

Studies on the biological functions and metabolism of
selenoneine

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General Introduction

Selenium (Se) is one of chalcogens as oxygen (O), sulfur (S), tellurium (Te), polonium (Po) and livermorium (Lv). Se is an essential trace element for animals including humans and responsible for an important role in the redox system in the body. Se has both inorganic and organic chemical forms and most of inorganic Se compounds exist as selenite and selenate in environment (Table 0). The inorganic Se compounds are incorporated into bacteria, yeast and plants, and they are converted various organic Se compounds (Böck, 2001; Kieliszek et al., 2015; Pilon-Smits and Quinn, 2010). Animals including humans can also convert such inorganic Se into organic Se compounds (Fig. 0, Yoshida, 2008). However, humans generally never take inorganic Se from their diet excluding supplementation for Se deficiency as parenteral or enteral nutrition, modified milk powder or breast milk substitutes added selenite as a food additive. Selenocysteine and selenomethionine (Table 0) are well-known organic Se compounds synthesized in such organisms, and animals including humans ingest them from foods (Fig. 0). Selenocysteine is a Se analog of cysteine and is incorporated into the primary structure of proteins in distinction from cysteine in higher eukaryotes, some bacteria and archaea (Fig. 0, Mihara et al., 2006). Such proteins containing one or more selenocysteines in their primary structure called “selenoprotein”. The insertions of selenocysteine in amino acid sequences of a selenoprotein are enabled by the specific transfer RNA for selenocysteine (Sec-tRNA, Fig. 0) and the mechanism of reading termination codon, the UGA codon, into

selenocysteine insertion by the selenocysteine insertion sequence (SECIS) located downstream of the termination codon (Mihara et al., 2006). Since SECIS is needed to synthesize selenoprotein, the number of selenoprotein in each species is predicted based on genetic analyses. Humans are speculated to have 25 types of selenoprotein (Kryukov et al., 2003). Some of selenoproteins in humans have also been identified their functions. For example, glutathione peroxidase (Gpx) family catalyzes the decomposition of hydrogen peroxide (H_2O_2) by selenocysteine located at the active center of the Gpx and reduced glutathione. Thioredoxin reductase (TrxR) family reduces thioredoxin which reduces disulfide bond of protein. Selenoprotein P (SelP) is one of a major secretory type selenoprotein synthesized in liver and distributed in plasma. It has 10 selenocysteins in its structure and is considered to transport Se to whole body. Excessive SelP is reported to induce insulin resistance in the liver and skeletal muscle of mice and is considered to be a risk factor for the type 2 diabetes, recently (Misu et al., 2010; Saito, 2019). On the other hand, selenomethionine, a Se analog of methionine, is incorporated into proteins non-specifically to methionine. Structures and functions of the proteins are not affected by the replacement of methionine to selenomethionine. That's because Se in selenomethionine located between carbons and the atomic radiuses of Se and S in the molecules are not so different, those are 0.117 nm and 0.103 nm respectively (Huber and Criddle, 1967). Excretion of Se in animals has also been studied. In mammals, Se is excreted in urine as trimethylselenonium (TMSe) and selenosugar (Table 0, Fig. 0). Selenosugar is a major metabolite of Se in urine. Humans have three

types of selenosugars, methyl-2-acetamido-2-deoxy-1-seleno- β -D-galactopyranoside (selenosugar 1), methyl-2-acetamido-2-deoxy-1-seleno- β -D-glucopyranoside that is the epimer of selenosugar 1 (selenosugar 2), and methyl-2-amino-2-deoxy-1-seleno- β -D-galactopyranoside (selenosugar 3) (Table 0, Fairweather-Tait et al., 2011). TMSe and selenosugar are synthesized from selenide (HSe^- , Fig. 0), generated by reducing both organic and inorganic Se compounds (Yoshida, 2008). As mentioned above, the functions and metabolism of Se have been studied in the world since Se is an essential trace element for humans.

Marine organisms like tuna contain abundant Se in its edible part. Therefore, seafoods have been considered to be a major source of Se for humans. However, a part of Se in tuna was an unknown organic Se different from selenocysteine and selenomethionine (Yoshida, 2008), and it has not been identified until recent year. In 2010, Yamashita et al. identified the organic Se as 2-selenyl- $N_\alpha, N_\alpha, N_\alpha$ -trimethyl-L-histidine from the blood of the bluefin tuna, *Thunnus orientalis*, and it was named “selenoneine” (Table 0, Yamashita and Yamashita, 2010). Selenoneine is constructed by an imidazole ring, with a trimethyl amino group, carboxyl group and a Se atom (Table 0), and it is a Se analog of ergothioneine which is an antioxidant found in the ergot fungus, *Claviceps purpurea* and is contained in mushrooms abundantly (Tanret, 1909; Kalaras et al., 2017; Tang et al., 2018). Selenoneine has two monomer forms and one dimer form. The one of monomer forms is a selenoketone (selone) form and another form is selenol form (Table 0). They are changes alternatively by a keto-enol tautomerism (Yamashita and

Yamashita, 2010). The dimer form of selenoneine is stable oxidized dimer constructed by two molecules of selenoneine with Se-Se covalently bond like selenocystine (Table 0). Selenoneine has 895 and 463 times stronger radical scavenging capacity than ergothioneine and aqueous vitamin E called Trolox against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, respectively (Yamashita and Yamashita, 2010). Health functions of selenoneine have been studied. For example, selenoneine detoxicates the toxicity of methylmercury (MeHg) in zebrafish (Yamashita et al., 2013b). In the previous studies using mice, selenoneine reduces the pathology of experimental colorectal cancer (Masuda et al., 2018) and it ameliorates hepatic steatosis in nonalcoholic fatty liver disease (NAFLD) model mice (Miyata et al., 2020). Absorption, distribution and metabolism of selenoneine have also been studied. For example, selenoneine is incorporated into cells via the organic cation/carnitine transporter-1 (OCTN1/SLC22A4) identified as a transporter for ergothioneine (Gründemann et al., 2005; Yamashita et al., 2013b). Selenoneine distributes and accumulates in erythrocytes of humans who eat fish more frequently (Yamashita et al., 2013a). In human blood and urine, the methylated selenoneine, Se-methylselenoneine, has been identified and speculated to be a metabolite of selenoneine (Klein et al., 2011).

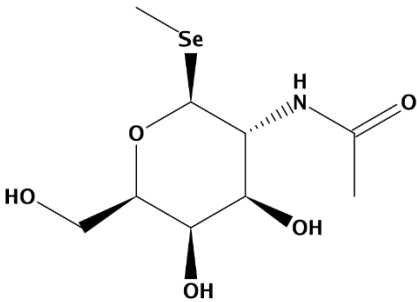
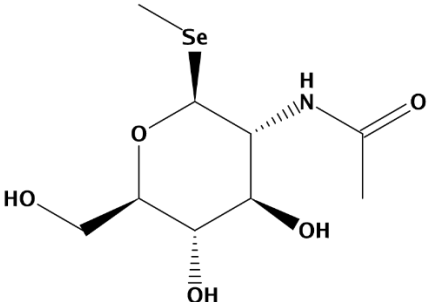
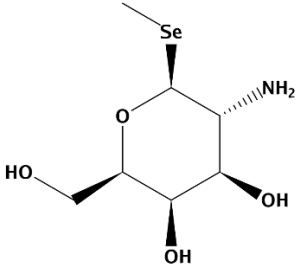
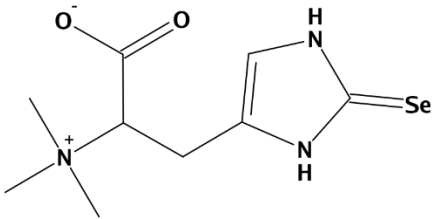
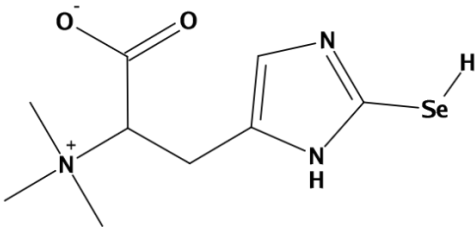
It has been more than decade since selenoneine was identified (Yamashita and Yamashita, 2010), questions as to health functions and metabolism of selenoneine have been unrevealed. That can be attributed to the difficulty of obtaining selenoneine because it is not available for purchase. Pluskal et al. developed genetically modified fission yeast producing

ergothioneine efficiently and they have succeeded to biosynthesize of selenoneine by culturing the fission yeast in the media containing selenate (Pluskal et al., 2014). Lim et al. have reported the total synthesis of selenoneine (Lim et al., 2019). Recently, a biosynthetic pathway of selenoneine has been identified in a species of bacteria, *Amycolatopsis palatopharyngis* (Kayrouz et al., 2022). The bacteria synthesize selenoneine from selenosugar using novel enzymes for selenoneine synthesis different from those for ergothioneine synthesis (Kayrouz et al., 2022). These studies as to synthesis of selenoneine could make it possible to obtain authentic selenoneine without the difficult process of purifying it from seafoods with high adulteration.

In this study, new functions of selenoneine were explored by *in vitro* and *in cellulo* experiments, a new method was developed for acquiring high purity selenoneine efficiently and distribution and metabolism of selenoneine *in vivo* were revealed by animal experiments using mice with the aim of accumulating nutritional knowledge of selenoneine.

Table 0. Chemical structures of representative Se compounds and ergothioneine

Name	Chemical structure
Selenite	
Selenate	
Selenocysteine	
Selenocystine	
Selenomethionine	
Trimethylselenonium	

Selenosugar 1	
Selenosugar 2	
Selenosugar 3	
Selenoneine (monomer, selenoketone, selone,)	
Selenoneine (monomer, selenol)	

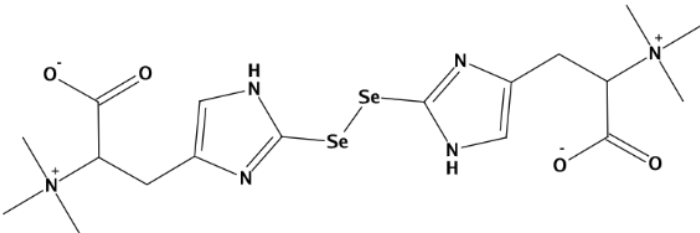
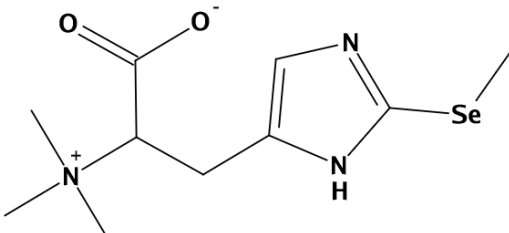
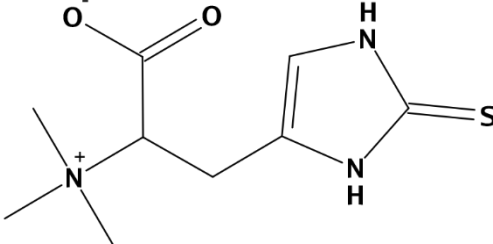
Selenoneine (dimer)	 The structure shows two selenoneine units linked by a diselenide bridge (-Se-Se-). Each unit consists of a 4-methyl-5-oxoimidazole-2-ylmethyl group attached to a 2-(trimethylammonio)ethyl side chain. The carboxylate group is shown as a carbonyl (C=O) and an oxygen with a negative charge (O⁻).
Se-methylselenoneine	 The structure shows a single selenoneine unit with a methyl group attached to the selenium atom. It features a 4-methyl-5-oxoimidazole-2-ylmethyl group and a 2-(trimethylammonio)ethyl side chain. The carboxylate group is shown as a carbonyl (C=O) and an oxygen with a negative charge (O⁻).
Ergothioneine	 The structure shows a single ergothioneine unit. It features a 2-(trimethylammonio)ethyl side chain attached to a 4-methyl-5-oxoimidazole-2-ylmethyl group. The carboxylate group is shown as a carbonyl (C=O) and an oxygen with a negative charge (O⁻). The imidazole ring has a double bond to a sulfur atom (C=S).

Fig. 0

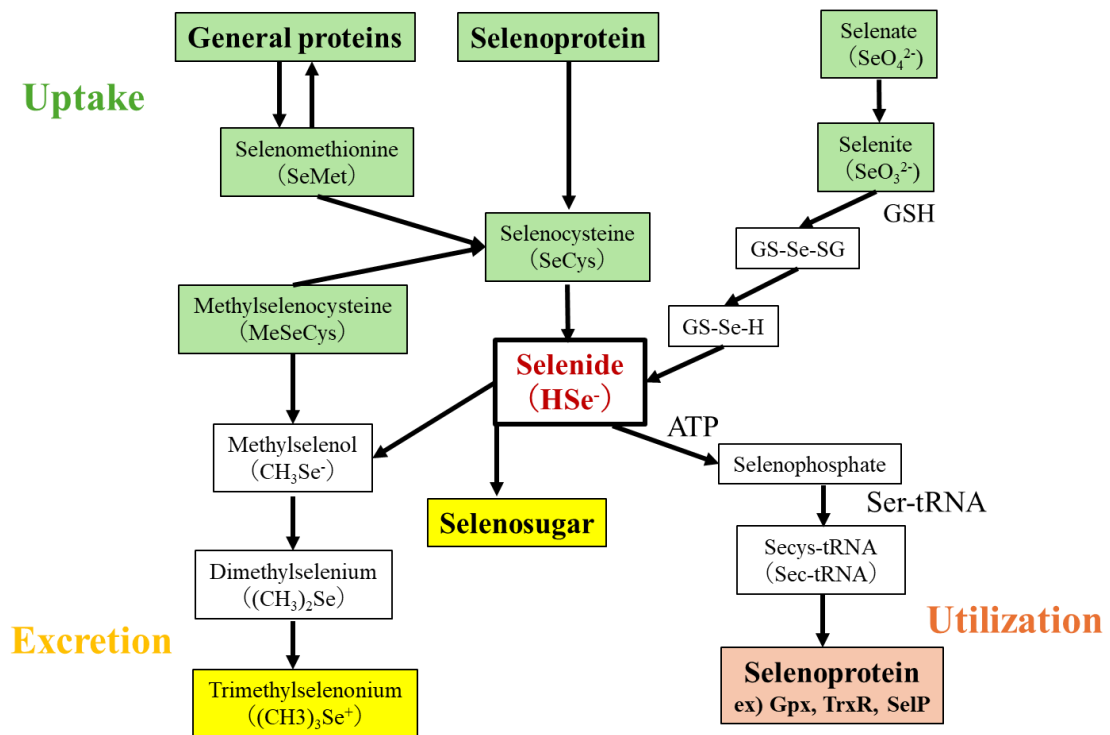


Fig. 0 The metabolic pathway of Se compounds in humans:

GS-Se-SG: Selenodiglutathione, GS-Se-H: Glutathioselenol, GSH: Reduced glutathione, ATP: Adenosine triphosphate, Ser-tRNA: Serine-transfer RNA

Chapter 1.

Inhibition of angiotensin-converting enzyme by selenoneine

1-1. Introduction

Selenoneine has been previously reported to be able to bind to and detoxify methylmercury (MeHg) by accelerating the excretion and demethylation of MeHg via secretory extracellular lysosomal vesicles that are found in animal cells and tissues (Yamashita et al., 2013b). These findings suggest that selenoneine can bind to mercury as well as other metals. A previous study reported that selones (selenoketones), the structural analogs of ketones where oxygen replaces Se, could detoxify organic mercurial compounds (Banerjee et al., 2015). Selenoketones have also been shown to react to metal halides such as copper (I) bromide and zinc chloride to form complexes (Yadav et al., 2017). Furthermore, it has also been suggested that selenoneine is able to react to the metal ion in a metalloenzyme.

Angiotensin-converting enzyme (ACE; EC 3.4.15.1), a zinc metalloprotease, is a component of the renin–angiotensin–aldosterone system. It catalyzes cleavage of the C-terminal dipeptide from angiotensin I to produce angiotensin II. This potent octapeptide plays a role in the elevation of blood pressure (Skeggs et al., 1956; Vickers et al., 2002).

The ACE inhibitors captopril and enalapril are used as antihypertensive medicines (Ondetti et al., 1977; Ferguson et al., 1982; Reynolds et al., 1984). Captopril attaches to the active center of ACE by direct interaction of its thiol group with the catalytic zinc ion of ACE (Natesh et al., 2004). ACE inhibitory

polypeptides can be produced from hydrolysates of fish meats such as sardines, in addition to also being used as source materials for functional foods and supplements (Matsufuji et al., 1994). Moreover, seleno-captopril, the Se analog of captopril, has been reported to inhibit ACE activity via the interaction of the selenol group with the zinc ion, similar to that seen in captopril (Bhuyan et al., 2011; Akif et al., 2011). A recent study has further confirmed that the selenol group does bind to the zinc ion (Banerjee et al., 2015). Therefore, I speculated that selenoneine might also be able to bind to metal ions and inhibit metalloenzymes.

In this chapter, the inhibitory effect of selenoneine on ACE activity was characterized based on *in vitro* enzymatic experiments. Enzyme kinetics revealed the type of inhibition of ACE by selenoneine. Moreover, *in silico* study predicted the location and molecular interactions of selenoneine at the active center of ACE.

1-2. Materials and Methods

1-2-1. Materials

Selenoneine was extracted from blood of tuna and purified by high performance liquid chromatography in accordance with a previous method (Yamashita and Yamashita, 2010). Selenoneine was then dissolved in Milli-Q water, with the solution adjusted to pH 6.8 using sodium hydroxide, and stored at 4 °C. Lung acetone powder from rabbit, *N*-hippuryl-L-histidyl-L-leucine hydrate (HHL), hippuric acid, L-valyl-L-tyrosine (Val-Tyr) and ergothioneine were all purchased from Sigma-Aldrich (MO, USA). Purified

bovine lung ACE was purchased from Life Laboratory (Yamagata, Japan). Sodium selenite was purchased from Wako Pure Chemical Industries (Osaka, Japan).

1-2-2. Enzyme solutions

The substrate solution consisted of 7.6 mM HHL in 200 mM borate buffer at pH 8.3, and contained 608 mM sodium chloride. To extract ACE from rabbit lung acetone powder, the powder (1.0 g) was homogenized in 10 mL of 200 mM borate buffer at 4 °C, and then centrifuged at $17,700 \times g$ for 10 min at 4 °C. The supernatant was collected and diluted by a factor of 5 using 200 mM borate buffer, and then used as the rabbit ACE solution. Purified bovine lung ACE was dissolved in 200 mM borate buffer (0.1 unit/mL), and then used as the bovine ACE solution. Inhibitor solution was made by dissolving Val-Tyr, selenoneine, ergothioneine and selenite in Milli-Q water.

1-2-3. Measurement of ACE activity

ACE activity was measured using a method previously reported with some modifications (Maruyama et al., 1987). An aliquot containing 30 μ L of inhibitor solution and 250 μ L of substrate solution was pre-incubated at 37 °C for 5 min. Subsequently, 100 μ L of rabbit ACE solution was added to the aliquot, followed by incubation at 37 °C for 20 min. To stop the reaction, 250 μ L of 1 M HCl was added to the reaction mixture. To extract hippuric acid, the product of this reaction, 1.5 mL of ethyl acetate was added to the solution, mixed and then centrifuged at $1750 \times g$ at 4 °C for 10 min, with 1.0 mL of the

supernatant then was collected. After completely desiccating the supernatant using a centrifugal evaporator, it was then diluted with 4 mL of Milli-Q water and used as the extracted hippuric acid solution. Hippuric acid was detected at an absorbance of 228 nm.

1-2-4. Kinetics studies

The kinetics of ACE with selenoneine were measured using bovine lung ACE. ACE activity was measured as per the above described method. The substrate solution was adjusted to a final concentration of 0.659–5.27 mM using a twofold serial dilution. Selenoneine was added to the reaction mixture to a final concentration of 0.5 μ M. The reaction velocity was calculated based on the amount of hippuric acid produced, which was quantified by a standard curve. Kinetic analysis was based on the Michaelis–Menten equation calculated by GraphPad Prism version 6 for Windows (GraphPad Software, CA, USA).

1-2-5. Docking simulation

A docking study was performed using selenoneine to examine the binding poses against human ACE. The crystal structure of ACE (2YDM) was obtained from the Protein Data Bank and used as the target structure for the docking study. This crystal structure was imported into the docking simulation software, Molecular Operating Environment (MOE; Chemical Computing Group, Montreal, Canada). Each of the selenoneine forms (Fig. 1) was drawn using the builder module in the MOE software. ACE and

selenoneine were prepared for docking using the Quick Prep command, and then charged and minimized under the AMBER 10 EHT force field in the MOE software.

1-2-6. Statistical analysis

Values are expressed as mean $\pm$ SD. Data were analyzed by one-way ANOVA with Tukey's multiple comparison test to identify any significant differences ($P < 0.05$) with GraphPad Prism version 6 for Windows.

1-3. Results

1-3-1. Inhibitory effect of selenoneine on ACE activity

The inhibition of rabbit lung ACE activity by the ACE inhibitor peptides and selenoneine was measured by the release of hippuric acid from the substrate peptide. Val-Tyr, which was added as the ACE inhibitory peptide, inhibited ACE activity at 2000 μ M. Selenoneine dose-dependently inhibited ACE activity at 0.4 and 2.0 μ M (Fig. 1-1a). ACE activity was not inhibited by ergothioneine and selenite at 2.0 μ M (Fig. 1-1b).

1-3-2. Kinetic study of ACE inhibition by selenoneine

Based on the enzyme kinetics, the $V_{\max}$ values were 4.27 ± 0.25 nmol/min and 4.22 ± 0.14 nmol/min, while the K_m values were 1.45 ± 0.12 mM and 0.63 ± 0.04 mM with or without selenoneine, respectively, in the experiment with purified bovine ACE (Table 1-1, Fig. 1-2). Although the K_m value in the presence of selenoneine was significantly higher than that

observed in the absence of selenoneine ($P < 0.01$), there was no change in the $V_{\max}$ values between the two groups. Lineweaver-Burk plot, the double-reciprocal plot of substrate concentrations and reaction velocities, was also presented that there was no change in the $1/V_{\max}$ values, the y-intercepts, but there was a change in the $-1/K_m$ values, the x-intercepts between the two groups (Fig. 1-2b). These results indicate that selenoneine acts via competitive inhibition.

1-3-3. Docking simulation study

In the docking simulation study, selenoneine was located in the active center of ACE. Different results were found for the selenoketone and selenol forms of selenoneine. The selenoketone form (Table 0) bound to the zinc ion (Zn1619) in ACE via the oxygen in the carbonyl group. The distance between the zinc and the oxygen was 0.221 nm. In this model, the selenoketone group interacted as a proton acceptor with glutamine (Gln-281), lysine (Lys-511) and histidine (His-513). The distances between the Se and these amino acids were 0.326, 0.318 and 0.339 nm, respectively (Fig. 1-3a, b). For this simulation, the docking score, which is the score of the affinity of a ligand and an enzyme, was -25.23 kJ/mol. In contrast, the selenol form (Table 0) interacted with other amino acids and did not bind to the zinc ion. The oxygen of the carboxyl group of selenoneine interacted with the lysine (Lys-511) and histidine (His-513) as a proton acceptor. The selenol group interacted as a proton donor with aspartic acid (Fig. 1-3c, d). For this simulation, the docking score was -21.21 kJ/mol.

1-4. Discussion

This study demonstrates that selenoneine inhibited ACE activity via competitive inhibition. The *in silico* docking simulation indicated that selenoneine inhibited ACE via the interaction between selenoneine and the zinc ion, namely stable selenoneine in the active center of ACE prevents the substrate from binding to ACE.

Previous studies have reported that some other peptides can also inhibit ACE activity. One of these, Val-Tyr, is found in the alkaline protease hydrolysate of sardine muscle. It inhibits ACE activity at a half maximal inhibitory concentration (IC_{50}) of 26.0 μM (Matsufuji et al., 1995). Selenoneine inhibited ACE activity more strongly than Val-Tyr and the IC_{50} value was 0.72 μM calculated from the result in Fig. 1-1a. Ergothioneine, which is a sulfur analog of selenoneine, and selenite did not inhibit ACE activity (Fig. 1-1b). These results suggest that the selenoketone group of selenoneine is associated with the inhibition of ACE. Since the properties of the selenketone group resemble those of the thiol group, I predicted that selenoneine would inhibit ACE in a similar way to that observed for some thiols like captopril. To clarify this, I conducted kinetic studies designed to elucidate how selenoneine inhibited ACE. The V_{max} values did not differ between the groups with or without selenoneine. In contrast, there were significantly different K_m values between these two groups (Table 1-1). These results demonstrate that the inhibition of ACE by selenoneine is competitive. In general, the K_i value is calculated to determine the potential inhibition of an inhibitor. Dixon's method to determine the K_i value requires a large

amount of selenoneine transiting stepwise from a low to high density (Dixon, 1953 and Dixon, 1972). In the current study, the K_i value, $0.37 \pm 0.09 \mu\text{M}$, was calculated by using the K_m values of the control group and the selenoneine-added group that were based on the enzyme kinetics in the presence of a competitive inhibitor $\{K_{m,\text{control}} = K_{m,\text{selenoneine}} (1 + [I]/K_i)\}$.

Previous studies have reported that seleno-captopril, a Se analog of captopril, inhibits ACE (Bhuyan et al., 2011; Akif et al., 2011). Captopril is a well-known ACE inhibitor; its thiol group chelates the catalytic zinc ion of ACE, thereby prevents substrates like angiotensin I from being cleaved (Natesh et al., 2004). Furthermore, it has also been reported that seleno-captopril inhibits ACE in the same way as reported for captopril (Akif et al., 2011). On the basis of these previous studies and my current results, I speculated that selenoneine also inhibits ACE via the chelation of the zinc ion through the selenoketone group. To test this hypothesis, I conducted an *in silico* docking simulation, which can predict the locations, forms and styles of small molecules by using enzymes. In this chapter, the crystallography of the testis ACE with seleno-captopril from the Protein Data Bank (ID 2YDM) was used for the docking simulation (Fig. 1-3). The simulation results showed that, although selenoneine was located at the active center of ACE, Se did not chelate the zinc ion. However, the oxygen in the carbonyl group chelated the zinc ion and the selenoketone group interacted with several amino acids (glutamine, lysine and histidine; Fig. 1-3b) as a proton acceptor. Fig. 1-3c, d shows the simulation results when selenoneine is the selenol form in the active center of ACE. I considered these results as reference findings since

selenoneine exists in the selenoketone form in the physiological condition (Yamashita and Yamashita, 2010). Based on these findings, I speculated that these interactions contributed to both the stability of selenoneine in the enzyme and the inhibitory effect.

The present findings also suggest that the inhibition of ACE in humans and animals might be a means of preventing hypertension *in vivo*. Thus, it is necessary to characterize the inhibition of ACE *in vivo* in conjunction with selenoneine intake. In addition, the findings that selenoneine exhibited interactions and has zinc-chelating abilities suggest that selenoneine may bind and inhibit other metalloenzymes, such as matrix metalloproteases and several cytochrome P450s.

In conclusion, this study found a novel biochemical function of Se in the inhibition of ACE activity by selenoneine. The results suggest that selenoneine might bind to the zinc ion at the active center of the enzyme.

1-5. Tables and Figures

Table. 1-1 $V_{\max}$ and K_m values of control and selenoneine-addition groups

	Control	Selenoneine
$V_{\max}$ (nmol/min)	4.22 ± 0.14	4.27 ± 0.25
K_m (mM)	0.63 ± 0.04	$1.45 \pm 0.12^*$

Inhibition of selenoneine against bovine ACE was measured. Values are mean $\pm$ SD of three replicates. Asterisks (*) indicate significant differences between groups ($P < 0.05$). Statistical comparison was made with Student's t-test.

Fig. 1-1

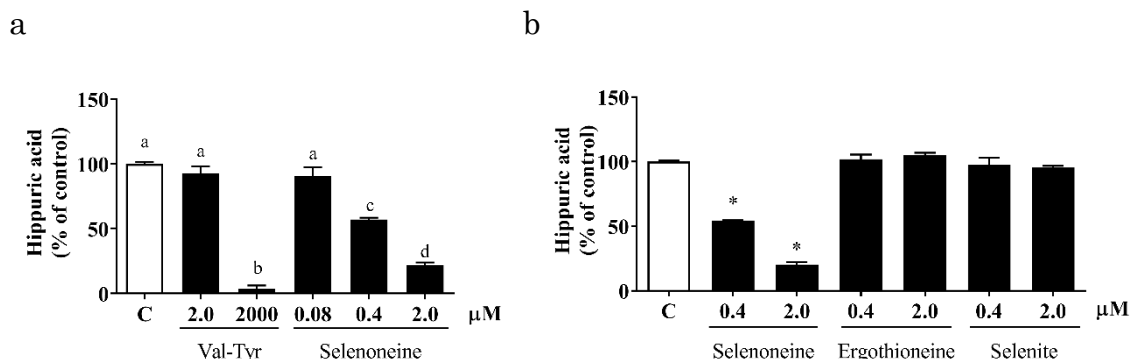


Fig. 1-1 Comparisons of the inhibitory effects of selenoneine and other compounds on rabbit angiotensin-converting enzyme (ACE) activity: a) Inhibitory effect of L-valyl-L-tyrosine and selenoneine against rabbit ACE. White bar: Value of the control (C) group. Values are mean $\pm$ SD of three replicates. Values with different letters are significantly different ($P < 0.05$). b) Inhibitory effect of selenoneine, ergothioneine and selenite. Values are mean $\pm$ SD of three replicates. Asterisks indicate significant differences between groups ($P < 0.05$). Statistical comparison was made with Tukey's multiple comparison test.

