Studies on biological activities of low dose of phenethylamine from hot water extract of *Chlorella pyrenoidosa*

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Contents

General Introduction

Chlorella is a fresh water unicellular green alga. It is rich in vitamins, minerals, dietary fiber, lipids, nucleic acids, all essential amino acids, and other bioactive substances, such as, polysaccharide, haemagglutinin and so on.[1-3] Dried chlorella, *Chlorella pyrenoidosa* and *Chlorella vulgaris,* has a long history of being used as a food supplement. To date, the world production of chlorella is approximately 5000 tons per year. With the development of functional food industry, the global market of chlorella ingredients probably reaches 7 billion US dollar by 2022.[4]

Human trials and animal experiments have demonstrated beneficial effects of dried *C. pyrenoidosa* by oral administration. Dried powder of *C. pyrenoidosa* ameliorated high fat diet-induced dyslipidemia in rats[5] and hyperglycemia-mediated oxidative stress in streptozotocin-induced diabetic nephropathic rats.[6] Some human trials have demonstrated that oral administration of *C. pyrenoidosa* reduced the risk of anemia, proteinuria, and edema in pregnant women[7] and significantly increased the secretion of salivary secretary immunoglobulin A, which play an important role in human immune function.[8] In addition to the dried form of *C. pyrenoidosa*, hot water extract of *C. pyrenoidosa* (WEC) was prepared to concentrate potential bioactive compounds, which has been referred to as chlorella growth factor (CGF) in some articles[1]. WEC was demonstrated to suppress ovariectomy-induced body weight gain and dyslipidemia in rats.[9] WEC extended the lifespan of tumor-bearing mice possibly by exhibiting immune modulatory activity.[10] Some clinical trials have demonstrated daily intake of WEC help to relieve fibromyalgia, hypertension and ulcerative colitis.[1]

These facts suggest that some water-soluble components of *C. pyrenoidosa* are responsible for these beneficial effects. Reported beneficial activities of WEC could not solely be attributed to the conventional nutrients, even though it contains relatively high contents of vitamins, amino acids, and other nutrients. Before this study, there was limited information on active compounds beyond conventional nutrients in WEC.

When underlying mechanism for the beneficial activity is understood, high throughput screening method using enzyme and cell culture systems could be developed. By using the high throughput screening method, activity-guided fractionation has been successfully used for identification of active compounds. However, it was difficult to detect active compounds in chlorella using *in vitro* assay, because the mechanism for the reported beneficial effects of chlorella was unknown. Animal experiments using rodents required a relatively large samples of fractions for evaluation of activity. In addition, animal experiment is time consuming. Therefore, it is also difficult to identify active compounds by animal experiment using rodent model. On the contrary, small insect such as *Drosophilia melanogaster* requires small samples and has short life span compared to rodents. In addition, oxidative stress can be loaded to *D. melanogaster* by knocking out of antioxidant enzyme such as superoxide dismutase (SOD)-1. Indeed, the superoxide dismutase *Sod1* mutant adults of *D. melanogaster* showed markedly short lifespan (10–20 days).[11-13] It was demonstrated that anti-oxidant vitamins and phytochemicals extended the lifespan of the *D. melanogaster* adults and this lifespan-elongation activity was associated with the health promoting activity of these food compounds.[13-16] However, the *Sod*1 mutant

adults of *D. melanogaster* has not been used for activity-guided fractionation.

In chapter 1, effect of WEC on life span of *Sod*1 mutant adults of *D. melanogaster* was examined. Consequently, it prolonged the life span. The activity-guided fractionation based on life span of *Sod*1 mutant adults of *D. melanogaster* and liquid chromatographic separation successfully identified phenethylamine as active compound in WEC.

As very low dose of phenethylamine (60 ng/g in diet) could extend life span of *Sod1* mutant adults of *D. melanogaster* (chapter 1), phenethylamine may ameliorate oxidative stress in mammalian. To confirm it, effects of low dose of phenethylamine on oxidation stress, anti-oxidant enzyme activity, and liver function of nonalcoholic fatty liver disease (NAFLD) mouse model was examined in Chapter 2. It has been demonstrated that high fat diet feeding caused liver damage in mice via increasing lipid peroxidation and decreasing SOD-like activity in the liver.[17,18] Consequently, oral administration of low dose (10 μg/kg body weight /day) of phenethylamine and WEC (100 mg/kg body weight /day) ameliorated high fat diet-induced oxidative stress and liver damage via increasing endogenous antioxidant enzyme activity.

Phenethylamine is classified as monoamine. It has been demonstrated that monoamines are generated by microorganisms during the storage and fermentation of certain foods and high concentrations of monoamine in spoiled food could cause some health problems.[19] However, biological activities by trace amounts of monoamines, which are demonstrated in Chapters 1 and 2, have not been reported before the present study. It has been demonstrated that very low contents of phenethylamine were found in some food items, such as, cheeses (0–3 μg/g), a chocolate (2.6 μg/g),

and wines (approximately 1 μg/mL)[20-22], and some fermented foods.[23] To find phenethylamine rich foods having been eaten long times, contents of phenethylamine in some fermented foods used in Japan were examined in Chapter 3. Consequently, some foods in one or few serving can provide amounts of phenethylamine, which could exert beneficial activities in animal model as shown in Chapter 2.

The present study first sheds light on the function of trace amounts of phenethylamine found in food.

Chapter 1. Phenethylamine in hot water extract of *Chlorella pyrenoidosa* expands lifespan of *SOD*1 mutant adults of *Drosophila melanogaster* at very low dose

1-1 Introduction

As mentioned in General Introduction, *C. pyrenoidosa* and its hot water extract (WEC) are rich in nutrients and has long history as food ingredient. Animal experiments and human clinical trials have demonstrated that oral administration of *C. pyrenoidosa* and WEC exert beneficial effects beyond conventional nutritional value. *In vitro* experiments also demonstrated that WEC stimulated cytokine production in human peripheral blood mononuclear cells.[24] It was demonstrated that polysaccharide extracted from chlorella contributes to immune modulatory activities.[3] There is, however, limited information on active compounds responsible for other beneficial activities on ingestion of *C. pyrenoidosa* and WEC.

The objective of the study in this chapter is to identify active compounds responsible for the beneficial effects of WEC. For this purpose, *Sod*1 mutant adults of *Drosophilia melanogaster* was used to detect biological activity as mentioned in General Introduction. The active compounds in WEC, were identified by the activity guided fractionation based on life span assay by using *Sod1* mutant adults of *D. melanogaste* and fractionation by solid phase chromatography, size exclusion

chromatography, and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ) precolumn derivatization followed by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis. By using this approach, phenethylamine, decarboxylated phenylalanine, was identified as active compound.

1-2. Materials and methods

Materials

Dried *C. pyrenoidosa,* and WEC were prepared and supplied by Sun Chlorella (Kyoto, Japan). Isopentylamine, 2-methylbutylamine, and phenethylamine were purchased from Nacalai Tesque (Kyoto, Japan) and Tokyo Chemical Industry (Tokyo, Japan), and 6-aminoquinolyl- *N*-hydroxysuccinimidyl carbamate (AccQ) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Acetonitrile (HPLC grade) was purchased from Nacalai Tesque. WST-1 SOD assay kit was purchased from Dojindo laboratory (Kumamoto, Japan).

Chromatographic fractionation

Sep-Pak C18 35 cc Vac Cartridge (Waters, Miliford, MA) was successively pretreated with acetonitrile (50 mL) and water (50 mL). WEC was dissolved in distilled water to obtain 5 % (w/v) solution. After centrifugation of the solution at 3000 *g* for 10 min, the supernatant was collected. The supernatant (300 mL) was loaded onto the Sep-Pak C18 35 cc Vac Cartridge, and the unabsorbed fraction was

collected. The cartridge was then washed with distilled water (50 mL). The compounds absorbed by the column were successively eluted using 10, 30, 100% (v/v) of acetonitrile, acetone, and hexan (50 mL). The unabsorbed fraction and fractions eluted with 10% and 30% acetonitrile were referred as the UA, 10ACN, and 30ACN fractions, respectively. These fractions were freeze-dried. Effluents with acetonitrile, acetone, and hexan were combined and dried in a rotary evaporator, and the resultant fraction was referred as the AAH fraction. These fractions were then evaluated for lifespan-elongation activity towards *Sod1* mutant adults of *D. melanogaster*.

Based on the results of the first Sep-Pak C18 fractionation, compounds in WEC were re-fractionated. WEC (1% water solution, 100 mL) was loaded onto the Sep-Pak C18 35 cc Vac Cartridge. After the UA fraction was eluted, the cartridge was washed with distilled water (100 mL). The compounds absorbed by the column were eluted by 30 % (v/v) acetonitrile (100 mL). The effluent was freeze-dried and dissolved in distilled water to obtain a 1% (w/v) solution. The solution was filtered through a Cosmonice filter W (0.45 μm, Nacalai Tesque). Aliquot (200 μL) of the filtrate was loaded onto the size exclusion chromatography (SEC) column (Superdex Peptide, 10/300, GE Healthcare, Buckinghamshire, UK), which was equilibrated with 30% acetonitrile containing 0.1% formic acid. Elution was performed at 0.5 mL/min. The Fractions were collected every minute, and these steps were repeated 10 times.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses

Compounds in the SEC fraction were resolved by reversed phase-high performance liquid chromatography (RP-HPLC) and detected by electrospray ionization-mass spectrometry (ESI-MS) in positive and negative modes. However, only a few peaks of

nucleoside and amino acids were detected (data not shown). To improve resolution and detection of compounds with primary and secondary amines, derivatization with AccQ, followed by LC-MS/MS, was performed (Ejima *et al*., 2018). Aliquots (200 μL) of SEC fractions were dried under vacuum. The residue was dissolved in distilled water (20 μL), and 0.3% AccQ acetonitrile solution (20 μL) and 50 mM sodium borate buffer (60 μL) (pH 8.8) were added. The resultant solution was kept at 50°C for 10 min. The reactant was clarified by passing it through a Cosmonice filter W. The filtrate (20 μL) was analyzed by LC-MS at precursor ion scan mode with an LCMS 8040 (Shimadzu, Kyoto, Japan) equipped with RP-HPLC column (Cosmosil 5C_{18} MS-II, Nacalai Tesque), targeting the
AccQ-derived product ion (*m/z* 171.1) at collision energy -35 eV. A binary linear gradient, with 0.1% formic acid (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent B), was used at a flow rate of 0.2 mL/min. The gradient program was as follows: 0–15 min, 0–50% B; 15–20 min, 50–100% B; 20–25 min, 100% B; 25–25.1 min, 100–0% B; 25.1–35 min, 0% B. The column was maintained at 40°C.

Determination of monoamines in WEC and crude chlorella

Monoamines were dissolved in distilled water or ethanol to obtain 10 μM solutions, which were used as standard solutions. Crude chlorella and WEC powders were suspended in distilled water to obtain 1% (w/v) solutions, which were vigorously stirred and then centrifuged at 15000 *g* for 10 min. The supernatants were collected. Monoamines in standard and sample solutions were derivatized with AccQ, as described above. The AccQ-derivatives were determined by LC-MS/MS at

multiple-reaction monitoring (MRM) mode. The elution conditions were the same as described above. MRM conditions for the AccQ-derivatives of standard monoamines were optimized using LabSolutions Version 5.65 (Shimadzu, Kyoto, Japan).

Survival assay using Sod1 mutant D. melanogaster flies

The *Sod*^{n1}*1* mutant flies were prepared by a method as described previously.[13] This mutant showed a considerably declined SOD-1 activity due to an amino acid substitution without the reduced mRNA level.[12] The young adult mutant males, homozygous for *Sod1*^{n1} were collected within 24 hrs after eclosion and 20 flies were added into the tube (100 mm in height and 15 mm in diameter) with medium containing sample at suitable contents and incubated at 25°C and 50-55% humidity. The assay was repeated three to five times (using 60–100 *Sod1* mutant flies). Dead adults in each vial were counted every 12 h for the *Sod1* mutants. Food vials were changed every three days.

