbance of the zero-time control sample. The IC_{50} value is defined as the concentration producing 50% inhibition of ACE activity.

Pronase digestion of the mustard extract

This was performed using the method of Yamada et al. (23). The butanol fraction (10 mg) was dissolved in 50 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$, and then pronase (2,000 units) was added. The reaction mixture was incubated at 37°C for 48 hr. After being boiled for 5 min to stop the reaction, the solution was centrifuged to remove insoluble precipitates. The supernatant was lyophilized and used to assay for ACE inhibitory effect.

Measurement of protein concentration

The protein concentration was determined by Lowry method using bovine serum albumin (type V, Sigma) as a standard (24).

Purification of ACE inhibitors from *Sinapis alba*

After mustard seeds were milled, the powder was extracted with hexane and distilled water, successively and fractionated with distilled water-butanol (1:1). The butanol fraction obtained was subjected to column chromatography on a Sephadex LH-20 (2.3×41 cm), which was equilibrated with 50% methanol and then eluted successively with the same solvent. The fractions were lyophilized and assayed the ACE inhibitory activity. The active fractions were pooled and applied to an ODS (μbondapak C_{18}, 4×250 mm) column equilibrated with deionized water. A linear gradient was performed by increasing the concentration of acetonitrile from 0 to 80% in water. The flow rate was 1 ml/min. The active fractions were pooled and applied on a silica column (Dynamax, 4.6×150 mm), which was performed at a flow rate of 1 ml/min using hexane/isopropanol (9:1) as an elution solvent. The active fractions were pooled and purified on Superdex Peptide HR 10/30 column (10×300 mm) equilibrated with 0.02 M phosphate buffer, pH 7.2, containing 0.25 M NaCl, which was performed at a flow rate of 0.25 ml/min using the same buffer as an elution solvent.

Determination of ACE inhibition pattern

ACE inhibition pattern was determined as follows; 10 μg and 20 μg of inhibitors were added to the reaction mixture, respectively and the enzyme activity was measured in the several concentrations of substrate. The kinetics of ACE in the presence of inhibitor was determined by plotting the Lineweaver-Burk plot.

Determination of molecular weight

The molecular weight was determined by gel filtration with Superdex Peptide HR 10/30 column (10×300 mm). The standard markers were lysozyme (Mw 14,300), aprotinin (Mw 6,500), and bradykinin (Mw 1,060).

Isolated rat uterus contraction assay

Uterine preparations were obtained from virgin rats (Sprague-Dowley, 150~200 g) treated with diethyl stilbesterol (0.1 mg/ml ethanol/kg, s.c.) at 24 hr before the experiments. An uterine strip was suspended in 10 ml of organ bath containing modified De Jalon's solution (composition, g/L; NaCl 9.0, KCl 0.42, $CaCl_2$ 0.045, $NaHCO_3$ 0.5, $MgCl_2$ 0.005, glucose 1.98) at 31°C and bubbled with 95% O_2 - 5% CO_2. After a resting period of about 30~60 min, isotonic contractions induced by the addition of 0.1 ml of the purified peptide (30 μg/ml) were measured under a resting load 0.5 g and recorded on a physiograph (25). The effect of the test compound was evaluated comparing with the contraction induced by Bradykinin.

RESULTS AND DISCUSSION

Screening of ACE inhibitors

We screened 285 extracts of 95 edible plants, herbs, and spices for ACE inhibitory activity. Among them, *Sinapis alba* L. (mustard seeds) showed the most potent anti-ACE activity. ACE inhibitory activity of mustard seed has not been reported so far. The milled mustard seeds were treated with hexane to remove lipid materials in the membrane before the solvent extraction. In the experiment of the effect of extraction solvents on ACE inhibitory activities, the hot water extract showed relatively higher inhibitory activity than others (Fig. 1), suggesting that the active component could be a polar one. The optimal extraction temperature for ACE inhibitory component extraction was examined. The highest inhibitory effect was shown in the extract which was extracted at 60°C (Fig. 2). That means the active component is very thermostable. The water extract was continuously fractionated with the various organic solvents in the direction of the increasing polarity degree. The butanol fraction contained the compounds having the highest inhibitory activity (Fig. 3).

