Anal Bioanal Chem (2004) 379: 861–866
DOI 10.1007/s00216-004-2670-6

ORIGINAL PAPER

**Yasumitsu Ogra · Kazuya Ishiwata
Jorge Ruiz Encinar · Ryszard Łobiński
Kazuo T. Suzuki**

Speciation of selenium in selenium-enriched shiitake mushroom, *Lentinula edodes*

Received: 13 February 2004 / Revised: 16 April 2004 / Accepted: 29 April 2004 / Published online: 17 June 2004

Abstract The major selenium compound in an aqueous extract of the most popular mushroom in Eastern Asian countries, shiitake (*Lentinula edodes*), fortified with selenium (Se) was identified by means of hyphenated techniques, i.e. HPLC-inductively coupled argon plasma mass spectrometry and HPLC-electrospray ionization mass spectrometry (HPLC–ICP MS and HPLC–ESI MS). Sixty-eight per cent of the total Se in the selenized shiitake was extracted with water, and 49.8% of the Se in the water extract was eluted in the high molecular mass fraction (>40,000 kDa) before incubation at 37 °C. After incubation, 40.6% of the Se in the water extract was eluted in a lower molecular mass fraction and the Se eluted in the high molecular mass fraction had decreased to 14.0%, suggesting that the major selenium compound in the water extract was initially in a form bound to macromolecule(s) and was then enzymatically liberated from the macromolecule(s). The retention time of the liberated selenium compound in HPLC–ICP MS matched that of selenomethionine (SeMet), and the masses of molecular and fragment ions detected by HPLC–ESI MS also suggested that the selenium compound was SeMet. The selenized shiitake accumulated Se as SeMet, and SeMet might be bound to the water extractable high molecular mass protein(s).

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00216-004-2670-6

Y. Ogra (✉) · K. Ishiwata · K. T. Suzuki
Graduate School of Pharmaceutical Sciences,
Chiba University, Chuo, Chiba 260-8675, Japan
E-mail: ogra@p.chiba-u.ac.jp
Tel.: +81-43-2262866
Fax: +81-43-2262866

J. Ruiz Encinar · R. Łobiński
CNRS UMR 5034, Hélioparc, 2, av. Pr. Angot,
64053 Pau, France

R. Łobiński
Department of Chemistry,
Warsaw University of Technology, ul. Noakowskiego 3,
00-664 Warsaw, Poland

Keywords Selenium · Speciation · *Lentinula edodes* · ICP MS · ESI MS · Selenomethionine

Introduction

Selenium (Se) is an ultra-trace essential element for mammals, and at least 15 different selenoproteins and/or selenoenzymes have been found [1]. Recently, various foods, supplements, and cosmetics fortified with Se have been available in the market. The chemical forms of Se ingested are important factors determining their toxicity, nutritional importance, and metabolic fate [2]. Whanger reviewed the chemical forms of Se in fortified vegetables and supplements [3]. According to this review, one group of selenized vegetables, broccoli, tomato leaves, and cucumbers, accumulate Se as selenate whereas another group, wheat grain, corn, rice, soybeans, and yeast, predominantly accumulate Se as selenomethionine (SeMet) and the third group, garlic, onions, sprouts, and leeks, accumulate Se as Se-methylselenocysteine (MeSeCys) and γ-glutamyl-Se-methylselenocysteine [4–6].

In addition to the vegetables mentioned above, some edible mushrooms are also known to be Se-accumulators. *Agaricus bisporus* is one edible mushroom that accumulates Se [7, 8]. After sequential enzymatic digestion the selenium compounds, i.e. selenite, selenocystin and SeMet, in this selenized mushroom were detected by means of the hyphenated technique HPLC–inductively coupled argon plasma mass spectrometry (HPLC–ICP MS) [9]. Another edible mushroom, shiitake (*Lentinula edodes*), can also accumulate Se. Shiitake is the most popular and widely used mushroom in eastern Asian countries such as Japan, Korea, and China. Shiitake in both fresh and dried forms, especially the dried form, is used for Japanese soup stock because of its particular flavor. It has been reported that the flavor components of shiitake are sulfur-containing compounds such as lenthionine and other members of its family [10]. Interestingly, these compounds are biologically transformed

from the sulfur-containing amino acid derivative, lenthinic acid [11]. However, there have been no reports addressing the chemical forms of selenium compounds in selenized shiitake.

