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An Antioxidant Hispidin from the Mycelial Cultures of *Phellinus linteus*

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In the course of screening for reactive oxygen species scavengers from natural products, an antioxidant was isolated from the mycelial culture broth of *Phellinus linteus* and identified as hispidin. The hispidin content was reached its maximum level at 12 days after onset of inoculation. About 2.5 mg/mL of hispidin was produced by *P. linteus* in a yeast-malt medium (pH 5.8, 25°C). Hispidin inhibited 22.6 and 56.8% of the super oxide anion radical, 79.4 and 95.3% of the hydroxyl radical, and 28.1 and 85.5% of the DPPH radical at 0.1 and 1.0 mM, respectively. The positive control α-tocopherol scavenged 25.6 and 60.3%, 74.6 and 96.3%, and 32.7 and 77.5% of each radical, respectively, at the same concentrations. However, hispidin showed no significant activity on the hydrogen peroxide radical.

Key words: *Phellinus linteus*, Hymenochaetaceae, Mycelial culture, Phenylpropanoid, Hispidin, Antioxidative effect

INTRODUCTION

Free radicals can be defined as species with an unpaired electron. The reactivity of free radicals varies from relatively low, as in the case of the oxygen molecule itself, to very high, as in the case of the short-lived and highly reactive hydroxyl radical (·OH) (Wettasinghe and Shahidi, 2000; Packer, 1994). Oxygen and reactive oxygen species (ROS) are among the major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro*. The triplet state oxygen can react with other molecules to yield ROS such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot -}$), and hydroxyl radicals (·OH) (Borg *et al.*, 1993).

ROS have been implicated in the development and progression of cancer (Slaga *et al.*, 1978) as well as inflammation and aging (Rohrdanz and Kahl, 1998). On the other hand, recent evidence in the field of Alzheimer's disease (AD) research has highlighted the importance of the oxidative process in its pathogenesis. Based on laboratory and clinical studies, it appears that ROS and reactive nitrogen species (RNS) that are generated extracellulary and intracellulary by various mechanisms are among the major intermediary risk factors that initiate and promote neurodegeneration in idiopathic AD (Prasad *et al.*, 2000). Thus, antioxidant supplements could be useful in the prevention of AD, and as an adjunct to standard therapy in the treatment of AD.

In the course of screening natural products for ROS scavengers, a broth of mycelial cultures of *Phellinus linteus* was found to have high antioxidative activity. We here report on the production, isolation, and structure elucidation of an active compound, hispidin, and on its inhibitory activity on several ROS's.

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MATERIALS AND METHODS

General

Optical density was measured by a Bio-TEK ELx 808 (VT, USA). ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance Digital 400 spectrometer (Karlsruhe,

Germany) at 400 and 100 MHz, respectively. Chemical shifts were given in δ (ppm) from TMS. EIMS was recorded on a Micromass VK QUATTRO II (Hertsfordshire, UK). TLC was performed on a precoated silica gel plate (Kieselgel 60F254, Merck, NJ, USA). Silica gel column chromatography was carried out using a Kiegelgel 60 (Merck, NJ, USA). Authentic hispidin and other chemicals were purchased from Sigma (MO, USA).

Reactive oxygen species scavenging activity

Hydrogen peroxide scavenging activity was measured according to Müller's method (Müller, 1985). Hydroxyl and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity were analyzed by the methods of Chung (Chung *et al.*, 1997) and Blois (Blois, 1958), respectively. The xanthine-xanthine oxidase system was used for the evaluation of superoxide anion scavenging activity (Iio *et al.*, 1985).

Microorganism, fermentation, extraction, and isolation

A mycelium of *P. linteus* Teng (Strain No. KCTC 6190, originally from ATCC 26719, USA) belonging to Hymenochaetaceae was obtained from KCTC (Korean Collection for Type Culture), Daejon, Korea. The stock culture was deposited and maintained at KCTC and the Innovative Research Laboratory of Natural Product Medicine, Kyungpook National University, Daegu, Korea. The strain was inoculated into 100 ml of a yeast-malt (YM) or potato-dextrose (PD) medium (pH 5.8, 25°C, and 100 rpm). Five ml of the above seed culture was transferred to 100 mL of the same medium in a 500 mL Erlenmeyer flask and cultured for 12 days. For plotting growth curves, cells were harvested and measured every two days after the onset of inoculation. Mycelia were filtered through filter paper and a suction flask; then the mass of harvested cells were promptly measured (fresh weight) or weighed after they were dried for 48 h at 110°C in a dry oven (dry weight). One ml of each culture broth was filtered through a 0.45 μm membrane filter and the 10 μL of filtrate was introduced to be analyzed for its antioxidative activity on hydroxyl radical. The culture broth of *P. linteus* (5 L) was partitioned with 12 L of EtOAc. The EtOAc-soluble fraction (2.3 g) was chromatographed on a silica gel column [5.5 ×60 cm, benzene/EtOAc/HOAc (each 400 mL of 25:1:1, 20:1:1, 10:1:1, 5:1:1, and 2:1:1)] to give fr. 1 to fr. 3. HPLC [μBondapak C18, Waters, MA, USA, 7.8×300 mm, 1% HOAc in 25% MeOH, UV254 nm, 1.8 mL min^{-1}] of the active fraction (fr 2, 522.0 mg) afforded 17.5 mg of compound **1**. ^{1}H and ^{13}C-NMR: see Table I.

