Dietary bovine lactoferrin enhances tolerance to high temperature stress in Japanese flounder *Paralichthys olivaceus*

Saichiro Yokoyama[a], Shunsuke Koshio*[a,b], Natsuko Takakura[c], Kyoichi Oshida[c], Manabu Ishikawa[b], Francisco J. Gallardo-Cigarroa[a,d], Shin-ichi Teshima[a,b]

[a] Sciences of Marine Resources, The United Graduate School of Agricultural Sciences, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan

[b] Laboratory of Aquatic Animal Nutrition, Faculty of Fisheries, Kagoshima University, 4-50-20 Shimoarata, Kagoshima 890-0056, Japan

[c] Nutritional Science Laboratory, Morinaga Milk Industry Co., Ltd, 5-1-83 Higashihara, Zama, Kanagawa 228-8583, Japan

[d] Present address: Yamaha Nutreco Aquatech Co., Ltd, 12-1-3 Hakata, Fukuoka-City, Fukuoka 812-0013, Japan

*Corresponding author. Tel.: +81-99-286-4181; Fax: +81-99-286-4184; E-mail: Koshio@fish.kagoshima-u.ac.jp

Abstract

Bovine lactoferrin (LF) was orally administrated to Japanese flounder juveniles to investigate the effect of LF on tolerance to high temperature stress. Four different concentrations of LF (0, 400, 1000 and 2000 mg LF / kg diet) were supplemented in test diets and fed to juveniles (mean body weight = 11.8 g) at 26.7 °C. After feeding for 30 days, lethal time to 50 % mortality (LT_{50}) after exposed to 34.0 °C seawater (lethal stress test) and the levels of heat shock protein 70 family (HSP70s) in skin and liver after exposed to 31.0 °C seawater (sub-lethal stress test) were measured as parameters of tolerance to high temperature stress. In the lethal stress test, the fish fed the diet with 1000 mg LF supplemented group showed significant longer LT_{50} compare to that of 0 mg LF group, but other LF supplemented groups did not show any significant differences on the LT_{50} from 0 mg LF group. In the sub-lethal stress test, significant higher level of HSP70s was measured in skin collected from the fish fed the diet with 1000 mg LF supplemented group. The fish fed 400 and 1000 mg LF supplemented diets showed significant higher levels of liver HSP70s compare to that of 0 mg LF group. In conclusion, dietary LF supplementation enhances the tolerance to high temperature stress in Japanese flounder juveniles.

key words: Lactoferrin; Japanese flounder; Heat shock protein; High temperature stress

1. Introduction

Ever since aquaculture became intensive, increase in mass mortality caused by environmental fluctuations and outbreak of disease has been considered as a serious problem in Japan (Muroga, 2001). The feasibility of disease and infection is facilitated by various stressors (Wademeyer, 1996; Davis et al., 2002). It is, therefore, important to enhance the tolerance against stress for cultured fish. In recent years, some components which can stimulate defense systems and stress response of fish and crustaceans, called immunostimulant, have been isolated from plants, animals and micro organisms (Sakai, 1999). The use of immunostimulants has been suggested to be an effective means to control biomass in aquaculture.

Lactoferrin (LF) is a family of iron-binding glycoproteins with molecular weight of 80 kDa that originates from some secretions of mammals. It can be regarded as a kind of immunostimulant, and has a lot of biological functions e.g., iron absorption and transportation (Hagiwara et al., 1997), bacteriostatic effects (Nemet and Simonovits, 1985) and enhancement of mucosal immunity systems in mammals (Wang et al., 2000). Orally administrated LF has been shown to enhance nonspecific defense systems and stress resistance of fish. Namely, LF enhanced mucus secretion in juvenile red seabream (Kakuta et al., 1996) and phagocytic activity in rainbow trout (Sakai et al., 1993, 1995). LF also enhanced defensive effect against *Cryptocaryon irritans* for red seabream (Kakuta and Kurukura, 1995) and *Ichthyophthirius multifiliis* for goldfish (Kakuta, 1996b). Some reports also showed that orally administrated LF could enhance tolerance to physicochemical stress. Juvenile ayu treated with dietary LF appeared to have higher tolerance to stress when exposed to low oxygen water, formalin and copper sulfate solutions (Kakuta et al., 1998), and decrease plasma cortisol level in goldfish (Kakuta, 1996a) by LF administration. It has also been reported that dietary LF enhances tolerance to physiological stressors, such as, air exposed stress in juvenile

