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The dietary effects of a fermented vegetable product on glutathione peroxidase activity and lipid peroxidation of Japanese flounder *Paralichthys olivaceus*

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ABSTRACT: To study the dietary effects of fermented vegetable product (FVP) on the protection against lipid peroxidation of various tissues in Japanese flounder, *Paralichthys olivaceus*. The fish were fed on experimental diets with or without FVP for 4 weeks. The glutathione concentrations in serum or liver had a tendency to increase on FVP-feeding fish. The FVP-feeding fish showed higher glutathione peroxidase (GPx) activity of liver than the control fish, and the GPx activity was increased by the administration of 6 mg/kg body weight/day FVP ($P < 0.05$). Conversely, fish fed on FVP containing diets exhibited significantly ($P < 0.05$) lower lipid peroxidants of serum and liver than the control fish. The FVP is suggested to suppress lipid peroxidation in the administrated fish, which led to enhancement of antioxidant effect against cultured fish.

KEY WORDS: **fermented vegetable product, glutathione, glutathione peroxidase, lipid peroxidation.**

INTRODUCTION

In fish culture, the nutrition plays a very important role in the maintenance of health and growth. Lipids are an important component of the diet, which are an element of the increase of fish body weight. Fish have many *n*-3 polyunsaturated fatty acid, such as eicosapentaenoic acid and docosahexaenoic acid, which are essential substance for marine animals.[1] However, these substances are known easily to be oxidized *in vivo*.[2] In fish, lipid peroxidation is a principal cause of jaundice,[3,4] and impairs their value as food. Murata *et al.*[5] discussed that cultured fish may suffer more oxidative stress than wild fish. We reported that the infected fish exposed to oxidative stress and these conditions were induced heat shock proteins[6,7] and antioxidant enzymes in tissues.[8,9] Sekiya *et al.*[10] reported that the suppression of lipid peroxidant may be associated with the possible enhancement of fish biological protective ability against the disease.

Manda (Manda Fermentation, Hiroshima, Japan) is a fermented vegetable product (FVP) made by natural fermentation of fruits, plant roots, cereals, marine algae, and *kokuto*, a type of non-antifugal cane sugar. These raw materials were crushed and fermented by lactobacillus and yeast generated spontaneously from raw materials at room temperature for more than 3 years and 3 months. The products are known to be one of the natural health food in Japan for a number of years. The FVP is a sweet, black-brown and paste substance that contains 38.1% water, 2.5% protein, 0.2% lipid, 57.2% carbohydrate and 2% ash. Shimada *et al.* reported that FVP protected on fat desoption and bone metabolism in ovariectomized rats.[11] The substance showed a free radical scavenging action[12] and the suspension of lipid peroxidation with *tert*-butyl hydroperoxide *in vitro*.[13] FVP-feeding fish had suppressed thiobarbituric acid reactive substance (TBARS) value in their tissues, and the result showed that with increasing feeding dose of FVP. The feeding of FVP (3 mg/kg body weight) suppressed the value approximately 50% as compared with control. However, FVP contains a few antioxidant vitamins.[14,15] Glutathione peroxidase (GPx) activity is believed to play an important role in cellular antioxidant defense by reducing hydrogen peroxide and various hydroperoxides using glutathione as a reducing agent to form water.[16]

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Therefore, the authors investigated that the effect of FVP is dependent on between GPx activity and the suppression of lipid peroxidant protection.

MATERIALS AND METHODS

Chemicals

A fermented vegetable product, Manda, was obtained from Manda Fermentation (Hiroshima, Japan). Total glutathione (T-GSH) quantification kit was purchased from Dojindo Molecular Technologies (Tokyo, Japan). Glutathione (GSH), 2-thiobarbituric acid, β-NADPH, 5-sulfosalicylic acid and glutathione reductase (GR) were obtained from Wako Pure Chemical Ind. (Osaka, Japan). Bicinchonic acid protein assay reagent was obtained from Pierce Biotechnology (Rockford, IL, USA). Other chemicals were commercial product of the highest purity available.

Fish

Japanese flounder *Paralichthys olivaceus* were obtained from Kaneto Suisan Corporation (Hiroshima, Japan). After transportation to the laboratory, they were maintained in circulating filtered seawater with aeration. The fish were fed daily with Super Ex Diet (Nippon-nosanko, Tokyo, Japan). The diet contains 67.1 mg vitamin E/100 g diet as alpha–tocopherol equivalent. At the beginning of the experiment the fish weighed 302 ± 49 g.