SOD-like activity assay

Phenethylamine was dissolved in water to obtain the resultant solution (0.6–600 ng/mL). SOD-like activity was examined by using a WST SOD assay kit. In addition, wild *D. melanogaster* was homogenized with phosphate buffer saline (PBS) (10 μL/individual). The homogenates were mixed with the same volume of water or phenethylamine above and incubated at 25°C for 30 min. The mixture was then centrifuged at 15000 *g* for 10 min. The supernatants were collected, and SOD-like activity was examined.

Statistics

A survival curve was calculated based on Kaplan-Meier survival estimation and analyzed by log-rank test between the drug-treated and control groups. For comparison of the two groups, we used the Student's t-test. One-way ANOVA with post-hoc Tukey's test was applied to assess the differences among the groups. Data were considered significant at p-values < 0.05.

1-3. Results

Lifespan-elongation activity of WEC

As shown in Figure 1-1, WEC extended the survival period of the *Sod1* mutant flies in a dose dependent manner to 800 μg/mL in diet. WEC (3200 μg/mL), however, showed a survival period similar to that of WEC (200 μg/mL) and shorter than that of WEC (800 μg/mL).

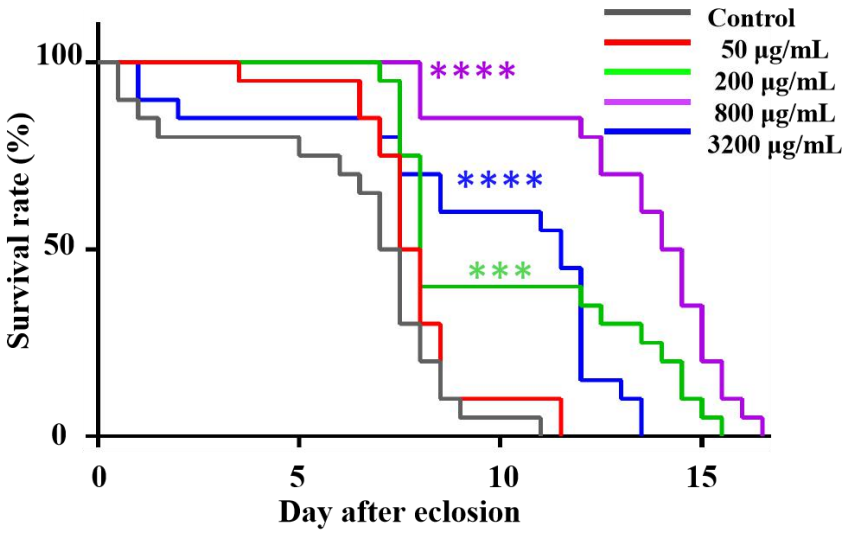

Figure 1-1 Effect of different concentration of WEC on survival rate of *Sod*1 mutant adults of *Drosophilia melanogaster*

Table 1-1. Recovery of solid phase extraction fractions using Sep-Pac C18 cartilage

Fraction	Dry weight (g)	Recovery to WEC (%)
unabsorbed	10.3	68.67
10% acetonitrile	0.6	4
30% acetonitrile	0.59	3.9
AAH	0.06	0.404

Identification of compounds with life span elongation activity

WEC was first fractionated by solid phase extraction using a Sep-Pak C18. As shown in Table 1-1, the UA fraction accounted for approximately 70% (w/w) of the initial materials. The 10ACN and 30ACN fractions approximately accounted for 4% of the initial materials, respectively, while only small amounts of compounds were recovered into the AAH fraction. As shown in Figure 1-2, until day 9, survival rates of all groups treated with WEC and its fractions were higher than that of the control group. Thereafter, survival rate of the AAH group drastically decreased. After day 10, survival rate of the UA group also decreased. On the contrary, the 10ACN and 30ACN groups showed survival curves similar to that of crude WEC. Based on these results, active compounds in WEC were eluted with 30% acetonitrile from the solid phase column after washing the cartilage with water.

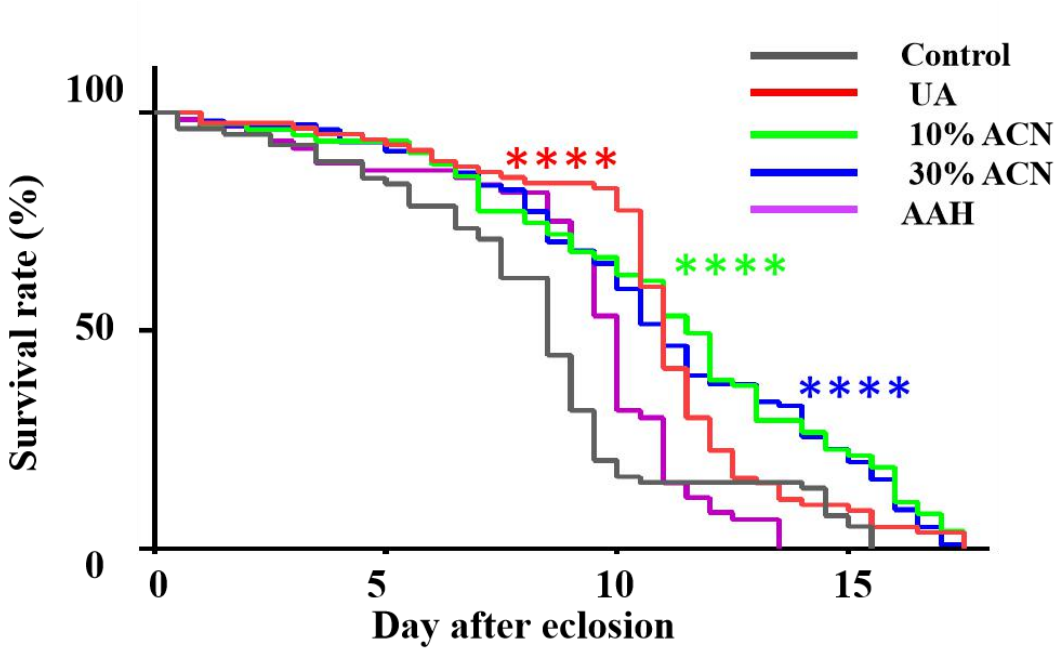

Figure 1-2 Effect of WEC and its fractions on survival rate of *Sod*1 mutant adults of *Drosophilia melanogaster*

The active fraction was further fractionated using SEC. As shown in Figure 1-3A, fractions 16–35, 36–40, 41–44, 45–50, and 51–70 were collected and referred as SEC Fr. I, II, III, IV, and V, respectively. Lifespan-elongation activity of these fractions was evaluated. As shown in Figure 1-3B, SEC Fr. III and V (200 μg/g)-administered fractions, compared to the control, showed a decreased survival rate after day 7. SEC Fr. II-administered group, compared to the control, showed a reduced survival rate between days 2 and 7. Before day 8, SEC Fr. I and IV-administered fractions showed survival curves similar to each other and survival rates higher than that of the control. However, after day 8, survival of *D. melanogaster* of the SEC Fr. I group decreased. SEC Fr. IV-administered group, compared to the control, showed an increased survival rate and lifespan of *D. melanogaster* throughout the experiment ($P<0.001$). Thus, SEC Fr. IV was used as the active fraction for the following experiments.

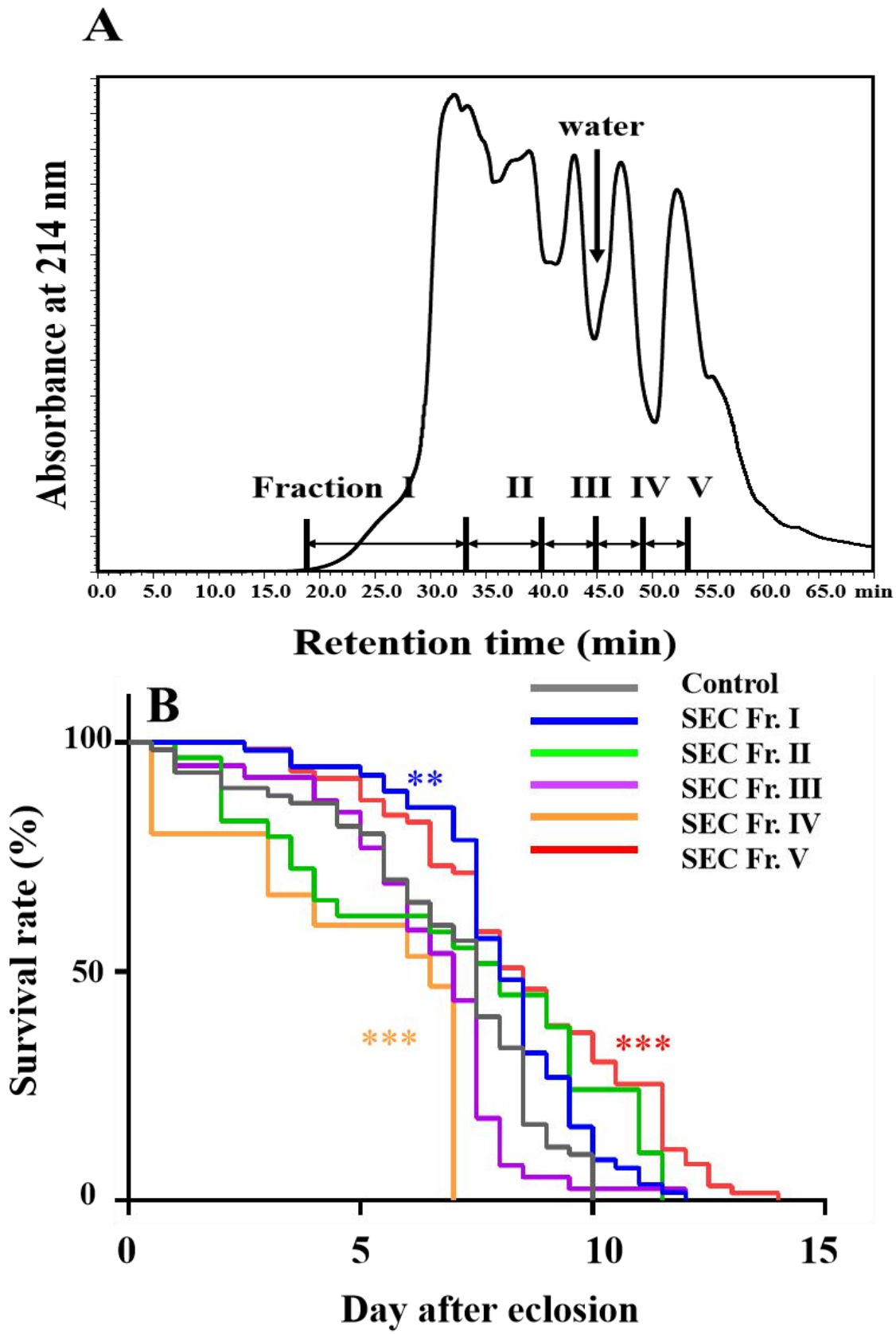

Figure 1-3. Elution pattern of size exclusion chromatography of 30% acetonitrile fraction (A) and the effect of SEC fractions on survival rate of *Sod1* mutant flies (B).

