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RELATIONSHIP BETWEEN THE SELENIUM, SELENOMETHIONINE, AND SELENOCYSTEINE CONTENT OF SUBMERGED CULTIVATED MYCELIUM OF *Lentinula edodes* (Berk.) ◈

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SUMMARY

To obtain extracts rich in the organic forms of selenium with putative cancer-preventive properties more effective than those of selenized-yeast, *Lentinula edodes* mycelia were cultivated in media enriched with selenium at concentrations from 0 to 20 μg mL^{-1}, by addition of sodium selenite either before inoculation or after three days of mycelial growth. The total selenium content of submerged cultivated mycelial biomass was determined by atomic absorption spectroscopy (AAS) and concentrations of selenomethionine and selenocysteine were determined by RP-HPLC. The concentration of selenium in the mycelia increased from 23 μg g^{-1} dry weight (d.w.) for mycelia cultivated in media not enriched in selenium to 1800 μg g^{-1} (d.w.) for mycelia cultivated in medium enriched with 20 μg mL^{-1} selenium. The amount of selenomethionine in the cultivated biomass increased in proportion to the selenium content of the mycelium (concentration range 23–289 μg g^{-1} d.w.) but the percentage of selenium accumulated as the selenoamino acid decreased. For selenium concentrations greater than 1 μg mL^{-1} the selenocysteine content of the mycelial biomass does not depend directly on the concentration of selenium in the medium.

INTRODUCTION

Selenium is a trace element of fundamental importance to human health, part of the antioxidant enzymes that protect cells against the effects

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of free radicals [1,2]. One important health effect of selenium not exclusively linked to its enzymatic functions is connected with functioning of the immune system. Selenium seems to be a key nutrient in cancer prevention and in inhibiting progression of HIV to AIDS [3–5]. It must be kept in mind that the biological activity of selenium is determined by its chemical form and the dose ingested. Selenium supplements in the form of inorganic compounds may not be as bioavailable as organically bound Se [6,7]. Clark et al. reported a putative role of selenized yeast in cancer prevention [8]. Supplementation of people's diet with selenized yeast (Se-yeast) is capable of reducing overall cancer morbidity by almost 50%. Analytical speciation studies have shown that the bulk of selenium (approx. 85%) in Se-yeast was in form of selenomethionine [9].

For higher mushrooms selenium accumulation is comparable with that of yeast [10]. Similarly to yeast, selenomethionine was the main selenium compound detected in aqueous extracts of the fruit bodies of *Agaricus bisporus* and *Lentinula edodes* grown on medium (compost) supplemented with selenium [11,12]. Cell cultures of higher mushrooms, cultivated in selenium-enriched media, are potentially as good a source of the highly bioactive and safe organic species of selenium as selenized yeast or the fruit bodies of higher mushrooms. Our previous work has shown that the submerged cultivated mycelium of *Lentinula edodes* very effectively accumulates selenium from cultivation medium. The total concentration of selenium in submerged cultivated mycelial biomass, as determined by atomic absorption spectroscopy (AAS), expressed in mg per 100 g mycelial dry weight (d.w.), rose from 0.1 mg (mycelium cultivated in unmodified medium) to 5000 mg (mycelium cultivated in medium enriched with 100 μg mL^{-1} selenium) [13]. Speciation investigations of selenium at these high concentrations accumulated in selenated mycelial biomass, and determination of the relationship between the total selenium content of the cultivated mycelium and the concentration of individual Se compounds seem to be interesting problems.

Several different techniques have been used for speciation of selenium compounds [14,15]. Because our objective was to determine the relationship between the concentrations of three amino acids (selenomethionine, methionine, and selenocysteine) in mycelia containing different total amounts of selenium, we adapted an RP-HPLC method with *o*-phthaldialdehyde derivatization (OPA method) widely used for analysis of amino acids. The main advantages of this method are good selectivity and efficiency and low detection limit [16–18].

