Composition of Chemical Species of Selenium Contained in Selenium-Enriched Shiitake Mushroom and Vegetables Determined by High Performance Liquid Chromatography with Inductively Coupled Plasma Mass Spectrometry

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(Received October 18, 2004)

Summary Selenium (Se) species in Se-enriched shiitake mushroom (Lentinula edodes) were identified and quantified by high performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICPMS). Two types of Se-enriched shiitake obtained from selenite- or selenate-fertilized mushroom beds were used. More than 80% of Se in both shiitake samples could not be extracted with 0.2M HCl. Protease digestion released a large amount of selenomethionine from the shiitake enriched with selenite. However, most of the Se in the shiitake enriched with selenate was not released by protease but was released by a cell wall digestive enzyme and most of the Se released was identified as selenate. These results indicate that the main Se species in the shiitake enriched with selenite or selenate is selenomethionine bound to protein or selenate bound to polysaccharides in the cell wall, respectively. Several Se-enriched vegetables grown on a soil fertilized with selenate were also analyzed by HPLC-ICPMS. Four Se species, selenate, Se-methylselenocysteine, selenomethionine, γ-glutamyl-Se-methylselenocysteine, and an unknown Se compound were detected in the vegetables. The composition of Se species varied with the kinds or parts of vegetables. The main Se species in bulbs, leaves or flowers of the Se-enriched garlic, onions, cabbage and ashitaba were selenate, Se-methylselenocysteine or γ-glutamyl-Se-methylselenocysteine, while those in fruit bodies of the peppers and pumpkin were selenomethionine bound to protein. Bioavailabilities of Se in the shiitake mushroom enriched with selenite and the vegetables enriched with selenate are expected to be high, but that in shiitake enriched with selenate may be low.

Key Words selenium, selenomethionine, Se-methylselenocysteine, shiitake mushroom, vegetables

Selenium (Se) is an essential trace element in human and animal nutrition and plays several important functions in the form of selenoenzymes (1). In addition to its function as an essential nutrient, Se is thought to be associated with cancer prevention from the results of epidemiological studies (2, 3). The anticarcinogenic effect of Se has also been confirmed in numerous animal studies (4, 5), and monomethylated selenoamino acids such as selenomethionine, Se-methylselenocysteine and γ-glutamyl-Se-methylselenocysteine have been found to show higher anticancer activities than selenite, selenate or selenocysteine (6, 7). These monomethylated selenoamino acids have been identified in several Se-enriched vegetables belonging to the Allium or Brassica genus (7–10) or Se-enriched yeast (7, 11, 12).

In a previous report, we described how Se-enriched sprouts of various plants could be easily prepared and the main Se species in these high-Se sprouts was identified as Se-methylselenocysteine irrespective of the kind of plant species (13). This suggests that vegetables other than Allium or Brassica species can also synthesize Se-methylselenocysteine.

Recently, enrichment of Se has been applied to various foods including mushrooms (14–16) and several vegetables other than Allium or Brassica species (17). However, the Se species in these novel high-Se foods were not completely identified. In the present study, we attempted to determine composition of the Se species in several Se-enriched vegetables and shiitake mushroom and to examine the Se metabolism in plants and mushrooms using high performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICPMS).

MATERIALS AND METHODS

Reagents. Nitric acid (metal-free grade), methanol (HPLC grade), water (HPLC grade), malonic acid, sodium 1-butanesulfonate, sodium selenate, sodium selenite and DL-selenomethionine were purchased from...
Wako Pure Chemical (Osaka, Japan). Tetramethylammonium hydroxide was purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA). DL-Selenocystine, DL-Se-methylselenocysteine and Protease XIV (from Streptomyces griseus, formerly known as Pronase or Actinase E) were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Westase® (a digestive enzyme of cell walls of yeast, from Streptomyces rochei DB-34) was purchased from Takara Bio Inc. (Otsu, Japan). Stock solutions of selenoamino acids were prepared in 0.2 M HCl and stored in the dark at 0–4 ºC.

