Characterization of Selenium Species in Extract from Niboshi (a Processed Japanese Anchovy)

Sakura Yoshida, Mamoru Haratake,* Takeshi Fuchigami, and Morio Nakayama*

Graduate School of Biomedical Sciences, Nagasaki University; 1–14 Bunkyo-machi, Nagasaki 852–8521, Japan.
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Fish are selenium rich foodstuffs and a major selenium source for the Japanese population. Niboshi is processed from Japanese anchovy (*Engraulis japonicus*) and commonly used to prepare soup stock for Japanese dishes. In this study, we characterized selenium species in the Niboshi extract by ultrafiltration, ion-exchange chromatography and mass spectrometry. Selenium species in the Niboshi were more extractable by polar solvents (water and ethanol) than an apolar one (hexane) along with amino acids and proteinous species. Selenium in the water-extract from the Niboshi was mostly ascribed to organoselenium compounds with a molecular mass less than 5 kDa. Although selenoamino acids and selenoproteins and their fragments were involved in the extract, a large portion of the selenium species appeared to be low-molecular-mass organoselenium compounds other than selenoamino acids and their derivatives. Ion-exchange chromatographic separations revealed that most of the selenium species in the extract possess anionic and/or amphoteric characteristics. One of these selenium species from the Niboshi extract was detected at *m*/*z* 577 for ^{80}Se by mass spectrometry subsequent to ion-pair extraction.

Key words selenium; fish; seafood; mass spectrometry; ion-exchange

Selenium is an essential trace element with a fundamental importance to human health, such as redox and immune regulations.[1,2] This mineral nutrient required for maintaining normal human health comes from daily meals; *i.e.*, cereal, meat, eggs and fish are known to be higher in selenium content than other foodstuffs.[3] The selenium status is dependent not only on the selenium content, but also on the chemical form of the selenium species in consumed foodstuffs. On the basis of many reports on the cancer preventive effect of selenium,[4] selenium-containing supplements, such as selenized yeast, are commonly utilized in western countries,[5] although the required amount of selenium should be taken all from diets unless there are no causes of its deficiency. A recent ironic fact that long-term supplementation with selenized yeast could cause type 2 diabetes demonstrates the difficulty of effective selenium supplementation.[6] Such a cancer preventive effect would be responsible for the chemical forms of the selenium species, as well as bioavailability.[7] It will be even more important to elucidate the chemical structures of selenium species in food and supplements from the viewpoints of the safety and benefits of this element.

A speciation analysis of selenium in various foodstuffs has been performed during the past few decades. Inorganic selenium species, selenomethionine (SeMet) and their metabolites have been identified in plant foodstuffs.[8] Such speciation studies have been mostly performed with selenium-enriched foodstuffs, for example, selenized mushroom.[9] There are quite few reports on the speciation analysis of selenium in natural foodstuffs, probably due to their extremely low selenium contents in comparison to the selenium-enriched ones; selenium contents in various natural foodstuffs including fish are generally less than 1 μgSe/g,[3] on the contrary, selenized mushroom contains 355 μgSe/g in wet state[9] and selenized yeast can accumulate selenium up to 3000 μgSe/g.[10]

Selenocysteine (SeCys or Sec) is generally thought to be one of the major selenium species in animal foodstuffs, since most of them involve SeCys that is incorporated into the selenoproteins by the genetic code. Most studies by chromatographic separation techniques using the limited standard materials could identify only several selenium species, while unidentified peaks of which their retention times were not consistent with those of the standard materials still need to be evaluated.[11] Although SeMet, selenocystine (SeCyst) and trimethylselenonium ion (TMS) were detected in fish and shellfish,[12] other organoselenium species are still unknown. Actually, water-soluble and low-molecular-mass selenium species other than SeCys and SeMet were found in many flat fish, such as plaice, but their chemical structures have not yet been elucidated.[13] Selenium from certain fish meat was reported to be less bioavailable due to the complex formation with heavy metal species, such as mercury.[14—16] On the contrary, the bioavailability of selenium from several fish, such as flounder, was reported to be comparable to inorganic selenious acid that can effectively restore the selenium-dependent glutathione peroxidase (GPx) activity of tissues in experimental animals.[17] The bioavailability of selenium from fish and seafood materials still appears to remain controversial, which may be related to the species-specific chemical form of the selenium compounds.

