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Identification of molecular species of selenium contained in several animal foods

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Summary

To elucidate the molecular species of selenium ingested by usual animal foods, molecular species of selenium in several animal food samples were identified by HPLC-inductively coupled plasma mass spectrometry (ICPMS). The samples used were as follows: dark muscle of yellowfin tuna, fish meat (skipjack, horse mackerel, seabream, and yellowfin tuna), shellfish (asari clams, shijimi clams, and oysters), chicken tender, chicken and bovine liver, and hen's whole eggs. An HCl extract and a protease extract were prepared for each sample. Except for the dark muscle, no peaks of selenium compounds could be detected in the analysis by HPLC-ICPMS for the HCl extracts. In contrast, the protease extracts from fish meat and shellfish showed a peak identified as selenomethionine in the HPLC-ICPMS chromatogram. Furthermore, it was confirmed that selenocystine was present in addition to selenomethionine in the protease extract from animal foods derived from poultry and livestock. These results indicate that the intake of general seafood leads to the intake of selenomethionine, and the intake of animal foods derived from livestock and poultry leads to the intake of selenomethionine and selenocystine.

Selenium is an essential trace element in mammals, and it has various functions in the form of selenoprotein having selenocysteine residues[1]. Selenium deficiency will affect the function of important organs of the human body, leading to the occurrence of characteristic diseases[2]. Epidemiologically, it has been reported that the risk of various chronic diseases including cancer increases when the intake of selenium falls below a certain level[3]. In animal experiments, it is also known that tumor formation caused by various chemical substances is suppressed by supranutritional administration of selenium compounds[4] or selenium-enriched plant materials[5].

Selenium is also considered to have different nutritional or supranutritional availability depending on the food, because the chemical forms of selenium are diverse[6]. Therefore, identifying the molecular species of selenium in food is important for predicting the physiological function of selenium contained in food. Foods containing a relatively large amount (0.1 ppm or more) of selenium include seafood, livestock products, and grains[7]. Selenomethionine has been shown to be present in the proteins of ordinary wheat[8] and soybeans[9]. Most of the selenium in selenium yeast used in selenium supplements is also selenomethionine combined with protein[10]. Free unique selenoamino acids were found in selenium-enriched plant materials[11–15]. In recent years, a low-molecular-weight selenium compound called selenoneine has also been identified in tuna blood, organs, and dark muscle[16]. However, few studies have rigorously identified the molecular species of selenium in animal foods. In this study, molecular species of selenium contained in several animal foods were identified by HPLC-inductively coupled plasma mass spectrometry (ICPMS) measurement.

Materials and Methods

Sample preparation. Dark muscle of yellowfin tuna was purchased from MARUEI-SHOJI (Naha). Other animal foods used as analytical samples were purchased from local retail stores in and around Osaka City and are listed in Table 1. Each sample was freeze-dried and then pulverized. To facilitate pulverization, the liver samples were defatted with hexane before freeze-drying. Fifty mg of each powdered sample was suspended in 1.92 mL of sodium phosphate buffer (10 mM, pH 7.1), stirred sufficiently, and then 0.08 mL of 5 M HCl was added. After centrifug-

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Table 1 Selenium content in several animal foods used as analytical samples

Sample	Selenium content (μg/g dry weight)
Dark muscle of fish	
Yellowfin tuna, *Thunnus albacares*	12.50
Fish meat (fish muscle)	
Skipjack, *Katsuwonus pelamis*	3.01
Japanese horse mackerel (Jack mackerel), *Trachurus japonicus*	1.05
Japanese red seabream, *Pagrus major*	0.84
Yellowfin tuna, *Thunnus albacares*	8.55
Shellfish	
Asari clam (Short-necked clam), *Ruditapes philippinarum*	2.78
Shijimi clam (Freshwater clam), *Corbicula japonica*	1.84
Oyster, *Crassostrea gigas*	6.50
Poultry and livestock	
Chicken tender	0.35
Chicken liver	1.16
Bovine liver	6.25
Hen's whole egg	0.56

ing the mixture (1,500 × *g*, 60 min), the supernatant was collected and filtered through a 0.45 μm membrane filter. This filtrate was used as an HCl extract. To another 50 mg of each sample, 1.92 mL of the phosphate buffer and 5 mg of protease (type XIV derived from *Streptomyces griseus* (Merck KGaA, Darmstadt)) were added, and the mixture was thoroughly stirred and shaken at 37℃. After 24 hours, 0.08 mL of 5 M HCl was added and the mixture was centrifuged at 1,500 × *g* for 60 min. The supernatant was collected and filtered through a 0.45 μm membrane filter. This filtrate was used as a protease extract.

