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Production of Antihypertensive Angiotensin I-Converting Enzyme Inhibitor from *Malassezia pachydermatis* G-14

Seung Chan Jeong, Jae Ho Kim, Na-Mi Kim[1] and Jong Soo Lee*

Department of Genetic Engineering and Bio-medicinal Resource Research Center, Paichai University, Daejeon 302-735, Korea
[1]Central Research Institute, KT & G, Daejeon 305-805, Korea

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To produce a novel antihypertensive angiotensin I-converting enzyme (ACE) inhibitor from yeast, a yeast isolate, designated G-14 showing the highest ACE inhibitory activity was obtained and identified as *Malassezia pachydermatis* based on morphological, biochemical and cultural characteristics. The maximal extracellular ACE inhibitor production was obtained from *M. pachydermatis* G-14 when the strain was cultured in YEPD medium containing 0.5% yeast extract, 3.0% peptone and 2.0% glucose at 30°C for 24 h and the final ACE inhibitory activity was 48.9% under the above condition.

KEYWORDS: Angiotensin I-converting enzyme inhibitor, Antihypertensive, *Malassezia pachydermatis*

Angiotensin I-converting enzyme (ACE, dipeptidyl carboxypeptidase I, kininase II, EC. 3.4.15.1) is a multifunctional, zinc-containing enzyme located in different tissues which plays a key physiological role in the control of blood pressure by virtue of rennin-angiotensin system (Fujita, 2000; Ondetti, 1982; Rencland, 1994). ACE converts the inactive decapeptide, angiotensin I, to the potent vasopressor octapeptide, angiotensin II, and inactivates bradykinin (Ukeda, 1991).

Several ACE inhibitors show antihypertensive effects and may also have beneficial effects on glucose and lipid metabolism (Choi, 2001; Oshima, 1979; Pal, 1995; Pollare, 1989), decreasing insulin requirements in diabetes and increasing exercise tolerance (Pollare, 1989; Gohlke, 1994; Tomiyama, 1994).

Since the original discovery of ACE inhibitors in snake venom (Elisseeva, 1971), 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 (Ondetti, 1977). However, even though synthetic ACE inhibitors including captopril are remarkably effective as antihypertensive drugs, they have certain side effects such as cough, allergies, taste disturbance, skin rashes, etc. Therefore, research and development on safer, innovative, and economical ACE inhibitors is necessary for the prevention and remedy of hypertension.

Many research groups have screened for novel ACE inhibitors from natural products and microbial sources (Demain, 1989) including *Doratomyces putredinis*, *Nocardia orientalis*, *Streptomycetes*, *Actinomycetes*, *Actinomadura* spp. Food-derived ACE inhibitory peptides have been isolated from food or enzymatic digestion of food proteins (Fujita, 2000) including gelatin (Ondetti, 1982), casein (Morigiwa, 1986), fish (Matsumura, 1993; Sugiyama, 1991; Sun, 1997), fig tree latex (Maruyama, 1989) and α-zein (Miyoshi, 1991). Other ACE inhibitors were found from sake and its by-products (Saito, 1994), Korean traditional rice wines and liquors (Kim, 2002), cereals and legumes (Rhyu, 1996), and microbes such yeasts (Kim, 2003) and *Basidiomycetes* (Lee, 2003).

In this paper, we described the isolation and characterization of a novel yeast that produced potent extracellular ACE inhibitor.

Materials and Methods

Yeast strains, enzyme and reagents. Several Meju yeasts (Lee, 1997), industrial yeasts and other yeasts were obtained from the Lab. of Biotechnology, Paichai University, Korea Culture Center of Microorganism (KCCM) and Korea Collection for Types Cultures (KCTC).

The angiotensin I-converting enzyme (ACE) used in this experiment was extracted from rabbit lung acetone powder (Sigma Chemical Co., St. Louise, MO., USA) and its activity was determined using Hippuric acid-Histidine-Leucine (Hip-His-Leu) (Sigma Chemical Co.) as substrate. One unit was defined as the amount catalyzing the formation of 1 μM hippuric acid from Hip-His-Leu in 1 minute at 37°C under standard assay condition (Ukeda, 1991). Unless otherwise specified, all chemicals and solvents were analytical grade. Pepsin, trypsin, trifluoroacetic acid and acetonitril were purchased from Sigma Chemical Co.

