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Characterization of Antihypertensive Angiotensin I-Converting Enzyme Inhibitor from *Saccharomyces cerevisiae*

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Abstract This study describes the purification and characterization of a novel antihypertensive angiotensin I-converting enzyme (ACE) inhibitory peptide from *Saccharomyces cerevisiae*. Maximal production of the ACE inhibitor from *Saccharomyces cerevisiae* was obtained from 24 h of cultivation at 30°C and its ACE inhibitory activity was increased by about 1.5 times after treatment of the cell-free extract with pepsin. After the purification of ACE inhibitory peptides with ultrafiltration, Sephadex G-25 column chromatography, and reverse-phase HPLC, an active fraction with an IC_{50} of 0.07 mg and 3.5% yield was obtained. The purified peptide was a novel decapeptide, showing very low similarity to other ACE inhibitory peptide sequences, and its amino acid sequence was Tyr-Asp-Gly-Gly-Val-Phe-Arg-Val-Tyr-Thr. The purified inhibitor competitively inhibited ACE and also showed a clear antihypertensive effect in spontaneously hypertensive rats (SHR) at a dosage of 1 mg/kg body weight.

Key words: Antihypertension, *Saccharomyces cerevisiae*, angiotensin I-converting enzyme inhibitor

Angiotensin I-converting enzyme (ACE, dipeptidyl carboxypeptidase I, kininase II, EC. 3.4.15.1) is a multifunctional, zinc-containing enzyme that is located in different tissues. By virtue of the rennin-angiotensin system, this enzyme plays a key physiological role in the control of blood pressure [8]. ACE converts the inactive decapeptide, angiotensin I, to the potent vasopressor octapeptide, angiotensin II, and inactivates bradykinin [23].

Several ACE inhibitors show antihypertensive effects and may also have beneficial effects on glucose and lipid metabolism [5, 29], decreasing insulin requirements in diabetes, increasing exercise tolerance, as well as other beneficial effects [10]. Since the original discovery of ACE inhibitors in snake venom [7], captopril (d-3-mercapto-2-methylpranoryl-proline), enalapril, and lisinopril, an effective oral ACE inhibitor, have been developed, and they are currently used as clinical antihypertensive drugs [22]. Although synthetic ACE inhibitors, including captopril, are remarkably effective as antihypertensive drugs, they cause adverse side effects, including coughing, allergic reactions, taste disturbances, and skin rashes. Therefore, research and development to find safer, more innovative, and more economical ACE inhibitors are necessary for the prevention and remedy of hypertension.

Many antihypertensive ACE inhibitors have been isolated and characterized from foods [3], enzymatic hydrolysates of proteins [28], sake and sake lees [27], Korean traditional rice wines and liquors [12], flavonoid of citrus and fruits [9, 17], and cathecin of tea [19]. Many research groups have also screened for ACE inhibitors with microbial origins such as *Doratomyces putredinis*, *Nocardia orientalis* [1], *Virgaria nigra* [2], *Actinomycetes* [11], Baker's yeast [13], *E. coli* [19] and *Basidiomycetes* [14]. WF-10129, obtained from *Doratomyces putredinis*, is an ACE inhibitor resembling the potent synthetic ACE inhibitor enalaprilat, and is a substituted N-carboxymethyl dipeptide. WF-10129 inhibits ACE in a dose-dependent manner with IC_{50} of 14 nM, indicating that it is one of the most potent ACE inhibitors with microbial origins. Kohama *et al.* [13] isolated 3 kinds of ACE inhibitory peptides from Baker's yeast including YG-1(Gly-His-Lys-Ile-Ala-Thr-Phe-Gln-Glu-Arg), Morigiwa *et al.* [18] isolated strong antihypertensive triterpene compounds such as ganoderal A, ganoderols A and B, and ganoderic acids K and S from 70% methanol extract of *Ganoderma lucidum*, and Lee *et al.* [15] recently, isolated

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an ACE inhibitory peptide from *Tricholoma gigantum*. The ACE inhibitory peptide is a novel tripeptide with a sequence of Gly-Glu-Pro, that shows very little similarity to the other ACE inhibitory peptides sequences.