Japanese flounder (Gallardo-Cigarroa et al., 2000), against high stocking density stress in rainbow trout (Kakuta, 1997) and common carp (Kakuta, 1998), and low salinity stress in shrimp (Koshio et al., 2000). LF administration seems to enhance tolerance against chemical and physiological stressors in marine and freshwater species. However, it is still unclear how LF stimulates the tolerance to continuous high temperature stress and primary stress response of finfish. Thus, we investigated the effect of orally administrated LF on tolerance to continuous high temperature stress of Japanese flounder, which is an important culture species in Japan, by measuring the heat shock protein (HSP) and survival at high temperature condition as a biochemical criterion of tolerance to high temperature stress.

2. Materials and mthods

2.1. *Test diet and LF analysis*

The basal composition of test diets (Table. 1) was designed to satisfy the requirements of protein, lipid, carbohydrate and other nutrients for Japanese flounder (Kosutarak et al., 1995; Gallardo-Cigarroa et al., 2000). The test diets contained four different levels (0, 400, 1000 and 2000 mg LF / kg) of LF (Bovine Lactoferrin, Morinaga Milk Industry Co., Ltd, Tokyo, Japan). The test diets were pelleted with a meat grinder and dried below 39 °C for 2 h to prevent LF activity from being inactivated, and stored at −16 °C until use. The content of bovine LF in the test diets (mg / kg) was determined by sandwich enzyme-linked immunosorbent assay (ELISA) with goat anti-bovine LF as a primary, and goat anti-bovine LF-HRP conjugate as a secondary antibody (E10-126, Bethyl Laboratories Inc., TX, USA). Carbon coloidal blocking buffer (Super Block^{TM} in PBS, Pierce, Rockford, IL, USA) was used to avoid nonspecific binding on binding

sites on microplate. TMB (3,3',5,5' tetramethylbenzidine, Immunopure TMB Substrate, Pierce, IL, USA) colorimetric reaction was stopped with H_2SO_4 then the absorbance of each well was measured at 450 nm using a microplate reader (Immuno Mini NJ-2300, Nalge Nunc International, Rochester, N.Y. USA). LF content in the sample solutions was calculated using standard curve. Chemical composition of the test diets was determined by the methods described in AOAC (1990), chemical composition and content of LF in the test diets are shown in Table 2.

2.2. *Fish and rearing*

Japanese flounder (*Paralichthys olivaceus*) juveniles (mean body weight = 11.8 g) were obtained from a local hatchery (Matsumoto Suisan, Miyazaki, Japan), and transported to the Faculty of Fisheries, Kagoshima University, Japan, and fed a commercial diet for Japanese flounder (Higashimaru Co., Ltd, Kagoshima, Japan) until the start of feeding test diets. Triplicate groups of 25 fish were maintained in 100-l black-colored polypropylene columnar tanks with flowing (26.7 ± 0.7 °C) filtered seawater. Test diets were fed to the fish at a ration size of 5 % of body weight (wet weight basis, dose levels of LF: 0, 20, 50 and 100 mg LF / kg body weight / day) divided into three feedings (9:00, 13;00, and 17:00) for 30 days.

