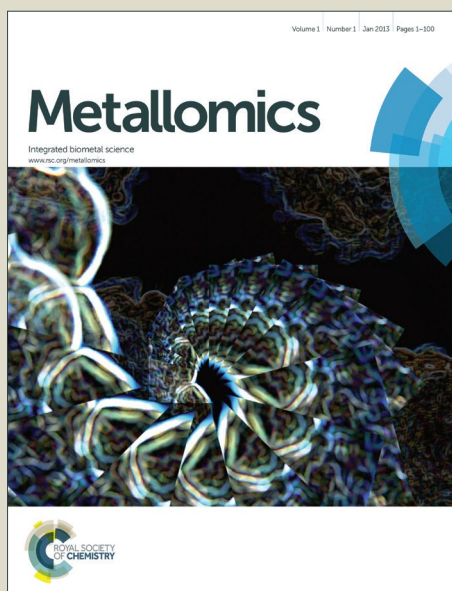


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5 **Comparison of metabolism of inorganic and organic selenium species between two**
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7 **selenium accumulator plants, garlic and Indian mustard**
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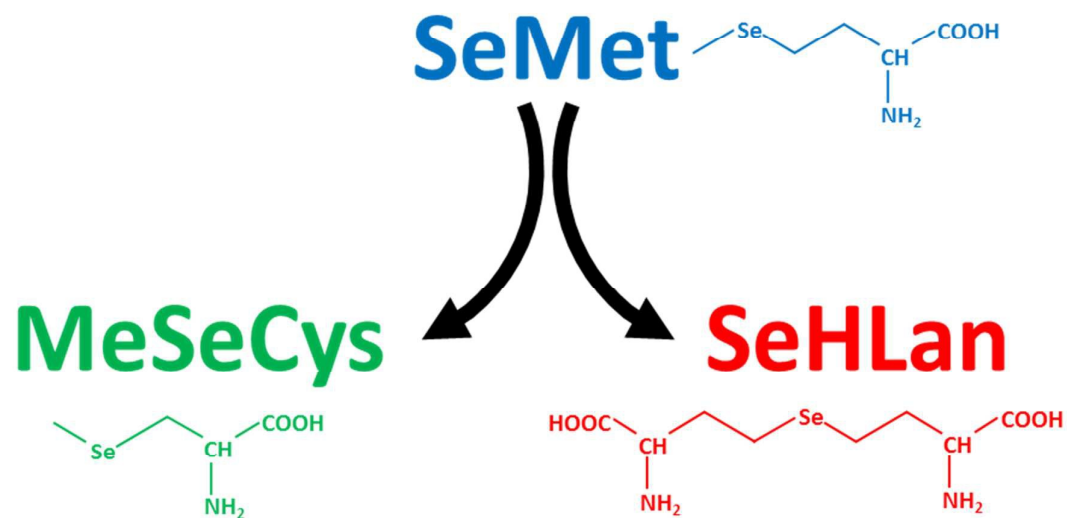
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Table of contents entry



Selenomethionine (SeMet) can be metabolized into other selenoamino acids such as *Se*-methylselenocysteine and selenohomolanthionine in selenium-accumulator plants.

ABSTRACT

The metabolism of selenomethionine (SeMet) in two major selenium (Se) accumulator plants, garlic and Indian mustard, was compared to that of stable isotope labeled selenate. Indian mustard more efficiently transported Se from roots to leaves than garlic. In addition, Indian mustard accumulated larger amounts of Se than garlic. γ -Glutamyl-Se-methylselenocysteine (γ -GluMeSeCys) and Se-methylselenocysteine (MeSeCys) were the common metabolites of selenate and SeMet in garlic and Indian mustard. Indian mustard had a specific metabolic pathway to selenohomolanthionine (SeHLan) from both inorganic and organic Se species. SeMet was a more effective fertilizer for cultivating Se-enriched plants than selenate in terms of the production of selenoamino acids.

Keywords: selenium, garlic, Indian mustard, speciation, selenate, selenomethionine, stable isotope

Significance to metalloomics

Selenium (Se) is an essential element in animals but not plants. Nevertheless, Se metabolism in plants is an exciting topic because Se metabolites in plants are beneficial to human health. Considering the environmental circulation of Se, plants may be exposed to not only inorganic but also organic Se species, particularly, selenomethionine (SeMet), a naturally occurring selenoamino acid. In this study, the metabolism of SeMet in two major Se accumulator plants, garlic and Indian mustard, was compared to that of selenate. As a result, we clearly showed the differences in Se metabolism between SeMet and selenate in the plants.