Fig. 1-2

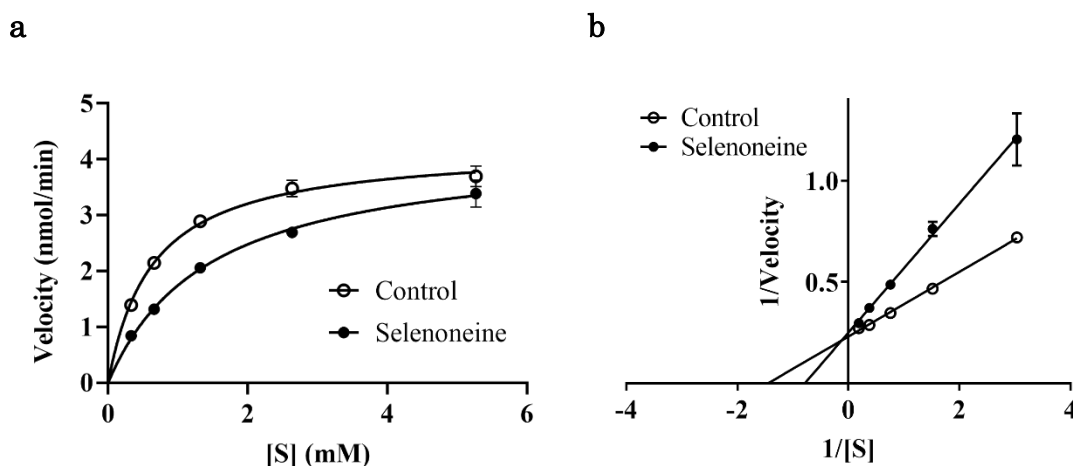


Fig. 1-2 Kinetic analysis of ACE with or without selenoneine: a) Activity of bovine ACE in the presence of selenoneine. Velocity was calculated from the amount of hippuric acid produced per minute. b) Lineweaver-Burk plot of activity of ACE with or without selenoneine. Values are mean $\pm$ SD of three replicates.

Fig. 1-3

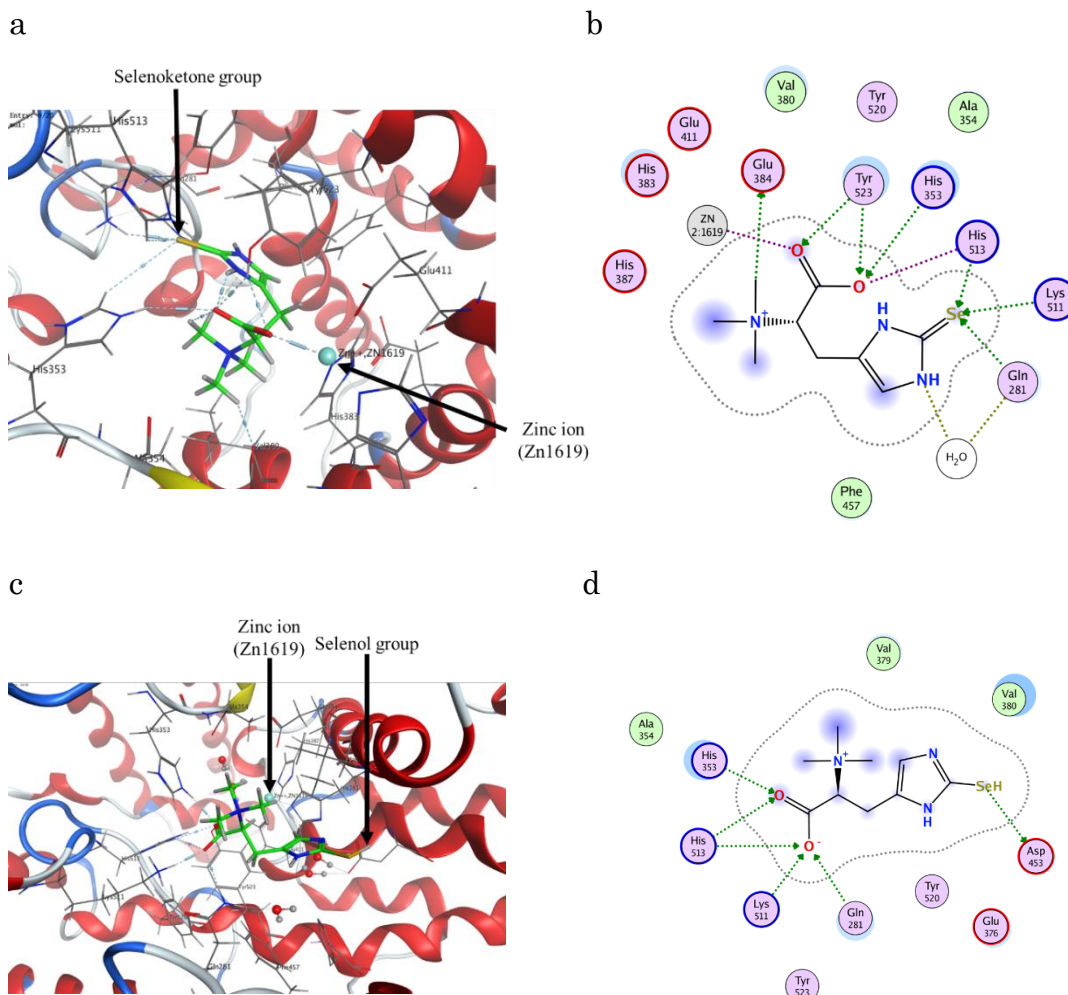


Fig. 1-3 Results of the *in silico* molecular docking simulation study: a) One of the stable positions and structures of selenoneine, the selenoketone form (docking score of -25.2 kJ/mol) at the active center of ACE (identifier 2YDM). b) Protein–ligand interaction of this simulation. c, d) Results of the simulation for the selenol form of selenoneine (docking score -21.2 kJ/mol). Purple dotted line indicates metal or ion contact. Green dotted line indicates side chain proton acceptor/donor. Amino acids circled in blue are basic amino acids; amino acids circled in red are acidic amino acids. Background disks behind certain amino acids, e.g., Tyr-523 in a), denote their solvent exposure. Blue shading behind certain ligand atoms also denotes their solvent exposure. Amino acids or ligand atoms with larger background disks or deeper shading were more exposed to solvent. Pictures and figures were predicted and described with MOE software.

Chapter. 2

Selenoneine suppresses melanin synthesis by inhibiting tyrosinase in murine B16 melanoma cells and human 3D epidermal model containing functional melanocytes

2-1. Introduction

In Chapter 1, selenoneine inhibited ACE activity by its binding to the zinc ion at the active center of ACE (Fig. 1-3, Seko et al., 2019) and selenoneine is predicted to be a metal-binding small molecule. Previous studies showed that Se has metal-binding functions (Akif et al., 2011; Raymond et al., 2011). For example, Se analog of captopril chelates zinc ion in ACE (Akif et al., 2011) and Se has a high-binding affinity to mercury (Raymond et al., 2011). In experimental rats fed both methylmercury (MeHg) and sodium selenite, Se intake reduced MeHg toxicity effects such as weight loss and hind-limb crossing (Ralston et al., 2008). In a zebrafish toxicity assay, selenoneine detoxified MeHg by accelerating the excretion and demethylation of MeHg (Yamashita et al., 2013b). The excretion is predicted to be caused by forming a complex of MeHg with selenoneine (Yamashita et al., 2013b) because selenoketones can be used to detoxify organic mercurials (Banerjee et al., 2015). Moreover, selenoketones react with metal halides, such as CuBr and ZnCl₂, to produce complexes (Yadav et al., 2017). Therefore, selenoneine is predicted to bond to copper (Cu) ions.

Tyrosinase (EC 1.14.18.1) is a metalloenzyme that has two Cu ions in its active center (Solomon et al., 1996) and is essential in the melanin

biosynthetic pathway (Oetting et al., 2000). Tyrosinase catalyzes hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and dehydrogenation of L-DOPA to DOPA quinone (Cooksey et al., 1997). DOPA quinone is then converted to melanin (Oetting et al., 2000). Because melanin is a cause of pigmentation in the skin, inhibition of tyrosinase activity is effective for the prevention of stains and freckles. For example, hydroquinone, arbutin, kojic acid, and ellagic acid, which are used as typical skin-whitening agents, reduce the melanin content in cells or the skin of animals (Jimbow et al., 1974; Battaini et al., 2000; Shimogaki et al., 2000; Sugimoto et al., 2004; Lajis et al., 2012). Moreover, these compounds and ergothioneine inhibit tyrosinase activity (Jimbow et al., 1974; Battaini et al., 2000; Shimogaki et al., 2000; Sugimoto et al., 2004; Lajis et al., 2012; Liao et al., 2012). In particular, kojic acid and ellagic acid are predicted to competitively inhibit tyrosinase activity by chelating copper ions (Shimogaki et al., 2000; Battaini et al., 2000; Lima et al., 2014), suggesting that low molecular weight compounds reactive to metal ions may inhibit tyrosinase activity. Hence, selenoneine may act as a metal-binding molecule to inhibit tyrosinase activity and decrease melanin production and accumulation.

In this chapter, I report novel biological functions of selenoneine for inhibiting melanin synthesis based on enzymatic and cellular experiments. The biological activity of selenoneine was compared with that of other antioxidants, such as a thiol-compound, ergothioneine, and Se supplement for cell culture, sodium selenite, in murine B16 melanoma cells and normal human epidermal melanocytes as an alternative experiment using mammals.

In this chapter, the inhibitory activity of selenoneine against tyrosinase was characterized by an enzymatic experiment using purified mushroom tyrosinase. Additionally, *in silico* docking simulation suggested that selenoneine inhibited the copper enzyme tyrosinase due to binding to a copper ion in the active center of the enzyme.

2-2. Materials and Methods

2-2-1. Materials

Selenoneine was extracted from tuna, purified by high-performance liquid chromatography at the National Research Institute of Fisheries Science (Yamashita and Yamashita, 2010), and dissolved in Milli-Q water. The pH of the solution was adjusted to 6.8 with NaOH and stored at 4 °C. α -Melanocyte stimulating hormone (α -MSH) was purchased from Phoenix Pharmaceuticals, Inc. (CA, USA), dissolved in Milli-Q water, and stored at 4 °C (100 μ M). Synthetic melanin was purchased from MP Biomedicals, Inc. (CA, USA). Mushroom tyrosinase, L-DOPA, ergothioneine, and arbutin were purchased from Sigma-Aldrich Co. (MO, USA). Sodium selenite and L-ascorbic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2-2-2. Cell culture

A murine melanoma cell line, B16 melanoma 4A5 (RCB0557), was obtained from the RIKEN BioResource Research Center (Tsukuba, Japan). The B16 cells were maintained in Dulbecco's modified Eagle's medium

(DMEM; GE Healthcare, IL, USA) with 10% fetal bovine serum (FBS, Thermo Scientific, MA, USA) and 1% penicillin–streptomycin solution (Wako, Osaka, Japan). Cells were cultured in a CO₂ incubator (37 °C, 5% CO₂).

2-2-3. Cell viability assay for B16 melanoma cells

Cells (1.0×10^4) were seeded in a 96-well plate with DMEM + 10% FBS and incubated for 24 h. Selenoneine was added by changing to new DMEM medium with a final concentration of 0–5000 nM of selenoneine, and cells were incubated for 24 h. Cell viability was then assayed with the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

2-2-4. Induction of melanin synthesis and measurement of melanin for B16 melanoma cells

Cells (2.0×10^5) were seeded in a 12-well plate with DMEM + 10% FBS and incubated for 24 h. Melanin synthesis was induced by incubation in 1.0 μ M α -MSH for 72 h. Cells were treated with selenoneine (0.01, 0.1, and 1.0 μ M), ergothioneine, sodium selenite, ascorbic acid, or arbutin (1.0 μ M) at the same time a final concentration of 1.0 μ M α -MSH was added. Melanin content was measured according to a previous method (Ohno et al., 2010). Cells were washed with phosphate buffered saline (PBS) and dissolved in 250 μ L of 1 N NaOH for 5 min at 100 °C, and cell lysates were sonicated on ice. Cell lysates (100 μ L) were added to a 96-well plate, and the absorbance at 405 nm was measured by a microplate reader (SpectraMax 190, Molecular Devices

Corporation, CA, USA). Melanin content was calculated using a standard curve of synthetic melanin.

2-2-5. 3D culture assay for human skin

MEL-300-B (Kurabo, Osaka, Japan) was used as a human skin model and was constructed with normal human epidermal keratinocytes and normal human epidermal melanocytes from a Black donor. Cells were grown in 3D conditions. The human skin model was maintained in special medium to induce melanin synthesis by α -MSH (EPI-100LLMM, Kurabo, Osaka, Japan). Selenoneine was added in the medium at 0.2–5.0 μ M throughout the time the cells were in culture. Cells were cultured in a CO₂ incubator (37 °C, 5% CO₂) for 16 days. Melanin was extracted as described above for B16 melanoma 4A5 cells.

2-2-6. Inhibition assay for cellular tyrosinase activity in B16 melanoma cells

Cellular tyrosinase activity was measured according to a previous study (Ohguchi et al., 2006) with some modifications. B16 melanoma 4A5 cells (2.0×10^5) were seeded in a 12-well plate and incubated for 24 h. Melanin synthesis was induced with 1.0 μ M α -MSH for 72 h, and 1.0 μ M selenoneine was added simultaneously. The cells were washed with cold PBS and lysed in 500 μ L radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris–HCl, pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA) containing protease inhibitors (Wako, Osaka, Japan). The lysates were collected in 1.5 mL microtubes and centrifuged at $10,627 \times g$ for 10 min.

The supernatant (100 μ L) as a source of tyrosinase was mixed with 50 μ L of 0.1 M phosphate buffer, pH 6.8, in a 96-well microplate, and 50 μ L of 25 mM L-DOPA was then added. The reaction was monitored at 475 nm every 5 min for 60 min at 37 °C.

2-2-7. Inhibition assay for mushroom tyrosinase activity

Tyrosinase activity was measured according to a previous study (Kubo et al., 1999). Selenoneine aqueous solution (20 μ L at 0, 10, and 100 μ M) was mixed with 100 μ L of 1 M phosphate buffer (pH 6.8) in a 96-well plate. Tyrosinase aqueous solution (40 μ L at 40 U/mL) from mushrooms was added to each well, and the plate was pre-incubated for 10 min at 20 °C. Then, 40 μ L L-DOPA (5.0 mM) or Milli-Q water was added to each well, and reactions were monitored at 475 nm every 5 min for 60 min at 27 °C. The tyrosinase inhibition activity was calculated as absorbance of A–B (A: the absorbance of test sample, B: the absorbance of test sample without L-DOPA).

2-2-8. Docking simulation

A docking study was performed on selenoneine to examine its binding pose against tyrosinase. The crystal structure of tyrosinase from mushrooms was obtained from the Protein Data Bank (ID: 2Y9X) and used as the target structure for the docking study. This crystal structure was imported to the docking simulation software, Molecular Operating Environment (MOE, Chemical Computing Group, Montreal, Canada). Selenoneine and kojic acid were drawn using the builder module in MOE. According to Chapter 1 (Fig.

1-3; Seko et al., 2019), tyrosinase and selenoneine were prepared for docking using the MOE Quick-Prep command. The protein and ligands were charged and minimized under the AMBER10EHT force field in MOE. Atoms of the protein farther than 0.8 nm from ligands were fixed. Water molecules farther than 0.45 nm from ligand and receptor were deleted. This is the standard setting of this software.

2-2-9. Statistical analysis

Data were analyzed by one-way analysis of variance with Tukey's multiple comparison test to identify any significant differences ($P < 0.05$) using GraphPad Prism version 6 software for Windows.

2-3. Results

2-3-1. The effect of selenoneine on cell viability of B16 melanoma cells

Cell viability of B16 melanoma 4A5 was not affected by 0–5000 nM selenoneine (Fig. 2-1). Therefore, in the following cell experiments, selenoneine was added up to 5000 nM.

2-3-2. Inhibition of melanin synthesis by selenoneine in B16 melanoma cells

The melanin content was measured in the presence or absence of selenoneine in B16 cells. Melanin synthesis was induced by addition of 1.0 μ M α -MSH. In the presence of α -MSH, melanin content was 4.6 times higher than that of the control group. In cells cultured in the presence of 0.10 and 1.0 μ M selenoneine, the melanin content was significantly lower than that of

cells cultured with 1.0 μM α -MSH, indicating that selenoneine inhibited melanin synthesis in a dose-dependent manner (Fig. 2-2a). When we compared melanin content between cells treated with 1.0 μM selenoneine and arbutin, both compounds exhibited similar melanin levels, indicating inhibition of melanin synthesis. In contrast, other antioxidants including 1.0 μM ergothioneine, selenite, and ascorbic acid led to higher melanin content than selenoneine (Fig. 2-2b).

2-3-3. The effect of selenoneine on human 3D epidermal model containing functional melanocytes

To confirm the inhibitory effect of selenoneine on melanin synthesis under conditions that mimic *in vivo* conditions, human 3D epidermal model containing functional melanocytes (MEL-300-B) were examined. The control cells showed a brown color and 1.22 ± 0.08 $\mu\text{g}/\text{well}$ melanin. The cells treated with 1.0 and 5.0 μM selenoneine showed the strongest suppression of pigmentation (Fig. 2-3a). Melanin content was significantly lower in the cells cultured with 1.0 and 5.0 μM selenoneine and 5.0 μM arbutin than in control cells (Fig. 2-3b). In the presence of 1.0 μM selenoneine, the melanin content was 0.483 ± 0.027 $\mu\text{g}/\text{well}$, and that with 5.0 μM selenoneine was 0.280 ± 0.007 $\mu\text{g}/\text{well}$. Compared with control cells, 1.0 and 5.0 μM selenoneine reduced melanin to 39.7% and 23.0%, respectively. The melanin content of cells treated with 5.0 μM arbutin was 72.3% of the control. These findings show that both selenoneine and arbutin inhibited melanin accumulation.

2-3-4. Inhibition of cellular tyrosinase activity in B16 melanoma cells

For melanin generation, tyrosinase catalyzes oxidation reactions of tyrosine to DOPA, and DOPA to DOPA quinone. This enzyme is highly active in melanin-synthesizing cells (Ohguchi et al., 2006). When I measured tyrosinase activity in B16 melanoma cells, α -MSH-treated cells showed higher tyrosinase activity, and cells treated with 1.0 μ M selenoneine showed lower activity than that of α -MSH-treated cells (Fig. 2-4a). In differences of absorbance from 0 to 60 min, α -MSH-treated cells exhibited significantly higher tyrosinase activity than control cells with the increase of 30.9%, and there is no significance between control cells and selenoneine-treated cells with the increase of only 3.96% (Fig. 2-4b).

2-3-5. Inhibitory activity for mushroom tyrosinase

To characterize the mechanism of melanin synthesis suppression by selenoneine, tyrosinase activity was measured with purified mushroom tyrosinase. Tyrosinase activity in cells was not inhibited in the presence of 1.0 μ M selenoneine, whereas 60% of tyrosinase activity was inhibited in the presence of 10 μ M selenoneine (Fig. 2-5).

2-3-6. Docking simulation study

In the docking simulation study, selenoneine was located in the active center of tyrosinase. The selenoketone form (Table 0) of selenoneine interacted with copper ions (Cu400 and Cu401) in tyrosinase through the oxygen in the carbonyl group (Fig. 2-6a, b). The distances between the copper

ions (Cu400 and Cu401) and the Se were 0.281 nm and 0.261 nm, respectively. Asparagine (Asn-260) and valine (Val-283) contributed to the stability of selenoneine at the active center as a side chain acceptor and by inducing arene-H interaction, respectively. The docking score, which is a score of the affinity of a ligand for an enzyme, in this simulation was -29.5 kJ/mol. Kojic acid was simulated under the same conditions, and the docking score was -22.8 kJ/mol (Fig. 2-6c).

2-4. Discussion

In this chapter, selenoneine inhibited tyrosinase activity and decreased the melanin content in 2D- and 3D-cultured cells. These results may be due not only to the antioxidant function of selenoneine but also to its inhibitory effect on tyrosinase. The mechanism of tyrosinase inhibition was speculated to be binding of selenoneine to the copper ions in the active center of tyrosinase, and bound selenoneine may have interrupted the ability of tyrosinase to hydroxylate tyrosine and dehydrogenate DOPA.

In a previous study, the number of B16 cells was slightly decreased by 1.0 μ M sodium selenite, and significantly decreased by 1.0 μ M selenocystine (Siwek et al., 1994). Because selenoneine had no cytotoxicity up to 5000 nM (Fig. 2-1), it can be used for Se supplementation in cultures of B16 cells and human 3D epidermal model containing functional melanocytes. In contrast, sodium selenite and selenocystine are toxic to cultured cells. Selenite reacts with reduced glutathione (GSH) and leads to the formation of selenodiglutathione (GS-Se-SG, Fig. 0). GS-Se-SG is a toxic Se species that

induces apoptosis by GS-Se-SG-mediated oxidative DNA damage and depends on coexistence with thiol compounds (Tobe et al., 2015). Moreover, selenocystine also induces apoptosis via oxidative stress (Chen and Wong 2009).

Selenoneine decreased the melanin content in B16 melanoma cells in a dose-dependent manner (Fig. 2-2a). In addition, the inhibitory effect on tyrosinase was observed with 5.0 μ M selenoneine in human 3D epidermal model containing functional melanocytes as an alternative experiment to the *in vivo* animal test (Fig. 3). Selenoneine apparently reduced the melanin content in comparison with other types of antioxidants, including ergothioneine, ascorbic acid, and inorganic Se, selenite (Fig. 2-2b). Arbutin at 1.0 μ M also reduced the melanin content (Fig. 2-2b). In a previous report, a kinetic analysis showed the ability of ergothioneine to inhibit tyrosinase, but the concentration required to suppress cellular melanin synthesis remains unclear (Liao et al., 2012). Arbutin, 4-Hydroxyphenyl β -D-Glucopyranoside, has been used as a strong skin whitening agent because it inhibits tyrosinase activity and suppresses melanin synthesis (Akiu et al., 1991).

Many compounds have been reported to decrease melanin content, and the mechanisms of this effect vary among compounds. For example, radical scavengers such as ascorbic acid suppress melanin synthesis by reducing reactive oxygen species (Panich et al., 2011). Sulforaphane downregulates tyrosinase expression by modulating mitogen-activated protein kinase pathways (Shirasugi et al., 2010). Kojic acid and ellagic acid inhibit tyrosinase activity and thus reduce melanin content (Lajis et al., 2012;

Mishima et al., 1988; Shimogaki et al., 2000). Selenoneine is incorporated into cells via the transporter, OCTN1 (Yamashita et al., 2013b), and may also inhibit endogenous tyrosinase. Alternatively, selenoneine may reduce oxidative stress conditions in melanocytes, and repress indirect mechanisms of melanin formation similar to the reducing agent glutathione (Matsuki et al., 2008). Moreover, selenoneine is thought to suppress tyrosinase expression like sulforaphane. To confirm whether tyrosinase expression is changed by selenoneine, the amount of tyrosinase protein should be determined.

When *in vitro* tests using enzymes were conducted, the crude enzymes from cells treated with α -MSH demonstrated significantly higher tyrosinase activity than enzymes from control cells and selenoneine inhibited tyrosinase activity at the control level (Fig. 2-4). In the experiment, selenoneine was added while culturing cells, and we assume that selenoneine inhibited endogenic tyrosinase directly and reduced the ability of tyrosinase to catalyze DOPA and DOPA quinone in the cell lysate. To verify that, however, further experiments are needed in which selenoneine is added in a dose-dependent manner into the cells and lysates after cell culturing and tyrosinase activity is compared, since tyrosinase is included in melanosomes in a melanocyte (Marks and Seabra 2001). Although the localization of selenoneine in a cell remains unclear, if selenoneine is incorporated into melanosomes, selenoneine may inhibit tyrosinase at a higher concentration than selenoneine added into cell lysates. On the other hand, selenoneine may directly chelate and bind copper ions like kojic acid (Lajis et al., 2012; Mishima et al., 1988). Such metal-binding function of selenoneine is

supported by the results in Chapter 1 that selenoneine inhibits ACE activity by binding to the zinc ion in the active center (Fig. 1-3; Seko et al., 2019). When I used purified mushroom tyrosinase having two copper ions in the active center as a model of eukaryotic tyrosinase (Ochiai et al., 2016), selenoneine inhibited its activity at 10 μ M and is suggested to suppress melanin synthesis by chelating copper ions and inhibiting tyrosinase. However, selenoneine did not inhibited tyrosinase at 1.0 μ M (Fig. 2-5), at which murine and human cells were suppressed melanin synthesis (Fig. 2-2, 2-3). The difference was assumed to be caused by not only selenoneine affected to melanin synthesis pathways except tyrosine oxidation by tyrosinase but the difference of structures between mushroom tyrosinase and human or murine tyrosinase. A previous study reported that tyrosinase from mushroom and from human share only about 23% of their sequences, and some inhibitors showed different inhibitory activities depending on the origin of the enzyme (Oyama et al., 2022).

In order to clarify the inhibition pattern between selenoneine and tyrosinase, assays based on enzyme kinetics are needed. In Chapter 1, selenoneine inhibited ACE activity competitively by the Michaelis–Menten equation and the Lineweaver-Burk plot (Fig. 1-2; Seko et al., 2019). However, I failed to reveal how selenoneine inhibits tyrosinase by the experiment using selenoneine and purified tyrosinase from mushroom because the solvents were colored when the concentrations of DOPA as a substrate were low. Accordingly, a docking simulation study was conducted to infer the inhibition pattern between selenoneine and tyrosinase compared with the result of a

competitive inhibitor of tyrosinase such as kojic acid. In addition, simulation study predicted the relation between selenoneine and metal ions. The binding affinity between selenoneine and tyrosinase was estimated with the docking score. The docking score represents the potential for binding calculated by the sum of interactions including electrostatic, van der Waals, and Coulombic interactions, and the formation of hydrogen bonds (Pagadala et al., 2017). In this simulation, the docking score was -29.5 kJ/mol, and that of kojic acid, a competitive inhibitor of tyrosinase, was estimated to be -22.8 kJ/mol under the same conditions. On the other hand, selenoneine may react with some metal ions in metalloenzymes because organic Se reacts with metal halides, such as CuBr and ZnCl_2 , to create complexes (Yadav et al., 2017). However, this study predicted that selenoneine chelated copper ions in the active center of tyrosinase not via Se but via the oxygen in the carbonyl group of selenoneine (Fig. 2-6). These findings suggest that selenoneine located in the ligand binding pocket is more stable than kojic acid and inhibited tyrosinase competitively. Compared to the results for ACE in Chapter 1, although the distances between oxygen and copper ions (0.281 nm and 0.261 nm) were greater than the distance between the oxygen in the carbonyl group of selenoneine and the zinc ion of ACE (0.221 nm), selenoneine might be more stable at the center of tyrosinase because of its lower docking score (-29.5 kJ/mol) than that of selenoneine and ACE (-25.2 kJ/mol) (Fig. 1-3, Seko et al., 2019). It is suggested that these differences are caused by the numbers and types of metal ions binding to selenoneine. Based on my findings, selenoneine inhibited tyrosinase and ACE. The mechanism of chelating metals in the

active center of a metalloenzyme and inhibiting enzyme activity may be a novel biological function of selenoneine.

In conclusion, results based on the cellular test, the alternative test of an animal experiment, the *in vitro* enzymatic experiment, and the simulation study in Chapter 2 indicate that selenoneine decreased melanin content and thus could be a candidate for use as a new skin-whitening agent.

2-5. Tables and Figures

Fig. 2-1

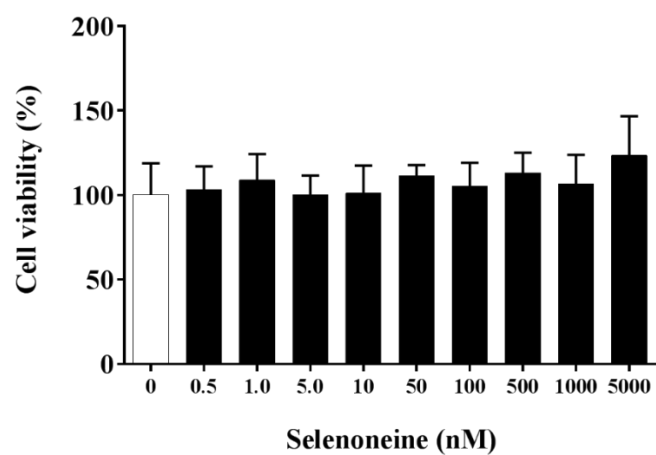


Fig. 2-1 The effect of selenoneine on the cell viability of B16 melanoma cells: Values are the mean $\pm$ standard deviation (SD) ($n = 5$ at 0–1000 nM and $n = 6$ at 5000 nM). No significant difference was found. Statistical comparison was made with Tukey's multiple comparison test.