*Corresponding author <E-mail: biotech8@mail.pcu.ac.kr>

Screening and identification of the extracellular ACE inhibitor-producing strain. Yeasts from several sources were inoculated in the YEPD medium (0.5% yeast extract, 3.0% peptone and 2.0% glucose) and cultured at 30°C for 72 h. After centrifugation at 15,000×g for 15 min, ACE inhibitory activities of the supernatants were determined. On the other side, the cells were suspended in the phosphate buffer (pH 7.0), washed, resuspended in phosphate buffer (pH 7.0), and then disrupted by glass bead. After centrifugation at 15,000×g (for 10 min at 4°C), ACE inhibitory activities of the cell free extract were determined.

Morphological, biochemical and cultural characteristics of the selected yeasts were investigated according to Taxonomy and Methods for the Identification of Microorganisms and Yeasts (Hasegawa, 1984) and The Yeast (Kreger-van, 1984).

Assay for ACE inhibitory activity. The activity of ACE inhibition was assayed by a modification of the method of Cushman and Cheung's (Ariyosh, 1993). A mixture containing a 100 mM sodium borate buffer (pH 8.3), 300 mM NaCl, 3 unit of ACE 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 terminated after 30 min of incubation by adding 250 μl of 1.0 N HCl. The hippuric acid liberated was extracted with 1 ml of ethyl acetate, and 0.8 ml of the extract was evaporated. 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 (Choi, 2001). The concentration of ACE inhibitor required to inhibit 50% of the ACE activity under the above assay conditions was defined as IC_{50}.

Results

Screening and identification of the extracellular ACE inhibitor-producing yeast. To select an extracellular ACE inhibitor-producing yeast, culture broth of 350 kinds of yeasts were tested for ACE inhibitory activities. Among them, strain G-14 showed the greatest ACE inhibitory activity. Therefore, G-14 was selected as a yeast for production of extracellular ACE inhibitor.

Morphological, biochemical and cultural characteristics of the G-14 strain are summarized in Table 1, 2 and Fig. 1. The selected G-14 strain was a spherical shaped yeast that did not form an ascospore. The strain formed a pseudomycelium and had no urease activity and also can not assimilate nitrate. A white color was shown in the TTC colorization test and the G+C content was 52.5 mol%. Although the strain assimilated almost of the sugars except soluble starch and xylose, it could not ferment all sugars. From these characteristics, the strain, was identified as *Malassezia pachydermatis* (Kreger-van, 1984).

Furthermore, the supernatants of *M. pachydermatis* G-14 culture broth were digested with pepsin and trypsin under each optimal reaction condition (Rhyu, 1996) and then ACE inhibitory activity of the hydrolysates was determined. Original ACE inhibitory activity did not de-

Table 1. Microbiological characteristics of the strain G-14

Classification	Characteristics
Cell shape	Spherical
Vegetative reproduction	Monopolar budding
Ascospore	–
Pseudomycelium	+
Growth in media: YEPD	+
2% olive oil/YEPD agar	+
Growth temp	25~40°C (opt.temp; 37°C)
Urease activity	–
Assimilation of nitrate	–
TTC colorization test	White
G+C content	52.5 mol%

Table 2. Assimilation and fermentability of carbon sources by the strain G-14

Carbon source	Assimilation[a]	Fermentability
Glucose	+	–
Fructose	+	–
Galactose	+	–
Sucrose	+	–
Lactose	+	–
Maltose	+	–
Raffinose	+	–
Soluble starch	–	–
Xylose	–	–
Rhamnose	+	–
Inositol	+	–
Inulin	–	–

[a]+; assimilatable or fermentable, –; not assimilatable or fermentable.

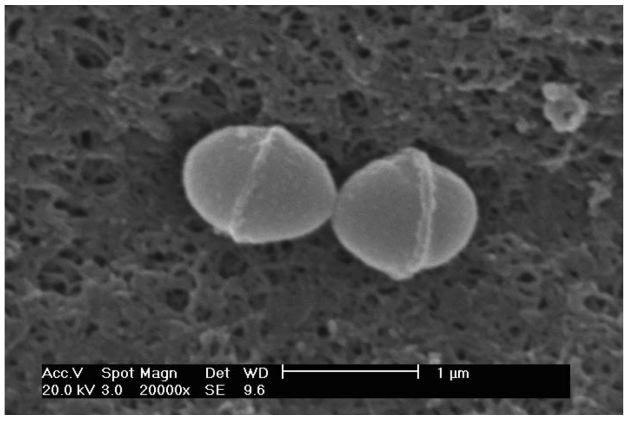

Fig. 1. Scanning electron micrograph of the strain G-14.

creased and scarcely maintained even after digestion by pepsin (42.5%) and trypsin (43.8%) (data not shown).