INTRODUCTION

Selenium (Se) is a trace essential element in animals, and 25 genes encoding selenoproteins have been found in human genome.¹ It has been proposed that Se reduces the risk of cancer and cardiovascular diseases.^{2,3} Meat and seafood are the main sources of Se. However, some vegetables can also be an appropriate Se source. Although Se is not an essential element in plants, Liliaceae family plants, such as garlic, onion, and leek, and Brassicaceae family plants, such as Indian mustard, are well-known Se accumulators.⁴ These plants have a large metabolic capacity for sulfur. As Se shares the same metabolic pathway with sulfur, which belongs to the same group in the periodic table as Se, the plants can produce several kinds of Se-containing amino acids (selenoamino acids) as secondary metabolites. For instance, *Se*-methylselenocysteine (MeSeCys), γ -glutamyl-*Se*-methylselenocysteine (γ -GluMeSeCys), and selenohomolanthionine (SeHLan) are produced in the plants via the sulfur assimilation pathway.⁵⁻⁷ These selenoamino acids are reported to be effective against tumors and sepsis.^{8,9} Therefore, selenized vegetables can function as chemopreventive or medicinal foods.

In a plethora of past studies, inorganic Se compounds, such as selenate and selenite, have been used for growing selenized vegetables because massive amounts of inorganic Se compounds are more easily available than organic Se compounds for the purpose of fortification.^{10,11} Microorganisms, in particular, fungi, are well known as Se accumulators.^{12,13} Contrary to Se accumulator plants, fungi accumulate Se as selenomethionine (SeMet).^{12,14} Hence, it can be assumed that plants are exposed to not only inorganic but also organic Se species in the ecosystem.

The bioavailability as well as the pharmacological and toxicological effects of Se in animals markedly depends on the chemical form of ingested Se. In plants, Se metabolism could also depend on the chemical form of fortified Se. Indeed, some differences in

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3 absorption, translocation, and metabolism by chemical form were reported.¹⁵⁻¹⁷ However,
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5 those differences were evaluated between inorganic species, i.e., selenite and selenate. At
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7 present, very few reports addressing Se metabolism in plants fortified with organic Se species
8
9 are available.¹⁸
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11 In this study, we aimed to reveal Se metabolism in two Se accumulator plants, *Allium*
12 *sativum* (garlic) and *Brassica juncea* (Indian mustard), exposed to SeMet. We used stable
13
14 isotope [⁷⁶Se]-labeled selenate for simultaneous exposure with non-labeled SeMet. This
15
16 experimental design allowed us to discuss the metabolism of SeMet in the plants by precise
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18 comparison to the metabolism of selenate. We also discussed differences in Se metabolism
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20 between garlic and Indian mustard.
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27 MATERIALS AND METHODS

28 *Chemicals*

29
30 Sodium selenate, glutathione reduced form (GSH), nitric acid, and ammonium
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32 acetate were purchased from Wako (Osaka, Japan). *Se*-L-methylselenocysteine (MeSeCys),
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34 L-selenomethionine (SeMet), and γ -glutamyl-*Se*-L-methylselenocysteine (γ -GluMeSeCys)
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36 were purchased from Acros Organics (Geel, Belgium), Sigma (St. Louis, MO, USA), and
37
38 Pharmase (Austin, TX, USA), respectively. Selenohomolanthionine (SeHLan) was
39
40 synthesized in our laboratory by a previously reported method.⁶ The metallic form of ⁷⁶Se
41
42 (99.9% enriched) was purchased from Isoflex USA (San Francisco, CA, USA). ⁷⁶Se-enriched
43
44 selenate was prepared by oxidation of ⁷⁶Se-enriched metallic Se. Metallic Se was oxidized by
45
46 dissolving in concentrated metal-free nitric acid containing hydrogen peroxide (H₂O₂:conc.
47
48 HNO₃, 4:1 v/v) and subsequently neutralizing with 1 M sodium hydroxide. Otsuka salt
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50 mixture-A for preparation of cultivation medium was purchased from Otsuka Agri Techno Co.,
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52 Ltd. (Tokyo, Japan). Deuterium gas (D₂, >95%) was purchased from Showa Denko (Tokyo,
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3 Japan). All reagents used in this study were of the highest or analytical grade. Deionized
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5 water (18.3 M Ω cm) was used throughout.
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8 9 ***Plant growth and sample preparation***

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11 *Brassica juncea* (Indian mustard) seeds and *Allium sativum* (garlic) bulbs were
12
13 purchased from Sakata (Kanagawa, Japan) and Sawada Farm, Inc. (Aomori, Japan),
14
15 respectively. The seeds and the bulbs were germinated and hydroponically grown in
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17 cultivation medium in a growth chamber (LH-55-RDS; Nihonkikai, Osaka, Japan) with a
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19 photoperiod of 14 h light (8000 lux)/10 h dark at 25.0 °C for 30 and 14 days for Indian
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21 mustard and garlic, respectively. The cultivation medium was sterilized prior to use. Then, the
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23 plants were exposed to medium containing ⁷⁶Se-enriched selenate and SeMet at the
24
25 concentration of 30 $\mu\text{mol L}^{-1}$ for 7 days. After the exposure, the plants were immediately
26
27 harvested, gently washed with deionized water, and divided into roots, leaves, and bulbs
28
29 (garlic only). Each part was lyophilized and milled to obtain homogenized powder. A 0.1 g
30
31 portion of the lyophilized root powder was incubated in 1.0 mL of deionized water for 1 h at
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33 room temperature. Water extracts were obtained by ultracentrifugation of the mixture at
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35 105,000 g for 60 min.
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43 ***Determination of Se concentration***

