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Isolation and Identification of an Antioxidant Enzyme Catalase Stimulatory Compound from *Garnoderma lucidum*

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Antioxidant enzymes are scavenger reactive-oxygen intermediates and are involved in many cellular defense systems. We previously reported that a crude extract of *Garnoderma lucidum*, a medicinally potent mushroom, profoundly increased the catalase gene expression and enzyme activities in mouse livers (Park *et al*., *J. Biochem. Mol. Biol*. 34. 144-149, 2001). In this study, we elucidated the detailed mechanism whereby *G. lucidum* stimulates the catalase activity and expression. The major active fraction was isolated from *G. lucidum* and methyl linoleate was considered the most major component of the fraction. In order to determine whether methyl linoleate increases mRNA and protein synthesis of catalase, Northern and Western blot analyses were performed *in vivo* with methyl linoleate-treated mouse liver homogenate after feeding methyl linoleate to the mice. Northern and Western blot analyses of the crude liver homogenates in the mice that were administered methyl linoleate revealed that the expression catalase was significantly increased when compared to the untreated controls. In addition, the catalase protein levels and enzymatic activities increased in the mouse liver homogenates. These results suggest that methyl linoleate that is produced by *G. lucidum* stimulates the catalase expression at the transcription level.

Keywords: Antioxidant, Catalase, *Garnoderma lucidum*, Methyl linoleate, Transcriptional regulation

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Introduction

A number of highly reactive-oxygen species (ROS), which include the superoxide anion (O_2^-), the hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2), are produced as unavoidable side products of the normal electron-transport processes in the respiratory chain and in numerous oxidase-catalyzed reactions. ROS damages macromolecules in cells, an event which has been implicated in the etiologies or manifestations of several pathological processes. Among the antioxidant enzymes, catalases are ubiquitous heme enzymes that are found in aerobic organisms, ranging from bacteria to higher plants and animals. Functionally, catalases are related to peroxidases; both promote H_2O_2 oxidation by mechanisms that involve ferryl intermediates (Deisseroth and Dounce, 1970; Dawson, 1988). However, catalases differ from peroxidases, with the exception of chloroperoxidase and myeloperoxidase, in that they have the ability to utilize H_2O_2 as both an electron acceptor and donor that yields a disproportionate reaction. Due to this catalytic activity, the catalases are believed to be involved in the protective destruction of H_2O_2 that is generated in respiring cells (Fita and Rossmann, 1985; Ikeda-Saito *et al*., 1985; Shaffer *et al*., 1987).

Catalase (EC 1.11.1.6; Hydrogen peroxide oxidoreductase) from bovine liver is a homotetrameric enzyme that is primarily located in peroxisomes (Chance *et al*., 1979). This enzyme catalyzes the decomposition of H_2O_2 to O_2 and H_2O, and thus provides protection against the toxic effects of the oxygen radical.

Moreover, the catalase role in deterring tumor promotion and enhancing cell survival during periods of oxidative stress

supports the antioxidant function of this enzyme (Aebi and Wyss, 1978; Jones *et al*., 1985; Lewis, 1985; Agar *et al*., 1986). The catalase can also oxidize a variety of compounds, such as alcohols, alkyl peroxides, and formic acid. This peroxidatic activity requires a hydrogen donor other than hydrogen peroxide: $H_2O_2 + AH_2 \rightarrow 2\ H_2O + A$.

Garnoderma lucidum is commonly known as a medicinally-potent mushroom. It has been widely used in China and other oriental countries for hundreds of years for the treatment of various diseases, including cancer. This mushroom is reported to have various biological activities, such as anti-tumor, antibacterial, and antiviral activities (Yoon *et al*., 1994; Wang *et al*., 1997; El-Mekkawy *et al*., 1998; Eo *et al*., 2000). It was also reported to have an anti-inflammatory and liver-protective effect in rats (Lin *et al*., 1993; Lin *et al*., 1995). It is now well established from *in vitro* and *in vivo* studies that the polysaccharide fraction of *G. lucidum* is largely responsible for its anti-tumor efficacy (Wang *et al*., 1997). Recently, the *G. lucidum* spores were shown to have a stimulatory effect on the canavalin A-induced mitogenic activity of T-lymphocytes (Bao *et al*., 2002). However, though *G. lucidum* induces various physiological effects, its antioxidant properties have not been extensively studied.

