Thesis for the Degree of Master of Science

The effects of quercetin on oxidative stress resistance and physiological responses in olive flounder, *Paralichthys olivaceus*

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February 2010

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by

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A dissertation submitted in partial fulfillment of the requirements

for the degree of

Master of Science

In the Department of Marine Bioscience and Environment,

the Graduate School of Korea Maritime University

February 2010

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December 2009

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요 약

어류는 독성 및 염분등과 같은 외부 인자에 의한 스트레스는 체내에서 유해 산소인 reactive oxygen species (ROS)를 생성·증가시켜 산화 스트레스를 유발시킨다. 따라서 생물체는 ROS에 의한 항산화 스트레스로부터 자신을 보호하고, 항상성을 유지하기 위하여 항산화 방어기작을 가지고 있는데 항산화 방어기작에 관여하는 대표적인 항산화 효소로는 superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) 및 glutathione S-transferase (GST)등이 존재하며, 항산화제로는 Metallothionein (MT), vitamin C 및 E 등이 항산화 작용을 하는 것으로 알려져 있다. 본 연구에서는 ROS 제거능이 우수한 천연 항산화제임과 동시에 면역 및 호르몬 증진 등 생리학적으로 뛰어나다고 알려져 있는 퀘르세틴을 넙치에게 공급한 후 독성물질인 Cd 및 저염분에 노출시켜 항산화 효소인 SOD와 CAT의 발현과 활성 변화를 조사하였으며, ROS의 일종인 H_2O_2농도, 지질 과산화(lipid peroxidation, LPO) 정도를 측정하였다. 퀘르세틴을 공급한 실험구와 일반 사료를 공급한 실험구를 Cd과 저염분에 노출시킨 후 항산화 효

소인 SOD와 CAT 발현량을 quantitative real-time PCR (QPCR)을 이용하여 분석한 결과, 퀘르세틴을 공급한 실험구에서 일반 사료를 공급한 실험구보다 발현량이 낮았으며, SOD와 CAT 활성 또한 낮은 값을 나타내었다. 이를 뒷받침하는 결과로 혈장 H_2O_2 농도 또한 퀘르세틴을 공급한 실험구에서 더 낮은 수치값을 보였다. 이와 같이 생성된 ROS는 지질 과산화를 일으켜 세포를 손상시킨다고 알려져 있어, 본 연구에서도 퀘르세틴을 공급한 실험구를 Cd과 저염분에 노출시킨 후 측정한 결과 LPO 정도가 일반 사료를 공급한 실험구에 비하여 더 낮은 값을 보여, 퀘르세틴에 의해 제거된 ROS로 인하여 지질 과산화 정도가 낮음을 보여주고 있다. 덧붙여 퀘르세틴의 항산화 능력뿐만 아니라 면역능을 파악하기 위해 퀘르세틴을 공급한 실험구의 체내 lysozyme 활성을 측정한 결과 일반 사료를 공급한 실험구보다 더 높은 값을 보여 퀘르세틴이 체내 면역능도 향상시킨다는 사실을 알 수 있었으며, 호르몬 증진 효과를 알아보기 위하여 T_3 농도를 측정한 결과 일반 사료를 공급한 실험구보다 높은 값을 보여 퀘르세틴이 체내 대사 작용 촉진을 위한 호르몬 증진에도 영향을 미친다는 것을 알 수 있었다.

I. General Introduction

Environmental factors affecting metabolism, growth, survival, osmolality, and immune function in fish (Britoa et al., 2000). Stress induced by environmental factors like xenobiotics and salinity change has been associated with enhanced reactive oxygen species (ROS) generation, which may seriously affect immune function and lead to oxidative stress (Fisher and Newell, 1986). ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical (HO^-), and singlet oxygen ($^1O_2$) (Kinnula et al., 1995). Overproduction of ROS by environmental factors can increase lipid peroxidation (LPO), the oxidation of nucleic acid and proteins, and DNA damage. It can also affect cell viability by causing membrane damage and enzyme inactivity, and can then accelerate cell senescence and apoptosis (Kim and Phyllis, 1998; Pandey et al., 2003). Complex antioxidant defense systems maintain homeostasis and protect aerobic organisms against ROS and the subsequent damage of oxidative stress. Antioxidants may be enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, and glutathione S-transferase, or compounds such as metallothionein, quercetin (Jayaraj et al., 2007), vitamin C, and vitamin E (α-tocopherol) (McFarland et al., 1999).