2.3. *Lethal stress test*

Lethal stress test was conducted to determine the lethal time to 50 % mortality (LT_{50}) at 34.0 °C. After feeding the test diets for 30 days, 5 fish from each rearing tank were randomly sampled and transferred to a black colored 30-l polyethylene columnar tank filled with 20-l filtered seawater which was aerated, maintained at constant temperature (34°C) by heating with a thermostat and aquarium heater. Number of dead fish was

recorded after 1, 2, 3 and 4 h. The evidence of death was judged by lack of gill movement. The test was repeated three times in each tank. Cumulative mortality (%) was transformed into common log % survival to fit a regression line and the mean LT_{50} was defined based on the regression equation (Borgmann et al., 1978).

2.4 *Sub-lethal stress test and quantitative analysis of heat shock proteins*

Sub-lethal stress tests were conducted to measure the levels of heat shock protein 70 family (HSP70s) as a biochemical indicator of tolerance to continuous high temperature condition. After feeding the test diets for 30 days, two fish were sampled from each rearing tank as no-stressed fish, and 8 fish were randomly sampled from each tank and transferred to a 30-l polyethylene columnar tank filled with 20-l of sub-lethal high temperature seawater (31.0 °C) with aeration. The seawater was kept at a constant temperature as the lethal stress test. After 12 h from first exposure, the seawater was renewed. Five fish were sampled after 24 h and immediately dissected to collect skin and liver. The levels of HSP70s, which cross-reacted with mouse HSP70 monoclonal antibody in the sampled organ, were analyzed by ELISA using a commercial quantitative kit (EKS-700; Stressgen Biotechnologies Corporation, Victoria, BC, Canada). This kit was designed for inducible HSP70 in humans, mice and rats. However, cDNA sequence of inducible HSP70 of Japanese flounder is highly conserved among various vertebrates even in bovine and humans (Yokoyama et al., 1998), Investigators have confirmed that monoclonal antibody originated from mammals cross-react with fish, shellfish crustacean and green alga HSP (Bierkens et al., 1998; Dubeau et al., 1998; Snyder et al., 2001; Cimino et al., 2002). Therefore, inducible HSP70s can be detect in these species. The HSP sample preparation was performed according to Lewis et al. (1999) with slight modification. Collected skin and liver were frozen immediately and then crushed in liquid nitrogen with a cold pestle

and mortar to a powder. An extraction buffer (provided in kit) with protease inhibitor (Complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics Corporation, USA) was added to the powdered sample and homogenized with a glass potter homogenizer in an ice bath. The homogenate was centrifuged ($17000 \times g$, 15 min, 4 ºC). Precipitate was discarded, and the supernatant was filtered with a cellulose acetate filter (DISMIC 3-CP filter unit; Advantec Toyo Kaisha, Tokyo, Japan) and subjected to analysis of HSP70s. Measurement of HSP70s level was performed a microplate reader. Total protein content of the supernatant was determined by the method of Lowry (1951) using bovine serum albumin (Nakarai tesque, Tokyo, Japan) as standard. The levels of HSP70s were expressed as levels of HSP70s in total protein in the sample solution.

2.5 *Statistical analysis*

  Data were statistically analyzed by one-way ANOVA followed by Tukey's HSD test to detect significant ($P < 0.05$) differences among treatments, using Kaleida Graph version 3.6 program for Macintosh (Synergy Software, PA, USA).

3. Results

  There was no mortality or abnormally behaved fish in each treatment during the feeding trial. In the lethal stress test (Table. 3), fish fed the diet supplemented with 1000 mg LF showed the greatest value of LT_{50}, which was significantly longer than that of fish fed the diet without LF (0 mg LF group). However, the values of the fish fed the diets with 400 and 2000 mg LF were not significantly different from that of 0 mg LF group. Quantitative HSP70s in liver of Japanese flounder exposed to sub-lethal high temperature seawater are shown in Fig. 1 for the fish fed the diets containing various

levels of LF. HSP70s was detected in each tissue collected from fish after exposed to the test solution but not detected in either liver or skin of no-stressed fish (data are not shown). Significantly higher levels of HSP70s were detected in liver from fish fed the diet with 400 and 1000 mg LF supplemented than that of 0 mg LF group. The level of HSP70s in liver from fish fed the diet containing the highest concentration of LF (2000 mg LF group) was not significantly different than that of the 0 mg LF group. Fig. 2 shows the quantitative HSP70s values from skin of Japanese flounder. Significantly higher HSP70 was detected in skin from fish fed the diet containing 1000 mg LF compared to that of the other treatment groups.

