

Identification of a Novel Selenium-containing Compound, Selenoneine, as the Predominant Chemical Form of Organic Selenium in the Blood of Bluefin Tuna^{*[S]}

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A novel selenium-containing compound having a selenium atom in the imidazole ring, 2-selenyl-*N*_α*N*_ω*N*_α-trimethyl-L-histidine, 3-(2-hydro-seleno-1*H*-imidazol-5-yl)-2-(trimethylammonio)propanoate, was identified from the blood and other tissues of the bluefin tuna, *Thunnus orientalis*. The selenium-containing compound was purified from the tuna blood in several chromatographic steps. High resolution mass spectrometry and nuclear magnetic resonance spectroscopy showed that the exact mass of the [M+H]⁺ ion of the compound was 533.0562 and the molecular formula was C₁₈H₂₉N₆O₄Se₂. Its gross structure was assigned as the oxidized dimeric form of an ergothioneine selenium analog in which the sulfur of ergothioneine is replaced by selenium. Therefore, we named this novel selenium-containing compound “selenoneine.” By speciation analysis of organic selenium compounds using liquid chromatography inductively coupled plasma mass spectrometry, selenoneine was found widely distributed in various tissues of the tuna, with the highest concentration in blood; mackerel blood contained similar levels. Selenoneine was measurable at 2–4 orders of magnitude lower concentration in a limited set of tissues from squid, tilapia, pig, and chicken. Quantitatively, selenoneine is the predominant form of organic selenium in tuna tissues.

Selenium, an essential micronutrient for humans and animals (1–3), is a trace element nutrient that functions as selenocysteine for the reduction of antioxidant enzymes such as glutathione peroxidases (4, 5), thioredoxin reductase (6), and thyroid hormone deiodinases (7). The entire selenoprotein gene population, designated the selenoproteome, has been identified in humans and rodents (8), and numerous selenoprotein genes have functions in development and health (9). Selenoproteins are also involved in different human genetic disor-

ders (9). The antioxidant activity of selenium plays a protective role in 50 human diseases, including prostate, lung, and intestine/colon cancer, immunodeficiency, and heart diseases (10–13). Selenium deficiency is also linked to animal diseases such as myopathy, exudative diathesis, and pancreatic degeneration in domestic animals and birds (9). Selenium also protects against mercury toxicity in many different organisms (14, 15).

Fish consumption is a major source of selenium in the human diet in Japan. Red muscle and other tissues of tuna contain organic selenium in excess of 1 ppm, making this the highest selenium content in food. However, the molecular mechanism of the antioxidant activity of dietary selenium in fish muscle has been poorly characterized. Here, we identified and characterized the chemical structure of the major organic selenium compound in the blood, muscles, and other tissues of bluefin tuna, *Thunnus orientalis*. A novel selenium-containing compound, 2-selenyl-*N*_α*N*_ω*N*_α-trimethyl-L-histidine, a selenium analog of ergothioneine we call “selenoneine,” was identified as a strong antioxidant in tuna tissues. This compound has a unique chemical structure and is the first example of a natural heterocyclic compound with a selenoketone derived from animals.

EXPERIMENTAL PROCEDURES

Materials—Blood and other tissues were collected from cultured and wild bluefin tuna, *T. orientalis*, and stored frozen at –80 °C until use. The cultured bluefin tuna (average body weight ~39 kg) were obtained at an aquaculture farm in the Kyushu Island. The wild bluefin tuna (average body weight 5.2 kg) were caught by set net in the Aomori Prefecture. Wild Pacific mackerel, *Scomber japonicus* (average body weight 843 g), were caught by set net in the Shizuoka Prefecture. Wild Japanese common squid, *Todarodes pacificus* (average body weight ~200 g), were caught by long line in the Sea of Japan. Tilapia, *Oreochromis niloticus* (average body weight 405 g), were reared with an artificial feed at 20 °C for 5 years in the National Research Institute of Fisheries Science before use. Fresh tissues of pig (average body weight ~115 kg) and chicken (average body weight ~3 kg) were obtained at the Yokohama Wholesale Market. Dithiothreitol (DTT),² reduced glutathione, selenite, 1-diphenyl-2-picrylhydrazyl (DPPH), and tetrahydrofuran (THF) were purchased from Wako Pure Chemical (Osaka, Japan). Acetonitrile was obtained from Kanto Chemical (Tokyo, Japan). Glutathione peroxidase (GPx) from bovine erythrocyte, l-selenocysteine, and l-ergothioneine were acquired from Sigma-Aldrich. l-Selenomethionine and 2,3-diaminonaphthalene were purchased from Tokyo Kasei (Tokyo, Japan). TroloxTM (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was bought from Roche Diagnostics (Tokyo, Japan).