Quantitative analysis of hispidin

The HPLC conditions were as follows: column; Nova-Pak C18 (Waters, 3.5×150 mm), mobile phase; 1% acetic acid in 30% MeOH, flow rate; 0.8 mL min^{-1}, detection; UV 370 nm. Standard curve: $y = 4704665x + 741788$ (r^2= 0.9972), where y equals the peak area and x is the amount of hispidin in μg. Every two days five ml of culture broth was filtered through a membrane filter (0.45 μm) and 10 μL of each filtrate was injected for HPLC analysis.

Table I. NMR data of hispidin (δ in ppm)

No.	Hispidin (**1**)	
	^{1}H (int., multi., *J*)[a]	^{13}C (multi.)[a]
2		171.0 (*s*)
3	5.28 (1H, *s*)	89.4 (*d*)
4		163.6 (*s*)
5	6.06 (1H, *s*)	101.5 (*d*)
6		159.9 (*s*)
7	6.55 (1H, *d*, 15.8)	116.1 (*d*)
8	7.16 (1H, *d*, 15.8)	134.7 (*d*)
9		127.2 (*s*)
10	7.01 (1H, *s*)	114.4 (*d*)
11		147.6 (*s*)
12		145.9 (*s*)
13	6.77 (1H, *d*, 7.8)	115.9 (*d*)
14	6.88(1H, *d*, 7.8)	120.3 (*d*)

[a] Integral, multiplicity, and coupling constants in Hz. NMR spectra were measured in methanol-d_4. Assignments were aided by DEPT, ^{13}C-^{1}H COSY, and COLOC.

RESULTS AND DISCUSSION

The mycelium of *P. multiplex* was cultured in a yeast-malt (YM) and potato-dextrose (PD) liquid medium, separately. Time course fermentation showed that YM was better than PD for cell growth (Fig. 1). Hydroxy

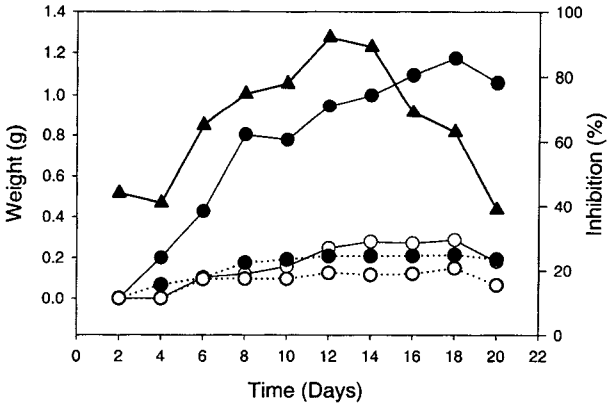

Fig. 1. Growth curves and time course radical scavenging activity by *Phellinus linteus*. Solid lines; fresh weight, broken lines; dry weight. The cultures were fermented either in YM (-●-) or PD (-○-) culture media. The closed triangle (-▲-) represents the relative scavenging activity of *P. linteus* grown in YM medium on hydroxyl radical.

radical scavenging activity reached its maximum level 12~13 days after the onset of inoculation in the YM medium (Fig. 1). The cultures were filtered through filter paper and the filtrate was partitioned with EtOAc. The EtOAc soluble fraction was chromatographed on a silica gel and a RP-HPLC column to afford **1**.

Compound **1** was obtained as a brown powder that was positive to the $FeCl_3$ reagent, suggesting that it had phenolic OH group(s) in its structure. Its molecular formula was determined to be $C_{13}H_{10}O_5$ on the basis of EI-MS and ^{13}C-NMR. Three major fragment ions at *m/z* 202 [$M^+ - CO_2$], 176 [$M^+ - C_3H_2O_2$], and 148 [$M^+ - (C_4H_3O_3 + H)$] were detected by EIMS (Fig. 2). In ^{1}H-NMR, two somewhat blunt singlets appeared at δ 5.28 (1H) and 6.60 (1H), suggesting a long range coupling of ^4J in a lactone ring system. The signals at δ 6.55 (1H, *d*, *J* = 15.8 Hz) and 7.16 (1H, *d*, *J* = 15.8 Hz) showed typical resonances of *trans*-olefinic protons. Three aromatic protons, which could be assigned to the protons of 1,3,4-tri-substituted benzene, were observed at δ 7.01 (1H, *s*), 6.77 (1H, *d*, *J* = 7.8 Hz), and 6.88 (1H, *d*, *J* = 7.8 Hz). In the ^{13}C-NMR and DEPT spectra, seven signals corresponding to the sp^2 methine carbons (δ 89.4, 101.5, 116.1, 134.7, 114.4, 115.9, and 120.3) and six quaternary carbons including a lactone carbonyl carbon (δ 163.6, 159.9, 127.2, 147.6, and 171.0) were observed. The NMR data are almost identical with those of 6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone (hispidin), which had been isolated from *P. pomaceus* (Klaar and Wolfgang, 1997) and studied as an anticancer agent due to its property as a selective PKC-βs inhibitor (Gonindard, 1997) and as an antiviral property (Awadh *et al.*, 2003). The final structure of **1** was re-confirmed by chemical synthesis starting from piperonal, according to the reported method (Adam *et al.*, 1994). The NMR data are presented in Table I and the structure is shown in Fig. 2.

