Appl Biochem Biotechnol (2009) 158:213–221
DOI 10.1007/s12010-008-8322-0

Supplementation of Methionine Enhanced the Ergothioneine Accumulation in the *Ganoderma neo-japonicum* Mycelia

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Received: 7 May 2008 / Accepted: 14 July 2008 /
Published online: 8 August 2008

Abstract The methods for increasing the production of ergothioneine (ERG) were investigated by using the mycelial culture of several mushroom species, primarily *Ganoderma neo-japonicum*. We first found that ERG was accumulated at the different levels in mycelia and fruiting bodies, respectively, depending on the mushroom species. As a result of adding various amino acids to the mycelial culture medium, methionine (Met) was shown to be the most effective additive. The most preferable condition of the additive was the combination of 4 mM Met and 1 g/l of yeast extract, and the maximum ERG production reached approximately 1.7 mg/l, which corresponds to 2.4 times (0.7 mg/l) that in the basal medium without Met. Although the supplementation of Met enhanced the ERG production, the mycelial growth was significantly inhibited. Furthermore, the analysis of amino acids in the culture medium revealed that the Met additive reduced the consumption rates of most amino acids tested, probably due to the decrease in mycelial growth. Taking these results into consideration, we suggest that the addition of Met to the mycelial culture medium is an efficient way to enhance the ERG production in economically important mushroom species.

Keywords Ergothioneine · Methionine · Additives · Mycelial culture · *Ganoderma neo-japonicum*

Introduction

A variety of mushroom species have been used in traditional medicines and become great interest in human diet due to their nutritional, medicinal, and pharmacological properties. Recent studies on edible mushrooms have demonstrated many interesting biological activities including antitumor [1, 2], anticarcinogenic [3, 4], and antioxidant effects [5–7]. Mushrooms contain a number of secondary metabolites, which have been shown to act as excellent antioxidants [8, 9].

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One such antioxidant, ergothioneine (ERG; 2-mercaptohistidine trimethylbetaine), is a naturally occurring amino acid, which is synthesized in some bacteria and fungi but not in animals [10]. In humans, ERG is probably absorbed primarily by intake of edible mushrooms and meat [11]. ERG is present in brain, red blood cells, liver, kidney, seminal fluids, and ocular tissues [12, 13]. Although the biological functions of ERG remain poorly understood, it is known to possess various beneficial effects including antioxidant activity and antimutagenic properties [14–19]. Furthermore, a number of rapid progresses have been achieved recently, mainly due to the development of an adequate quantification method using high-performance liquid chromatography (HPLC)-mass spectrometry [20].

In general, solid culture of mushrooms takes a long time to complete a fruiting body, and thus many attempts have been made to obtain useful and potent cellular or extracellular substances from a submerged mycelial culture for use in the formulation of nutraceuticals and functional foods [21]. Submerged culture gives rise to potential advantages of higher mycelial production in a compact space and shorter time with less chance of contamination.

While the functional importance and limited availability of ERG have long been known, the practical efforts to improve the ERG levels in mushrooms have never been attempted so far. In this study, the nutritional requirements and additives for the mycelial culture were investigated in an attempt to enhance the ERG production, primarily in the *Ganoderma neo-japonicum* mycelia.

Materials and Methods

Inoculum Preparation and Mycelial Culture Samples of the various mushrooms were obtained from mountains, local markets, and Korean Forest Research Institute. The collected mushrooms were frozen-dried and then stored at −70 °C. The mycelia isolated from each mushroom were maintained on the potato dextrose agar (PDA) medium. Each mycelium grown on PDA was inoculated to 100 ml fungal growth medium (FGM) [22] and then grown at 25 °C on a shaking incubator at 110 rpm for 10 days. This preculture was then homogenized at 13,000 rpm for 8 s in a homogenizer (Ingenieurbüro CAT. X1030D, M. Zipperer GmbH, Germany), and 5 ml of the homogenized culture was used as inoculum. Subsequently, the second culture (100 ml) was grown for 10 days under the same condition as used for the first preculture. Amino acids were added to the second culture in order to know their supplementation effect on the ERG enhancement. The cultured mycelia were isolated from the culture medium by centrifugation at 6,000 rpm, then freeze-dried and stored at −70 °C for further analysis.

