In vitro assessment of bioavailability of selenium from a processed Japanese anchovy, Niboshi

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ABSTRACT

Niboshi is a commonly used foodstuff that is processed from Japanese anchovy (*Engraulis Japonicus*) in Japanese cuisine. We previously demonstrated that Niboshi and its water extract contained highly bioavailable selenium for selenium deficient mice. In this study, we assessed the selenium bioavailability from the extract of the Niboshi using cultured cells. The activity of selenium-dependent glutathione peroxidase (GPx) of rat dorsal ganglion cells and human cervical carcinoma cells incubated with selenium from the Niboshi extract was over 2 times of that of the extract-free control cells and comparable to that of cells incubated with selenious acid of the same selenium concentration. These results suggested that selenium from the Niboshi extract was utilized for synthesis of the selenoprotein. Such *in vitro* selenium bioavailability probably reflected our previous results of *in vivo* assessment in mice.

Key words: seafood, dorsal root ganglion cells, HeLa cells, Japanese anchovy

1. Introduction

Selenocysteine (SeCys) is the major chemical form of selenium species in organisms and co-translationally incorporated into SeCys containing proteins, selenoproteins (Labunskyy, Hatfield, & Gladyshev, 2014). Selenium in the environment is ingested in the food chain through plants and animals, and almost all selenium required for the selenoprotein synthesis in humans is provided from the dietary food. Twenty-five human selenoproteins have been identified (Kryukov et al., 2003): glutathione peroxidases (GPxs) are the best-known selenoprotein family and critical enzymes in the antioxidative defense systems (Rayman, 2012). Because selenium is a micronutrient whose deficiency and toxic levels are close to each other, it is important to know its abundance and deficiency in foodstuffs. Additionally, food with a high-selenium content are not necessarily better selenium sources, but we should consider the bioavailability of selenium from food. Although fish and shellfish contain relatively higher concentrations of selenium, the number of reports on the selenium bioavailability from seafood are fewer than that from meats, cereals, vegetables, *etc* (Dumont, Vanhaecke, & Cornelis, 2006; Finley, 2006; Moreda-Piñeiro & Moreda-Piñeiro, 2015; Yoshida, Haratake, Fuchigami, & Nakayama, 2011). This is because selenium in certain fish meat was reported to be less bioavailable due to the complex formation with heavy metal species, such as mercury (Afonso et al., 2015; Ralston & Raymond, 2010). The selenium bioavailability in several tuna products were compared to that in wheat products, and a significantly lower activity of GPx in the tissue of tuna products-fed rats was observed in contrast to that of wheat products-fed rats (Alexander, Whanger, & Miller, 1983). Meanwhile, several papers described that the bioavailability of selenium from fish and shellfish was higher than that of inorganic selenium and selenized yeast, commonly used as nutritional supplements. Fox et al. (2004) assessed the bioavailability of selenium from

selenium-enriched trout fish and demonstrated that fish selenium was highly bioavailable in comparison to selenized yeast and sodium selenate. Hepatic cellular GPx activity of the mice fed with the diet supplemented by defatted dark muscle of tuna was higher than that of the mice fed with selenite-supplemented diet (Yoshida, Abe, Fukunaga, & Kikuchi, 2002). However, 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.

The selenium-deficient diseases and related pathologies have never been reported in Japan under normal nutritional conditions. This was thought to be attributed to the dietary habit that the Japanese population frequently have selenium-abundant fish and shellfish materials in their meals. Miyazaki, Koyama, Sasada, Satoh, Nojiri and Suzuki (2004) reported that fish and/or shellfish are the major dietary sources of selenium for the Japanese population (~60% of daily intake). In the Japanese diets, Niboshi (~1 μgSe/g) is a commonly used foodstuff that is processed from the Japanese anchovy (*Engraulis japonicus*), and its extract is used as a general base seasoning for a wide variety of Japanese cuisines, just like the *fumet de poisson* and/or *fond de veau* for the French cuisine. We have previously shown that selenium from the Niboshi and its extract could restore the activity of the hepatic cellular GPx in dietary selenium-deficient mice, and the ionic organic compound with a molecular mass of less than 5,000 was the major selenium species in the Niboshi extract (Haratake, Takahashi, Ono, & Nakayama, 2007; Yoshida, Haratake, Fuchigami, & Nakayama, 2012). In this study, the authors addressed the *in vitro* assessment of the bioavailability of partially purified selenium species from the Niboshi extract using cultured cells.

The bioavailability of selenium from foodstuffs is usually assessed using dietary selenium-deficient animals. However, the assessments using experimental animals are time-consuming and tedious. In addition, a large amount of selenium compounds is required for the animal experiments. Some researchers used cultured cells to assess the absorption

and utilization of selenium species. Zeng, Botnen and Johnson (2008) used selenium deficient human colon cancer Caco-2 cells as alternatives to selenium-deficient animals to assess the bioavailability of selenium species in selenized broccoli. Human hepatoma HepG2 cells are also used to evaluate the antioxidative effect of selenium compounds (Cuello et al., 2007; Marschall, Bornhorst, Kuehnelt, & Schwerdtle, 2016).

In this study, both primary-cultured dorsal root ganglion (DRG) cells and HeLa cells (human cancer cells) were used to investigate whether selenium from the Niboshi extract can be used for the synthesis of selenoproteins. Both cells were incubated with the partially purified selenium species in the Niboshi extract followed by determination of their cellular GPx activity.