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45 The lyophilized root powder (40 mg) and the water extract (100 μL) were
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47 wet-ashed with 200 μL of nitric acid overnight. The ashed samples were diluted with
48
49 deionized water, and Se concentration in each sample was determined by ICP-MS (Agilent
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51 7500ce, Agilent Technologies, Hachioji, Japan) equipped with an octopole reaction system
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53 (ORS) at m/z 76 and 77, with m/z 128 for tellurium as the internal standard. D₂ gas was used
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55 at the flow rate of 2.0 mL min⁻¹.¹⁹ Mass calibration and parameter optimization for ICP-MS
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3 were performed daily using a tuning solution containing 10 ng mL⁻¹ each of lithium, yttrium,
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5 cerium, and thallium (Agilent Technologies) prior to use. The concentration of each Se
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7 isotope was determined by an external calibration method. The concentration of Se
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9 originating from selenate was calculated from the signal intensity obtained by the following
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11 equation: [signal intensity of Se originating from selenate] = [total signal intensities of ⁷⁶Se] –
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13 [signal intensity of ⁷⁷Se from SeMet] x (0.0936/0.0763), whereas the concentration of Se
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15 originating from SeMet was calculated from the signal intensity at *m/z* 77. To validate our Se
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17 measurement, dogfish muscle (DORM2, National Research Council, Ottawa, Canada) was
18
19 used as standard reference material (SRM). We obtained 102.9 ± 8.6% to the certified value
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21 of the SRM.
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25 Data are presented as means ± S.D. Statistical analysis included the one-way
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27 analysis of variance followed by the Student's *t*-test. The level of significance was set at *p* <
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29 0.05 was indicated by an asterisk (*).
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34 ***Speciation analysis of Se in sample by HPLC-ICP-MS***

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36 A 20 µL aliquot of the water extract and 1.0 µg Se mL⁻¹ of the standard solution of
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38 selenate, SeMet, MeSeCys, SeHLan, and γ-GluMeSeCys were applied to an HPLC coupled
39
40 with the ICP-MS to analyze the distribution of Se. The HPLC system (Prominence, Shimadzu,
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42 Kyoto, Japan) consisted of an on-line degasser, an HPLC pump, a Rheodyne six-port injector,
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44 and a column. The eluate was directly introduced into the nebulizer of the ICP-MS to detect
45
46 Se. A multi-mode size exclusion column (Shodex Asahipak GS-320HQ, 7.5 i.d. x 300 mm
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48 with a guard column, 7.5 i.d. x 75 mm, Showa Denko, Tokyo) was mainly used in this study.
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50 This column was eluted with 50 mM ammonium acetate, pH 6.5, at the flow rate of 0.6 mL
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52 min⁻¹. A sample of plant exposed to ⁷⁶Se-enriched selenate was detected at *m/z* 76 and 77
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54 under the D₂ reaction mode, and the elution profile of Se originating in ⁷⁶Se-enriched selenate
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3 was depicted by the calculation mentioned above. A sample of plant exposed to SeMet was
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5 detected at m/z 82 without a reaction/collision mode. Triplicate data of the Se distribution in
6
7 the extracts were obtained, and typical one of them was shown.
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10 11 12 **RESULTS AND DISCUSSION**

13 14 **Comparison of Se accumulation in garlic and Indian mustard exposed to selenate and** 15 16 **SeMet**

