Digestibility and Chemical Species of Selenium Contained in High-Selenium Yeast

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Summary To evaluate the bioavailability of selenium (Se) in high-Se yeast (SeY), the digestibility and chemical species of Se in SeY were investigated. Both Se and nitrogen in SeY were readily released into the soluble fraction through trypsin digestion. In a Sephadex G-25 gel chromatography of the trypsin digest of SeY, the range in which Se was eluted was coincident with the range in which peptide fragments were eluted. Se was distributed almost uniformly within the range and there was no fraction that contained Se in a specifically high amount. A proteolytic enzyme extract of SeY was found to contain Se as selenomethionine (74.8%), selenocystine (9.9%), selenite (5.1%) and as at least three unknown Se compounds (10.2%) when analyzed using high-performance liquid chromatography—inductively coupled plasma mass spectrometry (HPLC-ICPMS). These results indicate that Se in SeY is mainly present as selenomethionine non-specifically incorporated into peptide chains and is highly digestible. Accordingly, it is concluded that the bioavailability of Se in SeY is high.

Key Words selenium, high-selenium yeast, selenomethionine, digestibility, high-performance liquid chromatography—inductively coupled plasma mass spectrometry

Selenium (Se) is an essential trace element in human nutrition. The World Health Organization (WHO) has presented values of 0.39 and 0.42 μg/kg body weight as the normative population requirements of Se for adult men and women, respectively (1). Recently, various materials have been studied for their potential as supplements or foods to improve nutrition and health. High-Se yeast or selenized yeast (SeY), baker's yeast grown in a low-sulfur medium supplemented with selenite, has been used for Se-enrichment of foods or sold as a Se supplement.

It has been established that Se in foods shows varying bioavailability (2). For example, fish Se has been reported to be poorly available (3, 4). In a previous report, we indicated that Se in SeY was more bioavailable than selenite Se using an animal nutritional assay (5). Se in food occurs in diverse chemical forms and its bioavailability is associated with the chemical form of Se. Accordingly, it is important to identify the chemical species of Se in food. Based on experimental results using several classical chromatographic techniques, the main chemical species of Se in SeY is believed to be a protein-bound selenomethionine (6). If Se in SeY is in a protein-bound form, its digestibility is a further factor in influencing bioavailability.

Recently, a conjunctive analytical technique, high performance liquid chromatography—inductively coupled argon plasma mass spectrometry (HPLC-ICPMS) has been developed as a powerful and unique technique for the identification and quantification of Se species in biological materials (7–11). However, the approach to Se species in SeY using this technique has only been described in a few reports and several different results were obtained concerning minor components (9, 11). In the present study, to confirm the bioavailability of Se in SeY, we investigated the in vitro digestibility of Se in SeY and attempted to clarify the quantitative composition of Se species in SeY using HPLC-ICPMS technique.

MATERIALS AND METHODS

Reagents. Nitric acid (metal-free grade), methanol (HPLC grade), malonic acid, sodium 1-butanesulfonate, iodomethane, crystalline trypsin (from porcine pancreas, 5600 USP trypsin unit/mg), sodium selenate, sodium selenite and DL-selenomethionine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Tetramethylammonium hydroxide was purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA). DL-Selenocystine and Protease XIV (from Streptomyces griseus, formerly known as Pronase or Actinase E) were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Se-Methyl-DL-selenocysteine was synthesized from DL-selenocystine and iodomethane (12). Stock solutions of selenoamino acids

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were prepared in 0.2 M HCl and stored in the dark at 0–4°C.

**Samples.** SeY was purchased from Bio Springer (Maisons-Alfort, Paris, France) through Koyo Mercantile (Tokyo, Japan). The SeY was a crude type and prepared by spray-drying after cultivation in a selenium-rich medium. Fluorometric analysis showed that the Se content of SeY was 1.214 µg/g (5).

**Trypsin digestion of SeY.** Two grams of SeY was incubated with 100 mL Tris-HNO₃ buffer (50 mM, pH 8.0) containing 10 mg crystalline porcine trypsin at 37°C for 24 h with shaking. During the incubation, aliquots of 3 mL of the reaction mixture were collected at intervals and centrifuged at 750×g for 20 min. The supernatant obtained was filtrated with a 0.20 µm membrane filter. After dilution with water, the total Se in the filtrate (i.e., soluble fraction) was determined by direct nebulization ICPMS using the operating conditions shown in Table 1. We confirmed that analytical values for Se in the soluble fraction, obtained by the present method without ashing, were paralleled to those obtained by HPLC-fluorometrical Se analysis (13) after treatment with nitric acid and perchloric acid. Nitrogen in the soluble fraction was measured by the Kjeldahl method.