Human selenium-deficient diseases were first recognized in China; an endemic cardiomyopathy (Keshan disease) and deforming arthritis (Kashin-Beck disease).[18,19] Selenium deficiency has also been found in other parts of the world, such as Finland and New Zealand, notable for their low soil selenium concentration. Selenium concentrations in the soil reflect the selenium status in human populations, since selenium enters the food chain through plants that take it up from the soil. The selenium deficiency and related pathologies have never been reported in Japan. Miyazaki *et al.* reported that fish and/or seafood are the major dietary sources of selenium for the Japanese population (*ca.* 60% of daily intake).[20] Niboshi is a commonly used foodstuff that is processed from Japanese anchovy (*Engraulis japonicus*), and its extract is used as a general base seasoning for a wide variety of Japanese cuisine, just like the *fumet de poisson* and/or *fond de veau* for the French cuisine. Recently, we have demonstrated that the Niboshi con-

* To whom correspondence should be addressed. e-mail: haratake@nagasaki-u.ac.jp; morio@nagasaki-u.ac.jp

tains a relatively high concentration of selenium (*ca.* 1 μgSe/g), and selenium from the Niboshi and its extract can restore the selenium concentration in the liver and activity of the hepatic cellular GPx in dietary selenium-deficient mice.[21] In this study, selenium species in the Niboshi extract were characterized by ultrafiltration, ion-exchange chromatographic and mass spectrometric techniques.

Experimental

Materials Japanese anchovies caught in the near shore of Japan were processed to the Niboshi products used in this study. The Niboshi products within 1 year after the processing were purchased at local grocery stores. Raw Japanese anchovies (almost size-matched) were obtained from a manufacturer of the Niboshi in Nagasaki, Japan. Water used throughout this study was generated using a Milli-Q Biocel system (Millipore Corp., Billerica, MA, U.S.A.). All other chemicals were of commercial reagent grade and used as received.

Preparation of Niboshi the Extracts The Niboshi (*ca.* 3 g) was placed in a non-woven fabric bag with or without pretreatment (cut into *ca.* 10 pieces in almost equal size or finely ground down using a mortar and a pestle). These samples were placed in 30 mL of water and then treated by the indicated procedures shown in Table 1. After passing through a paper filter (particle size cutoff, 4 μm), its volume was made up to 20 mL with water. The obtained yellow-colored solution was used as the Niboshi extract in this study. The concentrations of the selected substances in the extracts were calculated by the following equation; =(amount of substances in the extract used for analysis)/[(volume of the extract used for analysis)·(weight of the Niboshi used for extraction)] (ng or μg/mL/g-Niboshi)

Determination of Concentrations of Selenium, Amino Acids and Proteins The Niboshi and the Niboshi extract were digested using a 5 : 1 mixture by volume of nitric and perchloric acid. The selenium concentrations in the specimens were determined using 2,3-diaminonaphthalene (DAN, Tokyo Chemical Ind., Tokyo, Japan).[22] An FP-6600 spectrofluorometer (JASCO, Tokyo, Japan) was used for measurement of the piaselenol generated from DAN (working concentration range: 0.5—10.0 ngSe/sample). The selenium standard solution [1000 ppm as selenium (IV) dioxide in 0.1 M nitric acid] was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan).