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18 Se concentration in the roots was far higher than those in the other parts, such as
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20 bulbs and leaves, and there were no apparent differences between inorganic (selenate) and
21
22 organic (SeMet) Se concentrations in the roots, bulbs, and leaves of garlic (Fig. 1a). On the
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24 other hand, the difference in Se concentrations between the roots and leaves in Indian mustard
25
26 was not as large as that observed in garlic. The concentration of Se originating from SeMet
27
28 was significantly higher than that originating from selenate in the roots, but tended to be
29
30 lower than that originating from selenate in the leaves (Fig. 1b). Regardless of plant part, Se
31
32 concentrations in Indian mustard were higher than those in garlic. Taking biomass into
33
34 consideration, Se was found to accumulate more in Indian mustard than in garlic (Table 1).
35
36 These results indicate that Indian mustard has a larger accumulation capacity for Se than
37
38 garlic. In particular, the leaf accumulation capacity for Se is larger in Indian mustard than in
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40 garlic. However, the accumulation capacity for Se was species-specific in Indian mustard, that
41
42 is, SeMet was more preferably accumulated in the roots than selenate. It was reported that
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44 selenate was incorporated via sulfate transporter(s),^{20, 21} and SeMet was incorporated via
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46 amino acid transporter(s).^{22, 23} It is reported that Se is transformed into volatile species, such
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48 as dimethyldiselenide and dimethylselenide, in Se accumulator plants.^{24, 25} SeMet is
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50 efficiently transformed into volatile Se species in the leaves; thus, Se concentration
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52 originating from SeMet is higher than that from selenate in Indian mustard. In addition to the
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3 accumulation capacity for Se, the metabolism of selenate and SeMet in garlic and Indian
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5 mustard was also evaluated by speciation analyses.
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9 10 **Metabolism of Se from selenate and SeMet in garlic and Indian mustard**

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12 Three apparent peaks of Se originating from SeMet were detected in the water extract
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14 of garlic leaves at the retention times of 16.7 (**2**), 19.9 (**4**), and 20.9 (**5**) min (Fig. 2a). The
15
16 retention times matched those of authentic standards *Se*-methylselenocysteine (MeSeCys),
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18 and SeMet. The structures and retention times of γ -glutamyl-*Se*-methylselenocysteine
19
20 (γ -GluMeSeCys) found in garlic were confirmed by electrospray ionization mass
21
22 spectrometry (ESI-MS) in our previous study.⁷ Three peaks of Se originating from selenate
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24 were detected at the retention times of 15.6 (**1**), 16.7 (**2**), and 19.9 (**4**) min (Fig. 2b). The peak
25
26 at the retention time of 15.6 min corresponded to selenate; this was determined on the basis of
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28 the retention time that matched that of selenate authentic standard. The elution profiles of
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30 authentic standards of selenate, MeSeCys, SeMet and SeHLan were shown in Fig. S1. Hence,
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32 SeMet and selenate were commonly metabolized to γ -GluMeSeCys and MeSeCys. Contrary
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34 to garlic, an additional peak of Se originating from both SeMet and selenate was detected in
35
36 the water extracts of Indian mustard leaves at the retention time of 18.0 (**3**) min (Figs. 2c and
37
38 2d). The peak was assigned to selenohomolanthionine (SeHLan) because its retention time
39
40 matched that of SeHLan authentic standard (Fig. S1), and the structure of SeHLan found in a
41
42 *Brassicaceae* family plant was confirmed by ESI-MS in our previous literature.⁶ SeHLan is a
43
44 specific Se metabolite of Indian mustard and can be biosynthesized from both organic
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46 (SeMet) and inorganic (selenate) Se compounds. SeHLan was firstly identified in pungent
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48 radish, *Raphanus sativus*, a *Brassicaceae* family plant.⁶ This suggests that Indian mustard
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50 shows a specific metabolic pattern to SeHLan from SeMet and selenate. It is speculated that
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52 SeMet is demethylated to form selenohomocysteine (SeHCys), and SeHCys is reacted with
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3 *O*-acetylhomoserine for the biosynthesis of SeHLan.²⁶ In our previous work, we found that a
4 *Se*-methylselenyl moiety (CH₃Se-) was firstly cleaved from SeMet and then conjugated with
5 glutathione (GSH) to form GSSeCH₃ in another Se accumulator Brassicaceae family plant,
6
7 *Brassica rapa* var. *perviridis*.¹⁸ As an alternative pathway, SeMet is transformed into
8 MeSeCys via selenohomocysteine (SeHCys), selenocystathionine, and selenocysteine