Even though some ACE inhibitors have been produced and characterized from microbes, few ACE inhibitors are used commercially, because of their low antihypertensive action. The present study describes the purification and characterization of a novel ACE inhibitory peptide from alcohol fermentative *Saccharomyces cerevisiae*, which can be used as an antihypertensive drug.

MATERIALS AND METHODS

Strains and Enzymes

Several *Meju* yeasts [16], industrial yeasts, and other yeasts were obtained from the Dept. of Genetic Engineering at Paichai University, the Korea Culture Center of Microorganisms (KCCM), and the Korea Collection for Types Cultures (KCTC).

The angiotensin I-converting enzyme (ACE) used in this study was extracted from rabbit lung acetone powder with 100 mM sodium borate buffer (pH 8.3) containing 300 mM NaCl, and the extract was kept overnight at 4°C. Its activity was determined by using Hippuric acid-Histidine-Leucine (Hip-His-Leu) as a substrate. One unit was defined as the amount to catalyze the formation of 1 μM hippuric acid from Hip-His-Leu in 1 min at 37°C under standard assay condition [6]. Rabbit lung acetone powder for ACE, pepsin (4,150 units/mg), trypsin (1,200 units/mg), protease N (185 units/mg), and Hippuric acid-Histidine-Leucine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Spontaneously hypertensive male rats (SHR), Sam:TacN(SHR)fBR, were purchased from Samtaco Bio-Korea Co. (Osan, South Korea). Each rat, weighing 280-300 g, was 11 weeks old.

Assay of ACE Inhibitory Activity

The ACE inhibitory activity was assayed by a modification of the method of Cushman *et al.* [6]. A mixture, containing 100 mM sodium borate buffer (pH 8.3), 300 mM NaCl, 3 units of ACE from rabbit lung, and an appropriate amount of the inhibitor solution, was preincubated for 10 min at 37°C. The reaction was initiated by adding 50 μl of Hip-His-Leu at a final concentration of 5 mM, and was terminated after 30 min of incubation by adding 250 μl of 1.0 M HCl. The hippuric acid liberated was extracted with 1 ml of ethyl acetate, and 0.8 ml of the extract was evaporated by a Speed Vac Concentrator (EYELA Co., Japan). The residue was then dissolved in 1 ml of sodium borate buffer. Absorbance at 228 nm was measured to estimate the ACE inhibitory activity. The concentration of ACE inhibitor required to inhibit 50% of the ACE activity under the above assay conditions was defined as IC_{50}.

Table 1. ACE inhibitory activity of cell-free extracts of yeasts.

Yeasts*	ACE inhibitory activity (%)
Saccharomyces cerevisiae	42.1
Hansenula anomala	15.6
Hansenula capsulata	11.6
Pichia membranaefaciens	13.1
Thurammina sphaerica	14.6
Candida tropicalis	16.2
Candida edax	15.2
Kluyveryomyces lactis	11.8
Rhodotorula glutinis	15.1
Zygosacch. rouxii	18.3

*Yeasts which showed over 5.0% of ACE inhibitory activity in secondary screening tests.

Purification of ACE Inhibitors from *S. cerevisiae*

To increase ACE inhibitory activity, the pH of cell-free extracts from *S. cerevisiae* was adjusted to the optimum pH of each of the proteases described above, and these were digested with 1% (w/v) of pepsin (37°C), trypsin (25°C), and protease N (55°C) at an optimum temperature for 12 h. The reaction was terminated by heating in boiling water for 10 min, and the precipitate formed was separated by centrifugation. The precipitate was dissolved solution in 20 mM phosphate buffer, and was used as protein hydrolysate.

The protein hydrolysate of *S. cerevisiae* was ultrafiltrated with a 5 kDa cutoff filter (Labscale TFF System, Millipore Co., U.S.A.), and the ACE inhibitory activity of the filtrates was then determined. The active fraction was concentrated by lyophilization and was then applied to a Sephadex G-25 column (3.0×35 cm) equilibrated with distilled water, and the column was eluted with the same buffer at a flow rate of 12 ml/h. The fractions with ACE inhibitory activity were then applied to a preparative reverse phase high permeation liquid chromatography (μBondapak C_{18} column) equilibrated with acetonitrile (Table 1). A linear gradient formed with 0.1% trifluoroacetic acid (TFA) in water from 0 to 100% (v/v) was applied to the column. The active fractions were collected and lyophilized immediately [20].