HPLC–ICP MS is most frequently used for elemental speciation analysis [12–15]. Although ICP MS enables element-specific and highly sensitive detection, it cannot provide molecular information about a sample. Identification by means of HPLC–ICP MS relies on comparison of the retention times of a sample with those of commercially available or chemically synthesized standards. However, identification by HPLC–ICP MS using a size-exclusion column (SEC) is often unreliable, because of the lower theoretical plate number than other types of column, for example reversed-phase and ion-exchange. On the other hand, HPLC–electrospray ionization mass spectrometry (HPLC–ESI MS) can provide molecular information on a sample even with an SEC column, although a massive amount of sample is needed because the sensitivity is approximately two or three orders of magnitude less than that of ICP MS for detection of selenium compounds [16, 17].

In this study, the selenium compounds in selenized shiitake were extracted with water without enzymatic digestion, simulating the conditions in soup stock; the major selenium compound in the extract was then identified by both HPLC–ICP MS and HPLC–ESI MS.

Experimental

Reagents

The selenium compounds sodium selenite, sodium selenate, and L-selenomethionine (SeMet) were purchased from Wako Pure Chemical Industries (Osaka, Japan); Se-methyl-L-selenocysteine (MeSeCys) and trimethylselenonium iodide (TMSe) were purchased from Acros Organics (Geel, Belgium) and Trichemical Laboratories (Yamanashi, Japan), respectively. Se-Methyl-*N*-acetylselenogalactosamine (selenosugar) was synthesized in-house [18]. Other reagents such as pronase and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Trizma Base) were purchased from Sigma, St Louis, MO, USA. Analytical grade nitric acid and ammonium acetate were purchased from Wako Pure Chemical Industries.

Apparatus

An HP4500 inductively coupled argon plasma mass spectrometer (ICP MS; Yokogawa Analytical Systems, Hachiouji, Japan) and an API3000 triple quadrupole mass spectrometer (ESI MS–MS; Applied Biosystems, Tokyo) equipped with a turbo ion-spray ion source were used. The instrumental conditions used for ICP MS were: radio frequency (RF) forward power 1,250 W, plasma flow-rate 15.0 L min^{-1}, auxiliary flow-rate 1.15 L min^{-1}, and Babintong nebulizer flow-rate 1.0 L min^{-1}. The ESI MS was operated in the positive-ion mode under the conditions: spray voltage 4,200 V and turbo-gas temperature 450 °C. The two mass spectrometers were coupled as detectors to HPLC. The HPLC system consisted of an on-line degasser, an HPLC pump (PU610; GL Science, Tokyo), a Rheodyne six-port injector, an appropriate column, and a UV detector (UV620; GL Science). The eluate was introduced directly into the nebulizer of the ICP MS to detect Se at m/z 77 and 82 (HPLC–ICP MS method), or was introduced into the spray of the ESI MS to obtain a molecular mass spectrum (HPLC–ESI MS method).

Sample preparation

Shiitake mushrooms, *L. edodes*, fortified with sodium selenate were purchased from PhytoSelenium Research Laboratories (Kumamoto, Japan). The caps of the shiitake mushrooms were lyophilized, milled in a mortar, and then sieved to obtain a particle fraction of less than 0.5 mm. Deionized water (5 mL) was added to 200 mg selenized shiitake powder in a 15-mL polypropylene tube, the tube was placed under nitrogen atmosphere, and the sample was then vigorously stirred. The well-soaked sample in the tube was placed in an ultrasonic bath for 30 min at room temperature. After ultrasonication, the sample was centrifuged at 12,000*g* for 15 min at 4 °C. The supernatant was filtered through a 0.22 μm PVDF membrane filter (Millex-GV; Millipore, Tokyo, Japan) and then quickly chilled to −20 °C. Aliquots of the filtrate of the water extract (500 μL) were incubated for 3 h at 37 or 95 °C.