Fig. 2-2

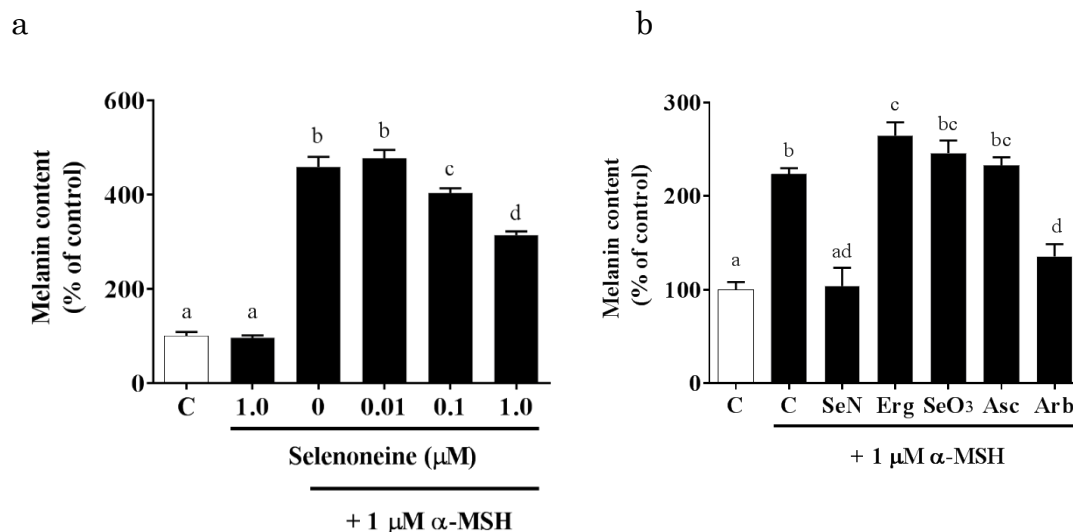


Fig. 2-2 The effect of selenoneine on melanin synthesis in B16 melanoma cells: a) Selenoneine reduced the melanin content in melanoma cells in a dose-dependent manner. C indicates the control group without selenoneine and α -MSH. Values are the mean $\pm$ SD (n = 4). b) Comparison of the effect of other compounds on melanin synthesis. C indicates control groups without test substances. SeN, Erg, SeO₃, Asc, and Arb indicate selenoneine, ergothioneine, sodium selenite, ascorbic acid, and arbutin, respectively. Values are the mean $\pm$ SD (n = 3). Values with different letters are significantly different ($P < 0.05$). Statistical comparison was made with Tukey's multiple comparison test.

Fig. 2-3

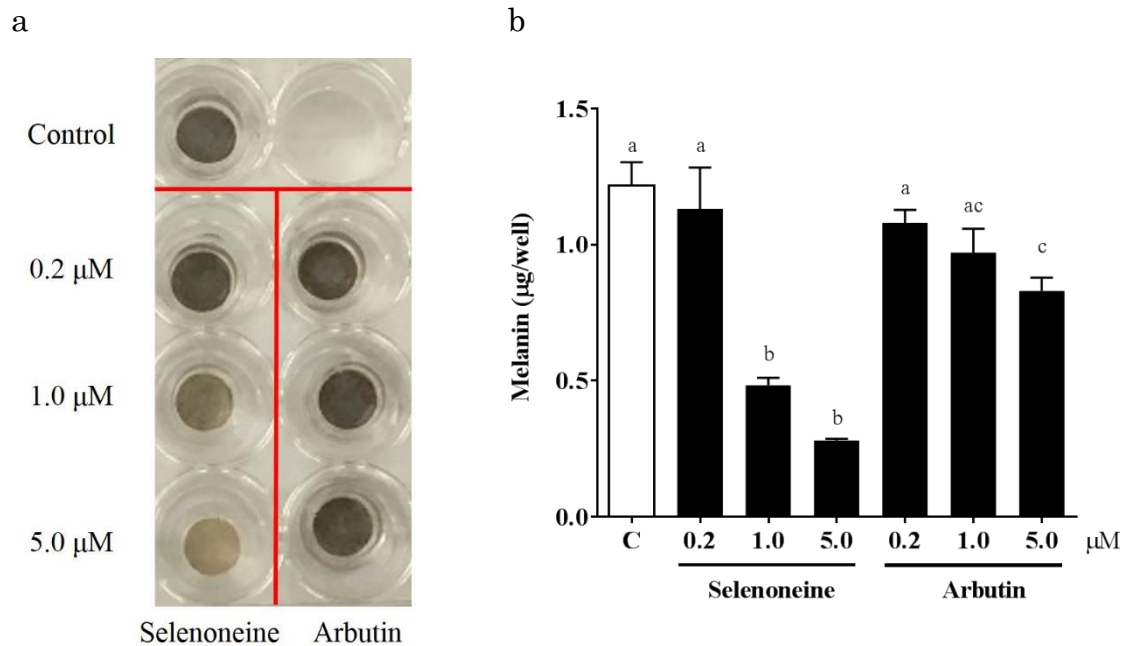


Fig. 2-3 Comparison of the effects of selenoneine and arbutin on melanogenesis in human 3D epidermal model containing functional melanocytes: a) The photograph shows that selenoneine and arbutin suppressed pigmentation in cells treated with 1.0 and 5.0 μM selenoneine and 5.0 μM arbutin after 16 days of incubation. b) Quantitative values of cellular melanin are shown as the mean $\pm$ SD ($n = 3$). C indicates the control group. Values with different letters are significantly different ($P < 0.05$). Statistical comparison was made with Tukey's multiple comparison test.

Fig. 2-4

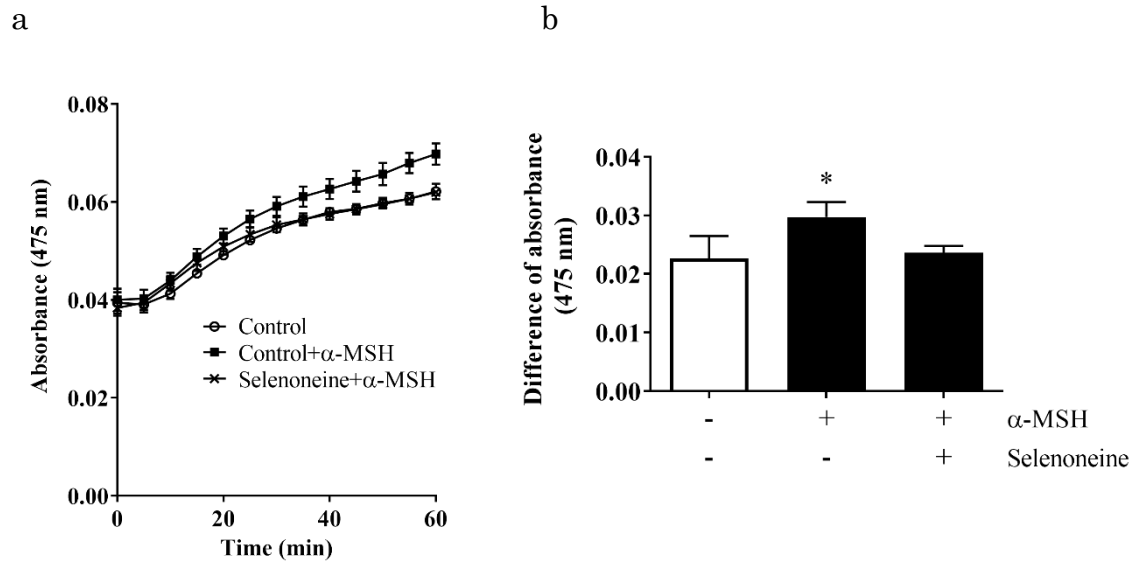


Fig. 2-4 Measurement of cellular tyrosinase activity: a) The activity of extracted cellular tyrosinase was tested by measuring DOPA quinone at an absorbance of 475 nm. b) Tyrosinase activity represented by differences of absorbance at 475 nm from 0 to 60 min. Cells were treated with or without 1.0 μ M selenoneine and 1.0 μ M α -MSH. Values are the mean $\pm$ SD (n = 3). A value with an asterisk is significantly different ($P < 0.05$). Statistical comparison was made with the Dunnett multiple comparison test.

Fig. 2-5

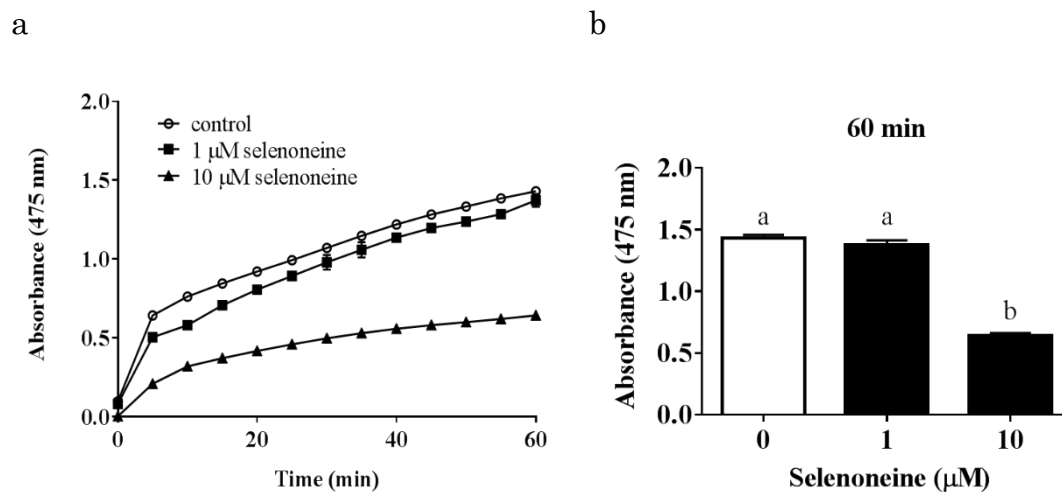


Fig. 2-5 Effect of selenoneine on mushroom tyrosinase activity by the oxidation of L-DOPA: a) The activity of mushroom tyrosinase was tested by measuring DOPA quinone. b) Mushroom tyrosinase activity after 60 min of incubation is shown in the bar graph. C indicates the control group. Values are the mean $\pm$ SD ($n = 3$). Values with different letters are significantly different ($P < 0.05$). Statistical comparison was made with Tukey's multiple comparison test.

Fig. 2-6

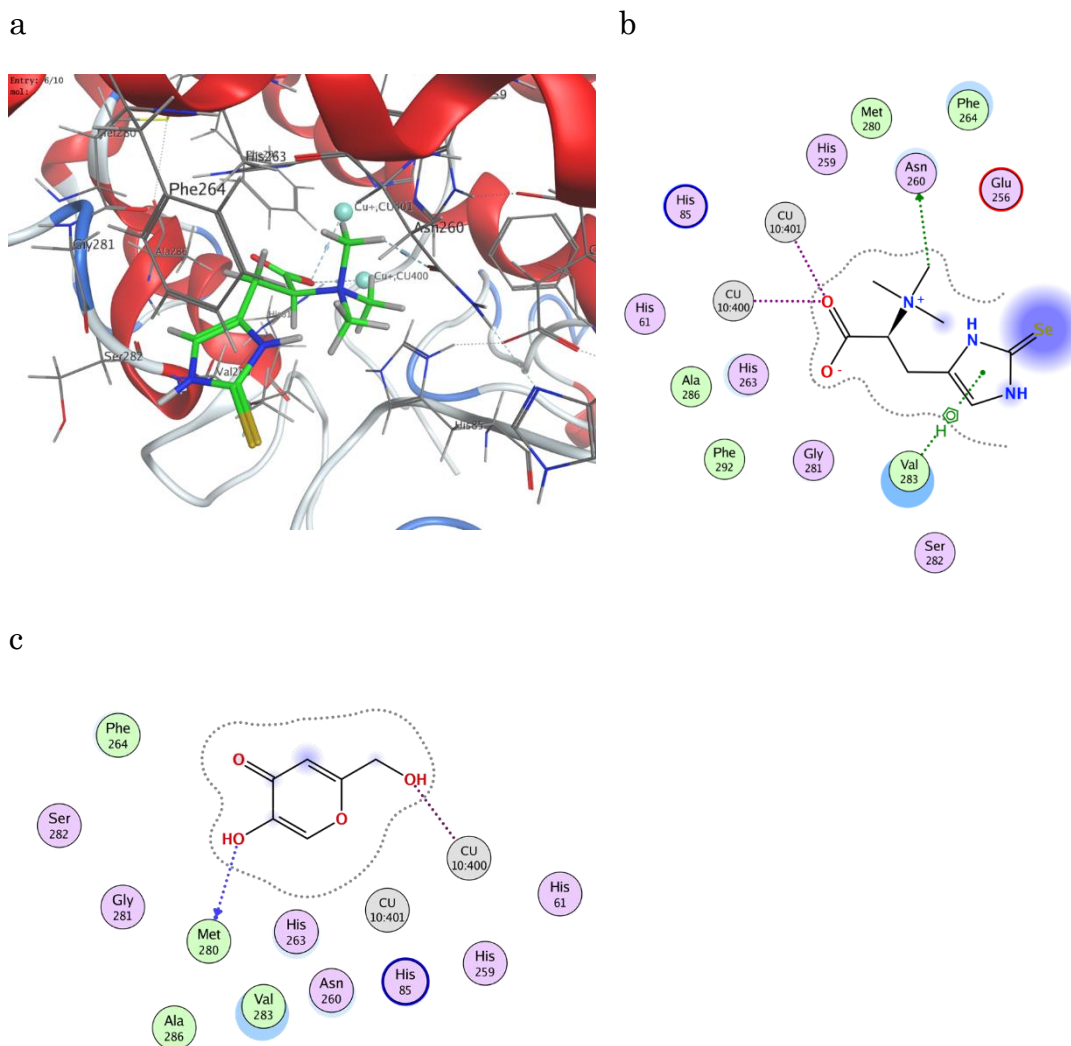


Fig. 2-6 The results of the *in silico* molecular docking simulation study: a) One of the stable positions and structures of the selenoketone form of selenoneine (Fig. 1) at the active center of mushroom tyrosinase (ID: 2Y9X). The docking score, which indicates the stability, was -29.5 kJ/mol. b, c) Protein–ligand interaction of this simulation by selenoneine and kojic acid. The docking score of kojic acid was -22.8 kJ/mol. The purple dotted lines indicate metal or ion contact. The green dotted lines indicate the side chain proton acceptor/donor, and the blue line indicates the basic chain proton acceptor/donor. Blue mist drawn behind some of the ligand atoms also denotes their solvent exposure. Amino acids or ligand atoms having larger disks or mist are more exposed to solvent. These pictures and figures were predicted and described using Molecular Operating Environment (MOE) software (Chemical Computing Group, Montreal, Canada).

Chapter. 3

Novel method for separating selenoneine reduced monomer and ergothioneine from fission yeast extracts

3-1. Introduction

In Chapter 1 and 2, selenoneine inhibited metalloenzymes such as ACE and tyrosinase, but the effects were observed just *in vitro* or *in cellulo* experiments (Seko et al., 2019; Seko et al., 2020). For investigations those inhibitory effects *in vivo*, the distribution, accumulation, metabolism and excretion of selenoneine in the body should be considered for determination of appropriate doses and administration methods of selenoneine. To reveal such dynamics of selenoneine in the body, an authentic selenoneine and isotope labeled one have been required. However, it is difficult to purify selenoneine from seafoods with high adulteration and is difficult to obtain labeled one because the synthetic pathway of selenoneine has not been identified in marine organisms. Moreover, selenoneine is not available for purchase. Therefore, a chemical synthesis procedure of selenoneine or a method to purify selenoneine from a bioresource with controllable Se supply and with low adulteration like microorganisms are needed.

The synthesis of selenoneine by a genetically modified fission yeast was recently reported (Pluskal et al., 2014). Replacement of the native promoter to the nmt1 promoter allows this yeast strain to overexpress egt1, a gene in the ergothioneine biosynthesis pathway (Pluskal et al., 2014). The strain produces ergothioneine abundantly in thiamine-free EMM-2 medium

(Pluskal et al., 2014). Addition of sodium selenate (Na_2SeO_4) to the medium results in the production of both selenoneine and ergothioneine in the yeast cells (Pluskal et al., 2014). Other genetically modified fungi were later reported to produce selenoneine in media containing selenite or selenocysteine as a Se source (Ichikawa et al., 2018).

Extracts of overexpressing yeast strain have been characterized and used as raw materials for the purification of selenoneine (Ichikawa et al., 2018). Recently, Turrini et al. reported a method for isolating selenoneine from methanol extracts of the genetically modified fission yeast strain FY25320 obtained from the Yeast Genetic Resource Center (Osaka, Japan) (Turrini et al., 2018). Using this method, they isolated oxidized selenoneine dimer from other components using an Atlantis dC18 reverse phase chromatography column and obtained it (Turrini et al., 2018). However, reduced selenoneine monomer has not been isolated yet due to its co-elution with ergothioneine (Kroepfl et al., 2019). A recent study reported that the total synthesis of selenoneine was succeeded, however, it was isolated as an oxidized dimer in 2% yield (Lim et al., 2019).

In this chapter, I report a novel method of separating selenoneine monomer and ergothioneine, and use this method to isolate selenoneine monomer for the first time. The proposed method enables the facile, reproducible preparation of selenoneine in high Se purity and enables to obtain authentic selenoneine necessary for the metabolism studies in following chapters.

3-2. Materials and Methods

3-2-1. Materials

Sodium selenate (Na_2SeO_4) and synthetic minimal medium (EMM-2) components were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). EMM-2 was prepared using a previously published method (Forsburg and Rhind, 2006). Ergothioneine was purchased from Sigma Aldrich (St. Louis, MO, USA). 2, 3-Diaminonaphthalene was purchased from Dojindo (Kumamoto, Japan). The genetically modified fission yeast strain (FY25320) developed in the previous study (Pluskal et al., 2014) was purchased from the National BioResource Project (Osaka, Japan).

3-2-2. Fission yeast culture

FY25320 was cultured by shaking at 28 °C in EMM-2 medium containing 10 μM sodium selenate, as previously described (Pluskal et al., 2014).

3-2-3. Preparation of water-soluble extract

After 48 h culture with sodium selenate, the yeast cells were separated from the medium by centrifugation ($307 \times g$, 15 min, 4 °C). The cell pellet was re-suspended in approximately 100 mL Milli-Q water, heated in a boiling water bath for 30 min, then the cellular suspension was centrifuged at $307 \times g$ for 15 min at 4 °C. The supernatant was filtered through a 0.22 μm membrane filter system (Iwaki, Shizuoka, Japan) and the filtrate was concentrated using a rotary evaporator for high performance liquid

chromatography (HPLC) analysis.

3-2-4. Partial purification of selenoneine monomer and ergothioneine

LC separation for partial purification was performed using an Agilent 1200 series HPLC system. Fractions containing selenoneine and ergothioneine were separated a Develosil C30-UG-5 column (4.6×50 mm, 5 μ m, Nomura Chemical, Aichi, Japan) equilibrated with 0.1% acetic acid at a flow rate of 0.25 mL/min at 40 °C using a column oven. The imidazole groups of selenoneine and ergothioneine were monitored by ultraviolet absorption at 260 nm. The binary gradient elution program using 0.1% acetic acid water (mobile phase A) and 0.1% acetic acid in acetonitrile (mobile phase B) is shown in Table 3-1. A 50 μ L aliquot of the concentrated extract was injected and the eluent from 2.6 to 6.0 min was collected by a fraction collector. The fractions between 2.6 and 6.0 min were combined and concentrated using a rotary evaporator. This partially purified fraction was then subjected to the final purification process.

3-2-5. Purification of selenoneine

The final purification process was conducted using the Agilent 1200 system with a Cosmosil PBr packed column (4.6×250 mm, 5 μ m, Nacalai Tesque, Kyoto, Japan) monitored at 260 nm. The column was equilibrated with water at a flow rate of 0.5 mL/min at 40 °C using a column oven. The binary gradient elution program using water (mobile phase C) and acetonitrile (mobile phase D) is shown in Table 3-2. A 30 μ L aliquot of

partially purified fraction was injected and the eluent from 16.1 to 18 min was collected as the final purified fractions by a fraction collector, which were combined and concentrated using a rotary evaporator.

3-2-6. LC-PDA-HRMS analysis

The purified selenoneine monomer was qualitatively analyzed by online liquid chromatography coupled with a photodiode array and high-resolution mass spectrometer (LC-PDA-HRMS) using an Ultimate 3000 LC-PDA system (Thermo Fisher Scientific, Waltham, MA, USA) and a time-of-flight micrOTOFQ II MS system (Bruker, Bremen, Germany). The crude extract was analyzed using a Develosil C30-UG-5 column (4.6×50 mm, 5 μ m, Nomura Chemical) equilibrated with 0.1% acetic acid at a flow rate of 0.25 mL/min at 40 °C using a column oven. The binary gradient elution program was the same as described above and shown in Table 3-1. The analyses of partially purified fraction and the final purified fraction were carried out on a Cosmosil PBr packed column (2.0 mm $\times$ 150 mm, 5 μ m, Nacalai Tesque) equilibrated with water at a flow rate of 0.3 mL/min at 30 °C using a column oven. PDA monitored the eluent from 195 to 800 nm. HRMS survey scans in positive ion mode detected ions from m/z 50 to m/z 800. The selenoneine monomer (Se-80) mass was extracted from the measurement data as m/z 278.0402, calculated from the composition formula for selenoneine ($C_9H_{15}O_2N_3Se$), with an extraction width of ± 0.01 .

3-2-7. LC-ICP-MS analysis

The purified selenoneine fraction was analyzed using online liquid chromatography (GL Science, Tokyo, Japan) with an inductively coupled plasma mass spectrometer (ICP-MS; ELAN DRC II, PerkinElmer, Inc., Waltham, MA, USA), a concentric quartz nebulizer (WE02-4371, PerkinElmer, Inc., Waltham, MA, USA), and a sample injector (2-mm inner diameter, quartz) using previously reported LC conditions (Yamashita and Yamashita, 2010) with some modifications. Separation was achieved on a gel permeation chromatography column, Ultrahydrogel-120 (7.8 × 300 mm, pore size 120 Å, molecular weight range 100–5000 Da Nihon Waters, Tokyo, Japan) equilibrated with 0.1 M ammonium acetate aqueous solution containing 0.1% IGEPAL (Sigma-Aldrich) at a flow rate of 1.0 mL/min at 40 °C using a column oven. The ICP-MS conditions were as previously described, with some modifications (Kroepfl et al., 2019). Sulfur and Se were detected as monoxides (SO and SeO) by introducing oxygen as a reaction gas into the dynamic reaction cell (DRC) at a flow rate of 0.45 L/min. The monitored masses were m/z 48 for $^{32}\text{SO}^+$ and m/z 96 for $^{80}\text{SeO}^+$. The plasma and auxiliary argon gas flow rates were 15 and 0.8 L/min, respectively. The nebulization argon gas flow rate was 1.13 L/min. The radio-frequency power was 1500 W. Using these LC-ICP-MS conditions, selenoneine eluted with a retention time of 9.6 min.

3-2-8. Calculation of peak resolution

The peak resolution (R_s) is a value representing the separation of one peak from another peak and was calculated by the retention time formula

$R=(tR_2-tR_1)/0.5 (W_1+W_2)$, where R is the resolution, tR is the retention time, and W is the width of the peak (IUPAC, 2006).

3-2-9. Total Se measurement

Total Se was measured using a previously described method (Yamashita et al., 2011). Samples were digested at 200–220 °C in 1 mL of a 1:2 mixture of nitric acid and perchloric acid. Se concentrations were measured by Walkinson's method, a fluorometric assay using 2, 3-diaminonaphthalene (Walkinson, 1966).

3-3. Results

3-3-1. Hydrophilic compounds containing selenoneine and ergothioneine purified from fission yeast extract

Fission yeast extract was applied to a Develosil C30-UG-5 reverse phase column to remove hydrophobic compounds. LC-MS analysis showed that selenoneine monomer and ergothioneine both eluted between 2.5 and 3.0 min (Fig. 3-1a). Considering the delay caused by connecting tubes, the eluent from 2.6 to 6.0 min described as a shaded area was collected (Fig. 3-1b).

3-3-2. Efficient PBr column separation of selenoneine monomer and ergothioneine

LC-PDA-HRMS analysis using a PBr column showed good separation of selenoneine monomer and ergothioneine. The peaks at 2.6 and 3.2 min in the PDA chromatogram monitored at 260 nm (Fig. 3-2a) were identified as

ergothioneine and selenoneine monomer, respectively, by HRMS (Fig. 3-2b).

3-3-3. Establishment of an HPLC-UV method for isolating selenoneine

I established a method using HPLC-ultraviolet (HPLC-UV) with a conventional PBr column (4.6×250 mm, $5 \mu\text{m}$) to isolate selenoneine on a large scale comparing to the conditions of LC-PDA-HRMS. The gradient program is presented in Table 3-2. Partially purified fraction, the concentrated fraction in the shaded area in Fig. 3-1b, was injected and the eluent from 16.6 to 17.5 min was collected as the purified selenoneine fraction (Fig. 3-3a). The peak resolution (R_s) between ergothioneine and selenoneine was 4.16 calculated from their retention times (13.4 and 17.0 min) and peak widths (1.03 and 0.70 min) (Fig. 3-3a). The collected fraction was analyzed by LC-PDA-HRMS. The peak with a retention time (R_t) of 3.2 min monitored at 260 nm (Fig. 3-3b) was identified as selenoneine monomer by its HRMS spectrum (Fig. 3-3c–f). In Fig. 3-3d, two types of selenoneine mass spectrum patterns were detected and identified as $[M+H]^+$ and $[2M+H]^+$ from their characteristic isotopic patterns of one or two Se containing compounds (Fig. 3-3e, f). $[2M+H]^+$ was a cluster ion which two selenoneine monomers were ionized by one proton (Fig. 3-3f). The PDA contour diagrams showed that the partially purified fraction contained many impurities, whereas only two impurities (R_t values of 0.9 and 1.7 min) were present in the purified selenoneine fraction (Fig. 3-3g, h).

3-3-4. ICP-MS and LC-HRMS analysis of purified selenoneine

ICP-MS analysis indicated that the above methodology separated selenoneine monomer from ergothioneine and other organic and inorganic Se compounds. Fig. 3-4a and b show ICP-MS chromatograms of 6.47 μM ergothioneine and purified selenoneine, respectively. No other sulfur or Se compounds were detected in the purified selenoneine fraction (Fig. 3-4b). In this ICP-MS condition, the value of limit of detection (LOD) and limit of quantification (LOQ) for ergothioneine were 1.39 and 4.63 μM calculated from the ratios of signal and noise (LOD: $S/N = 3$, LOQ: $S/N = 10$). LC-HRMS analysis also indicated that the above method separated selenoneine monomer (Fig. 3-5). However, separated selenoneine monomers were oxidized in the fraction and formed selenoneine dimer (Fig. 3-5).

3-3-5. Recovery efficiency of selenoneine

2.74 μmol Se and 2.42 μmol purified selenoneine were isolated from 1 L of genetically modified fission yeast culture grown for 48 h. The recovery efficiency calculated from the amount of selenoneine in the boiled water extract (4.59 μmol) was initially estimated to be 52.8% (Table 3-3).

3-4. Discussion

Selenoneine monomer and ergothioneine were purified from an aqueous extract of Se-supplemented fission yeast cells. Selenoneine monomer was separated from the sulfur analogue of selenoneine, called ergothioneine, by reverse phase chromatography using a Cosmosil PBr packed column. The

purity of the isolated selenoneine was 88.4% and the recovery efficiency was 52.8%. Previously, Yamashita and Yamashita reported the purification of selenoneine by a combination of reverse phase chromatography using an Atlantis dC18 silica gel column and gel-filtration on an Ultrahydrogel HP-120 GPC column; selenoneine was detected by monitoring Se-82 by ICP-MS (Yamashita and Yamashita, 2010). This method detected only Se-containing compounds, such as selenoproteins, selenite, selenomethionine, and selenoneine, and not ergothioneine and other sulfur-containing compounds (Yamashita and Yamashita, 2010). Recently, Kroepfl et al. simultaneously detected sulfur and Se compounds in human erythrocytes by monitoring $^{32}\text{SO}^+$ and $^{80}\text{SeO}^+$ using a C6 phenyl column (Kroepfl et al., 2019). Turrini et al. isolated selenoneine dimer from genetically modified fission yeast using a reverse phase Atlantis dC18 column (Turrini et al., 2018) by extracting selenoneine and ergothioneine in cold methanol, then separating the selenoneine dimer from ergothioneine by differences in their polarity. However, their HPLC method was unable to separate selenoneine monomer from ergothioneine.