Lentinula edodes (Shii-take mushroom), selected for our experiments, is a medicinal mushroom from which the extracted, highly purified, polysaccharide fraction (lentinan) is an approved drug used in cancer treatment and in AIDS research [19–23]. Hot water extracts of *L. edodes* mycelium (LEM) and culture media (LAP) have strong antitumor activity [24,25]. The objective of this study was, therefore, to optimize submerged culture conditions for good mycelium growth of *L. edodes* and high selenium content of the mycelial biomass, possibly in the form of the highly bioavailable selenoamino acids. We hoped that high concentrations of the organic forms of selenium in submerged cultivated mycelial biomass would enhance the anticancer activity and inhibition of HIV progression activity of mushroom extracts.

EXPERIMENTAL

Equipment

A GBC Avanta Ultra Z automatic, single-beam atomic-absorption spectrometer with autosampler, longitudinal Zeeman-effect background-correction system, and transversely heated graphite atomizer was used for determination of the total selenium content of cultivated mycelia. A coded 1.5-inch selenium hollow-cathode lamp (CPI Int.), operated at 10 mA, was used for determination of selenium at 196 nm; the slit width was 2.0 nm.

Microwave digestion was performed by use of a Plazmotronika BM-1s closed-vessel microwave system.

HPLC analysis of selenoamino acids was performed with a Shimadzu (USA) instrument including gradient system (SCL-10AVP controller, two LC-10AT vp pumps, CTO-10AC vp oven) and fluorescence detector (LaChrom L-7480; Merck–Hitachi, Darmstadt, Germany). Compounds were separated on a 250 mm × 4 mm Phenomenex Luna 2 C_{18} column with appropriate guard column.

Mushroom cultures were cultivated in a rotary shaker (New Brunswick Scientific, Edison, NY, USA).

Materials

L-Selenomethionine and an L-amino acid standard kit for use as external standards were obtained from Sigma (St Louis, MO, USA). A standard solution of carboxymethylselenocysteine was prepared from seleno-

L-cystine (Sigma) by using the same procedure for reductive carboxymethylation as for carboxymethylation of mycelial proteins.

HNO_3 solution (65%) and Suprapur quality water (Merck) were used for preparation of samples. Selenium atomic absorption standard solution (1020 μg mL^{-1} in 1% HNO_3; Aldrich) was used for preparation of selenium standards. Nickel(II) nitrate hexahydrate p.a. (Fluka) was used as a chemical modifier for selenium.

Sodium phosphate monobasic monohydrate, sodium hydroxide, boric acid, hydrochloric acid, sodium selenite, mercaptoethanol, (GR for analysis), acetonitrile (LC grade), and methanol (LC grade) were obtained from Merck.

Microorganism and Cultivation Media

The strain of *Lentinula edodes* used in this study was taken from the medicinal mushroom strain collection of the Department of Drug Technology of the Medical University of Warsaw (Poland). It originated in Korea. In nature it grew in the Pobwang Peak area, near Mt Myohyang.

The seed culture was grown in 500-mL flasks containing 150 mL basal medium (glucose 5% (*w*/*v*), yeast extract 1%, casein hydrolysate 1%, KH_2PO_4 0.1%), at 26°C, on a rotary shaker at 110 rev min^{-1} for seven days.

The fermentation medium selected for experiments on accumulation of selenium and for biosynthesis of selenoamino acids by *Lentinula edodes* submerged cultured mycelial biomass was partially composed of waste products from the food industry (beet molasses 10%, corn steep liquor 0.15%, grain wort 5%) and also contained 0.3% (*w*/*v*) KH_2PO_4.

Procedures

Fermentation

Culture medium was enriched in selenium by addition of sodium selenite (Na_2SeO_3). Concentrations of selenium were in the range 0 to 20 μg mL^{-1} (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 20 μg mL^{-1}). The sodium selenite was added to the medium either before inoculation or after 3 days of mycelial growth. The initial pH of the medium was 6.5.

Mycelia were grown in shake-flask culture in 500-mL flasks containing 200 mL medium. The fermentation medium was inoculated with 5% (*v*/*v*) seed culture. Cultures were incubated at 26°C in a rotary shaker at 120 rev min^{-1} for 14 days. Mycelia were harvested by filtration, washed three times with distilled water, and dried at 60°C. All experiments were conducted at least three times to determine reproducibility. Total selenium

content was determined for each sample of mycelium by use of the atomic-absorption spectrometry (AAS). Methionine and selenomethionine content were determined by high-performance liquid chromatography (HPLC).

