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Optimization of submerged culture conditions for the production of angiotensin converting enzyme inhibitor from *Flammulina velutipes*

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The angiotensin-converting enzyme (ACE) inhibitory effect was tested in the culture broth from submerged mycelial cultures of 20 basidiomycetes. The ACE inhibitory effect of culture broth from *Flammulina velutipes* strain 414 was the highest (52.8%), followed by *Lentinus edodes* strains 2 (44.4%) and 16 (41.3%). Nutritional requirements for the production of ACE inhibitory substance from *F. velutipes* were studied. Sucrose, ammonium acetate, and glutamic acid were chosen for the maximum production of ACE inhibitory substance. The optimal medium composition was (g/l): sucrose 20, ammonium acetate 5, glutamic acid 2, KH_2PO_4 3, $MgSO_4 \cdot 7H_2O$ 0.8, and yeast extract 0.5. Under optimal culture conditions, the ACE inhibitory effect was more than 80%.
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Introduction

In recent years, the number of identified zinc metallopeptidases has increased enormously. Members of this superfamily of enzymes are involved in many physiological and pathological processes such as blood pressure regulation, pain suppression and diuresis. Angiotensin-converting enzyme (ACE, EC 3.4.15.1) belongs to a group of enzymes which are important therapeutically and a focus of drug research. ACE inhibitors had been frequently used as anti-hypertensive agents and for an effective treatment of congestive heart failure [9] in the last decade. However, synthetic drugs are believed to have certain side effects such as cough, taste disturbances and skin rashes [2]. Therefore, for safe and economical use, the interest about food sources as ACE inhibitor involved in preventing hypertension has increased. ACE inhibitors from food sources are a peptide from corn gluten digested by pescalase [18], a peptide isolated from an acid extract of tuna [14], a peptide from cold water extract of mushroom [6], and various flavonoids isolated from leaves of persimmon [13].

Edible mushrooms including *Grifola frondosa*, *Lentinus edodes*, *Ganoderma lucidium* [3], *Pleuroteus sajorcaju* [19] and *Volvariella volvacea* [5] have been reported to have anti-hypertensive effects. Choi *et al* [6] reported the ACE inhibitory activities of a cold water extract from *G. frondosa*.

Plants and mushrooms are important sources for new enzyme inhibitors. Among them, mushrooms have been studied for the isolation of new compounds. But, more and more pharmacologically active metabolites from mushrooms are isolated and tested. Therefore, in the present study, ACE inhibitory activities were measured from several mycelial cultures of mushrooms, and optimal culture conditions for mycelial growth of *Flammulina velutipes* were studied for the production of ACE inhibitor as an anti-hypertensive substance.

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Materials and methods

Microorganism and cultivation

Twenty strains in seven types of basidiomycetes were donated from the National Forestry Cooperative Federation in Korea. The basal medium for mycelial growth contained (g/l): mannitol 20, ammonium nitrate 2, glutamic acid 4, KH_2PO_4 3, $MgSO_4 \cdot 7H_2O$ 0.8, yeast extract 0.5, pH was adjusted to 6.0. The submerged mycelial culture was incubated in 500-ml flasks containing 200 ml medium on a rotary shaker (120 rpm, 15 days) at 20°C. After centrifugation (8000×*g* for 20 min), mycelia were discarded and the supernatant was lyophilized to be used for analysis of the ACE inhibitory effect. ACE (rabbit lung acetone powder) and hippuric acid–histidine–leucine (Hip–His–Leu) were purchased from Sigma Chemical (St. Louis, MO, USA). The other chemicals were of analytical grade.

Assay of the ACE inhibitory effect

The ACE inhibitory effect was assayed by a modification of the method of Cushman and Cheung [7]. A mixture (300 μl) containing 100 mM sodium borate buffer (pH 8.3), 300 mM NaCl, 8 mU ACE (Sigma Chemical) 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 0.15 ml of Hip–His–Leu at a final concentration of 5 mM, and terminated after 30 min incubation by adding 0.5 ml of 1 M HCl. The hippuric acid liberated was extracted with 1.5 ml of ethyl acetate, and 1.0 ml of the extract was evaporated to dryness by a Speed Vac Concentrator (Savant, Holbrook, NY, USA). The residue was then

Table 1 ACE inhibitory effect of the cell-free culture broth from basidiomycetes

Basiodiomycete	Strain no.	ACE inhibitory effect (%)	IC_{50} (mg/ml)	Basiodiomycete	Strain no.	ACE inhibitory effect (%)	IC_{50} (mg/ml)
Grifola fronsoda	1	8.1	N.T.*	*Pholiota nameko*	1	45.4	27.9
	2	8.8	N.T.		2	25.4	43.2
	3	0.9	N.T.		3	7.9	N.T.
Lentinus edodes	2	44.4	18.4	*Gonoderma luicidum*	1	16.4	N.T.
	4	36.6	39.3		2	26.9	47.2
	6	31.1	38.2	*Flammulina velutipes*	414	52.8	22.6
	9	33.5	34.2		416	40.7	7.4
	16	41.3	23.4	*Pleurotus coccinea*	1	18.9	48.1
	17	35.9	35.1		2	24.9	40.1
Phellinus gilves	1	27.4	41.2		3	3.8	N.T.