Amino compounds in SEC Fr. III and IV were detected by derivatization with AccQ, followed by LC-MS/MS at precursor scan mode targeting product ions from AccQ (*m/z* = 171.1). As shown in Figure 1-4, some peaks marked with asterisk (*) appeared in both SEC Fr. III (inactive fraction) and SEC Fr. IV (active fraction). On the other hand, four peaks indicated as 1–4 that appeared in SEC Fr. IV were not present in SEC Fr. III. The mass to charge ratio (*m/z*) of compounds in peaks 1–4 were 336, 258, 258, and 292, respectively. The *m/z* 336 and 292 corresponded to protonated AccQ derivatives of phenylalanine and its decarboxylated form, phenethylamine, respectively. The *m/z* 258 corresponded to decarboxylated isoleucine (2-methylbutylamine) and leucine (isopentylamine). Retention times of peaks 1–4 coincided with those of AccQ derivatives of authentic phenylalanine, 2-methylbutylamine, isopentylamine, and phenethylamine. Product ion patterns of these peaks by LC-MS/MS also coincided with those of the authentic amines, respectively. Therefore, peaks 1-4 were identified as phenylalanine, 2-methylbutylamine, isopentylamine, and phenethylamine. Contents of 2-methylbutylamine, isopentylamine, and phenethylamine in WEC were 2.5 ± 0.2, 10.7 ± 0.2, and 11.7 ± 0.4 μg/g, respectively, and those in dry chlorella powder were 0.0 ± 0.00, 0.5 ± 0.02, and 0.8 ± 0.04 μg/g, respectively. As shown in Figure 1-1, WEC (800 μg/mL), which contained isopentylamine and phenethylamine at 8.6 ng/mL and 10 ng/mL, respectively, showed the best lifespan-elongation activity towards the *Sod1* mutant flies. As shown in Figure 1-5, administration of isopentylamine (8.6 ng/mL) did not significantly affect the survival rate. A higher dose (86 ng/mL) of isopentylamine showed survival rates higher than that of the

control before day 5. However, after day 6, the survival rate drastically decreased, and maximum lifespan was shorter than that of the control group. Phenethylamine (60 ng/mL) showed a survival rate significantly higher ($P<0.001$) than that of the control group and a maximum survival period approximately 150% of that of the control group. However, phenethylamine (600 ng/mL) showed a survival rate lower than that of the control group. On the other hand, pentylamine, a non-natural monoamine, exerted lifespan-elongation activity at doses of 20 and 200 ng/mL ($p<0.001$ and 0.0001 respectively).

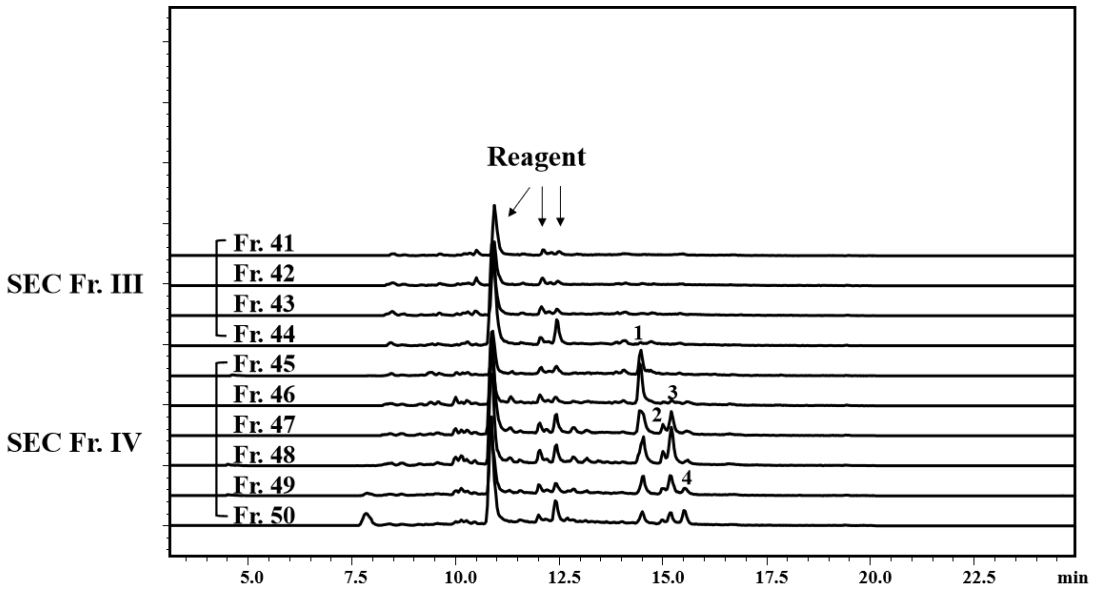

Figure 1-4. Precursor ion scan of AccQ derivatives in SEC fraction III and IV
***Shows reagent peak**

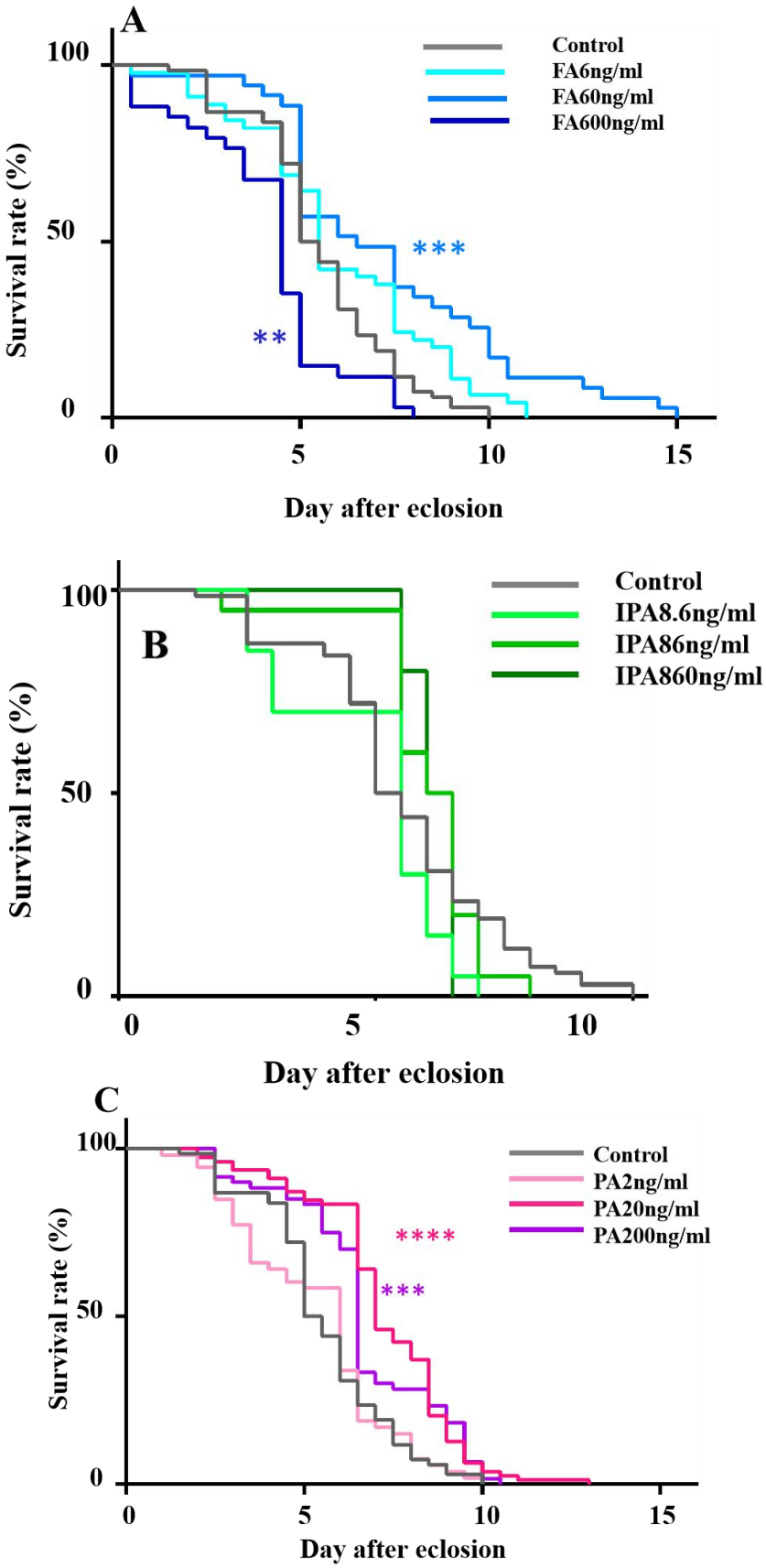

Figure 1-5. Effect of different concentration of phenethylamine (A) , isopentylamine (B) and pentylamine (C) on survival rate of *Sod1* mutant flies

1-4. Discussion

Phenethylamine is an endogenous trace amine, and is contained in some food items, such as cheese, a chocolate and wines as described in General Introduction. The present study demonstrated that WEC contained phenethylamine (10 μg/g of dry matter). It has been reported that oral supplementation of phenethylamine (10–60 mg/day) with selegiline (monoamine oxidase-B inhibitor, 10 mg/day) relieved depression.[25] On the other hand, high dose of phenethylamine (25–75 mg/kg body weight of mice) induced psychomotor dysfunction and decrease in striatal biogenic amines.[26] However, the amount of phenethylamine (10–100 μg) obtained from consumption of a few grams of WEC was similar or less than that obtained from one serving of cheese, chocolate, or wine. Therefore, such low doses of phenethylamine did not exert any adverse effects on human health.

It has been demonstrated that some antioxidant vitamins and food compounds extended the lifespan of *D. melanogaster* and *Caenorhabditis elegans*. Vitamin C (20 mM), lutein (0.1 mg/g), and apple polyphenols (10 mg/g) in the diet extended the lifespan of *D. melanogaster*, respectively.[27-29] Vitamin E (16.7 mg/g of diet) also extended the lifespan of the *Sod1* mutant flies.[27] Coenzyme Q10 (50 μg/g of diet) elongated the lifespan of *C. elegans*.[30] A strong superoxide dismutase/catalase mimetic, EUK-134, at a very low dose of 50 μM (ca.20 μg/g) in the diet, extended the lifespan of *C. elegans*.[31] The present study demonstrated that phenethylamine (6–60 ng/g of diet) expanded the lifespan of the *Sod1* mutant flies, and these doses were far less than those reported in the previous studies. On the contrary, isopentylamine did

not expand the lifespan of the *Sod1* mutant flies; however, pentylamine, a linear chain monoamine, did, suggesting that all hydrophobic monoamines did not always exhibit lifespan-elongation activity and structure of the hydrophobic moiety contributed to the elongation activity. To our best knowledge, there is no food compound, except some trace metals such as selenium, which exerts beneficial activity in such low doses.[32]

Inhibition of SOD-1 resulted in the accumulation of superoxide radicals (O_2^-).[33] Over production of O_2^- caused oxidation of some biomolecules, such as DNA, protein, and lipids,[34] damaging cells. However, phenethylamine (6-6000 ng/mL) did not show significant SOD-like activity. In addition, phenethylamine, which was treated with a homogenate of *D. melanogaster,* did not show significant SOD-like activity at the same dose. As mentioned above, the dose of phenethylamine for elongation of lifespan of the *Sod1* mutant flies was far less than that of vitamins E and C, which are strong antioxidants. It was, therefore, unlikely that antioxidant activities of phenethylamine and its metabolites contributed to the lifespan extension of the *Sod1* mutant flies. Although the mechanism of lifespan extension by phenethylamine remains unknown, it is possible that phenethylamine exerted this activity by binding to some cell surface receptors, intracellular transcription factors, or their regulators. It was demonstrated that some trace amines, such as tyramine and octopamine, exerted significant bioactivities via G protein-coupled receptors.[35] However, the specific receptor for phenethylamine was not identified. Now, further studies on target proteins of phenethylamine in *D. melanogaster* are being conducted.

Effects of oral administration of such low doses of trace amines, such as phenethylamine, on animal and human health have not been examined. In Chapter 2,

effects of low dose phenethylamine on high fat diet-induced dysfunction in mice are described.