Mass Spectrometry, Amino Acid Analysis, and Sequence Determination

The molecular mass of the purified ACE inhibitor was determined, using an LC/MS spectrometer (HP 1100 series LC/MSD, U.S.A.). The amino acid composition of the ACE inhibitor from *Saccharomyces cerevisiae* was analyzed with a Fluorometric Analysis System (SLM-AMINCO, U.S.A.) after hydrolysis for 24 h in 4 N methanesulfonic acid containing 0.2% of 3-(2-aminoethyl) indole at 110°C [4]. The amino acid sequence was determined by the Edman method [22], using an Applied Biosystems 491A automatic protein sequencer.

Determination of Inhibition Pattern on ACE

To investigate the inhibition pattern on ACE, 0.05 mg and 0.1 mg of the inhibitors were added to each reaction mixture [4]. The ACE inhibitory activities were measured with different concentrations of the substrate. The kinetics of ACE in the presence of the inhibitor was determined by using Lineweaver-Burk plots.

Antihypertensive Action in Spontaneously Hypertensive Rats (SHR)

A dose of the purified ACE inhibitor from *Saccharomyces cerevisiae*, 1 mg/kg body weight/rat, was orally administered. The systolic blood pressure of each rat was measured from each rat's tail before administration and thereafter at 15 min-6 h, by using a specially devised Blood Pressure Monitoring System (IWORX , U.S.A.).

Each group consisted of 4 SHR, and negative and positive control groups were provided. The positive control group was administered a commercial antihypertensive drug, captopril, at a dosage of 1 mg/kg/rat. The negative control group of rats was administered saline only. Prior to the administration of the purified ACE inhibitor, the blood pressures of the rats were measured four times during a one-week period and the test group rats were selected according to their average blood pressure. While the ACE inhibitor was being administered, the blood pressure of each rat was measured three times for every test. Data from these experiments were assessed by analyses of group differences and were considered statistically significant at $p<0.05$ by Tukey's test.

Results and Discussion

Production of ACE Inhibitor by *Saccharomyces cerevisiae*

Among the several ACE inhibitor-producing yeasts, *Saccaromyces cerevisiae* showed the highest ACE inhibition activity of 42.1% (Table 1). Therefore, *S. cerevisiae* was selected as a new producer of intracellular ACE inhibitor. To the best of our knowledge, this is the first report to show that alcohol fermentative *S. cerevisiae* produces a potent intracellular ACE inhibitor. Therefore, cultural conditions of *S. cerevisiae* for maximum ACE inhibitor production were investigated. Maximal cell growth was reached at 48 h of cultivation, whereas maximal production of the ACE inhibitor was obtained at 24 h of cultivation (Fig. 1).

Generally, many ACE inhibitors are known to be free peptides isolated [5] or peptides from protein hydrolysates [26, 27]. Therefore, in order to increase the productivity of the ACE inhibitor, cell-free extracts of *S. cerevisiae* were treated by various proteases under each optimal reaction condition, and the ACE inhibitory activity was then determined. The ACE inhibitory activity of *S. cerevisiae* increased by about 1.5 times after treatment of cell-free extracts with pepsin (64.4%) (Table 2). These results indicate that the ACE inhibitor of cell-free extract from *S. cerevisiae* is peptide and its contents was increased by protein hydrolysis. The results were similar to those of sake lee [27], cereals and legumes [26], in which ACE inhibitory activities were markedly increased by protein hydrolysis.

