Current Topics

Advances in Biology and Toxicology of Environmental Metals/Metalloids

Selenometabolomics Explored by Speciation

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Selenium (Se) belongs to the same group as sulfur in the periodic table but possesses certain chemical properties characteristic of a metal. It is an essential element in animals but becomes severely toxic when the amount ingested exceeds the required level. On the other hand, Se is not essential in plants although some plants are Se hyperaccumulators. Se changes into several chemical forms when metabolized. Thus, the identification of selenometabolites would enable us to formulate a metabolic chart of Se. Recently, speciation analysis by hyphenated techniques has contributed immensely to the study of selenometabolites, *i.e.*, the entirety of selenometabolites. Indeed, speciation has unveiled some unique selenometabolites in biological samples. The aim of this review is to present newly identified selenometabolites in animals and plants by speciation using hyphenated techniques and to delineate the perspectives of Se biology and toxicology from the viewpoint of speciation.

Key words selenium; speciation; inductively coupled plasma-mass spectrometry; selenoamino acid; selenosugar; hyphenated technique

1. INTRODUCTION

Selenium (Se) has chemically and biologically ambivalent characteristics. It has similar chemical properties to sulfur as a typical element, yet possesses certain chemical properties of a metal. Se is an essential element in animals but can be highly toxic when the amount ingested exceeds the nutritional level. The adequate nutritional range between deficient and excess doses is narrow¹⁾: it is one order of magnitude (0.1–1.0 µg/g diet or mL drinking water) in experimental animals. Se is required as the active center of selenoproteins that function as an antioxidant and participate in thyroid hormone production, DNA synthesis, and fertilization.^{2–5)} It acts as an active center in the formation of a selenol group (–SeH) on a selenocysteine (SeCys) residue in a selenoprotein sequence.^{6,7)} SeCys is incorporated into a peptide through the UGA codon on mRNA when a special motif, *i.e.*, SeCys insertion sequence (SECIS), appears in the 3' untranslated region of mRNA. Thus, SeCys is called the “the 21st amino acid.”^{8,9)} At present, 25 and 24 selenoproteins are identified in human and mouse, respectively, by bioinformatics search for SECIS in human and murine genomes.^{10,11)} In contrast to selenoproteins, proteins containing Se in the form of selenomethionine (SeMet) are called Se-containing proteins but not selenoproteins.¹²⁾ It is known that Se-containing proteins are translated without distinguishing SeMet from methionine (Met) by reading the AUG codon for Met. However, no specific effects of Se-containing proteins have been recognized so far.¹³⁾ Another biological significance of Se in animals is that Se can detoxify inorganic mercury (Hg) by directly interacting with it. It was reported that the administration of selenite at a lethal dose

antagonized the lethal dose of inorganic Hg in experimental animals.^{14,15)} Although the precise mechanism is still unclear, the toxicity of methylmercury (MeHg) is also ameliorated by Se ingestion.^{16–18)} In contrast to animals, Se is not essential in plants and exists as a “bystander” mineral. However, its beneficial effects on plant growth have been reported.^{19,20)} Some Se-containing compounds (selenocompounds), such as methylselenocysteine (MeSeCys), γ -glutamylmethylselenocysteine (GluMeSeCys), and SeMet, are biosynthesized in some plants^{21,22)} and fungi,^{23,24)} and they have anti-cancer and/or cancer-preventing effects.^{25,26)}

Due to its chemical properties, Se can form a carbon-Se bond through the metabolic pathway in animals and plants, and its biological and toxicological effects are very much dependent on its chemical form. Hence, the speciation of selenometabolites can provide important clues to elucidate the metabolic pathway of Se. Speciation studies of Se have provided integrated knowledge to understand the entire metabolic pathway of Se in animals and plants. Se has fascinated not only biologists but also analytical chemists.^{27–31)} This review focuses on some selenometabolites recently identified by speciation analysis and verifies their biological, pharmacological, and toxicological significance from the viewpoint of analytical chemistry.