2. Experimental

2. 1. Niboshi samples

Niboshi is a popular processed food product from Japanese anchovy. The size of 5–12 cm length of Japanese anchovy caught in the near shore of Japan were boiled in sea water for a few minutes immediately after unloading and dried in the sun or oven. Five hundred-gram packages of the Niboshi products were purchased at local grocery stores and stored at –20 ºC until use without any treatments.

2. 2. Preparation of the Niboshi extracts

Three pieces of almost size-matched Niboshi (~3 g) were placed in a non-woven fabric bag after cutting into ~10 pieces of almost equal size. These samples were placed in 30 mL of water, then heated and boiled for 10 minutes. The obtained solution was filtered through

105 paper (particle size cutoff, 4 μm) and water was added to make a final volume of 20 mL,
106 which was used as the Niboshi extract in this study. The concentrations of the selected
107 substances in the Niboshi extracts were calculated by the following equation: = [(amount of
108 substances in the extract used for analysis) / (volume of the extract used for analysis)] ·
109 [(Total volume of the extract prepared) / (weight of the Niboshi used for extraction)] (μg or
110 mg/g-Niboshi).

111

112 2. 2. Determination of selenium concentration

113 The selenium concentrations in the specimens were fluorometrically determined using
114 2,3-diaminonaphthalene (DAN, Tokyo Chemical Ind., Tokyo, Japan) after digestion with
115 nitric acid and perchloric acid (Watkinson, 1966). After the reaction with the digested
116 sample and 0.1% DAN-0.1 M HCl solution, the fluorescent intensity of the piaselenol
117 generated from DAN (the excitation wavelength: 375 nm, the emission wavelength: 520 nm,
118 working concentration range: 0.5–10000.0 ngSe/sample) was measured by a FP-6600
119 spectrofluorometer (JASCO, Tokyo, Japan). The selenium standard solution (Kanto
120 Chemical Co., Inc., Tokyo, Japan) [1000 ppm as selenium (IV) dioxide in 0.1 M nitric acid]
121 was used for preparation of the calibration curve.

122

123 2. 3. Determination of selected component concentrations

124 After the digestion of Niboshi and its extract with nitric acid and perchloric acid, the
125 specimens were diluted with nitric acid or water and adjusted to a 10 mL-volume in a
126 volumetric flask. These solutions were subjected to the elemental analysis after the proper
127 dilution. The sodium, magnesium, potassium and calcium contents in the specimens were
128 determined using an AAnalyst 200 flame atomic absorption spectrometer (Perkin-Elmer, Inc.,

Waltham, MA). Atomic absorption spectrometry grade standard solutions of sodium, magnesium, potassium and calcium (Kanto Chemical Co., Inc., Tokyo, Japan) were used for preparation of the calibration curves. The determination of the phosphorus content was based on vanadium (V) that is capable of forming a yellow-colored complex with the phosphate ion. After the addition of the 0.25% ammonium vanadate solution and 5% hexaammonium heptamolybdate tetrahydrate solution to digested samples, the color produced by the complexation was monitored at 440 nm by a V-660 UV-Visible spectrophotometer (JASCO, Tokyo, Japan) (Kiston & Mellon, 1944).

The chlorine content in the Niboshi extract was directly measured by the Mohr method. After a 4-fold dilution with deionized water, the Niboshi extract was mixed with potassium chromate and then titrated with 0.01 M silver nitrate solution.

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 100-fold volume of 1.8 mM *o*-phthalaldehyde and 6.9 mM dithiothreitol (DTT) in 0.02 M borate buffer (pH 9.5) to form a thio-substituted isoindole fluorophore (the excitation wavelength: 340 nm, the emission wavelength: 450 nm, working concentration range: 0.1–50 μM) (Jones, Owen, & Farrar, 2002). Glycine dissolved in water was used to make the calibration curve.

For the protein concentration determination, the sample was appropriately diluted with water, then directly measured by Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951). The diluted sample was mixed with a 5-fold volume of a 10 : 1 : 1 mixture of 2% Na_2CO_3-0.1 M NaOH, 1% $CuSO_4 \cdot 5H_2O$ and 2% sodium (+)-tartrate dihydrate and allowed to react for 10 min. A half volume sample of Folin-Ciocalteu's reagent solution diluted 2-fold with water was added and allowed to react for 30 min. The absorbance at 650 nm was monitored by a V-660 UV-Visible spectrophotometer (JASCO, Tokyo, Japan) and bovine serum albumin was used as the reference (working concentration range: 0.01−0.5 mg/mL).

155

156 2. 4. Ion-pair extraction and mass spectrometry

157 Hexadecyltrimethylammonium chloride (HTAC, Tokyo Chemical Ind., Tokyo, Japan)
158 dissolved in water was used for the ion-pair extraction. The HTAC solution was combined
159 with the Niboshi extract to make its final concentration 1 mM, followed by extraction with
160 chloroform. The obtained chloroform layer was concentrated *in vacuo* and subjected to fast
161 atom bombardment mass spectrometry (FAB-MS) or matrix assisted laser desorption
162 ionization time-of-flight mass spectrometry (MALDI TOF-MS). The mass spectra were
163 acquired in the linear positive ion mode by a JMS-700N (JEOL, Tokyo, Japan) using
164 *m*-nitrobenzyl alcohol as a matrix and an Ultraflex (Bruker Daltonics, Inc., USA) using
165 2,5-dihydroxybenzoic acid as a matrix.