Table 3. Effects of nitrogen sources on the production of the extracellular ACE inhibitor from *M. pachydermatis* G-14

Nitrogen sources[a]	Growth (A_{660})	ACE inhibitory activity (%)[c]
Yeast extract	0.99	11.0±0.5
Peptone	0.52	11.3±0.4
Casamino acid	0.23	32.3±0.2
+ Peptone	0.55	14.6±0.7
+ Yeast extract	1.07	14.5±0.1
+ Beef extract	0.99	n.d
Beef extract	0.78	n.d
$NaNO_3$	n.d[b]	n.d
KNO_3	n.d	n.d
$(NH_4)_2SO_4$	n.d	n.d
$(NH_4)H_2PO_4$	n.d	n.d
NH_4Cl	n.d	n.d
NH_4NO_3	n.d	n.d
Gelatin	n.d	n.d
Urea	n.d	n.d
Yeast extract-Peptone-Dextrose (YEPD)	1.44	43.8±0.6

[a]Each nitrogen source was added into in the basal medium containing 2% glucose and cultured *Malassezia pachydermatis* at 30°C for 2 days.
[b]n.d; not determined or below 10% of activity.
[c]Values are means±S.D. of three determinants.

Table 4. Effects of yeast extract and peptone of YEPD medium on the production of the extracellular ACE inhibitor from *M. pachydermatis* G-14

Nitrogen sources[a]		Growth (A_{660})	ACE inhibitory activity(%)[b]
Yeast extract	Peptone		
0.1%	0.5%	0.34	n.d*
	1.0%	0.60	13.9±0.4
	2.0%	0.85	12.7±0.3
	3.0%	1.26	29.6±0.4
0.5%	0.5%	0.35	n.d
	1.0%	0.63	26.8±0.1
	2.0%	0.93	37.1±0.8
	3.0%	1.27	46.8±0.5
1.0%	0.5%	0.53	10.5±0.5
	1.0%	0.78	15.2±0.1
	2.0%	1.03	22.0±0.6
	3.0%	1.43	22.4±0.7
2.0%	0.5%	0.74	15.9±0.2
	1.0%	0.99	22.1±0.3
	2.0%	1.22	29.3±0.2
	3.0%	1.55	22.1±0.4

[a]*M. pachydermatis* was cultured at 30°C for 2 days in YEPD medium containing 2.0% glucose and indicated concentration of yeast extracts and peptone, respectively.
[b]Values are means±S.D. of three determinants.

Optimal conditions for the extracellular ACE inhibitor production. The effects of nitrogen sources on the production of the ACE inhibitor were investigated in basal medium containing 2.0% glucose. As shown in Table 3, *M. pachydermatis* G-14 did not grow with inorganic nitrogen sources and ACE inhibitor was not produced. The maximum production of the ACE inhibitor was obtained in YPED medium and casamino acid was a good nitrogen source for the production of ACE inhibitor. Furthermore, optimum concentration of yeast extract and peptone for the production of ACE inhibitor was 0.5% and 3.0%, respectively and its ACE inhibitory activity was 46.8% (Table 4). The effects of culture temperature on the production of the ACE inhibitor were examined in various temperature (25~40°C). For its growth *M. pachydermatis* preferred 37°C, whereas optimal temperature for the ACE inhibitor production was 30°C (Fig. 2). Therefore, we decided to culture the strain at 30°C for mass production of ACE inhibitor.

Time course of the ACE inhibitor production was determined in flask culture under the optimal culture conditions. As shown in Fig. 3, ACE inhibitor production

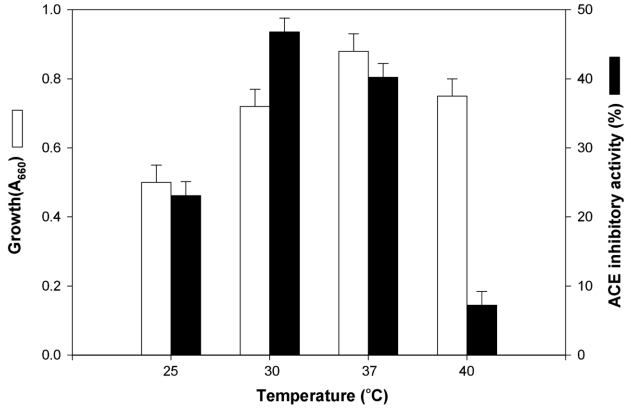

Fig. 2. Effect of temperature on the production of the extracellular ACE inhibitor from *M. pachydermatis* G-14.