2. HYPHENATED TECHNIQUES FOR SPECIATION OF SELENOMETABOLOMICS

2.1. Selenometabolomics “Selenometabolomics” is a newly coined word that means the metabolomics of Se. As mentioned above, in a biological system, Se exists in diverse

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chemical forms depending on the metabolic pathway, such as uptake, absorption, utilization, storage, and excretion. On the other hand, metabolome refers to the complete set of small-molecule metabolites, such as metabolic intermediates, hormones and other signaling molecules, and secondary metabolites in a biological system, and metabolomics is the study of the profiles of such small-molecule metabolites. Therefore, selenometabolomics refers to the systematic study of Se-containing metabolites (selenometabolites) processed in a biological cell, tissue, organ or organism.

2.2. Speciation The term "speciation" of an element (in particular, a metal and a metalloid) is defined by the distribution of an element amongst defined chemical species in a system, whereas speciation analysis is defined by the analytical activities involved in identifying and/or measuring the quantities of one or more individual chemical species in a sample.³²⁾ Selenometabolomes can be separated, identified, and then quantified by means of appropriate analytical methods. As selenometabolomes consist of diverse chemical forms in a biological system, each Se-containing metabolite should be separated by an appropriate separation technique and detected with a specific detector for Se.

2.3. Hyphenated Technique The hyphenated technique is the most frequently used analytical technique for Se speciation.^{33–35)} The hyphenated technique for metal/metalloid speciation consists of two analytical techniques: the separation technique and the detection technique. High-performance liquid chromatography (HPLC) is one of the most popular separation techniques used with such hyphenated techniques as gas chromatography, gel electrophoresis, and capillary electrophoresis. HPLC has several advantages over other separation techniques: diverse modes, such as gel filtration, ion exchange, affinity, and reversed phase, are available, and the separation conditions are similar to physiological conditions, particularly on a size-exclusion/gel filtration column. On the other hand, inductively coupled plasma-mass spectrometry (ICP-MS) has enabled multi-elemental detection with extremely high sensitivity and robustness to matrices, as well as isotope discrimination. In addition, the eluate of HPLC can be directly introduced into ICP-MS because the flow rate of regular bore HPLC is proportional to that for the introduction to ICP-MS.^{36,37)} Therefore, the coupling of HPLC with ICP-MS is the most widely used hyphenated technique for speciation. However, HPLC-ICP-MS has one crucial disadvantage: as ICP-MS provides only elemental information about selenometabolites, the identification by HPLC-ICP-MS is effective only when the retention times of samples could be matched with those of certified or authentic Se standards. Thus, HPLC-ICP-MS is practically useful for the screening and determination of column recovery and the monitoring of the purification efficiency of selenometabolites owing to its sensitivity and robustness.³⁸⁾ As a complementary technique to HPLC-ICP-MS, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI)-tandem mass spectrometry (MS-MS) have been used for the identification of unknown selenometabolites in yeast,^{39–41)} plant,⁴²⁾ and animal samples.^{43,44)} The fact that Se produces a characteristic cluster of signals that reflect its isotopic pattern (⁷⁴Se, 0.89%; ⁷⁶Se, 9.36%; ⁷⁷Se, 7.63%; ⁷⁸Se, 23.8%; ⁸⁰Se, 49.6%, and ⁸²Se, 8.73%) has facilitated the scanning of Se-containing species by MS. As ESI is a softer technique than ICP, it can provide

molecular information. In addition, MS-MS enables structure elucidation. Thus, ESI-MS-MS is a mandatory technique for the identification of unknown selenometabolites. However, ESI-MS-MS has a number of weak points compared with ICP-MS. First, the detection limit of ESI-MS-MS for seleno-compounds is inferior to that of ICP-MS. Second, ESI is severely affected by the sample matrix. To counter this problem, ESI-MS-MS is coupled with HPLC. Therefore, the complementary use of HPLC-ICP-MS and HPLC-ESI APCI-MS-MS is a powerful tool to explore selenometabolites by speciation.

