Regular Article

Characterization of Selenium Species in the Shijimi Clam

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Selenium is an essential trace element for humans and animals. Fish and shellfish are known to be rich in selenium and suppose to be an effective selenium source. In this study, we characterized the selenium species in the Shijimi clam (*Corbicula japonica*), which is a typical clam eaten in Japan. The Shijimi clam contains a relatively high concentration of selenium (3.5 μg-selenium/g-dry Shijimi). Approximately 30% of the total selenium in the Shijimi clam meat was extractable with water, while selenium in the Shijimi clam was hardly extracted with ethanol, chloroform and hexane. Based on an ultrafiltration study, the molecular mass of the major selenium species in the Shijimi water-extract was estimated to be less than 5000. Because amphoteric selenium species were contained in the Shijimi water-extract, which was indicated by ion-exchange chromatographic separation, an ion-pair reagent was utilized to extract the ionic selenium species into an organic solvent. A matrix assisted laser desorption ionization (MALDI) time of flight (TOF)-mass spectrometric analysis revealed the selenium isotopic pattern involving one selenium atom in a molecule with the ^{80}Se molecular ion peak at *m*/*z* 534. This selenium species was mainly found in the visceral part of the Shijimi clam by imaging mass spectrometry.

Key words selenium; seafood; imaging mass spectrometry

Selenium is an essential trace element for humans and animals.[1)] This element plays important roles as the active center of the selenocysteine (SeCys)-containing proteins (selenoproteins) in biological systems.[2)] Glutathione peroxidases (GPxs), the most abundant selenoproteins, catalyze the reduction of hydrogen peroxide and organic hydroperoxides into water and the corresponding alcohols using glutathione. The plasma GPx activity is commonly used to evaluate the individual selenium status.[3)] The WHO recommends taking 40 μg of selenium per day for adult men, which is the amount to achieve two-thirds of the maximum GPx activity of the plasma.[4)] The recommended dietary allowance for adults in Japan is 20–25 μg/d, and the actual amount of the mean selenium intake is reported to be approximately 100 μg/d in Japan.[5)] Cereals, meats, fishes and eggs are known to be rich in selenium.[6,7)] Miyazaki *et al*. reported that fish is the major selenium source for the Japanese population (more than 50% of daily intake).[5)] The bioavailability of selenium in foodstuffs is dependent not only on the selenium content, but also on the chemical form of this element. There have been many reports about the selenium role in cancer prevention.[8)] On the contrary, a recent large-scale trial to investigate the effect on prostate cancer risk by selenium and vitamin E revealed that 200 μg/d of seleno-L-methionine (SeMet) did not reduce the cancer risk but raised the risk of type 2 diabetes.[9,10)] This may be partially attributed to the chemical form of the selenium being used. Therefore, a speciation analysis of selenium in foodstuffs and supplements has been performed in the past few decades. SeMet was identified as the major selenium species in selenium-enriched supplements or foodstuffs such as selenized yeast and mushrooms, and SeCys derivatives were detected in selenized garlic and onion.[11–13)] The major chemical form of selenium species in animal foodstuffs is suppose to be SeCys, though other unknown selenium species have been detected.[14–17)] Because of the difficulty in purification and subsequent enrichment of selenium species in selenium-unreinforced natural foodstuffs, the chemical property of selenium species in fish and shellfish still remains largely unknown. Comparison of chromatographic retention times with standard materials after chromatographic separations is an effective method for identifying selenium species. However, this methodology is hardly applicable for unknown selenium species because of the limited number of standard materials.

SeCys is referred as the '21st amino acid' and incorporated in selenoproteins by genetic code UGA with a specific selenocysteine insertion sequence (SECIS) in the 3′ untranslated region of the selenoprotein mRNA.[18)] The size of the selenoproteome, a set of selenoproteins in organisms, is variable and some organisms (fungi and higher plants) have no selenoproteins or SECIS. In 2003, Kryukov *et al*. revealed that the human selenoproteome was composed of 25 selenoproteins.[19)] Fish selenoproteomes contain nearly 40 selenoproteins and is among the largest known.[20)] Large selenoproteomes also occur in other aquatic organisms, such as green algae, fish and crustaceans, compared to terrestrial animals.[21)]

