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SHORT PAPER

Effect of a dietary fermented vegetable product on the heat shock response of Japanese flounder *Paralichthys olivaceus*

Takayuki Ashida · Masahiro Takagaki · Yoshinori Matsuura · Eiji Okimasu

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Stress is considered a major problem in fish culture, because fish are farmed under such conditions as poor water conditions, the presence of chemical components, and with handling stress. These conditions can be a cause of growth reduction, fish diseases, and poor survival [1, 2]. Fish will respond to a stressors by inducing a generalized physical stress response, which is characterized by an increase in stress hormones and other substances. The responses are similar to those of other vertebrates and help maintain normal conditions or homeostatic state [3, 4]. Recently, protection from fish diseases and reduction of several stresses have become major interests in fish culture. To overcome these problems, vitamins and other substance have been tried in cultured fish. The use of natural compounds as supplements is safer for the cultured fish and more effective means of reducing stress response in aquatic organisms.

Manda is a fermented vegetable product (FVP) made by natural fermentation of fruits, vegetables, plant roots, marine algae, and *kokuto*, a kind of non-centrifugal cane sugar. These raw materials are crushed and fermented for more than three years and three months by lactobacillus, bacillus, and yeast generated spontaneously from the raw materials at room temperature. The products have been known as natural health foods in Japan for a number of years. The FVP is a sweet, black–brown, paste containing 32.9% water, 2.2% protein, 0.1% fat, 60.3% carbohydrate, and 1.9% ash, vitamins, and minerals [5]. Kawai and Matsuura reported that FVP inhibited emotional stress-induced stomach ulcers in rat [6]. The substance caused a reduction in lipid peroxidant formation [7], and activation of glutathione peroxidase [8] and the non-specific immune system in fish [9].

The heat shock protein (hsp) group belong to a family of proteins which are expressed in response to the several stressors. In the hsp family, hsp70 has been most widely studied and is involved in multiple cellular processes such as protein transcription, protein folding, and regulation of the stress response [10]. Dietz and Somero reported that hsp70 was induced in several tissues of marine teleost fishes by raising the temperature [11]. In this study, we investigated the effect of FVP on changes of stress protein (hsp70) and the concentration of serum cortisol and glucose.

Japanese flounder, *Paralichthys olivaceus*, which had been hatched and reared in our laboratory, were kept in 200-l tanks with a continuous supply of air (3.6 l/min) and sea water (3.0 l/min). Fish weighing 233 ± 27 g (mean ± SD) were randomly distributed into four groups of tanks. Water temperature varied from 20–24°C during the experiment. One group fed on Super Ex diet (Nihon-nosanko) served as control, and the others were fed on diet containing 3 or 15 mg/kg body weight/day of FVP. Each diet was fed to fish using a regime of 12 g/kg body weight of fish per day for 6 days a week. After 2 weeks, fish were transferred to high-water-temperature tanks (31°C) for 2 h, and then, were returned breeding tanks (21°C). Gills

T. Ashida (✉) · M. Takagaki · Y. Matsuura
Manda Fermentation Co. Ltd, 5800-95 Shigei, Onomichi-Innoshima, Hiroshima 722-2192, Japan
e-mail: takayuki_ashida@manda-kohso.co.jp

E. Okimasu
Department of Marine Biotechnology, Faculty of Life Science and Biotechnology, Fukuyama University, Fukuyama, Hiroshima 729-0292, Japan

removed from the fish before and after heat shock were added to ninefold the amount of 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride, then the samples were sonicated and centrifuged at 100,000×*g* for 30 min at 4°C. Proteins in the supernatants were separated by 10% SDS polyacrylamide gel electrophoresis (25 μg protein/lane). The separated protein bands on the gel were transferred to a poly(vinylidene difluoride) membrane, and then detected by anti-hsp70 mAb (Stressgen Bioreagents Columbia, Canada) with the Dr Western protein marker (Oriental Yeast, Tokyo, Japan). Arbitrary units (A.U.) were calculated using a NIH-image software (National Institute of Health, Bethesa, MD, USA). Blood samples from the experimental fish were centrifuged at 3,000×*g* for 10 min. The sample was stored at −30°C until required for analysis, and serum cortisol levels were analyzed using a commercial kit (Payer Medical, Tokyo, Japan) [12]. Serum glucose concentrations were determined by using a commercial kit according to the kit manual (Glucose CII-test Wako, Wako Pure Chemical Industries, Tokyo, Japan).

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

Fish were fed on experimental diets with or without FVP for 2 weeks. There were no mortalities during the feeding trial. At the end of this trial there were no significant differences among the groups in the body weight (data not shown).

