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Isolation of Antimicrobial Substances from *Hericium erinaceum*

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Mycelium of *Hericium erinaceum* isolate KU-1 was cultured in liquid medium (HL medium) and solid medium (Ko medium) at pH 4.0 in 28°C. 1.0% glucose or fructose was the most favorable carbon source, and 0.2% amonium acetate or $NaNO_3$ was an exellent nitrogen source for mycelial growth as well as production of antimicrobial substances. The mixture of saw dust 70% with rice bran 30% (SR medium) was the substrate for formation of sporophores. The active substrates in extracts from mycelium, culture filtrate and fruiting body were separated by TLC. The solvent for TLC was EtOAc : Chloroform : MeOH (10 : 5 : 10). Phenol-like substances appeared at Rf 0.5~0.9, and fatty acid-like substances appeared at Rf 0.1~0.2. The purified materials from the extracts showed antimicrobial effects to *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Aspergillus niger*, *Candida albicans* and *Microsporum gypseum*. The *S. aureus* was the most inhibited. Minimal inhibitory concentration (MIC) of purified white powder and the Hercenone derivatives against S. aureus were 5.65 μg/ml and 1.85 μg/ml, respectively.

KEYWORDS: ***Hericium erinaceum*, Antimicrobial substances, Hercenones, Hercerins**

Hericium erinaceum is an widely distributed edible mushroom. It is a saprophytic inhabitant on dead trunks of hardwoods such as narra (*Pterrocarpus indicus*), oak (*Cyclobalanopsis* spp.), beech (*Fagus crenata*), and Japanese walnut (*Juglans sieboldianan*). It is one of the wood-destroying fungi that cause white rot. In China, this mushroom has been used as an herbal medicine called "Houtou". The mushroom extraction with hot water, is considered as a health drink. The researches on nerve growth factors (NGF) in mycelium and fruiting body of this mushroom have been carried out by a few researchers (Aronone *et al.*, 1994; Kawagishi *et al.*, 1990; Kimura *et al.*, 1991; Kuwahara *et al.*, 1992). It has been demonstrated that *H. erinaceum* contains the other biologically active materials, which are hercenones (Kawagishi *et al.*, 1990), erinacine (Kawagishi *et al.*, 1996), polypeptides (Aronone *et al.*, 1994; Mizuno, 1995), fatty acids (Kawagishi *et al.*, 1994), polysaccharides (Mizuno *et al.*, 1992), lectins (Mizuno, 1995), phenols (Walker and Link, 1935) and hericerins (Kimura *et al.*, 1991). These phenolics and fatty acids exhibiting cytotoxi- city against HeLa cells (Kuwahara *et al.*, 1992), have inhi- bitory effect on tea or pine pollen growth (Martin, 1972), and have an antimicrobial effect against bacteria and fungi (Anke, 1977; Kuwahara *et al.*, 1992).

The incidence of various types of microbial human diseases has been increasing world-widely. There are high demands for antimicrobial drugs to combat against various life-threatening microbial diseases. Since the mid-20 century intensive researches on chemotherapeutics and antibiotics have made a great contribution to human welfare (Anke, 1977; Frear *et al.*, 1949; Goldsworthy, 1949; Hecht, 1978). Currently, the azole class of synthetic antimicrobial agents and microbial-originated antibiotics such as amphothericin B, nystatin and griseofulvin are widely used (Kelly *et al.*, 1994). However, due to the toxic side-effects of these agents and the appearance of resistant strains of the pathogenic microorganisms, the developments of safer and more effective new antimicrobial agents are required (Fox, 1993; Hector, 1993). About 60 types of antimicrobial compounds have been isolated from mushrooms (Anke, 1977; Mizuno, 1995), but none has been put into practical use because of their weak activities.

The objects of the present researches are to ascertain pharmacological effects of the compounds isolated from mycelium, fruiting bodies, and culture filtrate of *H. erinaceum* grown on various culture media, as well as to find out the cultural conditions for high yield of antimicrobial substrates.

