Characterization of Novel Antihypertensive Angiotensin I-converting Enzyme Inhibitory Peptides From Edible Mushrooms

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Abstract: This study describes the extraction and characterization of novel antihypertensive angiotensin I-converting enzyme (ACE) inhibitory peptides from *Tricholoma gigantum* and *Pholiota adiposa*. The maximum ACE inhibitory activity (IC_{50} : 0.31 mg) of *T. gigantum* was obtained when the fruiting body of commerical *T. gigantum* was extracted with distilled water at 30°C for 3 hours. After purification of ACE inhibitory peptides using ultrafiltration, Sephadex G-25 column chromatography and reverse phase HPLC, an active fraction with an IC_{50} of 0.04 mg and a yield of 0.3% was obtained. The ACE inhibitory peptide was a novel tripeptide that was sequenced as Gly-Glu-Pro. Water extracts of *P. adiposa* ASI 24012 fruiting bodies had potential ACE inhibitory activity of 66%. The maximum ACE inhibitory activity (IC_{50} ; 0.25mg) was obtained when the fruiting body of *P. adiposa* ASI 24012 was extracted with distilled water at 30°C for 12hr. After the purification of ACE inhibitory peptides using ultrafiltration, Sephadex G-25 column chromatography and reverse-phase HPLC, an active fraction with an IC_{50} of 0.044mg was obtained.

Key words: Antihypertensive peptide, angiotensin I-converting enzyme, *Tricholoma gigantum, Pholiota adiposa*, water extracts, inhibition

1 Introduction

Angiotensin I-converting enzyme (ACE, dipeptidyl carboxypeptidase I, kininase II, EC. 3.4.15.1) is a multi-functional enzyme which plays a key physiological role in the control of blood pressure by virtue of the rennin-angiotensin system.[1-3] ACE converts the inactive decapeptide, angiotensin I, to the potent vasopressor octapeptide, angiotensin II, and inactivates bradykinin.[4] Many research groups have screened for ACE inhibitors from natural products and microbial sources[5] including *Doratomyces putredinis, Nocardia orientalis, Streptomycetes, Actinomycetes, Actinomadura spiculosospora* and *Actinomadura* sp.. Food-derived ACE inhibitory peptides have been isolated from food or enzymatic digestion of food proteins[6] including gelatin,[2] casein,[7] fish,[8-10] fig tree latex[11] and α-zein.[12] Other ACE inhibitors were found from sake and its by-products,[13] Korean traditional rice wines and liquors,[14] cereals and legumes,[15] and microbes such yeasts[16] and Basidiomycetes.[17]
Recently, mushrooms have received attention because they are a nutritious food with health-stimulating properties and medicinal effects. There are some papers discussing the antihypertensive effect of the ACE inhibitors of *Grifola frondosa* and the other mushrooms.[18, 19] However, little work has been done on the pharmaceutical effects of *T. giganteum* except on its antitumor-active heteroglycans.[20, 21] Therefore, it has been mostly used as edible food. Since the original discovery of ACE inhibitors in snake venom,[22] captopril (d-3-mercapto-2-methylpranory-l-proline), enalapril and lisinopril, effective oral inhibitors, have been developed and are currently used as clinical antihypertensive drugs.[23] However, even though synthetic ACE inhibitors including captopril are remarkably effective as antihypertensive drugs, they have certain side effects including cough, allergies, taste disturbance and skin rashes. Therefore, research and development on safer, innovative, and economical ACE inhibitors is necessary for the prevention and remedy of hypertension.

This study describes the extraction and characterization of novel ACE inhibitory peptides from fruiting bodies of *T. giganteum* and *P. adiposa*, that can be used as antihypertensive drugs.