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² The abbreviations used are: DTT, dithiothreitol; DPPH, 1-diphenyl-2-picrylhydrazyl; THF, tetrahydrofuran; GPx, glutathione peroxidase; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; ICP, inductively coupled plasma; em, emission; ex, excitation.

Selenium Determination—To determine the total selenium content, each sample was digested in 1 ml of nitric acid and perchloric acid (1:2 in volume) at 200–220 °C. The selenium concentration was measured using a fluorometric assay with 2,3-diamino-naphthalene (16). Chromatographic separation was carried out using a high-performance liquid chromatography (HPLC) pump (712p, GL Sciences, Tokyo, Japan). The analytical column was an Ultrahydrogel 120 (7.8 × 250 mm, Nihon Waters, Tokyo, Japan) equilibrated with 100 mM ammonium formate buffer. The injection volume was fixed at 20 μl. The mobile phase was delivered at 1 ml/min isocratically, and selenium was detected using online liquid chromatography inductively coupled plasma mass spectrometry (LC-ICP-MS; ELAN DRC II, PerkinElmer Life Sciences), with a concentric nebulizer (WE02-4371, quartz) and a sample injector (2-mm inner diameter, quartz) monitoring ⁸²Se according to the method described previously (17). The plasma and auxiliary argon gas flow rates were 17 and 1.3 liters/min, respectively. The nebulization argon gas flow rate was 1.03 liters/min. The radio-frequency power was 1500 watts. During this separation, GPx, selenite, selenocysteine, selenomethionine, and selenoneine were eluted at retention times of 5.4, 7.4, 7.8, 9.8, and 10.5 min, respectively, and the selenium concentration was determined using the respective compounds as standards.

Purification of the Selenium-containing Compound—First, 0.1 g of DTT was added to 100 g of tuna blood and then homogenized in 10 volumes of acetonitrile. The homogenate was centrifuged at 6000 × g for 10 min, and the supernatant was then concentrated *in vacuo*. After adding acetonitrile/THF (1:1 in volume), the concentrated extract was solvent-partitioned to obtain acetonitrile-soluble organic fractions, and then the acetonitrile-soluble fraction was concentrated *in vacuo*. The concentrated extract was mixed with 5 ml of cold distilled water, and the water-soluble material was applied to an Atlantis dC18 300 Å column (19 × 150 mm, Nihon Waters) equilibrated with 0.1% acetic acid. The desired fraction containing total selenium at more than 10 μg/ml was eluted at an elution volume of 40–50 ml with a linear 0–50% acetonitrile gradient. The fraction containing selenium was applied to an Ultrahydrogel-120 column (7.8 × 300 mm, Nihon Waters) equilibrated with 0.1% acetic acid/30% acetonitrile, and the elution volume of 7.5–9.0 ml containing the selenium-containing compound was collected. The concentrated eluent was applied to an Ultrahydrogel-120 column equilibrated with 0.1% acetic acid, and the elution volume of 9.0–13 ml containing the selenium-containing compound was collected. The targeted component was also purified from the red muscles of tuna and swordfish and used in further experiments.

Spectroscopy and Spectrometry—UV spectra were recorded with a UV-visible spectrometer (DU640, Beckman-Coulter). Fluorescence spectra were recorded with a fluorescence spectrometer (model 850, Hitachi, Tokyo, Japan). The specific rotation was measured with a circular dichroism spectrometer (J-720 WI, JASCO, Tokyo, Japan). Mass spectrometry (MS) spectra were recorded on a Quattro II (Micromass, Manchester, UK) equipped with HPLC (HP 1100, Hewlett Packard, Palo Alto, CA) and a JMS-700 (JEOL, Tokyo, Japan). NMR spectra were recorded on a 500-MHz spectrometer (ECA500, JEOL) in

D₂O as the solvent. The signals of the deuterated solvents were used as references. The chemical shifts of the purified selenium-containing compound for both ¹H-NMR and ¹³C-NMR were observed: ¹H-NMR (D₂O) δ 7.00 (s), 3.80 (dd, 3.7 and 11.2), 3.16 (s), 3.08 (d, 3.4); ¹³C-NMR (D₂O) δ 170.1 (C-1), 155.8 (C-5'), 120.8 (C-2'), 115.3 (C-4'), 77.2 (C-2), 52.2 (N-CH₃), and 23.5 (C-3).