1-5. Conclusion

Oral administration of WEC (200 and 800 μg/g of diet) increased the lifespan of the *Sod1* mutant flies in a dose dependent manner. By *in vivo* activity-guided fractionation, phenethylamine was demonstrated to exhibit lifespan-elongation activity. Phenethylamine, compared to compounds including antioxidant vitamins and phytochemicals, increased life span at an extensively lower dose (6–60 ng/g of diet). Since phenethylamine did not show SOD-like activity, it did not exert lifespan elongation activity towards the *Sod1* mutant flies by only direct antioxidant activity. While there is no report on the beneficial effects of ingestion of trace amounts of monoamine, phenethylamine obtained from ingestion of chlorella and other foods might show some beneficial effects on human health. To examine the effects of trace amounts of phenethylamine, an animal experiment using NAFLD mouse model is necessary.

Chapter 2. Phenethylamine, found in water extract of *Chlorella pyrenoidosa*, attenuated high fat diet-induced oxidative stress in mice liver by oral administration at very low dose.

2-1 Introduction

In chapter 1, phenethylamine was identified as an active compound in water extract of chlorella (WEC), which extended the lifespan of superoxide dismutase-1 (*Sod1*) mutant adults of *Drosophila melanogaster* at very low dose (60 ng/g in diet). These facts suggest that low dose of phenthylamine might ameliorate oxidative stress in *D. melanogaster*, as short life span of *D. melanogaster* lacking SOD-1 was rescued. As mentioned in chapter 1, phenythylamine is classified as monoamine and trace amounts of phenethylamine are contained in some food items.[20-22] It has been reported that oral administration of relatively high doses of phenethylamine (10-75 mg/kg of body weight/day) exert pharmacologic and adverse effects.[25,26] However, biological activity of trace amounts of phenethylamine has not been examined in mammalians. The objective of the study in chapter 2, was to elucidate beneficial effects of trace amounts of phenythylamine on mammalian. Previous animal study demonstrated that dried powder of *C. pyrenoidosa* ameliorated high fat diet-induced dyslipidemia in rats[5]. It has been demonstrated that high fat diet feeding also causes liver damage in

rat via increasing lipid peroxidation and decreasing activity of antioxidant enzymes in liver [17,18]. In chapter 2, the effects of trace amounts of phenethylamine on the high fat diet-induced dyslipidemia and oxidative stress in mouse liver were examined.

2-2. Materials and methods

Materials and reagents

Hot water extract of Chlorella pyrenoidosa (WEC) was prepared and supplied by Sun Chlorella (Kyoto, Japan). Phenylethylamine, proteinase inhibitor cocktail, butylated hydroxytoluene (BHA), diethylenetriamine pentaacetic (DTPA), 1,1,3,3-tetraethoxypropane (TEP), thiobarbituric acid (TBA), glutathione (GSH), glutathione disulfide (GSSG), N-ethylmaleimide (NEM), and enhanced chemifluorescence (ECL) reagent were obtained from Nacalai Tesque (Kyoto, Japan). Heparin sodium was obtained from Nipro (Osaka, Japan). Cell lysis reagent was obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit immunoglobulin G (IgG) against mouse superoxide dismutase (SOD)-1 and mouse glutathione peroxidase (GPX) 1 was obtained from Abcam (Cambridge, UK). Mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, which can react to mouse GAPDH and β-actin, was obtained from Proteintech Group (Chicago, IL, USA) and Santa Cruz Biotechnology (Dallas, TX, USA). Goat IgG-horse-radish peroxidase (HRP) conjugates against rabbit and mouse IgG were obtained from Cell Signaling Technology (Danvers, MA, USA).

Animal experiment

All animals were treated and cared for in accordance with the guidelines of the National Institutes of Health (NIH) for the use of experimental animals. All experimental procedures were carried in Louis Pasteur Center for Medical Research after approval of the Animal Care Committee of Louis Pasteur Center for Medical Research (No. 20192). Male C57bl/6J mice (7 weeks old, 21-23 g) were purchased from Japan SLC (Shizuoka, Japan). Three mice were housed in one cage with a 12 h light–dark cycle at 22°C with free access to rodent chow (Certified Diet MF, Oriental Yeast, Osaka) and tap water for 3 days. Subsequently, all mice were divided into 6 groups randomly (n=6) as below. Mice received normal diet and tap water (ND group), high fat diet (60% in calorie base, CLEA Japan, Tokyo, Japan) and tap water (HFD group), the high fat diet and WEC in tap water at 100 mg/kg body weight (WEC group), and the high-fat diet and phenethylamine in tap water at 10 and 100 μg/kg body weight (PL and PH groups, respectively). All mice were sacrificed in the morning under isoflurane anesthesia after 12 weeks without fasting. Blood was collected from inferior vena cava with heparin sodium treated syringe. Plasma was prepared by centrifugation at 3500 rpm for 5 min. Liver was collected and blood in the liver was purged by infusing cold PBS into the portal vein. Plasma and liver were stored at -30°C.

Liver SOD-like activity

Liver tissues were homogenized in 5 volumes (w/v) of 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA by using a BioMasher II (Nippi, Tokyo, Japan). The homogenate was centrifuged at 10000 *g* for 60 min and supernatant was collected for SOD-like activity assay of soluble compounds in the liver. In addition, liver tissues were homogenized in same volumes (w/v) of PBS and then 6 volumes (w/v) ethanol was added. The resultant solution was centrifuged at 10000 *g* for 10 min and the supernatant were collected and used as low molecular weight compound fraction. The SOD-like activity in the supernatant above was assayed by using a WST-1 SOD assay kit (Dojindo, Kumamoto, Japan).

Blood plasma biochemical analysis

Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) contents were determined by outsourcing to Oriental Yeast. For determination of triglyceride in the liver, aliquots of liver tissues (20-30 mg) were homogenized in 200 μL of isopropanol by using a BioMasher II. The homogenate was centrifuged at 1100 *g* for 10 min. The supernatant was used for TG assay by using a triglyceride E-test Wako kit (Wako, Osaka, Japan).

2-thiobarbituric acid reactive substances (TBARS) assay

DTPA was dissolved in 1 M sodium acetate buffer (pH 3.5) to give 2 mM

solution. BHA was dissolved in methanol to give 10% (w/v) solution. Five milliliter of the DTPA solution, 45 mL hot water, and 100 μL BHA solution were added into 0.1 gram TBA to give 0.2% TBA solution. This solution was used as TBA reagent. Ten milliliter methanol was added into 4.8 μL TEP, a malondialdehyde precursor, to give 2 mM TEP solution. The TEP solution was diluted by methanol to 2-50 mM and used as standard solution. Liver tissues were homogenized in 9 volumes (w/v) of 1.15% KCl solution. The homogenate was centrifuged at 2000 *g* for 1 min. The supernatant or standard solution (25 μL) was mixed with TBA reagent (100 μL) and vortexed and then heated at 95°C for 60 min. Reaction was terminated by cooling on ice for 5 min. The reactant was centrifuged at 14200 *g* for 10 min. Absorbance at 515 nm of the supernatant was determined. TBARS value was expressed as μM of malondialdehyde.

Determination of glutathione and glutathione disulfide

Glutathione and its disulfide were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) following derivatization with NEM and AccQ by the method of Yamada et al.[36] Aliquots of 1.15% KCl extract of liver (50 μL) were dried under vacuum. Fifty micro liters of 20 mM NEM PBS solution were added into the residue then kept at room temperature for 10 min. Ethanol (150 μL) was added to the reactant. Precipitate was removed by centrifugation at 14200 *g* for 10 min. Supernatant (180 μL) was dried under vacuum. To the residue, 80 μL of 50 mM sodium borate buffer (pH 8.8) and 20 μL of 0.3% AccQ acetonitrile solution were added. The resultant solution was kept at 50°C for 10 min. The reactant was clarified by passing it through a Cosmonice filter W (0.45 μm, Nacalai Tesque).

AccQ-glutation-NEM derivative and AccQ-glutathione disulfide derivative in the filtrate (10 μL) were determined by LC-MS/MS at multiple-reaction monitoring (MRM) mode by using an LCMS 8040 (Shimadzu, Kyoto, Japan) equipped with RP-HPLC column (Inertstil ODS-3, GL sciences, Tokyo, Japan). A binary linear gradient using 0.1% formic acid (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent B) was used at a flow rate of 0.2 mL/ min. The gradient program was as follows: 0–15 min, 0–10% B; 15–18 min, 10–100% B; 18.1–25 min, 0% B. The column was maintained at 40°C.

Western blotting

Aliquots of liver (approximately 50 mg) were homogenized in 300 μL of cell lysis reagent containing proteinase inhibitor at 1% in a BioMasher II. After centrifugation at 12000 *g* for 15min (4°C), protein concentration in the supernatant was measured by a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) and adjusted to 10 μg/15 μL with same buffer. Proteins were resolved by SDS-PAGE using 12.5% gel and then transferred to a PVDF membrane (0.45 μm pore, GE healthcare Life Sciences, Chicago, IL, USA) by using a semi-dry blotting apparatus (WSE 4020, ATTO, Tokyo, Japan). The membranes were then blocked with 4% Block Ace (Megmilk Snow Brand, Sapporo, Japan) at room temperature for 30 min, followed by washed with 50 mM Tris-HCl buffer, pH 7.5 containing 2.68 mM KCl, 137 mM NaCl containing 0.05% (v/v) Tween 20 (TBST) for 5 min at room temperature (5 times). Primary antibody against SOD-1, GPX-1, β-actin, and GAPDH were diluted to 1:5000, 1:5000, 1:1000, and 1:8000 with 0.4% Block Ace solution

containing 0.05% (v/v) Tween 20, respectively. After overnight incubation with primary antibody, the membranes were washed with TBST for 5 min (5 times). HRP-secondary antibody conjugates were diluted to 1:10000 with 0.4% Block Ace containing 0.05% (v/v) Tween 20. The membranes were incubated with HRP-secondary antibody conjugates for 1h, followed by washing with TBST for 5 min (5 times) at room temperature. The membranes were soaked with ECL reagent for 1min and the bands were detected by using a Lumino graph I (ATTO).

Statistical analysis

The difference of band intensity of protein detected by western blot in same membrane between certain group and HFD group was analyzed by *t*-test. The statistical difference in other parameters between HFD group and other groups was analyzed by using one-way ANOVA, followed by Dunnett's test for multiple comparisons. The differences were considered significant at $p < 0.05$ and to have a certain tendency at $0.05 < p < 0.1$. GraphPad Prism 7 software (GraphPad Software, CA, USA) was used for the statistical analyses.

2-3. Results

Body weight and liver weight

As shown in Figure 2-1, body weight of groups fed high fat diet (HFD, WEC, PL, and PH groups) was significantly higher than that of group fed normal diet (ND group) after 4 weeks. However, administration of WEC and phenethylamine did not

significantly affect body weight gain. Liver weight in HFD group was significantly higher than that in ND group. Ratio of liver to body weight in HFD group tended to be higher than that in ND group, while there was no statistics difference. The ratio tended to decrease in the mice received phenethylamine (PL and PH groups) compared to HFD group ($p < 0.1$). Liver triglyceride content was significantly higher in HFD group than that in ND group. Administration of low dose of phenethylamine significantly decreased liver TG.