2 Materials and Methods

2.1 Materials

Korean mushrooms used in this experiment were obtained from National Institute of Agriculture Science and Technology in Suwon, South Korea. ACE was extracted from rabbit lung acetone powder (Sigma Chemical Co., St. Louis, MO, USA) and its activity determined by using hippuric acid-histidine-leucine (Sigma Chemical Co.) as substrate. One unit was defined as the amount catalyzing the formation of 1μM hippuric acid from Hip-His-Leu in 1 minute at 37°C under standard assay conditions.[4] Captopril, a commercial antihypertensive drug, was purchased from Kasei Kogyo Co.,(Japan). Unless otherwise specified, all chemicals and solvents were of analytical grade. Pepsin, trypsin, trifluoroacetic acid and acetonitrile were purchased from Sigma Chemical Co.

Male spontaneously hypertensive rats (SHR), Sam:TacN(SHR)fBR (280-300g, 11 weeks old), were purchased from Samtaco Bio-Korea Co.(Korea, O San City).

2.2 Extraction of ACE inhibitor

Dried fruiting bodies of mushrooms (5 g) were pulverized and added to 200 mL of water, ethanol and methanol. Water, ethanol, and methanol extraction were carried out at 30°C with stirring for 12 hours. The mixtures were centrifuged at 10,000 x g for 20 minutes and filtered through Whatman No. 41 filter paper. Each supernatant was lyophilized prior to analysis.

2.3 Assay of ACE inhibitory activity

The ACE inhibitory activity was assayed by a modification of the method of Cushman and Cheung.[24] 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 pre-incubated for 10 minutes at 37°C . The reaction was initiated by adding 50 μL of Hip-His-Leu at a final concentration of 5 mM, and terminated after 30 minutes 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 using a Speed Vac Concentrator (EYELA Co., Japan). The residue was then dissolved in 1 mL of sodium borate buffer. The 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}.

2.4 Purification of ACE inhibitor from *T. giganteum*

Water extract solutions were ultrafiltered using a 5,000 M.W. cut-off filter (Labscale TFF System, Millipore Co., USA) and the ACE inhibitory activities of the filtrates and a solution of the filter cake were determined. Active fractions were lyophilized, and 9 g was applied to a Sephadex G-25 column (3.0 × 35 cm; Pharmacia, Sweden) equilibrated with distilled water and eluted with the same buffer at a flow rate of 12 mL/hr. Active fractions were then applied to a preparative reverse phase high-performance liquid chromatography column (μ Bondapack C_{18} column) equilibrated with acetonitrile. The column was eluted with a linear gradient (0-100%) of 0.1% trifluoroacetic acid (TFA) in water. The active fractions were collected and lyophilized immediately. The active fractions obtained were then subjected to a first-reverse phase high-performance liquid chromatography (μ Bondapack C_{18} column) equilibrated with 0.1% TFA in water. The column was eluted with a linear gradient (0-100%) of 0.1% aqueous TFA in acetonitrile. The active fractions were again collected and lyophilized immediately. The active fractions obtained were then subjected to a second-reverse phase high-performance liquid chromatography (Nova-pak C_{18} column) equilibrated with 0.1% TFA in water. The column was eluted with a linear gradient (0-100%) of 0.1% aqueous TFA in acetonitrile.

2.5 Anti-hypertensive action in spontaneously hypertensive rats (SHR)

Doses of the purified ACE inhibitor from mushrooms, 1 mg/kg/rat, were orally administered and the systolic blood pressure was then measured before administration and after 0.15 h-6 h from the rat tail by using specially devised Blood Pressure Monitoring System (IWORX , USA).

Each group consisted of four SHRs, and negative and positive control groups. The positive control group was administered the commercial anti-hypertensive drug, captopril, at a dose of one mg/kg/rat. The negative control group was given only saline. Prior to administration of the purified ACE inhibitor, the rat blood pressures were measured four times during a one-week period, and test group rats were selected according to their average blood pressure. While the ACE inhibitosr were being administered, the blood pressures of each group were measured three times for every test.

