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Ergothioneine Contents in Fruiting Bodies and Their Enhancement in Mycelial Cultures by the Addition of Methionine

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The levels of ergothioneine (ERG), which have been shown to act as an excellent antioxidant, were determined in both fruiting bodies and mycelia of various mushroom species. We found that ERG accumulated at different levels in fruiting bodies of mushrooms and showed up to a 92.3-fold difference between mushrooms. We also found that ERG accumulated at higher levels in mycelia than in fruiting bodies of economically important mushroom species such as *Ganoderma neo-japonicum*, *G applanatum* and *Paecilomyces tenuipes*. The addition of 2 mM methionine (Met) to mycelial culture medium increased the ERG contents in most mushroom species tested, indicating that Met is a good additive to enhance the ERG levels in a variety of mushroom species. Taking these results into consideration, we suggest that the addition of Met to the mycelial culture medium is an efficient way to enhance the antioxidant properties in economically important mushroom species.

KEYWORDS : Antioxidant, Ergothioneine, Methionine, Mycelial culture

Mushrooms have been used as food and medicine for centuries in Korea and have become of great interest as an addition to the human diet because they have little fat and digestible carbohydrates but have higher protein contents than most vegetables. A number of studies on edible mushrooms have demonstrated many interesting biological activities, including antitumor (Chihara *et al*., 1970; Tabata *et al*., 1981), anticarcinogenic (Lee and Nishizawa, 2003; Pinheiro *et al*., 2003) and antioxidant effects (Fu and Shieh, 2002; Cheung *et al*., 2003; Yang *et al*., 2002). Mushrooms contain a number of secondary metabolites, including various phenolic compounds, which have been shown to act as excellent antioxidants (Mau *et al*., 2002).

Phenolic compounds are one of the most widely distributed secondary metabolites in mushrooms. One such phenolic compound, ergothioneine (ERG; 2-mercaptohistidine trimethylbetaine; see also Fig. 1), is a naturally occurring amino acid which is synthesized in some bacteria and fungi but not in animals (Melville *et al*., 1955). In humans, ERG is probably absorbed primarily by intake of edible mushrooms and meat (Jang *et al*., 2004). ERG is present in brain, red blood cells, liver, kidney, seminal fluids, and ocular tissues of human beings (Kaneko *et al*., 1980; Mitsuyama and May, 1999). Although the biological functions of ERG remain poorly understood, it is known to possess various beneficial effects, including antioxidant and antimutagenic properties (Asmus *et al*., 1996; Hartman and Hartman, 1987; Akanmu *et al*., 1991; Arduino *et al*., 1990; Aruoma *et al*., 1997, 1999). Furthermore, a number of rapid advances in the researches involved in physiological roles of ERG have been achieved recently, mainly due to the development of an adequate quantification method using HPLC-MS (Dubost *et al*., 2006).

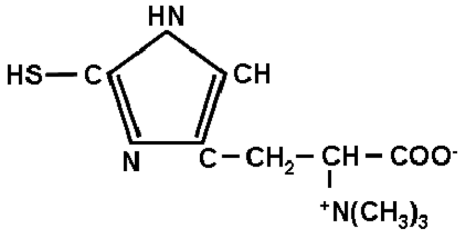

Fig. 1. Structure of ergothioneine.

In general, the production of a fruiting body using solid culture requires a long time period. Therefore, many attempts have been made to obtain useful and potent cellular or extracellular substances from submerged mycelial cultures for use in the formulation of nutraceuticals and functional foods (Shih *et al*., 2007). Submerged cultures are advantageous to solid culture because mycelia can be produced in a compact space and in a shorter time period with less chance of contamination. Therefore, submerged cultures of some isolates derived from a number of mushroom species have been developed (Gibbs *et al*., 2000; Kim *et al*., 2003; Wang *et al*., 1996). It is generally accepted that the medicinal merits of cultured mycelia are similar in effectiveness to those of mushroom species found in the wild (Koh *et al*., 2003).