Analysis of total selenium. To 100 to 500 mg of each dry powdered sample, 5 mL of nitric acid and 2 mL of perchloric acid were added and heated to incinerate. Ultrapure water was added to the ashed sample, the volume was raised to 10 mL, and the mixture was filtered through a 0.45 μm membrane filter. Then, selenium in the filtrate was quantified by ICPMS using ICPM2030 (Shimadzu. Kyoto). The analytical mass number of selenium in ICPMS was 82, and indium 115 was used as an internal standard.

Analysis of molecular species of selenium. Molecular species of selenium in the HCl or protease extract was analyzed by an HPLC-ICPMS system. The HPLC system consisted of a LC-20Ai multi-pump (Shimadzu. Kyoto), an DGU-20A3R on-line degasser (Shimadzu. Kyoto) and a reversed phase separation column (Develosil® RPAQUEOUS-AR column, 4.6 i.d. mm × 250 mm, Nomura Chemical, Seto, Japan). The mobile phase was the same as previously used[11]; methanol/distilled water (HPLC grade) (v/v = 0.05/99.95) containing 2.5 mM sodium 1-butanesulfonate, 4 mM malonic acid, and 15.9 mM tetramethylammonium hydroxide. The pH value of the mobile phase was adjusted to 2.3 by the dropwise addition of diluted nitric acid. Elution was performed isocratically at 0.5 mL/min at 30℃, and a sample aliquot of 20 μL was injected into the LC system. The eluate was directly led to the ICPMS nebulizing tube and monitored at ion intensities of m/z 82. The molecular species of selenium in the extract were identified by comparison with the retention times of standard selenium compounds (L-selenocystine (Sigma-Aldrich, St. Louis), *Se*-methylselenocysteine hydrochloride (Sigma-Aldrich, St. Louis), L-selenomethionine (Sigma-Aldrich, St. Louis), and selenohomolanthionine (kindly supplied by Prof. Ogra Y, Chiba University)).

Results

Selenium content in animal food samples. In Table 1, the total selenium content in the animal food samples used is described. Among the samples used, the selenium concentration of dark muscle was highest. Among other samples, selenium concentration of yellowfin tuna meat, oysters, and bovine liver were more than 5 μg/g dry weight, and the selenium concentration of yellowfin tuna meat reached 8.55 μg/g dry weight. Skipjack meat and asari clams had selenium at a level of about 3 μg/g dry weight, and the remaining samples had selenium concentrations of

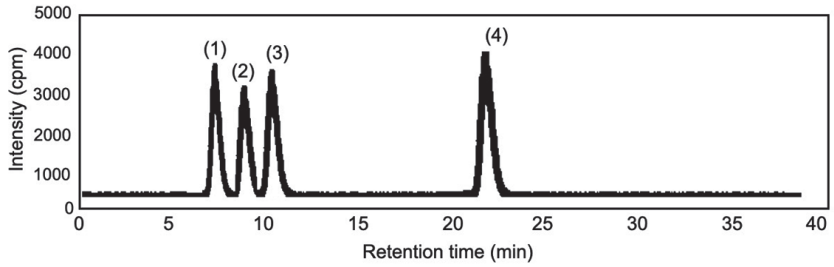

Fig. 1 Elution pattern of authentic selenium compounds in HPLC-ICPMS. (1) selenocystine, (2) selenohomolanthionine, (3) *Se*-methylselenocysteine, (4) selenomethionine.
The method of analysis is described in the text. The concentration of each compound was 10 μg Se/mL.

