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Running title:

~~Function~~ Safety and availability of mackerel peptide rich in selenoneine

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Abstract

Selenoneine has an enormous antioxidant action compared to ergothioneine, a traditional antioxidant ingredient, whereas the other selenium compounds such as selenious acid and seleno-L-methionine had no antioxidative property in several evaluations. We prepared hydrolysate of mackerel protein enzymatically (EMP) containing selenoneine. EMP also exhibited antioxidant action *in vitro*. EMP treatment modulated the secretion of interleukin-6 and -10, but not TNF-α, in cultured RAW264.7 macrophage cells. The safety of EMP-, i.e., selenium- feeding was confirmed in SD rats. Serum and hepatic triglyceride levels and serum insulin, leptin, and cholesteryl ester levels tended to lower with EMP feeding in SD rats. The elevated serum triglyceride, free fatty acid, insulin, and leptin levels also tended to be decreased with EMP feeding in KK-A^y mice. These results implied that EMP intake may involve in the improvement of lipid metabolism through the immune modulation by various antioxidants contained in EMP.

Keywords: selenoneine, mackerel meat hydrolysate, antioxidant, cytokine secretion, lipid metabolism

Introduction

Ergothioneine is a sulfur amino acid and a potent antioxidant ingredient mainly contained in Pleurotus citrinopileatus (*Pleurotus cornucopiae* var. *citrinopileatus*), which is a kind of mushrooms mainly cultivated in the north of Japan (Ito *et al.*, 2011; Pahila *et al.*, 2019). Ergothioneine is a derivative of histidine and has a thiocarbonyl group (C=S) in the keto form, so its antioxidant activity is exerted by reducing thiol group (C-SH) in the enol form (Figure 1; Ito *et al.*, 2011; Repine and Elkins, 2012). Ergothioneine is thought to exert anti-inflammatory effect through its antioxidative properties (Ito *et al.*, 2011; Repine and Elkins, 2012).

Selenoneine is an amino acid that the sulfur group in ergothioneine is replaced with a selenium group (Figure 1; Yamashita and Yamashita, 2010; Yamashita *et al.*, 2013a). The antioxidant activity of selenoneine evaluated by its radical scavenging ability has been reported to be stronger than that of ergothioneine (Yamashita *et al.*, 2013a). Selenoneine is widely found in the blood, muscle, and other tissues of migratory fishes such as tuna, mackerel, and yellowtail (Yamashita *et al.*, 2011; Yamashita *et al.*, 2013c). Selenoneine is thought to play important roles in the suppression of the auto-oxidation of heme iron, the detoxication of methylated mercury, and the repair of DNA damage (Tan *et al.*, 1999; Jackson and Combs, 2008; Yamashita *et al.*, 2013a). Thus, selenoneine is needed for resistance under a hypoxic environment and detoxication, especially important for these fish species.

Selenium is an essential micronutrient and dietary fishes are one of the major sources of selenium in the form of selenoneine for humans (Yamashita *et al.*, 2011; Yamashita *et al.*, 2013b). The recommended amount and tolerable upper limit of selenium for adult are determined as 25-30 and 350 μg intake/day in Dietary Reference Intakes for Japanese (2020). Dietary selenoneine as well as ergothioneine are thought to be incorporated into cells and tissues via organic cation/carnitine transporter 1 (OCTN1) (Yamashita *et al.*, 2013b). Dietary selenoneine may also be biosynthesized from another selenium source such as selenious acid and seleno-L-methionine (Figure 1) and be accumulated in the intercellular pool available for biosynthesis of seleno-proteins, increasing the seleno-redox effect (Yamashita *et al.*, 2013a). Selenoneine is expected to exert not only the detoxification, but also the prevention of cancer, cardiovascular disease, immunodeficiency, aging, and diabetes (Jackson and Combs, 2008; Matsuda *et al.*, 2018; Rayman and Stanges, 2013; Stranges *et al.*, 2007; Yamashita *et al.*, 2013a). On the other hand, there are safety concerns surrounding selenium due to the narrow range between therapeutic and toxic doses of selenium (Hadrup *et al.*, 2023; Kiełczykowska *et al.*, 2018).

The overproducing of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxy radical are responsible for the oxidative damage occurring via various biological processes such as energy production, enzyme reaction, and inflammation, and so on. The excess superoxide is converted to oxygen and hydrogen peroxide by superoxide dismutase (SOD), and then excess hydrogen peroxide is converted to oxygen and water by catalase and glutathione peroxidase. Even so, excess hydrogen peroxide reacts with iron ions contained in hemoglobin and other substances, leads to produce hydroxy radicals. When hydroxy radicals react with lipids to form lipid radicals,

they combine with oxygen and create lipid peroxyl radicals resulting in the production of lipid peroxide in a chain manner (Kalyanaraman, 2013). The antioxidant activity of selenoneine is thought to be due to radical scavenging (Yamashita *et al*, 2013a). However, the antioxidant action of substances should be evaluated with several indices since the redox pathway has several steps as described above.

In the present study, we confirmed the antioxidant activities of selenoneine and the other selenium compounds with several experimental methods. Dietary fishes containing selenoneine are thought to be useful for human health maintenance. So, we prepared peptide with enzymatic hydrolysis (protease digestion) from the edible part of mackerel meat rich in selenoneine (Yamashita *et al.*, 2011), and we also evaluated the antioxidant activity of the mackerel meat hydrolysate. And then, the effect of the mackerel meat hydrolysate on cytokine secretion from macrophage cells was examined since the immune modulation is one of the functions exerted through antioxidation. Furthermore, the safety and availability of the mackerel meat hydrolysate containing selenium as food was investigated using animal models.

Materials and Methods

1. Experimental sample

1-1. Preparation of mackerel meat hydrolysate (EMP)

EMP powder was provided by LS corporation (Tokyo, Japan). The edible part of chub mackerel (*Scomber japonicus*) and blue mackerel (*Scomber australasicus*) was powdered and heated at 90°C, and then was digested with proteases from *Bacillus subtilis* (A enzyme, Kyowachem-enzyme, Osaka, Japan) at 55-60°C for 120 min and *Aspergillus oryzae* (Sumichi-mu LP50D, Shin-nihon-Chemical Industry, Aichi, Japan) at 50-55°C for 120 min. After the subsequent processes of filtration, separation of oils and fats, concentration, removal of metals, and finally desiccation, approximately 100 g of EMP powder was obtained from 1,580 g of raw mackerel meat.