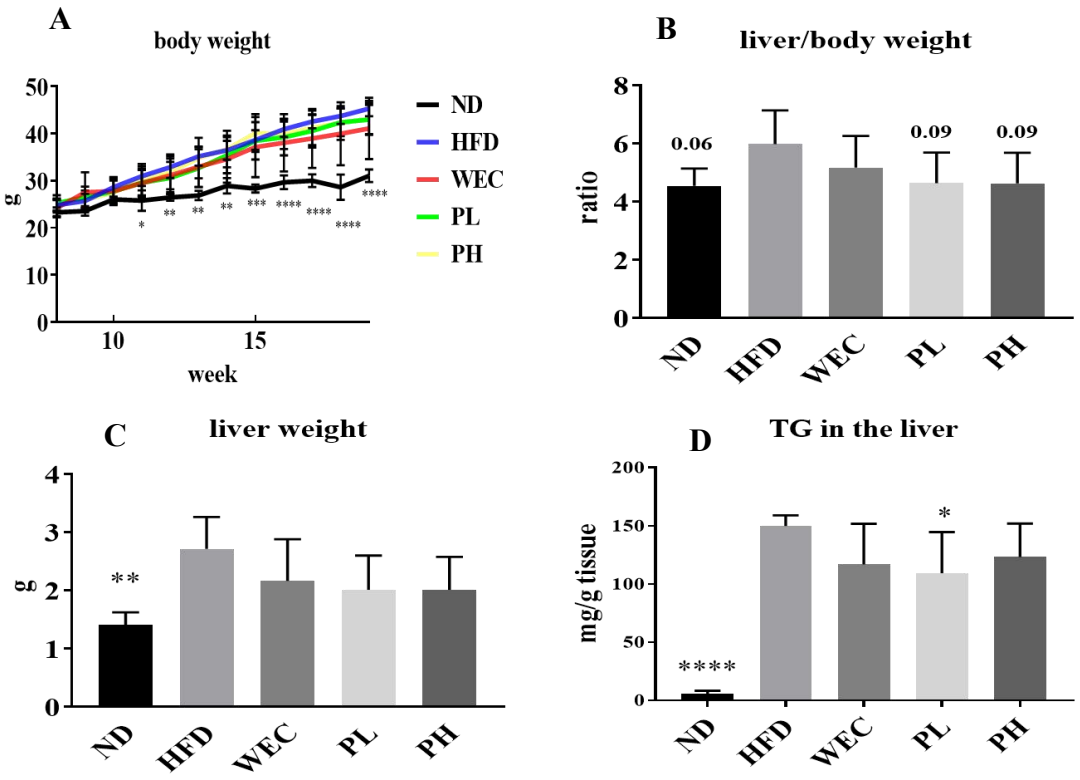

Figure 2-1. Body weight change over time, liver weight, the ratio of liver and body weight and TG in the liver in mice fed normal diet (ND), high fat diet (HFD), HFD+ low or high dose of phenethylamine (10 (PL) or 100 (PH) μg/kg), HFD+ water extract of *chlorella pyrenoidosa* (WEC). Data are shown as mean±S.D (n=6). Asterisks indicate significant differences (p<0.05; Dunnett's test).

Plasma biochemical parameters

lasma AST and ALT activities (Figure 2-2A and B) were significantly higher in HFD group than those in ND group, which indicates that liver damage was induced by high fat diet feeding. Administration of low dose of phenylethylamine (10 μg/kg/day, PL group) significantly reduced plasma AST and ALT activities compared with HFD group, while administration of high dose of phenethylamine (100 μg/kg/day, PH group) only showed tendency for decrease of ALT. On the other hand, administration of WEC did not significantly affect AST and ALT activities ($p>0.1$).

High fat diet feeding significantly increased plasma LDL-C, HDL-C, and T-CHO (Figure 2-2C,D and E) compared to normal diet feeding, which indicates that high fat diet feeding affected cholesterol metabolism. Administration of WEC and phenythylamine significantly decreased plasma LDL-C level compared to HFD group in spite of no significant effect on HDL-C level. Administration of WEC and phenethylamine also tended to decrease T-CHO in lesser extent compared to LDL-C. There is no dose dependency for phenylethylamine on cholesterol levels. No significant difference in plasma triglyceride level was observed between all groups (Figure 2F).

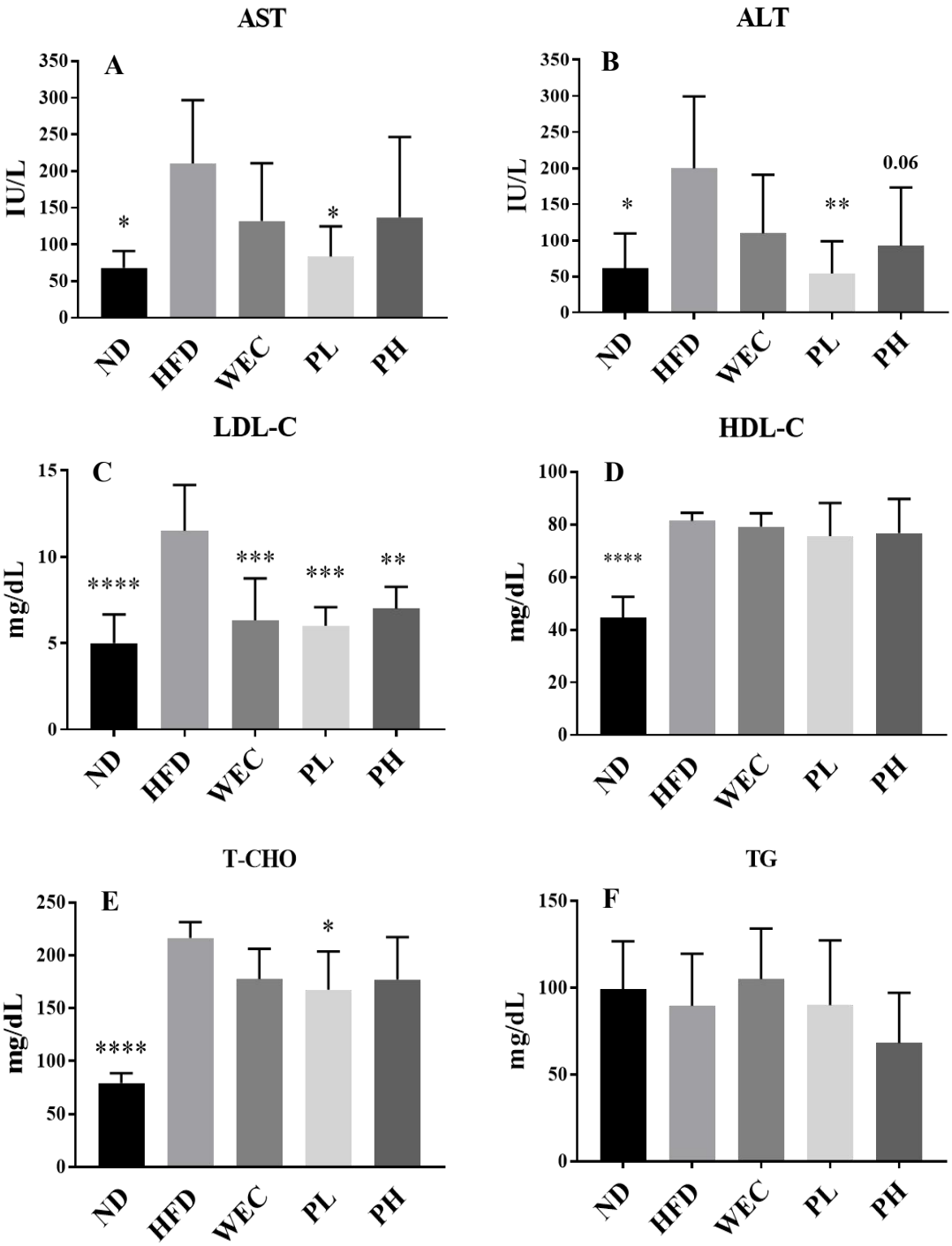

Figure 2-2. Plasm aspartate aminotransferase (AST) and alanine aminotransferase acitvities, low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), total cholesterol (T-CHO) and Triglyceride (TG) in mice fed normal diet (ND), high fat diet (HFD), HFD+ low or high dose of phenethylamine (10 (PL) or 100 (PH) μg/kg), HFD+ water extract of *chlorella pyrenoidosa* (WEC). Data are shown as mean±S.D (n=6). Asterisks indicate significant differences ($p<0.05$; Dunnett's test).

Oxidative stress in liver

High fat diet feeding (HFD group) significantly increased thiobarbituric acid reactive substances (TBARS, Figure 2-3A) value , an index of lipid peroxidation and decreased SOD-like activity (Figure 2-3C) in the liver extracts compared to normal diet feeding (ND group). Moreover, high fat diet feeding tended to reduce the ratio of glutathione to glutathione disulfide (GSH/GSSG, Figure 2-3B), an oxidative stress marker. These facts indicate that high fat diet increased oxidative stress in liver and decreased endogenous antioxidant system. Administration of WEC and phenylethylamine both at low and high doses significantly suppressed high fat diet-induced lipid oxidation. Administration of WEC and low dose of phenethylamine significantly increased SOD-like activity in the liver extract compared to HFD group, while high dose of phenylethylamine did not affect SOD-like activity in the liver. On the other hand, only negligible SOD-like activity was observed in the low molecular weight fraction (75% ethanol-soluble fraction, Figure 2-3D) of liver from all groups compared to the liver extract containing protein. GSH/GSSG in the liver also significantly increased in the PL group compared to HFD group, whereas no significant difference was observed between WEC, PH, and HFD groups.

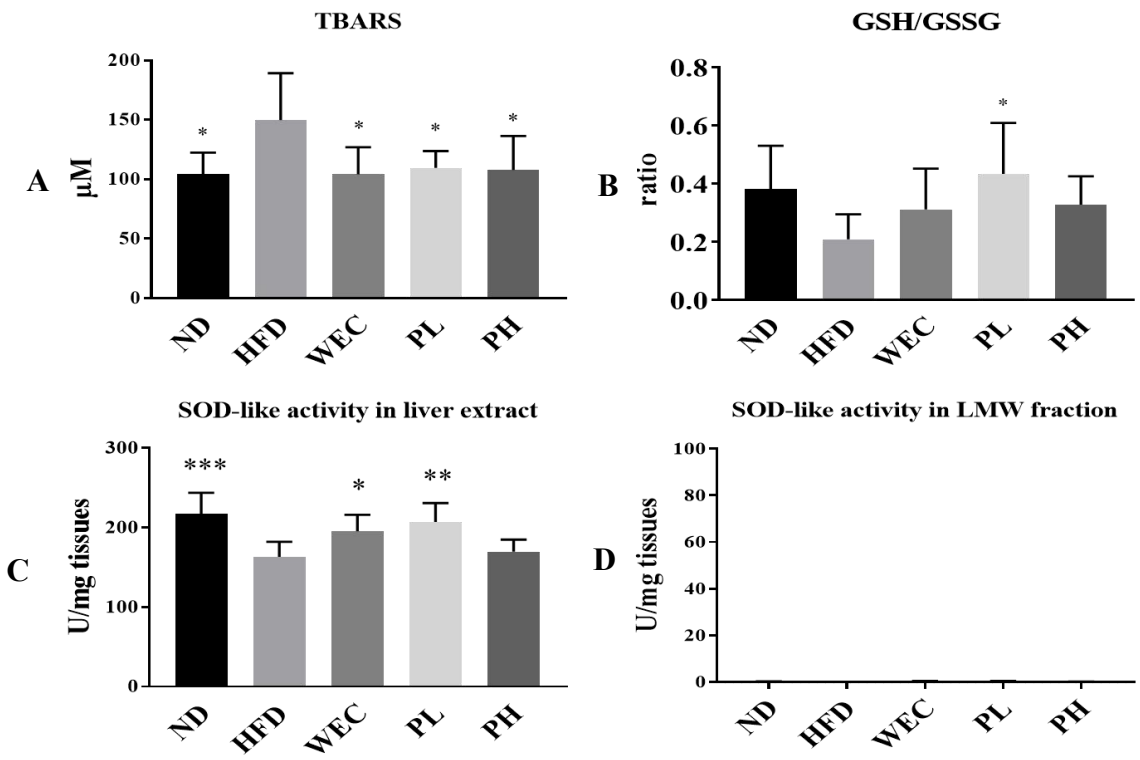

Figure 2-3. TBARS value, the ratio of reduced glutathione and oxidized glutathione, SOD-like activity in liver extract and SOD-like activity in low molecular weight fraction of liver extract in mice fed normal diet (ND), high fat diet (HFD), HFD+ low or high dose of phenethylamine (10 (PL) or 100 (PH) μg/kg), HFD+ water extract of *chlorella pyrenoidosa* (WEC). Data are shown as mean±S.D (n=6). Asterisks indicate significant differences ($p<0.05$; Dunnett's test).