Various seafood stuffs are reported to contain high concentrations of selenium, for example, 3.9 μg/g in tuna, and 2.2 μg/g in oyster.[17,22)] Fish and shellfish are the major selenium sources for the Japanese population because of their higher selenium contents compared to plant foodstuffs and the higher consuming amount of seafood in the Japanese cuisine.[5)] We previously reported that selenium in a processed Japanese anchovy of 5–7 cm length (Niboshi) was effective to restore the selenium content in the liver and the hepatic GPx activity of dietary selenium deficient mice.[23)] Niboshi containing low-molecular-mass organoselenium compounds possessed a hydrophilic and/or amphoteric character.[24)] Small fish and clams are thought to be effective selenium sources because

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the whole body, not excluding the abdominal parts with a large portion of selenium, is utilized for the cuisine. In this study, we addressed the physic-chemical characterization of the water-extractable selenium species from the Shijimi clam (*Corbicula japonica* Prime, Corbiculidae) that is a typically eaten bivalve in Japan.

Experimental

Preparation of the Shijimi Sample and Its Water-Extract Fresh Shijimi clams were purchased at local grocery stores in Nagasaki and used immediately for the sample preparation. The edible meat of the fresh Shijimi clam was separated from the shell and washed with water (Fig. 1A). The meats were lyophilized by a VD-800F freeze dryer (Taitec Corp., Saitama, Japan), then ground using a food processor (Tescom Co., Ltd., Tokyo, Japan) (Fig. 1B). About 2 g of the processed meat sample was placed in a non-woven fabric bag and boiled in 20 mL of water for 2 h. After centrifugation (7500×***g***, 4°C), the obtained supernatant was filtered using a disk filter with the membrane pore size of 0.45 μm, and the Shijimi water-extract was prepared by making the final volume of 20 mL with solvents used for the extraction (Fig. 1C). A Milli-Q Biocel system (Millipore Corp., Billerica, MA, U.S.A.) was utilized to generate the water (>18 MΩ·cm), which is used throughout this study.

Determination of Selenium and Protein Concentrations The selenium concentration in the specimens was fluorometrically determined using 2,3-diaminonaphthalene (DAN, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) after acid digestion using a mixture of nitric acid and perchloric acid.[25] A FP-6600 fluorometer (Jasco Corporation, Tokyo, Japan) was used to measure the fluorescence intensity [Ex: 375 nm, Em: 520 nm, working concentration range: 1–1000 ngSe/sample (RSD <10%)]. A selenium standard solution (1000 mg/L, Kanto Chemical Co., Inc., Tokyo, Japan) was used to make the calibration curve (Fig. S1). The protein concentration in the water-extract was determined by the Lowry method using bovine serum albumin as the reference material to prepare the calibration curve.[26] The UV absorbance at 650 nm was measured by a V-660 UV-Visible spectrophotometer (Jasco Corporation).

Ultrafiltration The Shijimi water-extract was centrifuged at 7500×***g*** and 4°C in a centrifugal tube (Amicon Ultra-4, Merck Millipore, Darmstadt, Germany) with the membrane molecular mass cutoff (MMCO) of 5 or 30 kDa. After centrifugation, the selenium and protein concentrations in filtered extract were measured.

Chromatography Sephadex G-50 (fine, particle diameter: 20–80 μm, exclusion limit: 1.5–30 kDa, Amersham Biosciences, Uppsala, Sweden) was used for the gel permeation chromatography. The Sephadex G-50 was immersed in water for more than 12 h at 4°C before use. Ion-exchange chromatography was performed using the Q Sepharose and SP Sepharose (particle diameter: 45–165 μm, capacity: 0.18–0.25 mEq/mL, Sigma Co., St. Louis, MO, U.S.A.). Columns packed with these resins were connected to a PUMP 560 and a UV-detector prep·UV254 (Yamazen Corporation, Osaka, Japan). The eluents from the Sephadex G-50, Q Sepharose and SP Sepharose columns were collected every 5 min, then subjected to the selenium determination.

Ion-Pair Extraction The cationic ion-pair reagent, hexadecyltrimethylammonium chloride (HTAC, Tokyo Chemical Industry Co., Ltd.) was dissolved in water and combined with the Shijimi water-extract to make its final concentration 1 mM. After 5 extractions with chloroform, the obtained organic layer was concentrated *in vacuo* and stored in a desiccator.