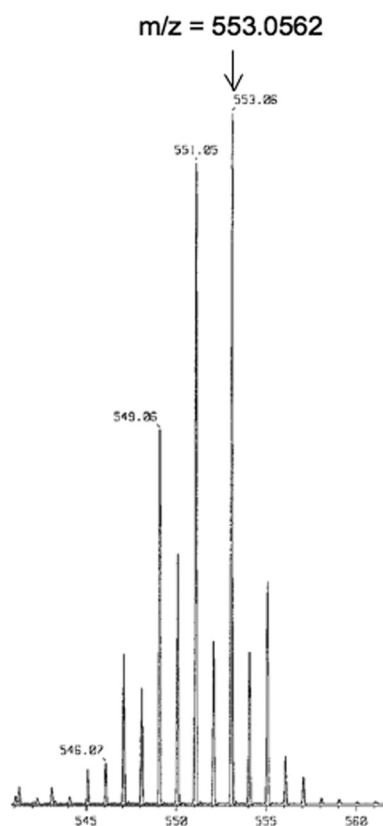
Antioxidant Assay—The radical scavenging activity against DPPH in 0.1 M sodium phosphate buffer (pH 6.8) was measured according to the method described by Oki *et al.* (18) using the water-soluble vitamin E-like compound Trolox as the standard antioxidant.

RESULTS

The selenium-containing compound in the tuna blood was purified in several chromatographic steps of C-18 reverse phase HPLC and gel filtration chromatography. A yellow oily compound of 0.2 mg was obtained from 100 g of the tuna blood. High resolution mass spectroscopy using electrospray ionization showed a molecular ion peak at *m/z* 553.0562, in agreement with the molecular formula C₁₈H₂₉N₆O₄Se₂ (calculated 553.0584) (Fig. 1). The isotopic pattern of the compound matched the theoretical isotopic pattern of two selenium atoms calculated from the natural abundance of selenium isotopes. MS/MS analysis showed fragment ions of 233.0422 predicted for trimethylammonioethyl-imidazole-selenolate (C₈H₁₅N₃Se), 173.9692 for ethyl-imidazole-selenolate (C₅H₆N₂Se), 79.9165 for selenium, and 59.0730 for trimethylamine (C₃H₉N), indicating that the compound contained a selenium-containing imidazole ring, a carboxyl group, and a trimethylamino group (supplemental Fig. S1). The molecular ion peak at *m/z* 553 was converted into that at *m/z* 278 by LC-electrospray ionization-MS after reducing the selenium compound in the presence of 10 mM DTT or 10 mM glutathione; the compound was found to be an oxidized dimeric form (supplemental Fig. S2). Therefore, from the molecular formula C₉H₁₅N₃O₂Se of the reduced monomeric form, this selenium compound was thought to be a selenium analog of ergothioneine (C₉H₁₅N₃O₂S) in which the sulfur was replaced by selenium. Because the monomeric form of the selenium compound was very unstable during extraction and chromatographic steps under both cold and room temperature conditions (data not shown), we treated the extracted material containing the selenoketone of the selenium compound from the tuna tissues with acetonitrile/THF (1:1 in volume) to accelerate selenoxo-selenol tautomerization (19) and formation of the oxidized dimeric form (Fig. 2). The dimeric form was stable at room temperature and was then used for the structural determination and chemical characterization.