In a previous paper, we reported that the *G. lucidum* crude extracts stimulate antioxidant catalase activity (Park *et al*., 2001) *in vivo* in a mouse model. Based on these earlier findings, we isolated the active fraction from the *G. lucidum* extract. The major component of the fraction was revealed to be methyl linoleate. We also investigated the stimulatory mechanism of this compound, including its regulatory effect, on the catalase gene expression and enzyme activity in mouse livers.

Materials and Methods

Materials *G. lucidum* spores were purchased from the local market in Chunchon. Male ICR mice (~6-8 weeks) were supplied from the experimental animal center at Hallym University. The NMR spectra were obtained with Unity Inova-400 (Varian, Palo Alto, USA) and GC/MS was analyzed with HP 5890 GC/JMS-AX505WA (Jeol, Tokyo, Japan). Hydrogen peroxide and methyl linoleate were purchased from the Sigma Chemical Co. (St. Louis, USA). All of the other reagents were of the highest quality generally available.

Isolation of active compound from *G. lucidum* The 500 g of *G. lucidum* was extracted in 80% aqueous MeOH (2 l× 2) at room temperature for 24 h. The solution was evaporated *in vacuo* and partitioned between H_2O (400 ml) and EtOAc (400 ml× 2). The organic phase was evaporated *in vacuo* to attain the EtOAc fraction (12.2 g). The EtOAc fraction was applied to the silica gel (250 g) column (6× 30 cm), eluting stepwise-gradiently [n-hexane : EtOAc = 3 : 1 (700 ml) → 1 : 1 (500 ml) → $CHCl_3$: EtOAc = 5 : 1 (500 ml) → $CHCl_3$: EtOH = 10 : 1 (500 ml) → 7 : 1 (500 ml) → 4 : 1 (750 ml)] to give 22 fractions (*G. lucidum* E-1 ~E-22), among which the second fraction (E-2, 272.6 mg) showed the highest antioxidant activity.

G. lucidum E-2: Yellowish oil ($CHCl_3$-EtOH), ^{1}H-NMR (400 MHz, $CDCl_3$, δ) 5.38-5.31 (4H, m, olefine), 3.65 (3H, s, methyl of methyl ester), 2.29 (2H, t, J = 8.0 Hz, H-2), 2.04 (2Hx2, m, allyl methylene), 1.62 (2H, m, allyl methylene), 1.36-1.26 (2Hx2, m, methylene), 0.88 (3H, t, , J = 6.8 Hz, H-18); ^{13}C-NMR (100 MHz, $CDCl_3$, δc) 173.72 (s, carbonyl of methyl ester), 129.81, 129.65, 127.77, 127.64 (all d, each olefine), 51.14 (q, methyl of methyl ester), 33.90, 31.44, 29.59, 29.50, 29.27, 29.09, 29.04, 29.00, 27.10, 25.52, 24.82, 22.61 (all t, methylene), 13.95 (q, terminal methyl).

GC/MS analysis of *G. lucidum* E-2 One mg of *G. lucidum* E-2 was dissolved in $CHCl_3$ and analyzed for GC/MS. The experimental conditions are as follows: DB-5 (J & W Scientific Co., Tokyo, Japan); Injector temp.: 300°C; Initial temp.: 200°C (5 min) → Elevation rate 10°C/min → Terminal temp.: 300°C (5 min). Ionization method in MS : EI (Electronic ionization, 70 eV). Retention time (relative area %): 4 min 30 sec (0.7, pentadecanoic acid methyl ester), 5 min 43 sec (9.8, methyl palmitate), 8 min 20 sec (72.1, methyl linoleate), 8 min 30 sec (15.7, methyl oleate), 8 min 55 sec (1.7, methyl stearate).

Catalase activity assay The catalase activity was spectrophotometrically assayed by measuring the disappearance of H_2O_2 at 240 nm (Cohen *et al*., 1970; Choi *et al*., 1999; Park *et al*., 2001; Kim *et al*., 2002b). One unit of enzyme activity was defined as 1 μmol of H_2O_2, decomposed per minute at 25°C. The protein concentration was determined by the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

Treatment of methyl linoleate in mice Five male ICR mice were used in each group. The animals were housed in a controlled environment of 23 ± 1°C, 55 ± 10% humidity with a 12 h light-dark cycle, and given regular food throughout the experimental period. Methyl linoleate was uniformly mixed into tap water and daily supplied to mice at a dose of 25 and 50 mg/kg/d for 2 wk. The control mice received tap water only. All of the operations were carried out a 4°C unless otherwise indicated. The liver was isolated quickly at the time of sacrifice, then the sliced liver tissue was homogenized in a 10 mM potassium phosphate buffer, pH 7.0 containing 1 mM β-mercaptoethanol, 0.1 mM EDTA. The homogenate was centrifuged at 10,000× g for 30 min, then the pellet was discarded. A supernatant solution was used to assay the enzyme activity. Production of the anti-catalase monoclonal antibodies was as follows: the monoclonal antibodies against human liver catalase were produced by the fusion experiments in our laboratory (Jin *et al*., 2003).