4. Discussion

In the lethal stress test, the LT_{50} of fish fed the diet supplemented with 1000 mg LF was significantly improved when compared to that of fish fed the 0 mg LF diet, suggesting the enhancement of a tolerance to high temperature stress. It is likely that the optimal range of dietary LF for the greatest stress tolerance would be between 400 and 1000 mg / kg diet. Although, the LT_{50} of fish fed the diet with 2000 mg LF did not show any difference from that of fish fed the diet without LF. Excessive and long-term administration of some immunostimulants may a cause decreased response of the defense system or a reverting to a previous state by negative feedback systems (Sakai, 1999). Kakuta (1996) has reported that growth was slightly lower and mucus secretion was decreased in red seabream fed the diet containing the highest concentration of LF (4% in diet) compared to that of fish fed the diet containing lowest concentration of LF. In this study, therefore, excessive administration of LF may have negatively affected tolerance to high temperature stress in Japanese flounder. There was no toxicity of LF in rats during 13 weeks of oral administration (Yamauchi et al., 2000). Further

comparative studies are required to investigate more details involving dosage and administration period of LF in fish.

HSP is a kind of stress protein which is synthesized when organisms are exposed to various stressors e.g., heat shock, cold shock, viral infection, and heavy metal exposure (Iwama et al., 1998). Administration of glutamine has induced expression of HSP25 and 72 in rats against endotoxin shock (Wischmeyer et al., 2001). The synthesized HSP improves tolerance to various stressors in finfish (Dilorio et al., 1996; Dubeau et al., 1998), finfish cell lines (Mosser and Bols, 1998) and shellfish (Brown et al., 1995). However, knowledge on the relationship between tissue HSP level and administration of immunostimulant in fish has scarcely been investigated. An increased expression of HSP60 in kidney tissue by oral administration of saponins was observed in yellowtail infected with *Enterococcus seriolicida* (Ashida et al., 1999). This report suggests that oral administration of an "immunostimulant" affects the expression of HSP synthesis in fish under stressful conditions.

The synthesis of HSP is a general primary cellular event which occurs during both exogenous and endogenous stress situation, and has an ability of mediate misfolded or denatured functional proteins caused by various stressors in the cell (Iwama et al., 1999). This protein is also known as a molecular chaperone (Ellis, 1999), and plays a role in novel protein synthesis. In the present study, HSP70s were not detected in either skin or liver of fish which were not exposed to high temperature.

Significantly higher levels of HSP70s were detected in liver tissue of fish fed the diet supplemented with 400 and 1000 mg LF compared to that of the 0 mg LF group. Liver is a representative organ to maintain vital functions of the body. HSP acts as a mediator of liver function under stressful situation (Kume et al., 1996; Bedirli et al., 2004; Lee et al., 2004). Consequently, it is expected that higher levels of synthesized HSP contribute to maintain liver and whole body vital functions. On the other hand, the level of HSP70s in skin of the 1000 mg LF supplemented group was significantly higher than

that of the other groups. This tendency agreed with the results of the lethal stress test. Thus, it is suggested that the improved LT_{50} in the 1000 mg LF supplemented group resulted from the establishment of homeostasis with some functional proteins which were protected by the newly synthesized HSP70s in skin.