Selenized shiitake powder (200 mg) was also extracted overnight, at 37 °C, with 15 mL 30 mmol L^{-1} Tris-HCl, pH 7.2, containing 20 mg pronase.

The lyophilized shiitake (10 mg) and 200-μL portion of the water extract were wet-ashed with 0.5 mL nitric acid and the concentration of Se was then determined by ICP MS at $m/z = 82$ by a standard addition method.

Procedures

HPLC–ICP MS

Aliquots (200 μL) of a sample of the selenized shiitake and a solution of SeMet were applied to SEC-HPLC coupled with ICP MS. The experimental conditions are summarized in Table 1. The eluate was introduced into the nebulizer of the ICP MS to monitor Se at m/z 77 and 82.

Preparation of the major selenium compound in the selenized shiitake for HPLC–ESI MS

Aliquots (200 μL) of the water extract of the selenized shiitake incubated at 37 °C for 30 min were centrifuged at 8,000*g* for 5 min and then applied to the GS-320 HQ column. The elution conditions are summarized in

Table 1 Experimental conditions for HPLC

SEC column	Asahipak GS-220 HQ[a]	Asahipak GS-320 HQ[a]	Asahipak GS-520 7G[a]
Exclusion size	> 3,000	> 40,000	> 300,000
Size (mm)	7.6×300	7.6×300	7.6×500
Guard column	GS-2B 7G[a] (7.6 mm×75 mm)	←[b]	←
Flow rate (mL min^{-1})	0.5	0.5	1.0
Injection volume (μL)	20	20 (analysis) / 200 (fractionation)	20
Eluent	10 mmol L^{-1} ammonium acetate, pH 6.5	←	←

[a]These columns were manufactured by Showa Denko, Tokyo, Japan
[b]The same

Table 1. An aliquot of the eluate was collected every 30 s to monitor the absorbance at 254 nm, and the Se concentration in each fraction was determined by ICP MS. The Se-containing fractions were concentrated to 36 μg Se mL^{-1} by lyophilization and 20-μL aliquots of the concentrate were applied to the GS-220 HQ column to determine the chemical species after the purification.

HPLC–ESI MS

The partially purified major selenium compound in the selenized shiitake was subjected to HPLC–ESI MS to determine its structure. Aliquots (10 μL) of the sample were applied to the GS-220HQ column and the column was then eluted with 10 mmol L^{-1} ammonium acetate, pH 6.5, at a flow rate of 0.5 mL min^{-1}. The eluate was introduced into the turbo ion spray of the ESI MS without splitting [19]. Introduction into the ESI MS was time-separated for Se elution by means of a four-way valve, i.e. the eluate was introduced from 22.6 to 23.0 min. The difference between retention times in HPLC–ICP MS and HPLC–ESI MS was compensated for by means of the retention time of 8.0 μg Se mL^{-1} Se-methyl-L-selenocysteine. Mass calibration and optimization of conditions for both mass spectrometers were performed daily before use.

Results

Effect of incubation conditions used for the water extract on production of the major selenium compound

The total selenium content of the lyophilized shiitake mushrooms was 355.7 ± 28.1 μg Se g^{-1} dry weight. The extraction efficiency for Se in the water extract was 68.0 ± 3.3%. The Se present in the water extract, 49.8%, was eluted at the void volume before incubation, 12.8 and 13.2% of the Se being eluted at retention times of 20.5 and 24.0 min, respectively (Fig. 1a). The exclusion size of this column (GS-320 HQ) is more than 40,000 kDa. Therefore, before incubation most of the Se in the water extract existed in forms bound to macromolecules such as proteins, glucans, and lignins. After incubation at 37 °C for 3 h, 40.6% of the Se present in the sample was eluted at a retention time of 24.0 min, Se eluted at the void volume having decreased to 14.0% (Fig. 1c). On the other hand, less Se was recovered from the sample treated at 95 °C for 3 h, because of precipitation with heat-unstable molecules (Fig. 1b). These results suggest that the major selenium compound in the water extract is initially present in a form bound to macromolecules and that the selenium compound is then liberated from the macromolecules by endogenous enzyme(s). The peak material at the retention time of 24.0 min might, therefore, be the minimum unit of the major selenium compound in the selenized shiitake. Of the selenium in the selenized shiitake 77.5% was extracted by pronase treatment.