Properties of the active compound

ACE inhibitors previously reported are classified in two groups, peptide and flavonoid inhibitors, mainly. We performed the digestion of the extract by pronase to investigate whether the active component is peptide or not. As shown in Fig. 4, ACE inhibitory effect of the pronase treated sample

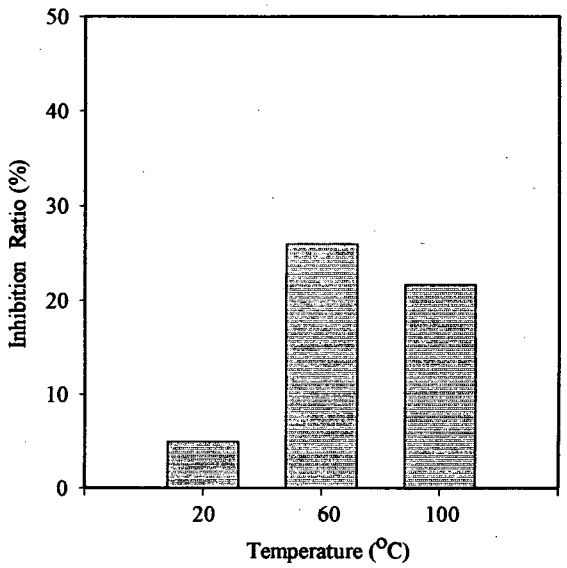

Fig. 2. Effect of water extraction temperature on ACE inhibitory activities.

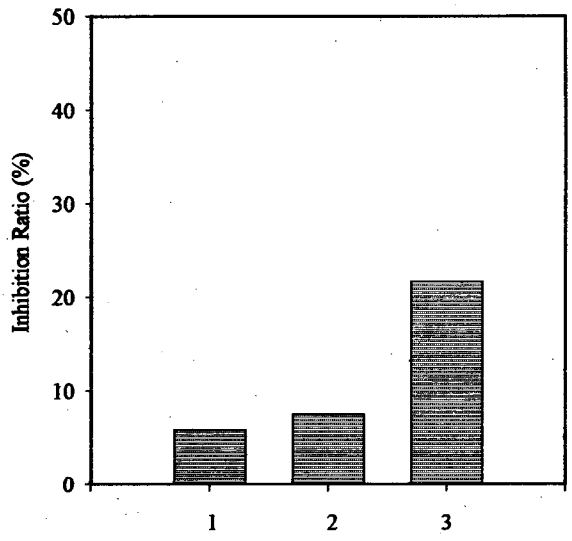

Fig. 1. Effect of various solvents for extraction on ACE inhibitory activities. 1, the extract with 70% acetone; 2, the extract with 70% methanol; 3, the extract with hot water.

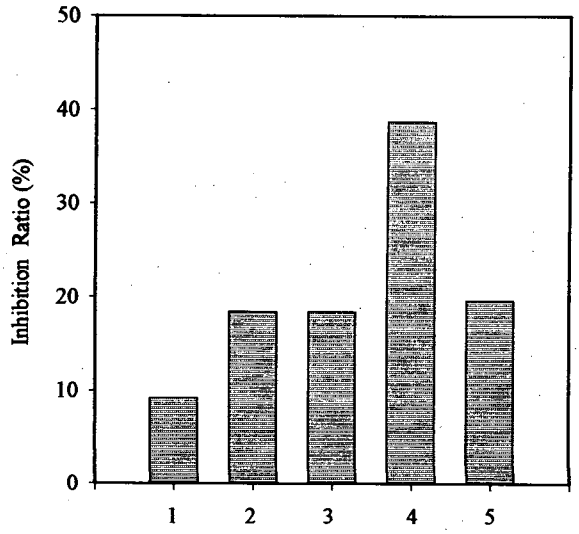

Fig. 3. Effect of organic solvent fractionation on the ACE inhibitory activities. 1, hexane fraction; 2, diethyl ether fraction; 3, ethyl acetate fraction; 4, butanol fraction; 5, aqueous layer.