1-2. ~~Nutritional composition~~ Protein content and selenium analysis of EMP

Crude protein content in EMP was measured using the Kjeldahl method, which was outsourced to Japan Food Research Laboratories (Tokyo, Japan). ~~The moisture content of EMP was determined as the loss in weight after drying at 105ºC for 24 h. The crude protein and fat contents were assayed by using the Kjeldahl method and the acid digestion-ether extraction method, respectively. The crude ash content was measured using the direct ignition method (heating at 540ºC for 24 h). The carbohydrate content was calculated by the subtraction of other component contents. The dietary fiber content was measured by using the enzymatic-gravimetric method (McCleary *et al.*, 2012). The natrium content was assayed using atomic absorption photometry and the sodium chloride equivalent was calculated.~~ The total selenium content was assayed using inductively coupling plasma mass spectrometry (ICP-MS), and the selenoneine content as selenium equivalent was measured using liquid chromatography (LC)-ICP-MS (Yamashita and Yamashita, 2010). The

selenium and selenoneine analyses were outsourced to Japan Fisheries Research and Education Agency.

1-3. Molecular distribution analysis of EMP

The molecular distribution of peptides in EMP was analyzed with sodium dodecyl sulfate (SDS)-poly acrylamide gel electrophoresis (PAGE) using 5-20% gel (GLX-271B, Gell International, Hong Kong), a voltage of 300 V (constant), Coomassie Brilliant Blue (CBB)-dyeing (SP-4011, APRO Science, Tokushima, Japan), a reduction using dithiothreitol, and heat treatment, but not alkylation. Polypeptide SDS-PAGE Molecular weight Standards (161-0326, Bio-Rad, Hercules, USA) was used.

2. Antioxidant evaluation for selenoneine, involved compounds, and EMP in vitro

Selenoneine was prepared as described previously and provided by Japan Fisheries Research and Education Agency (Yamashita and Yamashita, 2010). Ergothioneine (Sigma-Aldrich Japan, Tokyo, Japan), selenoneine, and low molecular weight selenium compounds in foods and supplements, such as selenious acid and seleno-L-methionine (Wako Pure Chemical, Osaka, Japan), were used for antioxidant evaluation and comparison. As sample solutions, 100 mg of each component and EMP were dissolved in 1 mL of deionized water, but seleno-L-methionine was dissolved in 0.1 M hydrochloric acid solution.

The power of antioxidation (PAO) was assessed as the reduction power from Cu^{2+} to Cu^{+} using an assay kit (KPA-050, Japan Institute for the Control of Aging, Shizuoka, Japan). 1, 1-Diphenyl- 2- picryhydrozyl (DPPH) radical scavenging activity was measured according to a previous microplate method at 490 nm and calculated as Trolox equivalent (Oki *et al.*, 2003). Superoxide dismutase (SOD)-like activity was measured using the WST method (Alam *et al.*, 2013) with an assay kit (S311, Dojindo Molecular Technologies, Inc., Kumamoto, Japan).

Values are expressed as mean ± standard deviation (SD) for triplicate assays. Statistical analysis was carried out with Dunnett's test (SPSS, 1993) and significant differences among groups were considered when the *p*-value was lower than 0.05.

3. The effect of EMP on cytokine secretion

RAW264.7 cells, a macrophage-like cell line derived from leucocytes in mice (RIKEN Bioresource Research Center, Tsukuba, Japan), were suspended at 1×10^6 cells/mL/well and inoculated in 24 well-plate and incubated with Dulbecco's modified Eagle's medium (DMEM) medium (high-glucose, Wako Pure Chemical, Osaka, Japan) containing 10% fetal bovine serum (FBS, Sigma-Aldrich Japan, Tokyo) and 50 µL of streptomycin-penicillin solution (×100, Wako Pure Chemical, Osaka, Japan) in 5% CO_2 atmosphere at 37ºC. ~~a~~After 3 days, the confluent cells were incubated with medium containing 0.1 µg/mL lipopolysaccharide (LPS, Wako Pure Chemical, Osaka, Japan) to stimulate cells and promote cytokine secretion. EMP solutions (100 mg/mL of sample powder) were filtrated aseptically and added to media at 1% (v/v) concentration. After incubation for 24 hours, each medium was collected and stocked at -80ºC until analysis. NO_2 and NO_3 production was measured using NO_2/NO_3 assay kit-C III (Dojindo Molecular Technologies,

Kumamoto, Japan) with Griess method. Tumor Necrosis Factor (TNF)-α production was measured using Quantikine ELISA Mouse TNF-α (R&D Systems, Minneapolis, USA). The production of interleukin (IL)-6 and IL-10 was analyzed using Mouse IL-6 Assay Kit-IBL (Immuno-Biological Laboratories, Gunma, Japan) and Quantikine ELISA Mouse IL-10 (R&D Systems, Tokyo, Japan), respectively. Statistical processing was followed the method as described above.

4. Repeated dose toxicity study and utility evaluation of EMP in Sprague-Dawley (SD) rats

The animal studies were carried out under the Guidelines for Animal Experiments of University of Nagasaki at the approved No. R1-04 (Nagasaki, Japan), and under Law No. 105 and Notification No. 6 of the Government of Japan. Male SD rats aged 4 weeks were purchased from Clea Japan (Tokyo, Japan), kept individually in cages under a controlled atmosphere (temperature, 23±1°C, humidity, 55±5%, light cycle, 08:00-20:00), and fed a commercial pellet diet (CE-2, Clea Japan, Tokyo) for a week.

The diet composition was shown in Table 2. A control diet was prepared according to AIN-93G composition (Reeves *et al.*, 1993). For safety and utility confirmation of EMP containing selenium, EMP as protein source was added at the level of 5 or 10% to the control diet reduced casein. The calculated protein contents for control, 5% EMP, and 10% EMP diets are 17.2, 17.4, and 17.6%, respectively since the protein levels of casein and EMP are 86 and 90%.