Determination of Selenium in Mycelium and Culture Media by Atomic-Absorption Spectrometry

Digestion Procedure

A sample (0.02 g) of mycelium dried at 100°C was placed in a Teflon crucible and digested with 3 mL 65% HNO_3 in microwave mineralizer according to the program. When cool the solution was diluted to 25 mL with 1% HNO_3 in deionized water. If necessary, the solutions were diluted several times in a few steps with 5 mg mL^{-1} $Ni(NO_3)_2$ in 1% HNO_3. A blank digest was performed in the same way. Selenium standard solutions (40, 100, and 200 ng mL^{-1} Se) were prepared under the same conditions.

Analytical Procedure

Selenium standard or sample solution (10 μL) was wet-injected into the graphite furnace. The time and temperature program is shown in Table I. Slow solution uptake and slow solution injection conditions were selected. Three standard additions (four replicates of each) and peak-height measurements were used for quantification [25].

Table I

Furnace program for determination of selenium in *Lentinula edodes* mycelium using Ni(II) as chemical modifier

Stage	Temperature (°C)	Ramp (° s^{-1})	Hold time (s)	Air flow (L min^{-1})
Drying	110	10	10	3.0
	130	1	10	3.0
Ashing	800	10	10	3.0
	800	1.0	1.0	0.0
Atomizing	2100	0.5	0.5	0.0
Cleaning	2300	0.1	0.9	3.0

Determination of Methionine and Selenomethionine in Mycelial Biomass

Sample Preparation

Because methionine partially decomposes during acidic hydrolysis of mycelial proteins in the presence of carbohydrates and heavy metal ca-

tions, alkaline hydrolysis in 5 M NaOH was preferred. NaOH solution (5 M, 10 mL) was added to the pulverized mycelium (0.1 g) in an ampoule and the mixture was purged with nitrogen for 30 min. The ampoule was sealed, full of nitrogen, then hydrolysis was performed at 110°C for 20 h. The mycelial hydrolysate was transferred to a 100-mL graduated flask and diluted to volume with LC-grade water. Derivatization was performed as described in the Merck application note [14].

RP-HPLC of Mycelial Hydrolysates

Selenomethionine and methionine in mycelial hydrolysates were determined by high-performance liquid chromatography of the *o*-phthaldialdehyde derivatives (OPA method) [16,17,26] by using retention time standards and standard addition to the samples.

The mobile phase was a gradient prepared from 100 mM sodium acetate buffer, pH 7.2, containing 0.1% acetonitrile (component A) and methanol (component B). The gradient breakpoints (min/%B) were: (0/45), (6/45), (12/50), (20/63), (38/63), (49/100), and (55/100). The temperature was 32°C, the injection volume 20 µL, and the flow rate 1.2 mL min^{-1}. The excitation and emission wavelengths were 340 and 435 nm. The retention times of methionine and selenomethionine were 34.7 and 40.2 min (Fig. 1).

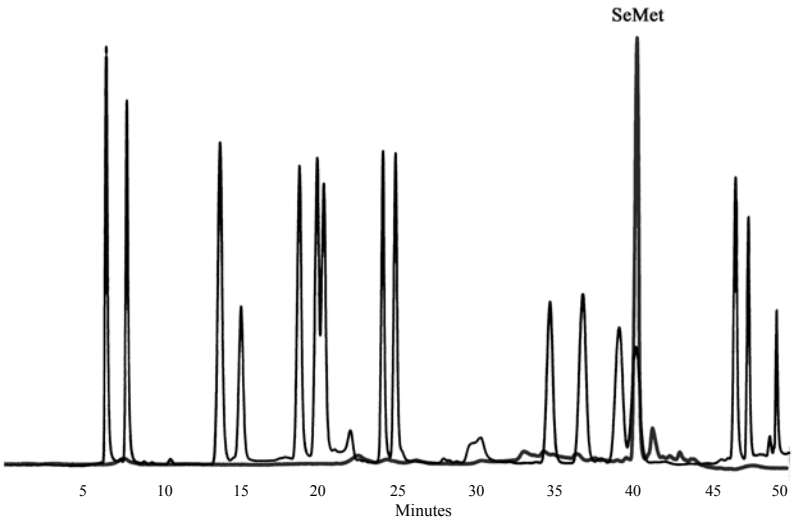

Fig. 1

Chromatograms obtained from selenomethionine standard solution (0.002 µmol mL^{-1}) and from a mixture of a mycelial hydrolysate and selenomethionine standard solution

