

# Formation of Selenium Adducts of Protein in Liver of Rats Administered Supranutritional Level of Selenium

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## Abstract

The formation of selenium (Se) adducts of protein in the liver of rats administered Se in excess of nutritional requirements was confirmed using high-performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICPMS). Male Wistar rats aged 4 weeks old were divided into three groups and fed a basal Se-deficient diet or basal diet supplemented with 0.2 or 2.0  $\mu\text{g Se/g}$  of selenite for 4 weeks, respectively. The liver Se concentration and GPX activity were markedly elevated in rats fed the Se-added diet; the 2.0  $\mu\text{g Se/g}$  group showed a higher Se concentration than the 0.2  $\mu\text{g Se/g}$  group, but GPX did not differ between the two groups. HPLC-ICPMS analysis of liver protease hydrolysates led to the detection of only selenocystine in the 0.2  $\mu\text{g Se/g}$  group, while the 2.0  $\mu\text{g Se/g}$  group showed the presence of four unknown Se compounds in addition of selenocystine. In another experiment, rats weighing 250 g and previously fed the Se-deficient diet for 4 weeks were intraperitoneally administered 50  $\mu\text{g Se/day}$  of selenite or L-selenomethionine for 7 days, and their liver protease hydrolysates were analyzed by HPLC-ICPMS. In selenite-treated rats, peaks of several unknown Se compounds other than selenocystine were detected. In selenomethionine-treated rats, selenomethionine was detected in addition to selenocystine. Unknown Se compounds were also present, but the number and height of peaks were smaller than in selenite-treated rats. These results indicate that with supranutritional Se, accumulation in organs occurs in the form of Se adducts on selenite exposure and mainly nonspecific insertion of selenomethionine into positions of methionine residues of proteins on selenomethionine exposure.

**Keywords:** selenite, selenomethionine, Se adducts, HPLC-ICPMS

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## Introduction

Selenium (Se) is an essential trace element for higher animals, including humans, but it is also highly toxic [1]. The mechanism of toxicity due to excess Se is considered to depend on the type of Se compound [2]. In the case of inorganic selenium compounds (selenate and selenite), oxidative stress is caused by reactive oxygen species generated during their reduction, and in that of selenomethionine, nonspecific insertion of selenomethionine into sites of methionine residues in general proteins is considered to be responsible for functional disorders caused by excess Se [3, 4]. For example, cataracts induced by oxidative stress derived from selenite administration are frequently used as a model for experimental cataracts, and it has

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been shown that oxidative stress also damages major organs including the liver, kidney, and brain in these animal models [5]. In addition to oxidative stress, selenide and methylselenol produced during metabolism of Se compounds are likely to add to various biomolecules. A selenosugar, the main Se molecular species excreted in urine when Se intake is within the normal range, is a compound of methylselenol added to *N*-acetyl-d-galactosamine [6] and can be regarded as a type of Se adduct. Se transport from plasma to organs is considered to involve selenotrisulfide, which is formed by the addition of Se to cysteine residues in albumin, and Se is likely to be added to thiol groups in proteins [7]. Furthermore, it has been reported that in *Arabidopsis thaliana* treated with a low level of selenate, Se is added to the cysteine residue of NAD-dependent glyceraldehyde-3-phosphate dehydrogenase, resulting in a highly active protein with selenol bound to sulfur of cysteine residues [8]. These cases involve physiologically adequate Se exposure, and the Se-adducts formed serve to maintain normal Se metabolism. However, excessive Se-loading is likely to affect cellular functions through nonspecific formation of various Se adducts by highly reactive selenide or methylselenol. Thus, such excessive Se-loading may lead to the formation of nonspecific Se adducts in biomolecules, including functional proteins and DNA. However, no studies have focused on such Se adducts or attempted to detect them. In addition, the amount of nonspecific Se adducts produced may serve as an indicator to determine the upper limit of Se intake or exposure.

In this study, we detected Se adducts in proteins produced in the liver of rats on administering a supranutritional dose of Se equivalent to 10-times their normal Se intake using high-performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICPMS).