166

167 2. 5. Cell culture

168 DRG cells were isolated from male 3−7 week old Wistar rats (weighing 35–280 g,
169 specific pathogen free) from Clea Japan, Inc. (Tokyo, Japan). Rats were housed 1−3 / cage
170 (PC, 270 × 440 ×187 mm, with sawdust for animal experiments) on a 12 h light-12 h dark
171 schedule at 23 ± 2 ºC and 60% relative humidity. A regular breeding diet CE-2 (Clea Japan,
172 nutritional composition of the diet can be seen at http://www.clea-japan.com/Feed/ce2.html)
173 and deionized water (>18 MΩ cm) were supplied *ad libitum*. The spinal column was
174 excised from rats after the decapitation under inhalational anesthesia with 2–5% of isoflurane.
175 All experiments with live animals were performed in compliance with the guidelines of the
176 Nagasaki University on Animal Care and Use and Fundamental Guidelines for Proper
177 Conduct of Animal Experiment and Related Activities in Academic Research Institutions
178 (Ministry of Education, Culture, Sports, Science and Technology), and the institutional

committee approved the experimental procedures. The dissected DRG were treated with 0.05% collagenase in Hank's solution at 37 °C for 2 h and subsequently with 0.05% trypsin plus 0.02% EDTA in Hank's solution at 37 °C for 1 h to disperse the cells. After the enzymatic treatments, the DRG cells were washed with 10% fetal bovine serum (FBS)-containing Dulbecco's modified Eagle medium (DMEM) and plated on polystyrene dishes (90 mmø, Nalge Nunc Int'l, Waltham, MA) coated with poly-L-lysine (molecular mass > 30 kDa, FIJIFILM Wako Pure Chem. Ind., Osaka, Japan). The DRG cells were cultured in 10% FBS-containing DMEM under humidified 5% CO_2-95% air (NU-4750D, NuAire, Inc., Plymouth, MN) for 7−10 days and subjected to experimentation. Eleven rats were used throughout this study and DRG cells from one rat were equally seeded into 3 culture dishes.

The HeLa cells were purchased from the Cell Bank and maintained in a culture flask (canted neck with a screw-threaded cap, usable surface area of 75 cm^2, Nalge Nunc Int'l., Waltham, MA) with 10% FBS-containing DMEM under humidified 5% CO_2-95% air. The cells were subcultured at 50–80% confluence. The HeLa cells at 70–80% confluence were used in the experiments. Phosphate-buffered saline (PBS, calcium and magnesium free) was used for the experiments involving the HeLa cells.

2. 6. Assessment of selenium absorption behavior by DRG and HeLa cells

A half-liter of the Niboshi extract was lyophilized and finely ground in a mortar. The powdered Niboshi extract was suspended in 200 mL of ethanol, then sonicated in a bath-type sonicator followed by filtration using filter paper (particle size cutoff, 4 μm). The ethanol was removed under reduced pressure and the obtained residue was dissolved in 10% FBS-containing DMEM to make a selenium concentration of 1 μM followed by filtration using a sterilized disk filter with the pore size of 0.20 μm. An aliquot (1 or 2 mL) of the filtrate was added to the 5% FBS-containing DMEM to make a final selenium concentration

of 0.025 or 0.05 μM. Selenious acid (SA, H_2SeO_3) and seleno-L-methionine (SeMet) were dissolved in Hank's solution for the DRG cells or PBS for the HeLa cells at the selenium concentration of 20 μM, then 50 or 100 μL of these solutions was added to 40 mL of the 5% FBS-containing DMEM.

2. 7. Determination of cellular GPx activity

The DRG and HeLa cells were cultured in 5% FBS-containing DMEM supplemented with selenium species from the Niboshi extract. After incubation for the indicated days, the cells were washed with Hank's solution or PBS and treated with 0.05% trypsin, 0.02% EDTA in Hank's solution or PBS for 5–20 min. Cells detached from the culture dishes were collected by centrifugation at 1,000 *g*, then washed three times by gentle pipetting in Hank's solution or PBS. A two-fold volume of Milli-Q water (> 18 MΩ ·cm) was added to the cell suspensions for the osmotic cell lysis. The cell suspension was further sonicated in a bath-type sonicator for 5 min and vortexed for another 5 min. The lysed samples were centrifuged at 2,000 *g* for 10 min. The protein concentration in the resulting supernatant was determined by Lowry's method. The supernatant was used for the determination of the cellular GPx activity.

The cytosolic fraction was combined with NADPH solution (3.6 mM), reduced glutathione (GSH) solution (18 mM) and glutathione reductase solution (9.36 unit/mL) in 66 mM phosphate buffer (pH 7.4). The reaction was initiated by the addition of a hydrogen peroxide solution (1 mM). Absorbance at 340 nm due to the NADPH was recorded every 1 min for 3 min just after mixing by inversion. The NADPH is consumed by glutathione reductase during the reduction of oxidized glutathione (GSSG) to be generated from the GPx catalytic reaction. The GPx activity was calculated using equation (1) as μmoles NADPH oxidized per minute, where ΔA_{SAM} is the decrease in the absorbance at 340 nm of the sample

solutions for 1 min between 15 and 195 sec after addition of the substrates, ΔA_{BLK} is the decrease in absorbance at 340 nm per minute of the solutions using Milli-Q water instead of the sample solutions, 20.6 is the dilution factor, ε_{mM} is the extinction coefficient for the 1 mM NADPH solution (6.22 mM^{-1} cm^{-1}), and c is the protein content (μg/mL) (Flohé & Günzler, 1984).

$$\text{GPx activity} = (\Delta A_{SAM} - \Delta A_{BLK}) \times 20.6 / \varepsilon_{mM} / c \cdots\cdots (1)$$

2. 8. Statistical analysis

Data were presented as the mean and standard error (S. E.) (n = 3 or 4). Statistical analyses were performed using PRISM 4 (GraphPad Software, Inc., La Jolla, CA, USA). The multiple mean values were compared by a one-way analysis of variance with a Tukey post-hoc test. Comparisons were considered to be statistically significant at $P < 0.05$.