This study investigated the effects of the natural antioxidant quercetin, which is almost ubiquitous in plants. Quercetin is a polyphenolic flavonoid compound with strong antioxidant capabilities. Quercetin chelates ROS induced by lipid peroxidation and metal ions, provides H^+ ions to prevent

lipid peroxidation in the cell membrane, and scavenges free radicals. Furthermore, quercetin converts ROS to energy and reduces metal concentrations and stress to protect cell membranes (Bors and Saran, 1987).

Also, we examined immune ability enhanced by quercetin through measuring plasma lysozyme activity. Lysozyme, a lysosomal enzyme implicated in the inflammatory process, is a non-specific humoral factor that acts under stressful conditions (Bols et al., 2001; Dautremepuits et al., 2004). It is released by leukocytes and plays an important role in antimicrobial activity (Eo and Lee, 2008). Many antioxidants are reported to enhance immune capacity as well as antioxidant capacity.

Therefore, we used olive flounder fed a diet containing quercetin for 30 or 60 days as an experimental model for studying (1) the effects of quercetin on enhancing the immune ability of olive flounder by measuring the plasma lysozyme activity and T_3 concentration. Then, we investigated the effect of quercetin pretreatment on the toxicity induced by Cd in the olive flounder by measuring the expression and activity of antioxidant enzymes (SOD and CAT), the plasma H_2O_2 concentration, and LPO as an oxidative stress parameter; (2) the plasma total cholesterol concentration to determine the ability of quercetin to reduce plasma cholesterol, and the mRNA expression and activities of the antioxidant enzymes SOD and CAT, the plasma H_2O_2 concentration, lysozyme activity, plasma cortisol concentration, and plasma osmolality to elucidate the effects of quercetin on the oxidative stress induced by changes in salinity.

II. Experiment 1

Effect of quercetin on the activity and mRNA expression of antioxidant enzymes and physiological responses in olive flounder, *Paralichthys olivaceus* exposed to cadmium

Abstract

We investigated the antioxidant efficacy of quercetin (0% Diet 1, 0.25% Diet 2, and 0.5% Diet 3) pretreatment for 30 and 60 days in cadmium (Cd) toxicity in the olive flounder, and measured the plasma lysozyme activity and T_3 concentration to understand the immune effects and hormone regulation of quercetin. The lysozyme activity and T_3 concentration with Diets 2 and 3 were higher than with Diet 1. Therefore, we postulated that quercetin increased the immune ability and maintained hormone homeostasis in the olive flounder. Then, we exposed olive flounder fed quercetin to Cd to understand the antioxidant role of quercetin and measured the expression and activity of antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)), and lipid peroxidation (LPO). With Diets 2 and 3, the expression and activity of antioxidant enzymes and the H_2O_2 concentration were lower than with Diet 1. In addition, the LPO levels were lower than with Diet 1, which protected the cell membrane. Therefore, quercetin removed the reactive oxygen species (ROS) produced by Cd, indicating that quercetin has antioxidant ability. In addition to its antioxidant ability, quercetin has immune effects and is a physiologically active material.

Keywords: Antioxidant, LPO, Lysozyme, Quercetin, ROS, T_3

1. Introduction

Cadmium (Cd) is a heavy metal that has natural and industrial sources. It is potentially highly toxic to aquatic organisms, humans, animals, and plants, even at low doses (Benavides et al., 2005). Living cells of aquatic organisms accumulate Cd (Rainbow and White, 1989). Cd increases the formation of reactive oxygen species (ROS) and provokes oxidative stress in organisms (Stohs et al., 2000). ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical (HO^-), and singlet oxygen ($^1O_2$) (Kinnula et al., 1995). Overproduction of ROS by Cd can increase lipid peroxidation (LPO), the oxidation of nucleic acid and proteins, and DNA damage. It can also affect cell viability by causing membrane damage and enzyme inactivity, and can then accelerate cell senescence and apoptosis (Kim and Phyllis, 1998; Pandey et al., 2003).