The ¹³C-NMR spectroscopy showed nine carbon signals, which were resolved into one carboxylic acid, three methyl, one methylene, three aromatic, and one sp³ methine carbons through distortionless enhancement in polarization transfer experiments. In the ¹³C-NMR spectrum, the three aromatic carbon signals (δ 155.8, 120.8, and 115.3) were ascribed to a two-substituted imidazole carbon, which was consistent with one proton signal at δ 7.00 (s) in the ¹H-NMR spectrum, indicating that the selenol group was within the imidazole ring (supplemental Fig. S3). The observed chemical shifts for both

A: observed ions



B: theoretical ion distribution

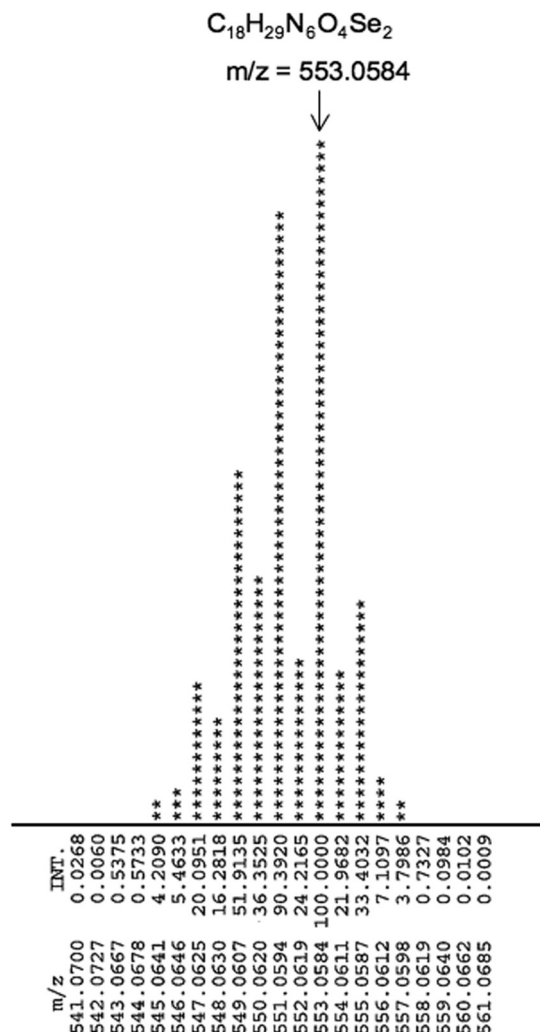


FIGURE 1. High resolution mass spectrometric identification of the selenium compound. The isotopic pattern of the purified compound (A) matched the theoretical isotopic pattern (B) of two selenium atoms calculated from the natural abundance of selenium isotopes. INT., intensity.

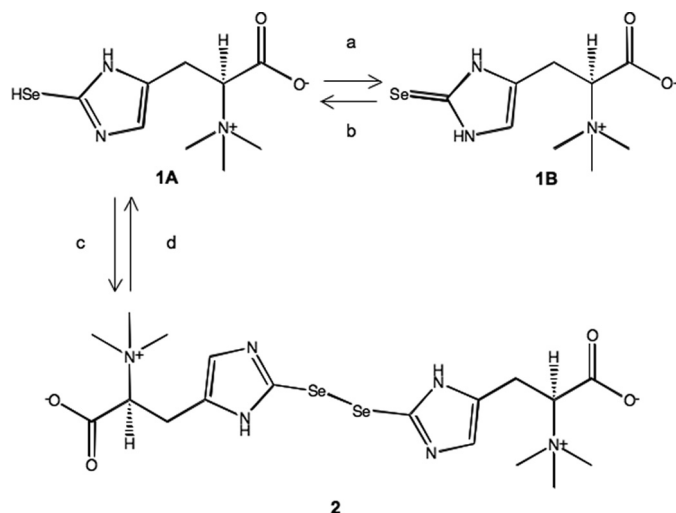


FIGURE 2. The chemical structure of the selenium-containing compound selenoneine (1A, 1B) and its autoxidized dimer (2). The reagents and conditions are as follows: a, water, methanol or acetonitrile, 0 °C; b, CH_2Cl_2 , or THF, -20 °C; c, CH_2Cl_2 , THF, room temperature; d, water, 10 mM DTT or GSH, room temperature, 30 min.

1H and ^{13}C were closely correlated with those of the structural analysis of ergothioneine: 1H -NMR (D_2O) δ 6.70, 3.83, 3.19, 3.10; ^{13}C -NMR (D_2O) δ 173.1 (C-1), 158.9 (C-5'), 126.7 (C-2'), 118.1 (C-4'), 79.8 (C-2), 54.3 (N- CH_3), and 25.4 (C-3), and the aromatic carbon signal of the selenium compound at C-2' (δ 120.8) was shifted lower than that of ergothioneine (δ 126.7) (20). Therefore, the selenium compound was confirmed to be the selenium analog of ergothioneine, in which the thiol at position C2' in the imidazole ring was replaced by selenol. The gross structure of the selenium-containing compound was assigned the chemical form shown in Fig. 2, in which a selenium-containing ergothioneine analog forms a novel selenol or selenoketone structure. We therefore named the compound selenoneine.