*N.T.: not tested due to lower ACE inhibitory effect.

dissolved in 1.0 ml of deionized water. The absorbance at 228 nm was measured to estimate the ACE inhibitory effect. The concentration of ACE inhibitor required to inhibit 50% of the ACE activity under the above assay conditions was defined as IC_{50}.

Optimal culture conditions

To find the optimal culture conditions, carbon sources, nitrogen sources, pH and temperature were studied in the basal medium. To select the optimal carbon source, the ACE inhibitory effect was determined after 15 days of incubation in the basal medium containing 2% of various carbon sources. The effect of nitrogen source on the ACE inhibitory effect was investigated in the basal medium containing 2% mannitol and 0.5% nitrogen source. The effect of various concentrations of glutamic acid on the ACE inhibitory effect was investigated in the basal medium containing 2% mannitol and 0.2% ammonium acetate. The effect of temperature and pH on the ACE inhibitory effect was also investigated in the optimal medium at different temperatures and pHs.

Results and discussion

ACE inhibitory effect of basidiomycetes

Twenty strains of seven different basidiomycetes were screened, using mycelium-free spent culture broth, to obtain the active substances for ACE inhibition. Table 1 shows the ACE inhibitory activities obtained. Five strains showed more than 40% ACE inhibitory effect, and only three basidiomycetes inhibited the ACE activity exclusively. The IC_{50} of strains 1 of *Pholiota nameko*, 2 and 16 of *L. edodes*, and 414 and 416 of *F. velutipes* were 26.8, 18.4, 23.4, 22.6, and 7.4 mg/ml, respectively. Strain 416 of *F. velutipes* had the highest potential ACE inhibitory effect. Therefore, in the present study, culture conditions for the mycelial growth of *F. velutipes* were optimized to produce the ACE inhibitory substances as anti-hypertensive substances.

F. velutipes, which belongs to the family Tricolomataceae (Hymenomycetes, Basidiomycota), is an edible mushroom frequently consumed fresh in Japan and Korea. A number of compounds isolated from the fruiting body of *F. velutipes* have antitumor and immunomodulatory activities. Most of the compounds are proteins, polysaccharides, or glycoproteins [21]. Lectin [20], sterol [23], and monoterpentriol [11] compounds also have been isolated from this mushroom. A few reports describe the hypotensive effects of mushrooms including *F. velutipes*.

Optimal culture conditions

To study the effect of carbon source, 20 g/l of each carbon source was added to the basal medium in place of mannitol (Table 2). In general, monosaccharides were better carbon sources than di- and polysaccharides for the mycelial growth of *F. velutipes*. The addition of glucose, fructose and mannose resulted in maximum mycelial growth, and the addition of sucrose resulted in higher ACE inhibitory effect than glucose, fructose or mannose.

Table 2 Effect of carbon sources on mycelial growth and ACE inhibitory effect of *F. velutipes* in shaken flask cultures

Sugar (2%)	Dry cell weight (mg/ml)	ACE inhibitory effect (%)
None	0.4	0.5
Xylose	4.3	2.2
Arabinose	2.4	6.7
Glucose	6.4	35.5
Fructose	5.9	24.4
Mannitol	2.8	51.6
Sorbitol	3.7	8.9
Galactose	3.4	0.2
Mannose	6.1	21.1
Sucrose	5.5	60.2
Lactose	1.2	0.0
Starch	4.9	36.1
CMC	1.5	2.4
Olive oil	2.6	0.5
Glycerol	1.1	0.0

Incubation was for 15 days at 20°C at an initial pH of 6.0.

Table 3 Effect of nitrogen sources on mycelial growth and ACE inhibitory effect of *F. velutipes* in shaken flask cultures

Nitrogen (0.5%)	Dry cell weight (mg/ml)	ACE inhibitory effect (%)
None	3.7	31.2
Ammonium citrate	8.4	2.3
Ammonium nitrate	5.7	60.2
Ammonium carbonate	7.2	40.2
Ammonium acetate	3.8	65.7
Potassium nitrate	8.0	2.5
Sodium nitrate	6.2	0.1
Casein	8.0	33.2
Tryptone	9.5	42.1
Proteose peptone	10.9	38.2
Urea	8.5	43.2
Peptone	8.5	41.1
Casamino acid	8.9	31.4

Incubation was for 15 days at 20°C at an initial pH of 6.0.