The Niboshi extract was appropriately diluted with water (typically 50—100-fold), and the total free amino acid concentrations were spectrofluorometrically determined after the reaction with *o*-phthalaldehyde and dithiothreitol (DTT) to form a thio-substituted isoindole fluorophore (the excitation wavelength: 340 nm, the emission wavelength: 450 nm, working concentration range: 0.1—50 μM).[23] For the protein concentration determination, the Niboshi extract was diluted 8—10-fold with water, and then directly measured by Lowry's method using bovine serum albumin as the reference and absorbance at 650 nm was monitored on a V-660 UV–Visible spectrophotometer (JASCO, Tokyo, Japan) (working concentration range: 0.01—0.5 mg/mL).[24]

Ultrafiltration The Niboshi extract was diluted five-fold with water and ultrafiltered using an Amicon Ultra-4 Ultracel-5K or 30K Centrifugal Filter [molecular mass cutoff (MMCO), 5 and 30 kDa, Millipore Corp., Billerica, MA, U.S.A.] at 7500 g and 20°C. The filtration rates of the MMCO 5 and 30 kDa membranes for lysozyme (molecular mass: 14.6 kDa, concentration: 0.4 mg/mL) under these conditions were 1.3±0.4 and 90.6±0.5%, respectively. The selenium and protein contents of the filtrate were determined by the DAN and Lowry's methods, respectively.

Ion-Exchange Chromatography Q Sepharose [particle size: 45—165 μm in wet state, capacity: 0.18—0.25 meq/mL, functional group: $-[OCH_2CH(OH)CH_2]_2N^+(CH_3)_3\cdot X^-$, Sigma Co., St. Louis, MO, U.S.A.] and SP Sepharose [particle size: 45—165 μm in wet state, capacity: 0.18—0.25 meq/mL, functional group: $-(CH_2)_3SO_3^-\cdot Y^+$, Sigma Co., St. Louis, MO, U.S.A.] were packed into a glass column. Q Sepharose in the OH form (X=OH) and SP Sepharose in the H form (Y=H)

Table 1. Selenium Species Extraction from the Niboshi by Various Procedures

Extraction method #	Pretreatment of Niboshi	Extraction procedure	Selenium concn.[a] (ngSe/mL/g-Niboshi)	Selenium extracted (%)
1	Finely ground down in mortar	Soaked in ethanol for 1 week at ambient temp.	3.953±0.021	6.5
2	Finely ground down in mortar	Soaked in hexane for 1 week at ambient temp.	0.637±0.008	1.1
3	None	Boiled in water for 10 min	6.360±0.025	10.5
4	Cut into *ca.* 10 pieces	Soaked in water for 12 h at ambient temp.	4.840±0.081	8.0
5	Cut into *ca.* 10 pieces	Soaked in water for 12 h at ambient temp. and then boiled for 10 min	7.215±0.120	11.9
6	Cut into *ca.* 10 pieces	Boiled in water for 10 min	5.869±0.156	9.7
7	Cut into *ca.* 10 pieces	Boiled in water for 30 min	3.795±0.006	6.3
8	Finely ground down in mortar	Boiled in water for 10 min	3.917±0.095	6.5
9	Finely ground down in mortar	Soaked in 1% (w/v) Tween 20 for 12 h at ambient temp.	3.473±0.059	5.8
10	Finely ground down in mortar	Digested with papain [0.2 mg/mL–50 mM phosphate buffer (pH 6.5)] for 1 h at 65°C	8.935±0.801	14.8

a) =(amount of substances in the extract used for analysis)/[(volume of the extract used for analysis)·(weight of the Niboshi used for extraction)], Mean±S.E. (n=3—5).

were prepared by conditioning with 0.1 M NaOH and 0.1 M HCl, respectively, followed by thoroughly washing with water until the pH of the eluate became neutral. A PUMP 560 (Yamazen, Kyoto, Japan) equipped with a prep·UV254 monitor was used for the ion-exchange chromatographic separations.

Ion-Pair Extraction and Mass Spectrometry An ion-pair reagent, hexadecyltrimethylammonium chloride (Tokyo Chemical Ind., Tokyo, Japan), dissolved in water was combined with the Niboshi extract to make its final concentration 1 mM, and then extracted with chloroform. The obtained chloroform layer was concentrated *in vacuo* (content of selenium was 22 μM) and subjected to FAB-MS. Mass spectra were acquired on a JMS-700N (JEOL, Tokyo, Japan) in linear positive ion mode using *m*-nitrobenzyl alcohol as a matrix. Seleno-L-methionine (SeMet, calcd for $C_5H_{11}NO_2{}^{80}Se$ 197.06) and seleno-L-cystine methyl ester (SeCyst-Me, calcd for $C_8H_{16}N_2O_4{}^{80}Se_2$ 364.23) solutions at the selenium concentration of 10 μM were used as the standard materials for detection of selenium-specific isotopic pattern.