Identification of the major selenium compound in the selenized shiitake by SEC HPLC–ICP MS

The retention time of the major selenium compound in the selenized shiitake was compared with those of authentic selenium compounds, i.e. selenite, selenate,

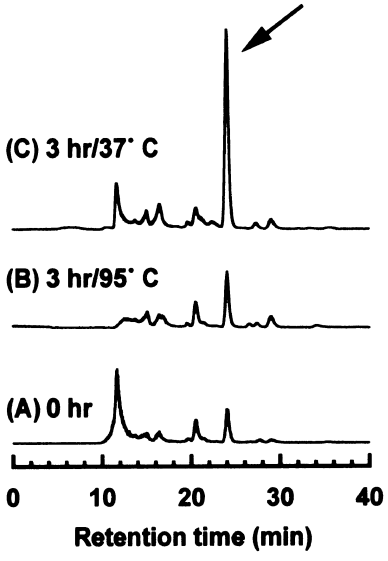


Fig. 1 Effect of incubation temperature on the distribution of selenium in the selenized shiitake mushroom extract. Aliquots (20 μL) of the extract without incubation (**a**) or with incubation for 3 h at 37 °C (**c**) or 95 °C (**b**) were applied to the GS-320 HQ column. The eluate was introduced into the nebulizer of the ICP MS to monitor Se at *m*/*z* 77 and 82. The elution profile of ^{82}Se is depicted. The *arrow* indicates the major selenium compound appearing after incubation at 37 °C for 3 h

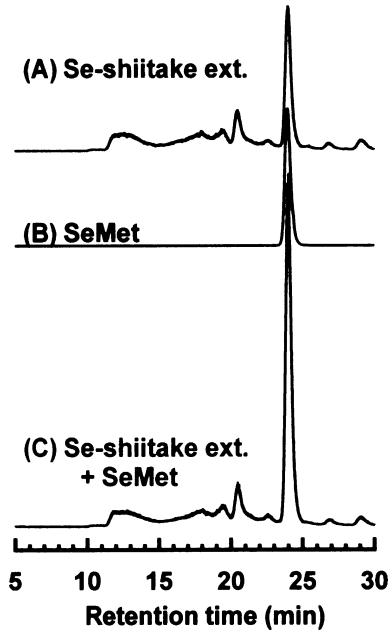


Fig. 2 Co-elution of the selenium compound in the selenized shiitake mushroom extract with selenomethionine on a GS-320 HQ column. Aliquots (20 μL) of the extract (**a**) or 2.0 μg Se mL^{-1} SeMet (**b**), or a mixture of both (**c**) were applied to the GS-320 HQ column. The eluate was monitored for Se at m/z 77 and 82. The elution profile of ^{82}Se is depicted

TMSe, SeMet, MeSeCys, and selenosugar. SeMet and MeSeCys were completely separated under the conditions used (see supplemental data in the electronic supplementary material). The retention time of the selenium compound in the selenized shiitake matched that of SeMet and it co-eluted with SeMet from the GS-320 HQ column (Fig. 2). Because identification by SEC-HPLC–ICP MS based on comparison of retention times was unreliable, HPLC–ESI MS was also used for identification of the selenium compound, as described below.