Different detection patterns of HSP70s levels in skin and liver have been reported to be due to specific patterns of HSP synthesis in each cell line (Dyer et al., 1991) against the same intensity of stressor. External exposure to a stressor can be assumed to first affect the body surface because it is directly exposed to the stressor. Therefore, skin or epidermis must have tolerance to stressors to maintain vital functions of the whole body. Skin is a potent organ to measure the levels of HSP as a parameter of the stress response (Burkhardt et al., 1998). It has been reported that oral administration of LF increased mucilage cells in juvenile ayu (Kakuta et al., 1998), and stimulated mucus secretion in red sea bream (Kakuta et al., 1996), gold fish (Kakuta, 1996a) and Japanese flounder (Gallardo et al., 2000). These results suggested that administrated LF have a stimulating action on epidermic cells.

The present study demonstrated that LF administration improved survival of Japanese flounder and enhanced synthesis of HSP under continuous high temperature conditions.

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Table 1

Basal diet composition

Ingredient	g / kg dry diet
Brown fish meal	300
Casein	100
Squid meal	100
Krill meal	100
Dextrin	20
α- Starch	40
Squid liver oil	80
Soybean lecithin	50
Vitamin mixture[*1]	40
Mineral mixture[*2]	40
Attractant[*3]	10
Activated gluten	50
α- Cellulose + Bovine lactoferrin[*4]	70

[*1] Vitamin mixture (mg / kg dry diet): β-Carotene 128.4; Vitamin D_3 12.9; Vitamin K_3 61.1; α-Tocopherol 513.3; Thiamin-HCl 77.0; Riboflavin 256.5; Biotin 7.7; Inositol 5132.2; Niacin 1026.3; Ca-Panthothenate 359.3; Folic acid 19.2; Choline chloride 10492.4; p-Aminobenzoic acid 511.0; L-Ascorbyl-2-phosphate-Mg 100.0.

[*2] Mineral mixture (mg / kg dry diet): NaCl 1437.2; $MgSO_4 \cdot 7H_2O$ 5066.9; $NaHPO_4$ 3225.4; KH_2PO_4 8869.3; $Ca(H_2PO4)_2 \cdot 2H_2O$ 5022.6; Fe Citrate 1098.4; Ca Lactate 12094.7; $Al(OH)_3$ 6.92; $ZnSO_4 \cdot 7H_2O$ 132.0; $CuSO_4$ 3.7; $MnSO_4 \cdot 5H_2O$ 29.6; $Ca(IO_3)_2$ 7.2; $CoSO_4 \cdot 7H_2O$ 36.9.

[*3] Attractant (g / kg dry diet): Alanine 2.0; Inosine 2.0; Taurine 2.0; Betaine 2.0; L-Proline 2.0.

[*4] Bovine lactoferrin: Lot No. 951110. Morinaga milk industry. Co. Ltd. Tokyo, Japan.

Table 2

Chemical composition and lactoferrin (LF) contents of test diets

LF supplemented (mg / kg)	Protein* (%)	Fat* (%)	Ash* (%)	Moisture (%)	LF content* (mg / kg)
0	42.2	15.1	12.5	12.1	0.0
400	42.5	15.0	12.4	12.0	350.7
1000	42.2	14.8	12.5	11.6	976.6
2000	41.5	15.2	12.5	11.2	1929.4

*Dry basis

Table 3

The result of lethal stress test on mean LT_{50} obtained by regression analysis [1, 2, 3.]

LF supplemented (mg / kg)	Slope coefficient[1] ($\times 10^{-3}$)	Correlation coefficient (R^2)	Mean LT_{50}[2, 3] (min)
0	3.28	0.95	119.7 ± 27.1a
400	3.06	0.98	128.6 ± 20.0a
1000	2.09	0.87	242.4 ± 31.5b
2000	2.93	0.99	119.3 ± 28.6a

[1] Slope coefficients are defined based on the mean log % survival in each observed time (n=3). [2] Values are expressed as mean ± standard deviation. The values with the same letters are not significantly different ($P < 0.05$). [3] LT_{50}: see the text.