Experimental diets and feeding conditions

The fish were divided into four groups of 10 fish/group and reared in 200 L tanks at 23.3 ± 1.4°C, with a recirculation sea water system. FVP was suspended in distilled water and added with Super Ex Diet (Nippon-nosanko) to Japanese flounder at doses of 3–15 mg/kg body weight per day. Control fish was fed on the diet without FVP. These experimental conditions were determined with a previous report.[14] Each experimental diet was fed to fish using a regime of 6.7 g diet/kg body weight of fish/day for 6 days a week. After 4 weeks, antioxidant index and lipid peroxidants from each dietary group was measured and is discussed in the following paragraphs.

Preparation of serum

At the end of the experimental feeding, the fish were fasted for 1 day, weighed and then blood was individually collected from the caudal vessel. The bloods were incubated for 1 h at room temperature, then overnight at 4°C, and centrifuged at 1800 × *g* for 30 min at 4°C.

Determination of total glutathione

Total glutathione (T-GSH) concentrations of serum and liver were determined by using a commercial kit according to the kit manual. The liver samples (0.1 g) were homogenized in 0.5% 5-sulfosalicylic acid (1 mL), using a tissue homogenizer at 4°C. The serum were added to the half volume of 0.5% 5-sulfosalicylic acid. The liver homogenates and serum mixture were centrifuged at 8000 × *g* for 10 min at 4°C. The supernatants were added to the co-enzyme solution and enzyme working solution in order, which was incubated at 30°C for 10 min. The substrate working solution was added to the mixture, and incubated at room temperature for 10 min, and then was monitored spectrophotometrially at 405 nm. The T-GSH content was determined by comparing the measured rate to a standard curve generated with a known amount of T-GSH.

Glutathione peroxidaseactivity and protein assay

To test the effect of FVP on GPx activity, the livers from five fish for each dietary group were removed. The samples were added ninefold 10 mM potassium phosphate buffer (pH 7.0), containing 1 mM EDTA and 1 mM NaN_3. The samples were sonicated and centrifuged at 100 000 × *g* for 30 min at 4°C.

GPx activity was determined using a modified method of Lawrence and Burk.[17] The mixtures containing 1.8 mL of 1 mM EDTA, 1 mM NaN_3, 1 mM GSH, 0.2 mM β-NADPH, and 0.2 U/mL GR in 10 mM potassium phosphate buffer (pH 7.0), 0.1 mL samples and 0.1 mL of 2 mM H_2O_2 was incubated at 25°C. Samples were measured and compared at OD_{340}; GPx activities were calculated as β-NADPH reduction. Protein content was determined using the Pierce Biotechnology Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA).

Measurement of lipidperoxidants

The lipid peroxidants in liver or serum were measured as the amounts of TBARS by using a spectrophotometer at 535 and 520 nm.[18]

Table 1 Growth profile of Japanese flounder fed on the experimental diets with or without fermented vegetable products

Experimental condition	Average body weight (g)		Weight gain (%)	Feed† efficiency (%)	Survival rate (%)
	Initial	Final			
Control	302.4 ± 37.8	313.9 ± 50.4	103.6	18.0	100.0
3 mg FVP	302.7 ± 54.5	316.9 ± 48.1	109.4	21.0	100.0
6 mg FVP	300.7 ± 43.9	329.5 ± 52.1	112.3	43.4	100.0
15 mg FVP	302.1 ± 54.7	337.6 ± 54.8	118.6	53.9	100.0

†Wet weight gain (g) × 100/dry feed intake (g). FVP, fermented vegetable products.

Statistical analysis

For statistical analysis, means for all test values were calculated, and ANOVA (Statview version 4.5; Abacus Concepts, Berkeley, CA, USA) was used to determine whether differences existed between two or more groups. Values of $P < 0.05$ were considered significant.

RESULTS

Fish were fed on experimental diets with or without FVP for 4 weeks. There were no mortalities during the feeding trial. At the end of the feeding trial, there were no significant differences among the groups in the body weight. The weight gain and feed efficiency depended upon FVP feeding dose (Table 1). The feeding effects of the FVP were evaluated using the T-GSH contents in serum or liver. As shown in Figure 1, serum or liver of FVP-feeding fish had not changed the T-GSH contents, but the difference was not significant. The effects of the FVP on GPx activity were measured. The GPx activity was significantly ($P < 0.05$) higher than control fish when the fish fed on 6 mg/kg body weight per day (Fig. 2).

The TBARS values of serum or liver from the four groups of fish are shown in Figure 3. FVP-fed fish showed significantly ($P < 0.05$) lower rates of both serum and liver than those in the control.