In this chapter, I produced selenoneine using a culture of Se-supplemented fission yeast according to a previous method (Pluskal et al., 2014), and extracted selenoneine and ergothioneine as boiling water soluble materials from the cultured yeast without the use of organic solvent to avoid the dimerization of selenoneine and establish a purification method for selenoneine monomer. This method is compatible with the generation of functional foods since organic solvent is not needed in the steps eluting

fractions containing selenoneine.

This study used a PBr column to separate selenoneine monomer and ergothioneine (Fig. 3-2a, b). The pentabromobenzyl group as the stationary phase can separate hydrophilic compounds under reverse phase conditions, including compounds with low hydrophobicity, due to London dispersion force interactions (Turowski et al., 2001), which depend on the number of extranuclear electrons (London, 1937). In this study, I initially speculated that the two hydrophilic compounds selenoneine monomer and ergothioneine were separated by London dispersion force interactions since the atomic weight and number of extranuclear electrons in Se (atomic weight: 78.96, number of extranuclear electrons: 34) differ greatly from those of sulfur (atomic weight: 32.065, number of extranuclear electrons: 16). A previous study reported that dispersion force decreases in organic molecules containing halides in the order $RF < RCl < RBr < RI$ (Schneider 2015). The PBr column can separate halogenated benzenes (F, Cl, Br, I) and pnictogen triphenyl (N, P, As, Sb, Bi), and thus may separate low molecular weight organic chalcogen compounds (Turowski et al., 2001). Further studies are needed to determine whether other sulfur and Se analogues, for example cystine and selenocystine, and methionine and selenomethionine, can be separated using a PBr column, and whether other columns utilizing dispersion force interactions can separate these analogues.

My proposed large-scale purification method for selenoneine monomer utilizes HPLC-UV with a conventional PBr column (4.6×250 mm). Selenoneine is eluted with Milli-Q water, which is attractive if the eluate is

to be used in bioassays involving cells or animals since it is free from organic solvents. The chromatogram indicates that selenoneine is completely separated from ergothioneine (Fig. 3-3a). The peak resolution (R_s) obtained for selenoneine and ergothioneine was 4.16, far greater than the value of 1.50 which indicates the complete separation of two peaks, as proposed by the Japanese Pharmacopoeia (The Japanese Pharmacopoeia, Seventeenth ed.).

The purity of the isolated selenoneine was confirmed by LC-PDA-HRMS. The 260 nm PDA chromatogram and the HRMS spectrum showed no or few peaks other than the selenoneine monomer (Fig. 3-3b–d), but compounds with absorption at 210 nm were detected at 0.9 min and 1.7 min in the PDA contour diagram (Fig. 3-3h). Although the level of ergothioneine was 4.11 times higher than that of selenoneine in the boiled water extract, no ergothioneine was detected by ICP-MS in the purified selenoneine (Table 3-3, Fig. 3-4b). Moreover, the ICP-MS chromatogram showed only a peak corresponding to selenoneine and the purity as a Se compound was 88.4%, calculated from the peak area of the chromatogram (Fig. 3-4b).

Using the above method, 2.42 μmol Se as selenoneine was purified from 1 L of genetically modified fission yeast culture grown for 48 h. This yield is slightly less than that reported for a previous method (Turrini et al., 2018). The recovery efficiency calculated from the amount of selenoneine in the boiled water extract (4.59 μmol) was initially estimated to be 52.8% (Table 3-3). The loss in recovery may be due to the formation of oxidized selenoneine dimer during concentration of the eluates.

Selenoneine monomer purified using this method can be used for not

only quantification analysis but bioassays to investigate its physiological function. However, in the purified selenoneine, monomer and dimer co-existed in uncontrol ratio (Fig. 3-5). Hence, Controlling the ratio of selenoneine monomer and dimer after the final purification is one of the most important research. In Chapter 1 and 2, selenoneine monomer inhibited metalloenzymes such as ACE and tyrosinase (Fig. 1-3, Fig. 2-6, Seko et al., 2019; Seko et al., 2020). Therefore, this chapter will greatly contribute to elucidating the chemical and biological properties of selenoneine.

3-5. Tables and Figures

Table 3-1 Gradient program for initial LC separation

Time (min)	Mobile phase (%)		Flow rate
	A	B	(mL/min)
0.00	100	0	0.25
10.00	100	0	0.25
15.00	0	100	1.00
20.00	0	100	1.00
22.00	100	0	0.25

Table 3-2 Gradient program for the purification of selenoneine by HPLC-UV

Time (min)	Mobile phase (%)		Flow rate
	C	D	(mL/min)
0.00	100	0	0.50
25.00	100	0	
30.00	0	100	
35.00	0	100	
40.00	100	0	

Table 3-3 Contents and a recovery efficiency of selenoneine in boiled water extract, and purified selenoneine

	Selenoneine content (μmol)	Ergothioneine content (μmol)	Ratio of ergothioneine to selenoneine	Recovery efficiency (%)
Boiled water extract	4.59	18.9	4.11	-
Purified selenoneine	2.42	N.D.	-	52.8

N. D. means not detected. ‘-’ means not calculated.

Fig. 3-1

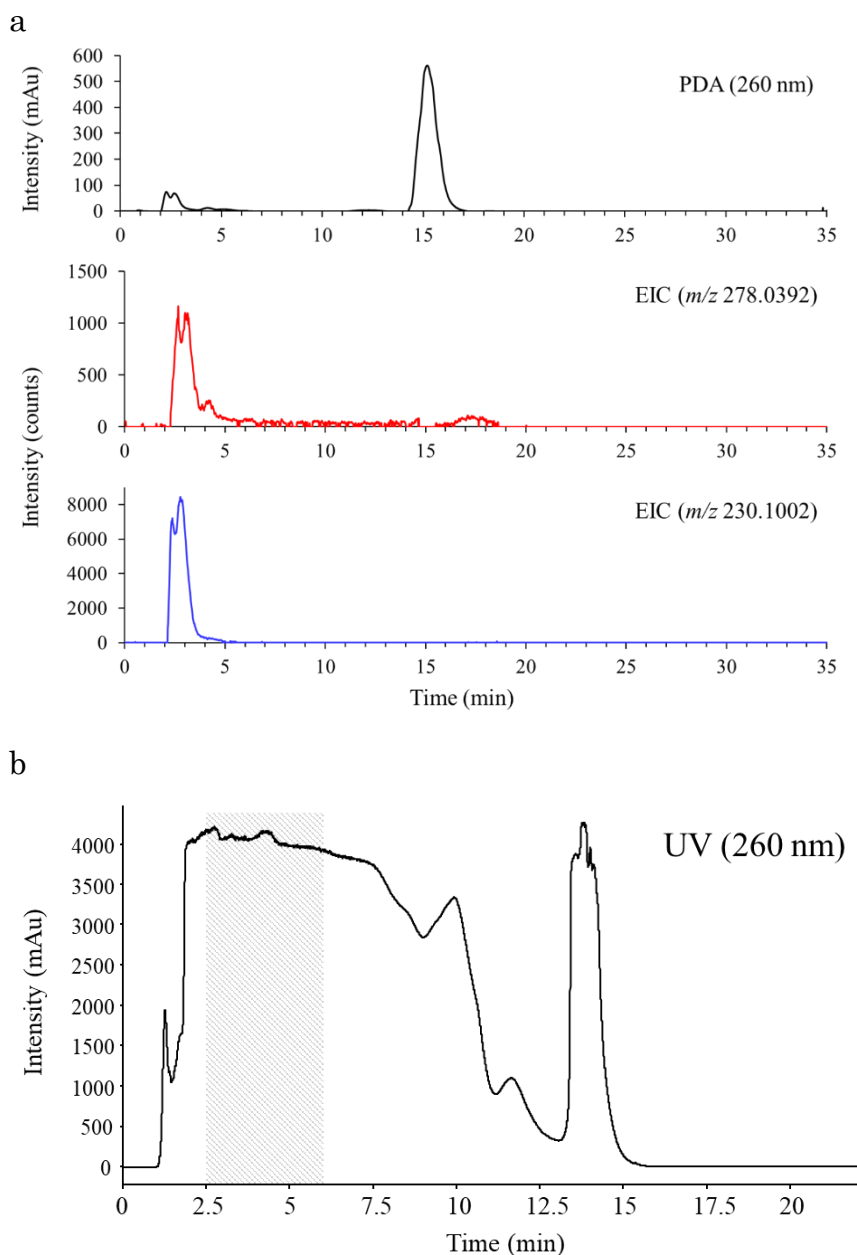


Fig. 3-1. Chromatograms of crude extracts. a) LC-PDA-HRMS chromatograms of crude extract. The upper panel shows the PDA chromatogram measured at 260 nm. The middle panel shows the extracted ion chromatogram (EIC) of selenoneine monomer (m/z 278.0392). The bottom panel shows the EIC of ergothioneine (m/z 230.1002). b) HPLC-UV chromatogram of crude extract monitored at 260 nm. The shaded area shows the eluate collected for purification of the selenoneine monomer; column: Develosil C30-UG-5 column 4.6×50 mm; mobile phase: 0.1% acetic acid and 0.1% acetic acid in acetonitrile; flow rate: from 0.25 to 1.0 mL/min.

Fig. 3-2

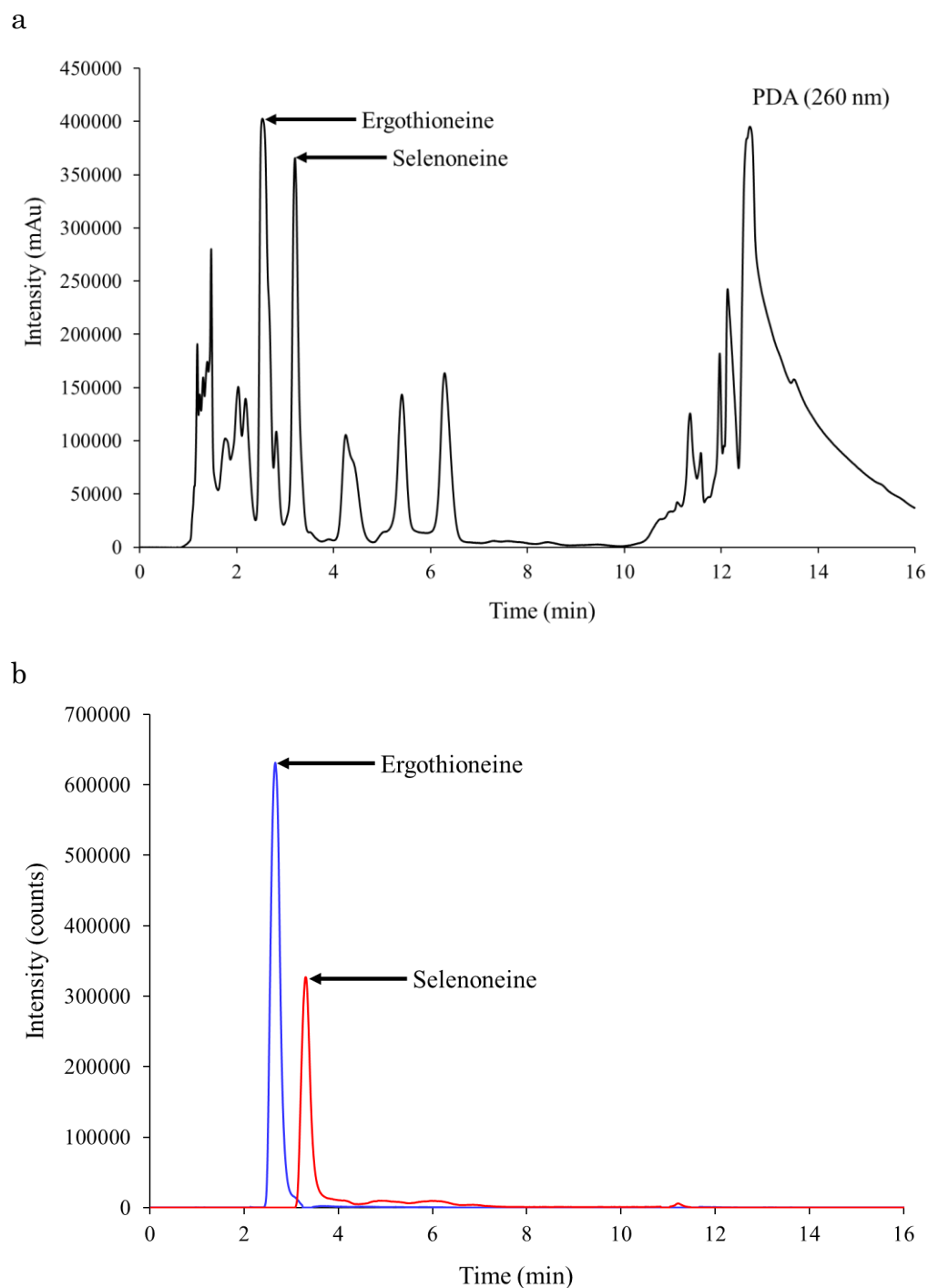
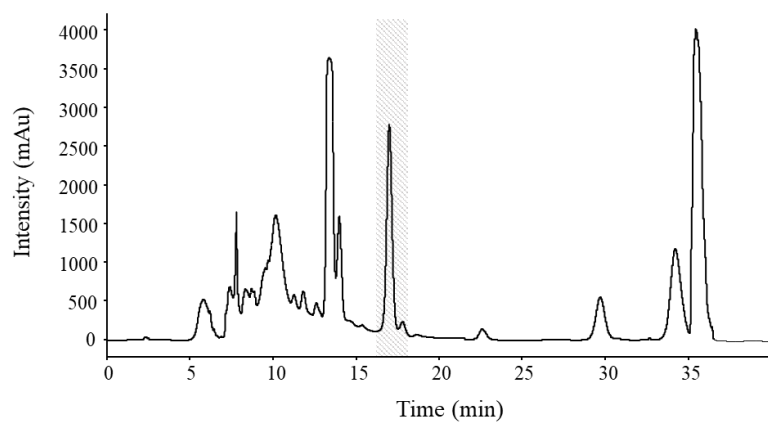


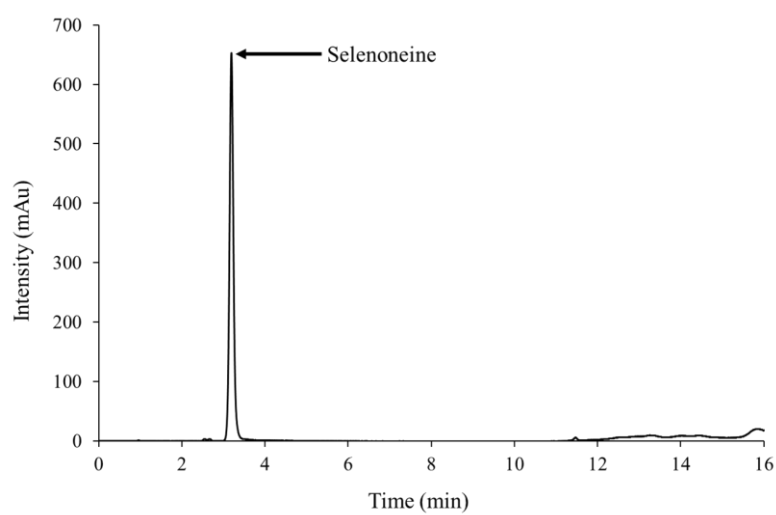
Fig. 3-2. LC-PDA-HRMS chromatograms using a PBr column: a) PDA chromatogram of the partially purified fraction monitored at 260 nm. b) EICs of ergothioneine (m/z 230.0903) and selenoneine monomer (m/z 278.0332); column: Cosmosil PBr packed column 2.0×150 mm; mobile phase: water; flow rate: 0.3 mL/min.

Fig. 3-3

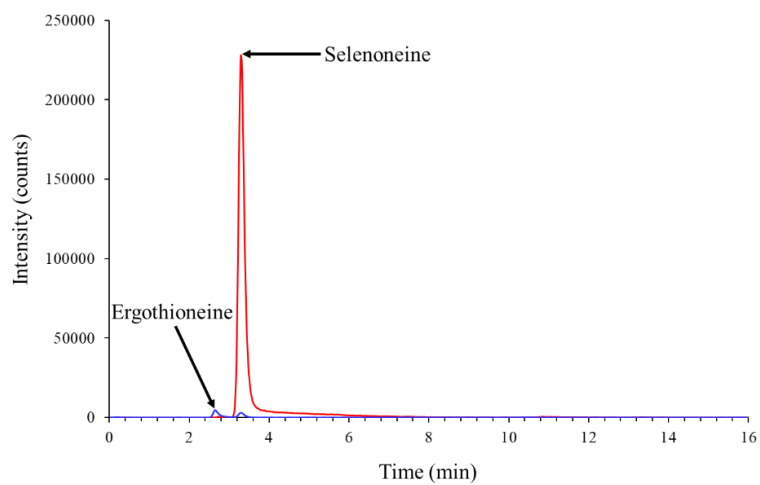
a



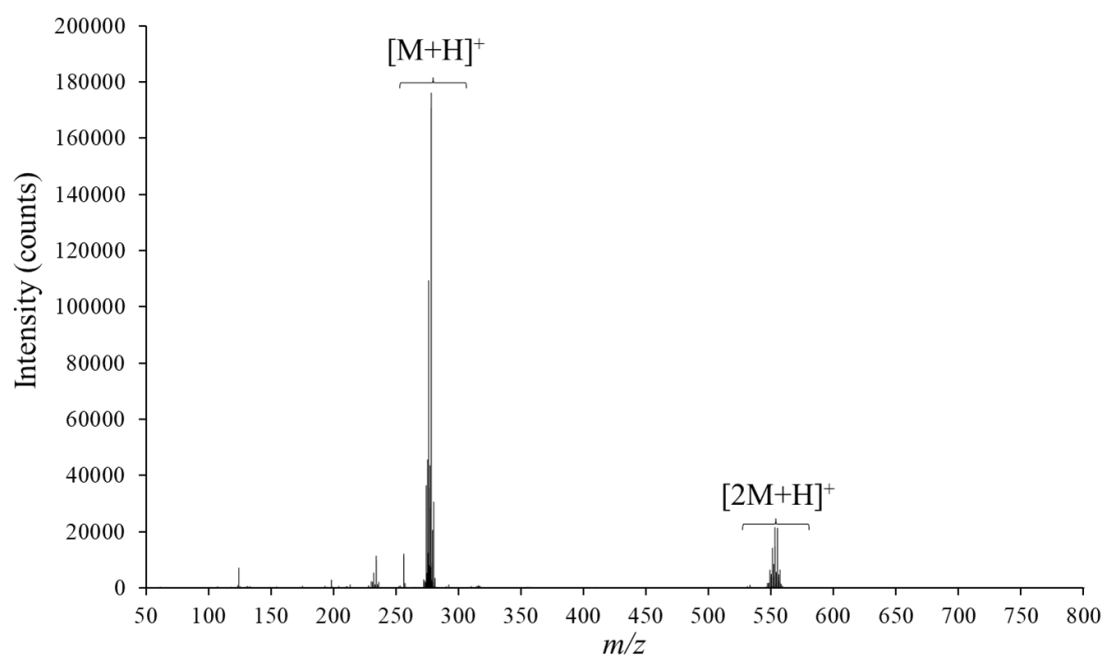
b



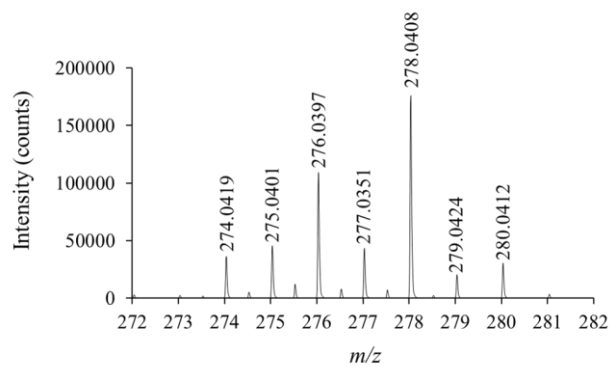
c



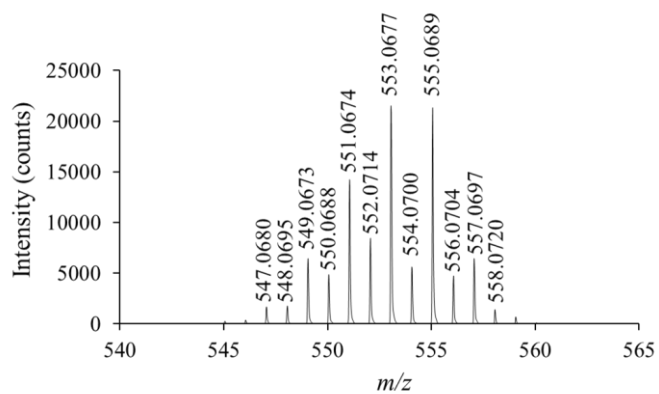
d



e



f



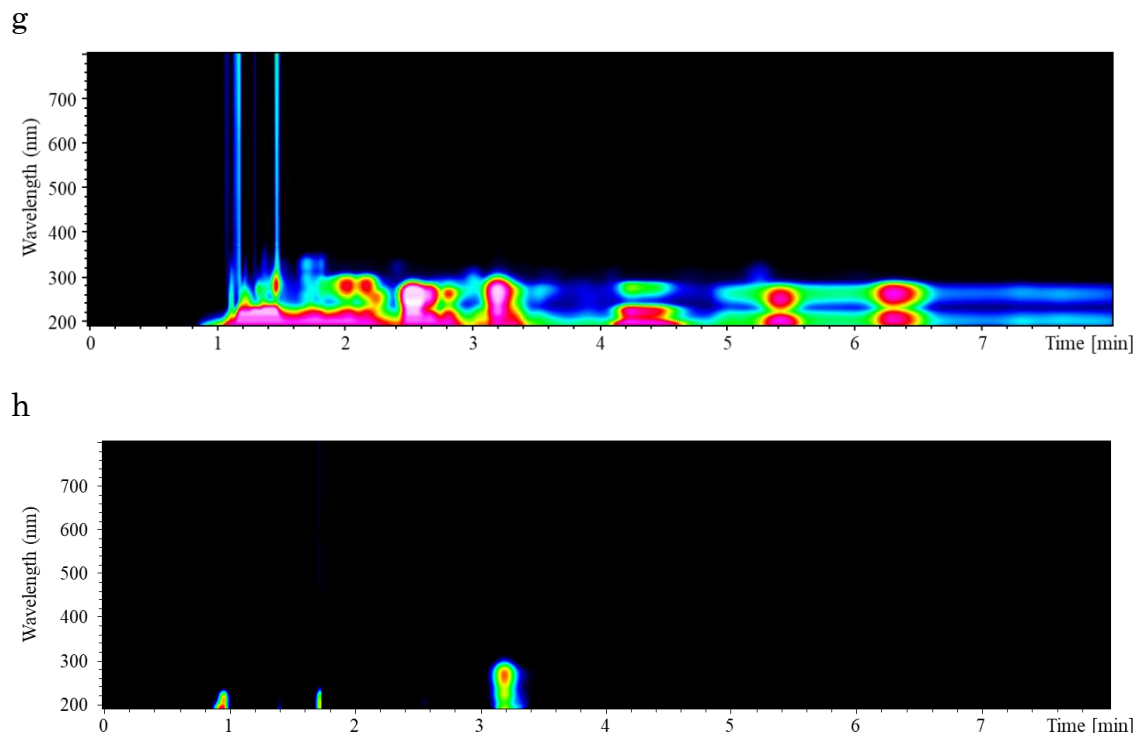
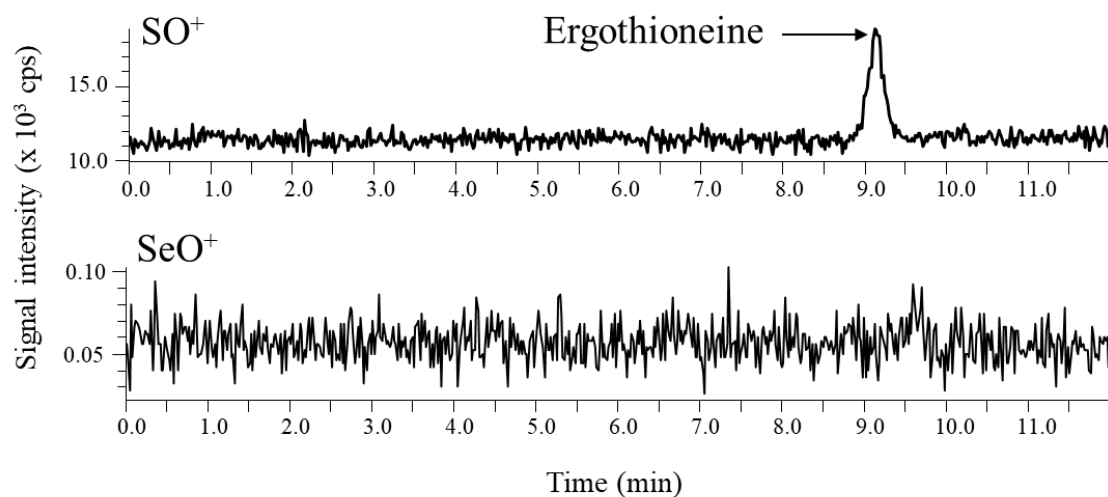


Fig. 3-3 Results of final purification processes: a) UV chromatogram of the partially purified fraction using a PBr column. The shaded area was collected as purified selenoneine. b) PDA chromatogram monitored at 260 nm of purified selenoneine using a short PBr column. c) EICs of ergothioneine (blue line, m/z 230.0903) and selenoneine monomer (red line, m/z 278.0346). d) Mass spectrum (m/z 50–800) of selenoneine monomer. e, f) Enlarged view of $[M+H]^+$ and $[2M+H]^+$ in Fig. 3-3d or exhibiting the characteristic isotopic patterns of one or two Se containing compounds. g, h) PDA contour diagrams of the partially purified fraction and of purified selenoneine. Colored areas correspond to compounds with absorption at the wavelength shown on the vertical axis. The magnitude of the absorption increases from blue to red; a) column: Cosmosil PBr packed column 4.6×250 mm; mobile phase: water and acetonitrile; flow rate: 0.5 mL/min. b–f) column: Cosmosil PBr packed column 2.0×150 mm; mobile phase: water; flow rate: 0.3 mL/min.

Fig. 3-4

a



b

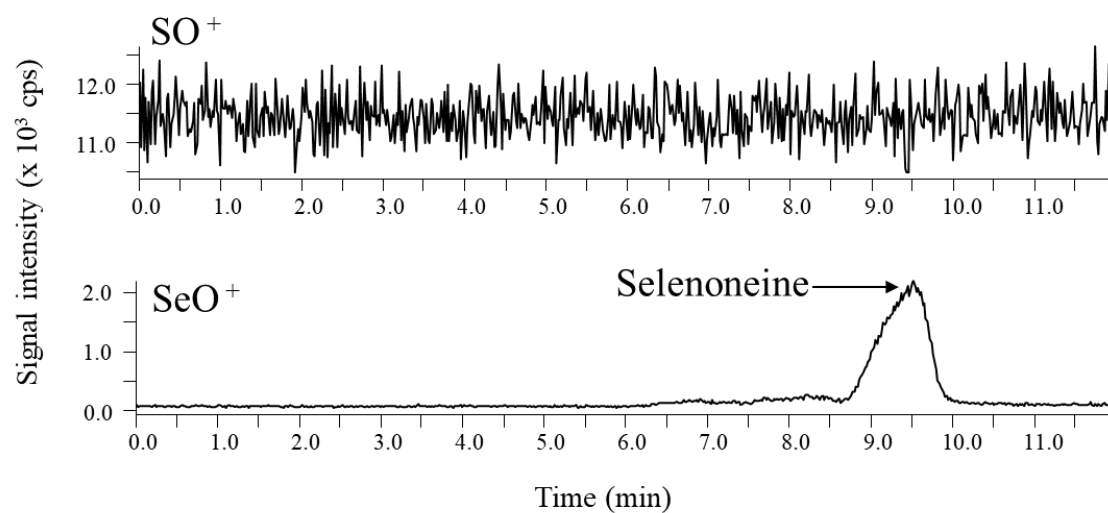


Fig. 3-4. LC-ICP-MS chromatograms of ergothioneine and selenoneine: a) SO^+ and SeO^+ in the ergothioneine standard were monitored. The peak in the SO^+ chromatogram corresponds to ergothioneine. The SeO^+ chromatogram showed no detectable Se compound. b) SO^+ and SeO^+ in purified selenoneine were monitored. These chromatograms showed that purified selenoneine was completely separated from ergothioneine and from other Se compounds; column: Ultrahydrogel-120 column 7.8×300 mm; mobile phase: 0.1 M ammonium acetate aqueous solution containing 0.1% IGEPAL; flow rate: 1.0 mL/min.