Immunoblot and Northern blot analyses For the Western blotting, the liver homogenates were subjected to 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Towbin *et al*., 1979; Kim *et al*., 2002a). Next, the blots were blocked with BLOTTO for 1 hr. After rinsing with TBS, the blots were incubated with anti-catalase monoclonal antibodies for 1 h. The blots were treated with HRP-conjugated goat anti-mouse IgG for 1 h and visualized by chemiluminescence (ECL; Amersham, Arlington Heights, USA).

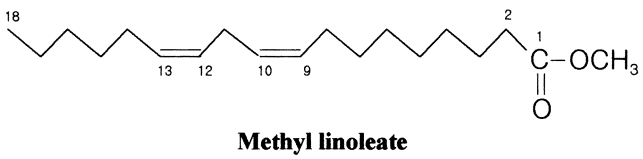
Total RNA was isolated from the liver tissues using a Trizol reagent (Gibco BRL, Grand Island, USA), following the manufacturers instructions. For the Northern blot analysis, 2 μg of poly(A)$^+$ mRNAs were electrophoresed on 1.2% agarose gels containing 1.1% formamide, then transferred to Hybond-N$^+$ nylon membranes (Amersham, Piscataway, USA). The RNA was cross-linked to the membrane by UV cross-linker. The membrane was pre-hybridized and hybridized in a Rapid-hyb blotting buffer from Amersham at 65°C for the recommended times. The blot was probed with the human catalase cDNA fragment, which was random primed-labeled with [α-^{32}P]dCTP to specific activity $> 5 \times 10^8$ cpm/μg. The hybridizing bands were visualized by autoradiography with Kodak XAR film. All of the Northern blots were stripped of probes and rehybridized with β-actin probes that were provided by Clontech (Palo Alto, USA). The blots were exposed and quantified using densitometric programs.

Results

Extracts of *G. lucidum* and GC/MS analyses To investigate the biochemical basis of the catalase stimulatory effect of the *G. lucidum* extracts, the identities of the fatty acids in the active fraction were determined by GC/MS. Silica gel column chromatography of the EtOAc fraction of *G. lucidum* afforded twenty-two fractions (data not shown). Antioxidant activity testing revealed that the second fraction, *G. lucidum* E-2, had the highest activity. The fraction produced one spot on silica gel and ODS TLC, no UV absorption, and the color changed from yellow-brown to violet on spraying the TLC plates with 10% aq. H_2SO_4 and heating. In the ^{1}H-NMR spectrum, olefins, a methoxy, a terminal methyl, and four allyl methylenes, along with several methylenes signals, were observed, which indicates that it was an unsaturated aliphatic compound. The ^{13}C-NMR spectrum showed one carbonyl, four olefine methines, one methoxy, one methyl, and twelve methylenes signals. Accordingly, *G. lucidum* E-2 was identified as a C_{18}-fatty acid with two double bonds, methyl linoleate.

G. lucidum E-2 was composed of methyl linoleate (72.1%), methyl palmitate (9.8%), methyl oleate (15.7%), methyl stearate (1.7%), and pentadecanoic acid methyl ester (0.7%). *G. lucidum* contained several fatty acids, and methyl linoleate accounted for 72.1% of its total fatty acid content (Fig. 1).

Activation of catalase activity by methyl linoleate in mouse livers To evaluate the effects of methyl linoleate on catalase activity *in vivo*, the mice were fed with 25 and 50 mg/kg/d of methyl linoleate for 2 wk. As shown in Fig. 2, in the methyl linoleate-treated group, the catalase activities were 1.5-2 times higher than in the control group, and the catalase activity increased in a dose-dependent manner. However, other antioxidant enzymes, including superoxide dismutase (SOD) and glutathione peroxidase (GPx), were unchanged under the same experimental conditions (data not shown).



Methyl linoleate

Fig. 1. The structure of methyl linoleate.