Determination of the Ergothioneine Content Levels of ERG were determined as described [23], with some modifications. Two hundred milligrams of freeze-dried samples was added into 20 ml of cold ethanolic extraction solution (10 mM dithiothreitol, 100 μM betaine, 100 μM 2-mercapto-1-methyl imidazole (MMI) in 70% ethanol) and mixed by vortexing and subsequent sonication for 3 min. A 1.0% ethanolic solution (4 ml) of sodium dodecyl sulfate was mixed by inverting and centrifuging. Ten milliliter of the supernatant was evaporated to dryness. The residue was then resuspended in 10 ml of distilled water (pH 7.3) and centrifuged. The resulting supernatant was injected into the HPLC (Thermo Electron C, Finnigan Surveyor System, Massachusetts, USA) equipped with Econosphere C18 column (4.6×250 mm, 5 μm; Alltech Associates, Illinois, USA). The mobile phase was 50 mM-sodium phosphate with 3% acetonitrile and 0.1% triethylamine adjusted to pH 7.3 with a flow rate of 0.7 ml/min. The ERG level was quantified by monitoring absorbance

at 254 nm with an ultraviolet (UV) detector, comparing the standard curve obtained from the authentic ERG (Sigma, St Louis, MO, USA).

Analysis of Amino Acids To determine the quantitative alterations of the amino acids in the liquid medium during the culture period, the mycelia were separated from the FGM medium by centrifugation at 4,500 rpm. Proteins were then removed from the medium by adding 10% w/v sulphosalicylic acid [24]. The contents of amino acids in the medium were quantified as described [25] using HPLC system equipped with Waters Pico-Tag column (3.9×150 mm, 5 μm; Waters, Massachusetts, USA) and UV detector.

Results and Discussions

ERG Accumulation in Mycelia and Fruiting Bodies

We compared the ERG levels in mycelia and fruiting bodies of the seven different mushroom species (Table 1). There were huge differences in the ERG contents of the seven mushrooms; the *Lentinus edodes* fruiting bodies contained approximately 30 times higher level of ERG than did *Ganoderma applanatum*. On the contrary, the ERG levels in mycelia were not different as much as shown in the fruiting bodies of the different mushroom species. Among the seven mushroom species, the mycelia of *G. neo-japonicum* and *Isaria japonica* produced the highest (0.72 mg/g DW) and lowest level (0.33 mg/g DW) of ERG, respectively, indicating that the differences of the ERG levels in mycelia of the different mushroom species were less than 2.1-fold at the maximum. It appeared that two *Ganoderma* species including *G. neo-japonicum* and *G. applanatum* produced higher level of ERG than did any other mushrooms tested. This result suggested that ERG was accumulated at the different levels in mycelia and fruiting bodies, respectively, depending on the mushroom species.

Various Effects of Methionine Supplementation on ERG Production

Various amino acids (16 mM) were added to the culture medium to examine their effects on the ERG production in the *G. neo-japonicum* mycelia (Table 2). The ERG contents were

Table 1 Ergothioneine contents in both mycelia and fruiting bodies of various mushroom species.

Mushroom species	Ergothioneine content (mg/g DW)	
	Mycelia	Fruiting bodies
Ganoderma neo-japonicum	0.72±0.10	0.07±0.00
Ganoderma applanata	0.69±0.02	0.06±0.02
Isaria japonica	0.33±0.05	0.25±0.02
Lentinus lepideus	0.49±0.01	1.37±0.02
Tricholoma matsutake	0.37±0.09	1.85±0.21
Sparassis crispa	0.42±0.04	1.10±0.09
Lentinus edodes	0.34±0.01	1.86±0.07

Mean±SD from three replications. The mycelia of seven mushroom species were cultured in the FGM medium for 10 days at 25 °C prior to determining the ergothioneine contents.

Table 2 Effect of various amino acids on the ergothioneine production in the *G. neo-japonicum* mycelia.

Amino acid	Ergothioneine content (mg/g DW)	Mycelia growth (g DW/L)	Total ergothioneine content (mg/L)
Control	0.72 ± 0.10^{c}	8.5 ± 0.2^{b}	6.08 ± 0.03^{c}
Asp	0.63 ± 0.12^{cd}	7.5 ± 1.1^{c}	4.75 ± 0.13^{cde}
Ser	0.48 ± 0.05^{d}	8.9 ± 0.2^{b}	4.24 ± 0.01^{de}
Cys	1.20 ± 0.16^{b}	8.4 ± 0.1^{b}	9.83 ± 0.02^{b}
Met	2.23 ± 0.06^{a}	6.1 ± 0.3^{d}	13.6 ± 0.02^{a}
His	0.79 ± 0.11^{c}	4.5 ± 0.0^{e}	3.58 ± 0.02^{e}
Leu	0.54 ± 0.02^{d}	9.9 ± 0.5^{a}	5.31 ± 0.01^{cd}
Pro	0.46 ± 0.08^{d}	9.0 ± 0.4^{b}	4.09 ± 0.04^{de}
Arg	0.19 ± 0.03^{e}	8.2 ± 0.0^{bc}	1.53 ± 0.00^{f}

