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Immunostimulatory effects of fermented vegetable product on the non-specific immunity of Japanese flounder *Paralichthys olivaceus*

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ABSTRACT: The stimulatory effect of fermented vegetable product (FVP) upon the phagocytic and superoxide generation of leukocytes was studied in the Japanese flounder *Paralichthys olivaceus*. The phagocytic activity of casein-induced, intraperitoneal leukocytes was investigated and quantified, that is the activity significantly increased ($P < 0.05$ or <0.01) by the addition of FVP beyond 3 mg/kg body weight. Further analysis investigated the effect of FVP on superoxide generation in leukocytes. Established *in vitro* cytochrome c reduction assay was used to measure superoxide generation; reduced levels of FVP in assay samples had a profound effect on superoxide generation. FVP was also incorporated in commercial diets and fed to Japanese flounder for 4 weeks. The phagocytic activities and superoxide generation of peritoneal induced leukocytes were significantly higher ($P < 0.05$, <0.01) in fish fed the FVP supplemented diet than fish fed the control diet. FVP feeding in fish had a significantly higher ($P < 0.05$) activity of lysozyme than in the control fish.

KEY WORDS: **fermented vegetable product, immunostimulation, Japanese flounder, non-specific immunity.**

INTRODUCTION

The fish farming industry has grown rapidly. However, as the culture of fish has advanced, the disease associated with fish is also increasing and the public has become increasingly aware of the many fish diseases. Non-specific immune systems are very important in the defense mechanisms of fish against pathogens or microorganisms. To overcome these diseases, several immunostimulants have been developed and found to be effective in fish and shellfish and these include chemical agents, bacterial components, polysaccharides, and animal or plant extracts.[1] It is tempting to speculate that the immunostimulant is safer on the cultured fish, and more effective means of treating disease in aquatic organisms than the variety of measures used to treat disease in nonaquatic animals.

Manda is a natural fermented food and is made by 50 types of fruits and vegetables fermented for 3 years and 3 months. Fermented vegetable product (FVP) is a sweet, black-brown paste which can be utilized in a wide range of applications including human health, agriculture, and livestock. FVP might improve natural killer cell activity in post-operative cancer patients,[2] and have anticancer properties and immunopotientiating effects *in vivo*.[3]

In this study, we examined the immunostimulatory effect of FVP *in vitro*, and also the effect of FVP-supplemented diets on growth and non-specific immunological index in Japanese flounder (*P. olivaceus*).

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MATERIALS AND METHODS

Chemicals

Manda (a FVP) is the product of Manda Fermentation Co. Ltd. (Hiroshima, Japan). FVP contains 38.1% water, 2.5% protein, 0.2% lipid, 57.2% carbohydrate, and 2% ash. Hemacolor was purchased from Merck (Darmstadt, Germany). Phorbol-12-myristate 13-acetate (PMA) and *Micrococcus lysodeikticus* were obtained from Sigma (St. Louis, MO, USA). Sodium casein was obtained from Across Organics (New Jersey, USA). Zymosan A, xanthine, and xanthine oxidase were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Other

chemicals used in this study were commercial products of the highest purity available.

Fish

Japanese flounder were hatched from eggs and reared in our laboratory aquaria with recirculating water. At the time of the experiment, 20 fish were 260–380 g in weight and maintained at 23–25°C.

Preparation of peritoneal induced leukocytes

Fish were intraperitoneal by injection with 2% casein in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) to obtain casein-induced leukocytes. Cells were collected 15 h after casein injection and centrifuged at 400 ×*g* for 10 min. The precipitate was washed with RPMI 1640 medium (Invitrogen Co., New York, USA) and resuspended to a concentration of 5×10^5 cells/mL in RPMI 1640 medium.

Phagocytic activity and superoxide generation assay

Intraperitoneal leukocytes were induced and prepared from five fish for each dietary group as described above. Phagocytic activity was determined using opsonized zymosan A (*Saccharomyces cereviciae*) following previously reported protocols.[4] Superoxide generation was quantified using cytochrome c reduction as a measure of phorbol-12-myristate-13-acetate (PMA)-stimulated superoxide generation as described previously.[5]

Superoxide scavenging assay of fermented vegetable product

To test the effect of FVP on superoxide scavenging activity, mixtures containing 1.8 mL of 50 μM xanthine and 5 μM cytochrome c in 50-mM potassium phosphate buffer (pH 7.4) and 2 μL of several concentrations of FVP and 16 mU xanthine oxidase (XOD) were incubated at 25°C. Samples were measured and compared at OD_{550}; superoxide generation was calculated as cytochrome c reduction.[6]

Experimental fish feeding system

Fish (302.0 ± 48.9 g) were divided into four groups of five fish each and kept in 200-L tanks with a continuous supply of air (3.6 L/min) and recirculating seawater (3.0 L/min). The rearing-water temperature ranged between 21 and 27°C during the experimental period.