Materials and Methods

Material

The isolate of *Hericium erinaceum* KU-1 was obtained from the tissue culture of fruiting bodies collected at Mt. Odae in Korea (Ko *et al.*, 1997). A voucher specimen and the mycelial culture are deposited at the Graduate School of Biotechnology at Korea University.

Mycelial culture

For basal media, Ko medium was used for solid culture

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and Hericium liquid medium (HL medium) was used for submerged culture. The composition of Ko medium (Ko *et al.*, 1997) is glucose 18.02 g, arginine 2.613 g, ammonium acetate 2.613 g, $CaCl_2$ 0.33 g, KH_2PO_4 8.5 g, $MgSO_4 \cdot 7H_2O$ 2.0 g, $FeSO_4 \cdot 7H_2O$ 0.02 g, $ZnSO_4 \cdot 7H_2O$ 0.02 g, $MnSO_4 \cdot 7H_2O$ 0.02 g, agar 18.0 g and distilled water 1 *l* at pH 4.5~5.0. The composition of HL medium is glucose 50.0 g, yeast extract 10.0 g, peptone 10.0 g, $MnSO_4 \cdot H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.02 g, $ZnSO_4 \cdot 7H_2O$ 0.004 g, $NaNO_3$ 0.87 g, $CaCl_2$ 0.3 g, $MnCl_2 \cdot 4H_2O$ 0.007 g, $CuSO_4 \cdot 5H_2O$ 0.001 g and distilled water 1 *l* at pH 4.5~5.0. The mycelium was cultivated in 1 *l* Erlenmeyer flasks containing 400 m*l* of HL medium in a shaking incubator at 28°C, 150 rpm for 12 days. To test the effects of carbon sources and nitrogen sources on mycelial growth and production of antibiotic substances, 1.0% of one of the six kinds of carbon sources, which were glucose, mannitol, starch, glycerol, mannose, and fructose, and 0.2% of one of the six kinds of nitrogen sources, which were peptone, ammonium acetate, $NaNO_3$, KNO_2, $NH_4H_2PO_4$, and arginine were supplied to the basal media. The mycelial growths in both solid culture and liquid culture were determined by dry weight. For fruiting body production, the mycelium of the mushroom was cultured on autoclaved SR medium (Oak sawdust : rice bran mixture = 7 : 3 (w/w)) in polyethylene bottles (500 m*l* in capacity) (Chiu, 1981; Horsfall *et al.*, 1940; Liu, 1981). The mycelium was inoculated in the bottle and incubated at 25°C. In 20 days after inoculation, temperature was lowered to 15°C. When primordia appeared, the plug was removed and the humidity was adjusted to 85~90% in dim light to produce fruiting body.

Extraction of fruiting bodies, mycelium, and culture filtrate

The 300 g of fruiting bodies or 100 g of mycelium on Ko medium were crushed with 5~6 volumes of 80% MeOH, and extracted twice with the MeOH for 24 hr. The extract was dried with vacuum evaporator (Eyela System, Type SB650, Tokyo Rikakikai Co. Ltd) at 40°C. The dried materials were saved for further analysis. The 400 m*l* of culture filtrate were harvested by filtration of liquid culture of the mycelium in HL medium with Buechner funnel with filter paper under reduced pressure. The mycelium was washed twice with EtOAc. After filtration, the culture filtrate and the EtOAc were combined. The acidity of the culture filtrate was adjusted to pH 3.0 with 0.12 M phosphate buffer. The filtrate was extracted by equal volume of EtOAc. The EtOAc fraction was collected by separatory funnel and evaporated in vacuum at 40°C for dryness.