Mass Spectrometry An Ultraflex TOF/TOF (Bruker Daltonics, Bremen, Germany) was employed for the matrix assisted laser desorption ionization (MALDI)-mass spectrometry using 2,5-dihydroxybenzoic acid (DHB) as the matrix to detect the selenium species from the Shijimi water-extract. Because selenium has 6 stable isotopes, the selenium species showed the characteristic isotopic pattern in their mass spectra. Mass spectra were acquired in the linear positive ion mode. The frozen Shijimi clam meat was cut into a 20-μm thick slice by a CM1950 cryostat (Leica Biosystems, Nussloch, Germany), then placed on an indium-tin oxide coated slide glass. Imaging mass spectrometry was carried out by an ultrafleXtreme (Bruker Daltonics) in the linear positive ion mode using DHB as the matrix.

Results and Discussion

Selenium Content in the Shijimi Clam and Its Extract The selenium concentrations in the lyophilized Shijimi clam meat and its water-extract were, respectively, 3.510±0.121 and 1.006±0.040 μg/g (Table 1). Moisture content of the fresh Shijimi clam meat was 79.5±0.66%, and selenium content of the Shijimi clam meat in wet state was calculated at 0.720 μg/g. This value is relatively higher than that of other foodstuffs.[6,7] The extraction rates into water were approximately 30% of the total selenium in the Shijimi clam, which is similar to those reported for other fish and shellfish such as oyster, mussel,

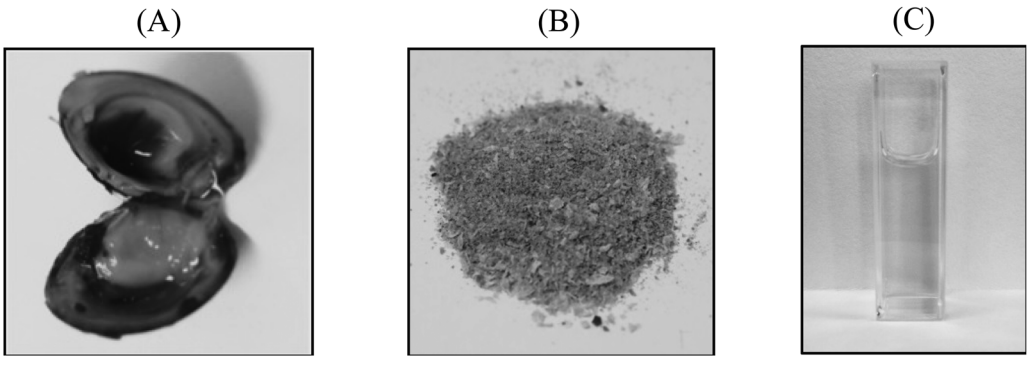

Fig. 1. Pictures of Shijimi Clam (A), Lyophilized and Ground Shijimi Clam Meat (B), and the Shijimi Water-Extract (C)

cod, herring, mackerel, tuna and krill.[14,15,17,22,27] No inorganic selenious acid was detected as the water-extract was directly determined with DAN that allows to generate the fluorescent piaselenol. The selenium in the Shijimi clam was unlikely to be present as an inorganic species. When the lyophilized clam meat was immersed in 20 mL of each organic solvent (ethanol, acetonitrile, chloroform, ethyl acetate and hexane) at 25°C for 5 h, the selenium extraction rates were less than 2%. This highly water-extractable selenium species is probably suitable to utilize for organisms and even smaller amount of selenium in the extract compared to enriched foodstuffs might be effective as selenium source because it is frequently eaten in Japanese cuisine. The protein concentration in the Shijimi water-extract was determined to be 51.72±4.028 mg/g-lyophilized Shijimi clam by the Lowry method.

Table 1. Selenium and Protein Concentrations in Shijimi Clam and Its Water-Extract[a)]

	Selenium (μg/g-lyophilised Shijimi)	Selenium extraction rate (%)[b)]	Protein (mg/g-lyophilised Shijimi)
Shijimi clam	3.510±0.121	—	—
Its water-extract	1.006±0.040[c)]	29.21±0.013	51.72±4.028[c)]

a) Values are mean±standard error, $n=4$–7. *b*) Selenium amount in lyophilized Shijimi clam meat used for extraction was defined as 100%. *c*) =(amount of each substance in the water-extract)/(weight of lyophilized Shijimi clam meat used for extraction).