3. SELENOMETABOLITES IDENTIFIED BY HY-PHENATED TECHNIQUES

3.1. Selenosugars Se is mostly excreted into urine. However, when an excessive amount of Se is ingested, Se is excreted into not only urine but also breath; namely, Se beyond the physiological or nutritional range can be exhaled into breath.⁴⁵⁾ Within the nutritional level to the low toxicity level, the major urinary metabolite is *Se*-methylseleno-*N*-acetylgalactosamine (MeSeGalNAc) as identified by HPLC-ICP-MS, ESI-MS-MS, and NMR⁴⁶⁾ (Fig. 1a). In addition to the major urinary selenosugar, MeSeGalNAc, minor selenosugars, such as *Se*-methylseleno-*N*-acetylglucosamine (MeSeGlcNAc) and *Se*-methylselenogalactosamine (MeSeGalNH₂), were also identified in urine by ESI-MS-MS.^{44,47)} *Se*-Glutathionylseleno-*N*-acetylgalactosamine (GSSeGalNAc) was also identified by ESI-MS-MS in the liver of experimental animals.⁴⁶⁾ This selenosugar is recognized as the precursor of the major urinary selenosugar, MeSeGalNAc. The glutathionyl precursors of minor selenosugars, MeSeGlcNAc and MeSeGalNH₂, have not been identified yet.

As mentioned above, Se has a characteristic cluster of signals that reflect its isotopic pattern (Fig. 2, ⁷⁴Se, 0.89%; ⁷⁶Se, 9.36%; ⁷⁷Se, 7.63%; ⁷⁸Se, 23.8%; ⁸⁰Se, 49.6%, and ⁸²Se, 8.73%), and this has facilitated the scanning of Se-containing species by MS. In the case of MeSeGalNAc, the characteristic cluster of signals were detected in urine at *m/z* 300 for ⁸⁰Se in the positive ion mode by ESI-MS. Fragment ions, *m/z* 298, 300, and 302, corresponding to ⁷⁸Se, ⁸⁰Se, and ⁸²Se, respectively, determined by MS-MS analysis indicate the structure of *Se*-methyl-*N*-acetylhexosamine. To determine the configuration of the Se-containing sugar, NMR was performed and the structure depicted in Fig. 1a was identified. This selenosugar was also detected in the livers of fish and reptiles.⁴⁸⁾ In addition to MeSeGalNAc, two Se-containing metabolites that have the characteristic cluster of signals were detected in the liver at *m/z* 591 and 753 for ⁸⁰Se in the positive ion mode by ESI-MS. In the negative ion mode, signals were also detected at *m/z* 589 and 751, indicating that 591 and 753 corresponded to [M+H]⁺, and 589 and 751 corresponded to [M-H]⁻. Thus, the molecular masses of the two selenometabolites in the liver were 590 and 752 for ⁸⁰Se. The small metabolite having a molecular mass of 590 is assigned to GSSeGalNAc on the basis of the fragment ions dissociated by ESI-MS-MS. Meanwhile, the large metabolite having a molecular mass of 752 has not been assigned yet due to its trace abundance. Although no further information and speculation of the unidentified selenometabolite except the molecular mass were provided, the molecular mass provided a valuable clue. The mass difference between GSSeGalNAc (molecular weight (Mw)=590) and the

Fig. 1. Selenometabolites Identified by Speciation Analyses

a, *Se*-methylseleno-*N*-acetylgalactosamine (MeSeGalNAc); b, selenomethionine (SeMet); c, *Se*-methylselenocysteine (MeSeCys); d, γ -glutamylmethylselenocysteine (GluMeSeCys); e, γ -glutamylselenocystathionine; f, selenohomolanthionine (SeHLan); g, selenoneine; h, methylmethionine selenonium (MeSeMet); i, *Se*-adenosylselenohomocysteine (AdoSeHcy); j, *Se*-adenosylselenomethionine (AdoSeMet).

