Antioxidant Activity of Ergosterol Peroxide (5,8-Epidioxy-5α,8α-ergosta-6,22E-dien-3β-ol) in *Armillariella mellea*

Sang-Wook Kim, Sang-Shin Park,† Tae-Jin Min, and Kook-Hyun Yu*

Department of Chemistry, Dongguk University, Seoul 100-715, Korea
†Department of Biochemistry, Dongguk University, Kyongju 780-714, Korea
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Antioxidant activities of various mushroom fruiting bodies were investigated *in vitro*. Among the mushroom extracts examined, *Armillariella mellea*, *Daedalea dickinsi*, *Fomitella fraxinea* and *Pleurotus cornusopiae* markedly exhibited inhibition on lipid peroxidaton of rat liver microsomes. Ergosterol peroxide (5,8-epidioxy-5α,8α-ergosta-6,22E-dien-3β-ol), antioxidant from *A. mellea*, was isolated by solvent extraction, silica gel column chromatography and recrystallization. The structure of the compound was determined by NMR, GC/MS and X-ray crystallography. Ergosterol peroxide showed potent inhibition on lipid peroxidation and exhibited higher antioxidant activity than well-known antioxidants, α-tocopherol and thiourea.

Introduction

It has been shown that singlet oxygen and oxygen radicals play an important role in numerous biological processes, such as enzymatic reactions occurring in the mitochondrial respiratory chain,[1,2] detoxifying reaction of the cytochrome P450 system[3] and prostaglandin synthesis.[4] Lipid peroxidation induced by free radicals may cause genetic damage due to cross-linking reactions of a peroxidation product, malondialdehyde (MDA), which can be used as validation criterion of lipid peroxidation with the amino group of DNA. This could lead not only to nuclear genetic and cancer injury but also to mutation or loss of the mitochondrial genome and aging of fixed post-mitotic cell. The main reason of the lipid peroxidation of membranes is their high contents of polyunsaturated fatty acids. The peroxidation of polyunsaturated fatty acids is easily initiated by free radicals.[5]

Antioxidants are effective in stopping oxidative reactions. Previous research discovered that fat-containing food was naturally protected by natural antioxidants, such as tocopherol in the lipid phase of the cell. Vitamin C, glutathione, and uric acid were classified as three main hydrosoluble antioxidants. The discovery of superoxide dismutase (SOD) enzyme spurred the identification of antioxidant mechanism.[6,7] This enzyme prevents the toxic action of the superoxide radicals by quenching them. Glutathione peroxidase shows another example of antioxidant mechanism. It removes H_2O_2 in the presence of selenium.[8,9] Since the diet contains many mutagens and carcinogens which generate many free radicals, the intake of adequate amount of antioxidants is essential to lengthen one's life and reduce various diseases.[10]

Therefore, the screening of antioxidant from edible mushrooms can be very advantageous in many aspects. The development of the bioactive compounds from mushrooms has been routinely studied. The study of screening on antioxidants from mushrooms is relatively unexhausted than those of other bioactive compounds. Until now it has been reported that some glycoproteins from *Coriolus versicolor* showed a similar antioxidant effect to SOD as a ion-radical scavenger[11] and that water-soluble extract from *Hypsizigus marmoreus* showed trap effect against peroxyl and alkoxyl radicals and antioxidants effect on lipid peroxidation.[12] We have recently reported that *Armillariella mellea*, *Daedalea dickinsi*, and *Fomitella fraxinea* show not only remarkable inhibition on lipid peroxidation of rat liver microsome and hepatic aldehyde oxidase but also exhibited slight stimulation on SOD activity.[13] The main steroid was determined to be a C_{28} sterol, ergosterol peroxide. It is a known natural product which has been obtained from a variety of fungi, lichens, sponges and marine organism.[14,15,16]

In this study we have performed isolation and identification of ergosterol peroxde from *A. mellea* and would like to report antioxidant activity of the compound on lipid peroxidation.

Experimental

Materials. Fruiting bodies of mushroom used were collected from Mt. Sokni and Mt. Kwanak. Thiobarbituric acid (TBA), α-tocopherol and L-ascorbic acid were purchased from Aldrich co. Butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), and dimethyl sulfoxide (DMSO) were purchased from Sigma co. Silica gel (70-230 mesh) and thin layer chromatography (TLC) plate (silicagel 60 F_{254}) were from Merck co. All the other reagents used were of analytical grade. All solvents were freshly distilled prior to use.