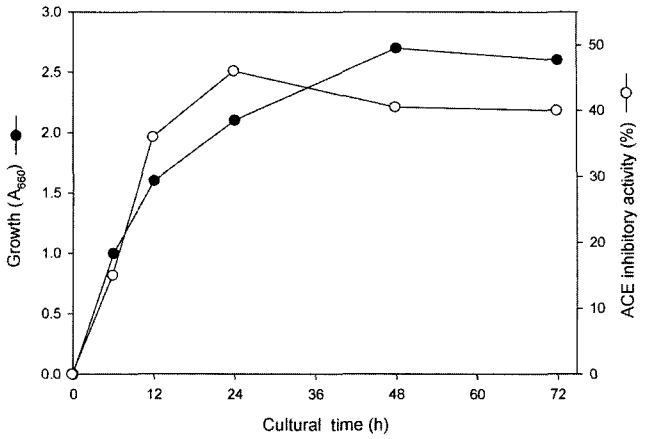

Fig. 1. Effects of *Saccharomyces cerevisiae* culture time on the production of the intracellular ACE inhibitor.

Purification of the ACE Inhibitor

The ACE inhibitor was purified from the pepsin-hydrolysates of cell-free extracts of *S. cerevisiae* by ultrafiltration, Sephadex G-25 Chromatography, and HPLC (Fig. 2). After the final purification step, the ACE inhibitor with an IC_{50} of 0.07 mg was obtained, and the yield was 3.5%. Its ACE inhibitory activity was stronger than that of *Ganoderma frondosa* (IC_{50}: 0.097 mg), but was lower than that of *Tricholoma gigantum* (IC_{50}: 0.04 mg), *Doratomyces putredinis* (IC_{50}: 14 nM), and captoprile, which was chemically synthesized (IC_{50}: 17.9 nM).

Even though the ACE inhibitory activity of the ACE inhibitor purified from *S. cerevisiae* was slightly lower than that of the commercial antihypertensive drug captopril, the ACE inhibitor from *S. cerevisiae* is considered to be a good candidate for antihypertensive drugs and functional foods, because it comes from edible yeast and does not have the side effects such as coughs and allergies that are associated with captopril [22].

Table 2. Effect of digestion of some proteases on the ACE inhibitory activity of cell-free extracts from *S. cerevisiae*.

Proteases	ACE inhibitory activity (%)
Control	42.1
Pepsin	64.4
Trypsin	30.9
Protease N	42.5

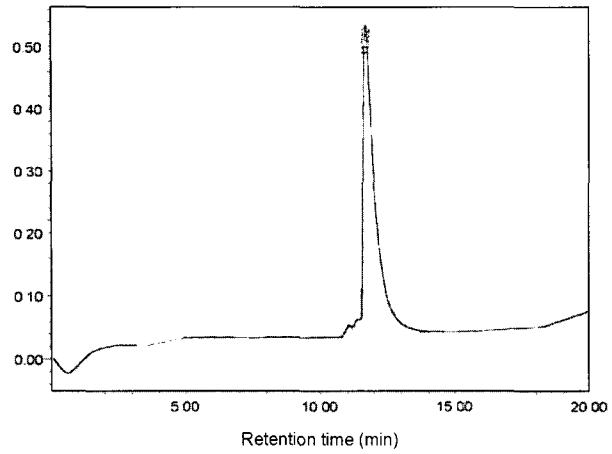

Fig. 2. HPLC elution profile of active fraction from reverse-phase μBondapak C_{18} column.

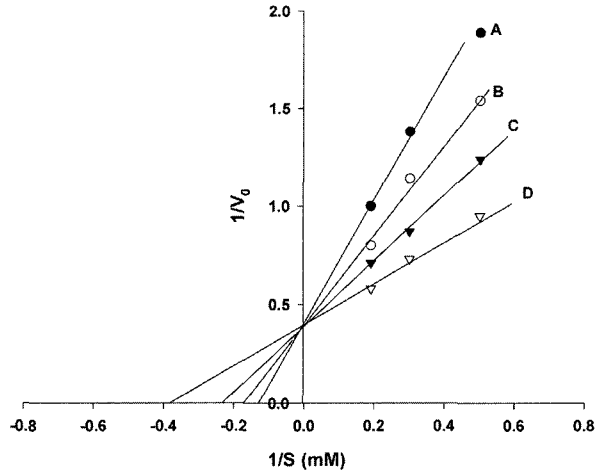

Fig. 4. Lineweaver-Burk plot of ACE activity in the presence of the inhibitor (A, ●) 10 μM, (B, ○) 7 μM, (C, ▼), 3 μM, (D, ▽) control.

