Selenium Accumulation in Mycelia of *Flammulina velutipes* during Fermentation Determined by RP-HPLC

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Z. Naturforsch. **64 c**, 382–386 (2009); received January 5/February 16, 2009

A method to estimate the content of selenium in organics was introduced based on reversed phase-high performance liquid chromatography (RP-HPLC). The maximum absorption peak of piazselenol was at 330 nm and the optimized temperature and pH value were 40 ºC and 2.8, respectively. The minimum detection concentration of selenium(IV) was 0.06 μg/mL and the measurable range was 0.12–12.0 μg/mL. The organic selenium accumulation in golden needle mushroom (*Flammulina velutipes*) mycelia was obtained by subtracting the amount of inorganic selenium from that of total selenium. The organic selenium accumulation of various inoculation amounts showed that organic selenium accumulation in a unit volume of the fermentation broth was positively related the inoculation amount. Compared with the methods reported previously, the method used here is simple, reliable and less toxic.

Key words: Flammulina velutipes, Mycelia, RP-HPLC, Selenium

Introduction

Selenoproteins, including selenium-dependent enzymes, play an important role in human health, particularly in relation to the immune response (McKenzie *et al.*, 1998), antioxidant action (Tapiero *et al.*, 2003), and cancer prevention (Abdulah *et al.*, 2005; Zeng and Combs Jr., 2008). So, the trace mineral selenium is of fundamental importance for organism and human health. Selenium consumed in foods and supplements exists in a number of inorganic and organic forms, and the latter is often preferred for its less acute toxicity (Rayman, 2000).

For the determination of selenium, many methods had been reported, such as gas chromatography-mass spectrometry (GC-MS) (Go'mez-Ariza *et al.*, 1999; Iscioglu and Henden, 2004), high performance liquid chromatography of acid ion-pairing agents combined with inductively coupled plasma and electrospray ionization mass spectrometry (HPLC-ICP-MS) (Kotrebai *et al.*, 2000), high performance liquid chromatography combined with hydride generation and atomic fluorescence spectrometry (HPLC-UV-HG-AFS) (Mazej *et al.*, 2006), electrothermal atomic absorption spectrometry (ET-AAS) (Izgi *et al.*, 2006), micellar liquid chromatography (Kulikov, 2007), and spectrophotometry (Kumar *et al.*, 2008). Of those, AAS has high detection limits but lacks precision, hydride generation (HG)-AAS suffers from copper and arsenic interference and also requires careful sample preparation (MacLeod *et al.*, 1996), spectrophotometry requires toxic reagents, such as 4-aminopyridine and NEDA (Kumar *et al.*, 2008), micellar liquid chromatography has limited measurable ranges (Kulikov, 2007), HPLC-ICP-MS and HPLC-UV-HG-AFS depend on complex instruments, and GC-MS depends on complex derivatization reactions, more than that, isobaric interference of $^{40}Ar_2$ with the most widespread isotope ^{80}Se (49.6%) is serious in MS analysis (Tie *et al.*, 2007). Here, we will introduce a simple method to determine the selenium content based on RP-HPLC.

Golden needle mushroom (*Flammulina velutipes*) is a good source of carbohydrates, proteins, fibers, essential amino acids and minerals (Smiderle *et al.*, 2008). Additionally, it contains many selenium compounds (Tie *et al.*, 2007). To assess the selenium accumulation capacity of golden needle mushroom mycelia during fermentation, it is necessary to determine the selenium content by a simple method.

0939–5075/2009/0500–0382 $ 06.00

Material and Methods

Instrumentation

A UNICO WFZ UV-2100 spectrophotometer with two 1.0-cm matched colorimetric dishes was used for all absorption measurements. A high performance liquid chromatograph (Agilent Model 1200, Santa Clara, USA) and an Agilent chromatographic column (XDB-C_{18}, 4.6 × 150 mm, 5 μm) were used for the analysis of selenium. A milestone digestion apparatus (ETHOS E, Italy Milestone Company, Milan) was used for dispelling selenium compounds in mycelia of golden needle mushroom.

Reagents

All chemicals used were of analytical reagent grade. All solutions were filtered through a membrane filter (0.45 μm) before HPLC. The methanol for HPLC was chromatographic pure.

A stock solution containing 1.0 mg/mL selenium was prepared by dispelling 100 mg selenium powder (AR Tientsin, China) in a solution of HNO_3 and H_2O_2 (8:1) reducing in hydrochloric acid. Then the residue was dissolved in 100 mL deionized double distilled water. A working solution of selenium(IV) was prepared for further dilution. A 2.0 mg/mL 1,2-diaminobenzene solution was prepared by dissolving 200 mg 1,2-diaminobenzene (AR Shanghai, China) in 100 mL double distilled water. The solution concentrations of disodium hydrogen phosphate and citric acid were 0.2 mol/L and 0.1 mol/L, respectively.