Extraction and Isolation of antioxidants. Mushroom extracts were prepared by the method described in our previous work.[13] In brief, dried mushrooms (3 kg) were extracted with 80% ethanol (2 L) for 2 or 3 weeks at room temperature and the filtrates of the extracts were evaporated. Among the 20 kinds of mushrooms tested, the extract (240 g) of *A. mellea* with antioxidant activity was successively extracted with 100% ethanol, chloroform and water. Chloroform fraction

was applied to silica gel column (3.5 × 60 cm) chromatography using hexane/ethyl acetate (2 : 1) as eluent. The fraction with antioxidant activity was repeatedly applied to silica gel column (3.5 × 60 cm) using ether/petroleum ether/triethyl amine (5/1/1). The antioxidative fraction (R_f = 0.55, ether/petroleum ether/triethyl amine, 5/1/1) was purified by recrystallization with acetonitrile. The mushroom extracts and the purified compound from *A. mellea* was prepared by dissolving in DMSO for antioxidant assay.

Structural analysis. Ultraviolet visible spectra were recorded on a HP-8452 spectrophotometer. Infrared spectra was recorded on a Bomem series 100 spectrophotometer using KBr-pellet method. Mass spectra (GC-MS) were obtained from Hewlett-Packard 5890 series-II equipped with mass dector. ^{1}H NMR spectra were recorded on a Varian 500 MHz. ^{13}C NMR spectra, DEPT, HETEROCOSY and HOMOCOSY were recored on a Varian 125 MHz in the $CDCl_3$. All chemical shifts were reported in ppm downfield from TMS. The crystallographic studies were measured on a MAC sciences MXC3 fourcircle diffractometer.

Preparation of rat liver microsome. Rats (200-250 g) were sacrificed and their liver was removed and homogenized. Microsomes of liver cell were prepared by differential centrifugation.[17] The tissue of rat liver was homogenized in pre-cooled 150 mM KCl/50 mM Tris-HCl buffer (pH 7.4) using glass homogenizer with electric motor. The homogenate was centrifuged at 10,000 × g for 20 min. The supernatant was further centrifuged at 100,000 × g for 60 min. The pellet, microsomal fraction, was suspended in 50 mM Tris-HCl buffer (pH 7.4) and used for antioxidant assay at concentration of 1 mg/mL microsomal protein. All operation was carried out below 4 °C. Protein concentration was determined according to Lowry methods[18] using bovine serum albumin as a standard.

Antioxidant assay. To measure the antioxidant activity of various mushroom extracts, lipid peroxidation of rat liver microsomes was carried out *in vitro* according to the Fe^{2+}/ascorbate method.[19] The reaction mixture containing 0.5 mL of rat liver microsome (1 mg/mL), 0.5 mL of 10 μM $FeSO_4$, 0.5 mL of 2 mM ascorbate, 0.1 mL of the sample in DMSO, and 0.4 mL of 150 mM KCl/50 mM Tris-HCl buffer solution (pH 7.4) was incubated at 37 °C for 60 min in a shaking water bath and the reaction was stopped by addition of 0.75 mL of 2.0 M TCA/1.7 M HCl. After centrifugation (4,000 rpm, 10 min), 0.5 mL of the supernatant was mixed with 1.5 mL of TBA and the mixture was heated at 95 °C for 10 min. After cooling, the quantity of MDA ($\varepsilon = 1.52 \times 10^5$ M^{-1} cm^{-1}) was determined by measuring the absorbance at 533 nm. Relative activity of the extracts and the purified compound was calculated as percent (%) in comparision with the activity (100%) of control group without the mushroom extracts.