3. Results and discussion

3. 1. Composition of the Niboshi extract

The extract used for the analysis was prepared by the 10-min boiling of the Niboshi that was cut into 10 pieces (quite similar to the typically used extraction procedure for the domestic cuisine). The selenium concentration in the extract was 0.1229 ± 0.0101 μg/g-Niboshi (indicated as the amount of selenium extracted from the Niboshi used for extraction) and this corresponded to approximately 10% of selenium in the Niboshi (1.21 ± 0.03 μg/g). The concentrations of other substances in the extract, such as minerals (Na, Mg, P, Cl, K and Ca), amino acids and proteins were determined and listed in Table 1 along with those for the Niboshi. The concentrations of the other minerals were in the order of

253 potassium > sodium > chlorine > phosphorous > magnesium, and incomparably higher than
254 the selenium concentration. These inorganic materials accounted for more than 50% by
255 weight of the lyophilized Niboshi extract sample. Calcium, the most abundant inorganic
256 material in the Niboshi, was not detected in the extract. Similarly, the second most abundant
257 phosphorous and magnesium were less extractable from the Niboshi than sodium and
258 potassium. The concentrations of the amino acids and proteins in the extract were 9.592 ±
259 0.051 and 11.73 ± 1.02 mg/g-Niboshi, respectively. Selenium was reported to interact with
260 several heavy metals such as mercury. Selenium in certain marine fish at levels capable of
261 forming an equimolar complex with mercury was reported to reduce the toxicity of mercury
262 (Afonso et al., 2015; Ralston & Raymond, 2010). This was thought to be one of the reasons
263 for the low bioavailability of selenium in fish as compared to that in plant food and meat.
264 Selenium in migratory fishes (*e.g.*, tuna) forms a complex with mercury and/or proteins for
265 detoxification, and such a complex selenium species seems to be poorly bioavailable as a
266 nutrient. The total mercury content in the Niboshi determined by cold vapor atomic
267 absorption spectrometry was 0.06 μg/g and thus molar ratio of mercury and selenium (Hg/Se)
268 was less than 0.02 (Haratake et al., 2007). Thus, such a biologically unavailable selenium
269 complex with mercury was thought to be negligible in the Niboshi.
270
271 3. 2. Separation of selenium species in the Niboshi extract

272 We reported that the Niboshi extract contained ionic organoselenium species with a
273 molecular mass less than 5,000 (Yoshida, et al., 2012). As a result of ion-pair extraction
274 with HTAC and the following mass spectrometric analysis, the selenium isotopic pattern at
275 *m/z* 577 with the ^{80}Se ion peak was detected. This selenium compound was presumed to be
276 one of the major selenium species in the Niboshi extract.
277 The extraction of selenium species with an ion-pair reagent into chloroform was an

effective way to prepare samples for mass spectrometry because inorganic salts and amino acids could be removed. These substances in the Niboshi extract can be toxic to cultured cells. However, the ion-pair reagent itself can also damage the integrity of the biological membranes. Thus, the selenium species in the Niboshi extract were extracted with an organic solvent without HTAC. Because the selenium extraction rate from the lyophilized Niboshi extract with ethanol (ethanol-extract) was higher than that with the other organic solvent, such as chloroform, ethyl acetate and hexane, it was used for the selenium absorption experiments (Fig. 1). The extraction rate of the selenium species was ~17% of the total selenium in the Niboshi extract, which was higher than that by the ion-pair extraction procedure. When the ethanol-extract was subjected to FAB and MALDI TOF mass spectrometry, no selenium specific isotopic pattern was found. Thus, the selenium species in the ethanol-extract was further subjected to the ion-pair extraction using HTAC (Fig. 1). The ^{80}Se-containing molecular ion peak at *m/z* 577 with the accompanying selenium isotopic pattern was observed by FAB mass spectrometry (Fig. 2). This indicated that treatment of the lyophilized Niboshi extract with ethanol was effective to extract the selenium species with the ^{80}Se-containing molecular ion peak at *m/z* 577. Thus, we used the ethanol extract of the lyophilized Niboshi extract for the subsequent experiment with the DRG and HeLa cells.

3. 3. *In vitro* assessment of selenium species in the Niboshi extract.