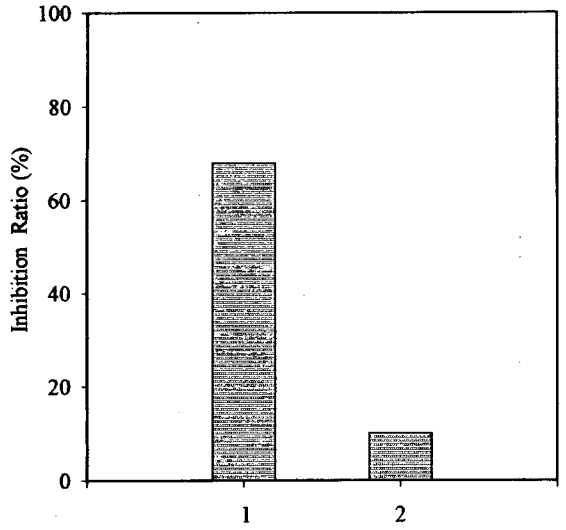

Fig. 4. Effect of the active extract digested with pronase on ACE inhibitory activities. 1, pronase untreated sample; 2, pronase treated sample.

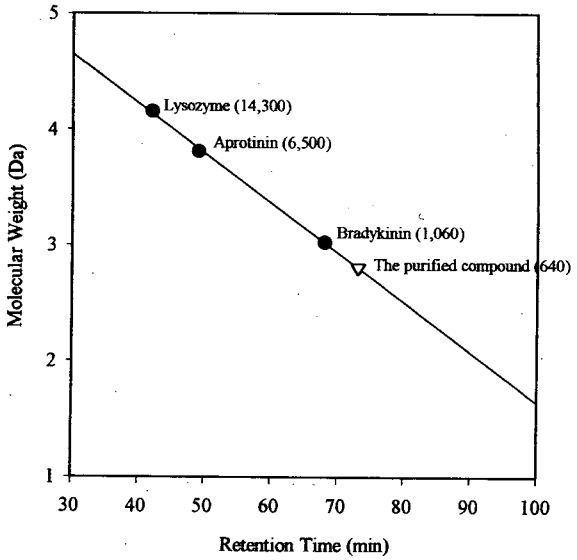

Fig. 6. Determination of the molecular weight of the purified compound by gel filtration using FPLC.

was decreased to about 75% compared with that of the untreated sample. The result suggests that ACE inhibitor from mustard seed could be a peptide. Peptide ACE inhibitors from spices have been rarely reported so far.

The active compound was purified with an overall yield of 0.02%. The purified compound was shown to be homogeneous by a normal phase HPLC (Fig. 5), showing a single peak by gel filtration using FPLC with Superdex peptide HQ 10/30 column as well.

The molecular weight was estimated by gel filtration using FPLC system and the result is summarized in Fig. 6. The molecular weight of the purified compound was calculated to be about 640, which suggests that the potent ACE inhibitor could be a short peptide. Amino acid sequencing is needed for further studies to understand the structure and function on the molecular level.

Determination of ACE inhibition pattern

The kinetic studies of inhibition of ACE by the purified compound were performed and investigated the inhibition pattern by Lineweaver-Burk plot. As shown in Fig. 7, ACE inhibition pattern of the purified compound was competitive. It was reported that peptide inhibitors produced competitive inhibition patterns (15) and flavonoid inhibitors showed non-competitive inhibition action (26). It has been reported that competitive inhibitors often closely resemble to the substrates. In addition to the result of pronase digestion experiment, this result strongly suggests as well that ACE inhibitor from *Sinapis alba* could be a peptide which has a structural similarity with the substrate. Further studies on structure-function relationship

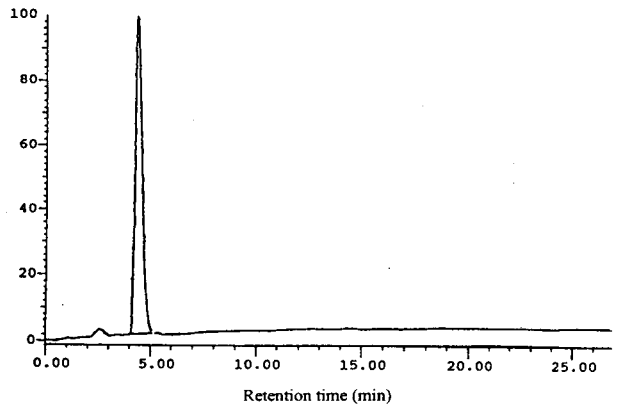

Fig. 5. Normal phase HPLC profile of the purified compound.