Results and Discussion

Inhibition effect of mushroom extracts on lipid peroxidation. The effect of various mushroom extracts on lipid peroxidation of rat liver microsomes was shown in Table 1. Among the 20 species of mushroom extracts, *A. mellea*, *D. dickinsi*, *F. fraxinea* and *P. cornusopiae* exhibited remarkable inhibition of lipid peroxidaton by 22.8%, 22.5%, 38.3%, and 19.5%, respectively, at concetration of 100 μg/mL compared with control group without mushroom extracts. The other mushroom extracts examined did not show significant inhibition effect. From these results, we decided to isolate and identify the antioxidant in *A. mellea*,

Table 1. Antioxidant activity of various mushroom extracts on lipid peroxidation of rat liver microsomes *in vitro*[13]

Mushrooms	Extract concentration (μg/mL)	MDA (μ)	Relative production (%)
Control (none)		13.6	100
Agricus arvensis	10	13.3	98.2
	100	13.5	99.3
Armillariella mellea	10	11.6	85.5
	100	10.5	77.2
Austroboletus fusisporus	10	13.4	98.5
	100	13.1	96.1
Boletus edulis	10	13.5	99.4
	100	13.3	98.3
Boletus griseus	10	13.2	97.2
	100	13.1	96.6
Boletus pseudocalopus	10	13.3	98.4
	100	13.1	96.9
Collybia butyracea	10	13.3	97.8
	100	13.5	99.6
Daedalea dickinsi	10	12.8	94.3
	100	10.5	77.5
Fomitella fraxinea	10	11.2	82.3
	100	8.4	61.7
Lactarius camphoratus	10	13.4	98.8
	100	13.1	96.4
Pleurotus cornusopiae	10	11.5	85.2
	100	10.9	80.5
Rhodophyllus crassipes	10	12.3	90.2
	100	11.5	84.5
Russula adusta	10	13.4	98.6
	100	13.1	96.2
Russula compacta	10	13.4	98.6
	100	13.2	97.1
Russula crustosa	10	13.1	96.5
	100	13.5	99.2
Russula delica	10	13.2	97.6
	100	13.4	98.6
Russula flavida	10	13.4	98.4
	100	13.2	96.9
Suillus bovinus	10	13.5	98.9
	100	13.2	97.1
Tyromyces sambuceus	10	13.5	99.3
	100	13.3	98.2
Xerocomus astaeicola	10	13.5	98.9
	100	13.3	98.1

Table 2. Color development test for the purified compound from *A. mellea* on TLC plate

Reagent	Response	Remark
Ninhydrin	Negative	For the detection of amino acid, amines
Dragendroff	Negative	For nitrogen containing compounds
Sulfuric acid	Positive	General detection
I_2 vapor	Positive	General detection
Anisaldehyde-sulfuric acid	Positive	For sugar, steroids, terpenes
Ferric chloride	Positive	For phenols and hydroxamic acid
Antimony chloride(III)-acetic acid	Positive	For steroids and diterpenes
Picric acid-perchloric acid	Positive	For hydroxy steroids
Phosphotungstic acid	Positive	For reducing compds., lipids and steroids
α-Naphthol-sulfuric acid	Positive	For sugars

which is not only edible but also easily secured large quantity. The extracts from *A. mellea* was successively extracted with chloroform, 100% ethanol and water. From the chloroform fraction, the compound with antioxidant activity was isolated by repeated silica gel column chromatography and recrystallization with acetonitrile.

The structure of antioxidant in *A. mellea*. The purified compound of 400 mg was obtained from *A. mellea* as a crystalline compound. Melting point of the purified compound was 181.5-183.8 °C (Previously published melting point of ergosterol peroxide is 181.5-183 °C [20]). In order to classify the compound, various kinds of color development test on TLC plate was investigated using Stahl's methods[21] and indicated that the purified compound was a steroid (Table 2). From the analysis of spectroscopic data, the compound was found to be ergosterol peroxide (5,8-epidioxy-5α,8α-ergosta-6,22E-dien-3β-ol) with molecular formular of $C_{28}H_{44}O_3$. In its EI/MS spectrum m/z 428, 410, 396 correspond to $[M]^+$, $[M-H_2O]^+$ and $[M-O_2]^+$, respectively. A strong absorption band at 3412 in the IR spectrum indicated the presence of hydroxyl group. The ^{13}C NMR spectrum showed carbon signals of two double bonds at δ 130.68 (C-7), δ 132.26 (C-23), δ 135.16 (C-22), δ 135.39 (C-6). Down field signals at δ 6.501, δ 6.241(AB quartet, J = 8, 2H, H-6, H-7) in the ^1H NMR spectrum revealed the presence of disubstitued double bond which were correlated with carbon signals of δ 135.39 (C-6), δ 130.68 (C-7) in HMBC spectrum. Other characteristic signals in ^1H NMR spectrum were δ 5.183 (m, 2H, H-22, H-23), δ 3.961 (m, 1H, -OH), δ 0.998 (d, J = 6, 3H, H-21), δ 0.908 (d, J = 7, 3H, H-28), δ 0.881 (s, 3H, H-19), δ 0.831 (d, J = 8, 3H, H-27), δ 0.818 (d, J = 8.5, 3H, H-26), and δ 0.815 (s, 3H, H-18). The single crystal structure of ergosterol peroxide is given in Figure 2. This analysis showed why the double bond (C6-C7, H6-H7) signals in ^1H, ^{13}C NMR spectrum appeared more downfield than those of ordinary alkene. The crystal structure of the compound was found to be monoclinic which consisted of three different sides, two of 90° angle and one leaning angle. The crystallographic data are listed in Table 3. Selected bond distances and angles are presented in Table 4. The bond length of O-O was 1.479 Å and from the data presented in Table 4, we reassured the presence of two double bonds C6-C7 (1.303 Å) and C22-C23 (1.314 Å) (Normal C-C bond length in this analysis was 1.5 Å).