Higher organisms requiring the selenium element can utilize a variety of inorganic and organic selenium compounds as selenium sources (Navarro-Alarcon & Cabrera-Vique, 2008; Rayman, 2012). In general, inorganic selenium compounds (*e.g.*, SA) are superior to organic ones regarding the bioavailability of selenium, while organoselenium (*e.g.*, SeMet) is less toxic than the inorganic forms (Marschall et al., 2016; Schrauzer, 2000; Weiller, Latta, Kresse, Lucas, & Wendel, 2004). In this study, inorganic SA and organic SeMet were used

304 as representative selenium sources for the absorption experiments to compare the
305 bioavailability of selenium from the Niboshi extract in the cultured cells, because these
306 compounds are most commonly used as references for the evaluation of nutritional effect and
307 toxicity of selenium compounds (Marschall et al., 2016; Zeng et al., 2008). To evaluate the
308 efficacy of absorbed selenium, the activity of the cellular GPx, which is the most abundant
309 intracellular selenoprotein, was measured. The activity of cellular GPx is reported to be
310 sensitive to the selenium content of the surroundings and frequently used to evaluate the
311 selenium availability (Labunskyy et al., 2014). DRG cells and HeLa cells were used for the
312 study of the selenium absorption behavior. DRG cells have the same biological features as
313 the brain neurons and are easily available from adult rodents. Some studies have pointed out
314 that decreases in the GPx activity in the brain are associated with neurodegenerative diseases
315 (Bellinger, Raman, Reeves, & Berry, 2009; Chen & Berry, 2003; Schweizer, Bräuer, Köhrle,
316 Nitsch, & Savaskan, 2004). On the other hand, HeLa cells are derived from human cervical
317 cancer cells in which the reactive oxygen species is actively generated (Benhar, Engelberg, &
318 Levitzki, 2002, Burdon, 1995). The role of the GPx activity in these cells appears even more
319 critical than that in other cells.

320 SA, SeMet or the ethanol-extract was mixed with 5% FBS-containing DMEM at the final
321 selenium concentration of 0.05 μM. DRG cells were incubated in the selenium
322 species-supplemented medium for 7 days followed by cell lysis and determination of the GPx
323 activity of the cell lysate. The appearance of the DRG cells under microscopic observation
324 did not change before and after the incubation with the ethanol-extract and no difference from
325 the control cells, such as detachment of cells from the bottom of the culture dish, was
326 observed (Fig. S1). The addition of the ethanol-extract to the culture media resulted in
327 increases in the GPx activity of the DRG cells and it was significantly more effective than that
328 of SeMet. In addition, the effect of the ethanol-extract was comparable to that of SA (Fig. 3),
329 which was consistent with the results of the bioavailability study using mice (Haratake et al.,

2007). The trend that SA showed a higher bioavailability than SeMet was also similar to that for the human colon carcinoma cell line (Caco-2) (Zeng et al., 2008). These data suggested that the selenium species from the ethanol-extract was utilized for the synthesis of selenoproteins in the DRG cells.

The supplementation effect of the ethanol-extract on the GPx activity was also examined using HeLa cells. When HeLa cells were incubated with the ethanol-extract at the selenium concentration of 0.05 μM, cell detachment from the bottom of the culture flask was observed. HeLa cells appeared to be susceptible to some constituents in the ethanol-extract, in which inorganic salts and various organic compounds were supposed to be present. When the selenium concentration was reduced to 0.025 μM, such morphological changes and the detachment of the cells during the incubation for 2 days were not observed (Fig. S2). Supplementation of the ethanol-extract into the culture media of HeLa cells resulted in an increase in the cellular GPx activity as well as in the case of the DRG cells (Fig. 4).

We previously assessed the bioavailability of selenium in the Niboshi and its extract using dietary selenium deficient mice; organ selenium contents and hepatic cellular GPx activity of the Niboshi extract-administered mice was comparable to those of SA-administered mice (Haratake et al., 2007). In the present study, the efficiency of each selenium species was examined using 2 different types of cells; one is the primary-cultured neuronal cells and the other is the established cell line of cervix carcinoma. In spite of the difference in cell type, a similar utilization tendency of selenium from the Niboshi extract was observed. Consequently, such *in vitro* selenium bioavailability probably reflected the previous results of *in vivo* assessment in the mice.

SA is usually used for the prevention and/or treatment of selenium deficient disease because of its high bioavailability. In our previous experiment, DRG cells cultured in SA-supplemented media showed a higher cellular GPx activity than that of cells incubated in non-selenium supplemented normal media (Haratake, Koga, Inoue, Fuchigami, & Nakayama,

2011). In this study, the supplementation effect of the ethanol-extracted Niboshi extract on an increase in the GPx activity was comparable to that of SA. Thus, it is suggested that the Niboshi extract contains nutritionally effective organoselenium species comparable to the highly bioavailable SA, and organoselenium species in the ethanol-extract supplemented in the culture media was thought to be utilized for the synthesis of the cellular GPx. The selenium species with the ^{80}Se-containing molecular ion peak at *m/z* 577 was thought to be one of the major selenium species in the Niboshi extract and it probably contributes to increases in the GPx activity of the DRG and HeLa cells.

The selenium concentration in natural foods is lower than that of selenium-enriched foods or supplements, such as selenized wheat or yeast, and this makes the speciation of selenium in natural food extremely difficult. It is questionable whether the chemical forms of selenium in artificially-enriched food are exactly the same as those in natural foods. It will be even more important to elucidate the chemical structures of the selenium species in food and supplements from the viewpoints of the safety and benefits of this element. Further structural analysis of the selenium species in the Niboshi extract will be conducted in future studies, which can also provide useful information for the development of safe and effective selenium supplements to exploit the health benefits of this element.