To protect themselves against ROS generating oxidative stress, aerobic organisms have evolved complex antioxidant defense systems. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione S-transferase (GST), and low-molecular-weight antioxidant materials such as metallothionein (MT), vitamin C, and vitamin E are found in the livers and kidneys of marine organisms (Basha and Rani, 2003; Hansen et al., 2006).

Previous studies have examined systems involving natural antioxidants. These

studies investigated the antioxidant effects of vitamin E supplements in the crab (*Chasmagnathus granulatus*) (Pinho et al., 2005), tilapia (*Oreochromis niloticus*) (Prieto et al., 2008), and mouse (*Mus musculus*) (Weng et al., 2007) against microcystin toxicity by analyzing CAT and GST activity. In isolated carp (*Cyprinus carpio* var. *Jian*) enterocytes, Chen et al. (2009) showed that the antioxidant effect of glutamine scavenging of ROS induced by H_2O_2 treatment occurred via the activity of the antioxidant enzymes SOD, CAT, and GPX. Qinghui et al. (2004) reported beneficial effects of vitamin C on immunological parameters, such as lysozyme, in the Japanese seabass (*Lateolabrax japonicus*).

Lysozyme, a lysosomal enzyme implicated in the inflammatory process, is a non-specific humoral factor that acts under stressful conditions (Bols et al., 2001; Dautremepuits et al., 2004). It is released by leukocytes and plays an important role in antimicrobial activity (Eo and Lee, 2008). Many antioxidants are reported to enhance immune capacity as well as antioxidant capacity.

This study investigated the effect of the natural antioxidant quercetin in olive flounder exposed to Cd. Quercetin is a polyphenolic flavonoid compound that is a strong antioxidant and is almost ubiquitous in plants. Broccoli and apples contain 7~110 mg/kg, while onions contain 284~486 mg/kg (Scalbert and Williamson, 2000). Quercetin chelates metal ions, has free radical scavenging activity, and reduces the concentration of metal to

protect cell membranes (Bors and Saran, 1987). Several studies have examined the antioxidant effects of quercetin. For example, it inhibits carcinogenesis in mice (Bors and Saran, 1987; Cook and Samman, 1996) and promotes the antioxidant system in response to induced oxidative stress caused by toxicity (Jayaraj et al., 2007; Bhatt and Flora, 2009). Most of these studies examined mammals, and no studies have examined its effects in fish.

2. Materials and methods

2.1. Experimental fish and conditions

Olive flounders (n = 800, length 10 ± 0.5 cm, weight 19.9 ± 1.3 g) were obtained from a commercial fish farm and acclimated to the experimental conditions for 2 weeks in nine 300 L circulation filter tanks in the laboratory. During the experiments, the water temperature and photoperiod were maintained at 20 ± 1 °C and a 12L:12D cycle, respectively. The fish were fed a commercial feed twice daily (09:00 and 17:00). In all, 800 flounders were chosen randomly and distributed in the nine 300 L flow-through tanks.

2.2. Experimental diets

Fish meal, dehulled soybean, and corn gluten meal were used as protein sources, and wheat flour and squid liver oil were used as carbohydrate and lipid sources, respectively. The ingredients of the experimental diets were mixed well with water in a ratio of 3:1 and then pelletized. The experiment diets were dried at room temperature and stored at −20 °C until required. In the experimental diets, the crude protein content ranged from 55.1~56.0%, and the crude lipid content from 9.1~9.7%. The estimated energy content was 4.1 kcal/g. The quercetin used in this study was extracted from discarded onion peels in a chromatogram column by Dongsun Industrial

(Gyunggi-do, Korea). The product was sterilized, and its quality was assured. The experimental diets contained 0% (Diet 1), 0.25% (Diet 2), or 0.5% (Diet 3) quercetin at the expense of 0, 0.25, or 0.5% wheat flour, respectively (Table 1).