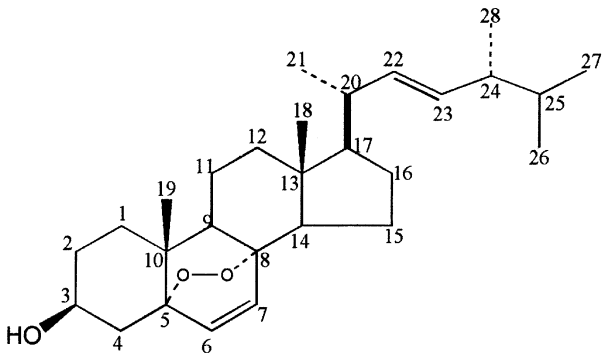

Figure 1. Structure of ergosterol peroxide from *Armillariella mellea.*

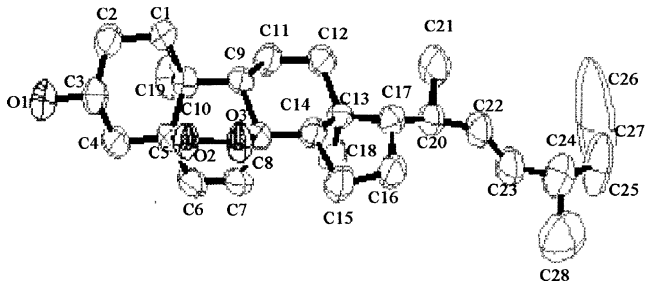

Figure 2. A molecular conformation with the atomic labelling scheme of 5,8-epidioxy-5α,8α-ergosta-6,22E-dien-3β-ol.

Antioxidant activity of ergosterol peroxide in *A. mellea*. Antioxidant activity of ergosterol peroxide from *A. mellea* was presented in Figure 3. The extent of inhibition of lipid peroxidation did not correspond with increasing concentration due to its solubility in aqueous solution for antiox-

Table 3. Structural data for ergosterol peroxide from *A. mellea*

Empirical Formular	$C_{28}H_{44}O_3$
Formular weight	428.3
Space group	$P2_1$
a (Å)	15.8040
b (Å)	10.8348
c (Å)	15.6372
α (deg)	90
β (deg)	101.807
γ (deg)	90
V (Å^3)	2621
Z	4
T(K)	293
Radiation	graphite monochromated Mokα λ = 0.71073 Å
Scan type	ω - 2θ
WR^2	0.1782

Table 4. Selected bond distances and bond angles for engosterol peroxide from *A. mellea*

Selected bond length (Å)		Selected bond angle (°)	
O-O	1.479	C_{10}-C_5-O	107.18
C_6-C_7	1.303	C_6-C_5-O	107.12
C_{22}-C_{23}	1.314	C_9-C_8-O	105.74
C_8-O	1.457	C_7-C_8-O	105.46
C_5-O	1.473		
C-C	1.5		
C-H	0.95		