Determination of Selenocysteine (SeCys) in Mycelium

Sample Preparation

Selenocysteine was determined in mycelium hydrolysate after derivatization with *o*-phthaldialdehyde [17,18]. Selenocysteine, similarly to cysteine, is very easily oxidized and decomposes during hydrolysis of mycelial proteins. The method normally used for cysteine – formation of cysteic acid by treatment with performic acid – results in complete decomposition of selenocysteine [17]. After carboxymethylation, however, selenocysteine is stable for several weeks. For carboxymethylation of mycelial proteins before acidic hydrolysis an adaptation of the method described by Jones [18] was used. Mycelial biomass homogenate (0.05 g), KBH_4 solution (2 mg mL^{-1}, 0.5 mL), mercaptoethanol (50 μL), and iodoacetic acid solution (200 mM, 2 mL) were stirred for 24 h under nitrogen in the dark. The mixture was then evaporated, HCl solution (6 M, 10 mL) was added to the dry residue, and the mixture was purged with nitrogen in an ampoule for 30 min. The ampoule was filled with nitrogen and sealed, and hydrolysis was performed at 110°C for 20 h.

RP-HPLC of Mycelial Hydrolysates

Carboxymethylselenocysteine in mycelial hydrolysates was determined in the same manner as selenomethionine, by reversed-phase HPLC of the *o*-phthaldialdehyde derivative (OPA method) [16–18,26], using retention time standards and standard additions to the sample.

A standard solution of carboxymethylselenocysteine was prepared from seleno-L-cystine (Sigma) using the same procedure as for reductive carboxymethylation [17,18].

The mobile phase was a gradient prepared from 100 mM sodium acetate buffer, pH 7, containing 0.1% acetonitrile (component A) and methanol (component B). The gradient breakpoints were (min/%B): (0/22), (15/22), (20/100), (22/100). The flow rate was 1.2 mL min^{-1}, the temperature 32°C, and the injection volume 20 μL. Under these conditions the retention time of carboxymethylselenocysteine was 8.7 min (Fig. 2.).

RESULTS

The efficacy of cultivation of mushroom mycelium in the medium selected was good. The biomass accumulation recorded after cultivation for 14 days (in medium not enriched with selenium) was 7–8 g d.w. L^{-1} cultu-

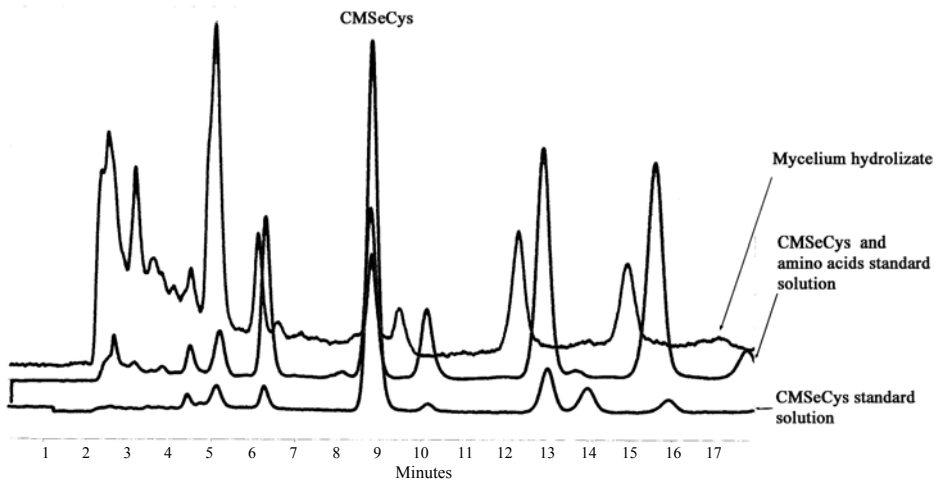

Fig. 2

Chromatograms obtained from carboxymethylselenocysteine standard solution (0.003 μmol mL^{-1}), a mixture of carboxymethylselenocysteine and protein amino acid standard solution, and a *Lentinula edodes* mycelium sample hydrolysed after reductive carboxymethylation

re medium. Mycelial growth was only weakly affected by increasing the concentration of selenium in the medium. Reduction of mycelial growth by selenium-containing compounds was observed only for cultivation in media enriched with selenium before inoculation (Tables II and III).