Table 1. Ingredients and nutrient composition of the experimental diets.

Ingredient (%)	Diet		
	Diet 1 (0%)	Diet 2 (0.25%)	Diet 3 (0.5%)
Fish meal[a]	45.00	45.00	45.00
Corn gluten meal	15.00	15.00	15.00
Wheat flour	**19.90**	**19.65**	**19.40**
Soybean meal	10.00	10.00	10.00
Fish oil – salmon	2.50	2.50	2.50
Squid meal	2.00	2.00	2.00
Krill meal	2.00	2.00	2.00
Lecithin	1.00	1.00	1.00
Mono-calcium Phosphate	1.00	1.00	1.00
Choline	1.00	1.00	1.00
VITAMIX[b]	0.20	0.20	0.20
MINEMIX[c]	0.20	0.20	0.20
Vitamin C	0.20	0.20	0.20
QUERCETIN	**0.00**	**0.25**	**0.50**
Total	**100**	**100**	**100**

[a] Imported from Chile.

[b] Vitamin premix contained the following ingredients (g/kg mix): L-ascorbic acid, 121.2; DL-α-tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003.

[c]Mineral premix contained the following ingredients (g/kg mix): $MgSO_4 \cdot 7H_2O$, 80.0; $NaH_2PO_4 \cdot 2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4 \cdot 7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3 \cdot 6H_2O$, 0.15; KI, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4 \cdot H_2O$, 2.0; $CoCl_2 \cdot 6H_2O$, 1.0.

2.3. Plasma lysozyme activity and T_3 analysis

To determine the lysozyme activity of olive flounder, 50 μL of plasma were added to 950 μl of a suspension of *Micrococcus lysodeikticus* (0.2 mg/mL) in 0.05 M sodium phosphate buffer (pH 6.2). The reactions were carried out at 25°C, and the absorbance at 530 nm was measured between 0.5 and 4.5 min. One lysozyme activity unit was defined as the amount of enzyme producing a 0.001/min decrease in absorbance. Plasma containing T3 was resuspended in enzyme immunoassay (EIA) buffer in one-half the original plasma volume before performing enzyme-linked immunosorbent assays (ELISAs). The total T_3 level was measured in plasma using commercial ELISA kits following the kit protocols (DSL Laboratories, Webster, TX).

2.4. Cd exposure

The experimental fish were exposed to $CdCl_2 \cdot 2.5H_2O$ (Kanto Chemical, Tokyo, Japan) dissolved in water to a Cd^{2+} concentration of 10 ppb in 50-L tanks for 0, 6, 12, 24, and 48 h. Four fish from each group (Diets 1, 2, and 3) were selected randomly for blood and tissue sampling. The fish were anesthetized with 200 mg/L tricaine methanesulfonate (MS-222, Sigma, USA) before blood collection. Blood was collected from the caudal vein using a 3-mL syringe coated with heparin. Plasma samples were separated by

centrifugation (4 °C, 10,000 rpm, 5 min), and stored at −80 °C until analysis. To collect liver tissue samples, fish were euthanized by spinal transection. Immediately after collection, the samples were frozen in liquid nitrogen and stored at −80 °C until total RNA was extracted for analysis.