unidentified selenometabolite ($M_w=752$) of 162 corresponds to the mass of a simple monosaccharide, such as glucose and galactose ($M_w=180$), minus water ($M_w=18$). Taken together, the structure of the unidentified selenometabolite is assumed to be *Se*-glutathionyl-disaccharide, which consists of *N*-acetylgalactosamine and a simple sugar. Solid identification of the unknown metabolite by molecular MS-MS and/or other techniques may give us novel insights into the metabolism of Se. Furthermore, there is no reasonable explanation of how selenosugars are biosynthesized, what enzymes participate in the biosynthesis of selenosugars, or why animals excrete Se as selenosugars. A more sensitive and robust molecular mass spectrometer than that currently in use is expected to provide the answers to these questions.

3.2. Selenoamino Acids in Se-Accumulating Plants

Although Se is not essential and exists as a “bystander” mineral in plants, some Se-accumulating plants are known and Se-enriched (selenized) plants have been developed for the following two reasons. One is nutritional supplementation to enable utilization of its pharmacological effects, such as cancer chemopreventive effects. It has been demonstrated that certain Se-containing species, such as SeMet (Fig. 1b), MeSeCys (Fig. 1c), and GluMeSeCys (Fig. 1d), reduce the incidence of breast, prostate, and colorectal cancers. Selenized yeasts and

Fig. 2. Isotope Ratio of Naturally Occurring Se

Allium plants belonging to Family Liliaceae, such as garlic, onion, wild leek, and shallot, have been developed for this purpose.^{49–51} On the other hand, Se is widely used in industry, such as glass and ceramic manufacturing and electronics, and is thus also known as an environmental contaminant. The other reason is the phytoremediation of Se to remove Se from

soil, mining, and other industrial sources. Phytoremediation is a low-cost and environmentally friendly technique to remove contaminants. The Indian mustard, *Brassica juncea*, has been extensively studied for this purpose because of its high Se-accumulating ability, rapid growth, and high biomass.⁵²⁾ Even though the plant is exposed to inorganic Se, such as selenite or selenate, the inorganic Se compounds are metabolized to form selenocompounds having an Se-C covalent bond to reduce its toxicity in plants.

In contrast to animals, the biosynthesis of SeCys-tRNA is not necessary because no selenoproteins exist; in other words, Se is a non-essential element in plants. However, SeCys may be a key metabolic intermediate for the synthesis of other selenoamino acids and volatile Se metabolites. SeCys is biosynthesized in accordance with the following scheme.⁵³⁾ Serine (Ser) is activated by acetyl-CoA *via* serine acetyltransferase to form *O*-acetyl-Ser.⁵⁴⁾ The activated Ser receives Se in the form of selenide to form SeCys.⁵³⁾ However, there are no selenoproteins in plants, *i.e.*, SeCys may not be required in a protein sequence. Hence, SeCys is recognized as a metabolic intermediate in plants to form other selenoamino acids and their derivatives, as mentioned below.

First, SeCys can enter the Met biosynthetic pathway to form SeMet *via* selenocystathionine and selenohomocysteine (SeHcy), *i.e.*, the “forward” trans-selenation pathway (from SeCys to SeMet).⁵⁵⁾ Selenocystathionine is formed by reacting SeCys with an activated homoserine, *O*-phosphohomoserine, in the presence of cystathionine- γ -synthase, and then selenocystathionine is decomposed by cystathionine- β -lyase to cleave SeHcy. It is reported that selenocystathionine also binds to glutamic acid to form γ -glutamylselenocystathionine (Fig. 1e) in monkeypot nuts (*Lecythis minor*), and γ -glutamylselenocystathionine was identified by HPLC-ESI-quadrupole and time-of-flight tandem MS (HPLC-ESI-Q-TOF).⁵⁶⁾ SeHcy is methylated by methyltransferase (Met synthase) to form SeMet with *N*⁵-methyltetrahydrofolate as the methyl group donor.⁵⁷⁾ As an alternate pathway, SeHcy is conjugated with another *O*-phosphohomoserine to form selenohomolanthionine (SeHLan, Fig. 1f) in Se-enriched Japanese pungent radish (*Raphanus sativus* L. cv. ‘Yukibijin’).⁴²⁾ SeHLan was also identified by HPLC-ESI-MS-MS. Consequently, some Se-accumulating plants accumulated Se as selenocystathionine or SeHLan. As mentioned later, methylated selenoamino acids, such as SeMet and MeSeCys, can be converted into volatile Se compounds to reduce Se accumulation in plants. In contrast, a geminal type of selenoamino acid, such as selenocystathionine or SeHLan, cannot be transformed into volatile Se compounds, resulting in Se accumulation.