9 (SeCys). This pathway is known as the reverse transselenation pathway.²⁶ In this pathway,
10 γ -GluMeSeCys is recognized as the final metabolite. Contrary to that pathway,
11 *S*-alkylcysteine is transformed from Cys via GSH and γ -GluCys via sulfur metabolism,²⁷ and
12 Se can share the same metabolic pathway with sulfur. If Se is metabolized in the pathway,
13 MeSeCys is the final metabolite. At present, it is difficult to decide which metabolite,
14 γ -GluMeSeCys or MeSeCys, is the final metabolite of Se in the plants. The peak
15 corresponding to selenate was detected in Indian mustard at *m/z* 76 (Fig. 2c). This seems to
16 originate not from SeMet but from [⁷⁶Se]-selenate contaminating [⁷⁷Se]-selenate. The
17 enrichment of [⁷⁶Se]-selenate used in this study was 99.9%. Because the signal intensity of
18 selenate at *m/z* 76 was one thousand times higher than that at *m/z* 77, the peak of selenate at
19 *m/z* 77 could originate from the contaminants (0.01%) of [⁷⁶Se]-selenate.
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38 Although Se concentrations in the roots were higher than those in the leaves, the
39 metabolisms quite resembled each other except the amount of γ -GluMeSeCys (Fig. 3), namely,
40 MeSeCys and γ -GluMeSeCys originating from SeMet and selenate were detected in garlic
41 and Indian mustard. The relative amount of γ -GluMeSeCys to that of MeSeCys in the roots
42 was smaller than that in the leaves. In addition, SeHLan was specifically detected in the water
43 extracts of Indian mustard roots (Figs. 3c and 3d). The peak corresponding to SeMet was
44 detected in the water extracts of Indian mustard roots at *m/z* 77. These indicate that selenate is
45 metabolized into SeCys, and a part of SeCys is transformed into metabolic intermediates,
46 such as selenocystathionine and SeHCys, via the forward transselenation pathway. Then,
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3 SeHCys is converted into SeHLan and SeMet. The forward transselenation pathway could be
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5 specific to Indian mustard.
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8 SeMet was more efficiently metabolized into selenoamino acid derivatives, such as
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10 γ -GluMeSeCys, MeSeCys, and SeHLan, than selenate in garlic and Indian mustard. It has
11
12 been reported that γ -GluMeSeCys and MeSeCys have anticarcinogenic and antitumor
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14 activities.² SeMet was also expected to be an anticarcinogenic agent; an intervention study,
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16 however, revealed that the effects of SeMet was ambiguous²⁸ A source of SeMet supplement
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18 is biologically synthesized in yeast. The results of this study show that pharmaceutically
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20 valuable Se species, such as γ -GluMeSeCys and MeSeCys, are efficiently biosynthesized in
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22 Se accumulator plants fortified with SeMet.
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26 Selenate concentration in the cultivation medium was decreased after 7 days due to
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28 absorption by the plant (Figs. 4a and b). No SeMet was detected in the cultivation medium
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30 after 7 days, although two peaks were detected at the retention times of 15.6 (**1**) and 17.1 min
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32 (Figs. 4c and 4d). Although the former one was assignable to selenate by its chromatographic
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34 behavior, the latter one was not identified. We also evaluated the stability of SeMet in the
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36 cultivation medium without any plants under the cultivation conditions. SeMet was stable for
37
38 7 days under the cultivation conditions (data not shown). This suggests that SeMet is not
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40 decomposed chemically or biologically by microorganisms in the cultivation medium, namely,
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42 selenate and the unidentified Se compound seem to be the products of SeMet degradation by
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44 the roots of Indian mustard or Se excreted by the roots. By clarifying the unknown Se
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46 metabolite, the precise metabolism of Se would be revealed.
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50 In conclusion, two major Se accumulator plants showed different Se accumulation
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52 characteristics. The proposed metabolic pathway was depicted in Fig. 5. Indian mustard more
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54 efficiently transported Se from the roots to the leaves than garlic. In addition, Indian mustard
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56 accumulated larger amounts of Se than garlic. γ -GluMeSeCys and MeSeCys were the
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3 common metabolites of selenate and SeMet in garlic and Indian mustard. Indian mustard
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5 showed a specific metabolic pattern to SeHLan from both inorganic and organic Se species.
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7 SeMet is a more effective fertilizer for cultivating Se-enriched plants than selenate in terms of
8
9 the production of selenoamino acids.
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23 24 25 **Notes**