2.5. Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression of SOD and CAT mRNA in total RNA extracted from the liver. Primers for QPCR were designed with reference to the known sequences of olive flounder as follows: SOD forward primer (5'-CGT TGG AGA CCT GGG GAA TGT G-3'), SOD reverse primer (5'-ATC GTC AGC CTT CTC GTG GAT C-3'), β-actin forward primer (5'-GCA AGA GAG GTA TCC TGA CC-3'), CAT forward primer (5'-GGC TGA GAA GTT CCA GTT CAA TCC-3'), CAT reverse primer (5'-CTC CAC CTC TGC AAA GTA GTT GAC-3'), and β -actin reverse primer (5'-CTC AGC TCG TTG TAG AAG G-3'). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, USA) and iQ™ SYBR Green Supermix (Bio-Rad, USA) according to the manufacturer's instructions. QPCR was performed as follows: denaturation at 95 ºC for 5 min; 40 cycles of denaturation at 95 ºC for 20 s, annealing at 55 ºC for 20 s. As an internal control, experiments were duplicated with β-actin, and all data were expressed as change with respect to corresponding β-actin calculated

threshold cycle (CT) levels.

2.6. SOD and CAT activity analysis

Tissues were homogenized in ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing 1 mM EDTA. The homogenates were centrifuged at 10,000 rpm for 15 min at 4 °C, the supernatant was removed, and the remaining sample was analyzed. SOD and CAT activities were determined using commercial kits supplied by Cayman Chemical (USA).

The SOD activity was assessed using a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The absorbance is read at 450 nm. Each assay was performed in duplicate, and enzyme units were recorded as U/mg protein.

The method for CAT activity is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen (Wheeler et al., 1990). Purpald specifically forms a bicyclic heterocycle with aldehydes and changes from colorless to purple on oxidation. The absorbance is read at 540 nm. Each assay was performed in duplicate, and the CAT activity was expressed in nmol of formaldehyde min/mg/protein.

2.7. H_2O_2 assay

H_2O_2 concentrations measured using the modified methods of Nouroozzadeh et al. (1994) and a Peroxidetect kit (Sigma, USA). 20 μL of olive flounder serum was added per well to flat bottom 96-well microtitre plates. Plates were left at room temperature for 20 min to allow serum to settle and adhere. A working color reagent was prepared by mixing 100 mL distilled water containing 100 mM sorbitol and 125 μM xylenol orange (Sigma, USA) with 1 mL of 25 mM ferrous ammonium sulphate prepared in 2.5 M sulphuric acid (Sigma-Aldrich). 200 μL of this reagent was then added to each well and allowed to incubate at room temperature for 1 h. Absorbance was read at 560 nm and concentration of H_2O_2 were interpolated from a standard curve. Concentrations are expressed as nM/mL.

2.8. LPO assay

The Cayman Chemical (Ann Arbor, MI, USA) lipid hydroperoxide assay kit was used to measure hydroperoxides directly, utilizing the redox reaction with ferrous ion. Hydroperoxides were extracted into chloroform and reacted with ferrous ions to produce ferric ions. The resulting ferric ions were detected using thiocyanate ion as the chromogen. The hydroperoxide concentration was determined based on the absorption at 500 nm.

2.9. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One way ANOVA followed by post hoc Duncan's multiple range test was used to compare the differences in the data (P<0.05).

3. Results

3.1. Plasma lysozyme activity and T_3 analysis

In Diets 2 and 3, the lysozyme activity was significantly higher than in Diet 1 after feeding flounder quercetin for 30 and 60 days. In addition, the lysozyme activities in Diets 1, 2, and 3 fed for 60 days were significantly higher than were those fed for 30 days (Fig. 1A). In Diets 2 and 3, the T_3 levels were significantly higher than in Diet 1 at 30 and 60 days. However, the T_3 levels with Diets 1 and 2 did not differ significantly between 30 and 60 days, although a significant difference was observed with Diet 3 (Fig. 1B).