The oxidized dimeric form of selenoneine was characterized as absorbing UV at 260 nm ($\epsilon_{260} = 525 M^{-1} cm^{-1}$) (supplemental Fig. S4) and fluorescing with maximum emission wavelengths at 240, 280, and 337 nm and a maximum excitation wavelength at 405 nm ($\epsilon_{em\ 240/ex\ 405} = 0.823 \times 10^3 M^{-1} cm^{-1}$, $\epsilon_{em\ 280/ex\ 405} = 1.51 \times 10^3 M^{-1} cm^{-1}$, $\epsilon_{em\ 337/ex\ 405} = 1.46 \times 10^3 M^{-1} cm^{-1}$). Selenoneine is an optically active substance based on the measured specific rotation,

$\alpha_D^{20} + 23^\circ$. The measured 50% radical scavenging concentration (RS_{50}) with DPPH for the water-soluble vitamin E-like antioxidant Trolox, l-ergothioneine, and the reduced selenoneine form was 880, 1700, and 1.9 μM , respectively, indicating that selenoneine has greater antioxidant activity than Trolox and ergothioneine.

To determine the tissue distribution of selenoneine, we developed a speciation analysis method for organic selenium in animal tissues based on monitoring ^{82}Se using LC-ICP-MS with a GPC column (Fig. 3). Selenium compounds, such as GPx, selenite, selenocysteine, selenomethionine, and selenoneine, were used as standards. The contents of selenoneine, selenoproteins including GPx and selenoprotein P, and other unidentified selenium compounds were determined in various animal tissues, whereas selenite, selenocysteine, and selenomethionine were not detected in the tissues that we examined in this study (Table 1, supplemental Figs. S5–S7). Because selenoneine was associated with the gel matrix, it was eluted 10.5 min after the bed volume of the column. The tuna red muscle contained selenoneine at 190 nmol of selenium/g and selenoproteins at 4.5