4. Conclusion

The Niboshi extract is comprised of various substances including selenium, such as minerals, amino acids and proteins. The selenium content in the Niboshi extract was 0.1229 ± 0.0101 μg/g-Niboshi and this corresponded to approximately 10% of selenium in the Niboshi. Selenium from the ethanol-extract of the Niboshi extract was absorbed by the DRG and HeLa cells. The cellular GPx activity of both cells increased after incubation with

selenium from the Niboshi extract, indicating that selenium from the Niboshi extract was effectively utilized for the synthesis of selenoproteins. Selenium from the Niboshi extract was more efficiently utilized than that from SeMet and comparable to that from SA regarding the improvement of the cellular GPx activity. The ethanol-extract involved the selenium species that gave the ^{80}Se-containing molecular ion peak at *m/z* 577. This selenium species was thought to be one of the nutritionally effective source compounds that increased the GPx activity of the DRG and HeLa cells.

References

Afonso, C., Costa, S., Cardoso, C., Oliveira, R., Lourenço, H. M., Viula, A., Batista, I., Coelho, I., & Nunes, M. L., (2015). Benefits and risks associated with consumption of raw, cooked, and canned tuna (Thunnus spp.) based on the bioaccessibility of selenium and methylmercury. *Environmental Research*, *143*, 130–137.

Alexander, A. R., Whanger, P. D., & Miller, L. T. (1983). Bioavailability to rats of selenium in various tuna and wheat products. *The Journal of Nutrition*, *113*(1), 196–204.

Bellinger, F. P., Raman, A. V, Reeves, M. A., & Berry, M. J. (2009). Regulation and function of selenoproteins in human disease. *The Biochemical Journal*, *422*(1), 11–22.

Benhar, M., Engelberg, D., & Levitzki, A. (2002). ROS, stress-activated kinases and stress signaling in cancer. *EMBO Reports*, *3*(5), 420–425.

Burdon, R. H. (1995). Superoxide and Hydrogen peroxide in Relation to Mammalian Cell Proliferation. *Free Radical Biology and Medicine*, *18*(4), 775–794.

Chen, J., & Berry, M. J. (2003). Selenium and selenoproteins in the brain and brain diseases. *Journal of Neurochemistry*, *86*(1), 1–12.

Cuello, S., Ramos, S., Mateos, R., Martín, M. A., Madrid, Y., Cámara, C., Bravo, L., Goya, L. (2007). Selenium methylselenocysteine protects human hepatoma HepG2 cells against oxidative stress induced by tert-butyl hydroperoxide. *Analytical and Bioanalytical Chemistry*, *389*(7–8), 2167–2178.

Dumont, E., Vanhaecke, F., & Cornelis, R. (2006). Selenium speciation from food source to metabolites: A critical review. *Analytical and Bioanalytical Chemistry*, *385*(7), 1304–1323.

Finley, J. W. (2006). Bioavailability of Selenium from Foods. *Nutrition Reviews*, *64*(3), 146–151.

Flohé, L., & Günzler, W. A. (1984). Assays of glutathione peroxidase. *Methods in Enzymology*, *105*, 114–121.

Fox, T. E., Van Den Heuvel, E. G., Atherton, C. A., Dainty, J. R., Lewis, D. J., Langford, N. J., Crews, H. M., Luten, J. B., Lorentzen, M., Sieling, F. W., van Aken-Schneyder, P., Hoek, M., Kotterman, M. J., van Dael, P., & Fairweather-Tait, S. J. (2004). Bioavailability of selenium from fish , yeast and selenate : a comparative study in humans using stable isotopes. *European Journal of Clinical Nutrition*, *58*(2), 343–349.

Haratake, M., Koga, K., Inoue, M., Fuchigami, T., & Nakayama, M. (2011). Absorption and

retention characteristics of selenium in dorsal root ganglion neurons. *Metallomics, 3*(10), 1019–1026.

Haratake, M., Takahashi, J., Ono, M., & Nakayama, M. (2007). An Assessment of Niboshi (a Processed Japanese Anchovy) as an Effective Food Source of Selenium. *Journal of Health Science*, *53*(4), 457–463.

Jones, D. L., Owen, A. G., & Farrar, J. F. (2002). Simple method to enable the high resolution determination of total free amino acids in soil solutions and soil extracts. *Soil Biology & Biochemistry*, *34*, 1893–1902.

Kiston, R. E., & Mellon, M. G. (1944). Colorimetric determination of phosphorus as molybdivanadophosphoric acid. *Industrial and Engineering Chemistry, Analytical Editionndustrial and Engineering Chemistry, Analytical Edition*, *16*, 379–383.

Kryukov, G. V., Castellano, S., Novoselov, S. V., Lobanov, A. V, Zehtab, O., Guigo, R., & Gladyshev, V. N. (2003). Characterization of Mammalian Selenoproteomes. *Science*, *300*(5624), 1439–1443.

Labunskyy, V. M., Hatfield, D. L., & Gladyshev, V. N. (2014). Selenoproteins: molecular pathways and physiological roles. *Physiological Reviews*, *94*(3), 739–777.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein Measurement with the Folin Phenol Reagent. *Journal of Biological Chemistry*, *193*, 265–275.

Marschall, T. A., Bornhorst, J., Kuehnelt, D., & Schwerdtle, T. (2016). Differing cytotoxicity and bioavailability of selenite, methylselenocysteine, selenomethionine, selenosugar 1 and trimethylselenonium ion and their underlying metabolic transformations in human cells. *Molecular Nutrition & Food Research*, *60*(12), 2622–2632.

Miyazaki, Y., Koyama, H., Sasada, Y., Satoh, H., Nojiri, M., & Suzuki, S. (2004). Dietary Habits and Selenium Intake of Residents in Moutain and Coastal Communities in Japan. *Journal of Nutritional Science and Vitaminology*, *50*, 309–319.