Table 2. Ultrafiltration Rate of Selenium and Protein in the Shijimi Water-Extract[a)]

MMCO (kDa)	Selenium filtered (%)[b)]	Protein filtered (%)[b)]
5	82.94±2.379	48.31±1.262
30	103.1±4.219	77.20±1.983

a) Values are mean±standard error, $n=5$–6. *b*) Selenium and protein content in the Shijimi water-extract used for ultrafiltration was defined as 100%.

Molecular Mass Estimation of Selenium Species in the Shijimi Water-Extract The molecular mass distribution of the selenium species in the Shijimi water-extract was studied by ultrafiltration. Selenium in the Shijimi water-extract completely passed through the membrane with a molecular mass cutoff (MMCO) of 30 kDa, hence 82.9% of the selenium was filtered through a membrane with an MMCO of 5 kDa (Table 2). The Shijimi clam contains a significant amount of water-soluble selenium species with a molecular mass less than 5 kDa. On the other hand, 48.3 and 77.2% of protein in the Shijimi water-extract were filtered through the membranes with the MMCO of 5 and 30 kDa, respectively. Since the expression of selenoproteins in invertebrates including clams have been reported,[28,29] selenium species in the Shijimi water-extract also probably come from selenoproteins in the Shijimi clam. Considering that the molecular mass of the already known eukaryotic selenoproteins is higher than 9 kDa,[30] the selenium species with a molecular mass less than 5 kDa appeared not to be intact selenoproteins, but fragmented selenoproteins, low-molecular-mass selenium species such as selenoamino acids and/or their derivatives.

Ion-Exchange Chromatographic Separation of the Shijimi Water-Extract Ion exchange chromatographic separation was performed to elucidate the ionic character of the selenium species in the Shijimi water-extract. Because the molecular mass of major selenium species in the Shijimi water-extract was less than 5000, the Shijimi water-extract was subjected to the gel permeation chromatography to remove high-molecular mass compounds before the ion-exchange chromatographic separation. Fractions separated by gel permeation chromatography using a Sephadex G-50 fine (Fig. S2. fractions #

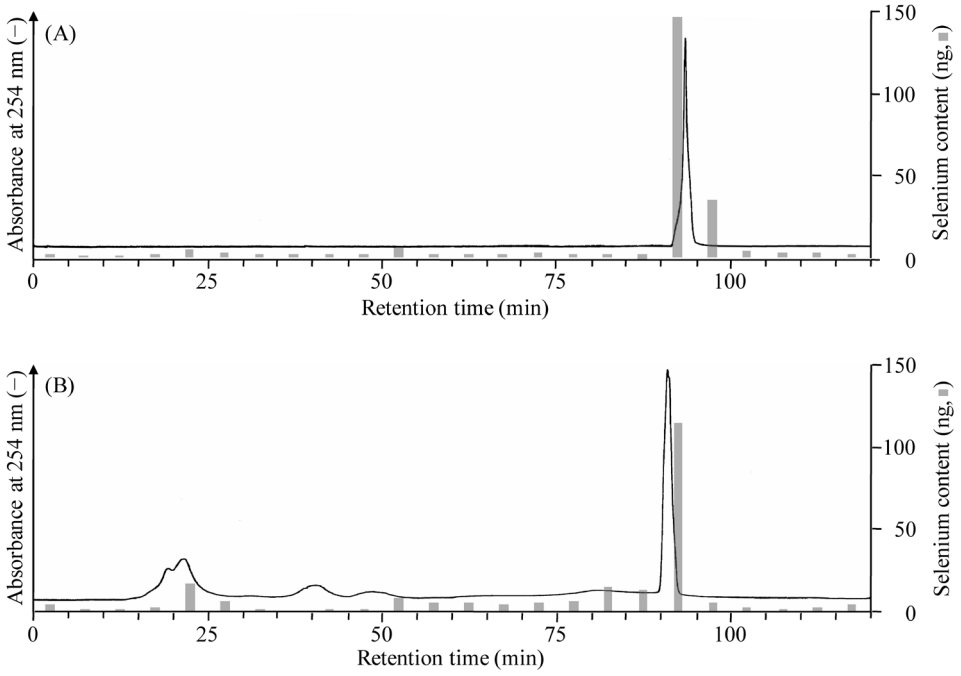

Fig. 2. Ion-Exchange Chromatographic Separation of the Shijimi Water-Extract on Q-Sepharose (A) and SP-Sepharose (B) Columns

Column dimension: 1.1 i.d. × 30 cm, Flow rate: 1.82 (A) and 1.84 (B) mL/min, Fraction volume: 9.1 (A) and 9.2 (B) mL, Injection sample volume: 0.25 mL, Mobile phase: 0–40 min; Milli-Q water, 40–120 min; 0.1 M HCl (A) and 0–40 min; Milli-Q water, 40–120 min; 0.1 M NaOH (B).