Administration of fermented vegetable product to experimental fish

FVP was suspended in distilled water and mixed with Super Ex diet (Nihon-nosanko Co. Ltd). One group fed on a commercial diet served as a control group, and the others were fed on diets containing 3, 6 and 15 mg/kg body weight/per day of FVP. Each diet was fed to fish using a regime of 15 g diet/kg body weight of fish per day for 6 days a week. After 4 weeks, non-specific immunological index from each dietary group was measured as described above.

Measurement of lysozyme activity

Lysozyme activities were determined following the method of Kusuda *et al.*[7] with pose modifications. Japanese flounder head kidney was removed, weighed and added with RPMI 1640 medium (2.5%, w/v). Then the samples were homogenized and centrifuged at 500 ×*g* for 10 min at 4°C. The supernatants and serum of five fish from each group was assayed and the lysozyme activity was measured by using 0.2 mg/mL *M. lysodeikticus* as a substrate in 0.04 M sodium phosphate buffer (pH 5.75). Serum or head kidney supernatant (40 μL) was added to 3 mL of suspension and reduction in absorbance at 540 nm was measured for 60 min at 20°C.

Statistical analysis

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

RESULTS

Phagocytic activity of intraperitoneal casein-induced leukocytes *in vitro* was measured by the addition of various concentrations of FVP at 20°C (Fig. 1). The addition of 10 μg/mL FVP was found to increase the phagocytic activity significantly ($P < 0.05$). High concentrations (2 mg/mL) of FVP

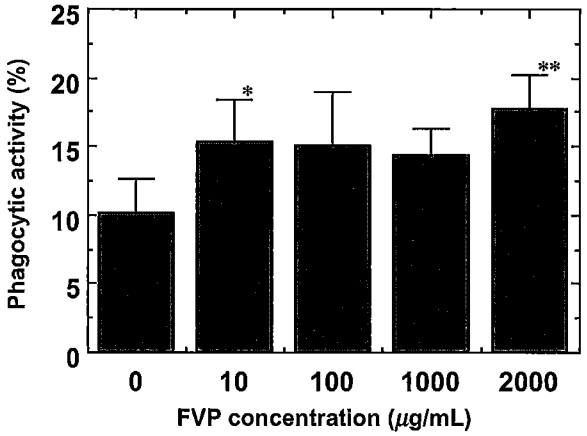

Fig. 1 Phagocytotic assay of intraperitoneal leukocytes treated with fermented vegetable product (FVP). Leukocytes prepared from Japanese flounder (5×10^5 cells/mL) were treated with 0–2 mg/mL of FVP for 1 h. Vertical bars show the standard deviation ($n = 3$). Significant differences from controls, as determined by ANOVA, are given by $^*P < 0.05$ or $^{**}P < 0.01$.

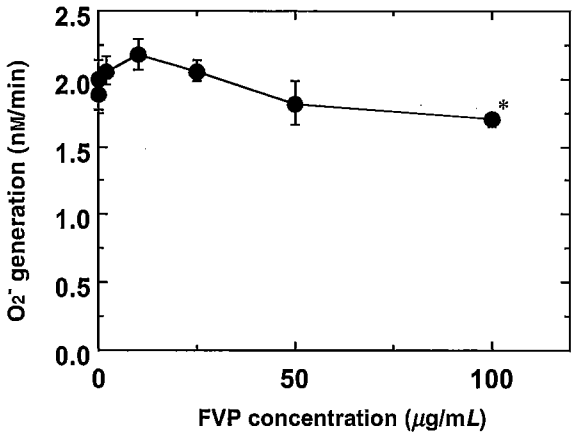

Fig. 2 The effect of fermented vegetable product (FVP) on the superoxide production in phorbol-12-myristate 13-acetate (PMA)-stimulated peritoneal leukocytes. Leukocytes (10^5 cells/mL) were incubated with PMA (25 nM) and various concentrations of FVP. The superoxide generation was calculated by cytochrome c reduction. Vertical bars indicate the standard deviation ($n = 3$). Significant differences from 0 μg, as determined by ANOVA, are indicated by $^*P < 0.05$.

increased the activity significantly ($P < 0.01$), with an increase by approximately 65% compared with that in the absence of FVP.