Se-enriched shiitake mushroom. Two types of Se-enriched Shiitake were purchased from PhytoSelenium Research Laboratories (Aso, Kumamoto, Japan). One was prepared by selenite and the other was prepared by selenium. Outlines of the procedures stated by the Laboratories were as follows. Mushroom beds (SK Bio Co., Miyakonojo, Japan), which were composed of sawdust, rice bran and mycelial pellets of shiitake mushroom (Lentinula edodes), were dried in a desiccator at 4 ºC. Each dried bed weighing 2.2 kg was steeped in 5 L of 1 mM sodium selenate or sodium selenite solution at 20 ºC for 14 h. The selenite- or selenate-fertilized bed was then left at room temperature for 9 d and fruit bodies thus grown were collected as Se-enriched shiitake mushroom. The samples of Se-enriched shiitake purchased were freeze-dried, milled and stored at −30 ºC until analysis.

Se-enriched vegetables. Onions (Allium cepa), garlic (Allium sativum), red peppers (Capsicum annuum), sweet peppers (Capsicum annuum), pumpkin (Cucurbita maxima), cabbage (Brassica oleracea (capitata group), peas (Pisum sativum) and ashitaba (Angelica keiskei) grown on a soil fertilized with Selsyte Ultra® (Se granules composed of sodium selenate and barium selenate, Crop Care Holdings Ltd, Richmond, New Zealand) were also purchased from PhytoSelenium Research Laboratories and used as Se-enriched vegetables. Cultivation procedures of these Se-enriched vegetables were described elsewhere (17). The resulting harvest was also freeze-dried, milled and stored at −30 ºC until analysis.

Determination of total Se in Se-enriched shiitake mushroom and vegetables. To extract low molecular weight Se species, 100 mg of each freeze-dried sample were homogenized with five volumes of 0.2 M HCl. The homogenate was centrifuged at 12,000 × g for 15 min, and the supernatant obtained was used as 0.2 M HCl extract for the speciation of Se after filtration with a 0.45 µm membrane.

In the case of Se-enriched shiitake mushroom, red peppers, sweet peppers, pumpkin and immature pea beans, another 0.2 M HCl extraction was performed on the samples hydrolyzed by a protease or a digestive enzyme of cell wall. The conditions of enzymatic hydrolysis were as follows. After 100 mg of each sample were mixed with 4.8 mL of deionized water containing 10 mg of Protease XIV® (for shiitake mushroom, red peppers, sweet peppers, pumpkin and immature pea beans) or Westase® (for shiitake mushroom), the mixture was shaken for 16 h at 30 ºC. After the enzymatic hydrolysis, 0.2 mL of 5 M HCl was added to the mixture, which was centrifuged at 12,000 × g for 15 min and filtered with a 0.45 µm membrane.

Separation and detection of Se species by HPLC-ICPMS. A part of each 0.2 M HCl extract was diluted with 1.2 M HNO3 and nebulized into ICPMS directly to determine Se. The Se species in the remaining 0.2 M HCl extracts were identified by an HPLC-ICPMS system (12). In this analytical system, selenate, selenite, selenocystine, S-
methylselenocysteine, selenomethionine and γ-glutamyl-Se-methylselenocysteine can be identified and determined (13). The HPLC system consisted of a CCPM-II multi-pump (Tosoh, Tokyo, Japan), an SD-8022 on-line degasser (Tosoh) and a Develosil RP-Aqueous column, 4.6 mm i.d. × 250 mm (Nomura Chemical, Seto, Japan). The mobile phase was methanol/water (v/v=0.05/99.95) containing 2.5 mM sodium 1-butanesulfonate, 4 mM malonic acid and 15.9 mM tetramethylammonium hydroxide. The pH value of the mobile phase was adjusted to 2.3 by dropwise addition of diluted nitric acid. Elution was performed isocratically at 1.0 mL/min at room temperature, and its aliquot was injected into the system by using a 20 µL sample loop. The eluate was directly led to the ICPMS nebulizing tube and monitored at ion intensities of m/z 82.

**RESULTS**

Se contents in Se-enriched shiitake mushroom and vegetables

Table 1 shows Se contents in the Se-enriched shiitake mushroom and vegetables used in the present study. The Se contents ranged from 40 to 561 µg/g dry weight. These values were more than 2,000 times higher than those obtained in ordinary mushrooms or vegetables.