Mycelia were cultured at 25 °C for 10 days in the FGM medium supplemented with 16 mM of amino acid, respectively. Data represent mean±SD from three replications. The same superscript letters indicate the treatments that do not differ statistically ($P<0.05$; Duncan's test).

significantly increased when 16 mM of either cysteine (Cys) or methionine (Met) was added to the culture medium, respectively. Particularly the addition of Met increased more than 3.1-fold of the ERG level per gram dry weight of mycelia compared to the control (no addition of amino acids). Although the mycelial growth was reduced 28% by the application of Met, total ERG content was 13.6 mg/l of culture medium, 2.2-fold increase compared to the control. This result suggested that the accumulation of ERG in the *G. neo-japonicum* mycelia was increased by the addition of Met, which is known to be involved in the biosynthesis of ERG [26].

It has long been known that ERG is synthesized from the amino acids such as histidine (His), Met, and Cys [26]. The intact His molecule is methylated to form hercynine, followed by sulfhydration of hercynine to form ERG [27]. His can also be transformed to thiolhistidine by Cys that is an immediate precursor of the sulfur atom of the thiolimidazole ring, followed by methylation of thiolhistidine to form ERG. Therefore, His is likely to be an essential molecule in the ERG biosynthesis. However, the addition of 16 mM His resulted in 45% reduction in total ERG content per liter culture compared to the control, due to the inhibition of the mycelia growth (Table 2). Therefore, we concluded that Met, among those three amino acids, might be the most efficient additive for the purpose of enhancing the ergothioneine (ERG) production in the *G. neo-japonicum* mycelia.

When various concentrations of methionine were added to the mycelial culture medium, the enhancing effect of the Met supplementation on the ERG level was observed at all concentrations of Met treated (Fig. 1a). Treatment with 0.5 mM Met showed 30% increase in the ERG production compared to the control (0.7 mg/g DW), and as the concentrations of Met increase up to 12 mM, the level of ERG was raised up to 2.3 mg/g DW. However, there was no significant difference between 4 and 12 mM Met treatments in terms of the ERG accumulation per gram DW of mycelia, suggesting that 4 mM Met might be the sufficient concentration to reach the maximum level of ERG in the *G. neo-japonicum* mycelia. On the other hand, the Met supplementation exhibited a negative effect on the mycelial growth (Fig. 1b). Although the low concentrations of Met (~1.0 mM) did not suppress the mycelial growth, more than 4 mM Met significantly inhibited their growth. Likewise, the inhibition of the mycelial growth was not significantly different between 4 and 12 mM Met treatments, resulting in total ERG contents reaching a plateau (Fig. 1c). Taking these results into consideration, we concluded that the addition of 4 mM Met was practically enough to pull up the ERG contents to the maximum level.