26
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Table 1. Se amounts in garlic and Indian mustard exposed to [⁷⁶Se]-enriched selenate and SeMet.

	Se amount (μg)	
	Garlic	Indian mustard
selenate origin	35 ± 18	208 ± 10
SeMet origin	42 ± 18	113 ± 1.0

Garlic and Indian mustard were cultivated in medium containing [⁷⁶Se]-enriched selenate and SeMet at the concentration of 30 μM for 7 days. Values are means ± standard deviations for 3 samples.

FIGURE LEGENDS

Figure 1. Se concentrations in roots, bulbs, and leaves of garlic and Indian mustard exposed to [⁷⁶Se]-enriched selenate and SeMet. Garlic (a) and Indian mustard (b) were cultivated in medium containing [⁷⁶Se]-enriched selenate and SeMet at the concentration of 30 μM for 7 days. Columns and bars indicate means ± standard deviations for 3 samples.

Figure 2. Elution profiles of Se in the water extracts of leaves of garlic and Indian mustard exposed to [⁷⁶Se]-enriched selenate and SeMet. Garlic (a and b) and Indian mustard (c and d) were hydroponically cultivated and then exposed to [⁷⁶Se]-enriched selenate and SeMet at the concentration of 30 μM for 7 days. A 20 μL aliquot of the standard solution of [⁷⁶Se]-enriched selenate and SeMet (each 1.0 μg Se mL⁻¹) and the water extract of leaves were applied to HPLC equipped with a multi-mode size exclusion HPLC column (GS-320HQ). The column was eluted with 50 mM ammonium acetate, pH 6.5, at the flow rate of 0.6 mL min⁻¹. The eluate was directly introduced into the nebulizer of ICP-MS to detect Se at *m/z* 76 and 77 for monitoring of Se originating in selenate (b and d) and SeMet (a and c), respectively, in the D₂ reaction mode. **1**: selenate, **2**: γ-GluMeSeCys, **3**: SeHLan, **4**: MeSeCys, **5**: SeMet.

Figure 3. Elution profiles of Se in the water extracts of roots of garlic and Indian mustard exposed to [⁷⁶Se]-enriched selenate and SeMet. Garlic (a and b) and Indian mustard (c and d) were hydroponically cultivated and then exposed to [⁷⁶Se]-enriched selenate and SeMet at the concentration of 30 μM for 7 days. A

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3 20 μL aliquot of the standard solution of [^{76}Se]-enriched selenate and SeMet
4 (each 1.0 $\mu\text{g Se mL}^{-1}$) and the water extract of roots were applied to HPLC
5 equipped with a multi-mode size exclusion HPLC column (GS-320HQ). The
6 column was eluted with 50 mM ammonium acetate, pH 6.5, at the flow rate of
7 0.6 mL min^{-1} . The eluate was directly introduced into the nebulizer of ICP-MS to
8 detect Se at m/z 76 and 77 for monitoring Se originating in selenate (b and d)
9 and SeMet (a and c), respectively, in the D_2 reaction mode. 1: selenate, 2:
10 γ -GluMeSeCys, 3: SeHLan, 4: MeSeCys, 5: SeMet.
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23 Figure 4. Time-dependent changes in elution profiles of Se in cultivation medium
24 containing [^{76}Se]-enriched selenate and SeMet. A small portion of the cultivation
25 medium of Indian mustard was collected on day 0 (a and c) and day 7 (b and d).
26 A 20 μL aliquot of the medium containing [^{76}Se]-enriched selenate and SeMet
27 was applied to HPLC equipped with a multi-mode size exclusion HPLC column
28 (GS-320HQ). The column was eluted with 50 mM ammonium acetate, pH 6.5, at
29 the flow rate of 0.6 mL min^{-1} . The eluate was directly introduced into the
30 nebulizer of the ICP-MS to detect Se at m/z 76 and 77 for monitoring of Se
31 originating in selenate (a and b) and SeMet (c and d), respectively, in the D_2
32 reaction mode. 1: selenate, 5: SeMet.
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47 Figure 5. Proposed metabolic pathways of SeMet and selenate in garlic and Indian
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Figure 1