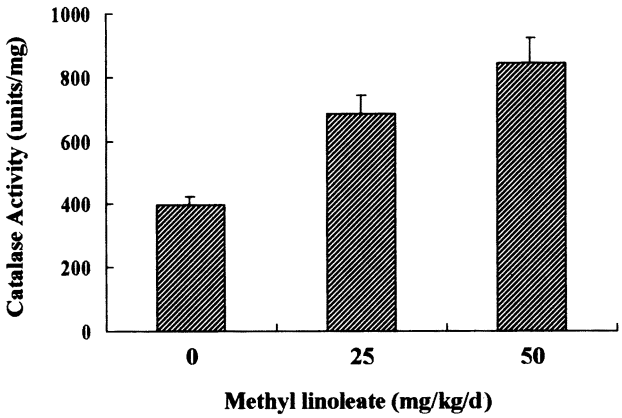


Fig. 2. Effect of methyl linoleate on catalase activity *in vivo*. Methyl linoleate (25-50 mg/kg/d) was daily supplied to mice for 2 wk. The homogenates from the mice livers were prepared and assayed for catalase activity using H_2O_2 as described in Materials and Methods.

Effects of methyl linoleate on the catalase expression levels in mice To investigate the methyl linoleate-induced catalase expression in the mice, we performed a Western blot using anti-catalase monoclonal antibodies. In the livers of the animals that were treated with methyl linoleate, the catalase expression level was about 2-fold higher than in the control livers (Fig. 3). Moreover, the catalase activity in the mouse liver extracts increased in a methyl linoleate dose-dependent manner.

Effects of methyl linoleate on the levels of catalase mRNA in the liver A Northern blot was used to examine the catalase mRNA expression patterns in mice. Catalase cDNA, cloned in our laboratory (Jin *et al*., 2003), was used as a probe. In the methyl linoleate-treated group, the catalase mRNA expression levels were 1.5-2.5 fold higher than in the control group. As shown in Fig. 4, methyl linoleate stimulated both the catalase protein and catalase mRNA expressions. These results suggest that the methyl linoleate in *G. lucidum* stimulates the catalase expression at the translation or transcription levels.

Discussion

For hundreds of years, *G. lucidum* has been widely used as a tonic to promote health and longevity in China and other oriental countries. However, its active principal and mode of

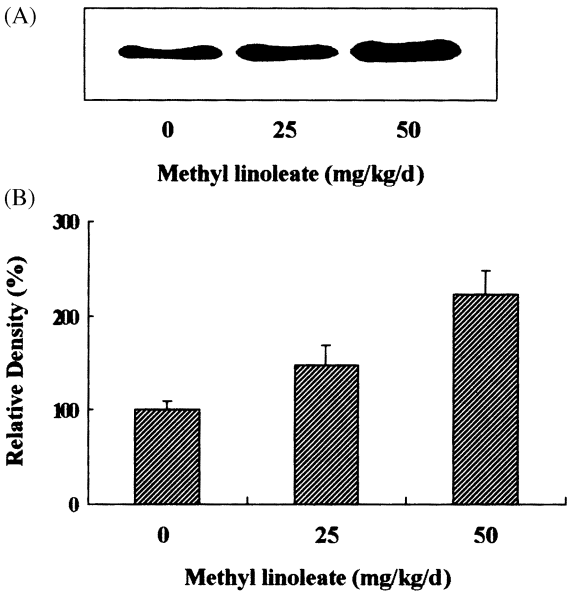


Fig. 3. Effect of methyl linoleate on the catalase expression in the mice livers. Immunoblot analysis of total proteins of the mice liver homogenates that were probed with anti-catalase mAb. Methyl linoleate (25-50 mg/kg/day) was daily supplied to the mice for 2 wk. Next, the mice livers homogenates were prepared and the proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (A). The relative magnitude of each band was calculated from the integrated area that was obtained from densitometry (B).