Molecular Weight and Amino Acid Sequence of the ACE Inhibitor

The molecular mass of the ACE inhibitor was estimated to be 1,178 Daltons by LC-MS analysis (Fig. 3). Because its molecular weight was much smaller than those of others [15], it appears to be suitable for absorption in the intestine. The amino acid sequence of the ACE inhibitor was found to be Tyr-Asp-Gly-Gly-Val-Phe-Arg-Val-Tyr-Thr by tandem LC-MS analysis [25]. Most of the ACE inhibitors are known as peptides in the range of dipeptides to oligopeptides, except for triterpene of *Ganoderma lucidum* [18] and cathecines. Therefore, the ACE inhibitor from *S. cerevisiae* with the size of decapeptide in this study appears to have no homology.

Determination of ACE Inhibition Pattern

The ACE inhibition pattern of the purified ACE inhibitor was investigated by Lineweave-Burk plot (Fig. 4). It was found to be a competitive inhibitor on ACE, suggesting that the ACE inhibitor from *S. cerevisiae* binds competitively with the substrate at the active site of ACE. This inhibition pattern was very similar to the patterns of those from *Tricholoma gigantum* [15] and *Ganoderma frondosa* [5].

Antihypertensive Action of the Purified ACE Inhibitor

As shown in Fig. 5, the average blood pressure of the ACE inhibitor group rats was found to be roughly 192 mmHg just before the administration. After 2 h of administration of the inhibitor at 1 mg/kg rat body weight, blood pressure decreased to 161 mmHg, slightly increasing later to average blood pressure. These results were similar to that of the commercial antihypertensive drug captopril (192 mmHg→ 162 mmHg), suggesting that the purified ACE inhibitor

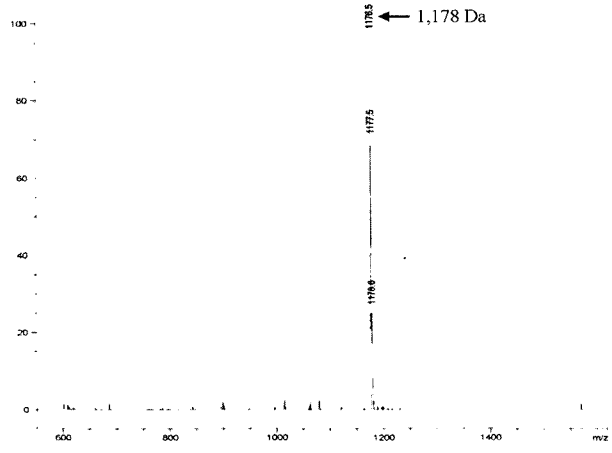

Fig. 3. Mass spectrum of the purified ACE inhibitor from *Saccharomyces cerevisiae.*

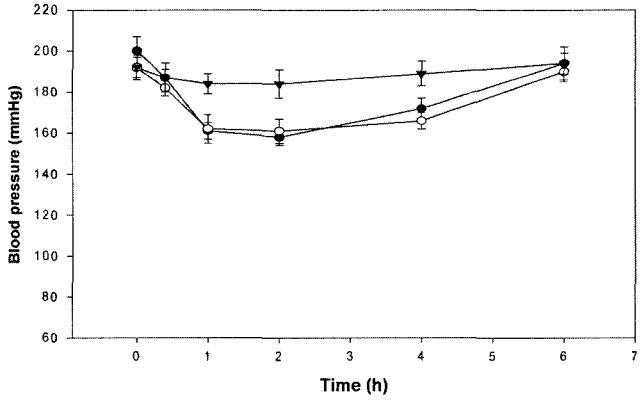

Fig. 5. Effect of the orally administered ACE inhibitor of *S. cerevisiae* on blood pressure in SHR.
●, ACE inhibitor 1 mg/kg body weight; ○, positive control (captopril) 1 mg/kg; ▼, negative control. *, ** significantly different from test group at $p<0.05$ by Tukey's test.

produces a clear antihypertensive effect in SHR at a dosage of 1 mg/kg rat body weight. Furthermore, no allergic reactions or coughing were observed for 1 day, and the inhibitor was also found to be nontoxic by the Ames test and the MTT assay (data not shown).

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