Results and Discussion

Extraction of Selenium Species from the Niboshi The Japanese anchovy of 5—7 cm in length is processed into the Niboshi by a several-minute boiling and subsequent drying. The selenium content of the Niboshi (1.21±0.03 μgSe/g) was comparable to that of freeze-dried raw Japanese anchovy (1.14±0.02 μgSe/g), which indicates that selenium in the raw Japanese anchovy is mostly retained in the Niboshi without extraction during the manufacturing process.

The selenium species in the Niboshi were extracted under various conditions (Table 1). The selenium species were more extractable with ethanol (method #1) than apolar hexane (method #2). The selenium species in the Niboshi appeared to possess polar characteristics rather than the hydrophobic one. The selenium species were extracted with water under various operating conditions. The selenium concentrations in the water-extracts by methods #3–8 were within the range from 3.795 to 7.215 ngSe/mL/g-Niboshi (The extraction rates were between 6 and 12% of the total selenium in the Niboshi). The extraction behavior of the selenium species into water was not markedly affected by the pretreatment (cutting and grinding) and extraction conditions of the Niboshi.

The effect of surfactant addition on the selenium extraction into water was examined. Several papers described that such a procedure was often effective for the extraction of selenium in biological samples.[25] The addition of the nonionic surfactant Tween 20 did not result in significant increases in the selenium concentration in the extract (method #9), as compared to those by methods #4 and #8. The effect of the protease treatment was also tested using papain that has a broad specificity for peptide bond cleavage (method #10). Selenium is an essential microelement for fish and shellfish species.[10] The selenoproteomes (sets of SeCys-containing proteins) of fish are greater in number than those of mammals (25 selenoproteins in human); at 30—37 selenoproteins, the selenoproteomes of fish are among the largest known. In addition, fish have several species-specific selenoproteins (fish 15 kDa selenoprotein-like protein, selenoprotein J and selenoprotein L) that are missing in mammals.[26] The digestion with proteases was reported for the extraction of selenium species from such proteinous materials in fish.[12,25,27—29] The digestion of the finely ground-Niboshi with papain gave at most a two-fold higher concentration of selenium than that by method #8, although the protein concentration in the extract increased eight-fold due to the enzymatic degradation.

Selenium was reported as a naturally occurring antagonist in certain marine fish at levels capable of forming an equimolar complex with a metal,[15,16] which was thought to be one of the reasons for the low bioavailability of selenium in fish as compared to that in plant food and meat. The total mercury content in the Niboshi was less than 2 mol% of selenium (0.06 μg/g) as determined by cold vapor atomic absorption spectrophotometry.[21] The extract used for the following experiments was prepared by the 10-min boiling of the Niboshi that was cut into 10 pieces (method #6 in Table 1, the most ordinarily used extraction procedure for the domestic cuisine). The selenium concentration in the extract was 0.006 μgSe/mL/g-Niboshi, in which no inorganic selenious acid was detected, as it was directly determined by the DAN method. The concentrations of the amino acid and protein in the extract were 451.3±27.1 and 568.1±56.9 μg/mL/g-Niboshi, respectively. The selenium in the extract appeared to be, in part, ascribed to selenoamino acids and selenoproteins and/or their fragments.

Molecular Mass Estimation of Selenium Species in the Extract The molecular mass of the selenium species in the extract was estimated by the ultrafiltration technique (Table 2). A membrane with a molecular mass cutoff (MMCO) of 30 kDa almost completely filtered out the selenium in the extract. In addition, nearly 90% of the selenium passed through the membrane with MMCO 5 kDa. Thus, the majority of the selenium species in the extract was postulated to be low-molecular-mass organoselenium compounds, including selenoamino acids and their derivatives. On the other hand, proteinous species in the extract were filtered at 65.3±5.6 and 79.2±1.3% by the membranes with MMCO 5 and 30 kDa, respectively. Combining these data on the ultrafiltration, the extract was also thought to contain proteinous selenium species.