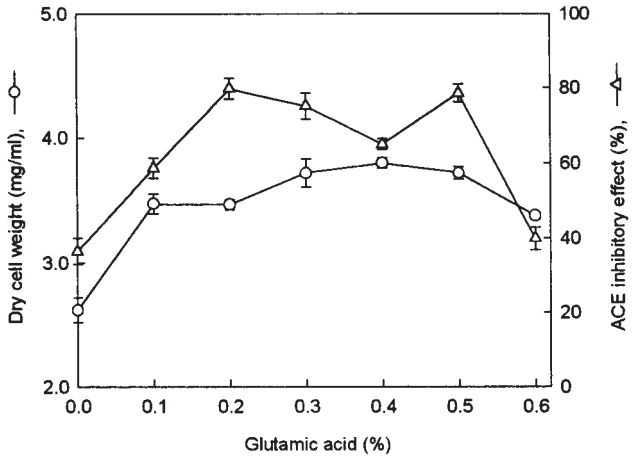

Figure 1 The optimal glutamic acid concentration on mycelial growth and ACE inhibitory effect by *F. velutipes* in shaken flask cultures. *F. velutipes* was inoculated in a medium containing (g/l) 20 sucrose, 5 ammonium acetate, 3 KH_2PO_4, 0.8 $MgSO_4 \cdot 7H_2O$, 0.5 yeast extract and various amounts of glutamic acid, and was cultivated at 20°C with initial pH 6.0 for 15 days.

The effect of nitrogen source on mycelial growth and ACE inhibitory effect was studied by incubating the organism in medium containing sucrose (20 g/l) and several nitrogen sources (5 g/l). As shown in Table 3, proteose peptone was the best nitrogen source for mycelial growth. The addition of ammonium acetate resulted in a high ACE inhibitory effect (65.7%), meanwhile the addition of protease peptone resulted in a 38% ACE inhibitory effect.

Glutamic acid was reported as the most effective amino acid for mycelial growth compared to 14 other amino acids (data were not shown). Without glutamic acid, the growth of mycelium was inhibited and finally stopped. Therefore, various concentrations of glutamic acid were added to the medium containing sucrose (20 g/l) and ammonium acetate (5 g/l) in the basal medium. Maximum mycelial growth (3.8 mg/ml) was observed in a medium containing 0.4% glutamic acid (Figure 1). The higher ACE inhibitory effects were obtained with the addition of 0.2% and 0.5% of glutamic acid (79.8% and 78.6%, respectively), and 0.2% glutamic acid was used in further work. However, IC_{50} values were slightly different between 0.2% and 0.5% glutamic acid: 2.3 and 3.4 mg/ml, respectively.

Components except sucrose, ammonium acetate, and glutamic acid in the basal medium, were not responsible for the increase of the ACE inhibitory effect (data not shown). The optimal medium composition was (g/l): sucrose 20, ammonium acetate 5, glutamic acid 2, KH_2PO_4 3, $MgSO_4 \cdot 7H_2O$ 0.8, and yeast extract 0.5.

Glucose, among the carbon sources, resulted in maximum mycelial growth, but this result differed from reports that mannitol was best for maximum mycelial growth [10,17]. These differences may be due to strain differences. Among the nitrogen sources tested, maximum mycelial growth was observed when ammonium formate was present. The relatively poor growth in medium containing nitrite and nitrate was similar to a common situation in fungi [8]. The effect of nitrite and nitrate could be the organism's ability to deaminate amino acid and interfere with sulphur metabolism since it is similar to the sulphite ion [16]. Although there was poor growth in the medium containing sucrose and ammonium acetate, they were the best carbon and nitrogen sources for production of ACE inhibitor. Moon *et al* [15] reported that *Streptomyces zoamyceticus* grew poorly in a medium containing rhamnose and tryptone, the highest ACE inhibitory effect was obtained, showing that nutrients for production of ACE inhibitor were not optimal for growth. Alanine, glycine, aspartic acid and glutamic acid were good nitrogen sources for mycelial growth, but lysine and methionine were not [12]. Ahn [1] also reported that glutamic acid was the best organic nitrogen source for mycelial growth and high ACE inhibitory effect.