## Methods

### Se administration to experimental animals

The experimental protocol followed the Guide for the Care and Use of Experimental Animals issued by the Prime Minister's Office of Japan and approved by the Animal Ethics Committee of Kansai University (Approval No. 2203).

In Experiment 1, eighteen 4-week-old male Wistar rats (mean  $\pm$  SD of body weight,  $80 \pm 3$  g) purchased from Shimizu Laboratory Supply Co. (Kyoto) were divided into three groups of equal body weight. One group was fed a *Torula* yeast-based Se-deficient basal diet, and the other two groups were fed the basal diet with sodium selenite at a level of 0.2 or 2.0  $\mu$ g Se/g, respectively, for 4 weeks.

In Experiment 2, eighteen male Wistar rats similar to those in experiment 1 were fed the Se-deficient basal diet for 4 weeks and then divided into three groups of equal body weight. The body weight (mean  $\pm$  SD) of rats at the time of initiating intraperitoneal administration was  $250 \pm 6$  g ( $n=18$ ). The first group received saline, the second group received saline containing sodium selenite, and the third group received saline containing L-selenomethionine intraperitoneally for 7 days. The dose of Se administered was 50  $\mu$ g/day. During the intraperitoneal administration period, all rats continued to receive the Se-deficient basal diet.

The composition of the basal Se-deficient diet was as follows: *Torula yeast*, 30.1%; sucrose, 56.9%; soybean oil, 8.0%; Se-free AIN93G mineral mixture, 3.5%; AIN93G vitamin mixture, 1.0%; choline bitartrate, 2.0%; DL-methionine, 3.0%. The Se concentration of the basal diet was 9 ng/g. The *Torula* yeast used was kindly supplied by Mitsubishi Corporation Life Science Limited (Tokyo, Japan).

After the end of the rearing or intraperitoneal administration period, the liver was collected under deep anesthesia with isoflurane (MSD Animal Health, Tokyo, Japan) under non-fasting conditions. The collected liver was stored at  $-30^{\circ}\text{C}$  until analysis.

### Analysis of total Se in liver and assay of glutathione peroxidase (GPX) activity

To approximately 500 mg of fresh liver, 5 mL of nitric acid and 2 mL of perchloric acid were added, and the sample was heated to incineration. Ultrapure water was added to the ashed sample, the volume was increased to 10 mL, and the mixture was passed through a 0.45- $\mu$ m membrane filter. Then, Se in the filtrate was quantified by ICPMS using ICPM2030 (Shimadzu, Kyoto). The analytical mass number of Se in ICPMS was 82, and indium 115 was used as an internal standard.

Approximately 1 g of fresh liver was homogenized with 9 volumes of saline to prepare a 10% homogenate. GPX activity in the homogenate was measured using *tert*-butyl hydroperoxide as a substrate and monitoring the amount of NADPH consumed in the reduction of the oxidized glutathione produced using glutathione reductase by the decrease in absorbance at 340 nm [9].