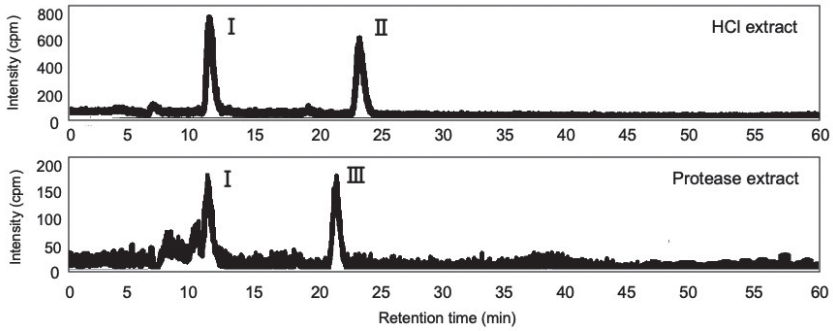

Fig. 2 Elution patterns of HCl extracts (upper side) and protease extracts (underside) from dark muscle of yellowfin tuna in HPLC-ICPMS

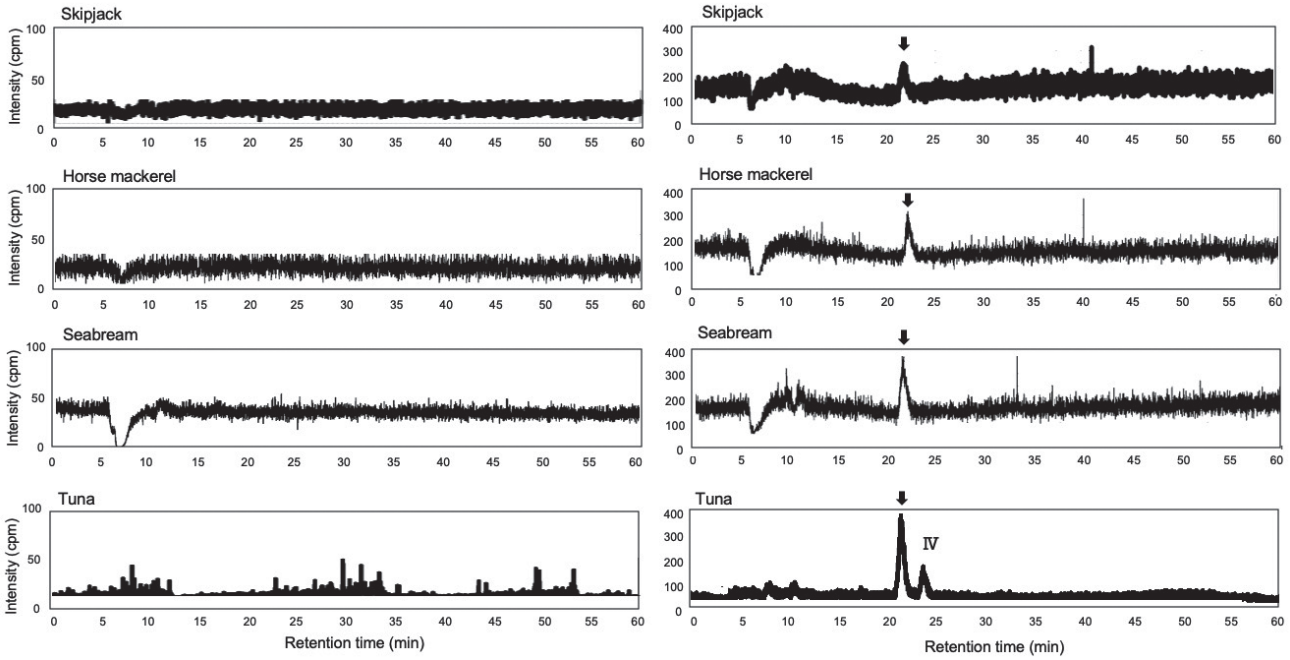

Fig. 3 Elution patterns of HCl extracts (left side) and protease extracts (right side) from several fish meat samples in HPLC-ICPMS
The method of analysis is described in the text. The black arrow indicates a peak with a retention time almost the same as that of selenomethionine.

less than 2 μg/g. In particular, the selenium concentration in chicken tender and hen's egg was only 0.35 and 0.56 μg/g dry weight, respectively.