Anti-oxidant enzymes level in liver

Protein levels of endogenous antioxidant enzymes, SOD-1 and GPX-1 (Figure 2-4), in liver were evaluated by Western blot analysis. To normalize band intensity of target protein obtained by different membrane, housekeeping proteins, such as β-actin, α-tublin, and GAPDH have been used. Surprisingly, high fat diet feeding and/or administration of WEC and phenethylamine significantly affected GAPDH and β-actin levels (Figure 2-5). In addition, band intensity of α-tublin was far less than target proteins. Therefore, the well-known internal standard proteins could not be used to normalize target proteins. The intensities of protein bands of each group were compared with those in HFD group, which were resolved on the same membrane, by t-test. No significant difference in SOD-1 protein level was observed between all groups although the SOD-like activity was significantly increased by WEC and low dose of phenylethylamine (Figure 2-3C). High fat diet feeding significantly decreased GPX-1 and β-actin protein level in the liver compared to ND group. Administration of low dose of phenylethylamine tended to increased GPX-1 ($p= 0.058$) and significantly increased β-actin level compared to HFD group, while no significant difference between HFD group and other groups was observed. In spite of no significant difference of GAPDH level between HFD and ND group, oral administration of phenethylamine and WEC significantly increased GAPDH level compared to HFD group.

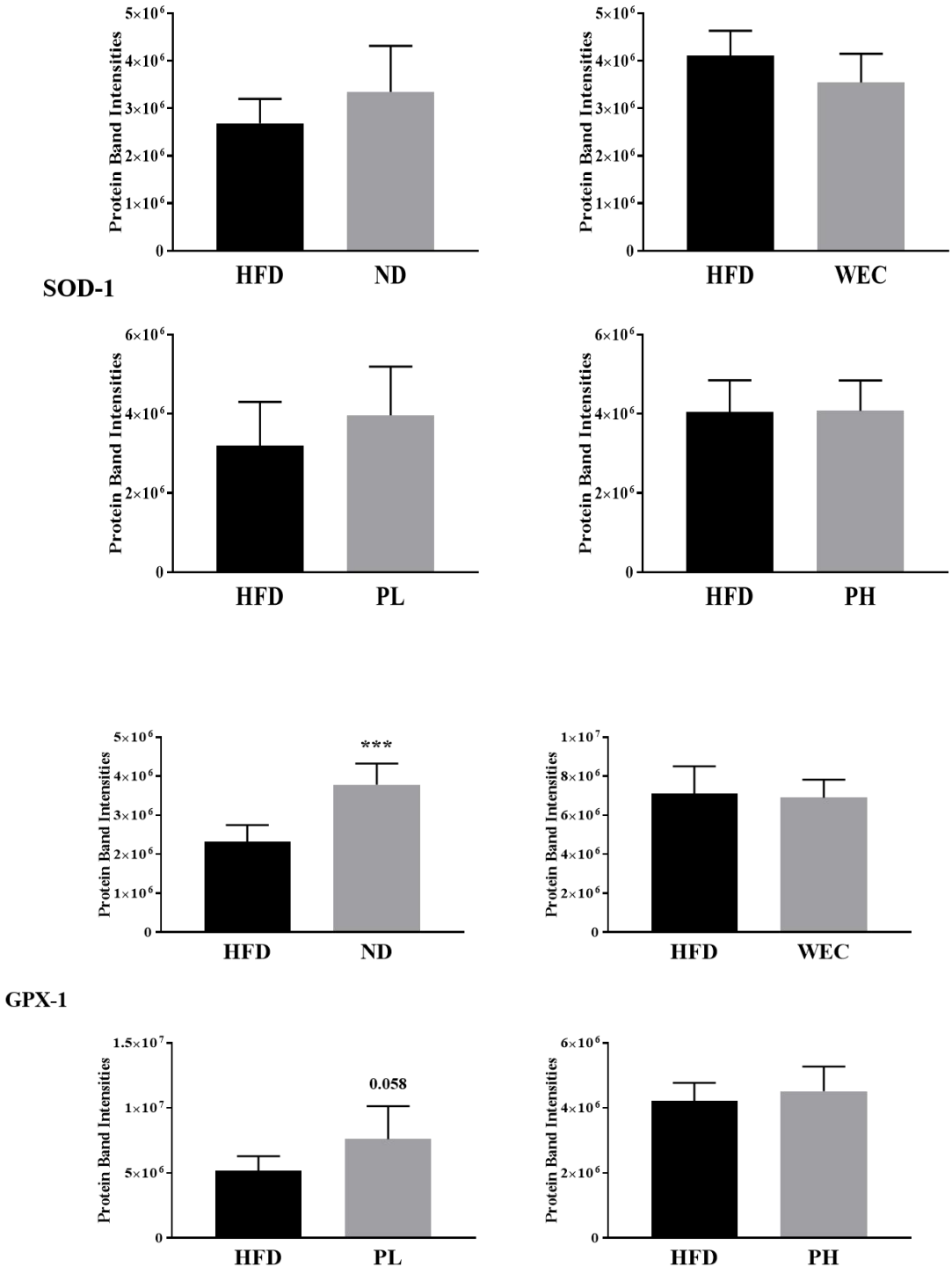

Figure 2-4. The protein level of SOD-1 (upper side) and GPX-1 (lower side) in liver in mice fed normal diet (ND), high fat diet (HFD), HFD+ low or high dose of phenethylamine (10 (PL) or 100 (PH) μg/kg), HFD+ water extract of *chlorella pyrenoidosa* (WEC). Data are shown as mean±S.D (n=6). Asterisks indicate significant differences ($p<0.05$; t test).

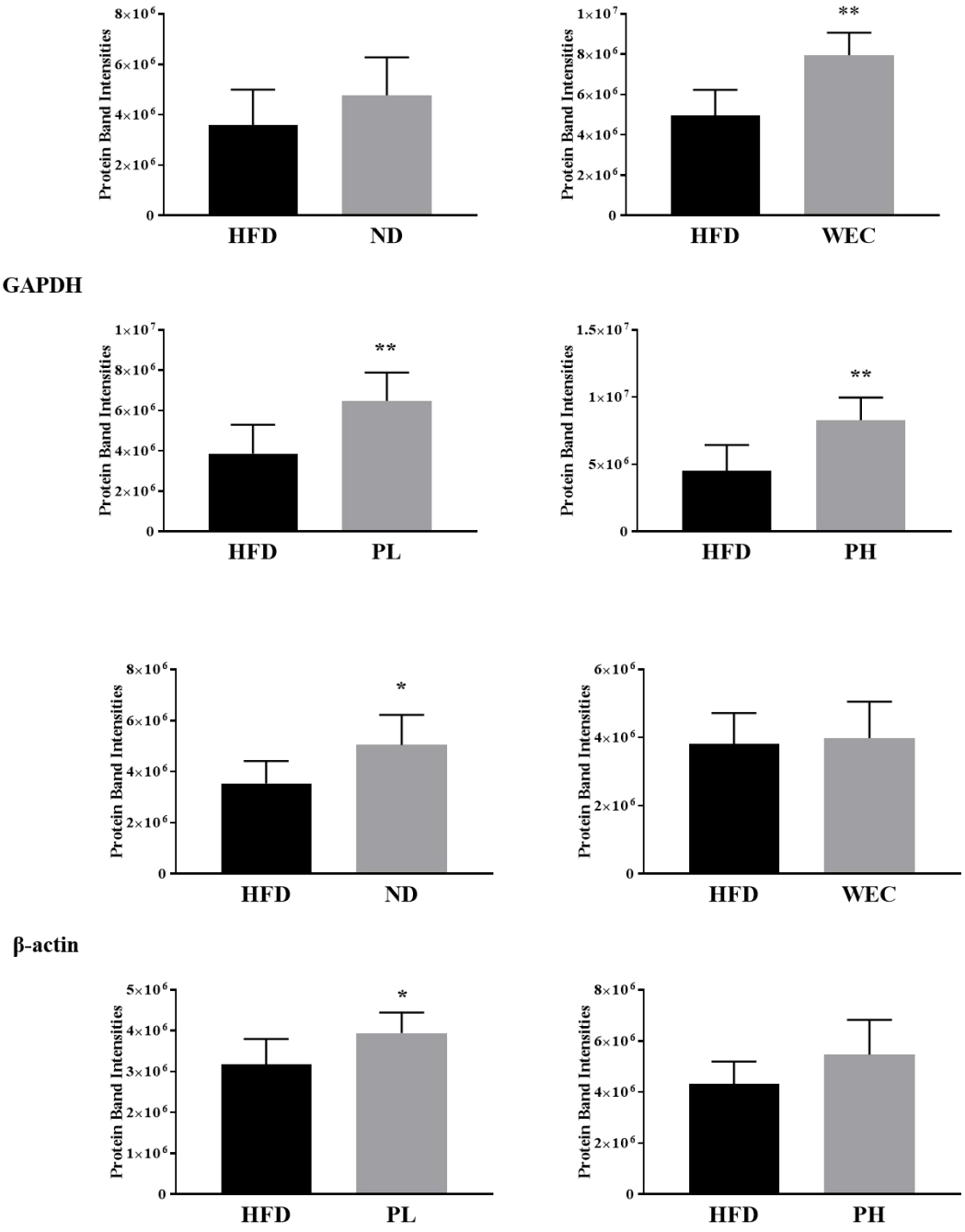

Figure 2-5. The protein level of GAPDH (upper side) and β-actin (lower side) in liver in mice fed normal diet (ND), high fat diet (HFD), HFD+ low or high dose of phenethylamine (10 (PL) or 100 (PH) μg/kg), HFD+ water extract of *chlorella pyrenoidosa* (WEC). Data are shown as mean±S.D (n=6). Asterisks indicate significant differences ($p<0.05$; t test).

2-4. Discussion

The study in chapter 1 demonstrated that very low dose of phenethylamine (60 ng/g of diet) extended *Sod1* mutant adult *Drosophila melanogaster*. On the other hand, it has been demonstrated that oral administration or injection of higher dose of phenethylamine (>25 mg/kg body weight) caused abnormal behaviors in rodents[37,38]. Furthermore, addition of phenethylamine in the culture medium at 10 mM strongly inhibited the growth of yeast strains via damaging respiratory function[39] and 1 mM phenethylamine generated reactive oxygen species in tobacco suspension culture.[40] However, it is very unlikely that trace amounts of phenethylamine in diet, which were used in chapter 1 and 2, show adverse effects, as such trace amounts of phenethylamine are obtained from daily diet as described in chapter 3. Before this study, beneficial biological activity of such low dose of phenethylamine has not been examined in mammalian.

In this study, dose for animal experiment was simply determined on the basis of content of phenethylamine in diet for *D. melanogaster* (60 ng/g of diet) and daily intake of mouse (approximately 4 g/20 g body weight/day), which corresponds to 60 × 4 ng/20 g body weight, namely 12 μg/kg body weight. Thus, 10 and 100 μg/kg of phenethylamine were administered to mouse via drinking water in the present study. Consequently, 10 μg/kg body weight phenethylamine (low dose) attenuated HFD-induced liver oxidative stress and damage. In comparison to the low dose of phenethylamine, the high dose of phenethylamine only partially exerted the beneficial effects. As described in chapter 1, high dose of phenethylamine (600 ng/g of diet), which was 10 times higher dose than the optimum dose (60 ng/g of diet), decreased

the lifespan of *D. melanogaster*. Thus, *D. melanogaster* and mice showed similar response to dosages of phenethylamine, which suggests that life span assay using *Sod1* mutant adults of *D. melanogaster* could be useful and rapid tool for screening health promoting compounds in foods with high sensitivity.