The second pathway from SeCys involves the formation of MeSeCys by methylation,^{58,59)} and MeSeCys is used for the biosynthesis of γ -glutamylmethylselenocysteine (GluMeSeCys).⁶⁰⁾ The knock-in of methyltransferase that catalyzes SeCys methylation increases tolerance to Se toxicity.^{61,62)} As Se-hyperaccumulating plants are available in nature or easily prepared by Se fortification, a larger number of selenometabolites are identified by MS in plants than in animals, in spite of the non-essentiality of Se in plants.

3.3. Selenoneine and Se-Methylselenoneine Selenoneine (Fig. 1g), 2-selenyl-*N*⁶,*N*⁷,*N*⁸-trimethyl-L-histidine, was identified for the first time in blood of bluefin tuna.⁶³⁾ Selenoneine was also identified in the liver of marine reptiles and

fishes.^{64,65)} However, its biological role is still ambiguous. It is suggested that selenoneine possesses antioxidative activity because of its unique structure—a selenoketone group attached to an imidazole ring.⁶⁴⁾ Further, as selenoneine was specifically detected in marine organisms, the antagonistic effect of selenoneine on inorganic and organic Hg compounds was speculated. However, at least *in vitro*, no direct interactions between inorganic or organic Hg and selenoneine were observed despite the fact that selenite directly interacted with Hg in the presence of glutathione (GSH).⁶⁶⁾ Marine organisms, in particular marine animals belonging to the higher hierarchy of the food chain, actually accumulate and detoxify Hg with Se. The effect of selenoneine on the detoxification of Hg compounds should be evaluated *in vivo* in future studies.

Recently, selenoneine and its methylated form, *i.e.*, Se-methylselenoneine, were detected in human blood cell lysate and urine by HPLC-ESI-hybrid linear ion trap-orbital ion trap MS.⁶⁷⁾ Selenoneine in those samples was initially derivatized with iodoacetamide prior to detection in order to mask the high reactivity of its selenoketone moiety. Although the biosynthetic pathway of selenoneine is still unclear, the authors postulated that selenoneine originated from the diet and was then methylated by methyltransferase because selenoneine appeared to be a predominant Se compound in the tissues of marine fishes; namely, the reactivity of selenoneine was weakened by a methyl group *in vivo*. This postulate seems to be in agreement with the hitherto accepted concept of Se detoxification *in vivo* because reactive Se moieties, such as an -SeH group, are actually masked with a methyl group, *e.g.*, SeMet, MeSeCys, and MeSeGalNAc, and then the masked Se compounds are excreted into urine. This result also indicates that selenoneine can react with Hg compounds *in vivo* due to its biological reactivity. Collectively, it would be exciting to explore the biological and toxicological significance of selenoneine in future studies.

3.4. SeMet Derivatives We have already reported that SeMet, which could not be discriminated from Met, was incorporated into proteins in an *in vitro* translation system using wheat germ extract (WGE). The fluorescence of green fluorescent protein (GFP) and the activity of JNK stimulatory phosphatase-1 (JSP-1) were not affected by the substitution of Met with SeMet by the *in vitro* translation.¹³⁾ On the other hand, although Met is used for not only protein synthesis but also metabolism into coenzymes, such as methylmethionine sulfonium (MeMet) and *S*-adenosylmethionine (AdoMet, SAM) in yeast, the metabolism of SeMet into such coenzymes in higher eukaryotes, *i.e.*, plants and animals, has yet to be revealed. Hence, we intended to reveal the biological transformation of SeMet into SeMet derivatives in a model system of higher eukaryotes *in vitro*, *i.e.*, WGE, by identifying Se-containing molecules with speciation analyses using HPLC-ICP-MS and HPLC-ESI-MS-MS.⁶⁸⁾