Speciation of Se in Se-enriched shiitake mushroom

Figure 1 shows elution patterns of 0.2 M HCl extracts from Se-enriched shiitake mushroom with or without enzymatic hydrolysis in HPLC-ICPMS. By comparison with an elution pattern of standard Se compounds (Fig. 2), peaks (1), (2) and (5) were identified as selenate, selenite and selenomethionine, respectively. However, other minor peaks were not identified; these peaks arose from unknown Se compounds. Table 2 shows composition of Se species in each original sample. Since almost 100% of Se injected was recovered in the HPLC, the percentage of each Se species to total Se in each original sample could be calculated from the extraction rate into 0.2 M HCl and peak ratio in the chromatogram. When the extraction from the shiitake was performed without enzyme hydrolysis, the extraction rate of Se was 10 to 15% and a large part of the Se could not be extracted.

**Fig. 1.** Elution patterns of 0.2 M HCl extracts from Se-enriched shiitake mushroom in HPLC-ICPMS. A unit of kcps is an abbreviation of “kilo-count per second.” The extracts were obtained from the shiitake prepared by selenite (a, b and c) or by selenate (d, e and f). The extraction with 0.2 M HCl was performed on the dried samples without any treatment (a and d), on those with Protease XIV® digestion (b and e) or those with Westase® digestion (c and f). Peaks (1), (2) and (5) were identified as selenate, selenite and selenomethionine, respectively.

**Fig. 2.** Elution pattern of standard Se compounds in HPLC-ICPMS. Concentration of each Se compound was 10 µg Se/mL. Peaks (1), (2), (3), (4) and (5) corresponded to selenate, selenite, selenocystine, Se-methylselenocysteine and seleno-methionine, respectively.
Table 2. Composition of Se species in Se-enriched shiitake mushrooms.

| Se species used<sup>1</sup> | Treatment<sup>2</sup> | Extraction rate (%)<sup>3</sup> | Composition of Se species (%)<sup>3</sup> |
|---------------------------|----------------------|-------------------------------|-----------------------------------|
| Selenite                  | None                 | 10.2                          | nd<sup>6</sup>                     |
| Selenite                  | Protease             | 83.2                          | nd                                |
| Selenite                  | Westase              | 41.0                          | 4.3                               |
| Selenite                  | None                 | 14.6                          | 4.5                               |
| Selenate                  | Protease             | 19.6                          | 24.3                              |
| Selenate                  | Westase              | 85.4                          | 72.9                              |

1 Se species used in preparation of Se-enriched shiitake mushroom.
2 Treatment before extraction with 0.2 M HCl.
3 Percentage to total Se in each original sample.
4 SeM: selenomethionine.
5 Unknown: sum of unknown Se compounds.
6 nd: not detected.

Fig. 3. Elution pattern of 0.2 M HCl extract from Se-enriched garlic bulb in HPLC-ICPMS. Each peak was identified as follows: (1) selenate; (4) Se-methylselenocysteine; (5) selenomethionine; (6) UKSe-1; (7) γ-glutamyl-Se-methylselenocysteine.

In the Se extracted, unchanged inorganic Se (selenite or selenate), selenomethionine and some unknown Se species were identified as described in Fig. 1a and d.

In the shiitake grown on the selenite-fertilized mushroom bed, the extraction rate of Se from the sample was elevated to more than 80% by digestion with a protease (Protease XIV<sup>6</sup>). Most of the Se released by the protease was identified as selenomethionine as described in Fig. 1b. On the other hand, in the shiitake grown on the selenate-fertilized mushroom bed, elevation of the extraction rate of Se was not caused by the protease but caused by a cell wall digestive enzyme (Westase<sup>6</sup>). The majority of the Se released by Westase<sup>6</sup> was identified as unchanged selenate as described in Fig. 1f.

Speciation of Se in Se-enriched vegetables

Figure 3 shows an elution pattern of 0.2 M HCl extract from Se-enriched garlic bulb in HPLC-ICPMS. Five peaks were observed in the chromatogram. Among the peaks, (1), (4) and (5) were identified as selenate, Se-methylselenocysteine and selenomethionine, respectively. When the garlic samples were treated with γ-glutamyltransferase before the extraction, peak (7) disappeared and the peak area of Se-methylselenocysteine increased. Accordingly, this peak was identified as γ-glutamyl-Se-methylselenocysteine. The remaining peak (6) observed at a retention time of 5.8 min was not coincident with any standard Se compounds; this peak arose from an unknown Se compound referred as “unknown Se compound 1 (UKSe1).” Among the five Se species, the major Se species contained in the Se-enriched garlic bulb were Se-methylselenocysteine and γ-glutamyl-Se-methylselenocysteine.