Moreda-Piñeiro, J., & Moreda-Piñeiro, A. (2015). Bioavailability of Selenium and Selenium Compounds in Foodstuff by *In Vivo* Testing. In V. R. Preedy (Eds.), Selenium Chemistry, Analysis, Function and Effects (pp. 173–196). Cambridge: Royal Society of Chemistry.

Navarro-Alarcon, M., & Cabrera-Vique, C. (2008). Selenium in food and the human body: A review. *Science of the Total Environment*, *400*(1–3), 115–141.

Ralston, N. V. C., & Raymond, L. J. (2010). Dietary selenium's protective effects against methylmercury toxicity. *Toxicology*, *278*(1), 112–123.

Rayman, M. P. (2012). Selenium and human health. *The Lancet*, *379*(9822), 1256–1268.

453 Schrauzer, G. N. (2000). Selenomethionine: a review of its nutritional significance,
454 metabolism and toxicity. *The Journal of Nutrition*, *130*(7), 1653–1656

455 Schweizer, U., Bräuer, A. U., Köhrle, J., Nitsch, R., & Savaskan, N. E. (2004). Selenium and
456 brain function: A poorly recognized liaison. *Brain Research Reviews*, *45*(3), 164–178.

457 Watkinson, J. H. (1966). Fluorometric Determination of Selenium in Biological Material with
458 2,3-Diaminonaphthalene. *Analytical Chemistry*, *38*(1), 92–97.

459 Weiller, M., Latta, M., Kresse, M., Lucas, R., & Wendel, A. (2004). Toxicity of nutritionally
460 available selenium compounds in primary and transformed hepatocytes. *Toxicology*,
461 *201*(1–3), 21–30.

462 Yoshida, M., Abe, M., Fukunaga, K., & Kikuchi, K. (2002). Bioavailability of selenium in the
463 defatted dark muscle of tuna. *Food Additives and Contaminants, 19*(10), 990–995.

464 Yoshida, S., Haratake, M., Fuchigami, T., & Nakayama, M. (2011). Selenium in Seafood
465 Materials. *Journal of Health Science*, *57*(3), 215–224.

466 Yoshida, S., Haratake, M., Fuchigami, T., & Nakayama, M. (2012). Characterization of
467 selenium species in extract from Niboshi (a processed Japanese anchovy). *Chemical &*
468 *Pharmaceutical Bulletin*, *60*(3), 348–353.

469 Zeng, H., Botnen, J. H., & Johnson, L. K. (2008). A selenium-deficient caco-2 cell model for
470 assessing differential incorporation of chemical or food selenium into glutathione
471 peroxidase. *Biological Trace Element Research*, *123*(1–3), 98–108.
472

473 Table 1 Contents of selected substances in the Niboshi and its extract

Substance	Niboshi (mg/g) [a)]	Niboshi extract (mg/g-Niboshi) [a,b)]
Selenium	0.00121 ± 0.00003	$1.229 \times 10^{-4} \pm 0.101 \times 10^{-4}$
Sodium	9.351 ± 0.679	14.34 ± 0.66
Magnesium	4.287 ± 0.190	0.495 ± 0.042
Phosphorous	21.75 ± 0.10	2.139 ± 0.088
Chlorine	– [c)]	11.55 ± 0.53
Potassium	15.01 ± 0.72	15.73 ± 0.98
Calcium	23.02 ± 0.75	< d. l. [d)]
Amino acid	–	9.592 ± 0.051
Protein	–	11.73 ± 1.02

474 *a)* Values are mean ± standard error (n = 3).

475 *b)* The concentrations of the selected substances in the Niboshi extracts = [(amount of
476 substances in the extract used for analysis) / (volume of the extract used for analysis)]
477 · [(Total volume of the extract prepared) / (weight of the Niboshi used for extraction)]
478 (μg or mg/g-Niboshi)

479 *c)* Not measurable due to the presence of perchloric acid used for the acid digestion.

480 *d)* Lower than the detection limit of the flame atomic absorption spectrometry.

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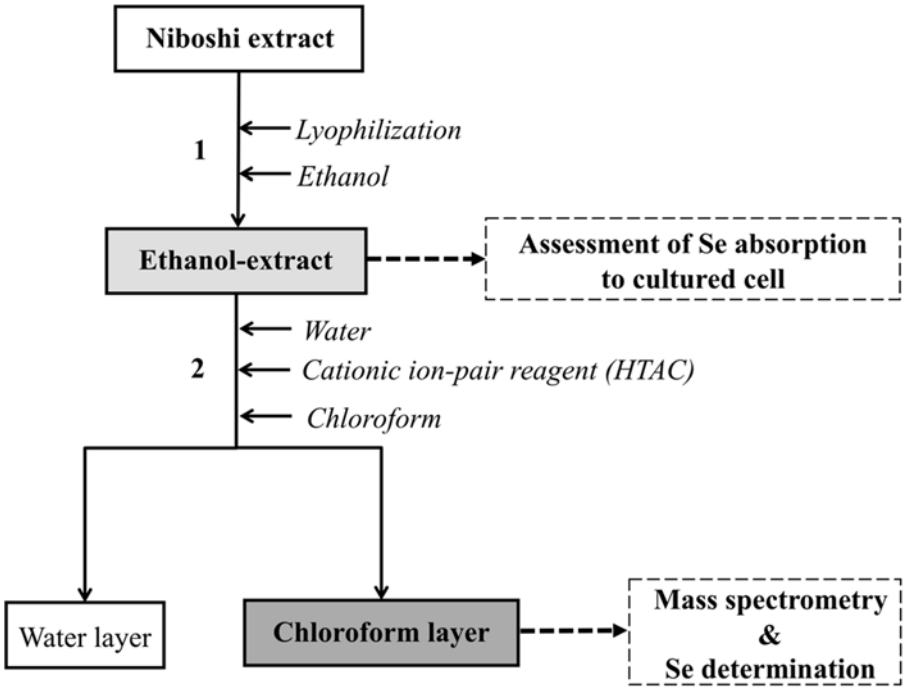
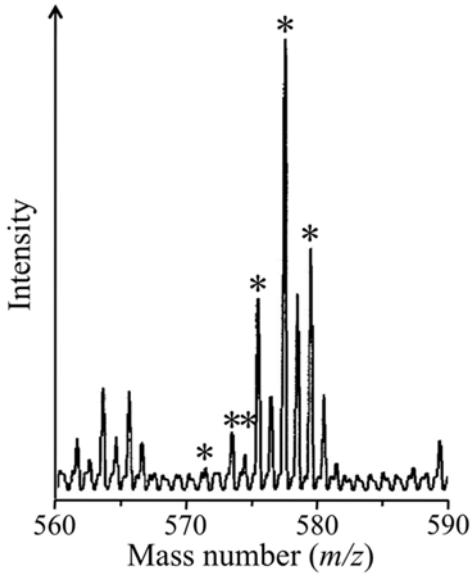