A single hsp70 band was detected using anti-hsp70 mAb (Fig. 1). The antibody exhibits cross-reactivity for several mammals, chicken, and fish such as carp, chinook salmon, chum salmon, and rainbow trout. The hsp70 band was detected between two markers at 68.4 and 81.8 kDa with a molecular mass of approximately 72 kDa. Yokoyama et al. [13] reported that heat-induced hsp70 corresponded to a predicted mass of 70.6 kDa in Japanese flounder, *P. olivaceus*. These results suggested that the detected hsp70 band was heat-induced hsp70 in Japanese flounder. In all the groups examined hsp70 was not detected in gills before giving high temperature-stress. In the control group and low-FVP feeding group, the levels of hsp70 were low for 72 h. In the high administration group (15 mg/kg b.w./day), however, a markedly increased high level ($P < 0.01$, 0.05) from 2 to 24 h after stress (Fig. 2) was followed by a decrease to the levels of the control group after 72 h. Thus, hsp70 expression was strongly induced by temperature stress when high-dose FVP was added to the diet. There is a possibility that hsp70 protects the gill tissues from subsequent damage in the FVP-treated fish. Previously, it has been reported that the several substances induced high hsp levels in fish tissues exposed to stress [14, 15]. When high-dose FVP feeding was used as hsp70 inducer against changes of environmental conditions in Japanese flounder gill, it induced a high level of hsp70 and seemed to contribute to reducing stress indicators such as cortisol and glucose levels.

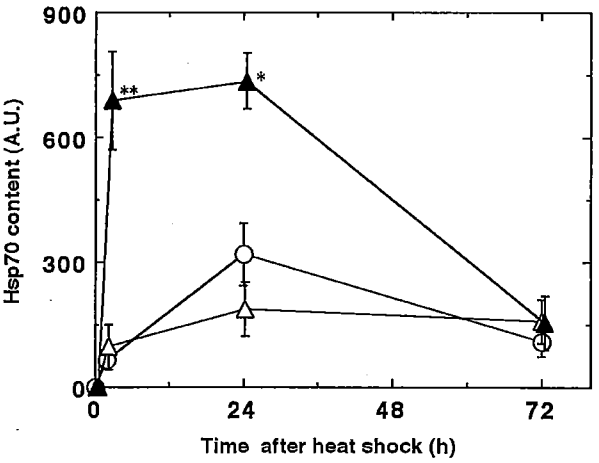

Fig. 2 Levels of gill hsp70 in Japanese flounder exposed to a heat stress. Samples were analyzed by SDS-PAGE and immunoblotting. FVP feeding doses were *circles*, 0 mg/kg b.w./day; *triangles*, 3 mg/kg b.w./day; *filled triangles*, 15 mg/kg b.w./day. *Vertical bars* show the standard deviation ($n = 3$). Significant differences from controls, as determined by ANOVA, are given by $^{**}P < 0.01$ or $^{*}P < 0.05$

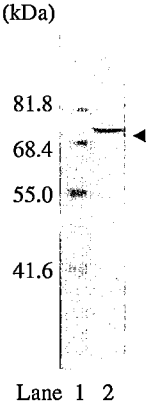

Fig. 1 Western blot analysis showing molecular weight markers (*lane 1*) and hsp70 in Japanese flounder gill (*lane 2*)

In the control group, plasma cortisol levels increased from 10 ng/ml (basal level) to more than 65 ng/ml after 2 h (Fig. 3a). On the other hand, plasma glucose concentrations were influenced by the change of plasma cortisol concentrations. In the FVP feeding groups, the cortisol levels decreased promptly with time; however, the glucose concentrations were maintained at the induced level after 72 h (Fig. 3b). The increase of several stressors, for