**Fractionation of trypsin digest.** After trypsin digestion for 24 h, 1 mL of the soluble fraction of the digest was fractionated using a Sephadex G-25 column (3.0 cm i.d.×40 cm). The digest was eluted with Tris-HNO₃ buffer (50 mM, pH 7.4) at a flow rate of 1.0 mL/min. Eluates were collected every 5 mL and subjected to direct Se determination using ICPMS.

**Sample preparation for HPLC-ICPMS analysis.** Enzymatic or hot-water extraction of SeY was performed using the procedure reported by Ip et al. (9). For hot-water extraction, 5 mL of deionized water was added to 0.2 g of SeY in a 10 mL centrifugation tube, and the tube was placed in a boiling water bath for 1 h. For the enzymatic extraction, 5 mL of deionized water was added to 0.2 g of SeY and 20 mg of Protease XIV in a 10 mL centrifugation tube, and shaken for 24 h at room temperature. After the extraction, the samples were centrifuged at 750×g for 20 min and filtrated with a 0.20 µm membrane filter.

**Separation and detection of Se species with HPLC-ICPMS.** Se species in the extracted samples were separated by an HPLC system that consisted of a CCPM-II multipump (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 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 a sample aliquot of 20 µL was injected into the LC system using a 20 µL sample loop. The Se species in the eluates were monitored by ICPMS using the operating conditions shown in Table 1.

**RESULTS**

**Trypsin digestion**

Figure 1 shows the release of soluble nitrogen and Se from SeY by trypsin digestion in vitro. Trypsin digestion released about 80% of Se into the soluble fraction within 8 h, and the release of Se paralleled that of nitrogen.

The elution pattern and Se distribution of the trypsin digest at the time of Sephadex G-25 gel chromatography are shown in Fig. 2. The range of fractions in which Se was detected was coincident with the range in which absorbance at 280 nm was observed. Within these range, Se was distributed almost equally in each fraction; there was no fraction that contained Se in a specifically high amount.

**HPLC-ICPMS**

More than 90% of the Se in SeY was extracted with proteolytic hydrolysis, whereas less than 5% was extracted using hot water. When the hot-water extract was analyzed using the HPLC-ICPMS, there was no peak on the chromatogram.
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Fig. 2. Elution pattern of trypsin digest of SeY by Sephadex G-25 gel chromatography. After trypsin digestion for 24 h, 1 mL of the soluble fraction of the digest was chromatographed on a Sephadex G-25 column (3.0 cm i.d. x 40 cm). The digest was eluted with Tris-HNO₃ buffer (50 mM, pH 7.4) at a flow rate of 1.0 mL/min. Eluates were collected every 5 mL and subjected to Se determination using ICPMS directly.

Fig. 3. HPLC-ICPMS chromatograms obtained from a mixed Se standard solution (broken line) and the enzymatic extract of SeY (solid line). A mixed Se standard solution contained selenate, selenite, DL-selenocystine, Se-methyl-DL-selenocysteine and DL-selenomethionine at a concentration of 10 μg Se/mL. On the chromatogram of the Se standard, each peak was as follows: a, selenate; b, selenite; c, DL-selenocystine; d, Se-methyl-DL-selenocysteine; and e, DL-selenomethionine. Chromatographic conditions were as follows: column, Develosil RP-AQUEOUS (4.6 mm i.d. x 250 mm); composition of mobile phase, 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; pH of mobile phase, 2.3 (adjusted with diluted nitric acid); flow rate, 1.0 mL/min; column temperature, ambient; and sample volume, 20 μL.