3 Results and Discussion

3.1 The ACE inhibitory activities of various extracts from mushrooms

Extracts (water, ethanol, methanol) of fruiting bodies of several commercial mushrooms were prepared to determine their ACE inhibitory activities. As shown in Table 1, aqueous extracts of all the mushrooms tested, except *Lentinus edodes*, had higher ACE inhibitory activities than those of other extracts. Aqueous extracts of *T. giganteum* ASI 14001 showed the greatest inhibitory activity (61.3%), and had a IC_{50} value of 0.47 mg. Therefore, *T. giganteum* ASI 14001 was selected as a producer of ACE inhibitor in commercial mushrooms. Optimal extraction conditions for the ACE inhibitor from *T. giganteum* were investigated over the range 10-70°C and 1-18 hrs. The highest ACE inhibitory activity of aqueous extracts of *T. giganteum* fruiting bodies (70.6%, IC_{50} ; 0.31mg) was obtained when the extraction was performed at 30°C for 3 hours; hot water extracts had lower activity.

Table 1. ACE inhibitory activities of the extracts from commercial mushrooms [a]

Mushrooms	ACE inhibitory activity (%)			
	ASI No	Water extract	Ethanol extract	Methanol extract
Pleurotus sajor-caju	S -1[b]	38.7±0.5	9.0±0.1	11.5±0.4
Pleurotus ostreatus	C-1	27.3±0.4	8.5±0.6	14.5±0.6
Flammulina velutipes	4065	16.4±0.2	N.D[c]	N.D
	4075	21.0±0.7	N.D	N.D
	V1	13.7±0.1	10.9±0.7	N.D
	4047	32.9±0.5	N.D	N.D
T. giganteum	14001	61.3±0.5	8.5±0.8	14.5±0.5
Agaricus bisporus	505	27.3±0.6	9.0±0.9	11.5±0.4
Poria cocos		34.0±0.1	N.D	N.D
Grifola umbellata		40.1±0.8	14.6±0.4	4.5±0.2
L. edodes		N.D	2.7±0.2	N.D

[a] Mushroom (1 g) was extracted using various methods. Values are means ±S.D of three determinations.
[b] No. of National Institute of Agricultural Science and Technology in Korea; [c] N.D; not detected

Extracts (water, ethanol, methanol) from the fruiting bodies of several RDA (Rural Development Administration of Korea) mushrooms were prepared to determine their ACE inhibitory activities. As shown in Table 2, the water extracts from *P. adiposa* ASI 24012 showed the greatest inhibitory activity with 0.25 mg of IC_{50}. Therefore, *P. adiposa* ASI 24012 was selected as a producer of ACE inhibitor in RDA mushrooms. Optimal extraction condition of ACE inhibitor from *P. adiposa* were investigated in range of 10°C-70°C and 1-18 hours The highest ACE inhibitory activity of the water extracts of *P. adiposa* fruiting body was obtained when the extraction was performed at 30°C for 12 hours (67.6%, IC_{50}, 0.25mg) and hot water extracts had lower activity.

Table 2. ACE inhibitory activities of the various extracts from mushrooms

Strain of *P. adiposa* (ASI)	ACE inhibitory activity (IC_{50}, mg)		
	Water extract	Ethanol extract	Methanol extract
24001	>1	>1	>1
24002	0.57	>1	>1
24004	0.49	>1	>1
24005	0.81	>1	>1
24007	0.76	>1	>1
24008	0.42	>1	>1
24010	0.95	>1	>1
24012	0.25	>1	>1
24017	0.28	>1	>1
24018	0.29	>1	>1
24022	0.38	>1	>1
24024	0.45	>1	>1
24027	0.31	>1	>1
5019	>1	>1	>1
500110	>1	>1	>1
500457	>1	>1	>1
500461	>1	>1	>1
500462	>1	>1	>1