Elution pattern of standard selenium compounds. Figure 1 shows the elution patterns of the four standard selenium compounds in HPLC-ICPMS. Under the analytical conditions used, the four compounds showed distinctly different retention times. The retention time for each was as follows: selenocystine, 7 min; selenohomolanthionine, 9 min; *Se*-methylselenocysteine, 10.5 min; and selenomethionine, 22 min.

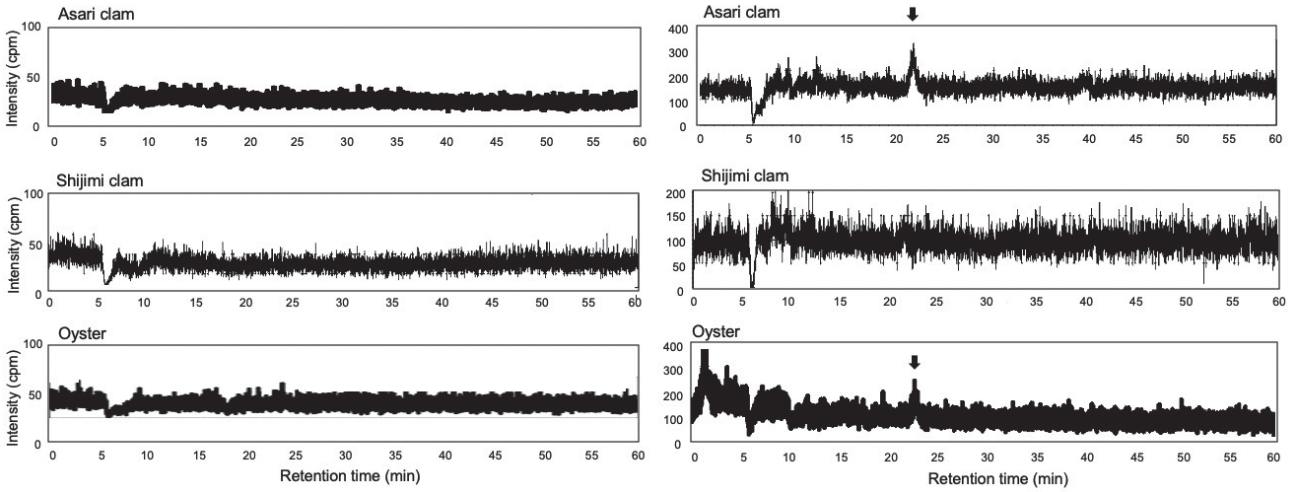

Fig. 4 Elution patterns of HCl extracts (left side) and protease extract (right side) from several shellfish samples in HPLC-ICPMS
The method of analysis is described in the text. The black arrow indicates a peak with a retention time almost the same as that of selenomethionine.