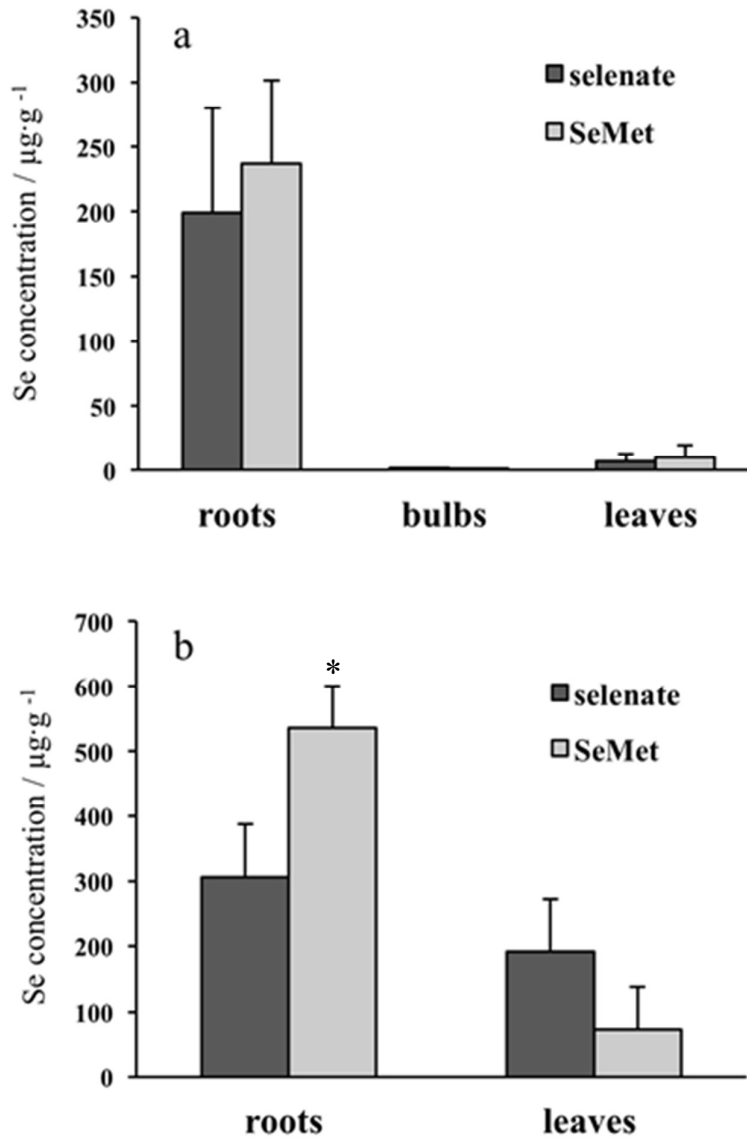


Figure 2

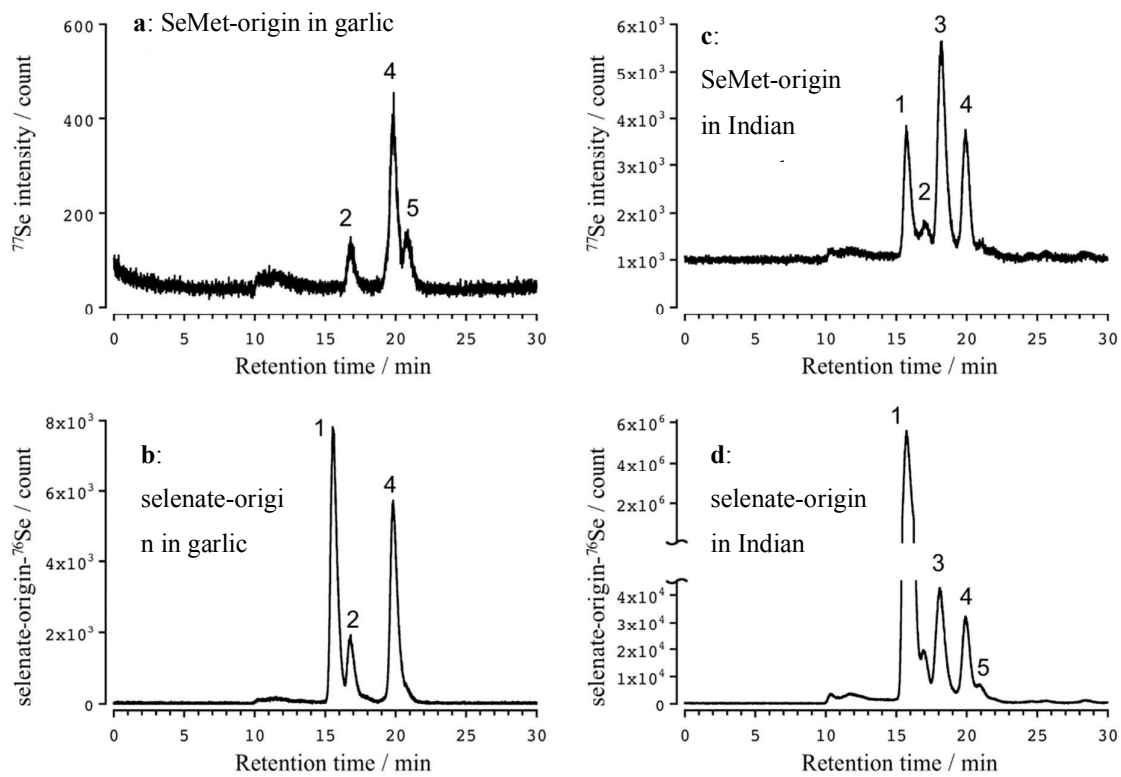


Figure 3

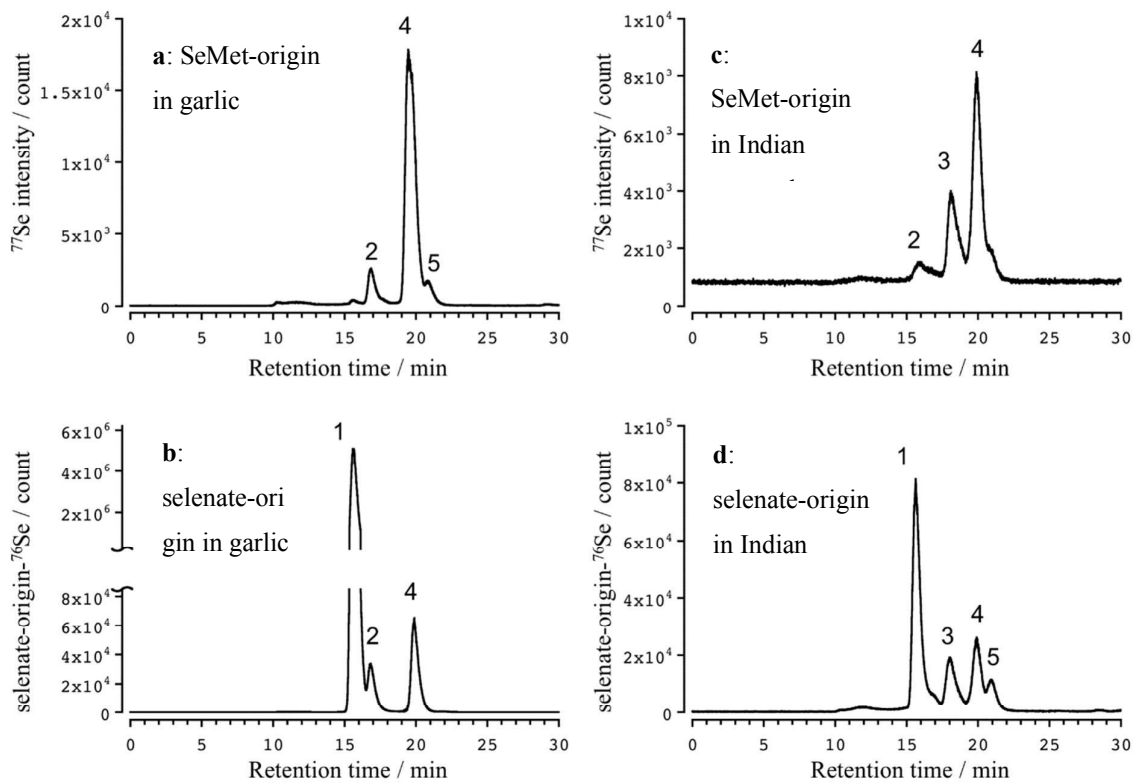