To investigate the characteristics of the proteinous selenium species, the extract was digested with papain, followed by ultrafiltration using a membrane with MMCO 5 kDa. The digestive treatment with papain resulted in the complete filtration of both the selenium and proteinous species in the extract.

Table 2. Selenium and Protein Concentrations in the Niboshi Extract before and after Ultrafiltration

Pretreatment of the Niboshi extract	MMCO (kDa)[a]	Selenium filtered (%)[b]	Protein filtered (%)[b]
None	5	87.3±6.4	65.3±5.6
	30	97.8±2.5	79.2±1.3
Papain (0.2 mg/mL) at 65°C for 1 h	5	99.9±0.2	103.0±2.9
Dithiothreitol (20 mM) at 37°C for 1 h	5	86.8±0.9	—[c]

a) MMCO: molecular mass cutoff. *b*) =100×(concentration in filtrate)/(concentration in extract before filtration) (%). *c*) Not measurable due to the presence of dithiothreitol.

These results showed that proteinous selenium species with a molecular mass ranging from 5 to 30 kDa were involved in the extract. To further explore the binding manner of selenium to proteinous species, the extract was treated with DTT, followed by ultrafiltration using a membrane with MMCO 5 kDa. If low-molecular-mass selenium species are bound to proteinous materials through selenenylsulfide (–Se–S–) or diselenide (–Se–Se–), such selenium-containing moieties are supposed to be cleaved by the DTT treatment and pass through the ultrafiltration membrane. Pretreatment of the Niboshi extract with DTT gave the same filtered rate of selenium as the non-treated sample. Therefore, the proteinous selenium species appear to involve not the attachment of low-molecular-mass selenium species through selenenylsulfide or diselenide, but selenoamino acids. Considering that the molecular mass of the known selenoproteins in mammals is higher than 9 kDa,[30] the proteinous selenium species in the Niboshi extract were thought to be mostly from fragments of selenoproteins with a molecular mass less than 5 kDa, not intact ones.

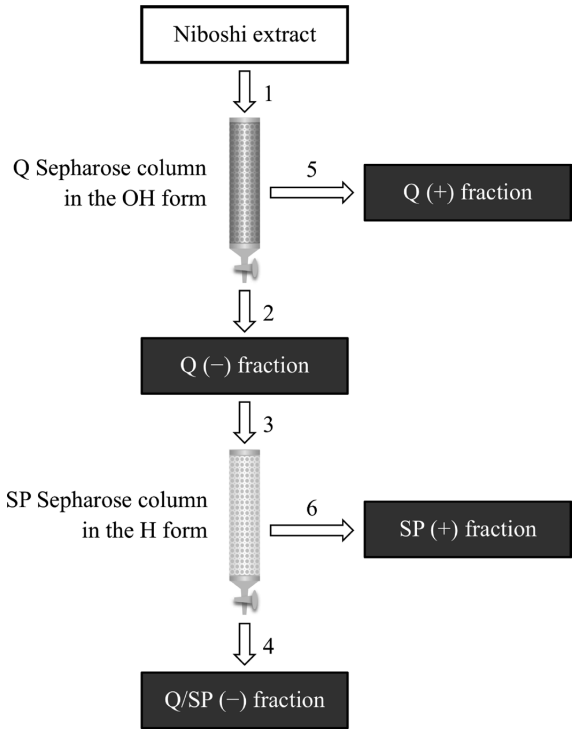

Fig. 1. A Procedure for Fractionation of Selenium Species in the Niboshi Extract by Q Sepharose in the OH Form and SP Sepharose in the H Form Columns

Column dimension: 23×5.1 i.d. cm (Q Sepharose), 21×4.1 i.d. cm (SP Sepharose). Injection sample volume to the Q Sepharose column: 200 mL. Operation 1: application of the Niboshi extract sample, 2: washing out with water, 3: application of lyophilized Q (−) fraction, 4: washing out with water, 5: flushing with 0.1 M HCl, 6: flushing with 0.1 M NaOH.