Fig. 3-5

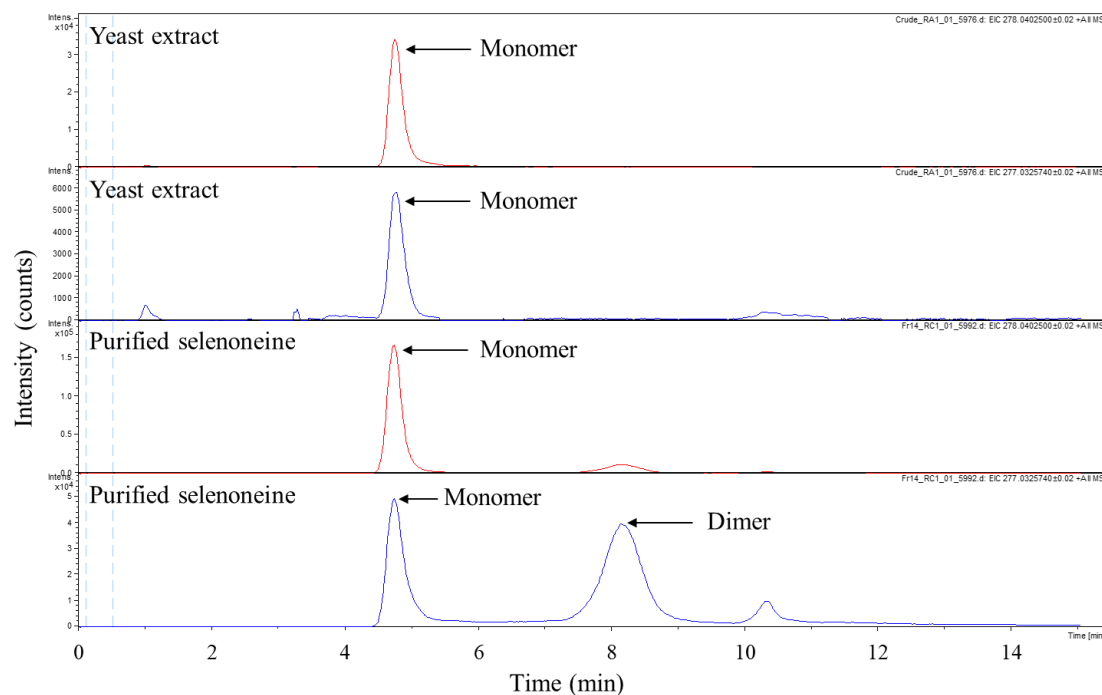


Fig. 3-5 The EIC chromatograms of the yeast extract and selenoneine purified by LC-PDA-HRMS using a PBr column: The two upper chromatograms show selenoneine in the yeast extract and the two lower chromatograms show selenoneine purified using a PBr column. The red line and blue line exhibited m/z 278.0402 as Se-80 selenoneine monomer and m/z 277.0326 as Se-80 selenoneine monomer dimerized just before the mass spectrometer from the column or selenoneine dimer occurred after purification.

Chapter. 4

Distribution and effects of selenoneine by ingestion of extract from mackerel processing residue in mice

4-1. Introduction

Accumulation of selenoneine in tissues *in vivo* has been reported severally so far. A study conducted on a remote island in Kagoshima Prefecture, Japan, found high concentrations of selenoneine in the erythrocytes of individuals who ate fish frequently (Yamashita et al., 2013a; Yamashita et al., 2013b). A Canadian study also reported accumulation of selenoneine in erythrocytes of individuals who ate seafood high in selenoneine (Achouba et al., 2019; Little et al., 2019). A study in which mice were fed purified selenoneine revealed accumulation of selenoneine in erythrocytes and the liver (Miyata et al., 2020). These reports suggest that selenoneine accumulates in the body following consumption of selenoneine-rich foods. Selenoneine is taken up into cells via an ergothioneine transporter known as OCTN1 (Yamashita et al., 2013a; Yamashita et al., 2013b). Thus, selenoneine is predicted to be taken up in tissues expressing OCTN1 and accumulate with a distribution similar to that of ergothioneine (Tang et al., 2018). However, the difference of accumulation ratios of selenoneine between organs and the difference of distribution ratios in organs between selenoneine and ergothioneine are not fully understood.

Some Japanese companies have recently developed selenoneine-containing ingredients for functional foods from fish residues such as tuna

dark muscle or mackerel heads and organs, as these sources contain high concentrations of selenoneine (Yamashita and Yamashita 2010; Yamashita et al., 2011). However, information regarding the accumulation, safety and health effects of selenoneine *in vivo* following consumption of these crude materials is lacking.

In this chapter, mice were fed a powdered extract prepared from the residues of mackerel used for canning process as a source of crude selenoneine. Mice were fed the extract for 32 days, after which the accumulation of selenoneine in the blood and tissues and the safety based on the body and tissue weights were examined. Biochemical analyses of plasma were conducted to identify new functions of selenoneine-rich extracts from mackerel residues.

4-2. Materials and Methods

4-2-1. Preparation of powdered extract from mackerel

Powdered extract from mackerel was prepared in reference to a patent (Yamashita et al., 2021) by BioChem. Corporation (Saitama, Japan). Fifty kilograms of frozen residue of mackerel, including head, mid-bone, and internal organs, was chopped and heated with 200 L of water at 80 °C for 10 min. The preparation was then filtered using a vibration sieve, 0.154-mm mesh, to remove solids. The resulting filtrate was mixed with 5% charcoal, 2% Japanese acid clay (Fujifilm Wako, Tokyo, Japan), and 2% silica gel at 55 °C and filtered with 6 kg of diatomite and a filter cloth. The filtrate was sterilized at 85 °C for 10 min. A total of 185 L of the filtrate was mixed with

200 mg of urease from Jack bean (Fujifilm Wako) for decomposing urea and trimethylamine oxide (TMAO), and an NF270 reverse osmosis membrane (Dow Chemical Co., MI, USA) was used to separate the filtrate into transparent and non-transparent fractions. The two fractions were re-mixed, and 100 mg of pronase (Roche, Basel, Switzerland) was added to the solution. The solution was separated using the reverse osmosis membrane, and the non-transparent fraction was concentrated. The concentrated fraction was filtered using 1.0 kg of silica gel and No. 5A filter paper (Advantech, Tokyo, Japan) to remove the precipitate. The filtrate was concentrated using an evaporator and powdered with dextrin using a spray dryer.

4-2-2. Chemical analysis of the powdered extract

The water, crude protein, crude fat, carbohydrate, and crude ash contents of the powdered extract were measured by Shokukanken Inc. (Gunma, Japan). Water, crude protein, crude fat, and crude ash were measured by air drying method at 105 °C for 5 hours, combustion method (modified Duma's method), acid hydrolysis method, and direct ashing method at 550 °C, respectively. Carbohydrates were calculated by the difference method, subtracting the total weight of water, crude protein, crude lipids, and crude ash. The methods were referenced from the Standard Tables of Food Composition in Japan (Ministry of Education, Culture, Sports, Science and Technology, Japan, 2015).

4-2-3. Free amino acid analysis

The free amino acid composition was analyzed according to previous studies (Murata et al., 1994; Murata et al., 2020), with slight modifications. Powdered extract (0.2 g) was dissolved in 20 mL of distilled water, and the solution was diluted twice with 0.04 mol/L HCl and filtered with a 0.2- μ m filter membrane (Millex-LG, Merck, Darmstadt, Germany) prior to analysis. Free amino acids were analyzed using an automatic amino acid analyzer (L-8900, High-Technologies Corp., Tokyo, Japan).

4-2-4. Total amino acid analysis

Total amino acids in the powdered extract were analyzed according to a previous study (Suzuki and Yasui, 1995), with slight modification. The powdered extract (80 mg) was dissolved in 1.0 mL of 6.0 mol/L HCl and heated at 145 °C for 4 h. The solution was filled with water to 50 mL and dried up with evaporator at 50 °C for removing HCl. It was dissolved by 0.02 mol/L HCl and filtered with 0.2- μ m filter membrane (Millex-LG, Merck, Darmstadt, Germany) prior to analysis. Amino acids in peptides were analyzed using an automatic amino acid analyzer (L-8900, High-Technologies Corp., Tokyo, Japan). Each analytical cycle was performed twice, and the mean of the two resulting values was used.

4-2-5. Main and trace element analysis

Main and trace elements analysis was conducted in reference to a previous study (Iguchi et al., 2014) with some modifications. The powdered

extract (30 mg) was dissolved in 5.0 mL of 61% HNO₃ and 3.0 mL of 30% H₂O₂ and heated using a microwave digestion system, NovaWAVE (SCP SCIENCE, Quebec, Canada). The digestion program was rising to 100 °C for 10 min from a room temperature, kept for 10 min, followed by a rate of 8 °C/min, kept at 180 °C for 10 min, and followed by a rate of 6 °C/min, kept at 240 °C for 10 min. The solutions were diluted with MilliQ water and placed into 50-mL tubes with Be, Sc, Ge, In, and Bi as internal standards to give a concentration of 10 µg/L. Elements in the solution were analyzed by an inductively coupled plasma–atomic emission spectrometry (ICP-AES, ICPE-9000, Shimadzu, Kyoto, Japan) and inductively coupled plasma–mass spectrometry (ICP-MS, ELAN DRC II, PerkinElmer, Inc., Waltham, MA, USA) as per a previous study (Iguchi et al., 2014). Na, Mg, Ca, and Fe were quantified by ICP-AES using an absolute calibration method. Trace elements (Li, V, Mn, Ni, Cu, Zn, As, Se, Rb, Mo, Cd, Ba, and Pb) were quantified by ICP-MS using an internal standard method.

4-2-6. LC-ICP-MS conditions for selenoneine analysis

Selenoneine in samples was quantified using the LC-ICP-MS under the same LC conditions presented in the materials and methods section in Chapter 3. Separation was achieved on an Ultrahydrogel-120 column (7.8 × 300 mm, Nihon Waters, Tokyo, Japan) equilibrated with 0.1 M ammonium acetate aqueous solution containing 0.1% IGEPAL (Sigma-Aldrich, MO, USA) at a flow rate of 1.0 mL/min; column temperature was maintained at 40 °C using a column oven. The ICP-MS conditions were as previously described

(Yamashita and Yamashita, 2010) with some modifications, and m/z 82 was monitored as Se-82. The nebulization argon gas flow rate was 1.13 L/min. The auxiliary argon gas flow rate and the plasma argon gas flow rate were 0.8 and 15 L/min, respectively. The radio-frequency power was 1500 W. Other parameters of ICP-MS were described in Table 4-1. Under these LC-ICP-MS conditions, selenoneine eluted at a retention time of 9.6 min.

4-2-7. Feed preparation

A standard rodent chow diet, powder form MF (Oriental Yeast Co., Tokyo, Japan), was used as the control diet (Control). Powdered extract was mixed with the MF standard rodent chow diet using Planetary Mixer 5DMr (DALTON Corporation, Tokyo, Japan) at 0.7% of total weight and designated the mackerel extract (ME) diet. The proportion of the extract in the diet was calculated that the amount of Se intake of the mice was lower than an equivalent of tolerable upper intake level of Se for Japanese (Health Service Bureau, 2019).

4-2-8. Animal experiments

Animal experiments were approved by the Fisheries Technology Institute, National Research and Development Agency, Kanagawa, Japan (Permission number: H29-1) and performed according to the Guideline for the Ethical Treatment of Laboratory Animals of the institute. Male and female 5-week-old mice (Slc: ICR) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The male and female mice were weighed and grouped according to

equivalent body weight and fed the control diet for 1 week before beginning the dietary modification experiment. Male and female mice were fed the control or ME diet for 32 days (control and ME mice, respectively). Both diets in the feeder were changed twice each week. Body weight and food consumption were monitored every 3 or 4 days. Mice were kept in an environmentally controlled room at a temperature of 22 °C and humidity of 55% with a 12-h light and dark cycle throughout the experiment.

4-2-9. Sample preparation

After feeding for 32 days, the mice were fasted for 12 h and dissected under isoflurane anesthesia (Pfizer, NY, USA). Blood was collected from the inferior vena cava using a 1-mL syringe rinsed with heparin lithium (Wako, Tokyo, Japan). The blood was centrifuged ($1000 \times g$, 10 min, 20 °C), and supernatant and precipitate (blood cells) were used as plasma and erythrocytes (major cellular composition of blood cells), respectively. The collected liver, spleen, and kidney were weighed. Plasma, erythrocytes and organs were stored at -80 °C until analysis.

4-2-10. Sample preparation for LC-ICP-MS

Erythrocytes and plasma were mixed with a tenfold volume of MilliQ water and then centrifuged ($1021 \times g$, 10 min, 4 °C). Tissues were diluted with a tenfold volume of MilliQ water, homogenized by pestle, and centrifuged ($1021 \times g$, 10 min, 4 °C). The supernatant was injected into the LC-ICP-MS instrument, and the concentration of selenoneine was calculated from a

standard curve of selenoneine purified from the genetically modified fission yeast extract by the method developed in Chapter 3 (Seko et al., 2021).

4-2-11. Biochemical examination of plasma

Total protein (TP), albumin (ALB), albumin-globulin ratio (A/G ratio), total bilirubin (T-Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total cholesterol (T-Cho), triglycerides (TGs), creatinine (Crea), calcium (Ca), glucose (Glc), sodium (Na), potassium (K), chloride (Cl), urea nitrogen (UN), low-density lipoprotein-cholesterol (LDL-Cho), and high-density lipoprotein-cholesterol (HDL-Cho) in plasma were analyzed by the Safety Research Institute for Chemical Compounds Co., Ltd. (Hokkaido, Japan). TP was measured using the Biuret method. ALB was measured using the BCG method. A/G ratio was calculated from TP and ALB ($A/G \text{ ratio} = ALB/[TP-ALB]$). T-Bil, T-Cho, and Crea were measured using enzymatic methods. AST, ALT, and ALP were measured according to the Japan Society of Clinical Chemistry method. TGs were measured using the free glycerol elimination method. Ca was measured using the ortho-cresolphthalein complexone (OCPC) method. Glc was measured using hexokinase method. Na, K, and Cl were measured using the ion selective electrode method. UN was measured using the urease/glutamate dehydrogenase method. LDL-Cho was measured using the selective solubilization method, and HDL-Cho was measured using the selective suppression method. These analyses were carried out on a Hitachi 7080 Chemistry Analyzer (Hitachi, Ltd., Tokyo, Japan).

4-2-12. Statistical analysis

Values are expressed as the mean $\pm$ SD. Differences in means were analyzed using the Student's *t*-test, $P < 0.05$ indicating significance. Data were analyzed using Excel in Microsoft 365 (Microsoft, WA, USA) or GraphPad Prism, version 9 (GraphPad Software, CA, USA).

4-3. Results

4-3-1. Components of powdered extract and feed for mice

The general components of the feed, including water, crude protein, crude fat, carbohydrates, and crude ash, are shown in Table 4-2; the main component was crude protein. The free amino acid and total amino acid compositions are shown in Table 4-3, and the total amount of free amino acids was 44.5 g/100 g and that of total amino acids was 63.4 g/100 g. Main and trace element compositions are shown in Table 3 and selenoneine concentration is 0.270 mg Se/100 g also exhibited (Table 4-4). In addition to arsenic and cadmium (Table 4-4), harmful chemicals regarded as problematic in seafood including histamine and mercury were quantified and shown in Table 4-5. Components of the control and ME diets are shown in Table 4-6. The components of control diet are in reference to the components of MF, as disclosed by Oriental Yeast Co. (Tokyo, Japan). The components of ME diet were calculated from those of the control diet and 0.7% powdered extract. There were no markedly differences between the diets except for selenoneine.

4-3-2. Food consumption and body weight gain

Daily food consumption did not differ between the control and ME diets in male and female mice (Fig. 4-1a). Daily body weight gain (%) from day 0 was not different between male and female mice on the ME diet (Fig. 4-1b). Total intakes of selenoneine in male and female mice were 3.8 and 3.2 μg Se/mouse, respectively.

4-3-3. Organ weights

Organ weights were evaluated based on the ratio (%) of organ to body weight (Fig. 4-2). The ME diet had no effect on the ratios of male and female liver, male and female spleen and female kidney (Fig. 4-2a–c). That of the kidney in male mice fed the ME diet was significantly lower than that of control mice (Fig. 4-2b).

4-3-4. Measurement of Se compounds

Se included in protein and selenoneine in the plasma, erythrocytes, liver, kidney ($n = 5$), and spleen ($n = 4$) were measured by LC-ICP-MS (Fig. 4-3). Se compounds in the spleen of one control mouse and one ME-fed mouse could not be quantified because good chromatograms could not be obtained due to poor storage condition of the spleens. High molecular weight-Se (HMW-Se) like Se in protein and selenoneine was quantified based on a standard curve of selenoneine purified by the method in Chapter 3. The values of limit of detection (LOD) and limit of quantification (LOQ) for HMW-Se and selenoneine were represented those for selenoneine. They were 0.259 nmol

Se/g and 1.30 nmol Se/g calculated from the ratios of signal and noise (LOD: S/N = 2, LOQ: S/N = 10). The levels of Se included in protein in plasma, erythrocytes, and organs were not affected by the ME diet (Table 4-7). Selenoneine was not detected in plasma or erythrocytes from any of the mice, but it was detected in the liver, kidney, and spleen in both male and female ME-fed mice (Table 4-8). Comparing the male and female mice fed the ME diet, the selenoneine level in the kidneys of female mice was 2.26-fold higher than that in male mice ($P < 0.05$). The ratios of total amount of selenoneine in the kidney and spleen to that of in the liver were calculated from the selenoneine concentration and organ weights (Table 4-8), and the results are shown in Table 4-9. The relative distributions (liver: kidney: spleen) were 1.00: 0.275 ± 0.079 : 0.198 ± 0.086 in male and 1.00: 0.434 ± 0.097 : 0.229 ± 0.083 in female mice (Table 4-9). The values for the kidney and spleen were not markedly different from those reported for ergothioneine calculated from a previous study (Table 4-9, Tang et al., 2018). The ratios of selenoneine accumulation in the organs relative to total intake of selenoneine for 32 days calculated from food consumption data (Fig. 4-1a) are shown in Table 4-9. The values in the liver were $1.60 \pm 0.43\%$ in male and $1.85 \pm 0.61\%$ in female mice. Those in kidney were $0.432 \pm 0.145\%$ in male and $0.768 \pm 0.128\%$ in female mice, significantly higher than that in male mice. The values in the spleen were $0.270 \pm 0.080\%$ in male and $0.377 \pm 0.154\%$ in female mice.

4-3-5. Biochemical analyses of plasma

Results of biochemical analyses of plasma are presented in Table 4-10.

The Glc level of male mice was significantly increased by ME diet feeding ($P = 0.039$), but that of female mice tended to decrease with ME diet feeding ($P = 0.055$). The LDL-Cho level in male mice was significantly decreased by the ME diet ($P = 0.037$) but not affected in female mice. The T-Cho level in male mice tended to decrease with ME diet feeding ($P = 0.071$).

4-4. Discussion

In this chapter, mice were fed a selenoneine-containing ME for 32 days, after which the accumulation of Se-containing protein and selenoneine in the blood and tissues was evaluated. The effects of the ME diet on the body were examined by biochemical analyses of plasma.

Crude protein was the main component of the powdered ME, at 77.8 g/100 g crude protein (Table 4-2), including 63.4 g/100 g amino acids (Table 4-3). Free amino acids except tryptophan were contained in the total amino acids, and the rest of total amino acids was considered to be derived from peptides because the extract did not contain proteins in the production procedures. The values of phenylalanine and tyrosine in total amino acids were lower than ones in free amino acids (Table 4-3). That was predicted to be caused by the hydrolysis using concentrated HCl for total amino acids analysis (Kenmochi and Tamura, 1967). The rest subtracted the total amino acids and the free tryptophan from the crude protein would be the non-protein nitrogen compounds such as taurine, anserine, ammonia and nucleic acids. The ME contained 0.270 mg/100 g (= 34.2 nmol/g) of selenoneine. Previous studies have reported that the blood and white muscle of Pacific mackerel

(*Scomber japonicus*) contain 437 ± 159 nmol/g and 0.6 ± 0.2 nmol/g of selenoneine, respectively (Yamashita and Yamashita, 2010; Yamashita et al., 2011). The extract concentrated selenoneine from mackerel residues, although the concentration of selenoneine was not as high as that in the blood. No histamine or mercury was detected in the extract (Table 4-5), but arsenic and cadmium were detected at 0.582 mg/100 g and 0.0277 mg/100 g, respectively (Table 4-4). These results suggest that arsenic and cadmium are also contained in mackerel residues and concentrate along with selenoneine. The concentrations of arsenic and cadmium in the ME diet were calculated at 0.0409 mg/kg and 0.00194 mg/kg, respectively. The concentration of arsenic was < 0.20 mg/kg, the maximum allowable level of inorganic arsenic in rice in the European Union (European Commission, 2006). Moreover, most arsenic found in marine fish is the safer organic form of arsenic, arsenobetaine, which has a lethal dose 50 value by oral administration of 10,000 mg/kg body weight in male mice (Francesconi, 2010, Joint FAO/WHO Expert Committee on Food Additives, 2011). The concentration of cadmium was < 0.003 mg/L, the maximum allowable level in natural mineral water (Codex Alimentarius, 2008). The total amount of main and trace elements was lower than that of crude ashes because phosphorus and sulfur, which would have been present in higher amounts, were not quantified (Table 4-2, Table 4-4). Thus, the ME diet was judged to be safe for mice as for the substances examined in this chapter. The components and energy of the ME differed in comparison to the control diet only in terms of selenoneine (Table 4-6). Indeed, food consumption and weight gain did not differ significantly

between mice fed the control and ME diet for each sex (Fig. 4-1).

The effect of the ME diet on major organs was evaluated based on the ratio of organ to body weight (Fig. 4-2). The proportional weight of the liver, spleen, and kidney in female mice was not affected by the ME diet, but that of the kidney in male mice decreased significantly (Fig. 4-2b). A decrease in kidney weight was also identified in a previous study in which KK-Ay mice were administered selenoneine at 62 $\mu\text{g}/100\text{ g}$ as a mackerel peptide for 7 weeks (Hase and Matsumoto, 2020). Another previous study showed that oral administration of 16 mg/kg sodium selenite from water led to a decrease in kidney weight of male mice and suppressed weight gain (National Toxicology Program, 1994). Those results suggested that selenoneine is more toxic than sodium selenite, as it exerted toxicity at a lower level, 1.89 $\mu\text{g Se}/100\text{ g}$ (= 0.0189 mg Se/kg), compared with sodium selenite. In Chapter 2, selenoneine is reportedly less cytotoxic to B16 melanoma cells than sodium selenite or selenocystine (Fig. 2-1, Siwek et al., 1994; Seko et al., 2020). Thus, the present result suggests that selenoneine is specifically toxic to the kidney in male mice, but I could not conclude that the effect was due only to selenoneine due to inconsistent results (Table 4-8) and the observation that the crude extracts contained compounds not only selenoneine. Compared with male mice, more selenoneine accumulated in the kidneys of female mice, but a decline in kidney weight was not observed in female mice (Table 4-8, Fig. 4-2b). The more accumulation of selenoneine in the female kidney than the male one is predicted to be caused by the gender differences in expression of OCTN1 in kidney, but a previous study reported that mRNA levels of OCTN1 in kidney

were not different between male and female in C57BL/6 mice (Alnouti et al., 2006). On the other hand, the kidney in male mice was shrunk by the ME diet but that in female mice was not (Fig. 4-2). This result suggested that the male kidney was more sensitive than the female one against the toxicity of the ME diet, and selenoneine accumulated in the male kidney was spent more for the protection of the kidney. However, the mechanism of kidney protection by selenoneine was not fully understood. Further experiments are needed using purified selenoneine and particular nephrotoxic substances.

The effects of Se compounds on the blood and organs were evaluated by Se quantitation and speciation using LC-ICP-MS with size exclusion chromatography (Yamashita and Yamashita, 2010). This method was used in a previous study to separate Se-containing compounds and identified two main peaks as HMW-Se and selenoneine in blood and organs (Yamashita et al., 2011). In the present study, the amount of HMW-Se was not significantly affected by the ME diet in either male or female mice (Table 4-7). In mammals, organic Se compounds such as selenomethionine and selenocysteine from foods are metabolized and incorporated into proteins (Yang et al., 2017). Selenomethionine substitutes non-specifically for methionine in proteins to form Se-containing proteins, and selenocysteine is inserted into the amino acid sequence to form the active center of selenoproteins (Thiry et al., 2012). Sodium selenite and selenomethionine are reportedly to have elevate selenoproteins in mice, but selenoneine did not elevate them in the present study. This result suggests that selenoneine undergoes a different route of metabolism than other Se compounds. Selenoneine was detected in the liver,

kidney, and spleen of the ME-fed mice, but it was not detected in the plasma (Fig. 4-3). In erythrocytes, a peak was detected at the same retention time of selenoneine in a trace level, but its concentration was lower than the value of LOD (Fig. 4-3). Although selenoneine is reported to be detected in erythrocytes in human (Yamashita et al., 2013a), results in this chapter indicated that selenoneine accumulated in organs preferentially to erythrocytes. Moreover, this is the first report of the detection of selenoneine in the kidney and spleen of mice (Fig. 4-3). Other studies have found selenoneine in porcine and seabird kidney and bluefin tuna spleen (Yamashita and Yamashita, 2010; El Hanafi et al., 2022), but it has not been reported in spleen of mammals, which is an important finding for evaluating the functionality of selenoneine in the body.

Ergothioneine is also distributed to the kidney and spleen (Tang et al., 2018). Selenoneine is predicted to accumulate in other organs and tissues in which ergothioneine accumulates, as selenoneine is incorporated into cells via OCTN1, similar to ergothioneine (Yamashita et al., 2013a; Yamashita et al., 2013b). Moreover, in the present study, selenoneine was detected in the male and female liver at 0.411 and 0.562 nmol/g, respectively, by feeding a diet containing 0.0189 mg Se/kg selenoneine for 32 days (Fig. 4-3 and Table 4-8). Those values were under the LOQ value because the baseline noise level of LC-ICP-MS were high but the chromatograms showed the existence of selenoneine obviously (Fig. 4-3 and Table 4-8). A previous study showed that selenoneine accumulated in the liver at a concentration of 8.11 nmol/g by feeding a diet containing 0.3 mg Se/kg selenoneine for 4 months (Miyata et

al., 2020). Therefore, the present results indicate that a lower amount of selenoneine in crude material can accumulate in the liver with shorter-term feeding of a lower concentration of selenoneine compared with the previous study (Miyata et al., 2020).

The distribution of selenoneine in the kidney and spleen relative to the liver was calculated from the total amount of selenoneine in organs, and the results are shown in Table 4-9. The values for the kidney and spleen were not markedly different from those reported for ergothioneine calculated from Tang's study (Table 4-9) (Tang et al., 2018). The total intakes of selenoneine for 32 days as calculated from food consumption data (Fig. 4-1a) were 3.8 μg Se/mouse (48 nmol/mouse) and 3.2 μg Se/mouse (40 nmol/mouse) in male and female mice, respectively. These amounts were 238 μg Se/day/man and 200 μg Se/day/woman in terms of daily intake for a human weighing 60 kg. These values are lower than the tolerable upper intake level of Se for Japanese (Health Service Bureau, 2019). The ratios of selenoneine accumulation in the organs relative to total intake are shown in Table 4-9. More than 95% of the selenoneine contained in the ME diet was not accumulated. Most of the non-accumulated selenoneine was thought to have been excreted into the urine or not absorbed in the body, as the relative distribution of ergothioneine was the highest in the liver (Tang et al., 2018) and selenoneine was also thought not to accumulate in other organs at a concentration higher than that in the liver. Moreover, selenoneine was not detected in the chromatograms of erythrocytes (Fig. 4-3) because the concentrations were lower than the LOD value contrary to previous studies (Yamashita and Yamashita 2010; Yamashita et al. 2013a;

Yamashita et al., 2013b; Miyata et al. 2020). However, peaks derived from selenoneine were observed around 9.6 min in a trace level in the chromatograms of erythrocytes (Fig. 4-3) and selenoneine was speculated to distribute in erythrocytes slightly. This result suggests that selenoneine preferentially accumulates in organs and is only distributed to erythrocytes later when animals are fed a low concentration of selenoneine.

Biochemical analyses of plasma revealed the effect of the ME diet on general health parameters of mice (Table 4-10). In male mice, blood glucose was significantly elevated by the ME diet ($P = 0.0394$). However, the blood glucose level of 202 ± 21 mg/dL did not meet the criterion for diabetes for general diabetes model mice (Yagihashi et al., 2010). Elevated blood glucose causes renal dysfunction and kidney hypertrophy (Gross et al., 2005), but the kidneys of male ME-fed mice in the present study became smaller (Fig. 4-1b). Thus, the plasma biochemical data could not explain this decrease in kidney size. In contrast to male mice, the blood glucose level in ME-fed female mice tended to decrease ($P = 0.0548$). The LDL-cholesterol level in male mice decreased with ME diet feeding ($P = 0.0370$). This represents a beneficial effect of the ME diet, as blood LDL is oxidized by reactive oxygen species, leading to arteriosclerosis (Steinbrecher et al., 1990). However, this effect was not observed in female mice. These results indicate that male mice are more sensitive to the ME diet than female mice.

In conclusion, selenoneine is distributed to the liver, kidney, and spleen in mice fed an ME diet containing a low concentration of selenoneine for a shorter term compared with previous studies. This is the first study to report

the distribution of selenoneine to the kidney and spleen in mice. Male mice were more sensitive to the effects of the ME diet on plasma parameters compared with female mice.