Figure legends

Fig. 1. Levels of HSP70s detected in liver from Japanese flounder exposed to sub-lethal high temperature (31.0 °C) seawater.

Fig. 2. Levels of HSP70s detected from skin in Japanese flounder exposed to sub-lethal high temperature (31.0 °C) seawater.
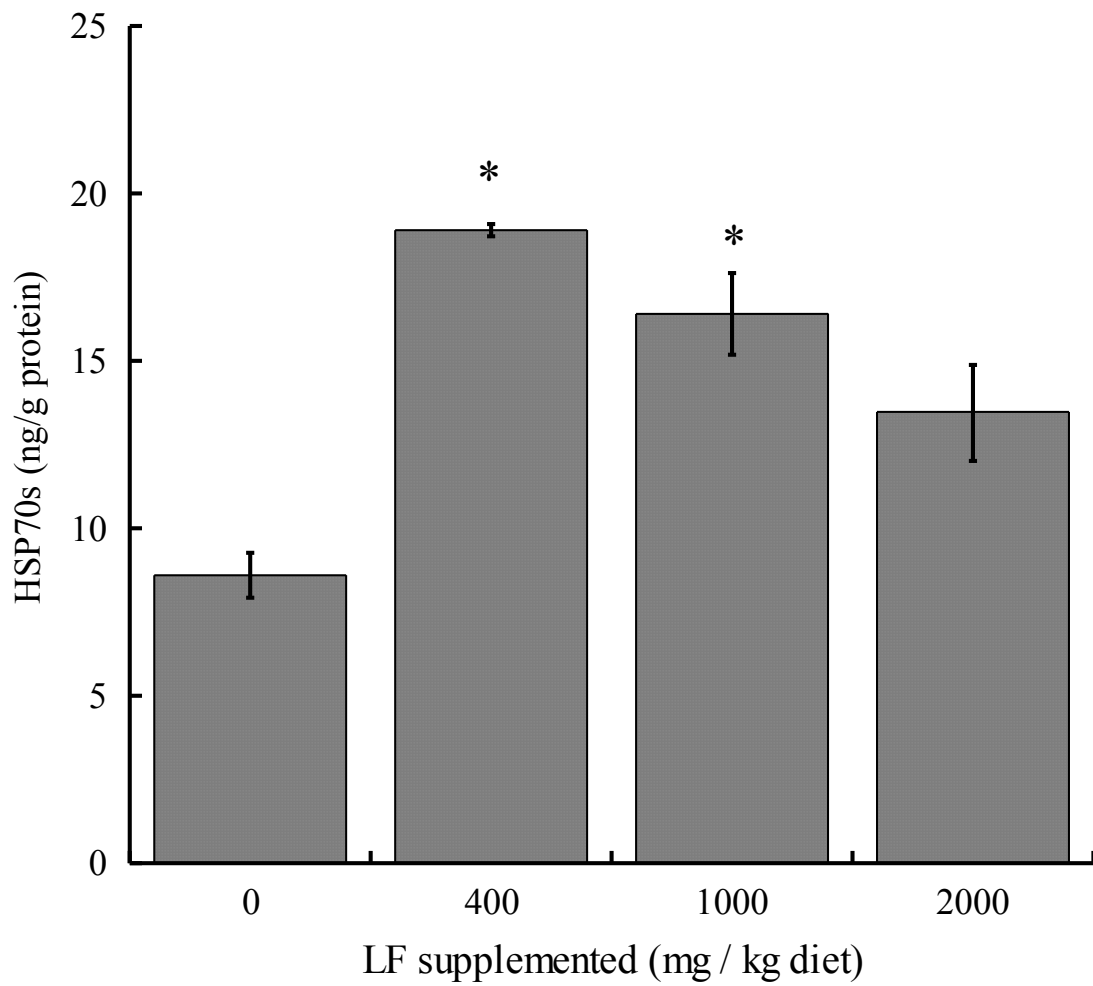
Fig. 1. Levels of HSP70s detected in liver from Japanese flounder exposed to sub-lethal high temperature (31.0 °C) seawater. Values are expressed as mean ± standard deviation (n=5). Asterisk indicates significant difference from 0 mg LF group (P<0.05).
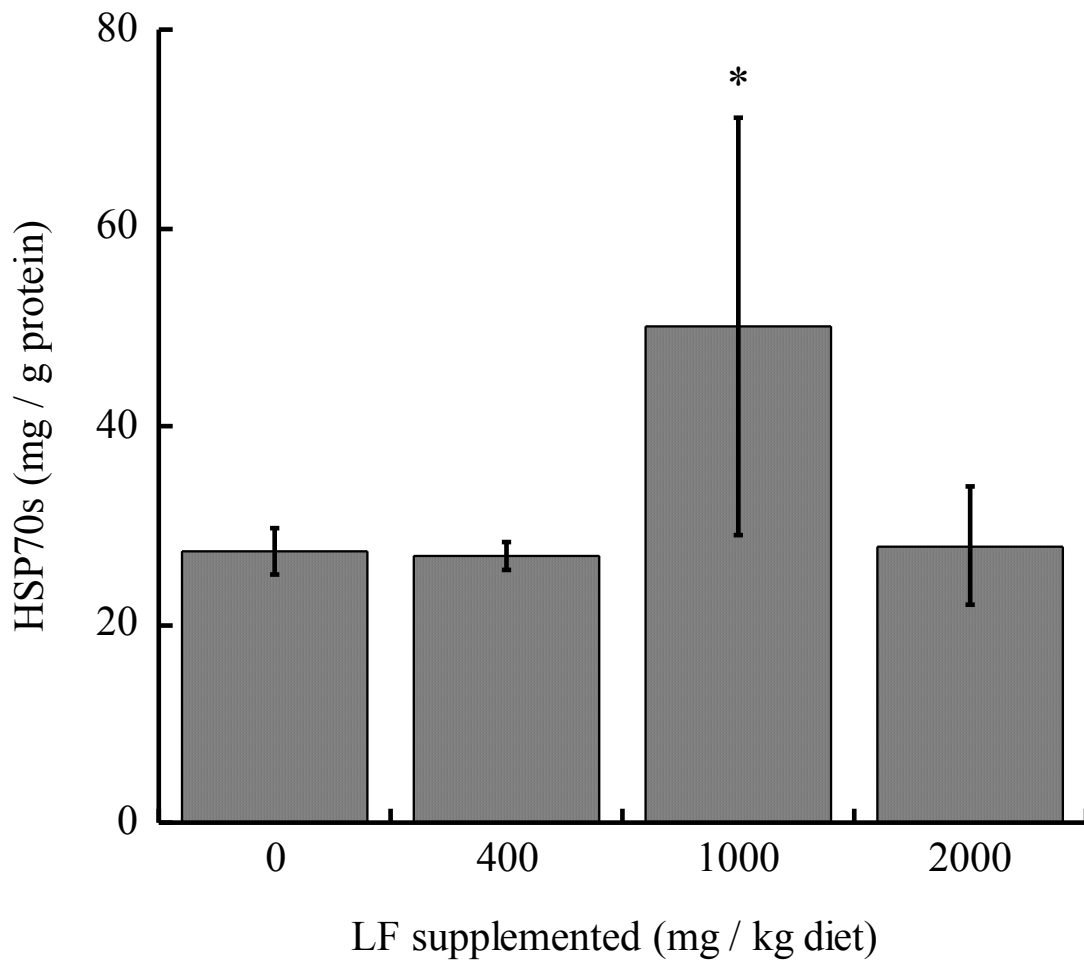
Fig. 2. Levels of HSP70s detected from skin in Japanese flounder exposed to sub-lethal high temperature (31.0 °C) seawater. Values are expressed as mean ± standard deviation (n=5). Asterisk indicates significant difference (P<0.05) from 0 mg LF group.