The effect of FVP on superoxide generation in leukocytes was measured. The highest levels of superoxide generation was observed when the leukocytes were incubated with 10 μg/mL FVP (Fig. 2).

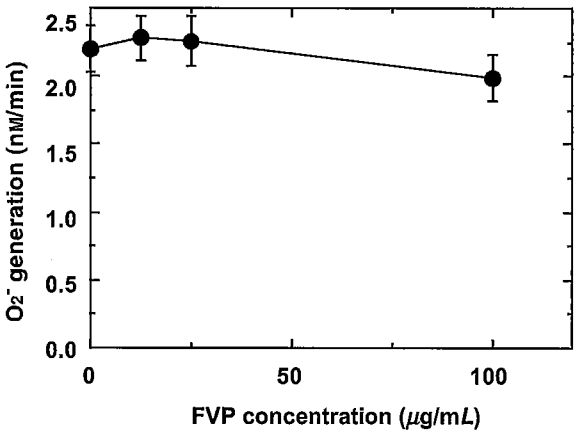

Fig. 3 The effect of fermented vegetable product (FVP) on the cytochrome c reduction-induced xanthine-xanthine oxidase system. Test tubes contained 1.8 mL of 50 μM xanthine and 8 mU xanthine oxidase in Krebs–Ringer-phosphate buffer. Vertical bars indicate the standard deviation ($n = 3$).

Thus, the bacteria killing activity of Japanese flounder leukocytes *in vitro* was activated by the addition of low concentrations of FVP. However, the presence of 100 μg/mL of FVP decreased the amount of superoxide generation by approximately 9.6% compared to that in the absence of FVP. The superoxide scavenging ability of FVP towards the xanthine-XOD (X-XOD) system was also studied and results are shown in Fig. 3. When lower concentrations of FVP were added to this reaction, each sample was found to be insensitive to the effect on cytochrome c reduction. However, the presence of high concentrations (100 μg/mL) of FVP appeared to suppress cytochrome c reduction by approximately 11%. Additionally, oxygen consumption by the X-XOD system during superoxide generation was also measured with an oxymeter. The addition of 100 μg/mL FVP to the X-XOD reaction did not suppress oxygen consumption (data not shown).

No differences in fish growth were observed between the groups of fish fed commercial and FVP-containing diets (Fig. 4). The feeding effects of the FVP were evaluated using the non-specific immunological index. The phagocytic activity of the control fish leukocytes (no FVP) was $15.3 \pm 0.6\%$. In contrast, the index of fish fed the FVP diets were $18.6 \pm 1.3\%$, $17.4 \pm 1.7\%$ and $17.3 \pm 1.6\%$, respectively. These rates were significantly higher ($P < 0.05$, <0.01) than the control fish (Fig. 5). The cytotoxic activity of leukocytes was determined by measuring superoxide generated by the leukocyte when stimulated with PMA (Fig. 6). After incubation with PMA, the leuko-

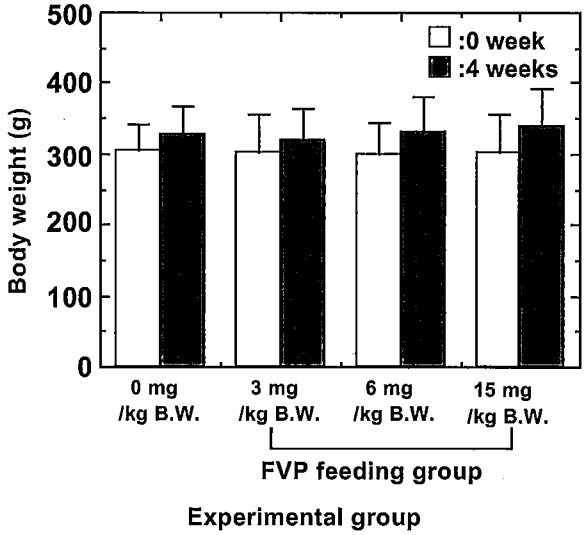

Fig. 4 Growth of Japanese flounder fed on the experimental diet. Fish were fed on several experimental diets for 4 weeks. Vertical bars indicate the standard deviation ($n = 5$).