9–12 eluted at the retention time of 40–60 min, which contained 68.2% of the selenium eluted from the column) were collected and concentrated by lyophilization followed by application to the Q Sepharose column in the OH form [P−$CH_2N^+(CH_3)_3 \cdot OH^-$]. Under this separation condition, not only anionic compounds, but also amphoteric compounds, such as selenoamino acids and/or proteins, can be retained on the column because the high pH environment in the Q Sepharose column can promote the dissociation of acidic groups in the amphoteric compounds (Fig. S3, left half). The component species in the Shijimi water-extract, including the selenium species, were mostly retained on the Q Sepharose column and eluted with 0.1 M HCl (Fig. 2A). The fractions between the retention times 90−100 min contained 75.9% of the total selenium eluted from the Q Sepharose column. These results suggested that the selenium species in the water-extract mostly have anionic and/or amphoteric characteristics. The fraction obtained by gel permeation chromatography was further separated by cation-exchange chromatography using a SP Sepharose column in the H form [P−$(CH_2)_3SO_3^- \cdot H^+$]. During this separation, the SP Sepharose column can retain not only cationic compounds, but also amphoteric ones, because a low pH environment in the SP Sepharose column can promote the dissociation of basic groups of the amphoteric compounds (Fig. S3, right half). A peak at the retention time of 16–24 min suggested the involvement of anionic species and not nonionic species (Fig. 2B), because no peak was observed in the Q Sepharose chromatogram as shown in Fig. 2A. The amount of selenium in this fraction was 7.8% of the eluted selenium from the SP Sepharose column. The amount of selenium in the later peak eluted with 0.1 M NaOH was 53.4% of the total selenium eluted from the SP Sepharose column. The elution profile of the proteinous species in the Shijimi water-extract was also similar to that of selenium (Table 3). The results of the ion-exchange chromatography indicated that the major selenium species in the Shijimi water-extract was amphoteric such as an amino acid or protein. A small amount of anionic selenium species was also included.

Table 3. Selenium and Protein Contents in Fractions Separated by Ion-Exchange Chromatography[a)]

Q Sepharose	Selenium eluted (%)	Protein eluted (%)
Eluted with Milli-Q water (Cationic/Nonionic fraction)	9.50	4.42
Eluted with 0.1 M HCl (Anionic/Amphoteric fraction)	74.8	90.7
SP Sepharose	**Selenium eluted (%)**	**Protein eluted (%)**
Eluted with Milli-Q water (Anionic/Nonionic fraction)	12.0	4.10
Eluted with 0.1 M NaOH (Cationic/Amphoteric fraction)	71.6	74.5

a) Total amounts of selenium and protein applied to each column were defined as 100% (n=2).

Ion-Pair Extraction and Mass Spectrometric Analysis Since selenium species in the Shijimi water-extract possess hydrophilic, low-molecular-mass and ionic characteristics, it was seemingly difficult to separate these species from inorganic salts and amino acids in the extract. Several chromatographic techniques, such as reverse-phase chromatography, did not provide a successful separation of the selenium species in the Shijimi water-extract (data not shown). To separate the amphoteric and anionic selenium species, ion-pair extraction with a cationic reagent, hexadecyltrimethylammonium chloride (HTAC), was performed. The selenium extraction rate was 14.3±1.76% (n=5) of the total selenium in the Shijimi water-extract. The obtained chloroform layer after the ion-pair extraction was concentrated *in vacuo* and analyzed by MALDI mass spectrometry using DHB as a matrix. A distinctive selenium isotopic pattern with ^{80}Se at m/z 534 appeared in the obtained MS (Fig. 3B). This isotopic pattern was almost identical to that of SeMet that has one selenium atom