Male SD rats were randomly divided into three groups and had free access to each diet and deionized water for 28 days. Seko *et al.* (2023) reported that male ICR mice were more sensitive to the effect of selenoneine intake on kidney weight and plasma parameters compared with female mice. Therefore, male experimental animals used in this study. Body weight and feed intake were measured every day. Feces were collected for 2 days before the end of feeding period and weighted. After feeding, rats were sacrificed by withdrawing blood from the aorta under the feeding status, and then serum was obtained from blood by centrifugation at 3,000 rpm for 20 min. The analysis of hemogram and hepatic function, glycemic, lipid except for free fatty acid, and mineral parameters in blood or serum were outsourced to SRL (Nishi Nihon office, Nagasaki, Japan). Serum free fatty acid level enzymatically measured using kit (NEFA C-Test, Wako Pure Chemical Industries, Osaka, Japan). Serum insulin and leptin levels were measured with ELISA assay (Morinaga Institute of Biological Science, Yokohama, Japan), and serum adiponectin level were also measured with ELISA assay (Otsuka Pharmaceutical, Tokyo, Japan). Serum PAO levels and SOD activity were measured described above.

The liver, spleen, kidney, testicle, cecum, and white adipose tissues were excised, rinsed, observed, and weighted. Total lipid in approximate 0.5 g of liver was extracted with chloroform: methanol (2:1, v/v) according to the previous method (Folch *et al.*, 1957). The extract was dried up with N_2 gas, purified with methanol, and dissolved in isopropanol. Hepatic levels of triglyceride and total cholesterol were enzymatically measured with a commercial kit (Triglyceride E-Test and Cholesterol E-Test, Wako Pure Chemical Industries, Osaka, Japan).

Data were shown as mean ± standard error (SE) for 5-6 rats. Statistical analysis was carried out with Tukey-Kramer test (SPSS, 1993) and significant differences among groups were considered at $p<0.05$.

5. The effect of EMP on lipid and carbohydrate metabolism in KK-A^y mice

The animal studies were carried out under the rule for Animal Experiments of Nakamura Gakuen University (approved No. 2017-7, Fukuoka, Japan). Four weeks old male KK-*A^y* mice were purchased and acclimated according to described above. Twelve KK-*A^y* mice were randomly divided into two groups and had free access to control or 10% EMP diet and deionized water for 48 days until hyperglycemia was developed. Blood glucose levels were measured weekly by flushing blood from the tail vein directly into the tip of a glucose pilot (Syntron Bioresearch, Inc., USA). Body weight and feed intake were measured every other day. After feeding period, mice were anesthetized, and blood was collected from the abdominal vena cava after laparotomy under feeding status. The anatomization and serum and tissue collection were performed according to described above. The analyses of serum and hepatic parameters were performed using commercial kits as described above.

Data were shown as mean ± SE for 6 mice. Statistical analysis with *t*-test ($p<0.05$) was performed.

Results

1. ~~Nutritional composition~~ Protein and selenium content and molecular distribution

~~Nutritional composition of EMP is shown in Table 1.~~ EMP powder contained 90 g of crude protein per 100 g, and 10.5 and 4.4 μg of selenium and selenoneine per gram.

SDS-PAGE analysis revealed that the molecular weight of peptides in EMP was lower than 6.5 kDa as shown in Figure 2. Except for the non-hydrolyzed fraction of EMP, 95% and 56% of the hydrolyzed part of EMP was lower than 3.0 and 0.5 kDa, respectively (data not shown).

2. Antioxidant activities

The superscript asterisks in Table 3 indicate the significant differences for ergothioneine with Dunnett. PAO value was measured and calculated as the reduction power for 2,186 μmol/L of copper ion per 1 mM uric acid (standard substrate) and then expressed per mg of each sample. PAO value for ergothioneine was comparable with that for Trolox used as a reference. Selenoneine indicated a significant potent reduction power, about 23-fold, compared to ergothioneine and Trolox. On the other hand, PAO value for selenious acid and seleno-L-methionine were significantly lower compared to ergothioneine, so these compounds had no reduction power. PAO value for EMP was significantly lower compared to ergothioneine. DPPH radical scavenging ability was measured and expressed as Trolox equivalent per mg samples. Ergothioneine had a potent radical scavenging ability. The ability of selenoneine was more potent and about 9-fold

compared to that of ergothioneine, but that of selenious acid was very low and that of seleno-L-methionine was under the detection limit. EMP had a radical scavenging ability lower than ergothioneine and selenoneine. SOD-like activity was measured as the inhibition against the chromogenic action that tetrazolium salt (WST-1) was converting to formazan-dye by superoxide and expressed as IC_{50} value (mg/mL sample solution). The SOD activities of ergothioneine and Trolox were comparable. Selenoneine showed a significant potent activity compared to ergothioneine. The activity of selenious acid tended to be lower than that of ergothioneine, and that of seleno-L-methionine was too low and could not be detected. The activity of EMP was also comparable with that of ergothioneine.

3. Cytokine secretion

The superscript asterisks in Table 4 indicate the significant differences for control (LPS (+)) with Dunnett. The EMP treatment did not affect the viability of RAW264.7 cells. LPS addition achieved the stimulation of cytokine secretions. NO, IL-6, and IL-10 secretions were significantly increased with EMP treatment compared to control (LPS (+))., but the effect of EMP treatment on TNF-α secretion was not observed obviously.

4. Repeated dose toxicity study and utility evaluation of EMP in SD rats

As shown in Table 5, EMP feeding did not influence body weight, feed intake, tissue weights, and adipose tissue weights. Cecum weight tended to increase in 10% EMP group compared with control group. Fecal excretion also showed increasing tendency in an EMP-dose dependent manner. There was no significant difference among groups in white adipose tissue weights. All parameters in hemogram had no abnormality. Although there was a significant difference in hematocrit values, all groups had normal values. Blood glucose and HbA1c levels and serum creatinine and uric acid levels were not affected by EMP feeding. Hepatic function markers in serum also indicated comparable levels among groups. As antioxidative parameters, serum PAO level had no significant differences among groups. Serum SOD activity (U/mL) was measured as the inhibition against the chromogenic action described above and was calculated as 1U which was indicating the serum dilution rate required to show IC_{50} value. There were no significant differences in serum SOD activity among groups. Serum triglyceride level tended to decrease in 10% EMP group compared with control group. Serum cholesteryl ester level significantly lowered in the 5% EMP group and tended to lower in the 10% EMP group than in the control group. Serum insulin level tended to lower in the 10% EMP group, and serum leptin level decreased to half of control value in the 10% EMP group. Serum adiponectin level increased to tendency in the 10% EMP group than in the control group. Serum mineral levels were comparable among groups. Hepatic triglyceride concentration showed decreasing tendency in an EMP-dose dependent manner, and the 10% EMP group indicated about half value compared to the control group. Thus, there was no abnormality with daily feeding of experimental diet containing 5 or 10% EMP powder for 28 d[ay]s on growth, feed intake, tissues, and biomarkers in SD rats.