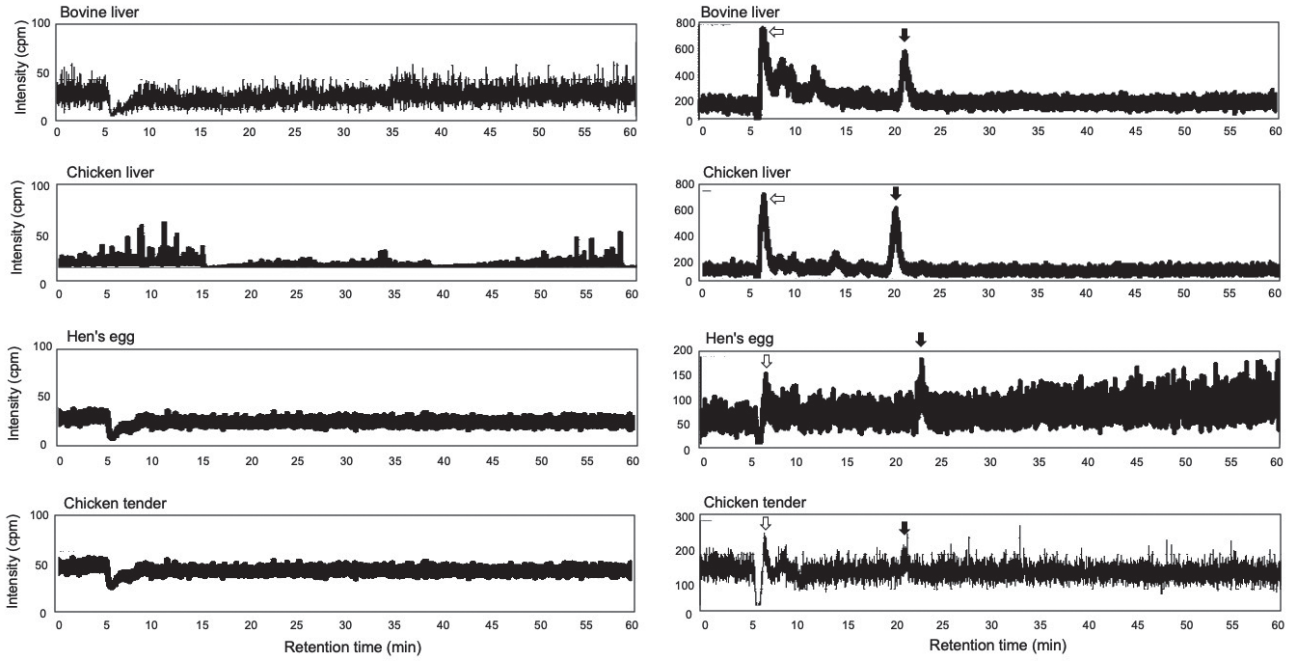

Fig. 5 Elution patterns of HCl extracts (left side) and protease extracts (right side) from other animal food samples in HPLC-ICPMS
The method of analysis is described in the text. The white and black arrows indicate peaks with retention times almost the same as those of selenocystine and selenomethionine, respectively.

Identification of selenium species in the extract. Figure 2 shows the elution pattern of the HCl extract and protease extract from dark muscle of tuna in HPLC-ICPMS. The HCl extract showed two large peaks (peaks I and II). The retention time was 12 min for peak I and 24 min for peak II, both of which were different from those of the four standard selenium compounds shown in Fig. 1. On the other hand, in the protease extract, the peak I shown in the HCl extract was also observed, but the peak II disappeared, and a new peak III with a retention time of 22 min, which was the same as that of selenomethionine, appeared.

Figure 3 shows the elution pattern of the HCl extracts (left side) and protease extracts (right side) from several fish meat samples in HPLC-ICPMS. In the HCl extract, no obvious chromatographic peak was detected. On the other hand, all the protease extracts showed a clear peak at the position with a retention time of 22 min, which was the same as selenomethionine. Furthermore, in the chromatogram of yellowfin tuna, two minor peaks appeared during the retention time of 7 to 11 min, and another peak (peak IV) was observed after the large 22 min peak.

Figure 4 shows elution patterns of the HCl extracts (left side) and protease extracts (right side) from several shellfish samples in HPLC-ICPMS. There was no valid chromatographic peak in the HCl extract. Similar to the fish meat, the protease extract from asari clam showed a clear

peak at a retention time of 22 min. In the oyster, the peak at a retention time of 22 min was very small and appeared to have a broad peak at a retention time of around 1.5 min, which corresponds to the dead volume of the analytical system. In the protease extract from shijimi clam, no clear peak could be detected.

Figure 5 show elution patterns of the HCl extract (left side) or protease extract (right side) from other animal food samples in HPLC-ICPMS. Similar to the fish meat and shellfish, no peak could be detected in the case of the HCl extract in any of the samples. On the other hand, in the case of the protease extract, different from fish meat and shellfish, multiple peaks were detected. In particular, among the peaks obtained with the extracts from bovine and chicken liver, the retention times of the two peaks were approximately 7 min and 22 min, which were consistent with those of selenocystine and selenomethionine, respectively. The 7 min and 22 min peaks were also observed in the protease extracts from whole hen's eggs and chicken tender, although they were very small. In addition to these two peaks, the presence of another peak was also confirmed in the protease extract from the liver. In particular, in the case of bovine liver, there were multiple other peaks, all of which were relatively large.