Chromatographic separation

The dried extracts were separated by silica gel column chromatography followed by thin-layer chromatography (TLC). For silica gel column chromatography, the dried extract was dissolved with 4 combinations of solvents. The ratios of four combinations of the solvents were EtOAc : chloroform : MeOH = (10 : 5 : 10), (10 : 5 : 5), (5 : 5 : 10), and (5 : 5 : 5) so that the polarity changes monotonically. The carrier of the silica gel column was Kieselgel 60, 70~230 mesh ASTM (Merk KGaA Inc., Germany). The samples were loaded on the silica gel column and the column was eluted with the solvent one another. 6 fractions with 10 m*l* each were collected from every solvent. A total of 24 fractions was obtained. Of these 24 fractions, the 6 fractions eluted with solvent; EtOAc : chloroform : MeOH = 10 : 5 : 10, were combined together to make 60 m*l*. 20 m*l* of solvent EtOAc : chloroform : MeOH = 5 : 5 : 5, were added to the 60 m*l* of elution sample to make up 80 m*l*. The 80 m*l* solvent was run on the same column chromatography and fractionated into 8 fractions of 10 m*l* each. Of the 8 fractions, antimicrobiotic substances were separated from the 5th and 6th fractions. The fractions were freeze dried. TLC was carried out by the ascending technique using spot film S-072 (Tokyo Kasei Kogyo Co., Ltd, Japan). The solvent system was EtOAc : chloroform : MeOH = 10 : 5 : 10.

Inhibition zone test

The every extracts from Ko medium and HL medium which were supplied with different sources of carbon and nitrogen were dissolved with 1 m*l* of 98% MeOH to perform inhibition zone test. The samples from gel chromatography (10~12 μg/m*l*) were serial diluted, 1/10, 1/50, 1/100 and 1/500, and antimicrobial activities of each dilute were determined by inhibition zone test (Heuberger, 1940; Horsfall *et al.*, 1940). The LB agar medium, pH 7.3, containing sodium chloride 10 g, tryptone 10 g, yeast extract 5 g, agar 16 g, and distilled water 1 *l* was prepared. 15 m*l* and 6 m*l* of the LB medium were placed in test tubes and sterilized by autoclave at 121°C for 20 min. The 15 m*l* medium was poured into a sterilized petri dish to make flat surface serving as base layer. Separately, the 6 m*l* of the medium was melted, cooled to 55~60°C, and inoculated with 50 μ*l* (2.5×10^6 cells/ml) of suspensions of 3 kinds of bacteria (*Escherichia coli* ATCC 8379, *Bacillus subtilis* NIHJ PIC 219P, and *Staphylococcus aureus* ATCC 6538) which had been cultured by shaking in LB liquid medium at 37°C for 16 hr. After being mixed well, this was poured on top of the base layer to be a test bacterial layer. Paper disks, with the diluted solution of each extract, were placed on top of these test bacterial layer by means of tweezers. After 24 hr incubation at 37°C in a thermostatic chamber, the diameter of the generated inhibition circle was measured to estimate antibacterial activity (Loo *et al.*, 1945; Thornberry, 1949).

Additionally, antifungal activities of the extracts against *Microsporum gypseum*, *Aspergillus niger* ATCC 10404 and *Candida albicans* ATCC 10231 were also measured in the similar method. The antimicrobial activities on TLC were assayed (TLC bioassay) (Gattani, 1954; Ko *et al.*, 1976). The TLC plates which were covered by thin layer of PD broth or Sabourouad Dextrose broth, were spray-inoculated with 50 μl of suspensions (2×10^7 CFU/m*l*) of fungal spore. After 3~5 days at 28°C, the locations with antimicrobial activity became visible as clean bands.

Minimum inhibitiory concentration

The materials of two fractions on TLC at Rf 0.1 to 0.2 and Rf 0.5 to 0.9 showing antimicrobial activity were dissolved with 98% MeOH and dried by freeze dry system to crystallize. The minimum inhibitory concentration of the two crystallized materials against to the bacteria and fungi was determined by spectrophotometer (DU-650UV/VIS Beckman).

Results

Effects of carbon and nitrogen sources on mycelial growth and antimicrobial activity of H. erinaceum

The nitrogen and carbon sources affected the mycelial growth and antimicrobial activities of *H. erinaceum* in both solid culture and liquid culture. The results indicated that 1.0% glucose or fructose as carbon source (Table 1) and 0.2% ammonium acetate or $NaNO_3$ as nitrogen source were best for the mycelial growth and antimicrobial activity to *S. aureus* ATCC 6538 (Table 2).