### Analysis of Se molecular species in liver

Livers were hydrolyzed with protease, and molecular species of Se released were analyzed using HPLC-ICPMS, as follows. Each liver sample was freeze-dried and then pulverized. To facilitate pulverization, the liver samples were defatted with hexane before freeze-drying. To 50 mg of each dried powder sample, 1.92 mL of phosphate buffer (10 mM, pH 7.1) and 5 mg of protease (type XIV derived from *Streptomyces griseus* (Merck KGaA, Darmstadt)) were added, and the mixture was thoroughly stirred and shaken at 37°C. After 24 hours, 0.08 mL of 5 M HCl was added and the mixture was centrifuged at  $1,500 \times g$  for 60 min. The supernatant was collected, passed through a 0.45- $\mu$ m membrane filter, and used as a protease extract. Molecular species of Se in the protease extract were analyzed by HPLC-ICPMS. The HPLC system consisted of an LC-20Ai multi-pump (Shimadzu, Kyoto, Japan), DGU-20A3R on-line degasser (Shimadzu, Kyoto, Japan), and a reversed phase separation column (Develosil<sup>®</sup> RPAQUEOUSAR column, 4.6 *i.d.* mm  $\times$  250 mm, Nomura Chemical, Seto, Japan). The mobile phase was the same as previously used [10]: methanol/distilled water (HPLC grade) (v/v = 0.05/99.95) containing 2.5 mM sodium 1-butanedisulfonate, 4 mM malonic acid, and 15.9 mM tetramethylammonium hydroxide. The pH of the mobile phase was adjusted to 2.3 by the dropwise addition of diluted nitric acid. Elution was performed isocratically at 0.5 mL/min and 30°C, and a sample aliquot of 20  $\mu$ L was injected into the LC system. The eluate was directly applied to the ICPMS nebulizing tube and monitored at ion intensities of *m/z* 82. Molecular species of Se in the extract were identified by comparison with the retention times of standard Se compounds (sodium selenite, L-selenocystine (Sigma-Aldrich, St. Louis, USA), Se-methylselenocysteine hydrochloride (Sigma-Aldrich, St. Louis, USA), L-selenomethionine (Sigma-Aldrich, St. Louis, USA), and selenohomolanthionine (kindly supplied by Prof. Ogra Y, Chiba University, Japan)).

### Statistical analysis

Measurements of the total liver Se concentration and GPX activity were tested using one-way ANOVA and Tukey's multiple comparisons. Statistical GraphPad Prism (GraphPad Software, San Diego, USA) was used for these analyses.

### Results

In both Experiments 1 and 2, there was no difference in body weight between the experimental groups. The body weight (mean  $\pm$  SD) was  $248 \pm 12$  (n=18) at the end of the feeding period in Experiment 1 and  $274 \pm 11$  (n=18) at the end of intraperitoneal administration of Se compound in Experiment 2. Thus, the different Se concentrations in the experimental diets (Experiment 1) and intraperitoneal administration of Se compounds (Experiment 2) had no effect on rat growth.

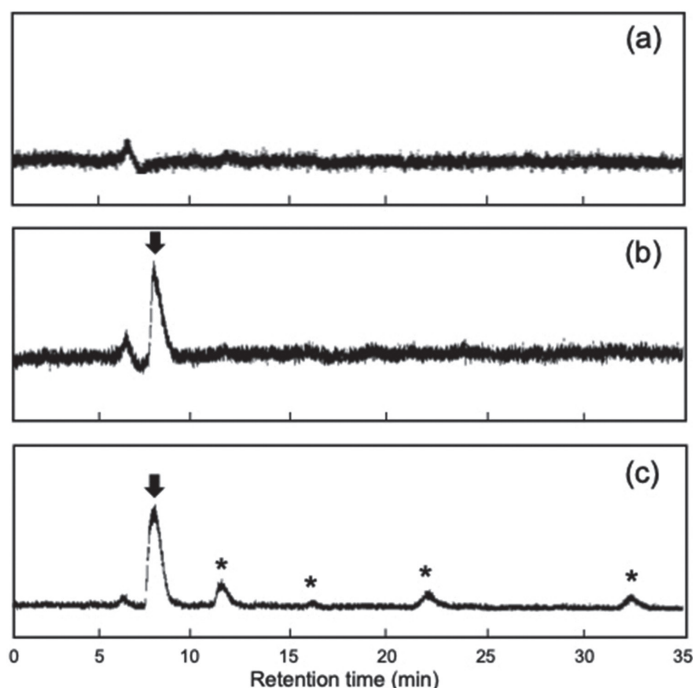
**Table 1** summarizes the Se concentration and GPX activity in the liver of each group of rats in Experiment 1. Se concentrations were proportional to the Se concentration of the experimental diet, with rats fed a diet containing a high level of 2.0  $\mu$ g Se/g having the highest values. In contrast, GPX activity was higher in the two groups fed the selenium-added than selenium-deficient diet, but the different levels of addition had no effect. Therefore, there was no difference between the 0.2 and 2.0  $\mu$ g Se/g diet group, indicating the saturation of enzyme activity.