Effect of pH and temperature

F. velutipes was cultivated in the optimal medium at different initial pHs in shaken cultures (Figure 2). The optimum pH for mycelial growth was from pH 4 to 7. The ACE inhibitory effect at pH 4–5.5 was higher than that at pH 6–7 with a maximum ACE inhibitory effect of 88.9% at pH 5.5. But, there were no significant

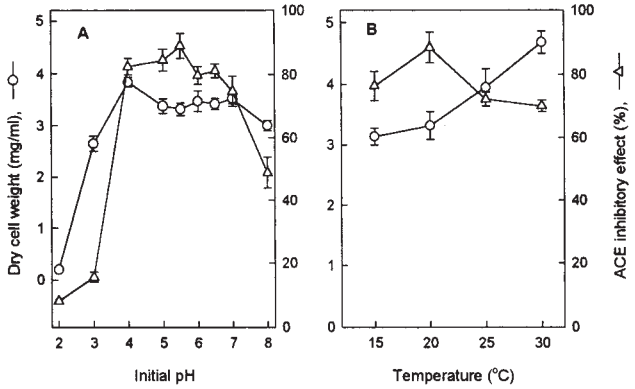

Figure 2 Effect of initial pH (A) and temperature (B) on mycelial growth and ACE inhibitory effect by *F. velutipes* in shaken flask cultures. *F. velutipes* was inoculated in a medium containing (g/l) 20 sucrose, 5 ammonium acetate, 2 glutamic acid, 3 KH_2PO_4, 0.8 $MgSO_4 \cdot 7H_2O$ and 0.5 yeast extract. Cultivation was carried out at 20°C with various initial pHs (A) or at various temperatures with initial pH 5.5 for 15 days.

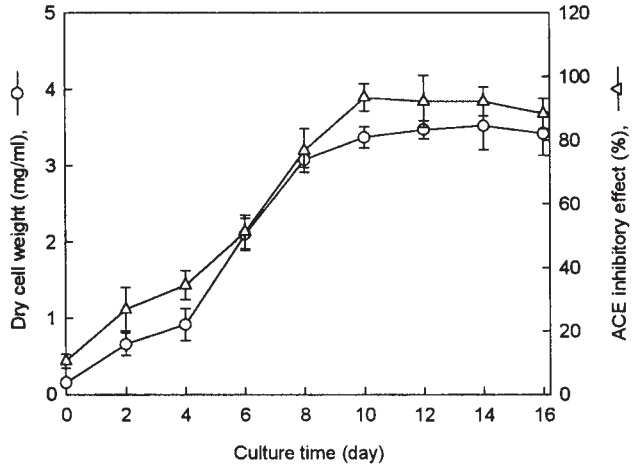

Figure 3 Mycelial growth and ACE inhibitory effect by *F. velutipes* in shaken flask cultures. *F. velutipes* was inoculated in a medium containing (g/l) 20 sucrose, 5 ammonium acetate, 2 glutamic acid, 3 KH_2PO_4, 0.8 $MgSO_4 \cdot 7H_2O$ and 0.5 yeast extract. Cultivation was carried out at 20°C with initial pH 5.5 for 16 days.

differences. Therefore, a high ACE inhibitory effect was obtained over a broad pH range (pH 4–7).

F. velutipes was cultivated at four different temperatures (15, 20, 25, and 30°C). The optimal temperature for mycelial growth was 30°C, but the ACE inhibitory effect at 30°C (72.2%) was lower than that at 20°C (88.4 %).

Cultivation under optimal culture conditions

Figure 3 shows various time courses of mycelial growth in flask cultures under optimal culture conditions. Mycelial dry weight reached a maximum of 3.37 mg/ml after 10 days of cultivation and the ACE inhibitory effect showed the same tendency. The maximum ACE inhibitory effect (93.4%) was observed at the late trophophase. There was no significant difference in ACE inhibitory effect after 10 days of cultivation. The ACE inhibitors are the primary products of metabolism in the trophophase [4].

To find the components of ACE inhibitor from *F. velutipes*, the inhibitor was hydrolyzed by pronase, a non-specific protease isolated from *Streptomyces griseus*. When the ACE inhibitor was treated with pronase, the inhibitory effect was greatly reduced from 88% to 3.4% (data not shown). The above result suggests that ACE inhibitor from *F. velutipes* was composed of peptides. Various ACE inhibitory peptides have been isolated from many food materials [22]. We reported a novel ACE inhibitor from *G. frondosa*, a peptide (Val-Ile-Glu-Lys-Tyr-Pro) composed of a hydrophobic amino acid at the amino-terminal, and a basic amino acid residue at the center, and proline at the carboxyl-terminal [6]. The use of culture broth as an ACE inhibitor has advantages such as economic mass production, simple extraction procedure, and shorter cultivation time of mycelium than for fruiting bodies. Development of the effective production of ACE inhibitor from *F. velutipes*, the investigation of the anti-hypertensive effect after oral administration, and its use as health-care foods need a further study.

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