Uptake of selenium by the submerged cultivated mycelium of *Lentinula edodes* was very effective. According to our previous observations the effect on the selenium uptake of the time of addition of the selenium-containing compound to the cultivation medium (i.e. before inoculation or after mycelial growth for 3 days) is significant only for concentrations of selenium in the medium greater than 30 μg mL^{-1} [13]. Thus when the concentration of selenium in the medium was relatively low (0.5 to 20 μg mL^{-1}), significant differences between concentrations of selenium in mycelia harvested from media enriched in selenium at different times was not observed (Tables II and III). Addition of 20 μg mL^{-1} selenium to the medium yielded 1815 μg selenium g^{-1} (d.w.) of mycelial growth (Fig. 3, Tables II and III).

The selenomethionine content of the cultivated mycelia increased as the amount of selenium added was increased (Fig. 3). For low concentrations of selenium in the mycelium (20 μg g^{-1}) almost 70% of the selenium content was accumulated as selenomethionine. For concentrations of selenium in the mycelium greater than 400 μg g^{-1}, however, the propor-

Table II

Relationship between the concentration of selenium in the cultivation medium and mycelial growth, and selenomethionine (SeMet) and methionine (Met) content of mycelia cultivated in media enriched in selenium before inoculation

Concn of Se in medium (μg mL^{-1})	Mycelial growth (g L^{-1}) (*RSD*)	Concn of Se in mycelia (μg g^{-1} d.w.) (*RSD*)	Concn of SeMet in mycelia (μg g^{-1} d.w.) (*RSD*)	Concn of Met in mycelia (μg g^{-1} d.w.) (*RSD*)	Amount of methionine transformed into selenomethionine* (%)
0	7.54 (0.31)	23.2 (0.13)	2.96 (0.35)	5933.0 (0.15)	0.35
0.5	5.16 (0.08)	60.6 (0.08)	54.9 (0.83)	5411.0 (0.11)	0.77
1	5.42 (0.19)	90.0 (0.28)	74.4 (0.14)	5256.2 (0.09)	1.08
3	5.42 (0.01)	416.7 (0.26)	88.9 (0.07)	5498.3 (0.02)	1.23
5	5.46 (0.27)	571.1 (0.14)	52.2 (0.35)	5192.9 (0.21)	0.76
7	8.63 (0.15)	637.7 (0.34)	101.4 (0.09)	5132.2 (0.04)	1.50
8	5.89 (0.16)	933.2 (0.25)	124.0 (0.29)	5310.2 (0.08)	1.78
9	6.22 (0.33)	1009.1 (0.08)	151.6 (0.16)	4278.5 (0.15)	2.69
10	5.21 (0.12)	1180.8 (0.26)	205.2 (0.29)	4533.7 (0.03)	3.44
20	5.74 (0.19)	1669.9 (0.37)	289.0 (0.21)	6135.0 (0.07)	3.58

Results are average values from three experiments

* Calculated on the basis of the number of moles of SeMet and Met in a sample

Table III

Relationship between the concentration of selenium in the cultivation medium and mycelial growth, and selenomethionine (SeMet) and methionine (Met) content of mycelia cultivated in media enriched in selenium after three days of cultivation