4-5. Tables and Figures

Table 4-1 LC-ICP-MS conditions for monitoring Se-82.

ICP-MS setting for monitoring Se-82	
Nebulization argon gas flow	1.13 L/min
Auxiliary gas flow	0.8 L/min
Plasma gas flow	15 L/min
Lens voltage	9.25 V
RF power	1500 W
Analog stage voltage	-1812 V
Pulse stage voltage	1100 V
Quadrupole rod offset	0 V
Cell rod offset	-6 V
Cell path voltage	-13 V

HPLC setting for isocratic elution	
Mobile phase	0.1 % IGEPAL in 0.1 M ammonium acetate
Flow rate	1.0 mL/min
Column	Ultrahydrogel-120 column (7.8 × 300 mm)
Column temperature	40°C
Injection volume	10 µL

Table 4-2 General composition of powdered mackerel extract

Components	g/100 g
Water	5.8
Crude protein	77.8
Crude fat	0.5
Carbohydrates	8.3
Crude ash	7.6

Table 4-3 Free and total amino acid composition of powdered mackerel extract

Amino acid	Free amino acid (g/100 g)	Total amino acid (g/100 g)
Isoleucine	2.69	3.39
Leucine	4.68	5.27
Lysine	3.36	4.69
Methionine	1.41	1.86
Cystine	0.320	0.418
Phenylalanine	2.65	2.52
Tyrosine	1.16	0.983
Threonine	2.30	3.35
Tryptophan	0.810	-
Valine	3.01	3.89
Arginine	4.48	5.04
Histidine	1.50	2.19
Alanine	3.43	4.55
Aspartic acid	3.65	6.14
Glutamic acid	3.54	8.56
Glycine	1.49	3.92
Proline	1.62	3.06
Serine	2.43	3.59
Total	44.5	63.4

“-“ means not analyzed.

Table 4-4 Main and trace elements composition in powdered mackerel extract

Main elements	g/100 g	Trace elements	mg/100 g
Na	0.633	Li	0.080
Mg	0.224	V	0.523
Ca	0.167	Mn	0.196
		Fe	13.5
		Ni	1.28
		Cu	0.190
		Zn	7.17
		As	0.582
		Se	0.684
		(selenoneine 0.270 mg Se/100g)	
		Rb	0.309
		Mo	0.190
		Cd	0.028
		Ba	0.133
		Pb	0.021
Total	1.03	Total	24.9

Table 4-5 Harmful chemicals regarded as problematic in seafood

Compounds	mg/100 g
Histamine	$< 1.00 \times 10^{-6}$
Total mercury	$< 1.00 \times 10^{-2}$

Table 4-6 Components of the control and ME diets

Components of feed (g/100g)	Control	ME	% of control
Water	7.90	7.89	99.8
Crude protein	23.1	23.5	102
Crude fat	5.10	5.01	99.4
Carbohydrates	58.1	57.7	99.3
Crude ash	5.80	5.82	100
Isoleucine	0.89	0.91	102
Leucine	1.74	1.77	101
Lysine	1.24	1.26	102
Methionine	0.44	0.45	102
Cystine	0.36	0.36	100
Phenylalanine	1.04	1.05	101
Tyrosine	0.68	0.68	100
Threonine	0.89	0.91	102
Tryptophan	0.28	0.28	101
Valine	1.08	1.10	102
Arginine	1.42	1.45	102
Histidine	0.60	0.61	102
Alanine	1.20	1.22	102
Aspartic acid	2.14	2.17	101
Glutamic acid	3.99	4.02	101
Glycine	1.18	1.20	102
Proline	1.31	1.32	101
Serine	1.11	1.13	102
Selenoneine (µg Se/100 g)	0.00	1.89	
Energy (kJ)	1502	1502	100.0

Table 4-7 Concentrations of HMW-Se in blood and tissues (plasma, erythrocytes, liver, and kidney: n = 5, spleen: n = 4)

	HMW-Se (nmol Se/g)			
	Male		Female	
	Control	ME	Control	ME
Plasma	16.0 ± 2.3	16.6 ± 1.8	15.0 ± 0.6	14.3 ± 1.0
Erythrocyte	11.6 ± 1.3	10.3 ± 1.8	13.5 ± 1.3	14.6 ± 1.5
Liver	17.3 ± 2.8	17.6 ± 1.1	19.8 ± 1.4	20.8 ± 1.1
Kidney	6.77 ± 0.91	6.39 ± 0.55	7.79 ± 0.64	7.57 ± 0.54
Spleen	9.24 ± 0.83	8.99 ± 1.07	9.27 ± 0.58	9.55 ± 0.56

Table 4-8 Concentrations of selenoneine in blood and tissues (plasma, erythrocytes, liver, and kidney: n = 5, spleen: n = 4)

	Selenoneine (nmol Se/g)			
	Male		Female	
	Control	ME	Control	ME
Plasma	-	-	-	-
Erythrocyte	-	-	-	-
Liver	-	0.411 ± 0.121	-	0.562 ± 0.203
Kidney	-	0.323 ± 0.091	-	*0.731 ± 0.151
Spleen	-	0.939 ± 0.092	-	1.10 ± 0.35

“-” means the peak of selenoneine was not detected. “*” means significant difference between male and female ($P < 0.05$). Statistical comparison was made with the Student's t-test. The LOD value was 0.259 nmol Se/g (S/N = 2).

Table 4-9 Relative distribution and ratio of selenoneine accumulation (liver and kidney: n = 5, spleen: n = 4)

		Selenoneine of ME fed mice		Ergothioneine (calculated from Tang et al., 2018)	
		Male	Female	Male (basal)	Male (maximum)
Relative distribution	Liver	1.00	1.00	1.00	1.00
	Kidney	0.275 ± 0.079	0.434 ± 0.097	0.284	0.539
	Spleen	0.198 ± 0.086	0.229 ± 0.083	0.231	0.388
Accumulation ratio (%)	Liver	1.60 ± 0.43	1.85 ± 0.61		
	Kidney	0.432 ± 0.145	*0.768 ± 0.128		
	Spleen	0.270 ± 0.080	0.377 ± 0.154		

Relative distributions of selenoneine were calculated from the total amount of selenoneine in organs. “*” means significant difference between male and female ($P < 0.05$). Statistical comparison was made with the Student’s t-test. Male (basal) means basal ergothioneine concentration in mice administered with single dose of saline and male (maximum) means maximum ergothioneine concentration in mice fed with 70 mg/kg/day ergothioneine for 28 days (Tang et al., 2018).

Table 4-10 Biochemical analyses of plasma from mice fed the control or ME diet (n = 5)

	Male			Female		
	Control	ME	<i>P</i> value	Control	ME	<i>P</i> value
TP (g/dL)	4.94 ± 0.31	5.06 ± 0.30	0.551	4.64 ± 0.21	4.66 ± 0.43	0.929
ALB (g/dL)	1.60 ± 0.07	1.64 ± 0.11	0.527	1.56 ± 0.05	1.56 ± 0.15	1.00
A/G ratio	0.480 ± 0.021	0.479 ± 0.017	0.960	0.507 ± 0.020	0.503 ± 0.022	0.786
T-Bil (mg/dL)	0.148 ± 0.059	0.108 ± 0.046	0.255	0.098 ± 0.018	0.118 ± 0.030	0.248
AST (units/L)	59.4 ± 10.4	49.0 ± 10.7	0.158	51.8 ± 4.3	52.2 ± 7.7	0.923
ALT (units/L)	26.0 ± 4.1	26.8 ± 3.0	0.732	24.2 ± 3.4	23.2 ± 3.8	0.675
ALP (units/L)	217 ± 60	243 ± 74	0.560	220 ± 61	253 ± 52	0.385
T-Cho (mg/dL)	134 ± 13	116 ± 13	0.071	73 ± 16	70 ± 23	0.831
TGs (mg/dL)	95 ± 60	103 ± 10	0.777	75 ± 17	57 ± 8	0.087
Crea (mg/dL)	0.096 ± 0.018	0.118 ± 0.047	0.369	0.064 ± 0.027	0.066 ± 0.021	0.899
Ca (mg/dL)	9.10 ± 0.72	9.16 ± 0.36	0.873	8.64 ± 0.29	8.66 ± 0.20	0.901
Glc (mg/dL)	169 ± 21	202 ± 21*	0.039	167 ± 20	140 ± 18	0.055
Na (mmol/L)	143 ± 2	144 ± 4	0.500	145 ± 4	145 ± 3	1.00
K (mmol/L)	8.43 ± 2.10	8.01 ± 1.25	0.715	5.64 ± 0.50	5.75 ± 1.12	0.848
Cl (mmol/L)	106 ± 3	107 ± 4	0.593	107 ± 3	108 ± 2	0.668
UN (mg/dL)	23.1 ± 3.5	24.0 ± 3.5	0.713	21.8 ± 4.2	25.1 ± 6.8	0.396
LDL-Cho (mg/dL)	17.0 ± 2.9	10.2 ± 5.0*	0.037	11.6 ± 3.0	11.6 ± 6.0	1.00
HDL-Cho (mg/dL)	117 ± 10	103 ± 13	0.101	59.4 ± 14.9	57.8 ± 20.4	0.891

“*” means significant difference between control and ME fed mice in each sex ($P < 0.05$).

Statistical comparisons were made with the Student's t-test.

Fig. 4-1

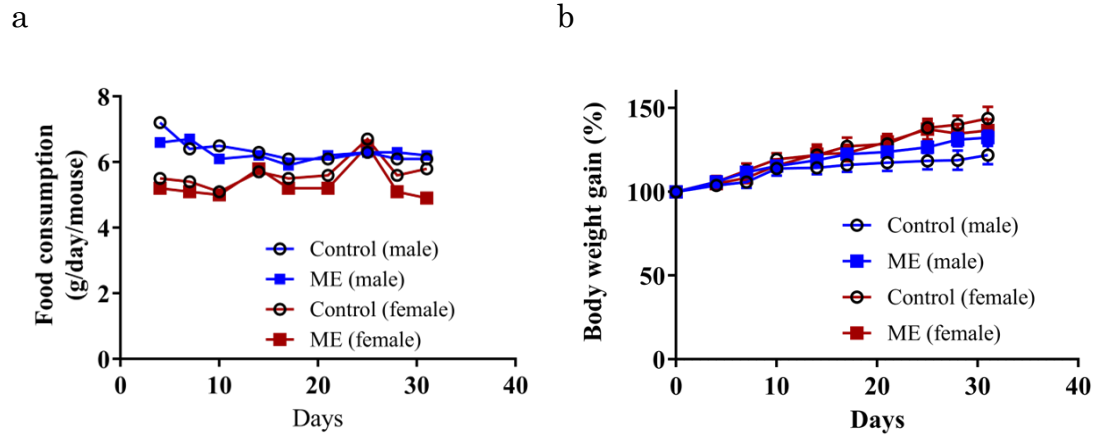


Fig. 4-1 Food consumption and body weight gain in mice: a) Food consumption (g/day/mouse) calculated from the weight loss of feed relative to cage weight. b) Body weight gain presented as percentile from day 0. Values are the mean $\pm$ SD ($n = 5$). Circles indicate control mice, and squares indicate ME-fed mice. Blue and red lines indicate male and female mice, respectively.

Fig. 4-2

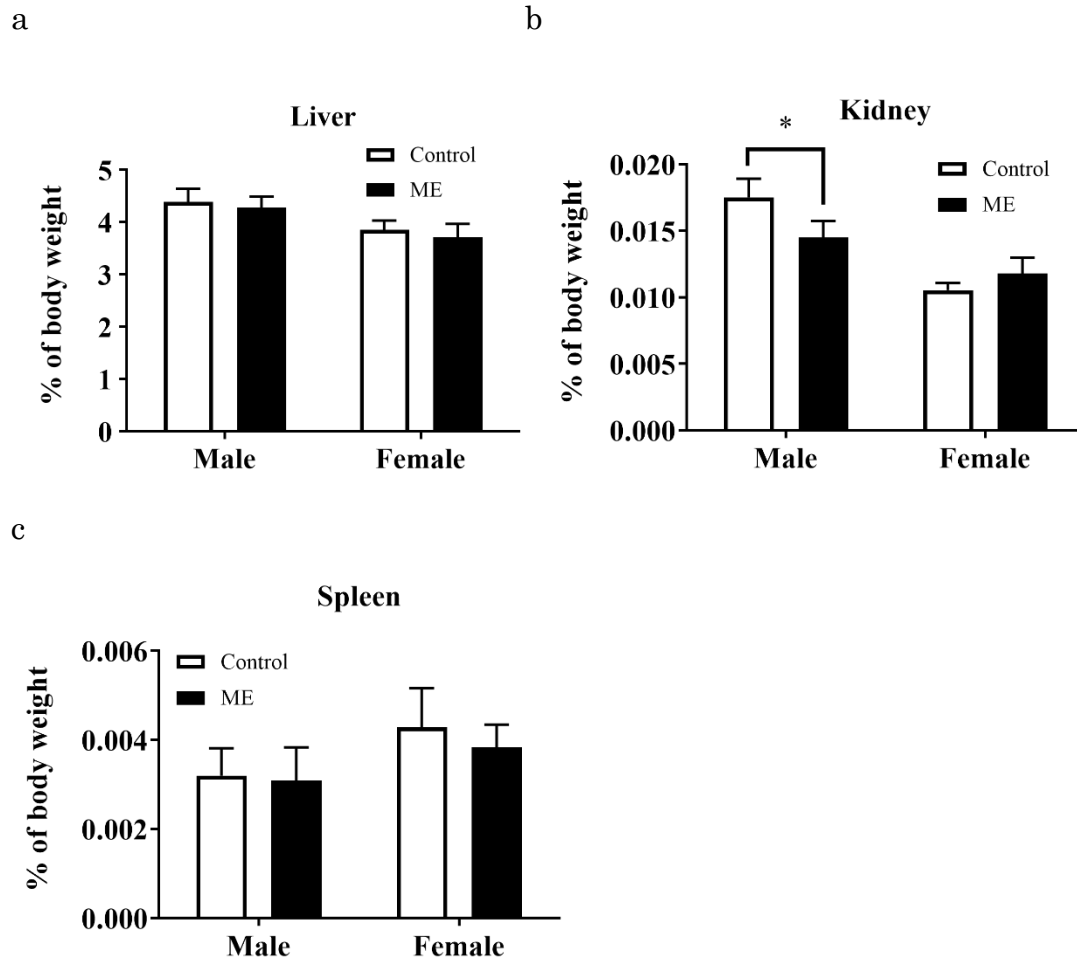


Fig. 4-2 Organ/body weight ratios (%) of mice fed the control or ME diet: a, c) Ratios for the liver and spleen did not differ between control and ME-fed mice. b) Ratio for the kidney of male mice was significantly decreased by ME diet ($P < 0.05$). Values are the mean SD ($n = 5$). A value with an asterisk is significantly different ($P < 0.05$). Statistical comparison was made with the Student's t-test.

Fig. 4-3

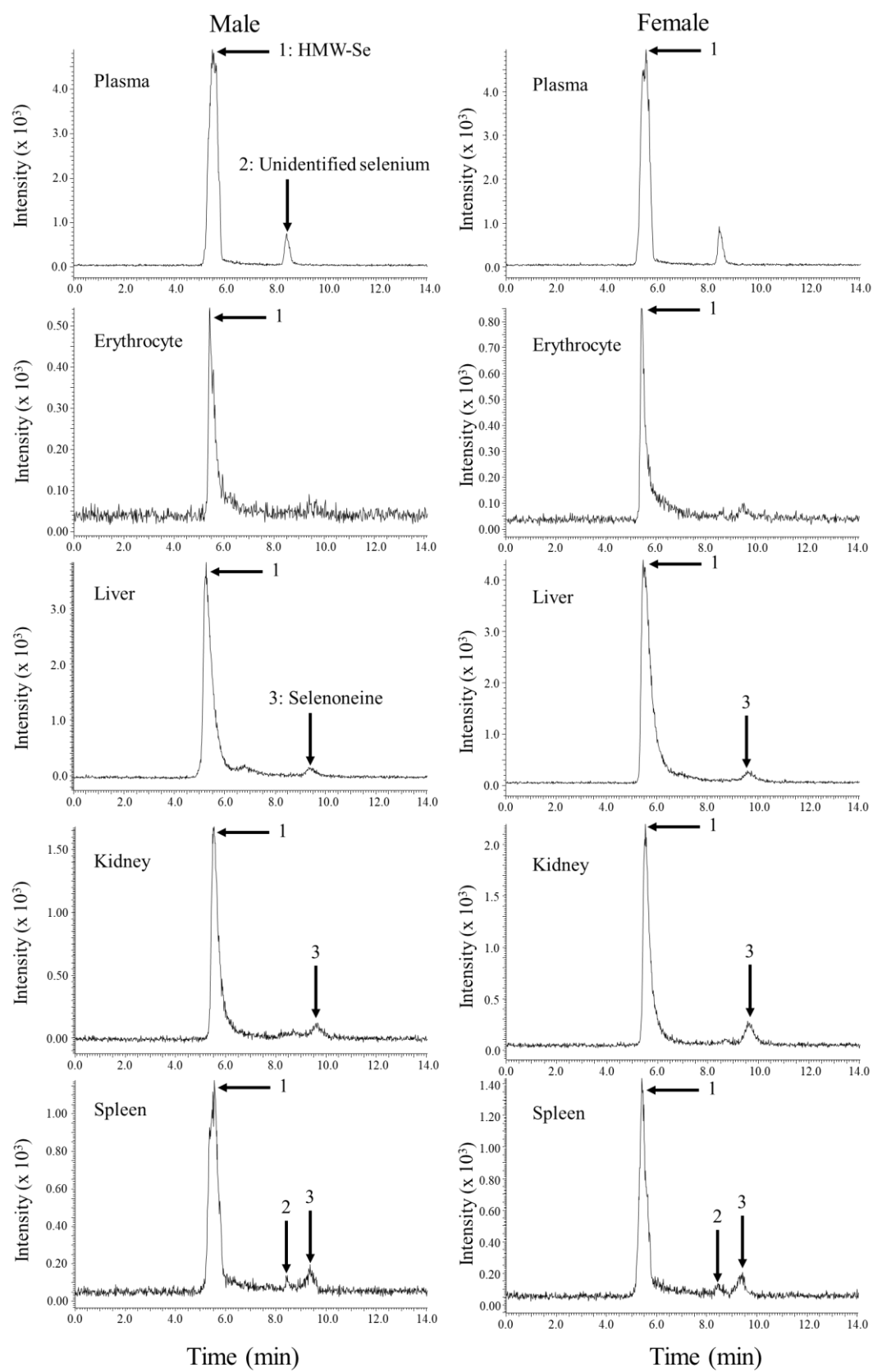


Fig. 4-3 LC-ICP-MS chromatograms of Se (Se-82) in the plasma, erythrocytes, liver, kidney, and spleen of ME-fed mice: Peak 1 was identified as HMW-Se and eluted with a retention time of 5.5 min. Peak 2 represents an unidentified Se compound detected in the plasma and spleen. Peak 3 was identified as selenoneine and eluted with a retention time of 9.5 min. Column: Ultrahydrogel-120, 7.8 × 300 mm; mobile phase: 0.1 M ammonium acetate aqueous solution containing 0.1% IGEPAL; flow rate: 1.0 mL/min.

Chapter 5.

Selenoneine is methylated in the bodies of mice and then excreted in urine as Se-methylselenoneine

5-1. Introduction

In the human urine, Se-methylselenoneine (Table 0) has been reported to be one of the major forms of excreted Se (Klein et al., 2011) in addition to selenosugars (Table 0, Fig. 0) including methyl-2-acetamido-2-deoxy-1-seleno- β -D-galactopyranoside (selenosugar 1) and methyl-2-amino-2-deoxy-1-seleno- β -D-galactopyranoside (selenosugar 3) (Kuehnelt et al., 2005). A study that examined a group of healthy volunteers reported finding that Se-methylselenoneine was consistently detected in the urine of all volunteers, with a significant metabolite found in one volunteer that contributed up to 24% of the total urinary Se (Lajin et al., 2016). Furthermore, another study stated that Se-methylselenoneine was predicted to be a metabolite of selenoneine and was generated in the liver and kidney by an unknown methyltransferase (Klein et al., 2011). In addition, as Se-methylselenoneine excretion is not stimulated by the selenite supplementation in healthy volunteers (Lajin et al., 2016), the pathway of Se-methylselenoneine synthesis is predicted to not be related to the main pathway of the Se excretion (Kobayashi et al., 2002). In contrast, Se-methylselenoneine could potentially be derived from food intake, as it has been detected in the muscles of fish such as, mackerel (*Scomber scombrus*), sardine (*Sardina pilchardus*), and tuna (*Thunnus albacares*) (Kroepfl et al., 2015). A recent study detected

Se-methylselenoneine in Caco-2 cells that had been cultured with selenoneine (Rohn et al., 2019). These results strongly support the supposition that Se-methylselenoneine is a metabolite of selenoneine. However, there has not been any experimental verification *in vivo* that Se-methylselenoneine is a metabolite of selenoneine.

In this chapter, after mice were administered purified selenoneine, urine samples were analyzed by liquid chromatography coupled with a photodiode array and high-resolution mass spectrometer (LC-PDA-HRMS) and a liquid chromatograph system with an inductively coupled plasma mass spectrometer (LC-ICP-MS) in order to verify whether Se-methylselenoneine was excreted after the selenoneine administration. In addition, selenoneine labeled with Se-76, which is one of the lower abundant stable isotopes of Se, was synthesized by the genetically modified fission yeast and purified using the method developed in Chapter 3. Then, it was administered to mice as a Se tracer in order to confirm whether or not Se-methylselenoneine was a metabolite of selenoneine. I also performed continuous administration of selenoneine in order to clarify the distributions of selenoneine in organs, and determine which organs in the bodies of mice methylate selenoneine.

5-2. Materials and Methods

5-2-1. Reagents

Selenoneine was extracted from the genetically modified fission yeast strain FY25320 and purified as per the method developed in Chapter 3 (Pluskal et al., 2014; Seko et al., 2021). Se purity was determined using a LC-

ICP-MS as per a LC condition presented in Chapter 3 (Seko et al., 2021) and the ICP-MS conditions were presented in Chapter 4 (Table 4-1). The purity of the selenoneine was greater than 99.5% based on Se. The selenoneine was dissolved in Milli-Q water and then stored at 4°C.

5-2-2. Se-76 selenoneine synthesis

Selenoneine labeled by the stable isotope Se-76 was synthesized using the genetically modified fission yeast, FY25320, which was cultured with Se-76 selenate. Se-76 selenate was synthesized from Se-76 selenite (the isotope purity was 99.8%) purchased from AMT Ventures Pte. Ltd. (Mackenzie Road, Singapore). Se-76 selenite was oxidized by H₂O₂ in 1.0 M NaOH at pH 11.0 that was heated at 70 °C resulting in conversion to Se-76 selenate, as per the details provided in the previous patent (Bjornberg et al., 1986). Residual H₂O₂ was decomposed by catalase (EC 1.11.1.6) from bovine liver (Wako, Tokyo, Japan), and the Se-76 selenate was extracted using ultrafiltration unit to remove substances with a molecular weight of 3000 (Vivaspin 500, Sartorius, Stonehouse, UK). Se-76 selenoneine was synthesized by the fission yeast in the media with 10 µM Se-76 selenate and then purified by HPLC by the method developed in Chapter 3 (Seko et al., 2021).

5-2-3. LC-ICP-MS conditions for Se-76 selenoneine analysis

Se-76 selenoneine was analyzed by the LC-ICP-MS in line with the LC conditions presented in Chapter 3 (Seko et al., 2021). Separation was achieved on an Ultrahydrogel-120 column (7.8 × 300 mm, Nihon Waters,

Tokyo, Japan) equilibrated with 0.1 M ammonium acetate aqueous solution containing 0.1% IGEPAL (Sigma- Aldrich) at a flow rate of 1.0 mL/min at 40 °C using a column oven (Table 5-1). Se-76 and Se-78 were detected by monitoring m/z 76 and m/z 78 by introducing ammonia gas as the reaction gas into the dynamic reaction cell (DRC) at a flow rate of 0.45 L/min (Table 5-1). Other LC-ICP-MS conditions were presented in Table 5-1. Using these LC-ICP-MS conditions, Se-76 selenoneine eluted with a retention time of 9.6 min.

5-2-4. LC-PDA-HRMS conditions for selenoneine and Se-methylselenoneine analysis

Selenoneine and Se-methylselenoneine were qualitatively analyzed by LC-PDA-HRMS in line with the parameters presented in Chapter 3 (Seko et al., 2021). Analyses of purified Se-76 selenoneine and mice urine samples were carried out on the Cosmosil PBr packed column (2.0 mm×150 mm, 5 μ m, Nacalai Tesque) equilibrated with 0.1% acetic acid at a flow rate of 0.3 mL/min at 30°C using a column oven (Table 5-2). A PDA monitored the eluent from 195 to 800 nm (Table 5-2). HRMS survey scans in the positive ion mode detected ions from m/z 50 to m/z 800 (Table 5-2). The Se-76 selenoneine dimer mass was extracted from the measurement data as m/z 273.03 of doubly charged ion $[M+2H]^{2+}$. The Se-methylselenoneine (Se-80) mass was extracted from the measurement data as m/z 292.05 of singly charged ion of $[M+H]^+$, which was calculated from the composition formula for Se-methylselenoneine as $[M+H]^+$ ($C_{10}H_{18}N_3O_2Se$), with an extraction width of ± 0.05 .

5-2-5. Animal experiments

Animal experiments were approved by the Fisheries Technology Institute, Japan Fisheries Research and Education Agency, Kanagawa, Japan (Permission number: H30-1 and H31-1) and performed in accordance with the Guidelines for the Ethical Treatment of Laboratory Animals of the institute. Male 4-week-old mice (Balb/c) were purchased from Japan SLC, Inc. (Shizuoka, Japan). They were fed the standard rodent chow diet, MF containing 0.26–0.49 mg/kg Se which value was suggested from the manufacture (Oriental Yeast Co., Tokyo, Japan), and kept in an environmentally controlled room at a temperature of 22°C and humidity of 55% with a 12-h light and dark cycle throughout the experiment.

5-2-6. Single dose administration of selenoneine

Male 5-week-old mice ($n = 3$) were administered 10 nmol Se as selenoneine per mouse or 10 nmol Se-76 as Se-76 selenoneine per mouse using the gastric sonde method. After the administration, each group of mice was moved into a metabolic cage with only water. Pooled urine samples were collected 480 min later. The urine samples were not diluted using water but just centrifuged ($1021 \times g$, 10 min, 4°C), and the supernatants were injected into the LC-PDA-HRMS and LC-ICP-MS with the PBr column.

5-2-7. Long-Term Administration of Selenoneine

Six male 5-week-old mice were divided into two groups of three mice, with the mice in each group moved to a metabolic cage. Mice in the metabolic

cages in both groups were fed the control diet, with the mice in one group administered selenoneine via the drinking water, which contained 10 μ M Se as selenoneine for 6 days. Mice were allowed to take the diet and the water freely. Pooled urine samples were collected every 24 h, and the Se-methylselenoneine was quantified using the LC-ICP-MS in the same LC and ICP-MS conditions in Chapter 4 (Table 4-1). Concentrations were standardized based on creatinine concentrations evaluated by colorimetry (Cayman Chemical, Ann Arbor, MI, USA). After 6 days, mice were dissected under isoflurane anesthesia (Pfizer, New York, NY, USA). Blood was collected from the inferior vena cava using a 1 mL syringe rinsed with heparin lithium (Wako, Tokyo, Japan). The blood was centrifuged ($1000 \times g$, 10 min, 20°C) and separated into plasma and erythrocytes. The collected liver, spleen, kidney and whole brain were weighed. Samples were stored at -80°C until analysis. High molecular weight Se species (HMW-Se) like Se-protein, selenoneine and Se-methylselenoneine in the blood and tissue samples were analyzed by the LC-ICP-MS. Peaks around the retention time at 5.5 min were presumed and were designated as HMW-Se based on the results in Chapter 4 (Fig. 4-3). All the Se compounds in this section were quantified from the standard curve determined for the purified selenoneine gained by the method developed in Chapter 3.

5-2-8. Sample preparation for LC-ICP-MS analysis

For the extraction of HMW-Se, selenoneine and Se-methylselenoneine in the blood and tissue samples, erythrocytes were diluted using a twofold

volume of MilliQ water. The liver, kidney, and spleen were diluted using a twofold volume of MilliQ water and homogenized by pestles. Brain was diluted 4-volume of MilliQ water and homogenized by pestles. The diluted erythrocytes and homogenates of tissues were centrifuged ($1021 \times g$, 10 min, 4°C). Plasma and urine were not diluted using water but just centrifuged ($1021 \times g$, 10 min, 4°C). The supernatants were injected into the LC-ICP-MS instrument in the same LC and ICP-MS conditions in Chapter 4 (Table 4-1). Recoveries were determined using bloods and tissues of a control mouse spiking with purified selenoneine. Bloods and tissues were spiked 2000 pmol Se as selenoneine and leaved in place for 3 min. Then, selenoneine was extracted from samples by the same procedures in this section and the supernatants were injected into the LC-ICP-MS in the same LC and ICP-MS conditions in Chapter 4 (Table 4-1).