Discussion

The analytical results of the HCl extract from the dark muscle of tuna showed that at least two selenium compounds (Fig. 2, peaks I and II) were present in this extract. It has been reported that selenoneine, a low molecular weight selenium compound, is present in the blood, organs, and dark muscle of tuna[16]. A compound corresponding peak I was also found in our previous studies in which HCl extract from dark muscle of tuna was analyzed[17] and is likely to be selenonein[16]. On the other hand, in the protease extract, peak I was also observed, but peak II disappeared, and a new peak (peak III) with the same retention time as selenomethionine appeared. From this, it is highly possible that Peak II is a peptide containing selenomethionine. The sample dark muscle was delivered to our laboratory in a refrigerated state rather than frozen, and myoglobin was oxidized and turned brown. Therefore, the selenium-containing peptide corresponding to peak II may appear by partially decomposing the protein in the dark muscle. Therefore, it is necessary to analyze a fresh dark muscle.

In the present experiment, HCl extracts obtained from other animal foods were also analyzed by HPLC-ICPMS, but no peaks derived from selenium compounds could be detected (Fig. 3 to 5). These results indicate that selenoneine is rarely found in general fish meat and shellfish that make up most of seafood. On the other hand, in the HPLC-ICPMS analysis of protease extracts from fish meat and shellfish, a selenium compound was eluted at the same retention time as selenomethionine (Fig. 3 and 4). This strongly suggests that most of the selenium in these extracts is present in the form of selenomethionine. Multiple peaks other than selenomethionine were observed in the protease extract from yellowfin tuna. Among them, since retention time of the peak IV is the same as that of peak II in Fig. 1, this would be a peptide bound with selenomethionine, which was produced due to insufficient hydrolysis by protease. Since organisms cannot distinguish between selenomethionine and methionine, selenomethionine is incorporated nonspecifically at the position of methionine residues during protein biosynthesis[18]. Accordingly, most of the selenium in fish muscle may be present as selenomethionine residues in proteins such as actomyosin. The existence of several specific selenoproteins containing selenium in the form of selenocysteine residue is also known in fish[19]. However, they may be present in blood and organs, and almost never in the edible muscles. Therefore, it can be concluded that the intake of seafood commonly eaten means the intake of selenomethionine and intake of selenoneine occurs only in unusual seafood.

In the case of oysters, which had the highest selenium concentration in shellfish, most of the selenium in the protease extract appeared to be eluted at the retention time corresponding to the dead volume of the analytical system. This suggests that most of the oyster selenium was released as a highly polar substance by the protease treatment. Therefore, it is necessary to further study the molecular species of selenium contained in oysters.

Unlike fish and shellfish, protease extracts from beef and chicken liver contained selenium compounds with the same retention time as selenocystine in addition to selenomethionine in the HPLC-ICPMS (Fig. 5). This result shows that selenocystine is contained in the protease extract. Since free selenocysteine is easily oxidized to selenocystine and the liver of mammals and birds is known to have many kinds of specific selenoproteins containing selenium in the form of selenocysteine residues[1], this selenocystine is thought to be derived from selenoproteins. Peaks of unknown selenium compounds other than selenomethionine or selenocystine were observed on the chromatogram of the protease extract from liver (Fig. 5). As with yellowfin tuna, it is presumed that these selenoamino acids bound peptide were produced, probably due to incomplete hydrolysis by the protease. These indicate that eat-

ing at least the offal of animals leads to the intake of selenocystine and selenomethionine. Since animals are unable to synthesize selenomethionine, the selenomethionine found in the liver may be derived from plants contained in animal feed. Hen's egg and chicken tender have a low selenium content (Table 1), so the peaks of selenocystine and selenomethionine in their protease extracts were traces (Fig. 5). Therefore, it cannot be asserted about the molecular species of selenium in eggs and common meat. However, like the liver, it is highly likely that they are selenocystine and selenomethionine. From the above, it is presumed that eating food obtained from livestock and poultry leads to the intake of selenomethionine and selenocystine.

In the present analysis, the selenium concentration in the sample was not necessarily proportional to the peak height of the selenium compound in the HPLC-ICPMS analysis of the protease extract. This indicates that the degree of hydrolysis by the protease may differ from sample to sample. Therefore, conditions of protease treatment need to be reexamined for a quantitative study of the molecular species of selenium in animal foods.

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