Concn of Se in medium (μg mL^{-1})	Mycelial growth (g L^{-1}) (*RSD*)	Concn of Se in mycelia (μg g^{-1} d.w.) (*RSD*)	Concn of SeMet in mycelia (μg g^{-1} d.w.) (*RSD*)	Concn of Met in mycelia (μg g^{-1} d.w.) (*RSD*)	Amount of methionine transformed into selenomethionine* (%)
0	7.87 (0.22)	27.0 (0.13)	2.30 (0.30)	6005.3 (0.05)	0.29
0.5	6.12 (0.40)	61.6 (0.09)	81.2 (0.15)	4490.7 (0.19)	1.37
1	8.01 (0.09)	90.4 (0.08)	72.5 (0.47)	6974.3 (0.09)	0.79
3	7.81 (0.17)	325.5 (0.21)	131.0 (0.16)	8096.4 (0.22)	1.23
5	10.86 (0.05)	415.1 (0.08)	164.9 (0.10)	6410.0 (0.03)	1.96
7	8.92 (0.27)	651.9 (0.22)	154.9 (0.38)	7855.9 (0.14)	1.50
8	9.98 (0.10)	644.2 (0.13)	203.6 (0.07)	8447.4 (0.28)	1.83
9	11.52 (0.17)	1173.5 (0.46)	203.5 (0.09)	6409.1 (0.15)	2.41
10	7.34 (0.53)	1289.0 (0.38)	198.8 (0.13)	6791.0 (0.03)	2.23
20	6.72 (0.30)	1815.0 (0.30)	265.4 (0.14)	5876.2 (0.18)	3.43

Results are average values from three experiments

* Calculated on the basis of the number of moles of SeMet and Met in a sample

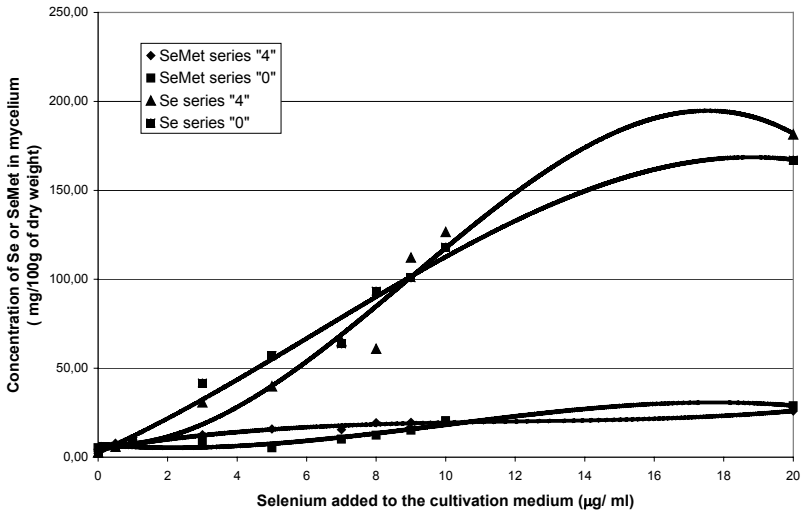

Fig. 3

Relationship between selenium and selenomethionine concentrations in mycelia from media enriched in selenium both before inoculation (series "0") and after cultivation for three days (series "4")

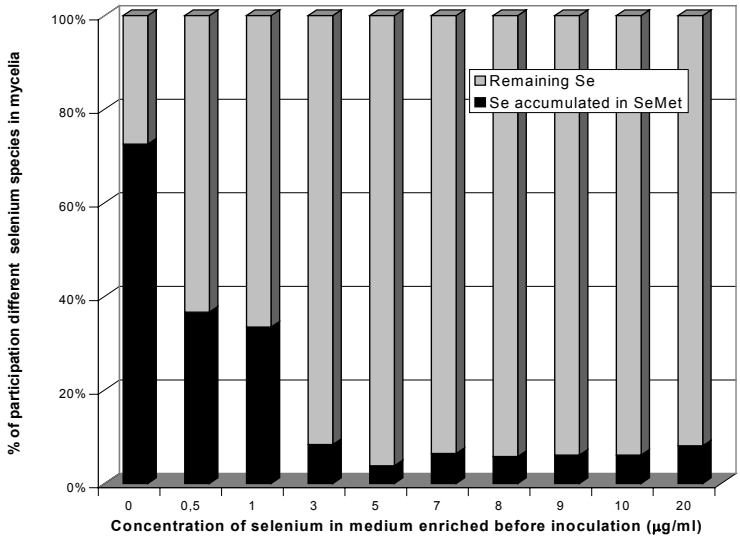

Fig. 4

Selenium accumulated in mycelium as selenomethionine as a percentage of total selenium content (series "0")

tion of selenomethionine was only almost 6% (Figs 4 and 5). The percentage of methionine transformed into selenomethionine rose in proportion to the total selenium content of *L. edodes* mycelial dry weight (Tables II and III) and reached almost 3%.