**Figure 1** shows typical chromatograms of protease hydrolysates from the livers of rats fed diets with different levels of selenium in HPLC-ICPMS. In rats fed the Se-deficient diet (a), only one noise-like peak was detected at around 7-min retention time. In rats fed the diet containing 0.2  $\mu$ g Se/g selenite (b), a distinct selenocystine peak and the noise-like peak described above were observed. In contrast, four unknown peaks were detected in addition to selenocystine in rats fed diets supplemented with 2.0  $\mu$ g/g selenite. The peak heights of selenocystine in **Figures 1b and 1c** are nearly identical.

**Table 1** | Total Se content and GPX activity in livers of rats fed diets with different levels of Se as sodium selenite

Level of Se addition	Se content (ng/g)	GPX activity (unit/g protein)
None	26 ± 3 <sup>a</sup>	44 ± 4 <sup>a</sup>
0.2 µg/g	236 ± 20 <sup>b</sup>	1219 ± 59 <sup>b</sup>
2.0 µg/g	743 ± 41 <sup>c</sup>	1181 ± 33 <sup>b</sup>

Rats were fed an Se-deficient basal diet, or the basal diet supplemented with selenite at 0.2 or 2.0 µg selenium/diet for 4 weeks. Values are means ± SEM (n=6). Means in the same column not sharing a common superscript differ significantly ( $p < 0.05$ ).

**Fig. 1** | Chromatograms of protease hydrolysates in livers of rats fed diets with different levels of selenium in HPLC-ICPMS

Rats were fed a selenium-deficient basal diet (a), or the basal diet supplemented with selenite at 0.2 µg selenium/diet (b) or 2.0 µg selenium/diet (c) for 4 weeks. Although the baseline deviation differs in each chromatogram due to the inconsistent sensitivity of the instrument, the vertical axis (ion intensity in ICPMS) is on the same scale. Black arrows and asterisks indicate the peaks of selenocystine and unknown Se species, respectively.

**Table 2** summarizes the Se concentrations and GPX activity in the livers of rats that received the Se compounds intraperitoneally for 1 week in Experiment 2. The Se concentration and GPX activity were markedly increased by administration of the Se compounds. Regarding the effect of the type of Se compound administered, both the Se concentration and GPX activity tended to be higher in rats treated with selenomethionine than in those receiving selenite, but the difference was not significant.

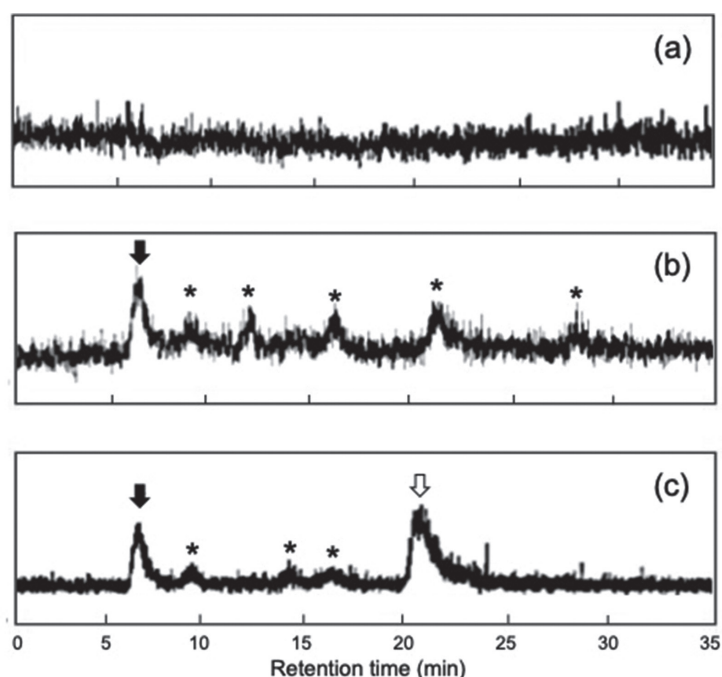
**Figure 2** shows typical chromatograms of protease hydrolysates from the livers of rats intraperitoneally administered selenium compounds in HPLC-ICPMS. No distinct peaks were observed in rats not treated with Se compound (a). In rats treated with selenite (b), several unknown Se compound peaks were noted in addition to the distinct selenocystine peak, as in rats fed the experimental diet with an Se concentration of 2.0 µg Se/g in Experiment 1. In rats treated with selenomethionine (c), distinct peaks of selenocystine and selenomethionine were detectable. In addition, unknown peaks were also present, but their number and heights were smaller than in selenite-treated rats.