5-3. Results

5-3-1. Determination of Se-methylselenoneine in the urine from a mouse administered selenoneine

Se compounds in the urine from a mouse administered selenoneine were analyzed by the LC-ICP-MS. In urine samples collected after 480 min of administration of selenoneine, an additional compound was detected at a retention time of 10.3 min (Fig. 5-1a). For the identification of the Se compound, the elution from 10.0 to 11.0 min was manually fractionated by switching the flow path in front of the ICP-MS and then analyzed by the LC-PDA-HRMS. Extracted ion chromatograms (EICs) revealed that the fraction

contained a compound having m/z 292.05 and being fragmented as m/z 278.04 (Fig. 5-1b). The m/z 278.04 and m/z 277.03 are the mass to charge ratios for detecting Se-80 selenoneine monomer and dimer, respectively (Fig. 3-5, Seko et al., 2021). A peak at 12.3 min was detected in the EIC monitoring m/z 278.04 and it was suspected to be selenoneine monomer contaminated in urine (Fig. 5-1b). However, selenoneine monomer is more hydrophilic than Se-methylselenoneine based on those structures and it is eluted faster than Se-methylselenoneine in the PBr column. Thus, the peak of m/z 278.04 detected at the same time of m/z 292.05 was suspected to be a demethylated fragment of m/z 292.05. The isotope pattern indicated that the unidentified compound contained one Se atom (Fig. 5-1c). The major mass containing Se-80 was extracted from the measurement data as m/z 292.0508 (Fig. 5-1c). This was identified as Se-methylselenoneine (Table 0) as $[M+H]^+$ from the calculated composition formula, $C_{10}H_{18}N_3O_2Se$, with an extraction width of ± 0.05 and the theoretical mass spectrum of Se-methylselenoneine (Fig. 5-1d).

5-3-2. Analysis of Se-76 selenoneine

Se-76 selenoneine labeled by the stable isotope, Se-76, was synthesized and purified in line with the results of Chapter 3 (Seko et al., 2021). The LC-ICP-MS analysis of Se-76 selenoneine revealed that compounds containing Se-78 were not detected in any of the chromatograms monitoring Se-78 while selenoneine was detected in the chromatogram that was monitoring Se-76 (Fig. 5-2a). The Se purity of isolated Se-76 selenoneine was 95.2% calculated from the chromatogram of Se-76 (Fig. 5-2a). The LC-PDA-HRMS analysis

determined that the Se-76 selenoneine was synthesized successfully from the EICs and the mass spectrum (Fig. 5-2b, c). The mass spectrum did not show the typical isotope pattern of stable isotopes of Se like the theoretical mass spectrum of the selenoneine dimer (Fig. 5-3b). The EICs not detected m/z 277.03 and m/z 278.04 derived from Se-80 selenoneine (Fig. 5-2b, Fig. 5-3a, b) and extracting the major mass spectrum of Se-76 selenoneine dimer as doubly charged ion m/z 273.0360 (Fig. 5-2c).

5-3-3. Se-76 Se-methylselenoneine detection in urine

To confirm whether Se-methylselenoneine found in the urine was a metabolite of selenoneine, mice were administered Se-76 selenoneine and urine samples were collected. In urine samples from the mice administered unlabeled selenoneine, Se-76, Se-78 and Se-80 Se-methylselenoneine were detected at a retention time of 11.8 min, depending on the isotope ratio of Se, by the LC-PDA-HRMS with the PBr column analysis (Fig. 5-4). In urine samples collected from the mice administered Se-76 selenoneine, there was little if any detection of Se-78 and 80 Se-methylselenoneine, while Se-76 Se-methylselenoneine was clearly detected (Fig. 5-4). Se-76 Se-methylselenoneine was also detected using LC-ICP-MS with the PBr column monitoring Se-76 and Se-78 with the DRC mode but Se-78 Se-methylselenoneine was not detected in the urine sample from Se-76 selenoneine administered mice (Fig. 5-5). These findings demonstrated that Se-methylselenoneine was a metabolite of selenoneine.

5-3-4. Monitoring of the time course of the Se-methylselenoneine excretion

Monitoring of the time course of the Se-methylselenoneine excretion was performed by continuous administration of selenoneine in the drinking water study over 6 days. Mice ingested a total of 27.7 mL of water per mouse over 6 days and ingested 277 nmol of Se as selenoneine per mouse. Body weights of mice were not affected by the continuous administration of selenoneine (Fig. 5-6a). Figure 5-6b shows that Se-methylselenoneine was hardly detected in the pooled urine sample collected during 0–24 h, but it was subsequently detected in the urine during 24–48 h and after (Fig. 5-6b). Selenoneine would elute at 9.2 min between the two peaks detected in the urine during 24–48 h and after in Fig. 5-6b because selenoneine was detected at the retention time with the same LC-ICP-MS conditions in Fig. 5-1a. Se-methylselenoneine was detected only in the group administered selenoneine, with the increase in the concentration found to be time-dependent (Fig. 5-6c). The peak present at all times in all urine samples was considered to be a major metabolite like selenosugars but unidentified (Fig. 5-6b). The concentrations of unidentified Se were not changed by selenoneine administration (Fig. 5-6d).

5-3-5. Accumulations of selenoneine and Se-methylselenoneine in the tissues

After 6 days, mice were dissected under isoflurane anesthesia and tissues were weighed (Table 5-3). HMW-Se, selenoneine and Se-methylselenoneine accumulations in the tissues were analyzed by the LC-ICP-MS (Fig. 5-7, Table 5-4). The values for the limit of detection (LOD) and the limit of quantification (LOQ) for HMW-Se, selenoneine and Se-

methylselenoneine were calculated from the ratios of the signal and noise (LOD: S/N = 3, LOQ: S/N = 10) of the purified selenoneine and they were 0.389 nmol/g and 1.30 nmol/g. Recoveries of selenoneine from plasma, erythrocyte, liver, kidney, spleen, and brain were 57.2%, 105%, 111%, 94.9%, 96.2% 107%, respectively. The concentrations of selenoneine in erythrocyte, liver, kidney, spleen, and brain were corrected by the recoveries. HMW-Se was detected in all of the tissues (Table 5-4). The concentrations did not differ significantly between the control and the group administered selenoneine (Table 5-4). In the plasma of mice administered selenoneine, while Se-methylselenoneine was barely detected, it could not be quantified as the concentration at 0.45 ± 0.02 nmol/g was lower than the LOQ value (Table 5-4). In the erythrocytes, while selenoneine was detected at 3.0 ± 0.1 nmol/g, Se-methylselenoneine was not detected (Table 5-4). In the liver, selenoneine was quantified at 16.1 ± 3.3 nmol/g, while Se-methylselenoneine was barely detected (Table 5-4). In the kidney, selenoneine was quantified at 13.1 ± 1.9 nmol/g, while Se-methylselenoneine was barely detected (Table 5-4). In the spleen, selenoneine was detected at 2.8 ± 0.5 nmol/g, while Se-methylselenoneine was not detected (Table 5-4). In the brain, both selenoneine and Se-methylselenoneine were not detected based on the LOD value, although selenoneine was detected in a chromatogram in traces (Table 5-4, Fig. 5-7).

5-4. Discussion

In the present study, Se-methylselenoneine was verified to be a metabolite of selenoneine through the administration of the stable isotope, Se-76, labeled selenoneine. Moreover, after the continuous administration of selenoneine to the mice, the chromatograms of the LC-ICP-MS demonstrated that selenoneine was shown to be distributed in the erythrocytes, liver, and kidney, which suggested that selenoneine was methylated in the liver and/or kidney.

Se-methylselenoneine, which was detected in the blood and urine, has been predicted to be a metabolite of selenoneine (Klein et al., 2011). This prediction is supported by the findings of a previous study that showed Se-methylselenoneine was detected in the Caco-2 cells after the addition of selenoneine (Rohn et al., 2019). In the present study, Se-methylselenoneine was detected in the urine after the administration of selenoneine to the mice (Fig. 5-1). Furthermore, the Se in Se-methylselenoneine was derived from selenoneine after the administration of the stable isotope, Se-76, labeled selenoneine and acted as a tracer (Fig. 5-4). At 480 min after a single dose administration of 100 μ M of the selenoneine dimer, a peak was detected at a retention time of 10.3 min in the LC-ICP-MS chromatogram (Fig. 5-1a). The peak was fractionated and identified as Se-methylselenoneine based on the measurement data as m/z 292.05 and the calculated composition formula, $C_{10}H_{18}N_3O_2Se$ using the LC-PDA-HRMS (Fig. 5-1b, c). In the EICs, m/z 278 was detected at a retention time of 12.0 min (Fig. 5-1b). This peak was considered as selenoneine monomer based on the mass but the retention time

of selenoneine monomer was 3.2 min referring in Chapter 3 (Fig. 3-2, 3-3, Seko et al., 2021). Thus, it was speculated to be a fragmented ion generated from demethylation of Se-methylselenoneine (Fig. 5-1b). These results indicated that mice generated Se-methylselenoneine and excreted it in urine after the intake of selenoneine.

Se is an element with multiple isotopes, and their abundance ratios vary according to the isotopes. The major isotope is Se-80, with an abundance of 49.61%, while the other isotope, Se-78 was at 23.78%. Se-76 is the third major isotope and had an abundance of 9.36%. I used this isotope for synthesizing the labeled selenoneine that was used as a tracer. Se-76 selenoneine was successfully synthesized through use of the genetically modified fission yeast and subsequent purification by HPLC (Fig. 5-2, Fig. 5-3). Although after administration of Se-76 Se-methylselenoneine it was detected in the urine, there was little detection of Se-78 and Se-80 Se-methylselenoneine (Fig. 5-4). If Se-methylselenoneine was the metabolite of both selenoneine and other Se compounds, Se-78 and Se-80 Se-methylselenoneine derived from endogenous Se should have been detected to a greater degree in the urine. In actuality, Se-78 and Se-80 Se-methylselenoneine was not detected in the urine of mice administered Se-76 selenoneine. Thus, these results suggest that Se-methylselenoneine is a metabolite of selenoneine.

Results in this chapter showed selenoneine was absorbed in the body and methylated for excretion, indicating that the selenoneine dimer was absorbed and reduced, or vice versa, in the body, as the administered Se-76 selenoneine was the oxidized dimer. Although the transporter of selenoneine,

OCTN1, can efficiently absorb the reduced selenoneine, it does not oxidize selenoneine (Lim et al., 2019; Gründemann et al., 2022). Therefore, oxidized selenoneine was predicted to be reduced in the digestive tract and absorbed into the body via small-intestinal epithelial cells that express OCTN1 on the apical side. Although the selenoneine dimer is reduced by glutathione or other reductants *in vitro* (Lim et al., 2019), the reduction mechanism *in vivo* has yet to be definitively determined.

Continuous administration of 10 μ M purified selenoneine for 6 days did not affect the body and organs weights of the mice (Fig. 5-6a, Table 5-3) and that suggests the administration does not exhibit negative effects on the bodies of the mice. A previous study using NAFLD model mice reported that 0.3 mg Se/kg purified selenoneine in a standard rodent chow did not change the body weight and suppressed the normally seen increase in the liver weight during the 4 months of feeding (Miyata et al., 2020). In this chapter, I used a higher concentration but shorter-term administration as compared to that of the previous study, and found that selenoneine did not affect the liver, kidney, spleen and brain weight (Table 5-3). This is important information, as there have been few reports on the safety of purified selenoneine. The variations in urinary concentrations of Se compounds over time were evaluated after correcting the compound concentrations for creatinine concentrations, as the rate of concentration and dilution of urine is variable. Se-methylselenoneine was not excreted in the urine until 24 h later and was excreted up until 48 h after the initial administration (Fig. 5-6b). A transwell test using Caco-2 cells demonstrated that selenoneine was detected after 24

h on the basolateral side after the addition of selenoneine on the apical side (Rohn et al., 2019). Se-methylselenoneine was detected at 72 h after the addition of selenoneine (Rohn et al., 2019). Although my results were comparable to the absorption time of selenoneine in the small intestine, the methylation of selenoneine was faster than that seen in a cellular experiment. These findings suggest that other cells or organs might more efficiently methylate selenoneine.

There was an increase in the Se-methylselenoneine excretion in a time-dependent manner (Fig. 5-6c). These results suggested that the continuous administration of 10 μ M selenoneine for 6 days (the total of 277 nmol/mouse) was excessive, as mice began to accumulate it in their body, which then led to it being actively methylated for the excretion in order to autonomously adjust the selenoneine concentration. On the other hand, the excretion of major Se species, selenosugar, in urine was not affected by selenoneine administration (Fig. 5-6d). Selenosugar is a metabolite for excretion of organic and inorganic Se compounds relate to the central metabolic pathway of Se for the selenoproteins synthesis (Fig. 0, Roman et al., 2014). Thus, the result that selenoneine not affected on selenosugar in urine is suggested that selenoneine did not related to the central metabolic pathway of Se.

To identify which tissues metabolized selenoneine, HMW-Se, selenoneine and Se-methylselenoneine in the blood and tissues were evaluated by the LC-ICP-MS (Table 5-4, Fig. 5-7). HMW-Se was detected in all of the tissues (Table 5-4, Fig. 5-7) and the concentrations did not differ significantly between the control and the group administered selenoneine

(Table 5-4). Most of HMW-Se is presumed to be selenoproteins like glutathione peroxidase based on a previous study (Yamashita and Yamashita, 2010) and their expressions were generally promoted by Se compounds (Burk and Hill, 2009) because Se compounds relate to the central metabolic pathway of Se for the selenoproteins synthesis (Fig. 0, Roman et al., 2014). This result also indicated that selenoneine was not related to the central metabolic pathway of Se. This suggestion is important to discuss the difference of the nutritional properties of selenoneine from other Se species, but further studies are needed like tracing Se atom in selenoneine *in vivo* precisely and quantitatively.

As seen in Table 5-4, selenoneine was distributed in liver, kidney and spleen as in Chapter 4 (Seko et al., 2023, Table 4-8, Fig. 4-3) and it was accumulated in erythrocytes. In the liver and the kidney, the accumulation of selenoneine was 16.1 ± 3.3 and 13.1 ± 1.9 nmol/g, respectively (Table 5-4). Se-methylselenoneine was also detected in the liver and kidney at 0.40 ± 0.19 and 0.48 ± 0.10 nmol/g, respectively, although these concentrations were below the LOQ value (Table 5-4, Fig. 5-7). In the erythrocytes and the spleen, accumulation of selenoneine was 3.0 ± 0.1 and 2.8 ± 0.5 nmol/g, respectively (Table 5-4), although Se-methylselenoneine was not detected (Fig. 5-7). The results indicate that selenoneine was methylated in the liver and/or the kidney. Moreover, Se-methylselenoneine was detected in the plasma (Fig. 5-7), but not detected in the erythrocytes. Previous studies have reported that Se-methylselenoneine was detected in human whole blood (Klein et al., 2011) and a study detect it in human erythrocytes (Kroepfl et al., 2019). In the

present study, selenoneine was not detected in plasma (Fig. 5-7). Thus, it was not considerable Se-methylselenoneine contaminated in plasma by the hemolysis of erythrocytes. It remains unclear whether selenoneine is methylated in plasma or erythrocyte. Moreover, these findings suggest that selenoneine was likely methylated in the liver and/or kidney and/or plasma enzymatically or non-enzymatically and relatively rapidly exported for excretion. Methylation is a major process in organisms and occurs via enzymes such as methyltransferases and the methyl donors like S-adenosylmethionine (SAM). For example, thiopurine S-methyltransferase (TPMT; EC 2.1.1.67) is known to be a methyltransferase against thiol compounds and is also involved in selenocysteine methylation (Weinshilboum et al., 1978; Weinshilboum and Sladek, 1980; Urbančič et al., 2019). Although TPMT is expressed in erythrocytes (Weinshilboum et al., 1978; Weinshilboum and Sladek, 1980), Se-methylselenoneine could not be detected in the erythrocytes in the present study (Fig. 5-7). While ergothioneine, the sulfur analog of selenoneine is methylated and excreted in urine, the mechanism of ergothioneine methylation has yet to be elucidated (López et al., 2021). Se-methylergothioneine was detected in whole blood, liver, kidney, brain, but not in the spleen and heart (Tang et al., 2018). Selenoneine was suspected to be methylated by the same mechanism as that for ergothioneine due to the similarity of the distributions of Se-methylselenoneine and Se-methylergothioneine (Fig. 5-7 and Table 5-4). As it is important that the mechanism of Se-methylselenoneine synthesis be understood, further studies that examine the selenoneine metabolism will need to be conducted.

In conclusion, Se-methylselenoneine in the urine was identified as the metabolite of selenoneine after the administration of selenoneine in mice. Moreover, selenoneine was predicted not to affect synthesis of selenoproteins. These results demonstrate that selenoneine is metabolized by a unique pathway unlike that observed for ordinary Se compounds.

5-5. Tables and Figures

Table 5-1 LC-ICP-MS conditions for monitoring Se-76 and Se-78

ICP-MS setting for monitoring Se-76 and Se-78 (DRC mode)	
Nebulization argon gas flow	1.13 L/min
Auxiliary gas flow	0.8 L/min
Plasma gas flow	15 L/min
Lens voltage	9.25 V
RF power	1500 W
Analog stage voltage	-1812 V
Pulse stage voltage	1100 V
Quadrupole rod offset	-8 V
Cell rod offset	-2 V
Cell path voltage	-19 V
Reaction gas	NH ₃ (>99.999 vol.%)
Reaction gas flow	0.45 L/min

HPLC setting for isocratic elution	
Mobile phase	0.1 % IGEPAL in 0.1 M ammonium acetate
Flow rate	1.0 mL/min
Column	Ultrasphere-120 column (7.8 × 300 mm)
Column temperature	40°C
Injection volume	10 µL

Table 5-2 LC-PDA-HRMS conditions.

HRMS setting	
Ion polarity	Positive
Data acquisition	Scan mode
Mass range	<i>m/z</i> 50-800
Ion source	
End plate offset	-500V
Capillary	4500V
Neblizer	160 kPa
Dry gas flow	10.0 L/min
Dry gas temperature	200°C
Transfer	
Funnel 1 RF power	200 Vp·p
Funnel 2 RF power	200 Vp·p
Hexapole RF power	70 Vp·p
Quadrupole	
Ion energy	3.0 eV
Low mass cut off	<i>m/z</i> 100.0
Collision Cell	
Collision energy	8.0 eV
Collision RF power	100 Vp·p
Transfer time	50 μ s
Pre puls strage	5.0 μ s

PDA setting	
monitoring wavelength	195-800 nm

HPLC setting for isocratic elution	
Mobile phase	0.1 % acetic acid
Flow rate	0.3 mL/min
Column	Cosmosil PBr packed column (2.0 mm × 150 mm, 5 μm)
Column temperature	30°C
Injection volume	5 μL

Table 5-3 Ratios of tissue weights according to body weights

	Ratio (%)			
	Liver	Kidney	Spleen	Brain
Control	4.75 ± 0.07	1.75 ± 0.07	0.384 ± 0.051	2.17 ± 0.21
Selenoneine	4.73 ± 0.12	1.75 ± 0.02	0.370 ± 0.015	2.40 ± 0.03

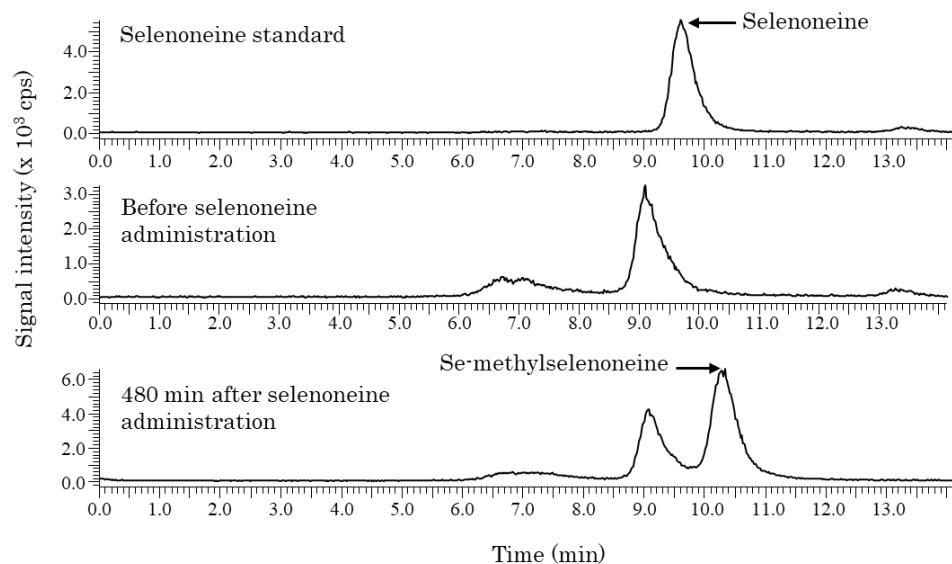
Table 5-4 Concentrations of Se compounds in tissues of mice

	HMW-Se (nmol Se/g)		Selenoneine (nmol Se/g)		Se-methylselenoneine (nmol Se/g)	
	Control	Selenoneine	Control	Selenoneine	Control	Selenoneine
Plasma	6.2 ± 1.8	7.8 ± 0.8	-	-	-	(0.45 ± 0.02)
Erythrocytes	15.7 ± 0.2	18.5 ± 3.3	-	3.0 ± 0.1	-	-
Liver	43.1 ± 2.6	43.7 ± 2.5	-	16.1 ± 3.3	-	(0.40 ± 0.19)
Kidney	12.8 ± 1.5	12.9 ± 1.2	-	13.1 ± 1.9	-	(0.48 ± 0.10)
Spleen	1.7 ± 0.4	1.5 ± 0.1	-	2.8 ± 0.5	-	-
Brain	(0.77 ± 0.06)	(0.89 ± 0.22)	-	-	-	-

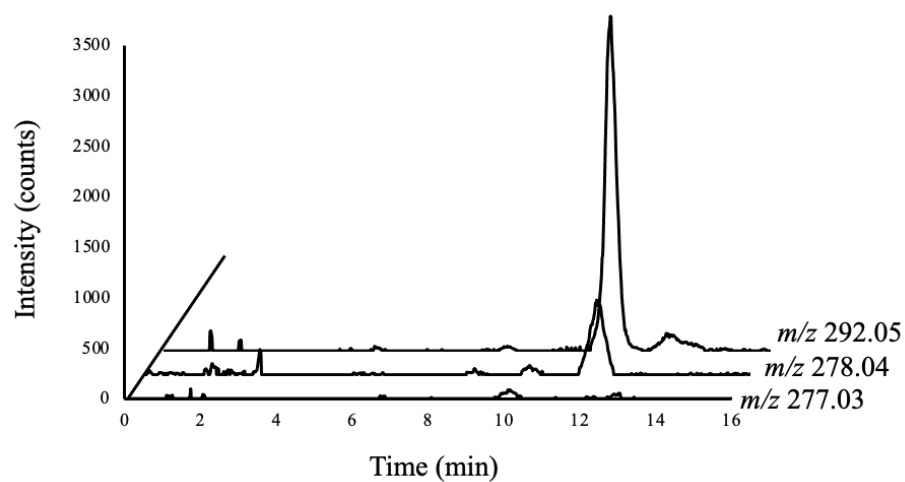
Values are expressed as mean ± standard deviation. “-” means not detected, and a value within parentheses means the value is lower than the limit of quantification but higher than the limit of detection.

Fig. 5-1

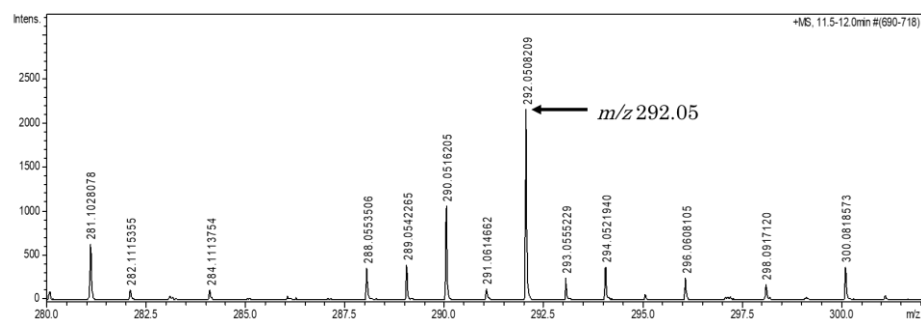
a



b



c



d

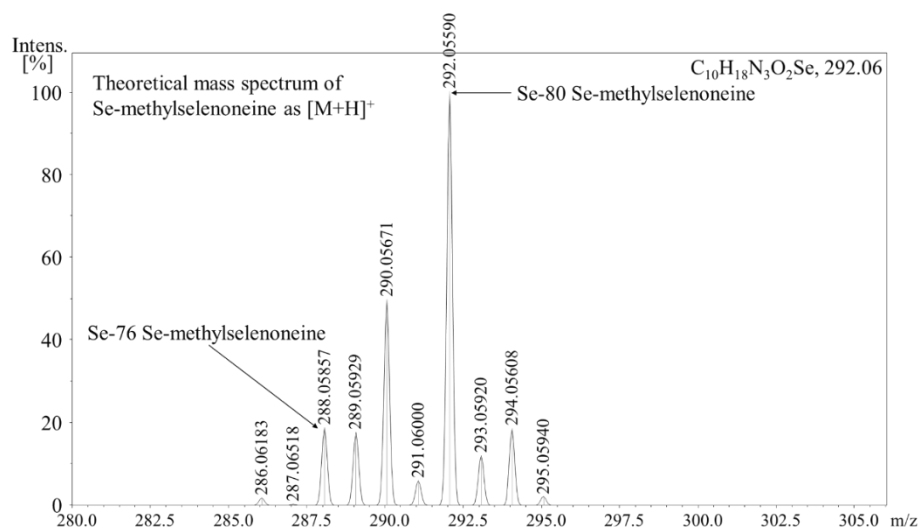
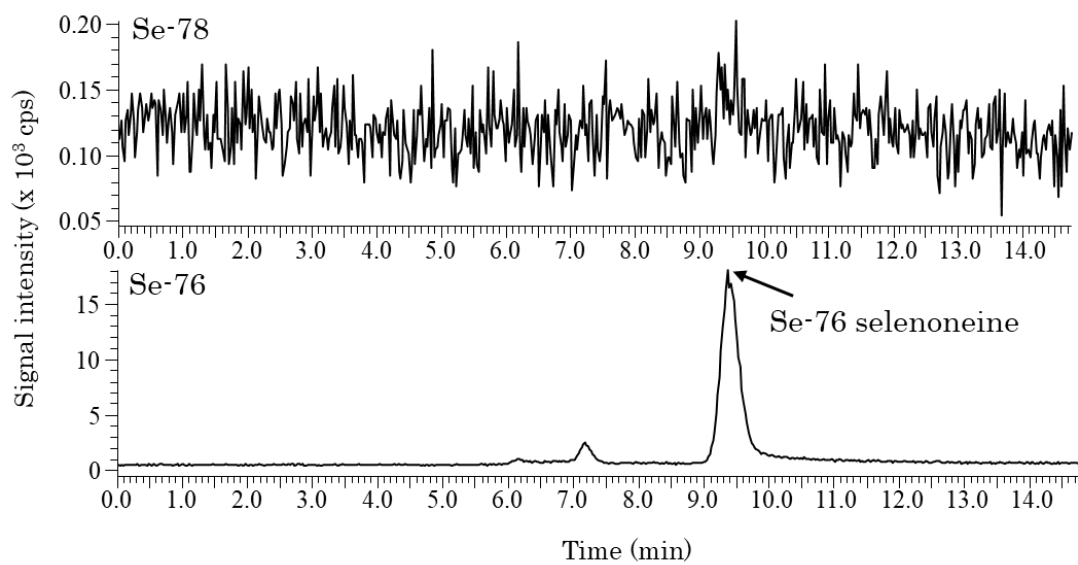


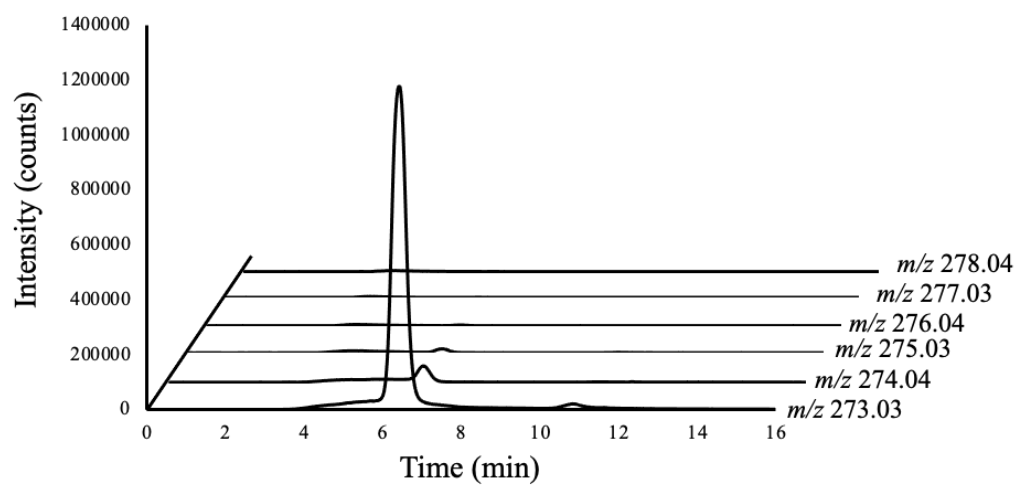
Fig. 5-1 Identification of Se-methylselenoneine in urine: a) LC-ICP-MS chromatograms monitoring Se-82 of the standard selenoneine and the urine sample of a mouse before administration of selenoneine and at 480 min after the administration. b) EICs using LC-PDA-HRMS of the fraction from the urine. The chromatograms exhibited ions, m/z 277.03 (selenoneine dimer), m/z 278.04 (selenoneine monomer) and m/z 292.05 (Se-methylselenoneine) with extraction widths of ± 0.05 relating to Se-78 selenoneine dimer mass, Se-78 monomer mass and Se-methylselenoneine mass, respectively. c) Mass spectrum using LC-PDA-HRMS of the Se-methylselenoneine fractionated from the urine sample at 480 min after administration. d) The theoretical mass spectrum of Se-methylselenoneine as $[M+H]^+$; a) column: Ultrahydrogel-120 column 7.8×300 mm; mobile phase: 0.1 M ammonium acetate aqueous solution containing 0.1% IGEPAL; flow rate: 1.0 mL/min. b, c) column: Cosmosil PBr packed column 2.0×150 mm; mobile phase: 0.1 % acetic acid; flow rate: 0.3 mL/min. Details provided in Material and Methods.