3.2 Purification and characterization of ACE inhibitor from *T. giganteum*

After the purification steps by ultrafiltration, Sephadex G-25 column chromatography and HPLC, the ACE inhibitor with an IC_{50} of 0.040 mg was obtained, and the yield was 0.3% (Figure 1). Even though the ACE inhibitory activity of *T. giganteum* was slightly lower than that of the commercial antihypertensive drug, captopril, which was synthesized chemically, the ACE inhibitor of *T. giganteum* was considered to be a good candidate for antihypertensive drug or functional foods because captopril has side effects such as cough and allergies.[23]

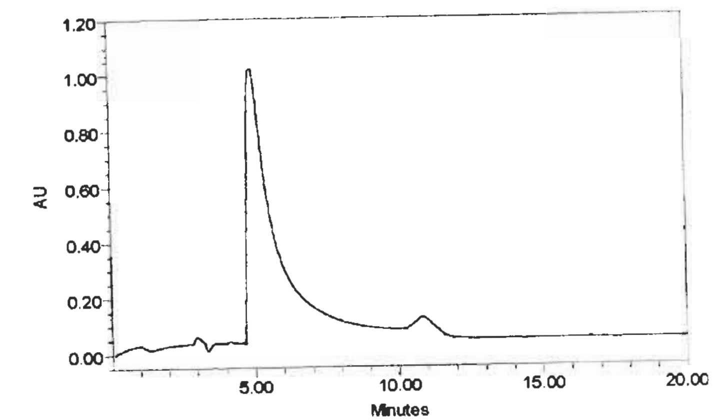

Figure 1. Reverse phase HPLC profile of active fraction from Nova-pak C18 column

The molecular mass of the purified ACE inhibitor from *T. giganteum* was estimated to be 301 daltons using LC/MS analysis (Figure 2). Although the molecular weight was very small compared to others, which were mostly oligopeptides, it was considered to be more suitable for absorption in the intestine. The amino acid composition of the ACE inhibitor was identified as Pro (40%), Glu (30%) and Gly (30%), and the sequence was found to be Gly-Glu-Pro.

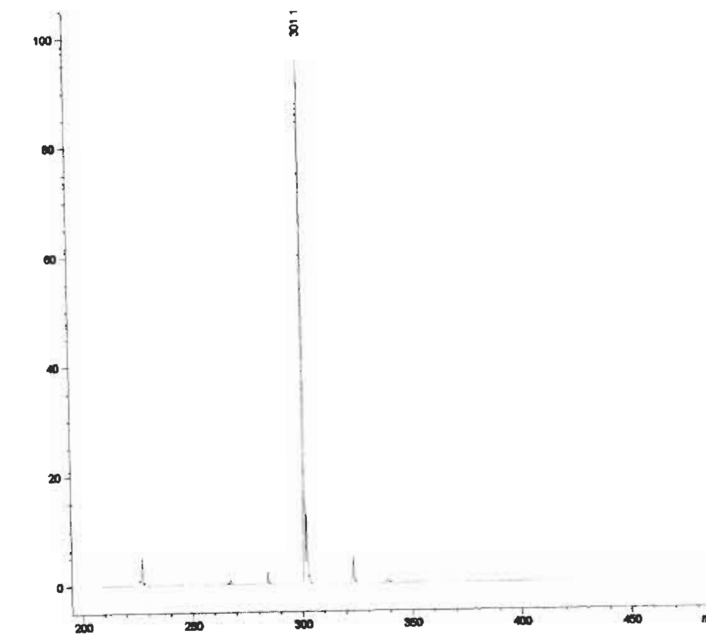

Figure 2. Mass spectrum of the purified ACE inhibitor from *T. giganteum*

The ACE inhibition pattern of the purified ACE inhibitor from water extracts of *T. giganteum* was investigated by Lineweaver-Burk plot (Figure 3). It was found to exhibit a competitive inhibition pattern on ACE, suggesting that the ACE inhibitor from *T. giganteum* binds competitively with the substrate at the active site of ACE. Therefore, it shows the same inhibition pattern as that of an inhibitor from *G. frondosa*[18] and others such as fibrinogen pentapeptides, casein fragment, porcine plasma tripeptides and tuna muscle octapeptide.[5, 18]