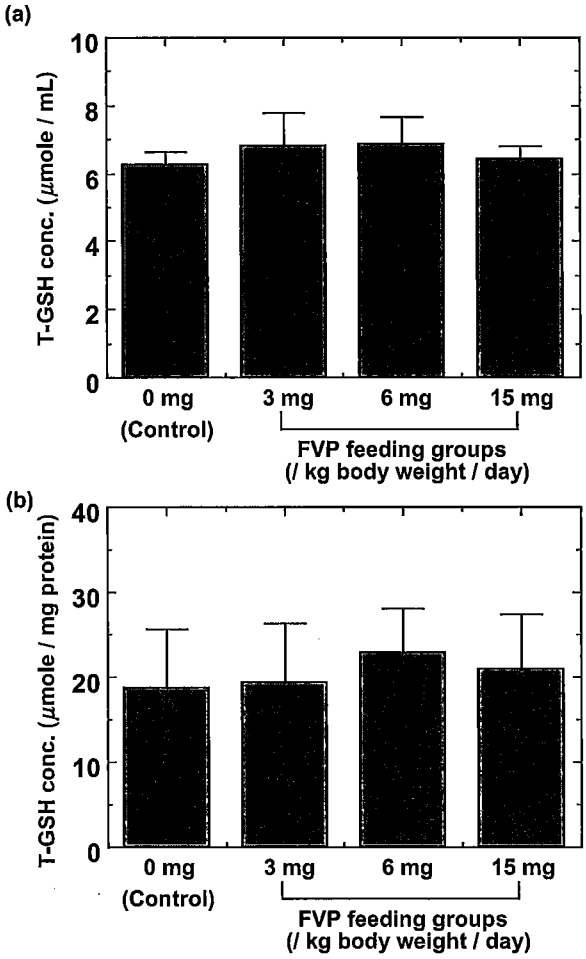

Fig. 1 Total glutathione content of the serum or liver of Japanese flounder, fed on the diet containing fermented vegetable products with several concentrations for 4 weeks. (a) Serum, (b) liver. Data points represent mean ± standard deviation ($n = 5$).

DISCUSSION

The authors demonstrated in this study that FVP, which is a known health food, comprised several antioxidant vitamins. Studies have been carried out on the relationship between vitamins and reduction of lipid peroxidation.[10,19] We reported that the feeding effect of the FVP reduced the lipid peroxidation level in the liver of the Japanese flounder.[14] However, the contents of antioxidant vitamins in FVP are much lower than previously reported. In the present study administrated FVP to fish and investigated the effect of the antioxidant substance other than vitamins and the antioxidant enzyme activity.

GSH is a known tripeptide component of L-γ-glutamil-L-cysteinyl glycine, which plays a crucial

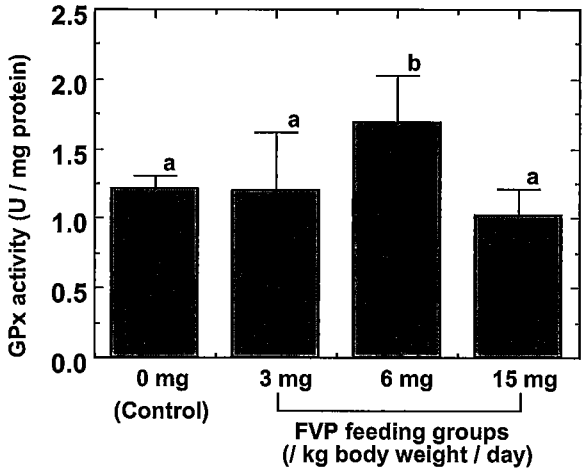

Fig. 2 Glutathione peroxidase (GPx) activity in the liver of Japanese flounder fed on the diet containing fermented vegetable products. GPx was determined with H_2O_2 as a substrate. Data points represent mean ± standard deviation. ($n = 5$). There are significant differences ($P < 0.05$) among different letters.