Purification of the major selenium compound in the selenized shiitake subjected to HPLC–ESI MS analysis

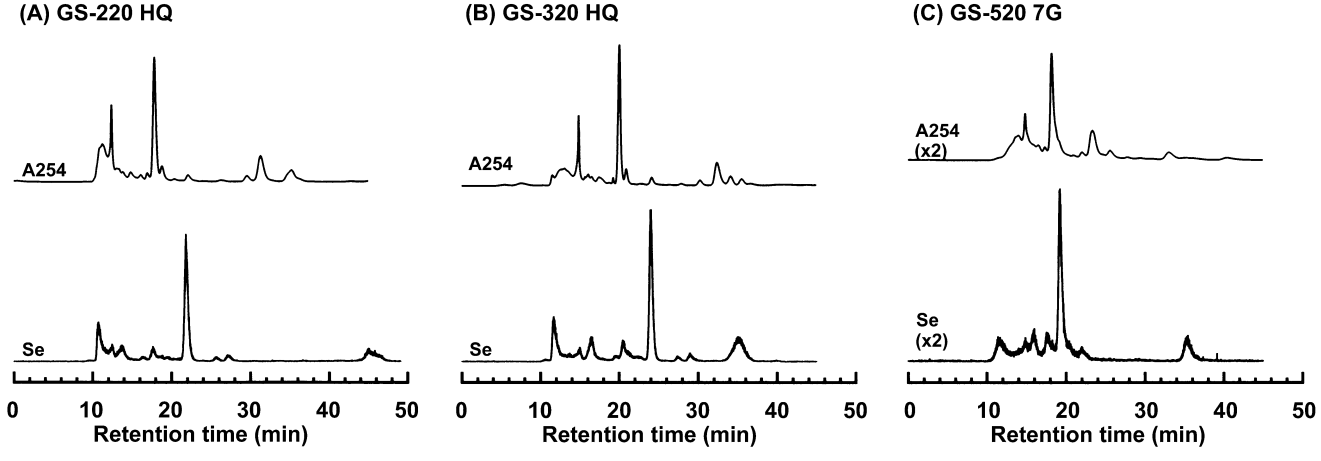
The optimum eluent for SEC HPLC–ESI MS was found to be 10 mmol L^{-1} ammonium acetate, pH 6.5, in our previous experiments [19]. Although all materials in the water extract were eluted within 40 min on the GS-320 HQ column, it took approximately 50 min to elute them completely from the GS-220 HQ column (Fig. 3). However, use of the GS-220 HQ column enabled better separation at the retention time of the selenium compound than use of GS-320 HQ. Therefore, GS-320 HQ and GS-220 HQ were used for purification and analysis, respectively, in HPLC–ESI MS. On the other hand, use of the GS-520 7G column resulted in separation worse than that from the other two SEC columns. In particular, the major selenium compound was not well separated from the main matrix of the selenized shiitake (Fig. 3). Thus, this column seemed to be inappropriate for both purification and analysis.

The major selenium compound was eluted at 32.5–33.0 min when a 200 μL portion of the water extract was applied to the GS-320 HQ column (Fig. 4). The selenium compound purified with GS-320 HQ was then eluted from the GS-220 HQ column coupled to ICP MS (Fig. 4, insert).

Identification of the major selenium compound in the selenized shiitake by HPLC–ESI MS analysis

Se consists of six isotopes, ^{74}Se (0.89%), ^{76}Se (9.36%), ^{77}Se (7.63%), ^{78}Se (23.78%), ^{80}Se (49.61%), and ^{82}Se (8.73%). The largest signals in the isotope pattern of Se were observed at approximately m/z 198, i.e. the most intense peak at m/z 198 was the ^{80}Se-containing peak

Fig. 3 Comparison of the elution profiles for absorbance at 254 nm and Se at m/z 82 among size exclusion columns. A 20-μL aliquot of the water extract after incubation at 37 °C for 3 h was applied to the SEC columns. The eluate was monitored for absorbance at 254 nm and Se at m/z 77 and 82. The elution profiles for absorbance at 254 nm and for ^{82}Se are depicted. The elution profiles for the GS-520 7G column are magnified twofold compared with the others because of the double flow rate