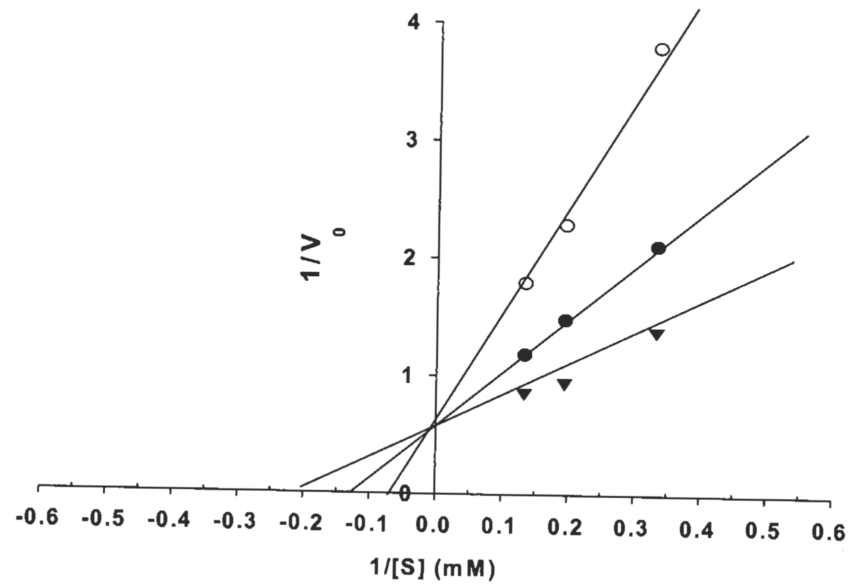

Figure 3. Lineweaver-Burk plot of ACE activity in the presence of the inhibitor, p1
(O , 0.1 mg of inhibitor, ● , 0.05 mg of inhibitor, ▲ , control)

When the ACE inhibitors were treated with pepsin, trypsin and protease N, the ACE inhibitory activities decreased only slightly, although the trypsin-treated ACE inhibitor was inactivated by approximately 11% (data not shown). These results indicated that when the ACE inhibitor peptide is orally treated, it would be stable in the stomach.