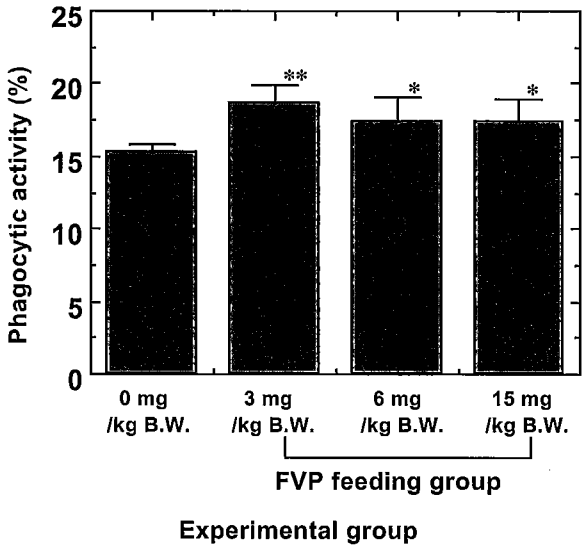

Fig. 5 Phagocytic activities of intraperitoneal leukocytes isolated from Japanese flounder fed on fermented vegetable product – containing diets. Leukocytes (5×10^5 cells/mL) were incubated with 20 μg/mL zymosan at 20°C for 1 h. Vertical bars indicate the standard deviation ($n = 5$). Significant differences from 0 mg, as determined by ANOVA, are indicated by $^*P < 0.05$ or $^{**}P < 0.01$.

cytes of control fish produced 5.3 ± 2.6 nM of superoxide. At a high concentration of FVP (6 mg and 15 mg/kg body weight), superoxide generation was significantly higher ($P < 0.05$, <0.01) than

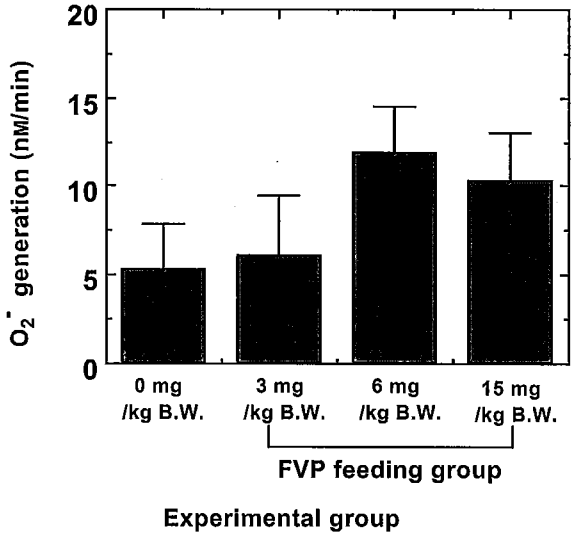

Fig. 6 Effect of feeding fermented vegetable product – containing diets to Japanese flounder on superoxide production from intraperitoneal leukocytes stimulated with phorbol-12-myristate 13-acetate (PMA). Leukocytes (10^6 cells/mL) were incubated with 25 nM PMA. Superoxide generation was determined by cytochrome c reduction. Vertical bars represent standard deviation ($n = 5$). Significant differences from 0 mg, as determined by ANOVA, are given by $^*P < 0.05$ or $^{**}P < 0.01$.

controls. The lysozyme activity of fish fed FVP significantly ($P < 0.05$) increased at 4 weeks (Fig. 7). The high activity was observed in the serum of fish treated with 3 or 15 mg/kg body weight of FVP.

DISCUSSION

Many useful products have been made from plants and food by the action of microorganisms. In biological studies, products made by fermentation have been shown to exhibit immunomodulatory effects. For example, rats fed with diets supplemented with fermentation extract of cabbage showed better immune function than controls.[8] LeBlanc *et al.*[9] reported that the peptidic fractions from fermented milk play important roles in the modulation of the cellular immune response. Yoshida *et al.*[10] reported that the fermented products of chicken eggs (EF203) led to increased resistance against bacterial infection.

The activation of non-specific immune systems is an important factor in protecting fish from pathogens, and several immunostimulatory chemicals which can be used in the prevention/treatment of fish diseases have been discussed.