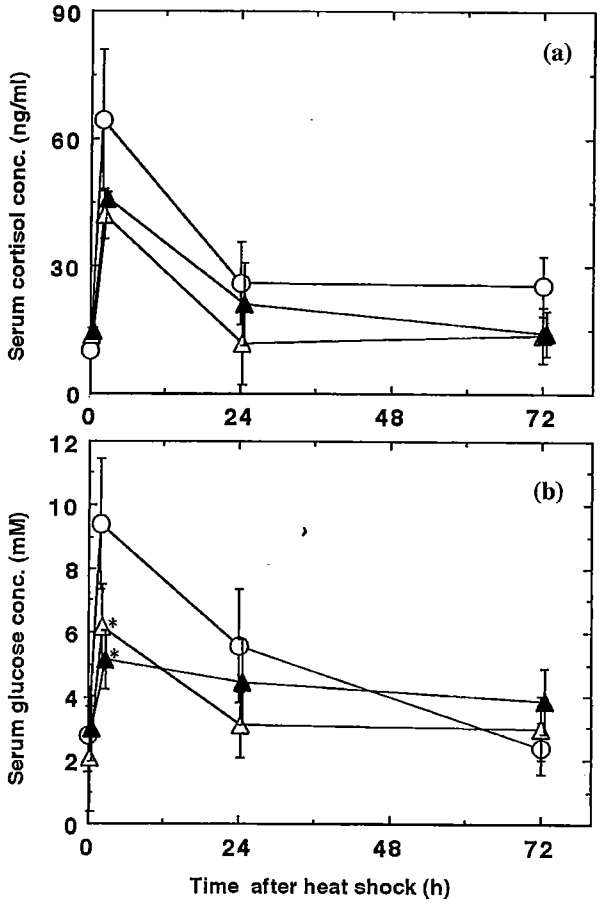

Fig. 3 Time course-related changes in plasma **a** cortisol and **b** glucose levels after heat shock in Japanese flounder. Symbols are same as in Fig. 1. *Vertical bars* indicate the standard deviation ($n = 3$). Significant differences from control as determined by ANOVA, are indicated by $^*P < 0.05$

example plasma cortisol and glucose level have been used as biochemical indicators after acute stress responses [16].

This study has demonstrated that dietary supplementation with FVP will help the cultured fish to induce the stress responses. Although FVP contains antioxidant substances [17], the compounds have not yet been isolated and identified. Further study is necessary to understand the mechanisms of hsp70 induction and the serum cortisol reduction initiated by feeding of FVP.

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References

1. Balm PHM (1997) Immune-endocrine interactions. In: Iwama GK, Pickering AD, Sumpter JP, Schreck CB (eds) Fish stress and health in aquaculture. Society for Experimental Biology, Cambridge, pp 195–221
2. Pankhurst NW, Van K (1997) Effect of stress on reproduction and growth of fish. In: Iwama GK, Pickering AD, Sumpter JP, Schreck CB (eds) Fish stress and health in aquaculture. Society for Experimental Biology, Cambridge, pp 73–93
3. Iwama GK, Vijayan MM, Forsyth RB, Ackerman PA (1999) Heat shock proteins and physiological stress in fish. Am Zool 39:901–909
4. Barton BA (2002) Stress in fish: a diversity of responses with particular reference to changes in circulating corticosteroids. Integr Comp Biol 42:517–525
5. Shimada T, Motonaga C, Matsuura S, Takagaki M, Ashida T, Okano T, Morii H (2004) Effects of a fermented vegetable product on fat deposition and bone metabolism in ovariectomized rats. J Nutr Sci Vitaminol 50:422–425
6. Kawai M, Matsuura S (1997) Manda suppresses emotional stress-induced stomach ulcers in rats. Int J Stress Manag 4:63–69
7. Ashida T, Okimasu E, Amemura A (2002) Effect of a fermented vegetable product on hemolysis and lipid peroxidation of Japanese flounder erythrocytes. Fish Sci 68:1324–1329
8. Ashida T, Takei Y, Takagaki M, Matsuura Y, Okimasu E (2006) The dietary effect of a fermented vegetable product on glutathione peroxidase activity and lipid peroxidation of Japanese flounder *Paralichthys olovaceus*. Fish Sci 72:179–184
9. Ashida T, Okimasu E (2005) Immunostimulatory effect of a fermented vegetable product on the non-specific immunity of Japanese flounder *Paralichthys olovaceus*. Fish Sci 71:257–262
10. Craing A, Baxter BK, Becker J, Halladay J, Zielhoffer T (1994) Progress and perspectives on the biology of heat shock proteins and molecular chaperones. In: Morimoto RI, Tissieres A, Ceorgopoilos C (eds) The biology of heat shock proteins and molecular chaperons. Cold Spring Harbor Laboratory Press, New York, pp 31–52
11. Dietz TJ, Somero GN (1993) Species- and tissue-specific synthesis patterns for heat-shock proteins HSP70 and HSP90 in several marine teleost fish. Physiol Zool 66:863–880
12. Endo M, Kumahara C, Yoshida T, Tabata M (2002) Reduced stress and increased immune responses in Nile tilapia kept under self-feeding conditions. Fish Sci 68:253–257
13. Yokoyama Y, Hashimoto H, Kubota S, Kinoshita M, Toyohara H, Sakaguchi M, Tanaka M, Seikai T, Kanamori M (1998) cDNA cloning of heat-inducible hsp70, a 70.6 kDa heat shock protein, in Japanese flounder *Paralichthys olivaceus*. Fish Sci 64:964–968
14. Basu N, Nakano T, Grau EG, Iwama GK (2001) The effects of cortisol on heat shock protein 70 levels in two fish species. Gen Comp Endocrinol 124:97–105
15. Whitham M, Walker GJ, Bishop NC (2006) Effect of caffeine supplementation on the extracellular heat shock protein 72 response to exercise. J Appl Physiol 101:1222–1227
16. Sjoerd E, Wendelaar B (1997) The stress response in fish. Physiol Rev 77:591–625
17. Kim DC, Hwang WI, In MJ (2003) In vitro antioxidant and anticancer activities of extracts from fermented food. J Food Biochem 27:449–459