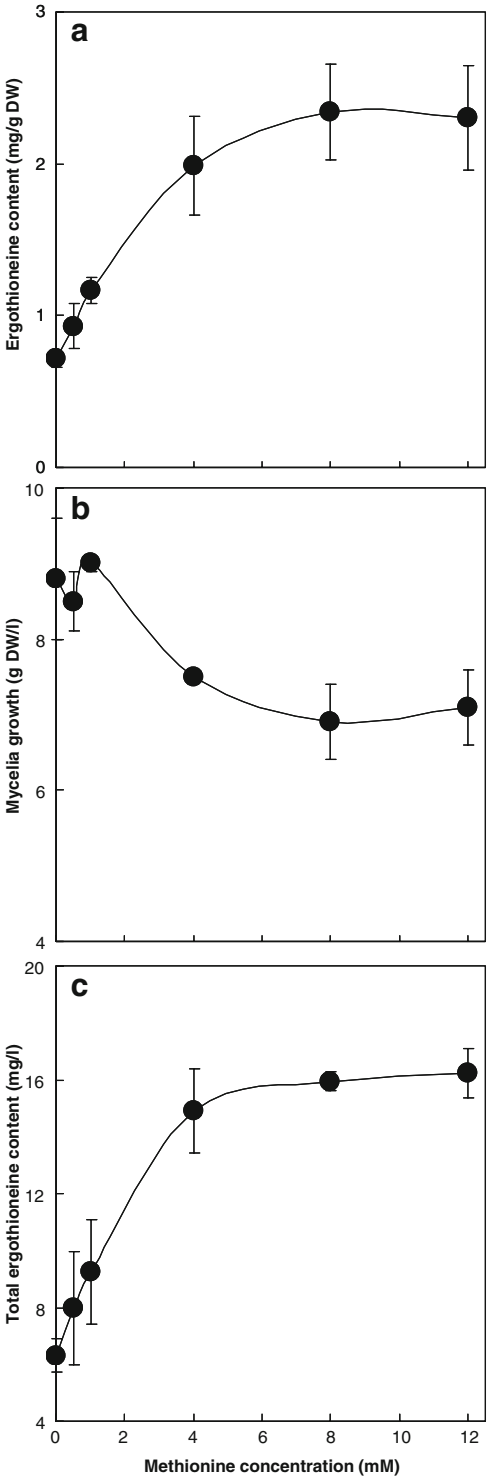

Fig. 1 Dose-dependent effects of methionine on both the ergothioneine production and the mycelia growth in the *G. neo-japonicum* mycelial culture. The mycelia were cultured in the FGM medium at 25 °C for 10 days before harvest. Total ERG content was calculated by multiplying the ERG content with the mycelial biomass. Data values indicate mean±SD from three replications

Since the ERG biosynthesis is affected primarily by three amino acids including Met, Cys, and His [27], those three amino acids (2 mM each) were added to the culture medium as single or combined treatments to find the best combination for enhancing the ERG production (Table 3). The control accumulated 6.4 mg of ERG per liter culture, while the single treatment of Met produced 13.2 mg of ERG per liter culture. The combined treatment of Met and Cys increased total ERG content to 15.2 mg/l, which was not significantly different from the Met treatment alone. In addition, the His treatment as single or combined had no effect on the ERG accumulation in the *G. neo-japonicum* mycelia. Therefore, this result indicated that the Met treatment alone enhanced the ERG level most efficiently compared to other treatments.

Effect of Yeast Extract on ERG Production

A number of studies have shown that most *basiomycetes* prefer complex organic nitrogen sources for their growth in submerged cultures [28]. Moreover, previous studies concerning the effect of C/N ratio (mass ratio) on cell growth showed that a higher C/N ratio was preferred for the *Cordyceps militaris* mycelial growth [21, 28]. Because the FGM medium contained yeast extract (YE; 2 g/l) as an organic nitrogen source, we tested the effect of YE on total ERG production in the mycelia grown in the medium supplemented with 4 mM Met (Fig. 2). Among various concentrations of YE, the reduced amount of YE (1 g/l) compared to the original level (2 g/l) yielded the higher level of total ERG, resulting in 15% increase in the mean value of total ERG production. However, the higher concentrations (3, 5, and 7 g/l) of YE decreased the ERG production. Therefore, when 4 mM Met was included in the FGM medium, the amount of YE needed to be reduced to increase total ERG production in *G. neo-japonicum* mycelia, indicating that C/N ratio might also impact on the ERG production.

Reduction in the Consumption Rates of Amino Acids by Met Supplementation

In order to know whether Met supplementation affected the consumption rates of other amino acids, we determined the levels of various amino acids remaining in the basal FGM medium after 10 days of the mycelial culture (Table 4). At day 0, the alanine concentration

Table 3 Combinational effects of three amino acids on the ergothioneine production in the *G. neo-japonicum* mycelia.

Amino acid	Ergothioneine content (mg/g DW)	Mycelia growth (g DW/L)	Total ergothioneine content (mg/L)
Control	0.76 ± 0.09^{b}	8.4 ± 0.2^{a}	6.37 ± 0.02^{c}
M	1.66 ± 0.21^{a}	8.3 ± 0.6^{a}	13.83 ± 0.13^{ab}
C	1.09 ± 0.09^{b}	8.4 ± 0.5^{a}	9.13 ± 0.44^{bc}
H	0.75 ± 0.05^{b}	8.4 ± 0.5^{a}	6.36 ± 0.02^{c}
MC	1.78 ± 0.19^{a}	8.5 ± 0.5^{a}	15.14 ± 0.10^{a}
MH	1.62 ± 0.38^{a}	8.2 ± 0.5^{a}	13.34 ± 0.18^{ab}
MCH	1.86 ± 0.53^{a}	8.3 ± 0.8^{a}	15.42 ± 0.41^{a}

Two millimolar of amino acids was added to the FGM medium, and the mycelia were then cultured at 25 °C for 10 days. Data represent mean±SD from three replications. The same superscript letters indicate the treatments that do not differ statistically ($P<0.05$; Duncan's test).