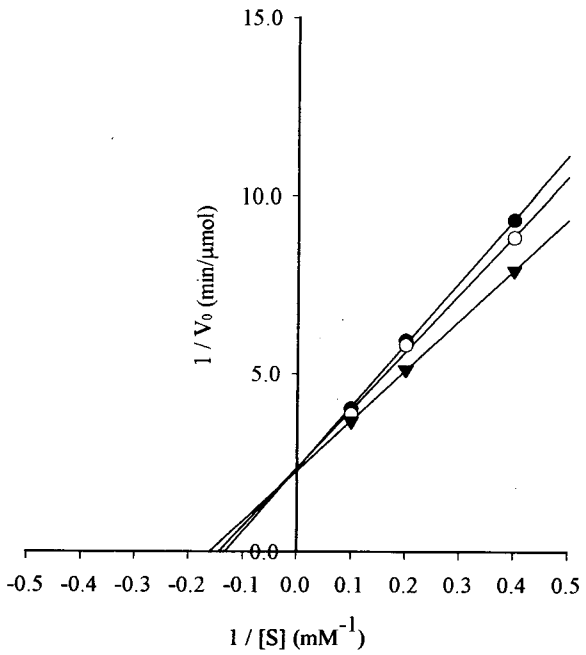

Fig. 7. Determination of ACE inhibition pattern. ●, Inhibitor (20 mg); ○, inhibitor (10 μg); ▼, without inhibitor.

Table 1. Inhibitory activities of compounds on ACE

Compound	IC_{50} (μg)
Enalapril	0.024
Badykinin	6.54
EGCG[1)]	181.61
The purified compound	79.59

[1)]Epigallocatechin gallate

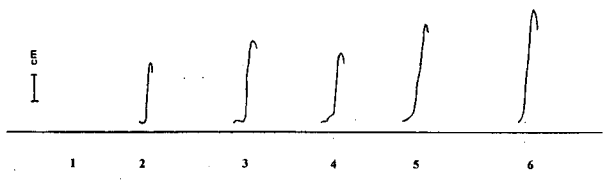

Fig. 8. Bradykinin agonist activity in the isolated rat uterus. 1, control; 2, the purified compound (30 μg/ml); 3, the purified compound (60 μg/ml); 4, Bradykinin (5×10^{-3} μg/ml); 5, Bradykinin (10×10^{-3} μg/ml); 6, Bradykinin (5×10^{-3} μg/ml) and the purified compound (30 μg/ml).

would be necessary. The concentration required for 50% inhibition of enzyme activity (IC_{50}) was 79 μg, which was higher than those of enalapril and bradykinin, whereas, the value was lower than that of EGCG (Table 1).

Bradykinin agonist activity in the isolated rat uterus

The regulatory function of ACE/kininase II connects the production of vasoconstrictor Angiotensin II and inactivation of vasodilator bradykinin. Linz and Scholkens (27) reported that inhibition of ACE (kininase II) resulted in the reduction of angiotensin II generation and kinin degradation, leading to beneficial cardiovascular effects. Thus, the isolated rat uterus contraction assay was performed with the purified compound to examine whether it has Bradykinin-related action. The experiment was performed to investigate the possibilities of the active substance as Bradykinin agonist (28), antagonist (29,30), or potentiation agent (31). The compound generated 6×10^{-3} μg bradykinin equivalents per ml against the rat uterus. T-kinin from rat plasma by trypsin showed 2.4×10^{-2} μg bradykinin equivalents per ml (28). Although the compound acted as a weak bradykinin agonist in rat uterus, it showed uterus contraction activity in isolated rat uterus by itself (Fig. 8). Furthermore, the increased contraction occurred in the treatment with Bradykinin and the purified compound, suggesting the compound is a Bradykinin agonist.

ACKNOWLEDGEMENTS

This work was supported by the grant from the Agricultural R&D Promotion Center of Korea.

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(Received March 6, 2000)