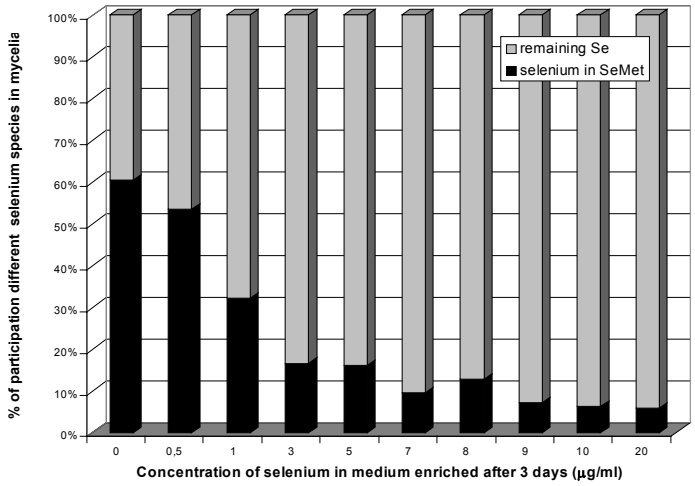

Fig. 5

Selenium accumulated in mycelium as selenomethionine as a percentage of total selenium content (series "4")

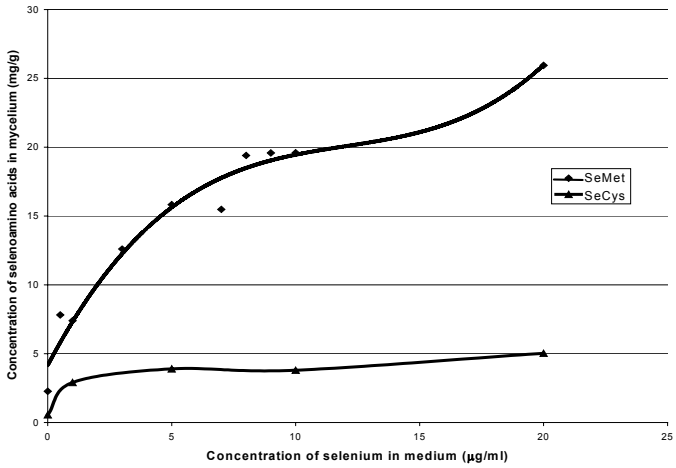

Fig. 6

Relationship between the concentration of selenium in the medium and the selenomethionine (SeMet) and selenocysteine (SeCys) content of mycelial dry weight

The selenocysteine content of mycelial dry weight seemed to be almost stable for mycelia cultivated in media enriched in selenium at concentrations higher than 1 μg mL^{-1}. The amount of selenocysteine in mycelium cultivated in medium containing 20 μg mL^{-1} selenium reached almost 50 μg g^{-1} d.w. (Fig. 6).

CONCLUSIONS

Amino acid analysis by RP-HPLC with *o*-phthaldialdehyde derivatization is a method with good selectivity and efficiency. The detection limit for most OPA amino acid derivatives is in range 25–50 fmol. The method can be successfully used for determination of selenomethionine and selenocysteine in hydrolysates of proteins. The problem of the oxidative decomposition of SeCys during protein hydrolysis can be avoided by carboxymethylation of proteins before hydrolysis. The standard HPLC equipment and relatively cheap C_{18} columns used are major advantages of this method. Selenomethionine and selenocysteine standards are commercially available. This method was successfully used for determination of the relationship between the selenium, selenomethionine, and selenocysteine content of submerged cultivated mycelium of the medicinal mushroom *Lentinula edodes*. The results indicate that in medium not fortified with selenium over 80% of the selenium in the mycelium is accumulated as selenoamino acids – over 70% as selenomethionine and approximately 10% as selenocysteine. Similar data were obtained for selenized yeast. For mycelia rich in selenium the proportion of selenium accumulated as selenoamino acids decreases. Speciation of these selenium compounds is the next important objective of this research because of their putative role in cancer prevention.

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