Fig. 5-2

a



b



c

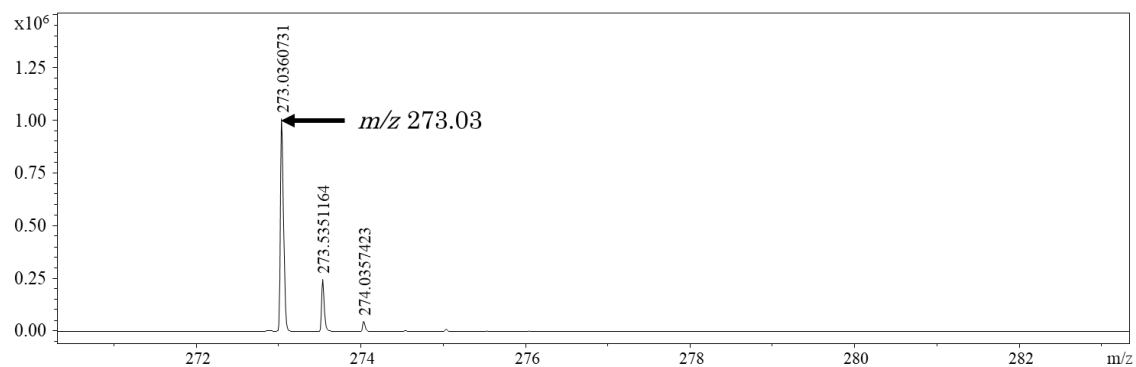
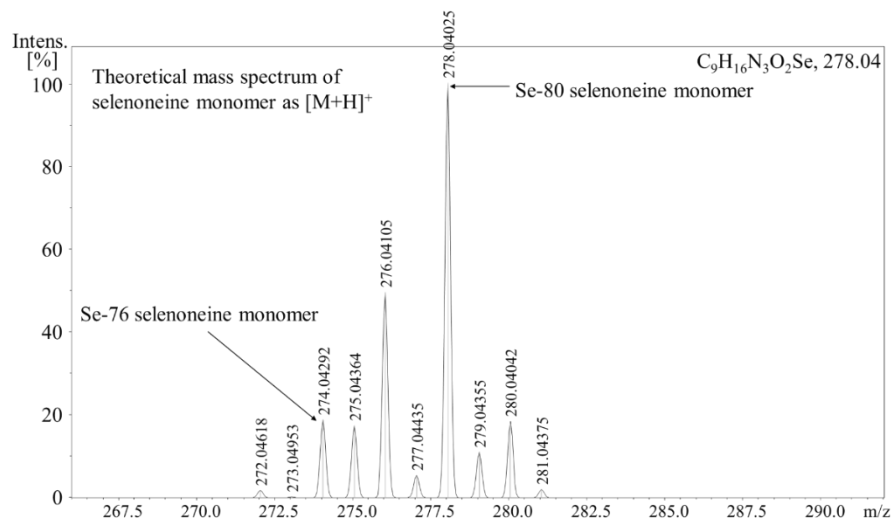


Fig. 5-2 confirmation of Se-76 labeled selenoneine: a) LC-ICP-MS chromatograms of synthesized Se-76 selenoneine. Se-78 was monitored in the upper chromatogram and Se-76 was monitored in the lower chromatogram. b) EICs using LC-PDA-HRMS of synthesized Se-76 selenoneine. The chromatograms exhibited ions, m/z 273.03, 274.04, 275.03, 276.04, 277.03, and 278.04 with extraction widths of ± 0.05 relating to Se-76 selenoneine dimer mass, Se-76 selenoneine monomer mass, Se-78 selenoneine dimer mass, Se-78 selenoneine monomer mass, Se-80 selenoneine dimer mass and Se-80 selenoneine monomer mass, respectively. c) Mass spectrum using LC-PDA-HR-MS of oxidized dimeric Se-76 selenoneine; a) column: Ultrahydrogel-120 column 7.8×300 mm; mobile phase: 0.1 M ammonium acetate aqueous solution containing 0.1% IGEPAL; flow rate: 1.0 mL/min. b, c) column: Cosmosil PBr packed column 2.0×150 mm; mobile phase: 0.1% acetic acid; flow rate: 0.3 mL/min. Details provided in Material and Methods.

Fig. 5-3

a



b

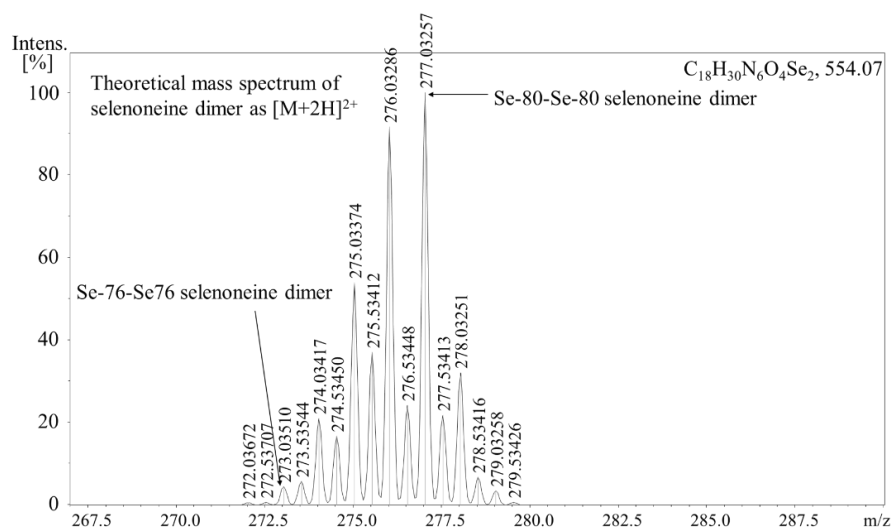
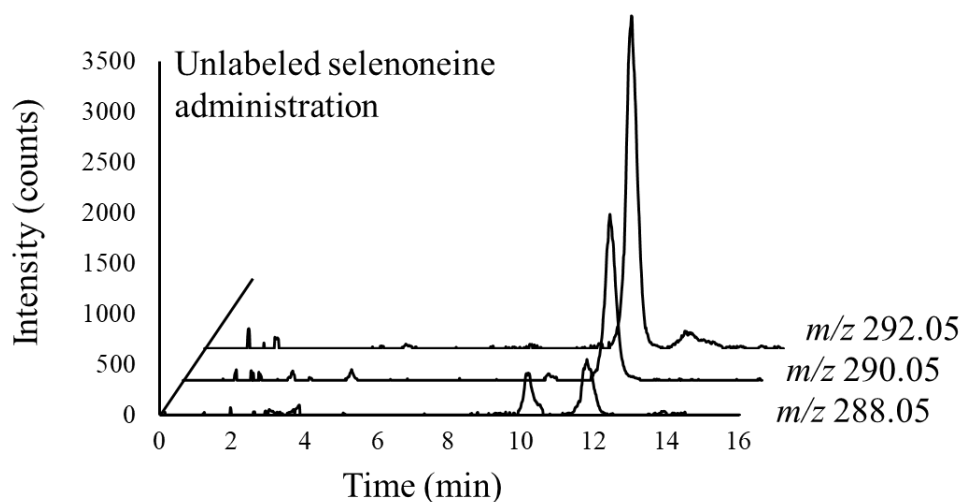


Fig. 5-3 Theoretical mass spectra of selenoneine monomer and dimer: a) A theoretical mass spectrum corresponding to Se-76 selenoneine monomer as $[M+H]^+$. b) A theoretical mass spectrum corresponding to Se-76-Se-76 selenoneine dimer as $[M+2H]^{2+}$.

Fig. 5-4

a



b

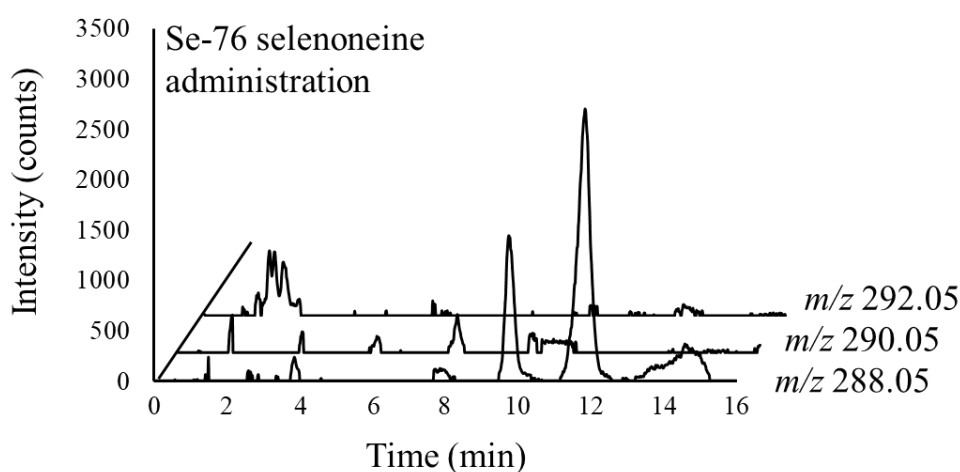


Fig. 5-4 EICs using LC-PDA-HRMS of the urine samples from the mice after 480 min administered 100 μ M Se as unlabeled selenoneine or Se-76-labeled selenoneine: a) EICs exhibit the urine sample from the mice administered unlabeled selenoneine and the right EICs exhibited that from the mice administered Se-76 selenoneine. b) Se-methylselenoneine is eluted at 11.8 min and peaks at 11.8 min in the EICs monitoring m/z 288.05, m/z 290.05 and m/z 292.05 are Se-76, 78 and 80 Se-methylselenoneine. The peak at 10.0 min in the EIC of m/z 288.05 was not identified; column: Cosmosil PBr packed column 2.0 $\times$ 150 mm; mobile phase: 0.1 % acetic acid; flow rate: 0.3 mL/min. Details provided in Material and Methods.

Fig. 5-5

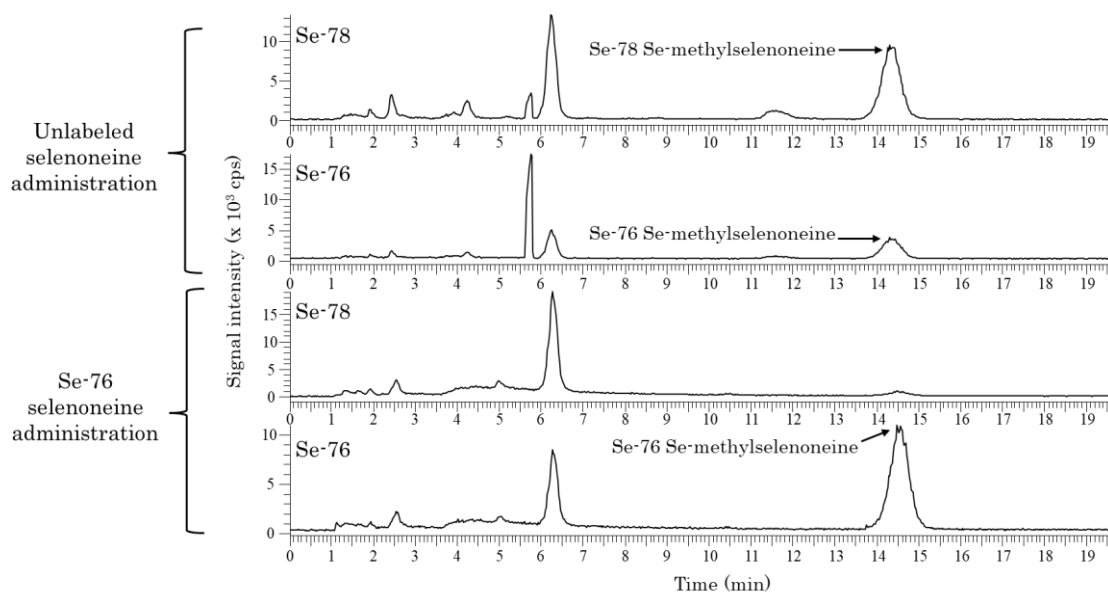
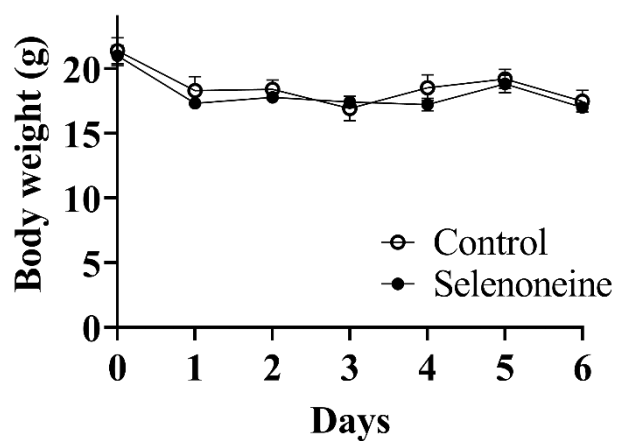


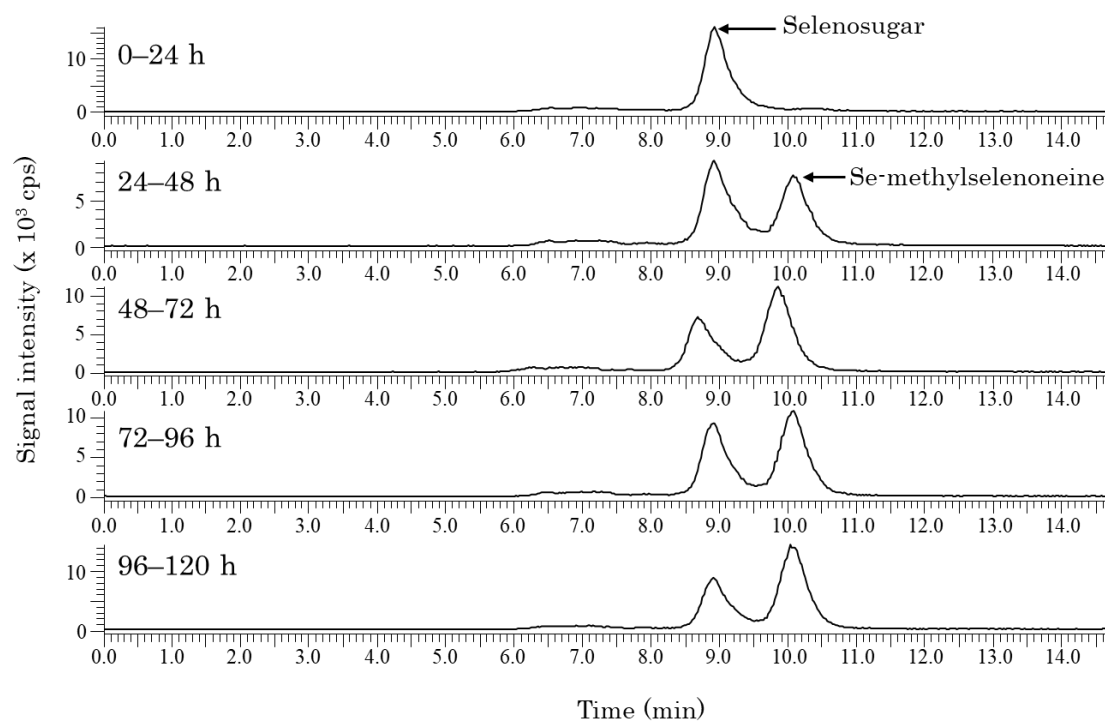
Fig. 5-5 LC-ICP-MS chromatograms of the urine samples from the mice after 480 min administered 100 μ M Se as unlabeled selenoneine or Se-76-labeled selenoneine: Chromatograms of the top and the third from the top express the results of the Se-78 monitoring. Those of second and fourth from the top express the results of Se-76 monitoring; column. The peaks at 14.4 min were considered to be Se-methylselenoneine based on the detection pattern of Se-76 and Se-78 and the EICs exhibited in Fig. 5-4: Cosmosil PBr packed column 2.0 $\times$ 150 mm; column temperature; 40 $^{\circ}$ C; injection volume, 10 μ L; mobile phase: 10 mM ammonium acetate; flow rate: 0.2 mL/min.

Fig. 5-6

a



b



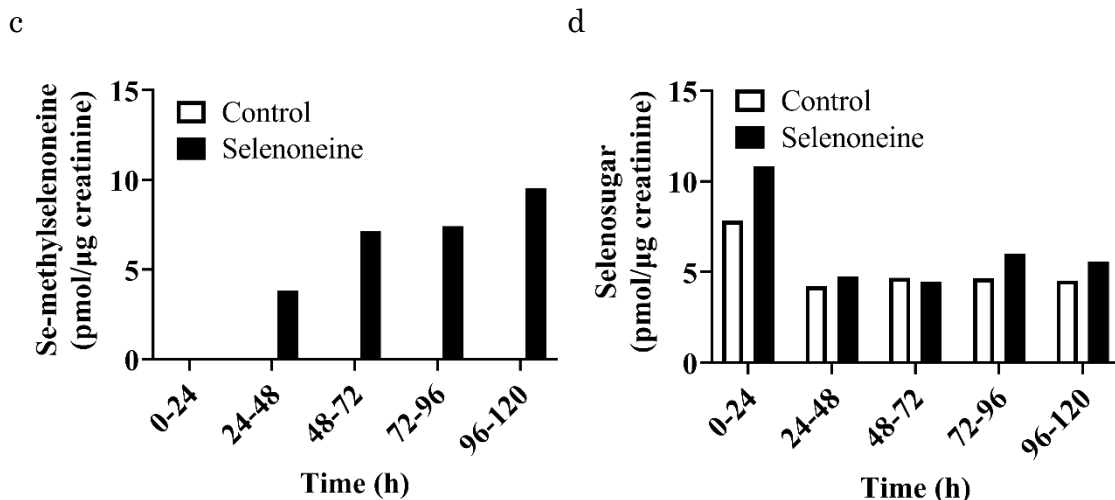


Fig. 5-6 body weight changes and excretions of Se-methylselenoneine of mice: a) Body weight changes of mice over 6 days. b) LC-ICP-MS chromatograms monitoring Se-82 of the urine sample from mice administered selenoneine. The peak eluted at 10.3 min contains Se-methylselenoneine from the results of Fig. 2a and b. c, d) The time course of Se-methylselenoneine and selenosugar concentrations in the pooled urine during the intake of selenoneine; column: Ultrahydrogel-120 column 7.8×300 mm; mobile phase: 0.1 M ammonium acetate aqueous solution containing 0.1% IGEPAL; flow rate: 1.0 mL/min. Details provided in Material and Methods.

Fig. 5-7

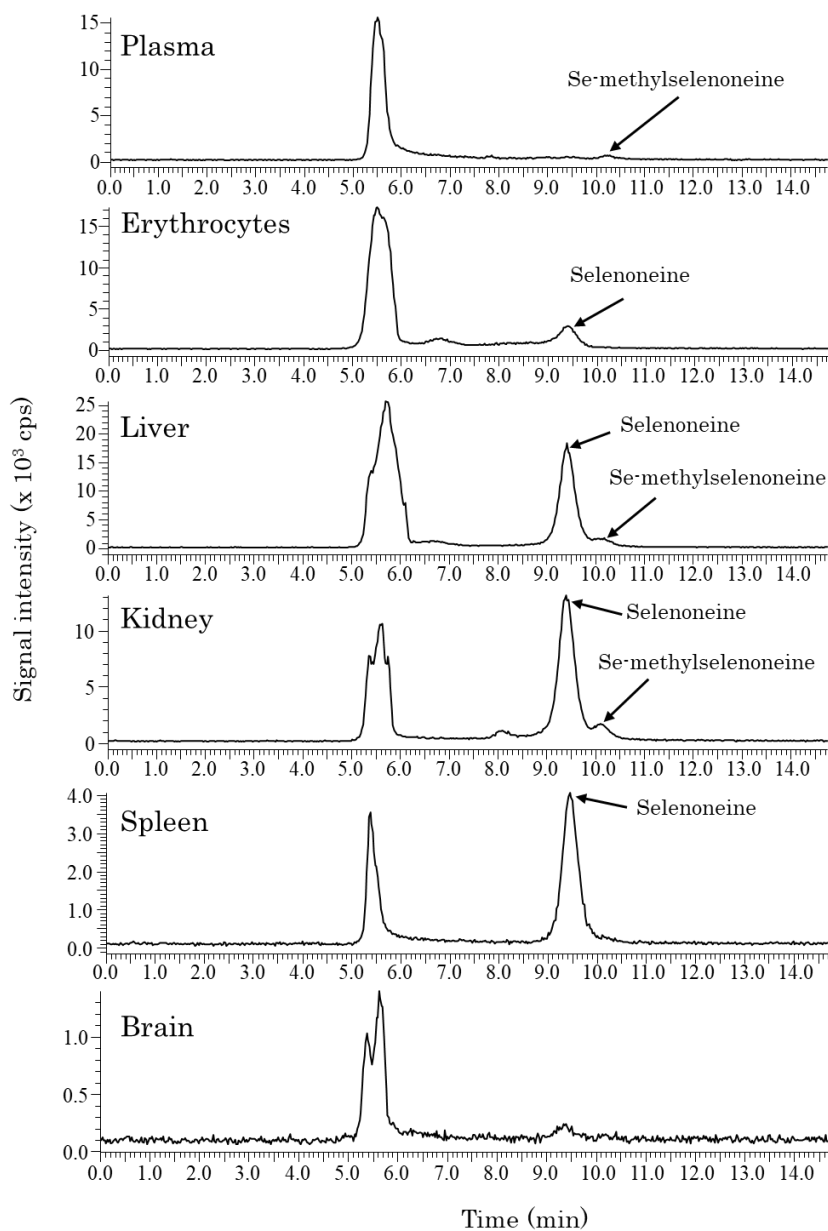


Fig. 5-7 LC-ICP-MS chromatograms monitoring Se-82 of tissues of a mouse administered selenoneine: The 5.5 min peak has been designated as high molecular weight Se species (HMW-Se) based on previous results (Yamashita and Yamashita, 2010). The second peak detected in the erythrocytes, liver, kidney, spleen and brain contains selenoneine. The third peak detected in the plasma, liver and kidney contains Se-methylselenoneine. The peaks containing selenoneine or Se-methylselenoneine were identified from the results of Fig.2a and b; column: Ultrahydrogel-120 column 7.8 × 300 mm; mobile phase: 0.1 M ammonium acetate aqueous solution containing 0.1% IGEPAL; flow rate: 1.0 mL/min. Details provided in Material and Methods.

Conclusion

Both organic and inorganic Se compounds have been studied all over the world since Se is an essential trace element for animals including humans. In 2010, selenoneine was identified as the major organic Se compound in marine organisms that had not been identified for many years. Although the discovery opened up a new research field in Se research, many of scientific phenomenon corresponding to selenoneine remains unresolved because of the short history. In this study, I explored new functions of selenoneine by *in vitro* and *in cellulo* experiments (Chapter 1, 2) for the purpose of accumulating new knowledge of selenoneine. In addition, I developed a new method for acquiring high purity selenoneine efficiently from genetically modified fission yeast (Chapter 3) and revealed the distribution and metabolism of selenoneine *in vivo* using the purified selenoneine and mice (Chapter 4, 5).

In Chapter 1, selenoneine exhibited inhibition of angiotensin converting enzyme (ACE) associated with hypertension *in vitro* experiments. The enzyme kinetics indicated that the inhibition of ACE by selenoneine is competitive, with competition for the active center with other substrates.

In Chapter 2, selenoneine suppressed melanin synthesis in the murine melanoma cells. It also suppressed melanin synthesis in the 3D-cultured normal human melanocytes generally used as an alternative experiment to the *in vivo* animal test. The mechanism of the melanin suppression by selenoneine was indicated that the inhibition of tyrosinase, the key enzyme of melanin synthesis, by the enzymatic experiments using purified tyrosinase

and selenoneine. Those inhibitions of enzymes in Chapter 1 and 2 suggest that selenoneine inhibits metalloenzymes such as ACE with a Zn ion and tyrosinase with Cu ions at their active centers.

In Chapter 3, a new method was developed to acquire highly purified selenoneine from genetically modified fission yeast. This method enabled us to separate ergothioneine and selenoneine monomer, which could not be separated before, using the PBr column separating substances based on the London dispersion force.

In Chapter 4, low dose administration of selenoneine in a mackerel extract revealed that selenoneine accumulated in the liver, kidney and spleen in mice. This result suggested that selenoneine prior to accumulate in such tissues than erythrocytes, where it had been reported to accumulate at a highly concentration previously. Moreover, high molecular weight Se including selenoproteins in tissues were not affected.

In Chapter 5, selenoneine was revealed to be methylated and excreted as Se-methylselenoneine in urine of mice using stable isotope (Se-76) labeled selenoneine. Selenoneine administration for 6 days did not effect on the major metabolite of Se, selenosugar, in urine and high molecular weight Se compounds in tissues.

Chapter 1 and 2 revealed that selenoneine inhibited metalloenzymes such as ACE and tyrosinase, but the effects was not examined *in vivo*. On the other hand, Chapter 4 and 5 revealed that selenoneine distributed to erythrocytes, liver, kidney and spleen. The distribution suggests that selenoneine is delivered into cells in organs where blood vessels widely

distribute. For example, ACE expresses in vascular endothelial cells and they are expressing the selenoneine transporter OCTN1. Therefore, selenoneine is predicted to exert its metalloenzyme inhibition against ACE and decrease the blood pressure *in vivo*. However, Chapter 4 revealed that the accumulation ratio of selenoneine is low and Chapter 5 exhibited that selenoneine is excreted into urine as Se-methylselenoneine. Thus, examinations using model animals or humans are needed to reveal that selenoneine exerts the functions found in Chapter 1 or 2 at a realistic dosage of selenoneine for mammals.

On the other hand, Chapter 5 provides an important suggestion for the metabolism of selenoneine and other Se compounds. Selenoneine did not affect on the selenosugar excretion but was methylated into Se-methylselenoneine. This result indicates that selenoneine does not go through the central metabolic pathway of Se compounds (Fig. 0) and that suggests selenoneine is not used for the synthesis of selenoproteins, the major utilized form of Se. That means selenoneine exerts its functions as an antioxidant or regulator of enzymes rather than a supply source of Se as a nutrition.

In conclusion, selenoneine is suggested to distribute and accumulate in the tissues and exert health effects by modulating enzyme functions like metalloenzymes. On the other hand, it is speculated to be methylated and excreted via the unique pathway different from other Se compounds.

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Yokohama, 2024

Takuya Seko

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List of Publications

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2. Seko, T., Imamura, S., Ishihara, K., Yamashita, Y., Yamashita, M. (2020). Selenoneine suppresses melanin synthesis by inhibiting tyrosinase in murine B16 melanoma cells and 3D-cultured human melanocytes. *Fisheries Science*, 86, 171–179.
3. Seko, T., Uchida, H., Yamashita, Y., Yamashita, M. (2021). Novel method for separating selenoneine reduced monomer and ergothioneine from fission yeast extracts. *Separation and Purification Technology*, 254, 117607.
4. Seko, T., Sato, Y., Kuniyoshi, M., Murata, Y., Ishihara, K., Yamashita, Y., Fujiwara, S., Ueda, T., Yamashita, M. (2023). Distribution and effects of selenoneine by ingestion of extract from mackerel processing residue in mice. *Marine Biotechnology*, 25, 1020–1030.
5. Seko, T., Uchida, H., Sato, Y., Imamura, S., Ishihara, K., Yamashita, Y., Yamashita, M. (2023). Selenoneine is methylated in the bodies of mice and then excreted in urine as Se-methylselenoneine. *Biological Trace Element Research*, 1–14.