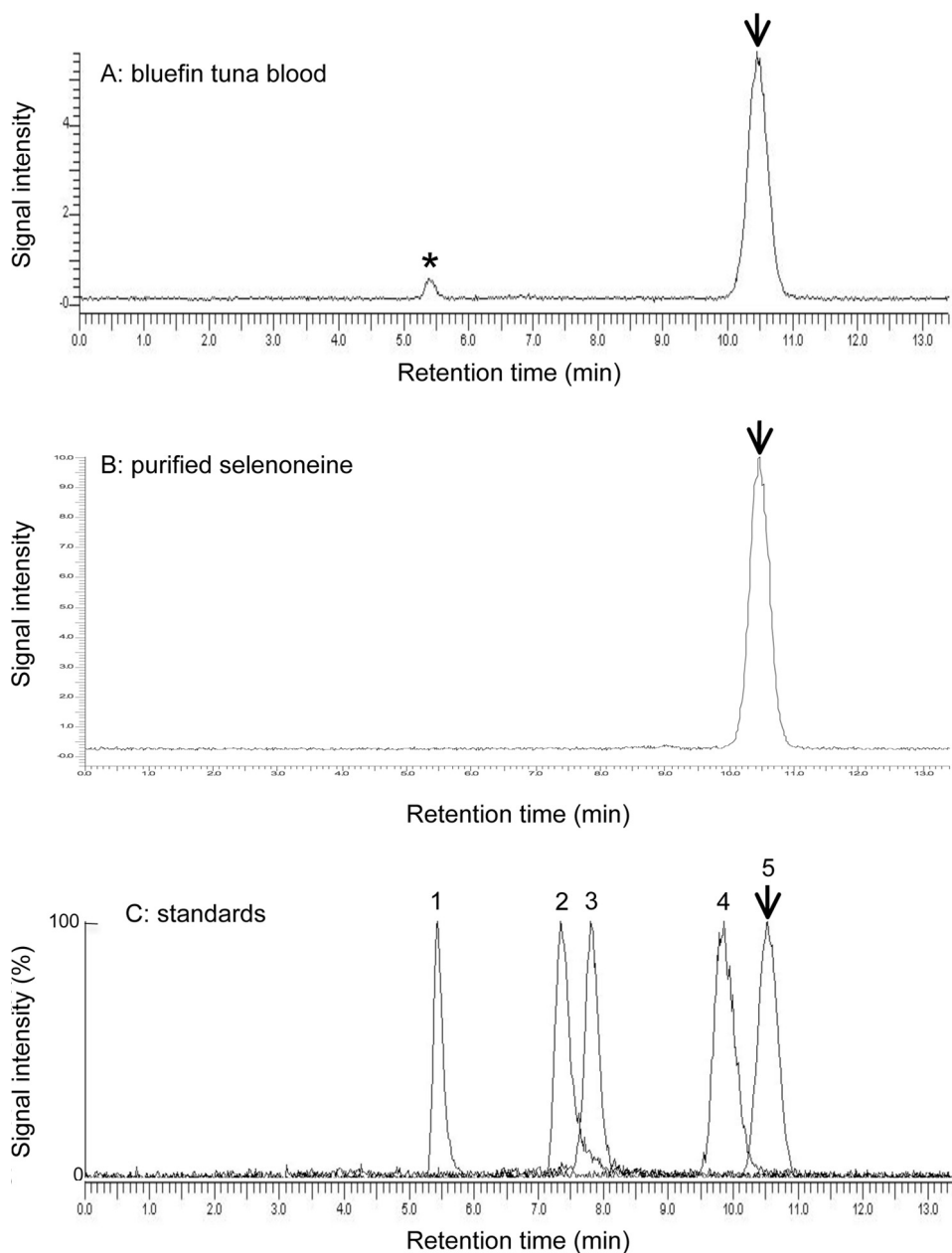


FIGURE 3. Speciation analysis of organic selenium in the fish and other animal tissues by LC-ICP-MS. The speciation of water-soluble selenium compounds in the tissues of bluefin tuna and other animal tissues based on LC-ICP-MS analysis is shown. 0.1 g of sample was homogenized with 5× water, and 20 μ l of supernatant was injected to LC-ICP-MS after 2–200× dilution with mobile phase (0.1 M ammonium formate). *A*, the blood of farm-raised bluefin tuna. *B*, purified selenoneine from the tuna blood. *C*, standards. For example, *peak 1*, bovine erythrocyte GPx; *peak 2*, selenite; *peak 3*, selenocysteine; *peak 4*, selenomethionine; *peak 5*, selenoneine. An Ultrahydrogel 120 (7.8 × 250 mm) column equilibrated with 0.1 M ammonium formate buffer (flow rate at 1 ml/min) was used. An asterisk indicates selenoproteins including GPx eluted near the void volume of the column. An arrow shows the elution of selenoneine.

nmol of selenium/g, indicating that almost all of the selenium (98%) was present as selenoneine. Other tissues, such as the spleen, hepatopancreas, heart, white muscle, and blood, also contained high levels of selenoneine at 2.4–430 nmol of selenium/g. Pacific mackerel blood, tilapia blood, porcine kidney, chicken heart, gizzard, liver, and squid hepatopancreas also contained selenoneine and selenoproteins, whereas porcine liver contained only selenoproteins and not selenoneine. In the tilapia blood, selenoneine was contained in erythrocytes, but not in serum (supplemental Fig. S6). Therefore, selenoneine is

distributed in various animal tissues and at especially high levels in tuna tissues, indicating that it is the predominant form of selenium in tuna tissues.

DISCUSSION

A novel selenium-containing compound was purified and identified from tuna blood. High resolution mass spectrometry and NMR spectroscopy showed that the compound was the oxidized dimeric form of the ergothioneine selenium analog in which the sulfur was replaced by selenium. Therefore, we propose that this selenium compound, 2-selenyl- $N_{\alpha},N_{\alpha},N_{\alpha}$ -trimethyl-L-histidine, be named selenoneine; this name is derived from the terms selenium and ergothioneine, which was previously identified in ergot (21) and blood (22).