5. The effect of EMP on lipid and carbohydrate metabolism in KK-A^{y} mice

The KK-A^y mouse develops type II diabetes with obesity due to overfeeding. As shown in Figure 3, blood glucose level elevated with growth up to 600 mg/dL, which is upper limit that the instrument can determine. No significant differences were observed in blood glucose levels among groups at any measured point. As shown in Table 6, EMP feeding did not influence body weight gain, feed intake, tissue weights except for kidney, and white adipose tissue weights. Kidney weight significantly decreased in EMP group compared to control group. Serum PAO level and SOD activity had no significant difference among two groups. Serum insulin level tended to decrease by half with EMP feeding compared to control, but not serum glucose level. Serum leptin level also tended to lower to 61.0% in the EMP group than in the control group, and serum adiponectin levels were comparable in control and EMP group. Serum triglyceride and free fatty acid levels showed decreasing tendency in 10% EPM group compared to control group, but not serum cholesteryl level. Hepatic triglyceride concentration tended to increase 10% EMP group.

Discussion

The antioxidant activities of ergothioneine, selenoneine, selenious acid, and seleno-L-methionine were measured with several index such as reduction power (PAO), DPPH radical scavenging ability, and SOD-like activity *in vitro* (Table 3). The antioxidant activity of ergothioneine reported by previous studies (Ito *et al*, 2011; Pahila *et al*., 2019) was also confirmed with various methods in this study. In addition, the antioxidant activity of selenoneine was enormous greater than that of ergothioneine, not only in DPPH radical scavenging ability reported previously (Yamashita *et al.*, 2013a), but also in PAO and SOD activity. These results suggest that selenoneine has a potent and various antioxidant action. Selenoneine is thought to exert an antioxidant action due to the reducing of seleno-group (C-SeH) as well as thiol group (C-SH) in ergothioneine (Figure 1). A selenium atom has a more potent antioxidant action than a sulfur atom (Yamashita *et al*., 2013a). On the other hand, selenious acid and seleno-L-methionine evidently exhibited no antioxidant action in this study. This finding suggests that these selenium compounds need to be metabolized to selenoneine, a potent antioxidant substrate, playing important roles in the detoxification and self-protection of fishes (Yamashita *et al.*, 2013a) and probably in the prevention of various diseases of experimental animals (Stranges *et al*., 2007; Jackson and Combs, 2008; Rayman and Stanges, 2013; Yamashita *et al.*, 2013a). In addition, EMP containing selenoneine at the ppm scale (Table 1) was thought to have an antioxidant action in this study. For reference, oxygen radical absorbance capacity (ORAC) was also analyzed using the reported method (Alam *et al*., 2013) in Japan Food Research Laboratories (Tokyo, Japan). ORAC value for EMP was 520 nmol-Trolox eq./mg sample. The antioxidant action of EMP is thought to be expressed by not only selenoneine, but also by antioxidative amino acids and peptides including an imidazole group, such as ergothioneine, histidine, anserine, and carnosine, contained in EMP. The content of these ingredients is thought to be larger than that of selenoneine in EMP. The correlation between each content and antioxidant power degree should be clarified thereafter. Regarding the antioxidant action of peptides *per se*,

the hydrolysates obtained from wheat gluten in pork meat were reported to suppress the lipid oxidation in foods (Park *et al.*, 2012). The acidic and basic fractions of plant proteins, gluten, and soy were also reported to have an antioxidant action in foods (Park *et al.*, 2008). So, EMP may also exhibit an antioxidant action.

We subsequently examined the effect of EMP on cytokine secretion using RAW 264.7 cells stimulated with LPS (Table 4). NO is a radical and toxic molecule produced by oxidative stress. EMP as an antioxidant compound could not suppress the NO production in this study. TNF-α is an essential pro-inflammatory mediator in the inflammation process, which can lead to another inflammatory molecule expression, such as IL-6. On the other hand, IL-10 is a potent anti-inflammatory cytokine which has immunomodulatory effects and causes the suppression of other pro-inflammatory cytokines, such as TNF-α (Sundaram *et al.*, 2015; Gutierrez and Hoyo-Vadillo, 2017). In the present study, the secretion of IL-10 increased, but that of TNF-α and IL-6 did not decrease with EMP treatment, respectively. Omagari *et al.* (2018) reported that mRNA expressions of ~~inflammation parameters, such as~~ TNF-α, IL-6, and IL-1β, tended to be higher in the liver of rats fed a diet containing EMP powder~~, corresponding to our observation~~. Meanwhile, the activation of nuclear factor-kappa B (NF-kB), an important transcription factor for inflammation which causes an inflammatory disease, tended to be lowered at the level of mRNA expression in the liver of rats fed EMP (Omagari *et al.*, 2018). Therefore, our results indicated that EMP is involved, at least, in the modulation of cytokine secretion.

As the example of protein hydrolysate, casein glycomacropeptide, which is a casein-derived whey peptide, was hydrolyzed with papain (GHP) (Cheng *et al.*, 2015). LPS-stimulated RAW264.7 macrophages incubated with 0.25, 0.5, 1, or 2 mg/mL of GHP for 24 hours indicated a significant dose-dependent decrease in TNF-α, IL-1β and iNOS mRNA expression (Cheng *et al.*, 2015). EMP powder, a mackerel protein hydrolysate, was added to the LPS-stimulated RAW264.7 cells at 1 mg/mL of final concentration and cultured for 24 hours in this study, since GHP sufficiently exerted the suppressing effect on these inflammatory cytokines secretion when added at 1 mg/mL or more (Cheng *et al.*, 2015). However, the effect of EMP on TNF-α and IL-6 secretions was unclear in the present study (Table 4), so higher concentration of EMP was thought to be required.