Bioassay and production of antimicrobial substances

From TLC bioassay with the 3 bacteria (*E. coli* ATCC 8379, *B. subtilis* NIHJ PIC 219P, and *S. aureus* ATCC 6538) and 3 fungi (*M. gypseum*, *A. niger* ATCC 10404 and *C. albicans* ATCC 10231), the extract of mycelia on Ko medium showed one large antimicrobial region at Rf 0.89 against above microorganism and the extract of culture filtrate of HL medium showed 3 inhibition zones at Rf 0.85, 0.76, and 0.63 against *S. aureus* ATCC 6538, *E. coli* ATCC 8379, *B. subtilis* NIHJ PIC 219P, *A. niger* ATCC 10404 and *M. gypseum*. The extracts of fruiting bodies showed two small inhibition spots at Rf 0.56 and 0.91 against *S. aureus* ATCC 6538, *E. coli* ATCC 8379, *B. subtilis* NIHJ PIC 219P and *M. gypseum*. The phenol-like substances of all extracts were at Rf 0.7 with 2.6 cm inhibition zone and the fatty acid-like substances were at Rf 0.15 with 1.5 cm inhibition zone against the *E. coli* ATCC 8379, *B. subtilis* NIHJ PIC 219P, and *S. aureus* ATCC 6538. Antifungal activity of all extracts were measured in similar manners against the *M. gypseum*, *A. niger* ATCC 10404 and *C. albicans* ATCC 10231. The diameter of inhibition zone was 0.8 cm against *M. gypseum* and 1.2 cm against *A. niger* ATCC 10404 and *C. albicans* ATCC 10231 at Rf 0.7 of the phenol-like substances (Fig. 1). The 1/2 diluted samples of column chromatography showed antibacterial activity to *S. aureus* ATCC 6538 with diameter of 4.3 cm inhibition zone, while the inhibition zone to *B. subtilis* NIHJ PIC 219P and *E. coli* ATCC 8379 were 3.2 cm and 2.8 cm, respectively. However, the antifungal activities to *M. gypseum*, *A. niger* ATCC 10404 and *C. albicans* ATCC 10231 were lower than that to *E. coli* ATCC 8379, *B. subtilis* NIHJ PIC 219P, and *S.*

Table 1. Effects of carbon sources on mycelial growth and antimicrobial activity

	Media	Carbon sources (1.0%)					
		glucose	manitol	starch	glycerol	mannose	fructose
Mycelial growth	Ko[a]	18.5	12.0	11.7	10.3	12.5	20.6
(mg)	HL	35.3	26.9	25.7	23.7	28.1	38.8
Antimicrobial activity	Ko	2.8	1.8	1.7	1.5	1.9	3.3
(cm)[b]	HL	3.8	2.9	2.5	1.9	2.4	4.7

[a]Ko medium (14 days), HL: HL medium (21 days).

Table 2. Effects of nitrogen sources on mycelial growth and antimicrobial activity.

	Media	Nitrogen sources (0.2%)					
		peptone	ammonium	$NaNO_3$	KNO_3	$NH_4H_2PO_4$	arginine
Mycelial growth	Ko[a]	9.8	15.3	11.9	10.4	10.0	11.1
(mg)	HL	19.7	28.4	37.3	26.7	19.8	22.8
Antimicro bial activity	Ko	1.7	4.2	3.8	2.4	1.6	2.5
(cm)[b]	HL	1.5	3.5	4.5	2.1	1.8	1.5

[a]Ko medium (14 days), HL: HL medium (21 days).