Figure 3 shows HPLC-ICPMS chromatograms obtained from a mixed Se standard solution (10 μg/mL each) and the enzymatic extract of SeY. In the present analysis, three Se isotopes, 77Se, 78Se and 82Se were monitored. Since the ion intensity at m/z 78 provides better sensitivity and signal-to-noise ratio than other Se isotopes, it was used for the quantification. According to the chromatogram of the standard compounds, it can be seen that the separation of five standard Se compounds (selenate, selenite, DL-selenocystine, Se-methyl-DL-selenocysteine and DL-selenomethionine) could be completed within 25 min. The peak area of each standard Se compound was almost equal and the detection limit was 0.2 μg Se/mL.

On the chromatogram for the enzymatic extract of SeY, at least six peaks were observed. Among these, three peaks were identified as selenite, selenocystine and selenomethionine through a comparison with standard Se compounds. The recovery in the eluted Se to the injected Se was estimated to be almost 100%. Based on the peak area, the existence ratio of Se species was estimated as shown in Table 2. The largest peak was identified as selenomethionine and corresponded to 74.8% of total Se in the proteolytic extract. Among the other five peaks, two peaks were identified as selenite and selenocystine and corresponded to 5.1 and 9.9% of the total Se, respectively. The remaining three peaks were derived from unknown Se compounds and corresponded to 10.2%.

Table 2. Existence ratio of Se compounds in enzymatic extract of SeY.

<table>
<thead>
<tr>
<th>Se compounds</th>
<th>Existence ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenomethionine</td>
<td>74.8</td>
</tr>
<tr>
<td>Selenite</td>
<td>5.1</td>
</tr>
<tr>
<td>Selenocystine</td>
<td>9.9</td>
</tr>
<tr>
<td>Unknown</td>
<td>10.2</td>
</tr>
</tbody>
</table>

The existence ratio of eluted total Se in the analysis of HPLC-ICPMS was estimated from the peak area of the chromatogram.

DISCUSSION

During the trypsin digestion of SeY in vitro, Se was readily released as a soluble fraction and its solubility paralleled that of nitrogen. This indicates that Se in SeY is bound to the peptide chain of protein and does not inhibit the enzymatic digestion of protein, so the digestibility of Se in SeY is high.

In the gel chromatography of the digest, the range of fractions in which Se was eluted was identical to the range in which the peptide fragments were eluted. In addition, the distribution of Se was almost uniform with that range, i.e., there was no peptide fragment containing Se at a specifically high level. Thus, the incorporation of Se into the peptide chain in SeY is considered to be mostly non-specific.

In the analysis of the hot-water extract of SeY by HPLC-ICPMS, no peak derived from any Se species was detected. This means that neither free selenoamino acids nor inorganic Se compounds were present in SeY. When SeY was treated with protease, six peaks were detected on the chromatogram of HPLC-ICPMS. The largest peak, corresponding to 74.8% of the total Se in the proteolytic extract, was identified as selenomethionine. Among the other five peaks, two peaks, correspond-
ing to 5.1 and 9.9% of the total Se were identified as selenite and selenocystine, respectively. The remaining three peaks, corresponding to 10.2% of the total Se, could not be identified. The release of selenite through hydrolysis with protease may indicate that this Se compound was adsorbed to the inner site of the protein molecule in SeY. Ip et al. showed that the bulk (85%) of Se in SeY was in the form of selenomethionine and the remaining fractions were selenite and several kinds of selenoamino acids including selenocystine, selenocystathionine, selenocystathionine, Se-methylselenocysteine, Se-adenosyl selenohomocysteine and γ-glutamyl-Se-methylselenocysteine (9). On the other hand, Zheng et al. showed that Se species in a Se-supplement consisted of SeY were selenocystine (7.8%), selenomethionine (73.6%) and unknown compounds (12.4%) (11). Thus, although the identification of minor Se species is not completed, it can be concluded that most Se in SeY is in the form of selenomethionine non-specifically incorporated into peptide chains and that selenocystine is also present in the protein of SeY irrespective of their origin. These two selenoamino acids are known to be highly bioavailable forms as equal to selenite (2). Thus, Se in SeY is nutritionally available from both the chemical form and digestibility aspects, and therefore, can be used as a dietary Se source to improve human Se status effectively.

Several functional selenoproteins have been discovered and the Se species in these proteins was identified as selenocysteine residue in the peptide chains (14, 15), i.e., selenocystine is specifically incorporated into the peptide chains of selenoprotein. Thus, the presence of selenocystine in SeY indicates that yeast may contain an unknown functional selenoprotein.

Acknowledgments

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REFERENCES