Figure 4

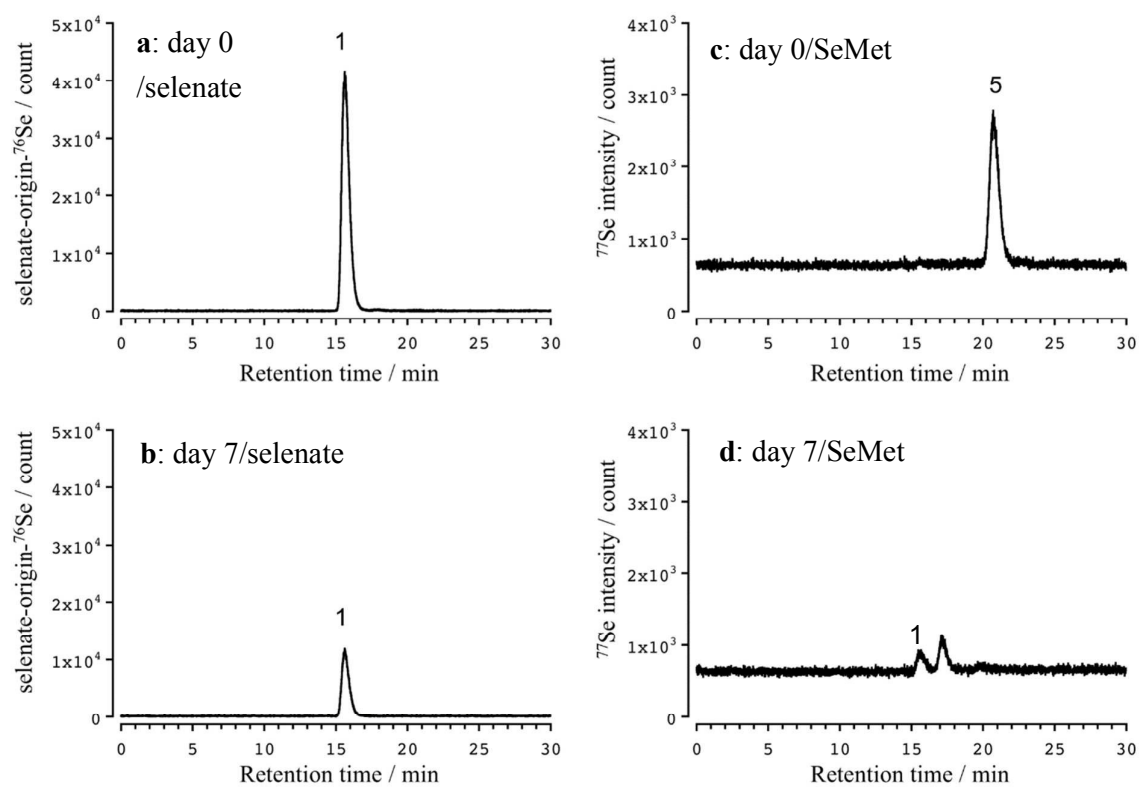
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Figure 5