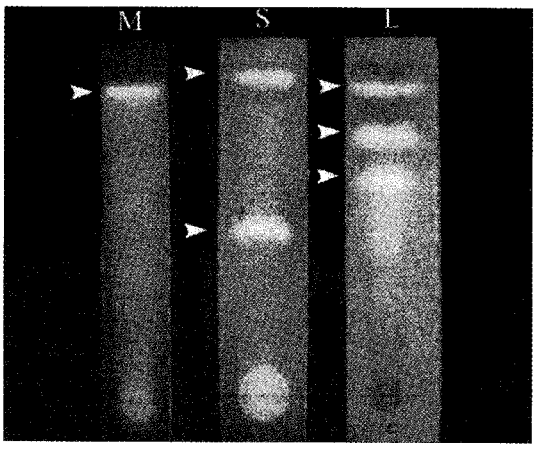

Fig. 1. The antimicrobial active bands of extract from mycelium of Ko medium (M: Rf 0.89), fruiting bodies of SR medium (S: Rf 0.56 and 0.91), and culture filtrate of HL medium (L: Rf 0.63, 0.76 and 0.85).

aureus ATCC 6538. The inhibition zones were 1.33 cm against *M. gypseum*, 1.43 cm against *A. niger* ATCC 10404 and 0.9 against *C. albicans* ATCC 10231. No zone of antimicrobial activity was detected in control with distilled water adjusted pH 3.0 (Table 3).

Productivity and physical properties of antimicrobial substance

The antimicrobial substance in culture filtrate was purified by silica gel column chromatography followed by freeze dried. It was white powder. The yield of the substrate was 15 mg per 1 *l* of culture filtrate. A further purification step made by preparative TLC yielded 8 different substance bands. All of these showed absorption maxima 216 and 260 nm and similar physiochemical properties (Table 4), thus this substance seems to be derivatives of same material, perhaps with a slight structural variation, for an example with different side chains. The most abundant compound at Rf 0.7 might be Hercenone derivatives that showed the most powerful antimicrobial activity (Fig. 2). Minimal inhibitory concentrations (MIC) of the white powder and the Hercenone derivatives against *S. aureus* ATCC 6538 were 5.65 μg/ml and 1.85 μg/ml, respestively.

Table 4. Physical properties of antimicrobial substance from *H. erinaceum*

▶ Crystal appearance	▷ White powder
▶ TLC (Silicagel 60F254)	▷ Rf 0.1~0.2, Rf 0.5~0.9
▶ Solubility: Soluble	▷ MeOH, EtOAc, Hexane, BuOH
Insoluble	▷ H_2O, Acetone
▶ Color reaction: Positive	▷ Iodine, Ninhydrin
Negative	▷ Dragendroff's regent

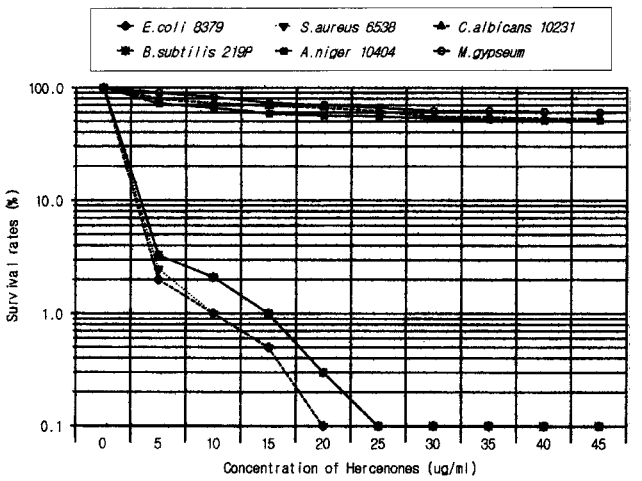

Fig. 2. Survial curve of bacterial and fungal strains treated with Hercenones (Rf 0.5~0.9) of culture filtrate.

Discussion

Secondary metabolites extracted from mushrooms are source of special chemicals for food and pharmaceutical industries. The market for mushroom-derives are estimated to be $9 billion per year in the United States alone. The large-scale production of such chemicals from mushrooms in field has been limited due to the dependency of climate (Ahn, 1992; Monaghan *et al.*, 1990; Omura, 1992). Thus numerous researchers have tried to improve mushroom cul-

Table 3. Antimicrobial activity in diluted of freeze dried samples of column chromatography