Fractionation of Selenium Species in the Extract by Ion-Exchange Chromatography To further characterize the selenium species in the Niboshi extract, the extract was sequentially separated by the Q Sepharose column in the OH form and by the SP Sepharose column in the H form (Fig. 1). During this separation manner, the Q Sepharose column can retain not only anionic species, but also amphoteric ones such as amino acids, because a high pH environment in the Q Sepharose beads can promote the dissociation of acid groups in amphoteric compounds. Subsequent flushing with water results in the elution of the cationic and nonionic species [coded by Q (−) fraction]. The Q (−) fraction is lyophilized and then applied to the SP Sepharose column in the H form. In contrast to the Q Sepharose column, cationic species are retained on the column and separated from the Q/SP (−) fraction containing nonionic species. For example, cationic TMS should be separated into the SP (+) fraction.

The selenium fractionation during the sequential separation by the ion-exchange chromatography is summarized in Table 3. Because 8.6±0.2% of the total selenium in the applied extract was eluted from the Q Sepharose column [Q (−) fraction], the rest of the selenium (91.4%) was retained on the column. In addition, amphoteric amino acid and proteinous species in the extract were mostly retained on this column. Subsequently, the Q (−) fraction was lyophilized and then applied to the SP Sepharose column in the H form. Retained on neither the Q nor the SP Sepharose column, the Q/SP (−) fraction contained 2.7±0.1% of the total selenium applied.

That retained on the Q Sepharose column was flushed with 0.1 M HCl, and 55.9±2.0% of the total applied selenium was eluted [Q (+) fraction]. Amino acids and proteins retained on the Q Sepharose column in the OH form were mostly recovered by the elution with 0.1 M HCl, whereas nearly 40% of the selenium species was left on the column. Under the same operating conditions, SeMet was quantitatively recovered from the Q Sepharose column [applied sample: 25 ngSe/mL, injection volume: 6 mL, recovery: 100.4% (n=2)]. Thus, the selenium species remaining on this column after the 0.1 M HCl flushing were thought to be chemically different from selenoamino acids and selenoproteins. Furthermore, 2.8±0.3% of the total selenium was recovered from the SP Sepharose column [SP (+) fraction], and that retained on this column was flushed with 0.1 M NaOH. The overall recovery of selenium, amino acid and protein throughout the consecutive ion-exchange chromatography were 61.5, 90.4 and 96.5%, respectively. Thus, a large portion of the selenium species in the Niboshi

Table 3. Selenium, Amino Acid and Protein Contents in Fractions Separated by Ion-Exchange Chromatography

Fraction	Content (%)[a]		
	Selenium	Amino acid	Protein
Not retained on Q Sepharose column [Q (−) fraction]	8.6±0.2	5.4±0.5	4.7±0.1
Retained on neither Q nor SP Sepharose column [Q/SP (−) fraction]	2.7±0.1	3.8±0.0	0.0±0.0
Eluted from Q Sepharose column with 0.1 M HCl [Q (+) fraction]	55.9±2.0	83.1±0.6	96.4±1.5
Eluted from SP Sepharose column with 0.1 M NaOH [SP (+) fraction]	2.8±0.3	3.5±0.0	0.1±0.0
Total recovery[b]	61.5	90.4	96.5

a) The amount of respective materials in the Niboshi extract sample applied to the Q Sepharose column was defined as 100%. *b*) The sum of the values for Q/SP (−) fraction, Q (+) fraction and SP (+) fraction.