Although three unknown Se peaks were detected by HPLC-ICP-MS measurement of the reaction mixture of WGE for *in vitro* metabolism of SeMet (Fig. 3a), Se standards having retention times that matched those of the three Se compounds were not available. Thus, these unknown selenometabolites were subjected to HPLC-ESI-MS-MS. Three groups of signals consisting of Se isotopes were detected at *m/z* 212, 433, and 477 as ⁸⁰Se (Figs. 3b–d). It has already been reported that the Se-containing compound in plant samples

Fig. 3. Speciation and Identification of Selenomethionine Metabolites

Elution profile of Se in the reaction mixture of wheat germ extract for the *in vitro* metabolism of SeMet (a). Absorbance at 254 nm (panel a, upper) and Se distribution at m/z 80 (panel b, lower) were measured. ESI-MS spectra of unknown Se compounds in the eluate of the reaction mixture of wheat germ extract for the *in vitro* metabolism of SeMet (b-d).

showed m/z 212 as its molecular ion peak,^{69,70} i.e., MeSeMet (Fig. 1h) was detected as $[M]^+$. Indeed, the fragment ions originating from m/z 212 could be assigned to MeSeMet. It has been reported that MeSeMet was further converted into the volatile Se compound, dimethylselenide, *via* dimethylselenoniopropionate to excrete the non-essential element, Se, in plants.^{71,72} Meanwhile, the fragment ions originating from m/z 433 and 447 indicated that the precursor ions were $[M+H]^+$ of *Se*-adenosylselenohomocysteine (AdoSeHcy, Fig. 1i) and $[M]^+$ of *Se*-adenosylselenomethionine (AdoSeMet, Fig. 1j). This result demonstrated for the first time that AdoSeMet is a metabolite of SeMet in a plant sample by direct measurements with HPLC-ESI-MS-MS. In addition, it was suggested that a trace amount of AdoSeMet also existed in rabbit reticulocyte lysate, a mammalian *in vitro* translation system, because the transformation of AdoSeMet from SeMet was completely inhibited by (2*S*)-2-amino-4,5-epoxypentanoic acid (AEPa), a potent inhibitor of AdoMet synthetase. Together, the results suggested that SeMet and Met shared a common metabolic pathway, namely, SeMet was not only incorporated into proteins in place of Met but also metabolized to AdoSeMet in higher eukaryotes.

4. CONCLUSION AND PERSPECTIVES

The evolution of instruments for speciation, particularly for the identification of target selenometabolites in trace amounts in complex matrices, has contributed immensely to the exploration of selenometabolomes. Some key selenometabolites,

such as selenosugars, selenoneine, and AdoSeMet, were identified with those instruments. However, several unanswered questions remain. For instance, why do animals excrete Se as selenosugars? What enzyme(s) participates in the biosynthesis of selenosugars? What is the biological role of selenoneine? To answer these questions, complementary and/or combined use of speciation and molecular biological techniques would be necessary. Today, the highly sensitive and robust detector, ICP-MS, is available, which is able to detect novel yet ultra-trace amounts of selenometabolites in diverse biological samples. Some important metabolic intermediates of Se, *e.g.*, selenophosphate and adenosine 5'-phosphoselenate (APSe), are an enigma for speciation chemists. Although their biological significance has been clearly established,^{73–76} their existence in *in vivo* samples has not been proved by speciation using conventional elemental or molecular MS. Hence, selenophosphate and APSe are the future targets of speciation chemists. In addition to further improving the detection limits of elemental and molecular MS, the techniques for sample preparation, fractionation, and separation of trace and fragile samples should be enhanced as well.

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