When the liver powder was analyzed without protease treatment, no peaks derived from Se compounds were observed on HPLC-ICPMS chromatograms in either Experiment 1 or 2.

**Table 2** | Total Se content and GPX activity in livers of rats receiving Se compounds intraperitoneally

Se compounds received	Se content (ng/g)	GPX activity (unit/g protein)
None	16 ± 2 <sup>a</sup>	42 ± 5 <sup>a</sup>
Sodium selenite	414 ± 49 <sup>b</sup>	917 ± 42 <sup>b</sup>
Selenomethionine	504 ± 48 <sup>b</sup>	1020 ± 98 <sup>b</sup>

Rats were fed a selenium-deficient basal diet. After 4-week feeding, rats received saline, sodium selenite, or L-selenomethionine intraperitoneally for 7 days. The dose of selenium administered was 50 µg/day. Values are means ± SEM (n=6). Means in the same column not sharing a common superscript differ significantly ( $p < 0.05$ ).



**Fig. 2** | Chromatograms of protease hydrolysates from livers of rats intraperitoneally administered selenium compounds in HPLC-ICPMS

Rats were fed a selenium-deficient basal diet. After 4-week feeding, rats were divided into three groups: the first group received saline (a), the second group received saline containing sodium selenite (b), and the third group received saline containing L-selenomethionine (c) intraperitoneally for 7 days. The dose of selenium administered was 50 µg/day. Although the baseline deviation differs in each chromatogram due to the inconsistent sensitivity of the instrument, the vertical axis (ion intensity in ICPMS) is on the same scale. Black arrows, white arrow and asterisks indicate the peak of selenocystine, selenomethionine and unknown Se species, respectively.

## Discussion

Considering that rats consume a diet equivalent to about 10% of their body weight per day, a diet with an Se concentration of 2.0 µg/g would result in Se intake of 50 µg/day for a rat weighing about 250 g. Thus, Se exposure levels of rats that received the Se compound intraperitoneally in Experiment 2 are comparable to those that consumed the 2.0 µg Se/g diet in Experiment 1. The Se concentration of AIN93G used as a standard semi-purified diet in nutritional studies with rats is 0.2 µg/g [11]. This means that the 0.2 µg Se/g diet in Experiment 1 provides a nutritionally sufficient Se intake. Therefore, the 2.0 µg Se/g diet leads to a supranutritional intake of Se.

In Experiment 1, when 2.0 and 0.2 µg Se/g diet groups were compared, the liver Se concentrations were higher in proportion to the dietary Se concentration, but GPX activity did not differ (Table 1). Although only GPX was measured, it is likely that in the 0.2 µg Se/g diet group, where nutritionally sufficient Se intake was achieved, the production of selenoproteins, which incorporate selenocysteine into the peptide chain, was already saturated. In addition, the similar peak height of selenocystine in Figures 1b and 1c also means that the selenoprotein production in the two groups is comparable. Therefore, it can be suggested that Se accumulates in the liver of rats

in the 2.0  $\mu\text{g Se/g}$  diet group in a form other than selenoprotein.