Composition of Se species in the various Se-enriched vegetables is summarized in Table 3. In all the Se-enriched vegetables examined, one or more peaks of the five observed in the Se-enriched garlic bulb were detected; no peak was observed except for the five. However, the composition of Se species varied with kinds or parts of the vegetables. For example, onion bulb contained three Se species including selenate, Se-methylselenocysteine and γ-glutamyl-Se-methylselenocysteine, but onion leaves contained only one species, i.e. selenate. Similarly to the onion leaves, leaves of garlic, sweet peppers and pea contained selenate as a major species, but leaves of cabbage and ashitaba contained Se-methylselenocysteine as another major species in addition to selenate. On the other hand, the garlic flower contained Se-methylselenocysteine alone, although the pea flower contained the four Se species other than γ-glutamyl-Se-methylselenocysteine.

Se in pepper or pumpkin fruits and beans could not be easily extracted with 0.2 M HCl. In particular, hardly any of the Se in the red pepper fruit was extracted. When the Se-enriched fruit vegetables and beans were digested with the protease, most of the Se became extractable with 0.2 M HCl and the Se released by the protease was identified as selenomethionine.

DISCUSSION

Se in foods occurs in diverse chemical forms and its bioavailability is associated with the chemical form of Se (18). Accordingly, it is important to identify the chemical species of Se in foods.

In the present study, we first attempted to identify Se
species in shiitake mushroom grown on selenite- or selenate-fertilized bed. When a direct extraction with 0.2 M HCl was performed on the shiitake samples, more than 80% of Se in both shiitake samples could not be extracted. Protease digestion released a large amount of selenomethionine from shiitake grown on the selenite-fertilized bed. However, most of the Se in shiitake grown on the selenate-fertilized bed was not released by protease but was released by a cell wall digestive enzyme and most of the Se thus released was identified as selenate. These results led us to conclude that the main Se species in the shiitake enriched with selenite or selenate on cultivation is selenomethionine bound to protein or selenate bound to polysaccharides in the cell wall, respectively.

Recently, two reports on Se species in Se-enriched mushroom were published. The first was made by Chinese researchers and has reported that a large part of the Se in Se-enriched mushroom species of Ganoderma lucidum grown on the selenite-fertilized bed was incorporated with the protein fraction and that selenocysteine might be the major Se species in the proteins (16). This is not consistent with the present result obtained from the Se-enriched shiitake grown on the selenite-fertilized bed. In this Chinese report, identification of Se species in the protein was performed after 6 M HCl hydrolysis of the Se-bound proteins, and since the selenomethionine identified accounted for only 8.2–18.3% of the total Se in the proteins, the remaining Se was estimated to be selenocysteine. However, because it has been pointed out that selenomethionine was also unstable during acid-hydrolysis with 6 M HCl (19), it is likely that a considerable amount of selenomethionine was decomposed before the identification. Thus, it is reasonable to consider that the main Se species in protein of mushroom is selenomethionine.

The second report described Se species in Se-enriched shiitake grown on the selenate-fertilized bed (20). In this report, a major Se species in the Se-enriched shiitake was found to be selenomethionine bound to protein. In the present study, a similar result was not obtained from Se-enriched shiitake grown on the selenate-fertilized bed, but in that prepared by selenite. The discrepancy between our results and the previous report is unclear. In the previous report, information supplied by PhytoSelenium Research Laboratories on the preparation method of Se-enriched shiitake might be inaccurate. Recently, we analyzed sprouts enriched with selenite or selenate and found that the reduction of selenate to selenite was a limiting step in the pathway of selenate to selenomethionine since unchanged inorganic Se was detected only in sprouts enriched with selenate (13). The results obtained in the present study are similar to those in our previous study and indicate that the reduction of selenate is a limiting step also in mushrooms.