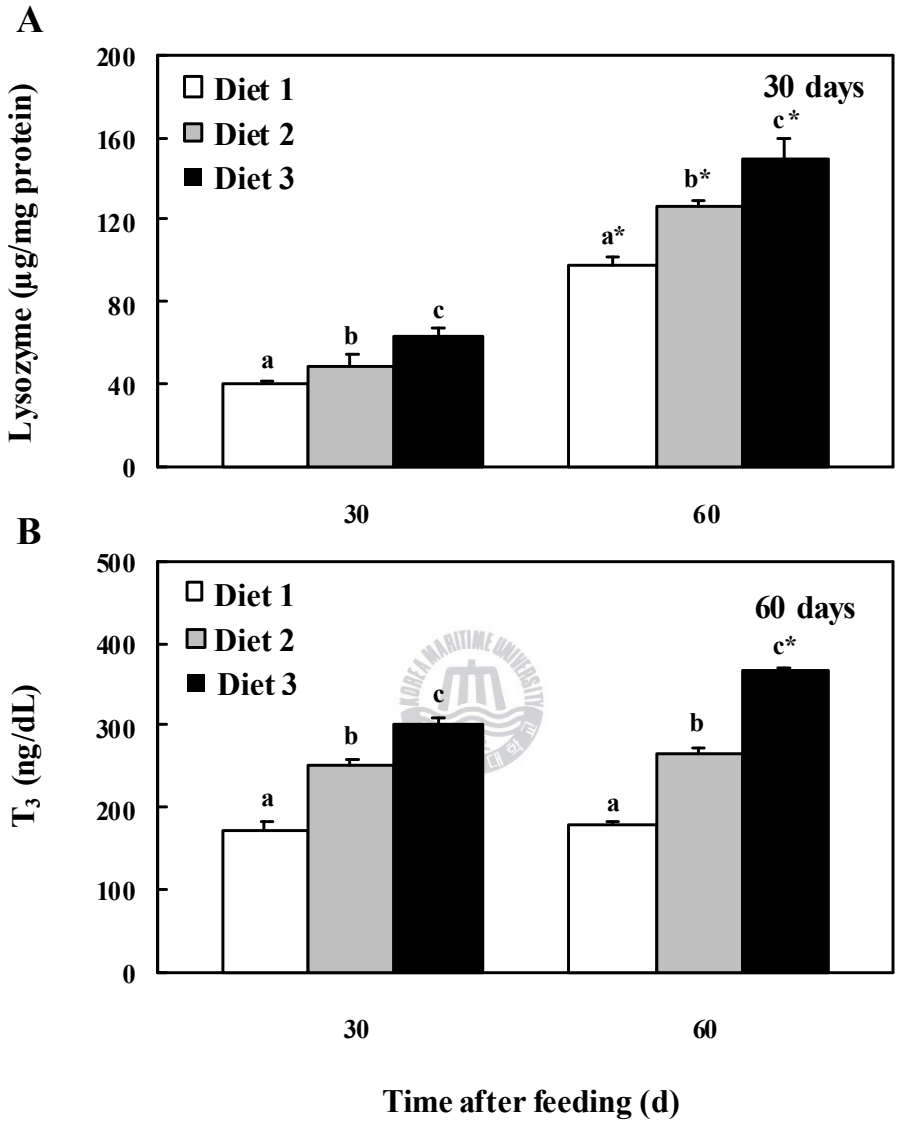
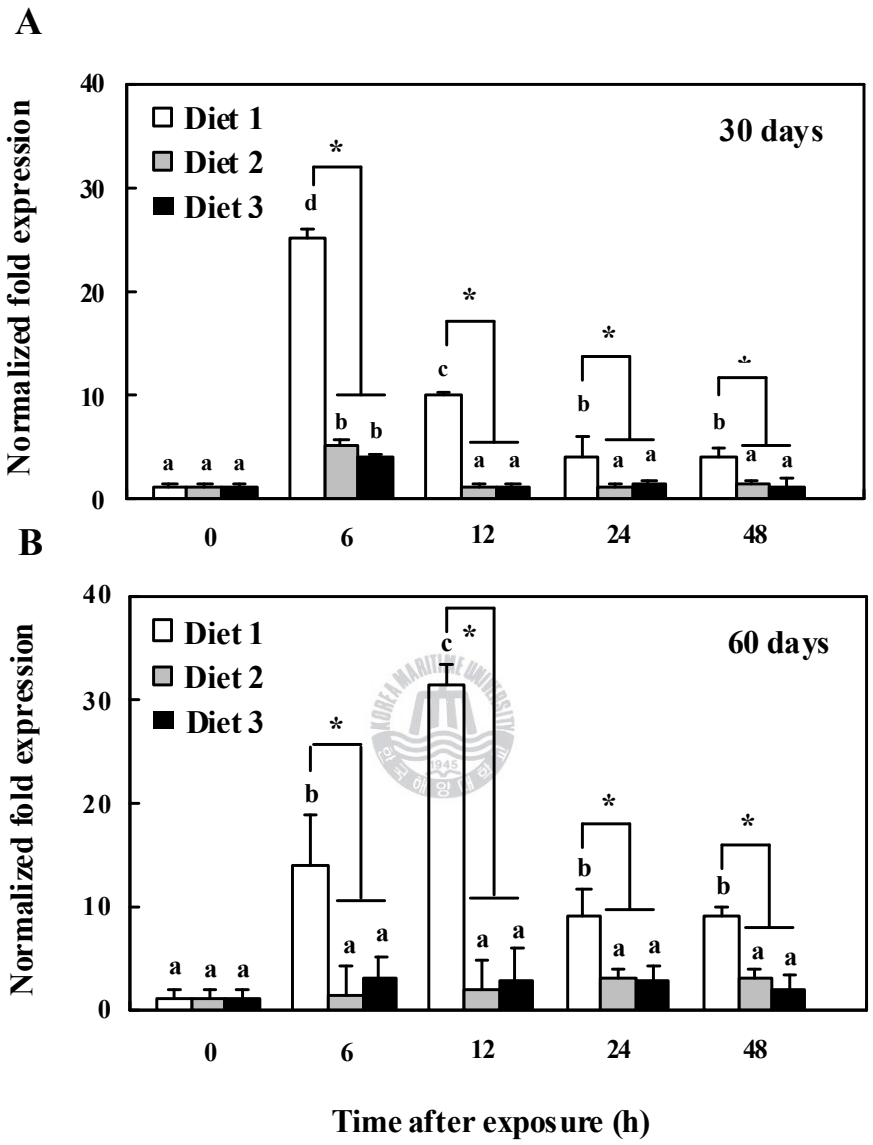