	Strains	Diameter of inhibition zone (cm)					
		1/1	1/10	1/50	1/100	1/500	Control[a]
Bacteria	*S. aureus* ATCC 6538	4.3	2.4	1.9	0.95	0.3	0.0
	B. subtilis NIHJ PIC 219P	3.2	2.1	1.5	0.52	0.2	0.0
	E. coli ATCC 8379	2.8	1.5	1.2	0.53	0.1	0.0
Fungi	*A. niger* ATCC 10404	1.43	0.58	0.2	0.08	0.0	0.0
	M. gypseum	1.33	0.4	0.1	0.0	0.0	0.0
	C. albicans ATCC 10231	0.9	0.32	0.08	0.0	0.0	0.0

[a]Distilled water.

tivation technique. As results of these efforts, the large-scale production of few substances such as polyacetylene derphenolquion, sesquiterpene, and triterpene have succeeded (Anke, 1977; Kuwahara *et al.*, 1992; Mizuno, 1995).

Recently, the development of the cultural media for *H. erinaceum* have been studied intensely because the importance of the secondary metabolites of the mushroom has been recognized (Ko *et al.*, 1996).

The hercerins, fatty acids constitute, one distinct group of secondary metabolites of the *H. erinaceum* has been interested by a few researches. Of these novel compounds phenol-like substances and fatty acid-like substances have stimulating effect on the synthesis of nerve growth factor (NGF), cytotoxicity against cancer cells, and antimicrobial activities against bacteria and fungi (Anke, 1977; Kawagishi *et al.*, 1991; Kimura *et al.*, 1991; Kuwahara *et al.*, 1992). To find out the effects of composition of cultural medium on production of antimicrobial substances, the mushroom was grown on different sources of carbon and nitrogen. The mushroom grew best on the medium containing glucose or fructose in combination with ammonium acetate or $NaNO_3$. The maximum growth rate was observed in 7.0% of the carbon and 1.4% the nitrogen sources of culture medium (Ko *et al.*, 1997). But the highest antimicrobial compounds was detected in medium with 1.0% carbon and 0.2% nitrogen Through the results of these tests, we found that the initial contents of carbon and nitrogen sources in culture medium greatly influenced the growth rate and production the antimicrobial substances. Chiu (1981) and some researchers (Garraway and Evans, 1984; Liu, 1981; Park *et al.*, 1992) reported the similar results.

It has been reported that some natural antimicrobial compounds and pharmacological active materials from mushrooms significantly inhibit the growth of *E. coli*, *B. subtilis*, *S. aureus*, *M. gypseum*, *A. niger* and *C. albicans* (Anke, 1977; Goldsworthy, 1949; Heuberger, 1940). The present results on TLC revealed that of extract of *H. erinaceum* contained (i) various acidic phenol-like compounds (Rf 0.5~0.9, named Hercenones), which are effective against pathogenic microorganisms, and (ii) neutral fatty acid-like compounds (Rf 0.1~0.2, named Y-A-2 and Hercerins), which showed antibacterial activity at low concentrations against *S. aureus*, *B. subtilis*, and *E. coli*. The yield of Hercenones was approximately 1.9% of fruiting body by weight. Until now, there have been few available antimicrobial compounds from mushrooms that were effective against both pathogenic and food spoilage bacteria and fungi. In contrast to the other antimicrobial substances, the extract was stable in chromatographic mobile phase with different polarities comprising. We found that various acidic antimicrobial compounds in the culture filtrate, which significantly inhibit the growth of pathogenic bacteria and fungi at low concentrations. These results were similar to those of Ram *et al.* (1973) who also showed that secondary products generally were more effective against bacterial and fungal growth in liquid suspension than in mycelium and fruiting bodies. These data suggest that the antimicrobial compounds could be mass producted in tank culture and those might be useful for natural food preservatives and pharmaceutic chemicals. Futhermore the extract was active against *A. niger*, *C. albicans*, and *M. gypseum* and amount to inhibit the organisms was 5.0, 6.0, and 10.0 μg/ml, respectively.

Since extracts have high acidic phenol-like compounds and neutral fatty acid-like compounds, it is possible that two groups of compounds might be essential antimicrobial agents for food industry.

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