In the present study, we also attempted to quantify the Se species in various vegetables enriched with selenate. As described in Table 3, five Se species, selenate, Se-methylselenocysteine, selenomethionine, γ-glutamyl-Se-methylselenocysteine and UKSe1, were detected in the vegetables. The composition of Se species varied

Table 3. Composition of Se species in Se-enriched vegetables.

| Samples                      | Extraction rate (%) | Composition of Se species (%) | Protease treatment |
|------------------------------|---------------------|------------------------------|--------------------|
|                              |                     | Selenate | MSeC | SeM | GMSeC | UKSe1 | Selenate | MSeC | SeM | GMSeC | UKSe1 |
| Onions, bulb                 | 109.3               | 37.4     | 50.8 | nd  | 21.1  | nd    | —        | —    | —    | —    | —    |
| Onions, leaves               | 95.3                | 95.3     | nd   | nd  | nd    | nd    | —        | —    | —    | —    | —    |
| Garlic, bulb                 | 109.5               | 5.9      | 42.7 | 11.6| 29.5  | 19.8  | —        | —    | —    | —    | —    |
| Garlic, leaves               | 95.1                | 95.1     | nd   | nd  | nd    | nd    | —        | —    | —    | —    | —    |
| Garlic, flower               | 95.3                | nd       | 95.3 | nd  | nd    | nd    | —        | —    | —    | —    | —    |
| Red peppers, fruit          | nd                  | nd       | nd   | nd  | nd    | nd    | 98.3     | nd   | nd   | 98.3 | nd   |
| Sweet peppers, fruit        | 21.7                | 13.5     | 8.2  | nd  | nd    | nd    | 104.4    | 15.3 | nd   | 89.1 | nd   |
| Sweet peppers, leaves       | 96.8                | 92.3     | nd   | 4.5 | nd    | nd    | 90.7     | 18.5 | 7.5  | 56.9 | 7.8  |
| Pumpkin, fruit              | 39.9                | 16.0     | 6.8  | 11.4| 5.7   | nd    | 96.5     | 27.1 | 35.9 | 27.6 | 5.9  |
| Cabbage, leaves             | 89.5                | 47.0     | 42.5 | nd  | nd    | nd    | 96.5     | 27.1 | 35.9 | 27.6 | 5.9  |
| Peas, immature bean         | 71.4                | 25.4     | 34.0 | 2.8 | nd    | 9.2   | 96.5     | 27.1 | 35.9 | 27.6 | 5.9  |
| Peas, immature pods         | 91.1                | 61.9     | 23.8 | nd  | 5.4   | 9.2   | 96.5     | 27.1 | 35.9 | 27.6 | 5.9  |
| Peas, leaves                | 89.0                | 77.7     | 3.2  | 3.2 | nd    | 4.9   | 96.5     | 27.1 | 35.9 | 27.6 | 5.9  |
| Peas, flower                | 92.7                | 33.2     | 20.7 | 17.9| nd    | 20.9  | 96.5     | 27.1 | 35.9 | 27.6 | 5.9  |
| Ashitaba, leaves            | 95.8                | 61.9     | 33.9 | nd  | nd    | nd    | 96.5     | 27.1 | 35.9 | 27.6 | 5.9  |

1 Values are expressed as percentage to total Se in each original sample.
2 MSeC, Se-methylselenocysteine; SeM, selenomethionine; GMSeC, γ-glutamyl-Se-methylselenocysteine.
3 UKSe1: unknown Se compound eluted at 5.8 min in the HPLC.
4 Protease treatment was not performed.
5 nd: not detected.
Se Species in High-Se Shiitake and Vegetables

with kinds or parts of vegetables. For example, Se-methylselenocysteine and its γ-glutamyl-derivative were the main species in bulbs of garlic and onions, flowers of ylscelena and its r-glutamyl-derivative were the main species in several Se-enriched vegetables belonging to the Allium or Brassica genus (7–10). The accumulation of selenate in some leaf vegetables may be caused by the difference in ability to reduce selenate. When the activity to reduce selenate is low, unchanged selenate could be accumulated.

A large part of the Se in fruit bodies of peppers and pumpkins was not extracted with 0.2 M HCl but was released by protease as selenomethionine; a major Se species in the Se-enriched fruit vegetables was protein-bound selenomethionine. In these fruit vegetables, selenocysteine synthesized from inorganic Se could be easily converted to selenomethionine and incorporated into protein because of its low methylation.

In view of the bioavailability including both nutritional and toxicological activities, Se in Se-enriched shiitake grown on the selenite-fertilized bed or in several Se-enriched vegetables are expected to have high availability because they were readily released by 0.2 M HCl or protease digestion and their main chemical species were identified as selenoamino acids or unchanged selenate. However, bioavailability of Se in shiitake enriched with selenate may be low since a large part of the Se was released neither by 0.2 M HCl nor by protease digestion.

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