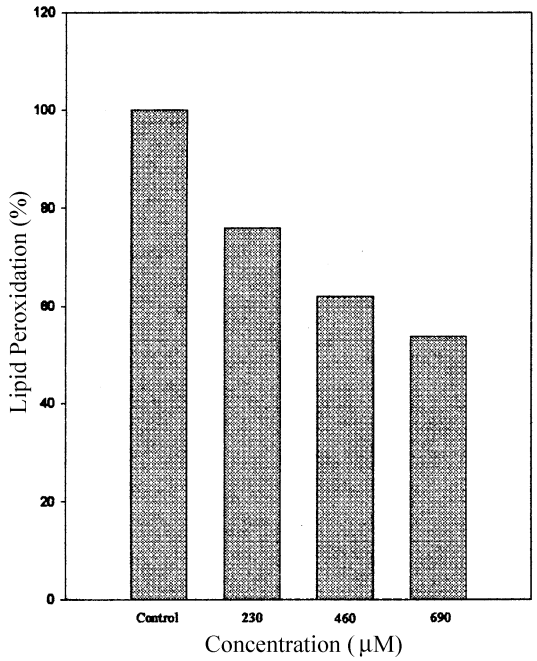

Figure 3. Antioxidant activity of ergosterol peroxide at various concentration on the lipid peroxidation of rat liver microsomes.

idant assay. However, the compound showed a tendency to inhibit lipid peroxidation with increasing its concentration, and it exhibited 46.3% inhibition of lipid peroxidation at concentration of 690 μM. Antioxidant activity of ergosterol peroxide was compared with those of well-known antioxidants in Figure 4. The ergosterol peroxide showed stronger inhibition than α-tocopherol and thiourea by 19.2%, and 21.5%, respectively, while less activity than BHT and BHA. Thus, the present results indicate that ergosterol peroxide from mushroom is expected to be effective as a natural antioxidants.

Ergosterol peroxide have been previously isolated and identified from *Inonotus obliquus*, common wood-rotting fungi in Filand,[22] *Lanum lyratum*,[23] and *Naematoloma fasciculare* which is toxic mushroom widely distributed in the world.[24] It was reported that ergosterol peroxide from *I. obliquus* not only inhibited the growth of cancer cells but also killed them in the anti-tumor tests. Recent study also revealed its inhibitory effects on induced inflammation and tumor promotion in mouse skin.[25] Antioxidative activity of ergosterol peroxide was elucidated from edible mushroom in

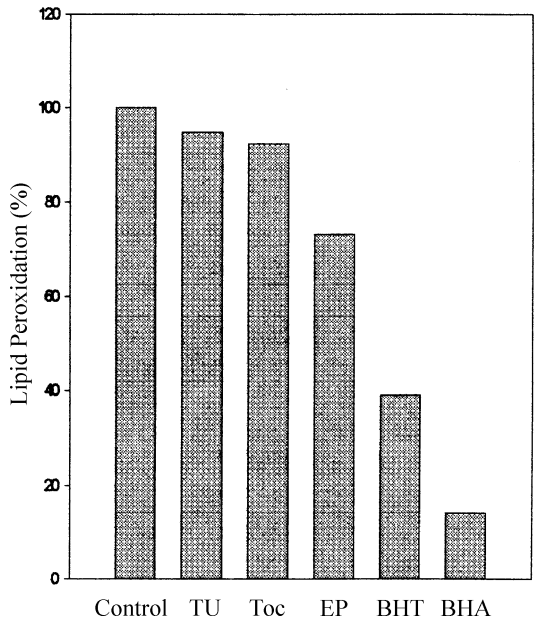

Figure 4. Antioxidative activity of ergosterol peroxide (EP) from A. mellea, α-tocopherol (Toc), thiourea (TU), Butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA) on lipid peroxidation of rat liver microsomes. Antioxidants of 290 μM were used for this experiments.

this study for the first time. However, since the experiment in this study have been carried out *in vitro*, further work is required to determine antioxidant activity of the compound *in vivo* system. Our previous study showed that various kinds of mushrooms inhibited hepatic aldehyde activity and have slight stimulation effect on SOD activity as well as inhibition effect on lipid peroxidation.[13] So, further investigations for antioxidant on aldehyde oxidase activity and SOD activity are in progress, and further identification of antioxidants from different kinds of mushrooms is required.

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