Present study demonstrated that oral administration of water extract of *Chlorella pyrenoidosa* (WEC) significantly decreased the plasma LDL-C, which is consistent with the previous research.[5] However, no significant difference in plasma TG levels was observed by administration of WEC, which is inconsistent with the previous study using the rat after overnight fastening. The present study did not fasten the animal before sample collection, which might arise the inconsistency. Both high and low dose of phenethylamine improved the HFD-induced high plasma LDL-C level, while there is no dose dependent manner between 10-100 μg/body weight. It is surprising that such low dose of food compound can decrease LDL-C; the dose is equivalent or less than medicine such as statins[41]. Administration of WEC at 100 mg/kg body weight provided phenethylamine approximately 1 μg/kg body weight. The phenethylamine in WEC might contribute to the LDL-C lowering activity by WEC. However, underlying mechanism remains to be solved.

Administration of WEC and low and high dose of phenethylamine decreased generation of malondialdehyde (TBARS value), which indicates that oxidative stress in liver was improved. It has been demonstrated that administration of Vitamin C (30 mg/kg body weight) attenuated oxidative stress and steatosis in liver induced by high fat diet.[42] Some antioxidant foods or antioxidant compounds from foods, such as onion oil (100 mg/kg body weight) and/or garlic tablets (500 mg/kg body weight)[43],

green tea extract (1 % w/w)[44], epigallocatechin gallate (EGCG, 1g/L via drinking water) or genistein (0.2 mg/kg body weight) can attenuate hepatic oxidative stress induced by long term HFD feeding in rodents.[45,46] However, the doses of antioxidants or antioxidant foods as described above are far larger than the dose of phenethylamine in present study (10 μg/kg body weight). In addition, only negligible SOD-like activity was observed in low molecular weight fraction of liver extract. These facts suggest that such low dose of phenethylamine unlikely exerts beneficial effects via direct antioxidant effect of phenethylamine. It likely exerts via modulation of expression of endogenous proteins.

The administration of WEC and low dose of phenethylamine increased SOD-like activity in the liver extract, while SOD-like activity in its low molecular weight fraction was very low compared to the total activity and did not significantly changed. These facts indicate that administration of WEC and low dose of phenethylamine increased endogenous SOD enzyme activity. In addition, low dose of phenethylamine tended to increase GPX-1 level. The effects of low dose phenethylamine on endogenous anti-oxidative enzymes could contribute to decrease of oxidative stress in liver. However, high dose of phenethylamine did not affect SOD-like activity or GPX-1 protein level in liver, while liver TBARS also decreased by administration of high dose of phenethylamine. Thus, increase of SOD-like activity and GPX-1 could not account for all decrease of TBARS in WEC, PL, and PH groups. Unexpectedly, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein level in the liver of WEC, PL, and PH groups significantly increased compared to HFD group. GAPDH is a key enzyme in glycolysis and usually used as internal standard, as GAPDH is

thought to be constantly expressed in tissues. Beisswenger et al. demonstrated that generation of methylglyoxal in human blood red cells is inversely correlated to GAPDH activity.[47] Mechylglyoxal, short chain aldehyde, has been considered to induce oxidative stress via glycation-derived free radicals.[48,49] Thus, increase of GAPDH in WEC, PL, and PH groups might suppress the glycation-induced oxidative stress, which consequently decreased TBARS. In PL group, increase of GAPDH coordinately with increase of SOD-like activity and GPX-1 level protected liver function against high fat diet-induced damage.

Some in vitro researches have suggested that mild oxidative stress increases expression of GAPDH[50-52]. On the other hand, high oxidative stress aggregates GAPDH, which induces apoptosis of cell.[53,54] Phenethylamine causes oxidative stress in yeast and plant.[39,40] There is, therefore, a possibility that low dose of phenethylamine might induce mild oxidative stress into cell and up-regulate GAPDH, which might suppress generation of methylglyoxal and consequently moderate glycation-induced oxidative stress.

In the present study, GAPDH was first determined for using as internal standard. Unexpectedly, it increased by oral administration of WEC and phenethylamine. There is a possibility that other proteins with significant biological activities might be affected by administration of WEC and phenethylamine. To solve this problem, comprehensive metabolome and proteome analyses are necessary.

2-5. Conclusion

Trace amounts of phenethylamine corresponding to doses used in chapter 1 also exert beneficial effects in mouse. Oral administration of WEC (100 mg/kg), 10 and 100 μg/kg body weight phenethylamine attenuated dyslipidemia and oxidative stress in liver, which were caused by high fat diet. High dose of phenethylamine only decreased TBARS and LDL-C, while low dose of phenethylamine also improved ALT, AST, SOD-like activity, and GPX-1 level. Thus, in addition to SOD and GPX, another anti-oxidant system might be also activated by WEC and phenethylamine. Unexpectedly, administration of phenethylamine and WEC increased GAPDH in protein level in liver, which indicates that phenethylamine or other compounds in WEC regulate glucose metabolism. It has been demonstrated that inhibition of GAPDH increases methylglyoxal, a reactive aldehyde. These facts suggest that phenethylamine or other compound in WEC might suppress glycation-induced oxidative stress via up-regulation of GAPDH in addition to increase of endogenous antioxidant enzymes at optimal dose. To confirm this hypothesis and understand detailed mechanism, further studies on effect of phenethylamine on liver metabolome and proteome are now in progress.

Chapter 3. Contents of monoamines in some unicellular algae and Japanese fermented foods.

3-1 Introduction

Phenethylamine, also referred to phenylethylamine, is classified as a trace amine for its low concentration in mammalian tissues (ng/g of tissues)[55]. Phenylethylamine regulates transportation and activity of other monoamine type neurotransmitters, such as, dopamine, serotonin, and norepinephrine through binding to trace amine associated receptor (TAAR)-1.[56] It has been demonstrated that high dose of monoamines can cause some health problems. For example, histamine can produce headaches, hypotension, and digestive problems, and tyramine is responsible for migraine and hypertension.[57] Thus, pharmacological and adverse effects of monoamines have been extensively examined.[57] On the other hand, before the present study, the beneficial effects of low dose of phenethylamine as described in chapter 1 and 2 (60 ng/g in diet for *D. melanogaster* and 10 μg/kg body weight for mice) have not been reported. However, the studies in chapters 1 and 2 also demonstrated that just ten times higher dose of phenethylamine to the optimum dose showed adverse effect (chapter 1) or less beneficial effects (chapter 2). Therefore, dose is critical whether phenethylamine exerts beneficial or adverse effect. Japan Food Safety Commission have suggested that daily intake of phenethylamine should not more than 540 μg, while, there is limited study on safety of low dose of phenethylamine. It is,

therefore, important to estimate daily intake of phenethylamine from normal daily foods having been eaten long times.

Some studies have demonstrated presence of phenethylamine in many foods especially fermented foods such as cheese ,[20] chocolate,[21] wine,[22] fermented soy paste (miso) (0.7–8.1 μg/g) ,[58] soy sauces (9.91–11.26 μg/g)[59] or natto (N.D. –51.5 μg/g).[60] In Japan, miso and soy sauce (shyou) have different types depending ingredients and processing methods with different taste, flavor, and color. The phenthylamine contents in different types of miso and shyou have not been examined. In addition, there is limited data of phenethylamine contents in Japanese rice wine (sake) and yogurt used in Japan.

The objective of the study in this chapter was to estimate intake of phenethylamine and other monoamines by consuming one serve of dish and beverage containing and consisting of fermented foods used in Japan. Consequently, one or few serves of some foods and beverage can provide phenethylamine equivalent or more than 10 μg/kg body weight, which exert beneficial activity in the mice fed high fat diet.

3-2 Materials and methods

Materials

Water extract of *Chlorella pyrenoidosa* were prepared and supplied by Sun Chlorella (Kyoto, Japan). Crude euglena was obtained from Euglena (Tokyo, Japan) Phenethylamine, isobutylamine, tyramine, tryptamine, isopentylamine, 2-methylbutylamine, gama-aminobutyric acid (GABA), formic acid, and acetonitrile (HPLC grade) were purchased from Nacalai Tesque (Kyoto, Japan) and Tokyo Chemical Industry (Tokyo, Japan) and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Rice wines (sake), Japanese fermented soy paste (miso), soy sauces (shoyu), yogurts, and beer were obtained from commercial sources.

Sake, miso, and shoyu were prepared by Japanese tradition methods. Four bottles of sake were made from steamed rice and water without adding alcohol and sugars (junmaisyu). Miso was prepared by using different ingredients such as steamed barley and soybean (A), steamed rice and soybean (B and D), and soybean only (C). Miso (B and C) was dark colored type after long aging periods (approximately 1-3 years). Others were light colored type by shorter aging periods (1-3 months). Shoyu A was light colored type and B-D were dark colored type. Light colored shoyu is prepared by fermentation in the presence of relatively high salt (20%) compared to dark colored type (15%). The high salt concentration suppresses fermentation and consequently Maillard reaction. Three Japanese yogurts (A-C) and one canned beer were prepared by modern industrial methods.

Determination of monoamines

Standard solution of monoamines (10 μM) were prepared by dissolving into distilled water or ethanol (phenethylamine and tyramine). Soy paste and yogurt were mixed with 3 volumes of water and vigorously stirred for a minuet and then centrifuged at 10000 *g* for 10 min. The supernatants and soy sauces, rice wines, and beer were diluted 10 times with distilled water and used for following analysis. Crude euglena and WEC powders were suspended in distilled water to obtain 1% (w/v), which were vigorously stirred and then centrifuged at 15000 *g* for 10 min. The supernatants were used for following analysis. Aliquots of samples or standards (20 μL), 20 μL of 0.3% AccQ acetonitrile solution, and 50 mM sodium borate buffer (60 μL) were mixed and then incubated at 50°C for 10 min. The same volume of 5 mM sodium phosphate buffer, pH 7.4, containing 5 % (v/v) acetonitrile was added into the reactants and the resultant solutions were clarified by passing it through a Cosmonice filter W. The filtrates (10 μL) were injected into LC-MS/MS under same conditions as described in chapter 1. Contents of monoamines were estimated by using external standards.

3-3 Results

Contents of monoamines in present samples are summarized in Table 3-1. Only negligible amounts of tryptamine (<1 μg/g) were detected. Relatively high contents of tyramine were contained in shoyu (A-D) and miso (C and D). Isobuthylamine, isopentylamine, and 2-methylbutylamine were abundantly contained in shoyu and miso. Gama-aminobutylic acid (GABA) was distributed in all samples. WEC and euglena contained relatively high amounts of GABA. Non-negligible amounts of

phenythylamine (3-40 μg/g) were present in the present samples except for euglena. Among them, miso C, long aging type of soybean miso, contained high amounts of phenythylamine (41.9 μg/g) with other monoamines except for tryptamine.

Table 3-2 shows average or recommended wight or volume of the fermented foods used in the present study in one serve of dish and beverage, while these values may differ by consumers. Ministry of Health, Labor and Welfare of Japan have recommend that consumption of sake and beer should be less than 160 and 500 mL per day[52]. On the basis of data in Table 3-1 and 3-2, the amounts of phenethylamine provided by consumption of average and recommended amounts of dish or beverage are estimated (Figure 3-1). Intake of phenethylamine from miso and shoyu are not so high except for miso C due to low intake amount. One cup of miso soup prepared from 13 g of miso C can provide 545 μg of phenethylamine, which is roughly corresponding to 10 μg/kg body weight, a dose used in chapter 2. On the other hand, relatively high amounts of phenethylamine can be obtained by consumption of one serve of yogurt (259–338 μg) due to relatively high intake amount, while contents of phenethylamine in yogurt are not so high. Surprisingly, consumption of recommended upper limits of sake (567–2856 μg /160 mL) and beer (1570 μg /500 mL) can provide large amounts of phenethylamine.