M Methionine, *C* cysteine, *H* histidine

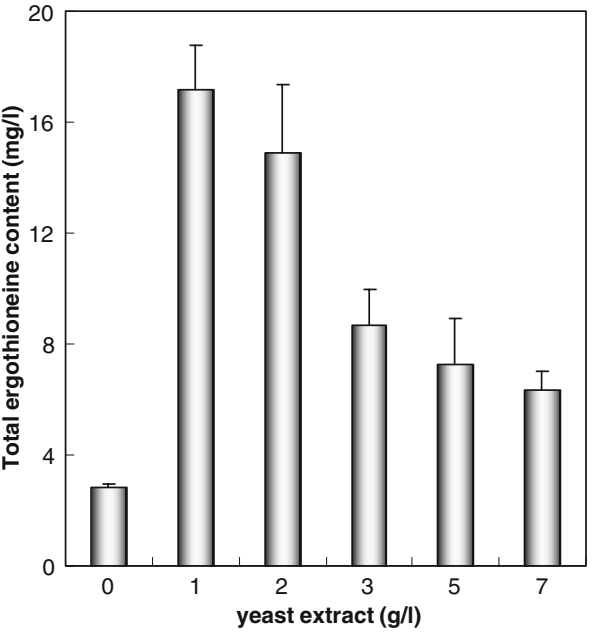

Fig. 2 Effects of yeast extract on total ergothioneine (ERG) production in the *G. neo-japonicum* mycelial culture, when 4 mM of methionine was supplemented. The mycelia were cultured in the FGM medium at 25 °C for 10 days before harvest. Total ERG content was calculated by multiplying the ERG content with the mycelial biomass. Data values indicate mean±SD from three replications

was the highest (1.0 mM) in the control, except for glutamic acid that was composed of the FGM medium as an essential nutrient. Among three amino acids involved in the ERG biosynthesis, the concentration of Met and His at day 0 was 0.2 and 0.07 mM, respectively, and Cys was negligible. Although 38% (1.56 mM) of the supplemented Met appeared to be consumed during 10 days of culture when compared to the Met level at day 0 (4.1 mM), the consumption rates of most amino acids were not as high as shown in the control culture, probably caused by the inhibition of mycelial growth. This is thus another indication that there is a negative correlation between mycelial growth and ERG production.

Table 4 Amino acid composition in the culture medium after 10 days of the *G. neo-japonicum* mycelial culture with or without 4 mM of methionine.

Amino acid	Control (0 mM Met; day 0)	Control (0 mM Met; day 10)	4 mM Met (day 10)
Asp	0.44±0.05[a]	0.13±0.01	0.27±0.01
Glu	6.54±0.16	1.41±0.03	4.51±0.01
Ser	0.67±0.02	0.10±0.03	0.37±0.00
His	0.07±0.01	0.00±0.00	0.09±0.01
Arg	0.23±0.02	0.00±0.00	0.02±0.00
Thr	0.15±0.02	0.03±0.01	0.04±0.00
Ala	1.01±0.04	0.02±0.00	0.21±0.00
Pro	0.25±0.13	0.09±0.00	0.24±0.00
Tyr	0.14±0.02	0.04±0.01	0.06±0.00
Val	0.62±0.03	0.31±0.01	0.42±0.01
Met	0.20±0.00	0.03±0.01	2.54±0.02
Cys	0.00±0.00	0.00±0.00	0.00±0.00
Ile	0.56±0.02	0.02±0.00	0.15±0.00
Leu	0.50±0.02	0.01±0.00	0.14±0.00

[a] Data (mM concentrations) represent mean±SD from three replications.

Conclusions

To the best of our knowledge, this was the first demonstration that the ERG production was enhanced via mycelial culture. We found that Met was an efficient additive for enhancing the ERG production through mycelial culture. Interestingly the supplementation of methionine increased the ERG levels but inhibited the mycelial growth, suggesting that there is a negative correlation between ERG production and mycelial growth. Furthermore, it provides the potential that this method is applicable to commercial-scale production of ERG through large-scale bioreactor.

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