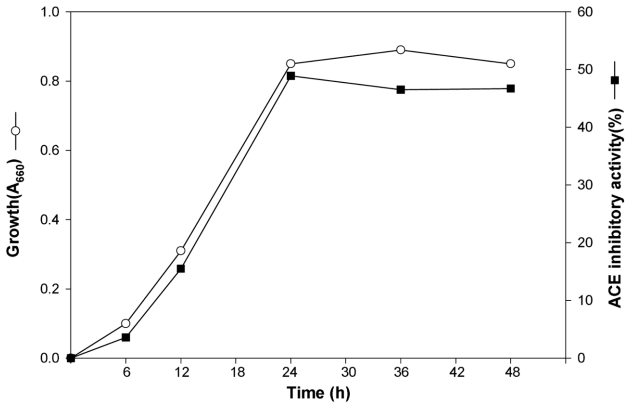

Fig. 3. Effect of culture time on the production of the extracellular ACE inhibitor from *M. pachydermatis* G-14.

increased with cell growth, and the maximum cell growth and ACE inhibitor production were obtained at 24 h of cultivation and its ACE inhibitory activity was 48.9%.

Considering the above results, the optimum culture condition for production of the ACE inhibitor was that medium was composed of 0.5% yeast extract, 3.0% peptone and 2.0% glucose, and culture temperature and time were 30°C and 24 h, respectively.

Discussion

There are two kinds of system in the regulation of blood pressure, that is, renin-angiotensin system and kallikrein-kinin system. Among them, angiotensin I-converting enzyme (ACE, dipeptidyl carboxy peptidase I, E.C 3.4.15.1) is the key enzyme in the renin-angiotensin system, which catalyzes production of active hypertensive hormone angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) from inactive pro-hormone angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu). This conversion action endows ACE with a very important role in regulating blood pressure through the direct action of angiotensin II on blood vessels, sympathetic nerves and adrenal glands (Folkow, 1961).

There are known some ways to remedy or prevent hypertension such as utilization of the ACE inhibitor or receptor blocker of angiotensin II. However, ACE inhibitors have long been used effectively because they inhibited both ACEs in renin-angiotensin system and kallikrein-kinin system, and their pharmaceutical effects are good and have less side-effect than chemically synthesized, antihypertensive drug, captopril. Since the ACE inhibitor was discovered for the first time from snake venom (Ferreira, 1970), many antihypertensive ACE inhibitors have been isolated and characterized from various natural sources such as foods, enzymatic hydrolysates of proteins, alcohol beverages and its by-products, cereals and legumes, etc (Fujita, 2000; Ondetti, 1982; Morigiwa, 1986; Matsumura, 1993; Sugiyama, 1991; Sun, 1997; Maruyama, 1989; Miyoshi, 1991; Saito, 1994).

Many research groups have been screened for novel ACE inhibitors of microbial origin such as *Doratomyces putredinis*, *Nocardia orientalis* (Ando, 1987), *Virgaria nigra* (Ando, 1988), *Actinomycetes* (Kido, 1983), baker's yeast (Kohama, 1990) and *Basidiomycetes* (Lee, 2003). WF-10129, obtained from *Doratomyces putredinis*, is an ACE inhibitor resembling to the potent synthetic ACE inhibitor, enalapril, and is a substituted N-carboxymethyl dipeptide. WF-10129 inhibited ACE in a dose-dependent manner and its IC_{50} was 14 nM. Kohama *et al.* (1990) isolated 3 kinds of ACE inhibitory peptides from baker's yeast glyceraldehyde-3-phosphate dehydrogenase. Among them, YG-1(Gly-His-Lys-Ile-Ala-Thr-Phe-Gln-Glu-Arg, IC_{50} : 0.4 μM) was the most potent yeast ACE inhibitor. Morigiwa *et al.* (1986) 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*. Recently, Lee *et al.* (2003) isolated an ACE inhibitory peptide from *Tricholoma gigantum*.

Even though a number of microbial ACE inhibitors were reported (Kim, 2003), almost of them were intracellular ACE inhibitors. However, *M. pachydermatis* G-14 was the first strain in yeast as a producer of extracellular ACE inhibitor.

That optimum growth temperature of *M. pachydermatis* (37°C) was higher than that of general yeast (25~30°C) and ACE inhibitory activity was also shown high at 37°C of cultivation, suggestsing that ACE inhibitor from *M. pachydermatis* may be thermostable.

Many natural ACE inhibitors and synthetic ACE inhibitors such as captopril, enalapril and lisinopril were known as remarkably effective antihypertensive drug (Ondetti, 1977). Since, *M. pachydermatis* G-14 produces as extracellular ACE inhibitor, its use will be easy and economic for the inhibitor production. Furthermore, the ACE inhibitor has high resistance to some proteases attack without side effects. Therefore, ACE inhibitor from *M. pachydermatis* G-14 might be useful in the preparation of antihypertensive drug or foods. Further purification and characterization of the ACE inhibitor are underway.

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