EMP, which is manufactured by limited degradation with microbially derived-protease, was found to contain resistant peptides for exo-form peptidase including hydroxyproline derived from collagen (Pro-Hyp and Leu-Hyp), proline (Leu-Pro and Gly-Pro), aspartate (Asp-Ile), and pyroglutamyl-peptides (pyroGlu-Leu, pyroGlu-Ile-Glu, and pyro-Glu-Glu) (Sato *et al.*, 2018). The present study indicated that most of peptides in EMP have a molecular weight between 200 and 300 (Figure 2), which is consistent to this finding. In particular, PyroGlu-Leu may exert the anti-inflammatory effect (Hirai *et al.*, 2014; Oishi *et al.*, 2015) and the preventive action of acute hepatitis (Sato *et al.*, 2013). PyroGlu-Leu seems to be generated by the processing and production of peptides and in fermented foods (Kiyono *et al.*, 2013), but not by the intake and digestion of proteins *in vivo* (Sato *et al.*, 1998). Therefore, pyroglutamyl-peptides such as pyroGlu-Leu may well move to blood flow and act after EMP intake due to its resistance.

The safety of EMP powder as food containing selenoneine and the other selenium compounds was confirmed in this study using male SD rats (Table 5). Rats were feeding 21.4 g of 10% EMP diet containing 22.5 μg of total selenium equivalent to 9.42 μg of selenoneine as an average daily intake for 28 days. As a result, there was no abnormality on growth, feed intake, tissues, and biomarkers. Seko *et al.* (2023) reported that selenoneine was distributed in liver, kidney, and spleen in male and female ICR mice fed a diet containing 0.7% of selenoneine for 32 days. Food consumption and body weight gain were reported to be not affected with selenoneine feeding. Kidney weight was reported to significantly decrease in male mice feeding 0.42 μg of selenoneine/day, but not in female mice feeding 0.35 μg of selenoneine/day. However, the selenoneine concentration in the kidney was reported to be higher in female than male mice. Thus, the decreased kidney weight in male mice was not explained due to only selenoneine-accumulation and toxicity (Seko *et al.*, 2023). The effect of selenoneine intake on kidney weight was not observed in male SD rats in this study. The effect on female SD rats also needs to be evaluated thereafter.

In terms of availability, EMP feeding tended to increase the cecum and fecal weight (Table 5), which is thought be due to improvement of intestinal environment with peptides in EMP. The intestinal environment seems to improve with fish peptide (Jin *et al.*, 2018) and fermented soybean meal (Wang *et al.*, 2020). Although antioxidants intake was expected to elevate serum PAO level and SOD activity, feeding of EMP containing selenoneine and other antioxidants did not affect these parameters (Table 5). SD rats, which are not disease model, may not require the improvement of these antioxidative parameters. Serum triglyceride, insulin, and leptin concentration tended to lower but not blood glucose level, and serum adiponectin level tended to be higher with EMP feeding in SD rats (Table 5). Insulin, leptin, and adiponectin are hormones involved in carbohydrate and lipid metabolism. Insulin can regulate and maintain blood glucose level, but concurrently stimulate lipogenesis and fat accumulation. As a result, insulin level elevates via its low sensitization and high resistance in obesity and/or type II diabetes subjects (Zhao *et al.*, 2019). The physiological role of leptin is thought to suppress food intake, promote energy expenditure, and modulate inflammatory responses. However, leptin also maintains a high level due to a lack of efficacy in those subjects (Zhao *et al.*, 2019). On the other hand, adiponectin is secreted from smaller adipose cells and can improve the insulin resistance. So, adiponectin level is high in lean subjects and low in obese subject (Fruhbeck *et al.*, 2018). Adiponectin has not been reported to exert its resistance unlike insulin and leptin. Therefore, the decreasing tendency in serum triglyceride level with EMP feeding may be caused, at least in part, through the modulation of hormone secretions such as insulin, leptin, and adiponectin in this study. These results may lead to the decreasing tendency in hepatic triglyceride concentration and liver weight. In addition, EMP intake was also thought to decrease serum cholesteryl ester level (Table 5). Total cholesterol level tended to be decreased, and LDL-cholesterol level was significantly decreased in male ICR mice feeding selenoneine (Seko *et al.*, 2023). So, the effect of EMP on cholesterol metabolism should be investigated in detail using animals fed a cholesterol-enriched diet in the future.

In conclusion, regarding safety, there seemed to be no problems with values related to organ weight, body weight, blood minerals, and liver function in this experiment using SD rats fed the diet containing EMP. Therefore, concerned toxicity of selenium and other antioxidant metals intake was not observed in this experiment.

Thus, EMP intake may be able to involve in lipid metabolism. So, we subsequently examined using male KK-*A*^{y} mice developing type II diabetes and obesity with disordered and elevated lipid and hormone levels. KK-*A*^{y} mice were feeding 4.91 g of 10% EMP diet containing 5.16 ng of total selenium equivalent to 2.16 ng of selenoneine as an average daily intake for 48 days. As shown in Table 6, kidney weight was significantly decreased, but serum glucose level was not affected with EMP feeding. Seko *et al.* (2023) reported that kidney weight was significantly decreased, and blood glucose level was significantly increased in male ICR mice feeding 0.42 μg of selenoneine/day. Elevated blood glucose causes renal dysfunction and kidney hypertrophy (Gross *et al.*, 2005), but the kidneys of male selenoneine-fed ICR mice became smaller (Seko *et al.*, 2023). Thus, they concluded that blood glucose data could not explain the decreased kidney size (Seko *et al.*, 2023). Therefore, increased kidney weight with developing diabetes was thought to be improved, not to be decreased due to toxicity, with EMP intake in the present study using KK-*A*^{y} mice.

Serum PAO level and SOD activity was slightly increased with EMP feeding (Table 6). Antioxidants such as vitamin C, vitamin E, and glutathione contained in serum are detected and reflected as PAO value, which is indicating reduction power or total antioxidation power. SOD, superoxide dismutase, is a radical scavenger and is an enzyme converting superoxide anions to hydrogen peroxide and carbon dioxide. Even so dietary antioxidants are exerting antioxidative action in vivo, the action seems to be not reflected directly to serum antioxidative parameters. In addition, PAO value in KK-*A*^{y} mice (Table 6) was higher than that in SD rats (Table 5) against expectation. The reason may that serum uric acid level reflected to PAO value is often high in diabetes. PAO value has been reported to decrease in Alzheimer disease subjects (Strafacea *et al.*, 2005) and coronary artery disease (Vassalle *et al.*, 2004) compared to normal subjects. Other parameters involved in antioxidative action such as catalase and glutathione peroxidase also should be investigated in the future.