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While the physiological importance of ERG in its antioxidant capacity has started to attract attention, the practical effort to increased ERG in mushrooms has not yet been attempted. In this study, nutritional requirements and additives for the mycelial culture were investigated in an attempt to enhance ERG production in the *Ganoderma neo-japonicum* mycelia.

Materials and Methods

Sample preparation. Samples of the various mushrooms were obtained from a number of mountains, local markets and the Korean Forest Research Institute (KFRI). A list of the fungi analyzed for the accumulation of ERG in their fruiting bodies is shown in Table 1. The mushrooms were freeze-dried and then stored at −70°C. The mycelia of various mushroom species were maintained on Potato Dextrose Agar (PDA) medium.

Inoculum preparation and mycelial culture. The mycelia grown on PDA was inoculated in 100 ml Fungal Growth Medium (FGM) (Lee *et al*., 2007) and then grown at 25°C on a shaking incubator at 110 rpm for 10 days. The culture was then homogenized at 13,000 rpm for 8 seconds in a homogenizer (Ingenieurbüro CAT. X1030D, M. Zipperer GmbH, Germany). Five ml of the homogenized culture was used as inoculum in 100 *ml* Fungal Growth Medium (FGM). This second culture was grown for 10 days under the same conditions as used for the first culture. Two mmol methionine (Met) was added to the second culture to determine its effect on the accumulation of ERG. The mycelia were isolated from the culture medium by the centrifugation at 6000 rpm. Mycelia were then freeze-dried and stored at −70°C for further analysis.

Determination of the ERG content. Fifty mg of freeze-dried mycelia was added to 20 *ml* of cold ethanolic extraction solution (10 mM DTT, 100 μM betaine, 100 μM MMI in 70% ethanol) and mixed by vortexing and subsequent sonication for 3 min. A ethanolic solution (4 *ml*) of sodium dodecyl sulfate (SDS) was mixed by inverting. The mixture was centrifuged at 4000 rpm for 15 min. Ten ml of the supernatant was evaporated using rotary vacuum evaporator (R-114, Büchi, Switzerland) to dryness. The residue resuspended in 10 *ml* of distilled water (pH 7.3) and centrifuged at 4000 rpm for 15 min.

Levels of ERG were determined using the method of Lee *et al*. (2007), with some modifications (Mondino *et al*., 1972). The resulting supernatant was injected into an HPLC (Thermo Electron C, Finnigan Surveyor System, Massachusetts USA) equipped with Econosphere C18 column (4.6*250 mm, 5 μm; Alltech Associates, IL. USA). The mobile phase was 50 mM sodium phosphate with 3% acetonitrile and 0.1% triethylamine adjusted to a pH 7.3 with a flow rate of 0.7 *ml* per min. The ERG level was quantified by monitoring absorbance at 254 nm with an UV detector and comparing the standard curve obtained from the authentic ERG (Sigma, St Louis, MO, USA).

DPPH radical scavenging activity. The scavenging activity of mushroom extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined using the method described by Huang *et al*. (2005b). A 1 *ml* aliquot of methanol extracts (20 mg/*ml*) from fruiting bodies was mixed with 1 *ml* of freshly prepared DPPH (80 μM) in methanol. The mixture was kept in the dark for 30 minutes. The absorbance was then measured at 517 nm using Lambda 1 UV-Vis Spectrophotometer (Perkin-Elmer, USA). The radical scavenging activity of gallic acid (10~160 μg/*ml*) was also determined. Percent activity was calculated