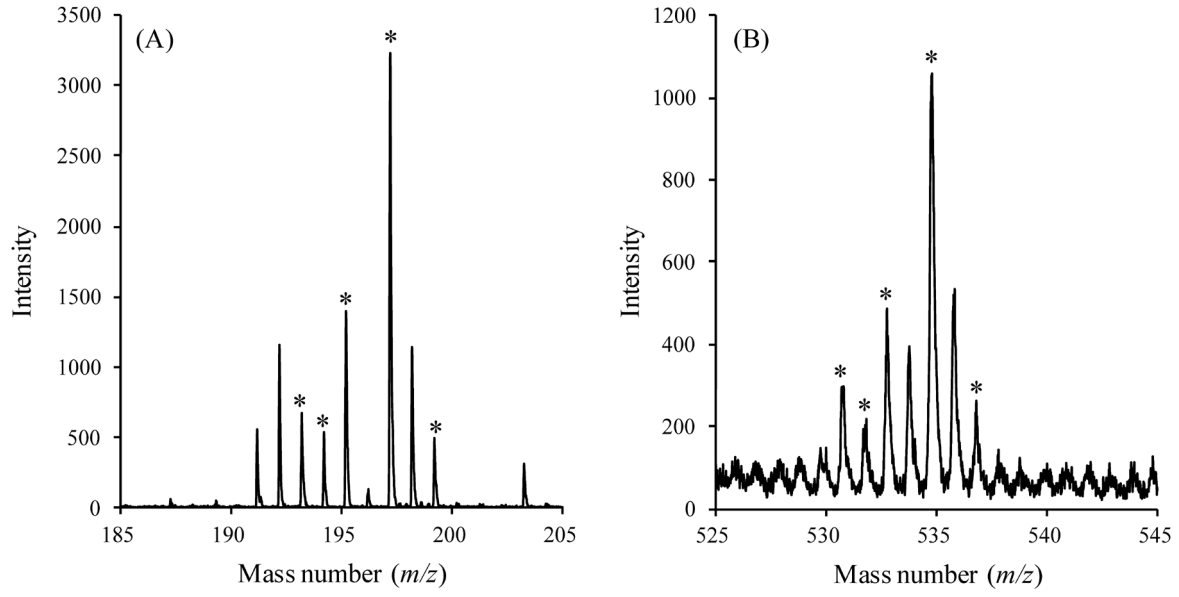

Fig. 3. Mass Spectra of SeMet (A) and a Selenium Species in the Shijimi Water-Extract with HTAC (B)

Peaks containing naturally occurring stable isotopes of selenium were indicated with asterisks.

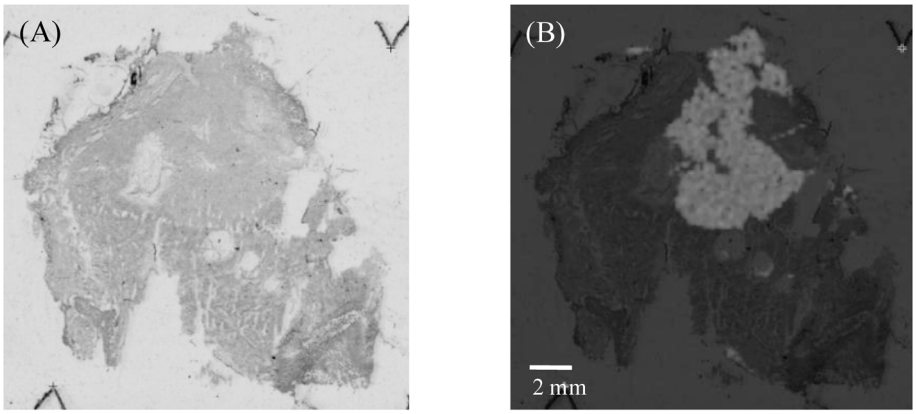

Fig. 4. Optical Image (A) and Mass Image of *m/z* 534 of Shijimi Clam Section by Imaging Mass Spectrometry (B)