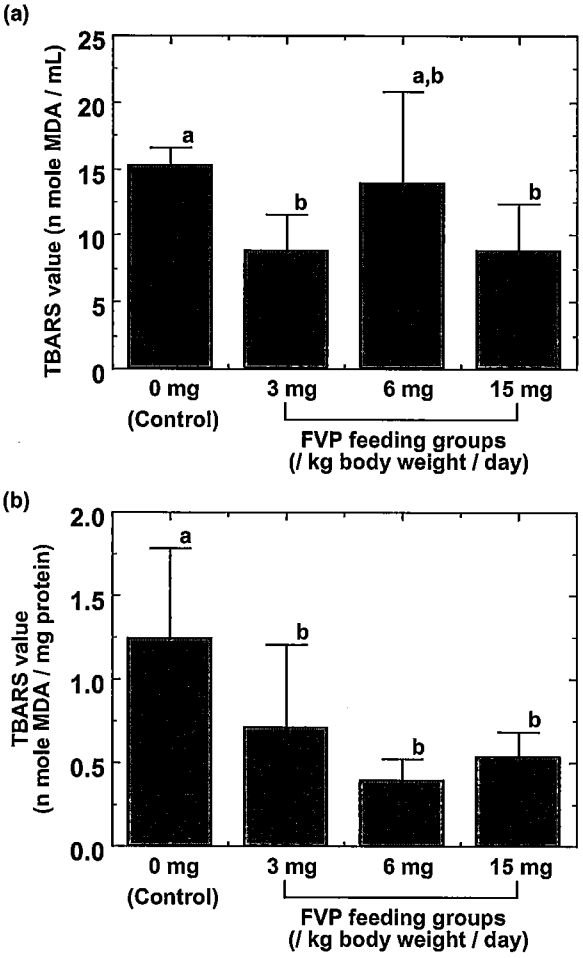

Fig. 3 Thiobarbituric acid reactive substance (TBARS) contents in Japanese flounder, fed on the diets containing fermented vegetable products for 4 weeks. (a) Serum, (b) liver. TBARS contents are expressed as nmole MDA/mL or nmole malondialdehyde (MDA)/mg protein. Data points represent mean ± standard deviation ($n = 5$). There are significant differences ($P < 0.05$) among different letters.

role in oxidative stress. GSH is oxidized by GPx to glutathione disulfide at the expense of hydrogen peroxide, and is also able to protect cell directly by scavenging radicals.[20] In fish, GSH is usually administrated with diets as an antioxidant substance in fish culture; liver serves as a storage tissue for GSH,[21] and erythrocytes are capable of producing GSH-dependent antioxidant enzymes. In the present experiment, the influence T-GSH concentrations of liver and serum by continuous oral administration of FVP to Japanese flounder is examined. The values had a tendency to increase on FVP-feeding fish, but the difference was not significant. These result suggest that FVP oral feeding has little effect on T-GSH concentration in serum or liver.

Fish contain large amounts of long-chain polyunsaturated fatty acids and these compounds easily induce lipid peroxidation. GPx is enzymatic defense, which is known to be induced in the fish tissues and protect from oxidative stress.[22] This type of GPx are widely distributed in several tissues, and it reduces fatty acid hydroperoxides and H_2O_2.[23] The FVP-feeding fish also showed some significant effects on the GPx activity of the liver. The GPx in fish fed with 3 mg/kg body weight FVP-containing diets for 4 weeks presented higher activity. However, the feeding of 15 mg of FVP decreased the GPx activity, compared to that in 6 mg feeding of FVP. FVP feeding dose may be suitable for GPx activation.

The effect on oral administration of FVP on TBARS value was therefore examined. In the present study, oral administration of FVP suppressed the TBARS values in Japanese flounder serum or liver. Since significant differences were apparent between control group and these FVP-feeding fish, the effect depended on the FVP dose can be used effectively in fish. GPx are known to detoxify H_2O_2 and organic hydroperoxides produced in lipid peroxidation in fish.[24] FVP contains low concentrations of antioxidant substances such as vitamin C and vitamin E,[15] and contains 0.5 g phenorics per 100 g as ferulic acid.[25] Naziroglu

reported that the GPx activity and GSH levels in the testes were significantly increased by administration of vitamins C and E, and selenium; the supplementation of the compounds reduced the high concentrations of TBARS value in the testes of diabetic rats.[26] These results suggest that FVP increased GPx activity, resulting in the suppression of lipid peroxidation in liver and serum. It is tempting to speculate that the suppression of lipid peroxidants decreases the diseases causing by reactive oxygen species in cultured fish.

The feeding of FVP increased the weight gain and feed efficiency, depending on FVP dose. FVP-fed fish had decreased TBARS values of serum and liver. These results suggested that the increase of growth profiles by FVP feeding might result from the improvement of oxidative stress. Conversely, Ishimine *et al.*[27] and Nakamura *et al.*[28] reported that FVP showed efficiency in increasing growth and yield of turmeric, however they have not identified the possible substance.

In present study, the relationships between dietary FVP, T-GSH, GPx activity and the amounts of TBARS values is demonstrated. The amounts of T-GSH in liver or serum were not related to the decrease of TBARS value. However, it seems that GPx activity has much to do with the suppression of TBARS values in serum and liver. FVP would help protect the cultured fish against lipid peroxidation. However, the protection of lipid peroxidation to other antioxidant enzymes, such as a superoxide dismutase or a catalase, remains unclear.

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