Table 1. The amounts of ergothionine (ERG) in fruit bodies of mushrooms

Mushroom species	ERG (mg/g DW)	Location	Mushroom species	ERG (mg/g DW)	Location
Sparassis crispa	2.37 ± 0.42	KFRI	*Hericium erinaceum*	0.96 ± 0.07	Hongcheon
Tremella foliacea	0.61 ± 0.03	Hongcheon	*Armillaria mellea*	1.94 ± 0.01	Hongcheon
Lepista nuda	5.54 ± 0.26	Kwangneung	*Neolentinus lepideus*	2.41 ± 0.09	Hongneung
Suillus luteus	2.27 ± 0.24	Hongcheon	*Hygrophorus russula*	4.98 ± 0.31	Hongcheon
Ramaria botrytis	0.29 ± 0.03	Hongcheon	*Cantharellus cibarius*	4.09 ± 0.20	Hongcheon
Tricholomopsis rutilns	2.50 ± 0.30	Goseong	*Polyozellus multilplex*	0.51 ± 0.01	Mungyeong
Tricholoma matsutake	0.74 ± 0.08	Hongcheon	*Boletus auripes*	2.40 ± 0.05	Hongneung
Suillus granulatus	0.09 ± 0.03	Hongreung	*Pleurotus ostreatus*	2.20 ± 0.13	Local market
Russula virescens	0.68 ± 0.04	Koseong	*Agaricus bisporus*	1.21 ± 0.82	Local market
Sarcodon aspratus	1.79 ± 0.02	Goryeong	*Lentinula edodes*	1.86 ± 0.73	Local market
Hydnum repandum	0.78 ± 0.02	Hongcheon	*Ganoderma applanatum*	0.06 ± 0.02	Hongcheon
Suillus bovinus	1.09 ± 0.07	Hongcheon	*Fomitopsis pinicola*	0.07 ± 0.01	Hongcheon
Lampteromyces japonicus	0.43 ± 0.16	Kwangneung	*Ganoderma lucidum*	0.08 ± 0.02	Hongcheon
Lactarius torminosus	0.82 ± 0.15	Hongneung	*Ganoderma neo-japonicum*	0.07 ± 0.00	KFRI

Data represent mean ± S.D. from three independent experiments.

using the equation

$$\% \text{ Activity} = (1 - (A_{Sample}/A_{Blank})) \times 100$$

The EC_{50} value, which is the sample concentration at 50% activity, was determined by interpolation. The test was run in duplicate and analysis of the samples was run in triplicate and averaged.

Statistical analyses. Regression analysis was completed to obtain a coefficient of determination (r) between the EC_{50} values and the contents of ERG. Multiple comparison tests were performed to examine significant differences of the ERG content in fruiting bodies by Duncan's test at $P < 0.05$. To determine if ERG contents in mycelial culture changed due to Met treatment, *t*-test analysis was completed ($P = 0.05$). Statistical procedures were carried out using the SAS program (SAS Institute, Inc., Cary, NC).

Results and Discussion

ERG accumulation in fruiting bodies. ERG is present in human tissues at concentrations up to 1~2 mM (Melville, 1958; Brummel, 1985; Hatman, 1990). The biological role of ERG is currently being explored and is under investigation for its impact on the inflammatory process and certain diseases. Dubost *et al*. (2007) developed a method to quantify the ERG in various genera of edible mushrooms. In this study, ERG concentrations in fruiting bodies of twenty-eight mushroom species ranged from 0.06 and 5.54 mg/g DW, a 92.3-fold difference (Table 1). Among the 28 different mushroom species tested, *Lepista nuda* contained the highest amount of ERG, while *Ganoderma applantum*, *G. lucidium* and *G. neo-japonicum* accumulated the lowest levels of ERG (0.06 and 0.08 mg/g DW).

Dubost *et al*. (2007) reported that antioxidant activity was significantly correlated with phenolic compound levels in various mushroom species but not with ERG levels. Previous studies conducted with mushrooms have also shown a positive correlationship between polyphenols and antioxidant capacity (Adamson *et al*., 1999; Kim *et al*., 2008). In this study, DPPH scavenging activity, which measures hydrogen-donating ability of antioxidants (Singh and Rajini, 2004), was evaluated with EC_{50} values The range of EC_{50} values is 0.03~6.16 mg/*ml* dry sample (Fig. 2). Although EC_{50} values were not correlated with ERG content ($r = 0.097$; $P = 0.484$) (Fig. 2), this does not provide evidence that there will be less biological activity from ERG.