Optimum procedure for determination of selenium(IV)

Selenium(IV) reacted with 1,2-diaminobenzene solution in acid medium. The range of scanning wavelength was 250 nm ~ 380 nm. The optimized CH_3OH/H_2O mixture had a ratio of 9:16 (v/v). The flow velocity was 0.8 mL/min. The effects of temperature (30–50 ºC), pH value (2.4–3.2), and various ions on the reaction (1 μg/mL Se) were evaluated by RP-HPLC.

Determination of selenium(IV) in mycelia of golden needle mushroom

Mycelia of golden needle mushroom (*Flammulina velutipes*) were from CCDM (Culture Collection of Department of Microbiology) of Huazhong Agricultural University, Wuhan, China. Various amounts (fresh weight) of golden needle mushroom mycelia were inoculated in potato liquid medium (20% potato, 2% glucose), after mycelia were activated, and sodium selenite (0.075 mM, by filter-sterilization) was added to the fermentation broth. Then mycelia were collected after a certain period of culture.

Digestion is an important step in the experiment, and the digestion efficiency influences the sensitivity of the determination of selenium(IV). Several pre-treatment methods were reported (Gao *et al.*, 2001; Schloske *et al.*, 2002), and the microwave-assisted wet digestion procedure based on the mixture HNO_3/H_2O_2 is an improved method (Lavilla *et al.*, 2007). Being dried to constant weight, fresh mycelia of golden needle mushroom were placed in the digestion apparatus with a solution of concentrated HNO_3/H_2O_2 (8:1, v/v, 9 mL) for a 20-min dispelling. Then this mixture was heated until it was clear under a fume hood. After adding 4 M HCl (10 mL), the solution was reheated until it was clear. Then deionized, double distilled water was added, and the solution was transferred into a 10-mL measuring flask.

Results and Discussion

Piazselenol, as the reaction product from 1,2-diaminobenzene and selenite, had a maximum absorption peak at 330 nm (Fig. 1), which had been used as the detection wavelength. It is worthwhile to note that piazselenol and 1,2-diaminobenzene were efficiently separated by RP-HPLC (Fig. 2), which implied that the interference of substrate with product was slight, and it is the basis for our motivation to use RP-HPLC as a simple method to determine the selenium content.

The optimal temperature and pH value are shown in Table I. It is obvious that increasing temperature and decreasing pH value are favourable to the formation of piazselenol; the optimized temperature and pH value were 40 ºC and 2.8, respectively. In addition, Table II shows the tolerance concentrations of different ions on the reaction with RSD ≤ 5%. From Table II, we can conclude that the main interfering ions in the reaction (as shown in Fig. 1) are Cr^{3+}, Fe^{2+}, and Cu^{2+}, which were not imported into the fermentation broth.

Based on the optimal conditions mentioned above, the selenium contents in selenized golden needle mushroom mycelia were determined by

Table I. The effect of temperature and pH value on the reaction.

Parameter		Peak area				
		25 min	50 min	75 min	100 min	125 min
Temperature	30 °C	135.21[b]	245.33	318.53	373.65	432.01
	40 °C	180.21	345.55	419.65	461.71	496.56
	50 °C	201.32	374.31	433.56	470.09	503.21
pH	3.2	126.35	236.73	340.36	416.32	460.89
	2.8	198.65	345.55	412.65	461.71	495.03
	2.4	216.35	362.36	428.32	472.55	501.32

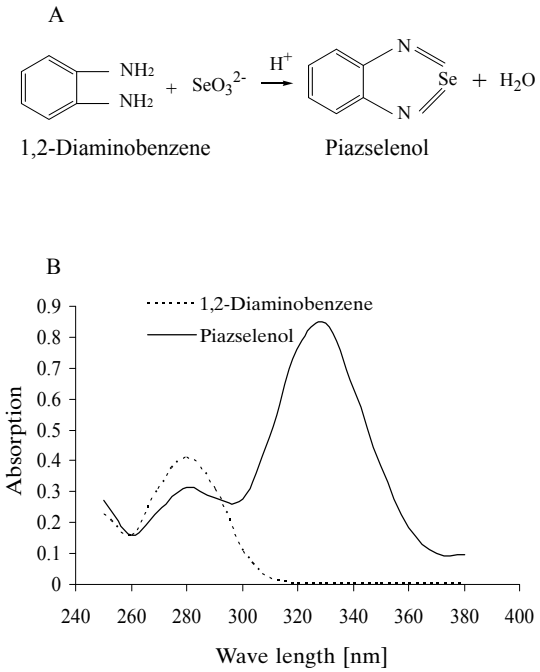

Fig. 1. (A) Synthesis of piazselenol by 1,2-diaminobenzene and selenite in acid medium. (B) Absorption spectrum of 1,2-diaminobenzene and piazselenol.