EMP intake tended to decrease the elevated serum insulin, leptin, triglyceride, and free fatty acid levels (Table 6), but could not suppress the elevated blood glucose level (Figure 3). Thus, EMP may indicate improvement tendency for disordered lipid metabolism and insulin-leptin resistance. However, hepatic triglyceride concentration tended to increase adversely with EMP intake. Omagari *et al.* (2018) had reported the effect of EMP feeding on nonalcoholic steatohepatitis (NASH) in male SD rats fed a high fat- and cholesterol- (HFC) diet. The additional level of EMP into HFC diet were 1, 2.5, or 5%, and the feeding period was from 9 to 18 weeks old. As a result, serum leptin and cholesteryl ester levels were reported to be decreased significantly in the EMP-dose dependent manner. ~~in SD rats, which~~ These results are corresponding to our study using SD rat (Table 5). The report has concluded that EMP could prevent NASH developing through the antioxidant action leading to suppress the inflammation (lowered NF-kB). ~~SD rats fed the HFC diet leading to lipid metabolism disorder are thought to be similar status with KK-*A*^{y} mice in this study.~~

Therefore, our findings implied that EMP intake has possibility to involve in the improvement of disordered lipid metabolism via an immune modulation by various antioxidants contained in EMP. On the other hand, there might be undesirable side that EMP feeding tended to increase TNF-α and IL-1β expressions and hepatic triglyceride concentration accompanying elevated fatty acid synthase (FAS) activity (Omagari *et al*, 2018). The elevated hepatic triglyceride level with EMP feeding~~, which~~ is also comparable to this study (Table 6). Further research is needed to resolve it.

Conflict of interest

Shizuka Hase-Tamaru received a research grand from LS Corporation for this works. The other authors declared no conflict of interest.

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633

Figures and Tables

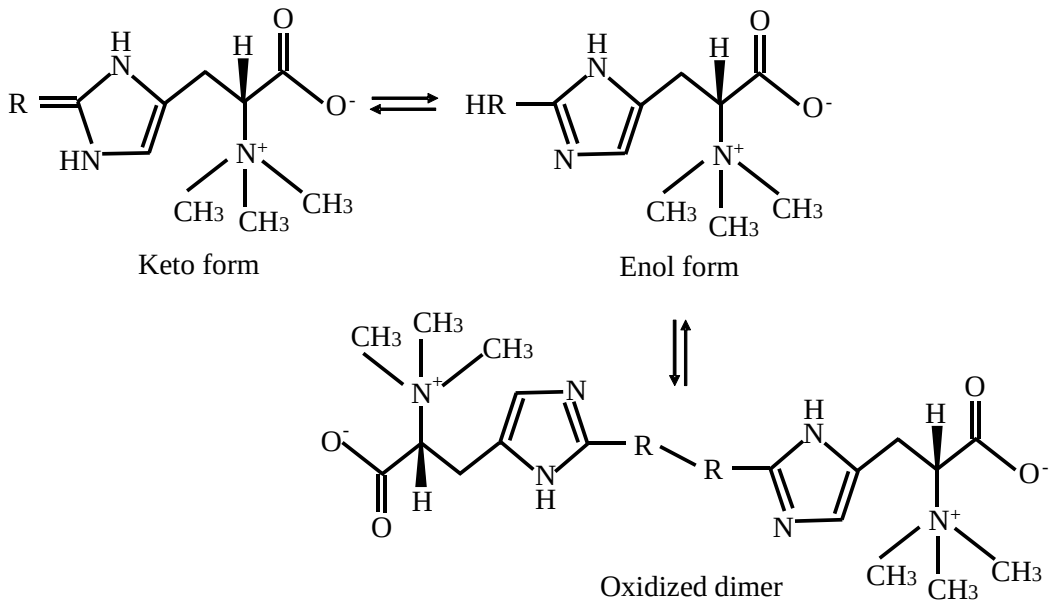

Figure 1. Chemical structures of ergothioneine, selenoneine, and the other selenium compounds

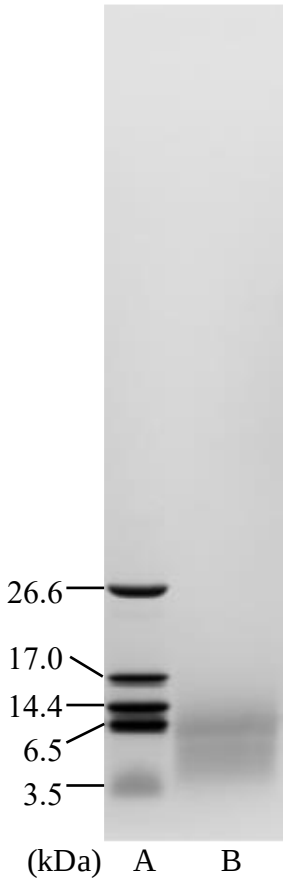

Figure 2. The molecular distribution of mackerel meat hydrolysate (EMP)
A, Polypeptide SDS-PAGE Molecular weight Standards; B, EMP powder

Table 1. Nutritional composition of the hydrolysate of mackerel protein (EMP)

Nutrients	(g/100 g EMP powder)
Moisture	2.5
Crude protein	90.0
Crude fat	0
Crude ash	8.9
Carbohydrate	0
Dietary fiber	0.6
Sodium	1.15
Sodium chloride equivalent	2.92
Energy (kcal)	361.2
Total selenium (μg/g)	10.5
Selenoneine (μg Se/g)	4.4

668 **Table 2**. Dietary composition for experimental animals (AIN-93G composition)