Fig. 1. A procedure for ethanol extraction followed by ion-pair extraction of selenium species in the Niboshi extract

Operation 1: The Niboshi extract was lyophilized and suspended in ethanol followed by filtration with paper and evaporation of ethanol. Operation 2: Obtained residue was dissolved in water and mixed with ion-pair reagents [hexadecyltrimethylammonium chloride (HTAC), final concentration 1.0 mM] and extracted with chloroform several times. After the evaporation of chloroform, the residual was subjected to mass spectrometry and selenium content determination.



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493 Fig. 2. Mass spectrum of selenium species in the ethanol-extract

494 Asterisks indicate peaks containing naturally-occurring stable isotopes of selenium.

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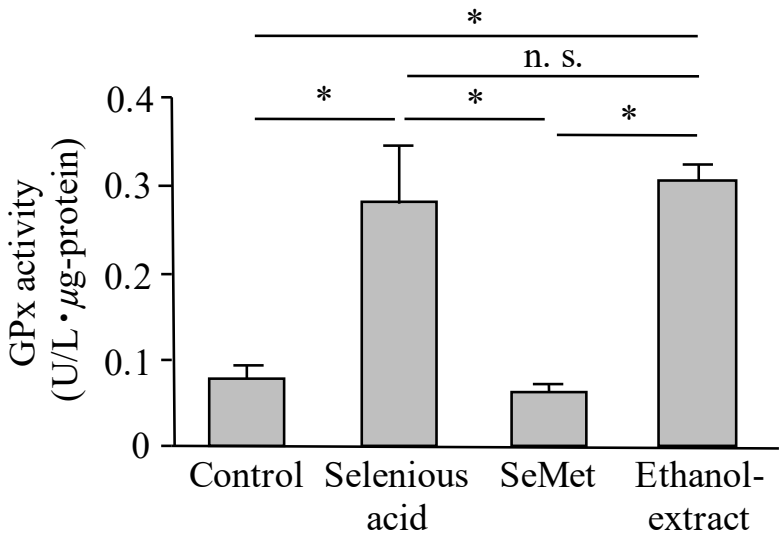

Fig. 3. GPx activity of DRG cells after incubation in 0.05 μM selenium species-supplemented culture medium for 7 days

Culture medium: 5% FBS-containing DMEM. Values are mean ± standard error (n = 3 or 4). *, Significantly different from each other with $P < 0.05$. n. s., Not significantly different from each other.

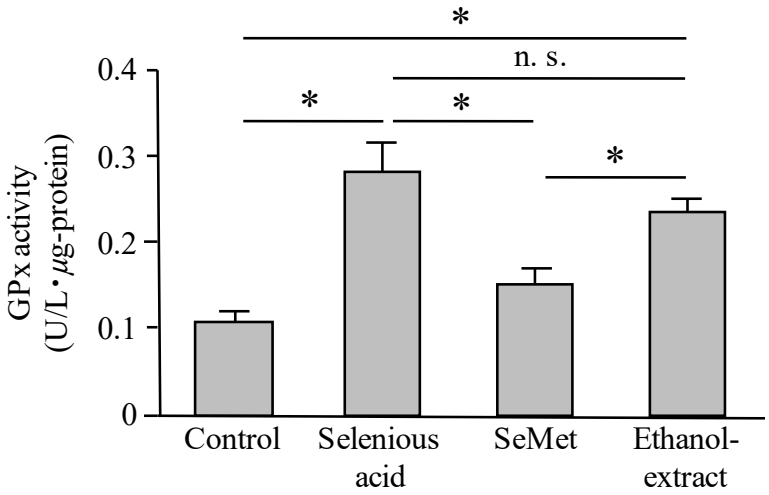

Fig. 4. GPx activity of HeLa cells after incubation in 0.025 μM selenium species-supplemented culture medium for 2 days

Culture medium: 5% FBS-containing DMEM. Values are mean ± standard error (n = 3 or 4). *, Significantly different from each other with $P < 0.05$. n. s., Not significantly different from each other.