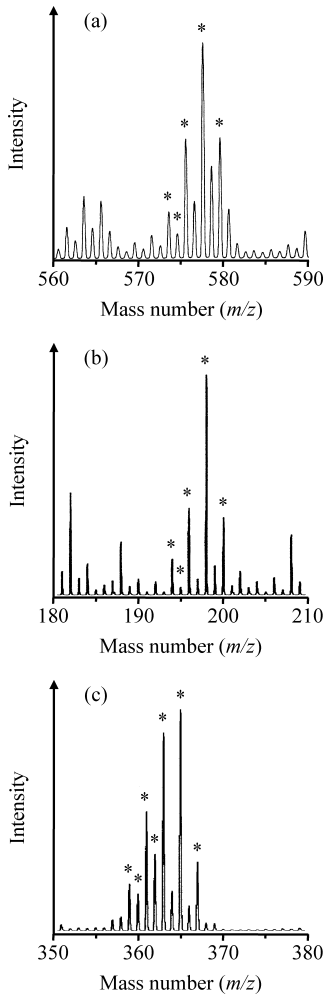

Fig. 2. Mass Spectra of a Selenium Species from the Niboshi Extract (a), Seleno-L-methionine (b) and Seleno-L-cystine Methyl Ester (c)

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

extract was strongly retained on the Q Sepharose column, in which anionic or amphoteric low-molecular-mass organoselenium compounds other than selenoamino acids and their derivatives appear to be involved.

Ion-Pair Extraction and Mass Spectrometry The anionic selenium species in the Niboshi extract were further extracted into chloroform by ion-pair extraction using a cationic ion-pair reagent, hexadecyltrimethylammonium chloride. When the concentrated extract sample was subjected to FAB-MS analysis, the selenium isotopic pattern involving one selenium atom in an ion was detected and ^{80}Se-containing ion peak was at *m*/*z* 577 (Fig. 2a). Because no selenium isotopic pattern was observed in the higher molecular mass range than *m*/*z* 577, the ^{80}Se ion peak at *m*/*z* 577 was thought to be a molecular ion peak, not a fragment one. Such selenium isotopic pattern was quite similar to that for SeMet (Fig. 2b), not for SeCyst-Me (Fig. 2c). These peaks were thought to be from one of the anionic or amphoteric selenium species from the Niboshi extract, which contains one selenium atom per one molecule.

So far, the selenium species in seafood are hardly known probably due to the extremely low selenium contents, as compared with such selenium-enriched food and supplements. In most of the speciation studies, only using the limited standard materials could identify several selenium species, whereas many low-molecular-mass non-proteinous species other than SeMet and the SeCys derivatives were detected in fish and shellfish samples.[12,13,27—29,31,32] Such unidentified low-molecular-mass species may be ascribed to fish diets and/or fish-specific metabolism.[33] More recently, a low-molecular-mass selenium compound, selenoneine (2-selenyl-N_α,N_α,N_α-trimethyl-L-histidine), was isolated from the blood of the bluefin tuna.[34] This was the first selenium compound that was detected in natural marine organisms. This compound was also detected in the liver of sea turtles.[35] Because the most abundant molecular ion peak for selenoneine in oxidized form is observed at *m*/*z* 553, the selenium species from the Niboshi extract seemed to be different from selenoneine. In addition, it should be noted that the selenoneine concentrations in both biological samples (several-tens of micromolar per gram-organ) were originally much higher than that in the Niboshi.

In conclusion, selenium species in the Niboshi were more extractable by a polar solvent (water and ethanol) than an apolar one (hexane) along with amino acids and proteinous species. Selenium in the water-extract from the Niboshi was mostly ascribed to non-proteinous organoselenium compounds with a molecular mass less than 5 kDa. Although selenoamino acids and selenoproteins and their fragments were involved in the extract, a large portion of the selenium species appeared to be low-molecular-mass organoselenium compounds other than the selenoamino acids and their derivatives. Ion-exchange chromatographic separation revealed that most of the selenium species in the extract possess anionic and/or amphoteric characteristics. Overall, the major selenium species in the Niboshi extract were ionic organic compounds with molecular mass less than 5 kDa and one of which was detected at *m*/*z* 577 for ^{80}Se by mass spectrometry subsequent to ion-pair extraction.

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