in a molecule (Fig. 3A). Because the matrix DHB itself gave no remarkable peaks in the range around *m/z* 534 (Fig. S4A), these peaks were thought to come from substances in the Shijimi water-extract, probably selenium compound concerning the characteristic isotopic pattern. The difference in the ratio of isotopic peak intensity between the observed MS and theoretical isotopic abundance was 2–27% (={[(peak intensity of the respective isotopes/sum of peak intensity of the five isotopes)×100]/natural abundance of the respective isotope}×100, natural abundance (%): 9.37 for ^{76}Se, 7.63 for ^{77}Se, 23.77 for ^{78}Se, 49.61 for ^{80}Se, 8.73 for ^{82}Se). We separately analyzed SeMet at the same selenium concentration as that in the Shijimi water-extract sample as the standard compound. The difference in the ratio of isotopic peak intensity for SeMet was 3−13%. A somewhat larger difference between theoretical and actual selenium isotopic pattern for the Shijimi-extract sample may be due to coexistence of low-molecular-mass compounds other than selenium species. A similar selenium isotopic pattern from the Shijimi water-extract was also detected when α-cyano-4-hydroxycinnamic acid was used as an alternative matrix for MALDI mass spectrometric analysis (Fig. S4B). To examine the tissue/organ distribution of the selenium species from the Shijimi water-extract, a section of the Shijimi clam meat was subjected to imaging mass spectrometry. It is a useful technique to visualize the location of the endogenous metabolites and drugs injected without probing materials such as a fluorescently-labeled antibody.[31,32] The imaging mass spectrometry demonstrated that this species with the molecular ion peak at *m/z* 534 was present in the visceral sites including the digestive tract and the mid-gut gland (Fig. 4). This selenium species detected from the Shijimi clam was probably ascribed to their feed such as organic substance suspended in water including phytoplankton or metabolites, *e.g.*, an amino acid derivative. Selenium species in fish and shellfish is hardly known because of the difficulty of enrichment compared to plant materials. Fish, shellfish and other marine animals are likely to have chemically diverse low-molecular-mass non-proteinous selenium species other than selenoamino acids and selenoproteins, which may be responsible for their diets and/or fish-specific metabolic pathways. In 2010, a selenium species, selenoneine (2-selenyl-N_α,N_α,N_α-trimethyl-L-histidine), was newly discovered in the blood of bluefin tuna.[33] This compound was then found in the liver of the sea turtle, various seafood stuffs and the blood of humans.[34,35] The molecular mass of the selenium species detected from the Shijimi water-extract was apparently different from the already reported selenium species including selenoneine. To the best of our knowledge, this selenium species is seemingly a novel selenium species from biological samples.

In summary, the Shijimi clam contained ≈3.5 μg-selenium/g and approximately 30% of that was extracted with water but hardly with organic solvents. The molecular mass of the water-extractable selenium species in the Shijimi clam was mostly estimated to be less than 5000. The selenium species in the water-extract was also likely to possess an amphoteric character. A major selenium species from the Shijimi water-extract was detected at *m/z* 534 for ^{80}Se by mass spectrometry. Finally, imaging mass spectrometry allowed visualizing its distribution in the Shijimi clam sample. Based on all the data, this selenium species may be one of the metabolites in the Shijimi clam. We are planning to evaluate the nutritional availability of this selenium species from the Shijimi clam in cultured cells and experimental animals.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

References

1) Rayman M. P., *Lancet*, **379**, 1256–1268 (2012).
2) Roman M., Jitaru P., Barbante C., *Metallomics*, **6**, 25–54 (2014).
3) Papp L. V., Lu J., Holmgren A., Khanna K. K., *Antioxid. Redox Signal.*, **9**, 775–806 (2007).
4) WHO, Food and Agriculture Organisation, International Atomic Energy Agency expert group. "Trace elements in human nutrition and health." Geneva, 1996.
5) Miyazaki Y., Koyama H., Sasada Y., Satoh H., Nojiri M., Suzuki S., *J. Nutr. Sci. Vitaminol.*, **50**, 309–319 (2004).
6) Navarro-Alarcon M., Cabrera-Vique C., *Sci. Total Environ.*, **400**, 115–141 (2008).
7) Pedrero Z., Madrid Y., *Anal. Chim. Acta*, **634**, 135–152 (2009).
8) Rayman M. P., *Proc. Nutr. Soc.*, **64**, 527–542 (2005).
9) Lippman S. M., Klein E. A., Goodman P. J., Lucia M. S., Thompson I. M., Ford L. G., Parnes H. L., Minasian L. M., Gaziano J. M., Hartline J. A., Parsons J. K., Bearden J. D. III, Crawford E. D., Goodman G. E., Claudio J., Winquist E., Cook E. D., Karp D. D., Walther P., Lieber M. M., Kristal A. R., Darke A. K., Arnold K. B.,