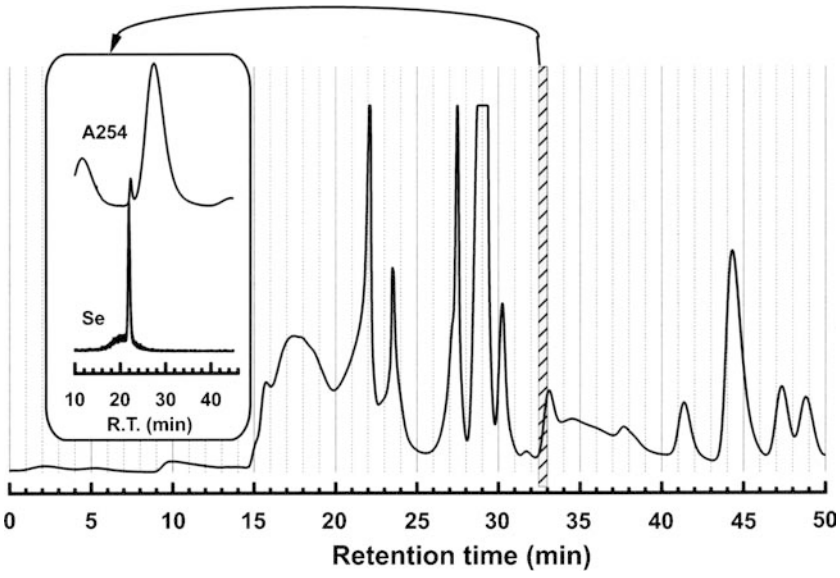


Fig. 4 Purification of the major selenium compound in selenized shiitake mushroom. Aliquots (200 μL) of the water extract of selenized shiitake mushroom were applied to the GS-320 HQ column. An aliquot of the eluate was collected every 30 s, to monitor the absorbance at 254 nm, and the Se concentration in each fraction was determined by ICP MS. The elution profile obtained by monitoring absorbance at 254 nm is depicted. The major selenium compound in the water extract was eluted in the hatched area. The insert shows the elution profiles obtained by monitoring absorbance at 254 nm and ^{82}Se for 20 μL aliquots of the purified selenium compound on the GS-220 HQ column

(Fig. 5). This ion corresponded to the molecular ion of SeMet. Furthermore, groups of peaks comprising the Se isotope pattern were also detected at approximately m/z 181, 122, 109, and 95 for ^{80}Se. These peaks were also assignable to fragments of SeMet depicted in Fig. 5. Thus, it was concluded that the major selenium compound in the selenized shiitake was SeMet.

Discussion

SeMet present as the major selenium compound in the water extract of selenized shiitake seemed to be liberated from heat-unstable macromolecules, e.g. proteins, by endogenous enzyme(s). Selenized yeasts also contain SeMet-containing medium and high molecular mass biomolecules such as Se-adenosylselenomethionine derivatives [20, 21], salt stress-induced protein 18 (SIP18), and heat-shock protein 12 (HSP12) [22, 23]. These SeMet-containing molecules might not easily decompose on incubation, especially the last two with SeMet in their primary structures, and thus SeMet-containing peptides would appear on treatment with protease. Almost all the selenized shiitake digests contained SeMet rather than SeMet-containing peptides, however, and SeMet seemed to be attached to high