Hispidin showed similar activity to positive controls, BHA (butyl hydroxyanisol) and α-tocopherol in the DPPH, superoxide anion, and the hydroxyl radical scavenging assays: however, its scavenging activity on the hydrogen peroxide radical was much less than that of the positive controls (Table II). The chemical structure of hispidin is very similar to those of resveratrol derivatives, strong antioxidants found in a number of plant species including grapes (Martin *et al.*, 2004). The strong radical scavenging activities of resveratol-like stilbenes from grapes are related to the dihydroxy group in A ring (Donald *et al.*, 2003). The antioxidative activity of hispidin also might be originated from the catechol moiety.

Unsaturated fatty acids are susceptible to attack by highly reactive oxygen species such as hydroxyl radical; hence, any reaction or process which forms ROS would stimulate lipid oxidation. In addition, the hydroxyl radical damages to deoxyribonucleic acid (DNA) (Packer and Glazer, 1990). In the process of trying to digest the material within plaques and tangles in AD patients, microglia release pro-inflammatory proteins and free radicals, which cause secondary damage in brain (Qin *et al.*, 2002). Accordingly, the scavengers of ROS are important not only in the food industry, but also as drug candidates such

Table II. Reactive oxygen species scavenging activity[a] of hispidin

Compounds (mM)		Inhibition (%)			
		DPPH[b]	H_2O_2	HR[c]	SOA[d]
Hispidin	0.01	25.5	8.0	71.4	8.1
	0.1	28.1	4.0	79.4	22.6
	1.0	85.5	20.4	95.3	56.8
BHA[e]	0.01	27.4	9.2	73.0	14.5
	0.1	30.7	9.0	79.4	22.0
	1.0	90.6	95.7	93.6	63.3
V-E[f]	0.01	26.5	6.9	75.6	22.1
	0.1	32.7	2.7	74.6	25.6
	1.0	77.5	63.2	96.3	60.3

[a]Presented as a mean inhibition±SE (%) of duplicated experiments.
[b]α,α-Diphenyl-β-picrylhydrazyl
[c]Hydroxyl radical
[d]Superoxide anion
[e]Butyl hydroxyanisol
[f]α-Tocopherol

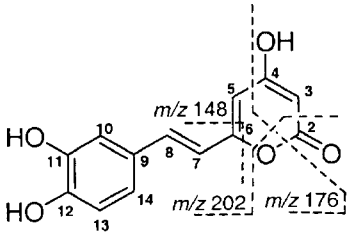

Fig. 2. Structure of hispidin. Broken lines indicate the fragment ions in EIMS

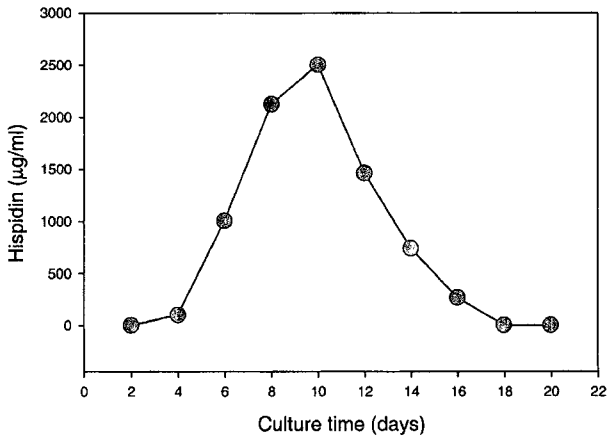

Fig. 3. Time course production of hispidin by *P. linteus*.

as anti-dementia, anti-carcinogenic and anti-aging agents.

Hispidin production was the highest after 10 days of incubation, while the inhibitory activity on hydroxyl radical was maximal at the 12th day of inoculation, suggesting the presence of ROS scavenger(s) besides hispidin (Fig. 3). The genus *Phellinus* is well known for its ability to produce laccase (Geiger *et al.*, 1986). Presumably, hispidin might be polymerized by the enzymatic oxidative coupling reaction after 12th day. In fact, a hispidin dimer has been isolated from the same family, Hymenochaetaceae (Fiasson, 1982). The hispidin content of the culture broth at the 10th day was *ca* 2.5 mg/mL, suggesting that *P. linteus* is an excellent source of the antioxidant, hispidin.

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