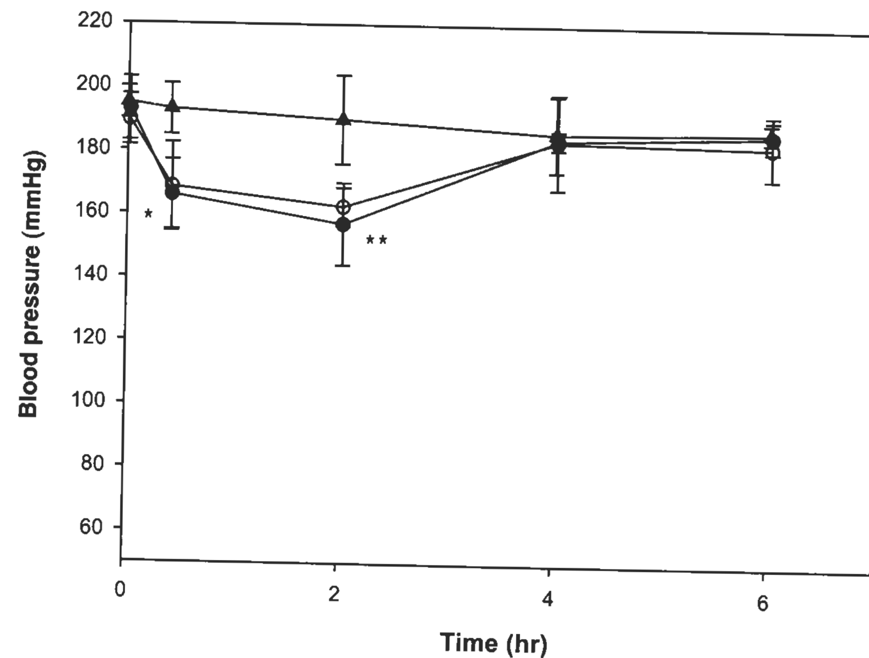

Figure 4. Effect of orally administered the ACE inhibitor from *T. giganteum* on blood pressure in SHR

●, ACE inhibitor 1 mg/kg, O, positive control(captopril) 1 mg/kg, ▲, negative control. *, ** significantly different from test group ($p < 0.05$, $p < 0.01$, respectively)

As shown in Figure 4, the average blood pressure of the ACE inhibitor group rats showed about 193mm Hg just before the administration. After 2 hour of administering the ACE inhibitor (1 mg/kg/rat), blood pressure decreased to 157mm Hg, and afterwards slightly increased to the average blood pressure. The pattern was similar to that of the commercial antihypertensive drug, captopril (190 mmHg →163 mmHg). This suggests that the purified ACE inhibitor produces clear antihypertensive effects in SHR at a dose of 1 mg/kg/rat.

3.3 Purification and characterization of ACE inhibitor from *P. adiposa*

The ACE inhibitory activity of the filtrates from 5,000 M.W. cut-off ultrafiltration of water extracts was 0.21 mg of IC_{50}. After Sephadex G-25 column chromatography, the active fraction had an IC_{50} value of 0.19 mg. Active fractions from the Sephadex column were collected and subjected to preparation reverse phase HPLC using a μ Bondapack C18 column. One peak containing ACE inhibitory activity was obtained (IC_{50}, 0.15 mg). Two reverse-phase HPLC separations were then performed on the active fraction. When subjected to reverse-phase HPLC using μVydac protein/peptide reversed-phase 218TP column, a single peak was eluted (Figure 5). After the purification steps, the ACE inhibitor with an IC_{50} of 0.044 mg was obtained.

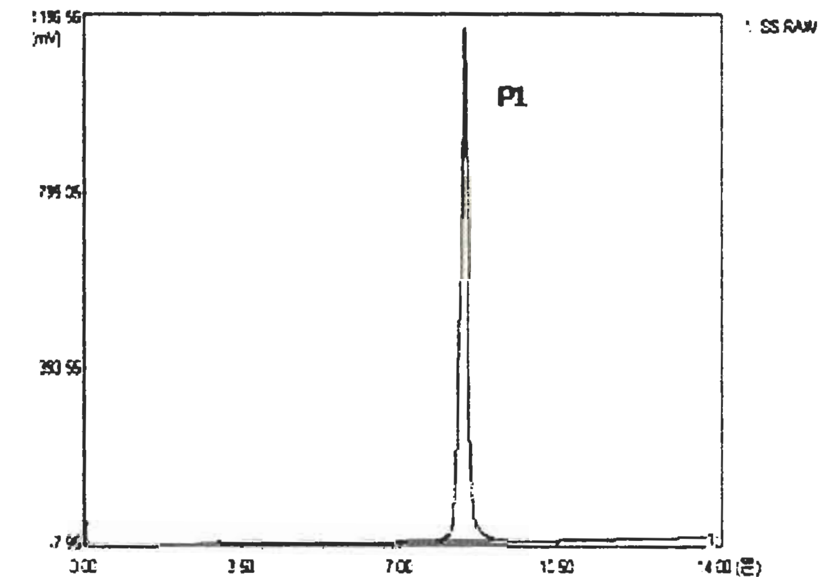

Figure 5. Reverse-phase HPLC chromatogram of the purified ACE inhibitor

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