Table II. The tolerance concentration of different ions.

Ion	Tolerance concentration [μg/mL]
K^+, Na^+, SO_3^{2-}, SO_4^{2-}, Cl^-	1000
Mn^{2+}, Fe^{3+}, Zn^{2+}, Ca^{2+}, Mg^{2+}	100
Cu^{2+}, Fe^{2+}	50
Cr^{3+}	20

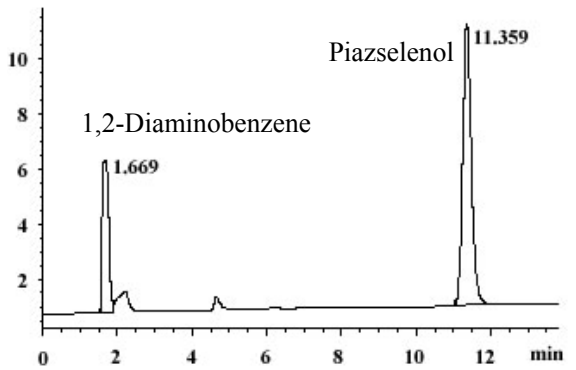

Fig. 2. Separation of piazselenol and 1,2-diaminobenzene by RP-HPLC.

RP-HPLC. The minimum detection concentration of selenium(IV) was 0.06 μg/mL (signal to noise ratio was 3). The standard curve of piazselenol containing 0.12–12.0 μg/mL selenium(IV) was drawn, and the regression equation was $y = 216.72x + 10.506$ with $R^2 = 0.9992$. Repeated experiments showed good reproducibility for peak area and transport time with RSD < 5% ($n = 6$), and a recovery ratio of 96.4% ~ 103.8%. The organic selenium content in mycelia was obtained by subtracting the amount of inorganic selenium from that of total selenium. The organic selenium accumulation of various inoculation amounts is shown in Fig. 3. As for the fermentation broth in unit volume, a significant positive correlation was observed between inoculation amount and organic selenium accumulation. The best organic selenium accumulation capacity (organic selenium in mycelia/selenium added to the fermentation broth, %) of golden needle mushroom mycelia is the inoculation amount with 5% (see Fig. 3). Higher inoculation amounts can lead to higher accumulation capacity, but the increment is slight. On the other hand, as shown in Fig. 3, the per-

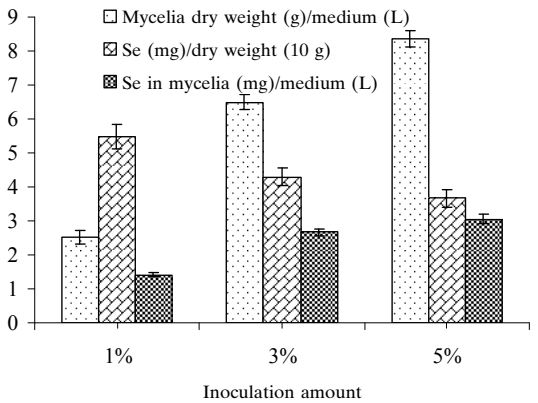

Fig. 3. Effects of inoculation amounts on selenium accumulation in golden needle mushroom mycelia.

centage content of organic selenium in fermentation products (*i.e.* mycelia) inversely correlated with the inoculation amount.

Up to this point, the selenium accumulation in golden needle mushroom mycelia was determined by a simple and reliable method based on RP-HPLC, which had good reproducibility and precision. Although many techniques selectively detecting and identifying selenium compounds and hyphenated techniques based on AAS or AFS became a trend (Capelo *et al.*, 2006; Uden *et al.*, 2004), in point of the total selenium determination, a simple, reliable and less toxic method is needed. Not like spectrophotometry (Kumar *et al.*, 2008), HPLC-ICP-MS, and HPLC-UV-HG-AFS, the method used here avoided complex instruments and toxic reagents and has a wide measuring range (0.12–12.0 μg/mL). More than that, it is a simple method to determine the whole organic selenium content including selenoprotein, Se–Met and Se–Cys by subtracting the content of inorganic selenium from that of total selenium.

Acknowledgement

This work was supported by Program for New Century Excellent Talents in University (NCET). The authors are grateful to Ms. Cheng, Analytical and Testing Centre, Huazhong University of Science and Technology, Wuhan, China for supplying the digestion apparatus.

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