HPLC-ICPMS analysis of the extract from the protease-treated liver in Experiment 1 detected only the selenocystine peak derived from selenoprotein in the 0.2  $\mu\text{g Se/g}$  diet group (**Figure 1b**), while four unknown peaks were detected in addition to selenocystine in the 2.0  $\mu\text{g Se/g}$  diet group (**Figure 1c**). Since these peaks were not detected in the absence of protease treatment, we consider that these unknown Se compounds were derived from Se added to the side chains of amino acids. Selenocysteine residues in selenoprotein are unstable, so it is possible that artificial Se species are produced in the process of preparing the protease extract. The amount of selenocysteine residues in the 0.2  $\mu\text{g/g}$  and 2.0  $\mu\text{g/g}$  groups was comparable because the production of selenoprotein was saturated. Therefore, if there was an artificial Se species derived from selenocysteines residues, a peak other than selenocystine should also occur in the 0.2  $\mu\text{g/g}$  group (**Figure 1b**). However, no such peak was observed. Thus, we conclude that Se-adducts of proteins were formed in the livers of rats fed the 2.0  $\mu\text{g Se/g}$  diet.

The peaks, which may be derived from the Se-adducts of proteins, were also observed in the liver of rats treated intraperitoneally with selenite in Experiment 2 (**Figure 2b**). Concerning selenomethionine administration, a large peak of selenomethionine was observed in addition to selenocystine (**Figure 2c**). Unknown peaks were also present, but they were clearly fewer in number and smaller in height than in the case of selenite administration. This means that most of the selenomethionine administered was inserted nonspecifically at sites of methionine residues of general proteins, resulting in low-level formation of Se-adducts of proteins.

The present results indicate that supranutritional Se intake causes saturation of selenoprotein formation, and that some of the Se not used for selenoprotein formation accumulates as Se-adducts of proteins in the case of selenite and at sites of methionine residues of proteins in the case of selenomethionine. If the formation of Se adducts is used as an index of Se toxicity, this result indicates that selenomethionine is less toxic than selenite. However, this conclusion is tentative because quantitative evaluation of the effect of the substitution of methionine residues in proteins for selenomethionine has not been completed.

The average Se intake of Japanese people is about 100  $\mu\text{g/day}$ , which is within the appropriate range [12]. In a study of chronic poisoning of Se in China, hair loss and changes in nail morphology were reported when Se intake of nearly 1000  $\mu\text{g/day}$ , or approximately 10-times the average Japanese Se intake, continued over a prolonged period [13]. In the present rat experiments, Se adducts were formed in liver proteins after oral or intraperitoneal administration of Se equivalent to 10 times the appropriate Se intake. Thus, it is possible that Se adducts were produced in the livers and other organs in these Chinese Se-poisoning cases.

In a study of mice, Se administered orally at 4 mg/kg body weight in the form of sodium selenite for 28 days resulted in an average increase in reactive oxygen species and malondialdehyde concentrations in livers, but no significant differences were observed [14]. The dose level in the present study (50  $\mu\text{g}/250\text{ g} = 200\ \mu\text{g/kg}$  for intraperitoneal administration) was less than one-tenth of this. Accordingly, oxidative stress at a toxic level is not considered to have occurred in the present study; Se adduct formation occurs at lower exposures than oxidative stress generation. Considering that the Se dose level in the present study was similar to exposure levels in human chronic-poisoning cases, it is possible that the formation of Se adducts, rather than oxidative stress caused by reactive oxygen species, is responsible for the symptoms of chronic Se-poisoning in humans, such as hair loss and changes in nail morphology.

The formation of Se adducts is not an oxidative stress, but may cause a loss of protein function via changes in protein conformation associated with Se addition. Se adducts could also be generated in DNA, in which case protein synthesis would be inhibited and more significant effects would occur. In the future, it will be necessary to focus on the formation of Se adducts when considering the pathogenic mechanism of Se poisoning.

We have identified selenomethionine as the major Se molecule species in protease hydrolysates of several seafood, chicken meat, and chicken eggs [15]. The present result that selenomethionine was detected only in the liver of rats that consumed selenomethionine indicates that selenomethionine in these animal products is not biosynthesized in the body of each animal species, but is derived from the food and feed of these animal species.

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