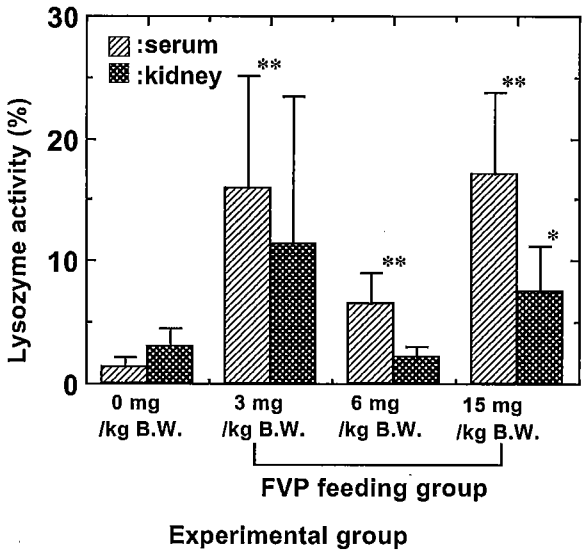

Fig. 7 Effect of feeding fermented vegetable product – containing diets on lysozyme activities in the serum and kidney of Japanese flounder. The lysozyme activities were determined by a reduction in absorbance at 540 nm measured for 60 min at 20°C. Vertical bars indicate the standard deviation ($n = 5$). Significant differences from 0 mg, as determined by ANOVA, are given by $^{*}P < 0.05$ or $^{**}P < 0.01$.

Sakai[1] reported that several immunostimulants could enhance the immune systems of fish. In the present study, we examined for the first time the effect of FVP enhancement on the non-specific immune response of Japanese flounder *in vitro*. To determine the effect of FVP on the non-specific immune response of fish, the phagocytic activity and superoxide generation of leukocytes induced by intraperitoneal injection of casein was evaluated. Increased phagocytic activity and superoxide production from leukocytes with the addition of low concentrations (< 10 μg/mL) of FVP was measured. However, the presence of FVP at higher concentrations (> 10 μg/mL) coincided with decreased superoxide production compared to that in the absence of FVP. Kawai *et al.*[11] reported that FVP exhibits a free radical scavenging action; it scavenges superoxide, hydroxyl and 1,1-diphenrl-2-picryl-hydrazyl radicals *in vitro*. In the present study, superoxide production decreased upon the addition of 100 μg/mL FVP to a suspension of leukocytes and it seemed likely that the superoxide generated was subsequently scavenged by FVP. Our results show that phagocytic activity and superoxide production from leukocytes obtained by intraperitoneal injection of casein were both increased *in vitro*. The activation of leukocytes is an important part of any immunostimulatory method that might be used to treat fish diseases. We believe that FVP might be used as an immunostimulant to enhance the non-specific immune system of Japanese flounder.

The effect of oral administration of FVP in Japanese flounder was investigated. Growth of fish was not affected in this study and this might be due to the short experimental exposure to FVP (4 weeks). It is known that *Photobacterium damselae* cells survive within the yellowtail macrophage.[12] Karczewski *et al.*[13] reported that the enhancement of superoxide production by leukocytes would cause increased resistance against fish diseases. The generation of reactive oxygen is considered to be important in the protection against bacterial infection. Kokoshis *et al.*[14] reported that mice increased their antibacterial defense and the degree of protection was correlated with the increase in lysozyme activity. Sakai reported that immunostimulants are valuable for the control of fish diseases, and fish treated with the substance increased lysozyme activity. Glucans are a type of high molecular substance which has been reported to enhance the non-specific immune systems of fish *in vitro*[15] and *in vivo*.[16] It seems that FVP might be able to protect fish against the disease. In our study, oral administration of FVP affected the non-specific immune systems in Japanese flounder, since significant differences were apparent between control groups and those fed high doses (>6 mg/kg body weight) of FVP. Thus, FVP appeared to enhance the phagocytic activity of leukocytes *in vitro and in vivo*. The oral administration of FVP at high doses (>6 mg/kg body weight) could be advisable for enhancing resistance in fish, for example before seasons known to be affected by disease outbreaks, and prior to transport. FVP is a natural fermented food, and LD_{50} value was determined to be 20 g/kg body weight in rats.[17] We previously reported that feeding FVP to Japanese flounder reduced lipid peroxidants in the liver[18] and similar results were shown when the FVP concentration was increased beyond 3 mg/kg body weight. It appears that this compound may be used more safely as an immunostimulant in fish given at 6 mg/kg body weight.

In conclusion, FVP enhances phagocytic activity and superoxide production of leukocytes *in vitro* and *in vivo*. The FVP-fed fish had increased lysozyme activity. The use of this substance may help protect fish against diseases. However, further studies are needed to investigate feeding dose and bacterial infection in applied aquaculture practices.

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