Ganz P. A., Santella R. M., Albanes D., Taylor P. R., Probstfield J. L., Jagpal T. J., Crowley J. J., Meyskens F. L. Jr., Baker L. H., Coltman C. A. Jr., *JAMA*, **301**, 39–51 (2009).
10) Stranges S., Marshall J. R., Natarajan R., Donahue R. P., Trevisan M., Combs G. F., Cappuccio F. P., Ceriello A., Reid M. E., *Ann. Intern. Med.*, **147**, 217–223 (2007).
11) B'Hymer C., Caruso J. A., *J. Anal. At. Spectrom.*, **15**, 1531–1539 (2000).
12) Ogra Y., Ishiwata K., Encinar J. R., Lobinski R., Suzuki K. T., *Anal. Bioanal. Chem.*, **379**, 861–866 (2004).
13) Arnault I., Auger J., *J. Chromatogr. A*, **1112**, 23–30 (2006).
14) Önning G., Bergdahl I. A., *Analyst*, **124**, 1435–1438 (1999).
15) Önning G., *Food Chem.*, **68**, 133–139 (2000).
16) Quijano M. A., Moreno P., Gutiérrez A. M., Pérez-Conde M. C., Cámara C., *J. Mass Spectrom.*, **35**, 878–884 (2000).
17) Moreno P., Quijano M. A., Gutiérrez A. M., Pérez-Conde M. C., Cámara C., *J. Anal. At. Spectrom.*, **16**, 1044–1050 (2001).
18) Lobanov A. V., Hatfield D. L., Gladyshev V. N., *Biochim. Biophys. Acta*, **1790**, 1424–1428 (2009).
19) Kryukov G. V., Castellano S., Novoselov S. V., Lobanov A. V., Zehtab O., Guigo R., Gladyshev V. N., *Science*, **300**, 1439–1443 (2003).
20) Mariotti M., Ridge P. G., Zhang Y., Lobanov A. V., Pringle T. H., Guigo R., Hatfield D. L., Gladyshev V. N., *PLOS ONE*, **7**, e33066 (2012).
21) Lobanov A. V., Fomenko D. E., Zhang Y., Sengupta A., Hatfield D. L., Gladyshev V. N., *Genome Biol.*, **8**, R198 (2007).
22) Moreno P., Quijano M. A., Gutiérrez A. M., Pérez-Conde M. C., Cámara C., *Anal. Chim. Acta*, **524**, 315–327 (2004).
23) Haratake M., Takahashi J., Ono M., Nakayama M., *J. Health Sci.*, **53**, 457–463 (2007).
24) Yoshida S., Haratake M., Fuchigami T., Nakayama M., *Chem. Pharm. Bull.*, **60**, 348–353 (2012).
25) Watkinson J. H., *Anal. Chem.*, **38**, 92–97 (1966).
26) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265–275 (1951).
27) Siwek M., Galunsky B., Niemeyer B., *Anal. Bioanal. Chem.*, **381**, 737–741 (2005).
28) De Zoysa M., Pushpamali W. A., Oh C., Whang I., Kim S. J., Lee J., *Fish Shellfish Immunol.*, **25**, 446–457 (2008).
29) Wu C., Mai K., Zhang W., Ai Q., Xu W., Wang X., Ma H., Liufu Z., *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.*, **152**, 121–132 (2010).
30) Labunskyy V. M., Hatfield D. L., Gladyshev V. N., *Physiol. Rev.*, **94**, 739–777 (2014).
31) McDonnell L. A., Heeren R. M. A., *Mass Spectrom. Rev.*, **26**, 606–643 (2007).
32) Rohner T. C., Staab D., Stoeckli M., *Mech. Ageing Dev.*, **126**, 177–185 (2005).
33) Yamashita Y., Yamashita M., *J. Biol. Chem.*, **285**, 18134–18138 (2010).
34) Anan Y., Ishiwata K., Suzuki N., Tanabe S., Ogra Y., *J. Anal. At. Spectrom.*, **26**, 80–85 (2011).
35) Yamashita M., Yamashita Y., Ando T., Wakamiya J., Akiba S., *Biol. Trace Elem. Res.*, **156**, 36–44 (2013).