It is also interesting to note the difference in antioxidant substances among the various genera of mushrooms. As shown earlier, the difference in the contents of ERG was up to 92.3-fold in fruiting bodies of various mushroom species. In general, genetics (species or strain), growing conditions, and environmental conditions can affect the amount of secondary metabolites produced by most living organisms. Furthermore, we collected three mushroom species including *Sparassis crispa*, *Tricholoma matsutake* and *Neolentinus lepideus* from various geographical locations in Korea and evaluated their contents of both ERG (Data not shown). Variances of the ERG contents in the same species were significantly different depending on the number of sample collecting sites. This indicates that mushrooms grown in different geographical locations accumulate different amounts of ERG, possibly due to different environmental conditions. Therefore, it is very likely that the contents of ERG can be altered by manipulating the growth condition.

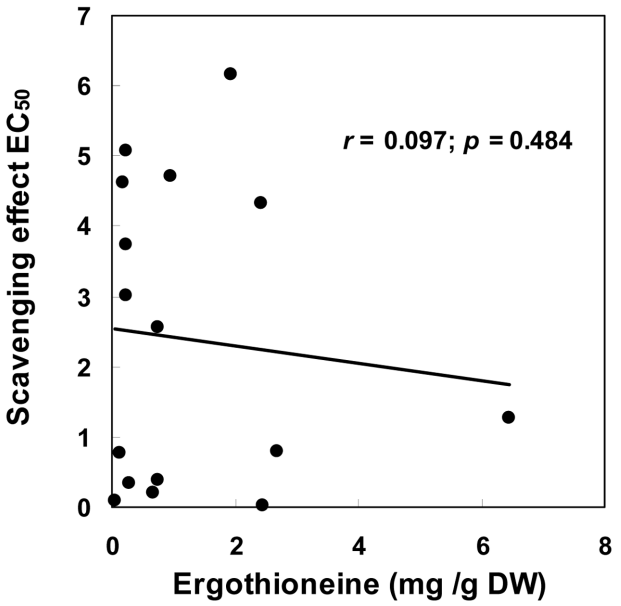

Fig. 2. Correlation between ergothioneine and the scavenging effect on DPPH radicals (mg GAE/g DW) in fruiting bodies of mushrooms.

ERG production in mycelial culture. We compared the ERG levels in between mycelia and fruiting bodies of eight different mushroom species (Fig. 3). There were large differences in the ERG contents of those mushrooms. The *Lentinula edodes* fruiting bodies contained a level of ERG approximately 30 times higher than those of *Ganoderma applanatum*. The ERG levels in mycelia did not differ as much as in the fruiting bodies. Among the eight mushroom species, the mycelia of *Ganoderma neo-japonicum* produced the highest level of ERG (0.72 mg/g DW) and *Paecilomyces tenuipes* produce the lowest level of ERG (0.33 mg/g DW), indicating that the differences of the ERG levels in mycelia of the different mushroom species were less than 2.1-fold. *G. neo-japonicum* and *G. applanatum* produced higher levels of ERG in their myce-