Group	Control	5% EMP	10% EMP
β-Corn starch	39.7486	39.7486	39.7486
Casein	20	15	10
α-Corn starch	13.2	13.2	13.2
Sucrose	10	10	10
Cellulose	5	5	5
Soybean oil	7	7	7
Mineral mix	3.5	3.5	3.5
Vitamin mix	1	1	1
L-Cystine	0.3	0.3	0.3
Choline btartrate	0.25	0.25	0.25
t-Butylhydroquinone	0.0014	0.0014	0.0014
EMP	0	5	10
Total	100	100	100

669 EMP, hydrolysate of mackerel protein

670

671 **Table 3**. Antioxidant activities of ergothioneine, selenoneine, the other selenium
672 compounds, and EMP *in vitro*

Samples	PAO (μmol Cu^{2+}/mg sample)	DPPH radical scavenging ability (μmol trolox eq./g sample)	SOD-like activity (IC_{50}, mg/mL)
Trolox (reference)	12.9±0.0	-	22.5±4.5
Ergothioneine	12.6±0.4	2,925±163	27.1±5.2
Selenoneine	300.4±9.6 *	26,626±2,050 *	0.0327±0.004 *
Selenious acid	0.000476±0.000388 *	1.25±0.02 *	54.3±1.2
Seleno-L-methionine	0.0170±0.00163 *	not detected	not detected
EMP	0.183±0.012 *	10.9±1.4 *	24.0±4.5

673 Data are expressed as mean ± SD for triplicate assays. *, Significant differences for
674 ergothioneine at $p<0.05$ calculated using Dunnett's test. EMP, hydrolysate of mackerel
675 protein; PAO, power of antioxidant; DPPH, 1, 1-diphenyl- 2- picryhydrozyl; SOD,
676 superoxide dismutase

Table 4. The effect of EMP on cytokine secretion from RAW264.7 cells into the medium

LPS-stimulation	Groups	NO (nmol/mL)	TNF-α (ng/mL)	IL-6 (ng/mL)	IL-10 (ng/mL)
-	Control	30.4 ± 0.7 *	1.35 ± 0.14 *	0.005 ± 0.004 *	not detected
+	Control	120.2 ± 3.9	1.99 ± 0.07	1.47 ± 0.013	0.22 ± 0.01
+	EMP	134.0 ± 1.4 *	1.97 ± 0.05	1.51 ± 0.004 *	0.35 ± 0.01 *

Data are expressed as mean ± SD for triplicate assays. *, Significant differences for Control (LPS (+)) at $p<0.05$ calculated using Dunnett's test. EMP, hydrolysate of mackerel protein; LPS, lipopolysaccharide; NO, nitric oxide; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-10, interleukin-10

683 **Table 5**. Growth parameters, tissue weights, and biomarkers in SD rats fed a diet
684 containing 0, 5, or 10% EMP for 28 days
685

Group	Control	5% EMP	10% EMP
Growth parameters			
Initial body weight (g)	119±2	118±2	117±1
Final body weight (g)	364±11	358±12	355±10
Body weight gain (g/day)	8.75±0.32	8.60±0.42	8.49±0.35
Feed intake (g/day)	21.5±0.7	23.9±1.0	21.4±1.1
Tissue weights (g)			
Liver	15.9±0.7	15.1±0.9	14.3±0.6
Spleen	0.83±0.05	0.84±0.06	0.77±0.04
Kidney	2.75±0.14	2.69±0.13	2.58±0.08
Testicle	3.35±0.11	3.19±0.05	3.37±0.02
Cecum (containing content)	3.42±0.32 ab	3.28±0.23 a	5.11±0.61b
Fecal excretion (g/day)	4.93±0.24	5.02±0.40	5.39±0.54
White adipose tissue weights (g)			
Perirenal	4.90±0.61	4.84±0.81	5.06±0.94
Epididymal	3.89±0.53	3.27±0.49	3.55±0.39
Mesenteric	3.44±0.41	3.60±0.45	3.30±0.49
Total	12.2±1.4	11.7±1.5	11.9±1.8
Blood parameters			
Hemogram			
Leukocyte (/μL)	4300±464	4133±789	3667±603
Erythrocyte ($\times 10^4$/μL)	690±14	712±5	680±15
Hemoglobin (g/dL)	13.3±0.2	13.9±0.2	13.3±0.2
Hematocrit (%)	43.5±0.6 a	45.9±0.5 b	43.9±0.7 ab
MCV (fL)	63.1±1.0	64.4±0.8	64.6±0.4
MCH (pg)	19.3±0.3	19.6±0.2	19.6±0.3
MCHC (%)	30.5±0.4	30.4±0.3	30.3±0.3
Platelet ($\times 10^4$/μL)	83.6±2.4	76.4±2.5	73.0±11.4
Reticulocyte (%)	33±2	31±3	30±3
Neutrophil (%)	15.2±2.4	16.8±2.3	13.8±2.0
Eosinophil (%)	0.8±0.2	1.5±0.3	1.5±0.2
Basophil (%)	0.0±0.0	0.0±0.0	0.2±0.2
Monocyte (%)	2.0±0.6	2.0±0.4	1.7±0.2
Lymphocyte (%)	81.2±2.9	79.7±2.6	82.8±2.2
Glycemic			
Glucose (mg/dL)	144±7	138±6	154±10
HbA1c (%)	5.1±0.1	5.1±0.1	4.9±0.1

686 Continued on next page
687

Group	Control	5% EMP	10% EMP
Serum parameters			
Hepatic function			
AST (GOT) (U/L)	113±12	112±15	112±10
ALT (GPT) (U/L)	21±1	24±2	21±1
ALP (U/L)	882±67	879±59	736±58
γ-GT (U/L)	0±0	0±0	0±0
Glycemic			
Creatinine (mg/dL)	0.29±0.05	0.35±0.04	0.37±0.02
Uric acid (mg/dL)	1.1±0.1	1.0±0.1	1.1±0.1
Antioxidative			
PAO (μM)	487±54	481±68	540±86
SOD activity (U/mL)	474±32	448±37	500±29
Lipid			
Triacylglycerol (mg/dL)	159±53	155±34	120±25
Free fatty acid (mEq/L)	2.63±0.25	2.35±0.29	2.36±0.24
Total cholesterol (mg/dL)	78±7	61±4	71±5
Free cholesterol (mg/dL)	15±2	13±1	15±1
Cholesteryl ester (mg/dL) #	62±5 b	48±3 a	56±4 ab
HDL-cholesterol (mg/dL)	33±2	27±2	30±2
LDL-cholesterol (mg/dL)	6±1	6±0	7±1
LDL-/HDL- ratio #	0.18±0.02	0.22±0.03	0.26±0.03
Hormone			
Insulin (pg/mL)	397±21 ab	458±45 b	284±13 a
Leptin (pg/ml)	462±61	241±22	278±33
Adiponectin (ng/ml)	2.94±0.24	3.52±0.32	3.72±0.28
Mineral			
Sodium (mEq/L)	142±0.6	143±0.6	143±0.2
Chlorine (mEq/L)	99.3±0.5	99.5±0.7	99.3±0.4
Potassium (mEq/L)	4.5±0.1	4.4±0.2	4.5±0.2
Calcium (mg/dL)	10.5±0.1	10.5±0.1	10.3±0.1
Phosphorus (mg/dL)	10.2±0.2	26.6±16.8	10.6±0.4
Hepatic lipid			
Triacylglycerol (mg/liver)	208±44	176±43	111±22
Total cholesterol (mg/liver)	39.1±2.6	39.8±4.8	33.4±1.6