Table 3-1 the contents of amino acid and GABA in fermented food used in Japan

Foods	Contents of monoamines and GABA in fermented						
	PEA (F*)	TRP (W*)	TYR (Y*)	ISB (V*)	Iso+2ME (I*+L*)	GABA (E*)	total
Rice wine A	13.08	0.37	0.8	10.05	7.5	33.76	65.56
Rice wine B	7.89	0.26	0.9	7.32	4.33	19.56	40.26
Rice wine C	17.85	0.48	1.04	12.1	6.26	24.81	62.55
Rice wine D	3.55	0.42	0.63	6.15	2.27	31.69	44.71
Soy sauce A	15.32	0.58	6.21	106.2	55.04	26.91	210.31
Soy sauce B	7.65	0.33	170.1	75.97	4.73	15.37	274.17
Soy sauce C	4.65	0.49	1.19	16.74	3.39	31.17	57.58
Soy sauce D	7.38	0.39	11.99	105.55	3.17	7.79	136.27
Miso A	5.06	0.72	0.99	240.08	6.06	32.31	285.23
Miso B	4.85	0.76	0.8	273.2	10.61	70.38	360.64
Miso C	41.91	0.81	10	308.43	66.7	25.32	453.14
Miso D	3.34	0.89	2.66	268.06	2.55	28.54	306.04
Yogurt A	3.38	0.72	1.71	2.49	2.04	4.46	14.81
Yogurt B	3.06	0.6	0.92	1.17	1.31	25.76	32.79
Yogurt C	2.59	0.63	0.84	4.46	1.78	54.36	64.67
Beer	3.14	0.24	0.72	0.37	4.94	1.95	11.37
WEC	11.71	NT	19.39	5.1	13.05	98.29	147.54
euglena	0.32	NT	0.11	ND	0.37	137.85	138.65

PEA: phenethylamine; TRP: tryptamine; TYR: tyramine; ISB: isobutylamine; ISP+2-ME: ispentylamine+2-methylbutylamine; GABA: γ-aminobutyric acid. ND: not detected; NT: not tested. Asterisk indicates decarboxylated amino acid.

Table 3-2 the amounts of fermented foods used in one serve (g or mL)

shyou	Miso	Yogurt	Sake	beer	WEC	euglena
18[62]	13	100[63]	160[61]	500[61]	3	3

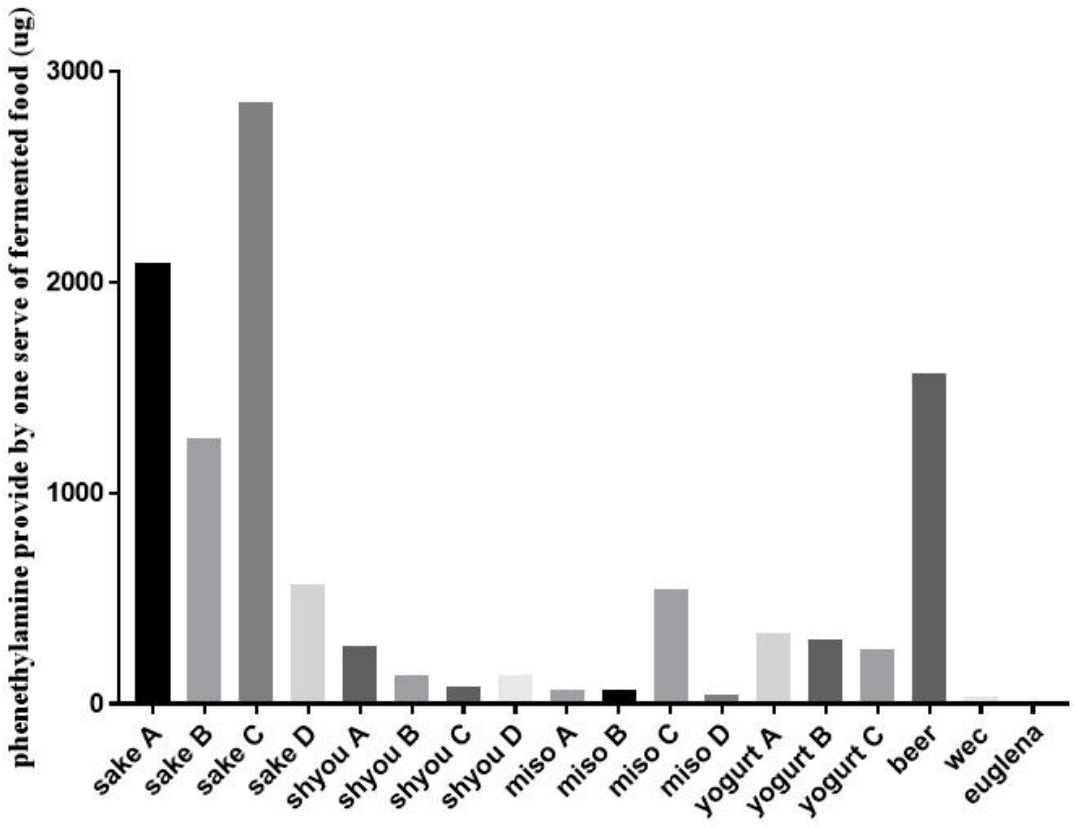

Figure 3-1 the amount of phenethylamine provided by one serve of fermented foods and alage supplement used in Japan (μg)

3-4 Discussion

Japan Food Safety Commission recommended that intake of phenethylamine should not exceed 540 μg/day. The present study demonstrates that significant amounts of phenethylamine (approximately 50-500 μg) can be obtained by consuming one dish containing miso and shoyu. In addition, daily intake of yogurt can provide 259–338 μg phenethylamine. Unexpectedly, large amounts of phenethylamine can be obtained by consumption of recommended upper limit of sake (567–2856 μg/160 mL) and also beer (1570 μg/500mL). Sake is not only used as alcoholic beverage but also used as seasoning (20–50 mL/one dish) in Japanese cuisine. Thus, approximately 1000 μg of phenethylamine is obtained per day by normal consumption of fermented foods used in Japan, for example, one cup of miso soup (40–545 μg) and one pack of yogurt (259–338 μg) and dishes seasoned with sake and shoyu (approximately 150–900 μg). In addition, from alcoholic beverage in recommended upper limit, more than 1000 μg of phenethylamine can be obtained. Consequently, more than 1000 μg of phenetylamine, which is higher than the upper limit recommended by Japan Food Safety Commission, has been daily taken in Japan. Therefore, it is necessary to re-examine safety of phenethylamine and other monoamines in the dose more than the present upper limit (540 or 1800 μg/g/day).

Japan is a nation with one of the highest average life-span in the world,[61] Japanese diet has been considered to be an important factor that contributes to this longevity. Tsuduki *et al*. prepared the representative meals of 2005, 1990, 1975, and 1960 and administered them to ICR mouse and senescence-accelerated mouse prone 8 (SAMP8). Consequently, administration of 1975 Japanese diet improved the lipid

metabolism in ICR mice.[64] In addition, the 1975 diet extended the lifespan and learning and memory ability of SAMP8 than other diets especially the diet 2005.[65] However, there was no significant difference in consumption of macronutrients between diet 1975 and 2005. Compared to Japanese diet other years, 1975 diet was rich in fermented soybean products, such as miso, natto and shoyu. In addition, sake has been used not only as alcoholic beverage but also as seasoning. Approximately 500-1500 μg of phenethylamine can be obtained by consumption of Japanese traditional foods. These values are equivalent or larger than the dose of phenethylamine used in chapter 2 (10 μg/kg body weight), which attenuated high fat diet induced liver damage and hyper LDL-cholesterol. While careful consideration is necessary to extrapolate the dose used for rodents to that in human, these facts suggest that phenethylamine in Japanese traditional foods might contribute to health promotion. However, beneficial effect of phenethylamine was demonstrated by only one rodent model (chapter 2). Further studies on effects of phenethylamine in the dose, which can be obtained by consumption of food, are necessary using other rodent models.

In addition, some Japanese fermented foods, such as sake and miso, are rich in peptides[69]. Pyroglutamine leucine (pEL) has been demonstrated to be present in these foods. pEL has demonstrated to attenuate dysbiosis via increasing endogenous antimicrobial peptide in the dose, which can be obtained by consumption of Japanese fermented foods.[69] The presence of phenethylamine in Japanese fermented food might coordinate with pEL and other peptides for improving human health. To prove this hypothesis, epidemiological and intervention studies are necessary.

3-5 Conclusion

Significant amounts of phenethylamine, tyramine, isopentylamine and 2-methylbutylamine and γ-aminobutyric acid were detected in Japanese fermented foods. Miso and soy shoyu contain higher content of monoamines than other fermented foods examined in present study. Especially, dark colored type of soybean miso contained higher amounts of monoamine than other types of miso. By consumption of dish and beverage containing Japanese fermented foods, phenythylamine can be taken in higher amounts than upper limit (540 μg/day) recommended by Japan Food Safety Commission. Therefore, safety of phenethylamine more than the upper limit should be re-examined. These doses obtained by consumption of fermented foods are equivalent to or higher than the dose exerting beneficial effects in mouse model as shown in chapter 2, which suggest that phenethylamine in the fermented foods and also supplements such as WEC might contribute to health promotion.

Conclusions and future prospect

It has been demonstrated that administration of crude *Chlorella pyrenoidosa* or its water extract exert many health promoting activities, such as improving dyslipidemia or immune modulatory activity in rodents.[5,8] However, there is limited information about active compounds in *C. pyrenoidosa.* In present study, by activity guided fractionation based on life span assay using *Sod1* mutant adults of *Drosophia melanogaster* and LC-MS/MS analysis, phenethylamine in water extract of *C. pyrenoidosa* (WEC) was identified as an active compound that exert life span elongation activity towards *D. melanogaster* at very low dose (60 μg/g of diet), while high dose of phenethylamine decreased the lifespan (600 μg/g of diet). In addition, a non-natural monoamine, pentylamine also exert the life span elongation activity at very low dose (20–200 μg/g of diet), while isopentylamine did not.

Phenethylamine belongs to trace amine. High dose of phenythylamine exerts pharmacological and adverse effects in animal model and also human. Thus, before the present study, the beneficial effects of trace amounts of phenethylamine have not been reported. Surprisingly, not only *D. melanogaster*, phenethylamine also exerts beneficial effects on mice at very low dose (10 μg/kg body weight/day). Administration of WEC (100mg/kg body weight/day) and phenethylamine (10 or 100 μg/kg body weight/day, PL and PH) attenuated high fat diet-induced hyper LDL-cholesterol, , oxidative stress without dose dependent manner. Only low dose of phenethylamine attenuated the high fat diet-induced liver damage. SOD-like activity and GPX-1 level were increased by administration of low dose of pheneythylamine,

which might contribute to attenuation of liver damage. Unexpectedly, β-actin and GAPDH, which are used as internal standards for western blot, changed by administration of HFD, WEC and phenethylamine. WEC and phenethylamine significantly increased the protein level of GAPDH in liver of mice fed on high fat diet. It has been demonstrated that decrease of GAPDH increases production of aldehydes such as methylglyoxal. Thus, phenethylamine might suppress glycation-induced oxidative stress via up-regulation of GAPDH. To confirm this hypothesis, the analysis of metabolome and proteome are necessary. In addition, the effects of phenethylamine should also be examined in other models.

The present study demonstrated that approximately 500-1500 μg of phenethylamine can be obtained by daily intake of fermented foods used in Japan, which exceed the upper limit recommended by Japan Food Safety Commission. Thus the upper limit of phenethylamine should be re-examined. The 1975 Japanese diet rich in fermented soybean foods, such as, miso or shoyu, has been demonstrated to improve dyslipidemia in mouse model and also increase life span of SAMP-8 compared to the 1960, 1990, and 2005 diets. The present study demonstrates that the amounts of phenethylamine by one or few serves of dish containing miso or shoyu is corresponding to the dose (10 μg/g) used in animal experiment (chapter 2). Thus, the presence of phenethylamine in Japanese fermented foods may contribute to the health promotion effects of Japanese fermented foods. To prove this hypothesis, epidemiological and intervention studies are necessary.

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