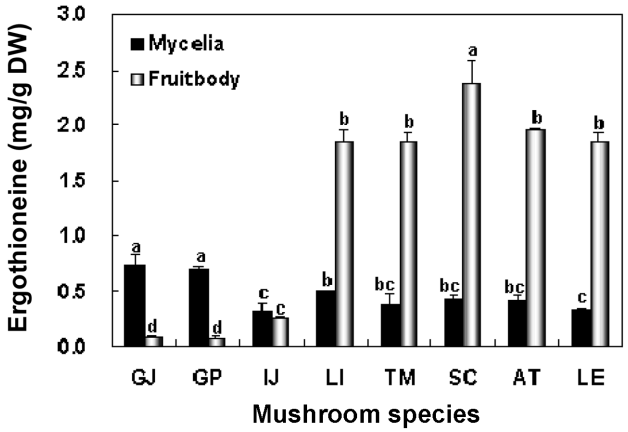

Fig. 3. Ergothioneine contents in mycelia and fruiting bodies of mushrooms. The mycelia of seven mushroom species were cultured in the FGM medium for 10 days at 25°C prior to determining the ergothioneine contents. All results are represented as means ± SE of results from three independent experiments. The same letters indicate the ERG contents that do not differ statistically ($P < 0.05$; Duncan's test). GJ, *Ganoderma neo-japonicum*; GA, *Ganoderma applanatum*; IJ, *Paecilomyces tenuipes* (= *Isaria japonica*); LI, *Neolentinus lepideus* (= *Lentinus lepideus*); TM, *Tricholoma matsutake*; SC, *Sparassis crispa*; AM, *Armillaria mellea*; LE, *Lentinula edodes*.

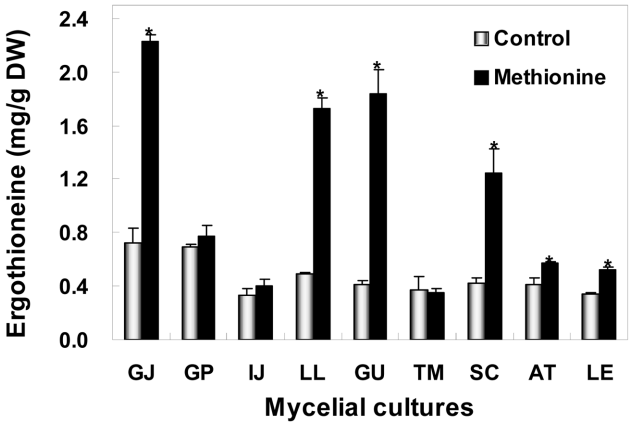

Fig. 4. Enhancement of ergothioneine accumulation in mycelial cultures of mushrooms by supplementation with methionine. The mycelia of nine mushroom species were cultured for 10 days at 25°C in the FGM medium supplemented with 2 mM of methionine prior to determining the ergothioneine contents. All results are represented as means ± SE of results from three independent experiments. The symbol '*' indicates a significant increase of the ERG contents in the Met treatment. GJ, *Ganoderma neo-japonicum*; GA, *Ganoderma applanatum*; IJ, *Paecilomyces tenuipes* (= *Isaria japonica)*; LI, *Neolentinus lepideus* (= *Lentinus lepideus*); GU, *Polyporus umbellatus* (= *Grifola umbellate*); TM, *Tricholoma matsutake*; SC, *Sparassis crispa*; AM, *Armillaria mellea*; LE, *Lentinula edodes*.

lia than did any other mushrooms tested. This suggested that ERG accumulated at the different levels in mycelia and fruiting bodies depending on the mushroom species.

Enhancement of ERG production by the Met additive. Previously, we have shown that the supplementation of Met in culture media increased the ERG production in the *Ganoderma neo-japonicum* mycelia (Lee *et al.*, In press). It has long been known that ERG is synthesized from the amino acids, such as histidine (His), Met, and Cysteine (Cys) (Askari and Melville, 1962). Cys and Met increased the ERG levels up to 1.7- and 3.1-fold, respectively, although His did not (Lee *et al.*, In press). In order to know if the Met supplementation also increases the ERG levels in other mushroom species, two mmol of Met was added to the mycelial culture medium. After 10 days, the ERG contents in six different mushroom species were significantly increased (Fig. 4). Interestingly, the *Ganoderma neo-japonicum* mycelia produced higher levels of ERG than the *G. applanatum* did, although they belong to the same Genus. This indicates that the Met treatment has different impacts on the activation of the ERG biosynthesis in two *Ganoderma* species.

In conclusion, we found that the ERG contents varied in the fruiting bodies of 28 mushroom species tested in this study, and that a number of genera could be a viable and economical source of antioxidants in the diet. ERG was also produced in mycelial cultures and the Met additive enhanced the ERG levels in economically important mushroom species. Therefore, the results of this study could provide valuable new opportunities for mushroom growers, since mushrooms can serve as an excellent source of antioxidants, specifically ERG, and provide new potential for commercial scale production of ERG through large-scale bioreactors.

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