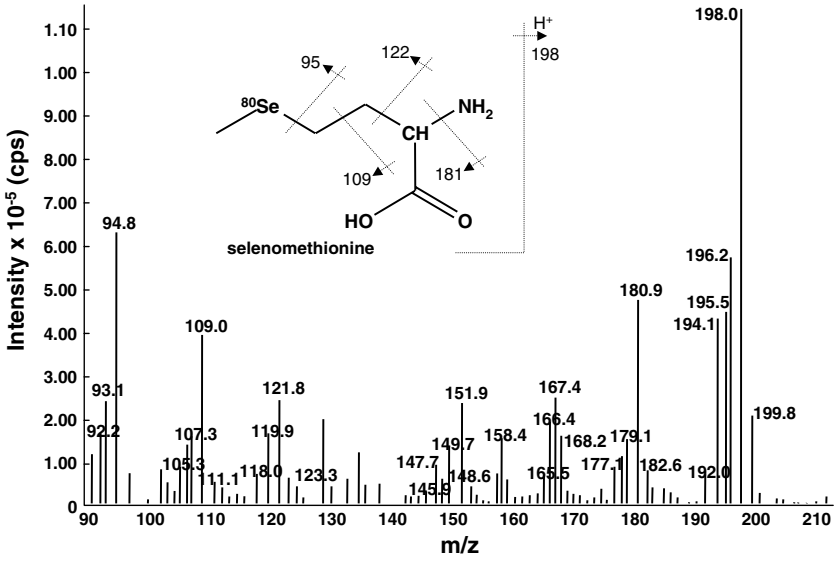


Fig. 5 Electrospray positive-ion mass spectrum of the eluate at retention times from 22.6 to 23.0 min for the partially purified selenium compound in the selenized shiitake mushroom. Aliquots (10 μL) of the sample were applied to the GS-220 HQ column. The eluate was introduced into the turbo ion spray of the ESI MS without splitting. Introduction into the ESI MS was time-separated for Se elution using a four-way valve, i.e. the eluate was introduced from 22.6 to 23.0 min

molecular weight protein(s) by labile bonds. Moreover, proteolytic digestion was effective for extracting selenium compounds from selenized *A. bisporus* and yeasts [9, 23]. For selenized *A. bisporus*, pronase treatment increased the efficiency of extraction twofold compared with water extract (ca 42–78%) [9]. On the other hand, for selenized shiitake in this study pronase treatment had little effect compared with water extraction (68.0 ± 3.3 to 77.5%). Although pronase treatment might be effective for extraction of SeMet, SeMet was almost completely extracted by endogenous enzyme(s) under the conditions adopted for the pronase treatment (37 °C, overnight). Therefore, unlike for selenized *A. bisporus* and yeasts, selenized shiitake was efficiently extracted with water alone (68.0 ± 3.3%). These results suggest that the shiitake stores SeMet in different biological form(s) from *A. bisporus* and yeast, i.e., it is speculated that the high molecular weight protein(s) in the selenized shiitake are not SeMet-containing proteins but SeMet-binding ones. Consequently, SeMet can be extracted into the soup stock of selenized shiitake with warm water, even though the selenized shiitake contains SeMet in a form bound to macromolecule(s). Thus, soup stock is a more effective source of SeMet than the entire shiitake.

It is known that selenium compounds in selenized garlic such as γ-glutamyl-Se-methylselenocysteine and Se-methylselenocysteine (MeSeCys) are potent anti-tumor agents [5, 24]. The pharmacological mechanism of selenium compounds involves reactive oxygen species (ROS), i.e., MeSeCys is not incorporated into proteins during protein synthesis but acts as a biological precursor of methylselenol (CH_3SeH), owing to β-elimination of MeSeCys, and then the methylselenol generates ROS in tumor cells resulting in the induction of apoptosis [25]. MeSeCys is twice as active as SeMet in antitumorigenesis in rodents [26]. On the other hand, the chemopreventive property of SeMet was reported recently [27]. The pharmacological mechanism of SeMet was quite different from that of MeSeCys, i.e. SeMet activated the tumor suppressor protein p53 in human lung cancer cells by transformation of the p53 from the oxidized to the reduced form [27]. As a result, MeSeCys induces oxidative stress and SeMet induces protein reduction. There is currently no reasonable explanation why these methylated selenoaminoacids have opposite effects. Further studies are needed from the viewpoint of biological transformation of selenium compounds to reveal the anti-tumor effects of selenoaminoacids. However, it is interesting to study whether the combination of SeMet and MeSeCys is synergistic or antagonistic in cancer chemoprevention.