Continued on next page

690 Data are expressed as mean ± SE for 5-6 rats.
691 a,b,Values not sharing a common letter within a column are significantly different at
692 $p<0.05$ (Tukey-Kramer).
693 EMP, hydrolysate of mackerel protein; PAO, power of antioxidant; SOD, superoxide
694 dismutase; #, calculated value; cholesteryl ester = total cholesterol - free cholesterol; LDL-
695 /HDL- ratio = LDL-cholesterol/HDL-cholesterol
696

Table 6. Growth parameters, tissue weights, and biomarkers in KK-A^y mice fed a diet containing 0 or 10% EMP for 48 days

Group	Control	10% EMP
Growth parameters		
Initial body weight (g)	27.2±0.7	27.4±0.3
Final body weight (g)	38.9±1.1	38.6±0.9
Body weight gain (g/day)	0.25±0.03	0.23±0.02
Feed intake (g/day)	4.01±0.52	4.91±0.24
Tissue weights (g/100 g body weight)		
Liver	5.07±0.26	4.88±0.30
Spleen	0.24±0.01	0.20±0.03
Kidney	1.59±0.05	1.28±0.02*
Cecum (containing content)	0.75±0.05	0.89±0.09
White adipose tissue weights (g/100 g body weight)		
Perirenal	1.70±0.08	1.72±0.13
Epididymal	3.91±0.08	3.97±0.12
Total	5.61±0.16	5.69±0.25

Group	Control	10% EMP
Serum parameters		
Antioxidative		
PAO (μM)	818±214	971±150
SOD activity (U/mL)	381±21	475±17
Carbohydrate and lipid metabolism		
Glucose (mg/dL)	564±13	555±17
Insulin (ng/mL)	10.7±1.9	5.1±1.2
Leptin (ng/mL)	20.5±0.3	12.5±0.1
Adiponectin (μg/mL)	50.2±6.3	55.3±8.9
Triacylglycerol (mg/dL)	271±45	177±41
Free fatty acid (mEq/L)	2.11±0.33	1.56±0.33
Total cholesterol (mg/dL)	148±32	147±22
Hepatic lipid		
Triacylglycerol (mg/liver)	83.9±17.0	134.3±24.8
Total cholesterol (mg/liver)	11.7±0.9	16.3±2.4

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Growth parameters		
Initial body weight (g)	27.2±0.7	27.4±0.3
Final body weight (g)	38.9±1.1	38.6±0.9
Body weight gain (g/day)	0.25±0.03	0.23±0.02
Feed intake (g/day)	4.01±0.52	4.91±0.24
Tissue weights (g)		
Liver	1.98±0.16	1.89±0.15
Spleen	0.09±0.01	0.08±0.01
Kidney	0.62±0.04	0.49±0.01*
Cecum (containing content)	0.29±0.03	0.34±0.04
White adipose tissue weights (g)		
Perirenal	0.66±0.03	0.67±0.06
Epididymal	1.52±0.07	1.54±0.07
Total	2.18±0.10	2.21±0.13

Group	Control	10% EMP
Serum parameters		
Antioxidative		
PAO (μM)	818±214	971±150
SOD activity (IC_{50}, U/mL)	381±21	475±17
Carbohydrate and lipid metabolism		
Glucose (mg/dL)	564±13	555±17
Insulin (ng/mL)	10.7±1.9	5.1±1.2
Leptin (ng/mL)	20.5±0.3	12.5±0.1
Adiponectin (μg/mL)	50.2±6.3	55.3±8.9
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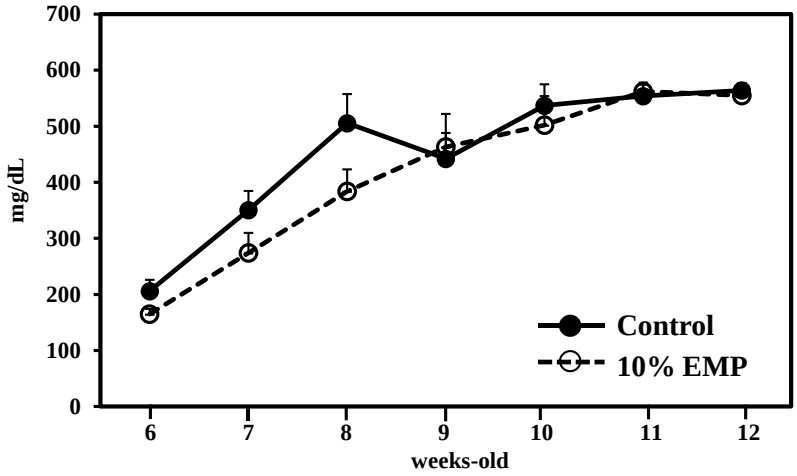
Data are expressed as mean ± SE for 6 mice.

*, Significant difference from Control group (p<0.05, t-test); EMP, hydrolysate of mackerel protein; PAO, power of antioxidant; SOD, superoxide dismutase



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705 **Figure 3**. Change of blood glucose level in KK-*A*^{y} mice fed a diet containing 0 or 10%
706 EMP for 48 days

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708 Data are expressed as mean ± SE for 6 mice.
709 EMP, hydrolysate of mackerel protein