Selenium speciation analysis using LC-ICP-MS indicated that selenoneine is the major selenium compound and is widely distributed in animal tissues, including the blood, hepatopancreas, spleen, heart, and skeletal muscle of the tuna, mackerel and tilapia blood, porcine kidney, chicken gizzard, liver, heart, and squid hepatopancreas. The tuna and mackerel blood contained high levels of selenium as selenoneine at 430–437 nmol of selenium/g. Further study is needed to examine whether selenoneine might be a non-toxic organic selenium in animal tissues and cells, in comparison with the high toxicity of known selenium compounds such as selenocysteine (LD_{50} 35.8 mg/kg), selenomethionine (LD_{50} 4.3 mg/kg), and selenite (LD_{50} 3.5 mg/kg) (23–26). Selenoneine was converted from the reduced form to the oxidized dimer in the presence of the nonpolar organic solvent tetrahydrofuran, whereas the oxidized dimeric form was reduced to the monomer in the presence of glutathione or DTT at a concentration of 10 mM. Furthermore, selenoneine had strong radical-scavenging activity against DPPH *in vitro*. Therefore, selenoneine may be an important member of the redox cycle in animal cells. Our unpublished data³ suggest that selenoneine binds to the heme

³ Y. Yamashita, T. Yabu, and M. Yamashita, unpublished data.

TABLE 1
Speciation of water-soluble selenium compounds in the tissues of bluefin tuna and other animals based on LC-ICP-MS analysis

Contents of selenoneine, selenoproteins including GPx and selenoprotein P, and unidentified selenium compounds in the tissues of bluefin tuna, Pacific mackerel, tilapia, chicken, pig, and Japanese common squid are shown. 0.1 g of sample was homogenized with 5× water, and 20 μl of supernatant was injected to LC-ICP-MS after 2–200× dilution with mobile phase (0.1 M ammonium formate). Values are the mean ± S.D. of three individuals.

| Species and tissue | Selenoneine | Selenoproteins ^a | Unidentified selenium compounds |
|----------------------------------|-----------------|-----------------------------|---------------------------------|
| | nmol of Se/g | nmol of Se/g | nmol of Se/g |
| Bluefin tuna, farm-raised | | | |
| Blood | 430 ± 82 | 24 ± 6.1 | ND ^b |
| Spleen | 41 ± 16 | 14 ± 2.5 | 4.0 ± 1.5 |
| Hepatopancreas | 39 ± 7.5 | 28 ± 12 | 2.2 ± 0.6 |
| Heart | 15 ± 15 | 4.8 ± 2.3 | 1.5 ± 1.0 |
| Red muscle | 190 ± 8.0 | 4.5 ± 0.5 | 5.3 ± 1.7 |
| White muscle | 2.4 ± 0.3 | 1.4 ± 0.2 | 0.5 ± 0.1 |
| Bluefin tuna, wild | | | |
| Blood | 83 ± 29 | 28 ± 6.5 | ND ^b |
| Pacific mackerel | | | |
| Blood | 437 ± 159 | 56 ± 10 | ND ^b |
| Tilapia | | | |
| Blood | 0.9 ± 0.6 | 6.8 ± 3.2 | 0.55 ± 0.36 |
| Chicken | | | |
| Liver | 0.3 ± 0.1 | 6.7 ± 1.0 | ND ^b |
| Heart | ND ^b | 2.0 ± 0.4 | 1.8 ± 0.4 |
| Gizzard | ND ^b | 2.7 ± 0.5 | ND ^b |
| Pig | | | |
| Liver | ND ^b | 8.0 ± 1.2 | 5.0 ± 1.1 |
| Kidney | 0.36 ± 0.1 | 3.3 ± 1.5 | ND ^b |
| Japanese common squid | | | |
| Hepatopancreas | 9.3 ± 3.5 | 5.0 ± 1.1 | 7.0 ± 1.1 |

^a Selenoproteins eluted near the void volume of the column were estimated using bovine GPx as the standard.

^b ND, lower than detection limit at 0.25 nmol of Se/g.

of hemoglobin and myoglobin to protect it from the autoxidation of iron ions under low oxygen conditions. Previously, GPx and other selenoproteins, which are induced by selenium intake, were believed to enhance the antioxidant ability in animal tissues and cells (1–5, 14, 15). However, because selenoneine is the predominant chemical form in tuna tissues, selenoneine derived from a dietary intake of fish may play the major role as a strong radical scavenger in a variety of physiological and nutritional functions found in previous medical and biochemical studies (1–15). Further studies of the bioaccumulation of selenium as selenoneine, its metabolism, and biological function in the free radical detoxification mechanism using selenoneine as the selenium source should help to characterize the

essential role of selenium as selenoneine as a micronutrient for humans and animals.

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