In conclusion, the major selenium compound in a water extract of selenized shiitake was identified as SeMet by HPLC–ICP MS and HPLC–ESI MS. SeMet in the water extract might be bound to SeMet-binding protein(s) and then liberated from them by endogenous enzyme(s). The original cooking method for obtaining a shiitake soup stock is a reasonable one for separating SeMet from selenized shiitake. Selenized shiitake is a bifunctional foodstuff fortified with Se, i.e. it might be effective both nutritionally and chemotherapeutically in cancer treatment.

Acknowledgements Financial support from the Japan–Europe (France) Research Cooperative Program of the Japan Society for the Promotion of Science is gratefully acknowledged. KTS and YO also wish to acknowledge the Grant-in-Aid from the Ministry of Education, Culture, Science, Sports and Technology of Japan (Grant No. 14657587).

References

1. Birringer M, Pilawa S, Flohé L (2002) Nat Prod Rep 19:693–718
2. Shiobara Y, Yoshida T, Suzuki KT (1998) Toxicol Appl Pharmacol 152:309–314
3. Whanger PD (2002) J Am Col Nutr 21:223–232
4. Uden PC, Bird SM, Kotrebai M, Nolibos P, Tyson JF, Block E, Denoyer E (1998) Fresenius J Anal Chem 362:447–456
5. Ip C, Birringer M, Block E, Kotrebai M, Tyson JF, Uden PC, Lisk DJ (2000) J Agric Food Chem 48:2062–2070
6. McSheehy S, Yang WJ, Pannier F, Szpunar J, Łobiński R, Auger J, Potin-Gautier M (2000) Anal Chim Acta 421:147–153
7. van Elteren JT, Woroniecka UD, Kroon KJ (1998) Chemosphere 36:1787–1798
8. Rácz L, Bumbálová A, Tölgyessy J, Tomeček O (2000) J Radioanal Nucl Chem 245:611–614
9. Dernovics M, Stefánka ZS, Fodor P (2002) Anal Bioanal Chem 372:473–480
10. Morita K, Kobayashi S (1967) Chem Pharm Bull 15:988–993
11. Yasumoto K, Iwami K, Mitsuda H (1971) Agric Biol Chem 35:2070–2080
12. Szpunar J (2004) Anal Bioanal Chem 378:54–56
13. Szpunar J, Łobiński R, Prange A (2003) Appl Spectrosc 57:102A–111A
14. Łobiński R, Edmonds J, Suzuki KT, Uden PC (2000) Pure Appl Chem 72:447–461
15. Suzuki KT (1998) Analusis 26:57–60
16. Uden PC (2002) Anal Bioanal Chem 373:422–431
17. Szpunar J (2000) Analyst 125:963–988
18. Kobayashi Y, Ogra Y, Ishiwata K, Takayama H, Aimi N, Suzuki KT (2002) Proc Natl Acad Sci U S A 99:15932–15936
19. Ogra Y, Ishiwata K, Takayama H, Aimi N, Suzuki KT (2002) J Chromatogr B 767:301–312
20. McSheehy S, Pannier F, Szpunar J, Potin-Gautier M, Łobiński R (2002) Analyst 127:223–229
21. McSheehy S, Szpunar J, Haldys V, Tortajada J (2002) J Anal At Spectrom 17:507–514
22. Ruiz Encinar J, Ouerdane L, Buchmann W, Tortajada J, Łobiński R, Szpunar J (2003) Anal Chem 75:3765–3774
23. Ruiz Encinar J, Śliwka-Kaszyńska M, Poatajko A, Vacchina V, Szpunar J (2003) Anal Chem Acta 500:171–183
24. Dong Y, Lisk D, Block E, Ip C (2001) Cancer Res 61:2923–2928
25. Jung U, Zheng X, Yoon S-O, Chung A-S (2001) Free Rad Biol Med 31:479–489
26. Ip C, Dong Y, Ganther HE (2002) Cancer Metas Rev 21:281–289
27. Seo YR, Kelley MR, Smith M (2002) Proc Natl Acad Sci U S A 99:14548–14553