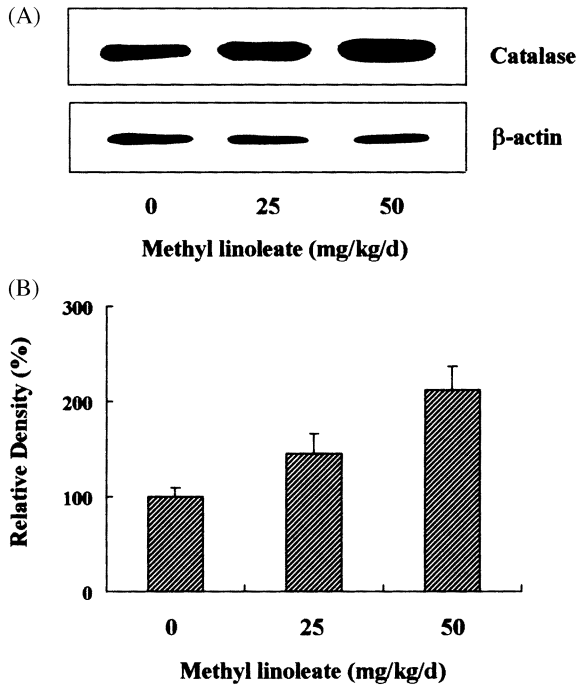


Fig. 4. Effect of methyl linoleate on the catalase mRNA expression in the mice livers. Methyl linoleate (25-50 mg/kg/d) was daily supplied to the mice for 2 wk. Poly(A)$^+$ RNA was isolated from the mice liver. The catalase mRNA expression level was measured by a Northern blot analysis (A). All of the blots were stripped and rehybridized with a β-actin probe to check RNA levels between the lanes. The relative magnitude of each band was calculated from the integrated area that was obtained from densitometry (B).

action are substantially unknown. In a previous study, we found that the crude extracts of *G. lucidum* increased the expression of the antioxidant enzyme catalase (Park *et al*., 2001). In this study, we identified the active principal in *G. lucidum* and its mode of action at the transcriptional level.

During the isolation of the components in the extract of *G. lucidum*, we observed the highest antioxidant enzyme activity in the second fractions. This fraction was further analyzed by GC/MS. The active fractions of *G. lucidum* were mainly composed of polyunsaturated fatty acids (PUFAa), primarily methyl linoleate (Fig. 1).

Polyunsaturated fatty acids (PUFAs), which cannot be synthesized by the human body, must be obtained from the diet in order to avoid the symptoms that are caused by a shortage. They can be considered as vitamin factors; linoleic acids should be particularly considered since they are PUFAa that are necessary for the normal development and functioning of human tissues, in fact, they are known as essential fatty acids (Moreno *et al*., 1999). Methyl linoleate, an essential fatty acid and a predominant PUFA, is a major membrane fatty acid (Kang and Leaf, 1994) and of endothelial cell triglyceride stores (Spindler *et al*., 1996). Despite the fact that methyl linoleate is known to act as an anti-oxidant, its physiological action and those of its metabolites are unknown.

As shown in Fig. 2, the catalase activity was significantly increased by feeding mice with methyl linoleate in a dose-dependent manner. However, methyl linoleate had no effect on the other anti-oxidant enzymes, superoxide dismutase (SOD), and glutathione peroxidase (GPx). Since a previous study reported that the extract of *G. lucidum* has a stimulatory effect on catalase, but not on SOD or GPx (Park *et al*., 2001), we postulated that methyl linoleate may be one of the activators of catalase activity.

To investigate in more detail the nature of the mechanism of stimulation, methyl linoleate was fed to the mice and changes in catalase mRNA and protein levels were monitored by Northern and Western blots. The protein expression levels of catalase in the livers of treated mice are shown in Fig. 3. Catalase protein levels in the livers of mice that were fed methyl linoleate were twofold higher than those of the untreated controls. This result suggests that methyl linoleate is a major enhancer of the catalase protein expression at the translational level. To provide a more complete description of this increase in the catalase level, the mRNA levels of the catalase in the livers of mice that were treated with methyl linoleate were determined. As shown in Fig. 4, the catalase mRNA levels in these liver homogenates were significantly higher than those of the untreated controls. This result suggests that methyl linoleate regulates the catalase gene at

the transcriptional and translational levels. These regulatory roles confirm our previous findings on the liver-protective and free radical-scavenging effects of *G. lucidum* in rats (Lin *et al*., 1993; 1995).

In summary, the results that were obtained in this study show that methyl linoleate, which is a known major component of PUFAs, has a stimulatory effect on the catalase expression and activity *in vivo*. There is still a need for further examination to discover whether methyl linoleate binds directly to the catalase gene or stimulates other intracellular molecules to act as second messengers.

Natural traditional medicines sometimes have significant therapeutic activity against ROS-related human disorders. Therefore, our hope is that methyl linoleate makes therapeutic or pharmacological contributions in the treatment of antioxidant enzyme-related diseases.

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