A
Lysozyme (μg/mg protein)
200
160
120
80
40
0
Diet 1
Diet 2
Diet 3
30 days
a
b
c
a*
b*
c*
30
60
B
T3 (ng/dL)
500
400
300
200
100
0
Diet 1
Diet 2
Diet 3
60 days
a
b
c
a
b
c*
30
60
Time after feeding (d)

Fig. 1. Lysozyme activity (A) and T_3 concentration (B) in plasma of olive flounder fed diets containing 0, 0.25, and 0.5% quercetin for 30 and 60 days. Values with different letters indicate significant differences from Diet 1, and asterisks indicate significant differences between results for 30 and 60 days with the same diet concentration ($P < 0.05$). All values are means ± SD (n=5).

3.2. QPCR for SOD and CAT mRNA expression

Using a quantitative polymerase chain reaction (QPCR), we examined the changes in SOD and CAT mRNA expression when olive flounder fed a diet containing quercetin were exposed to Cd. After feeding for 30 days, for Diet 1, the SOD mRNA expression was increased significantly at 24 h and then decreased, whereas the expression levels with Diets 2 and 3 were lower than with Diet 1 (Fig. 2A); the CAT mRNA was increased at 6 h and then decreased, whereas the expression levels with Diets 2 and 3 were lower than with Diet 1 (Fig. 2C). After feeding for 60 days, with Diet 1, the SOD mRNA expression was increased significantly at 12 h and then decreased, whereas the expression levels with Diets 2 and 3 were lower than with Diet 1 (Fig. 2B); the CAT mRNA was increased at 12 h and then decreased, whereas the expression levels with Diets 2 and 3 were lower than with Diet 1 (Fig. 2D).


A
Normalized fold expression
Diet 1
Diet 2
Diet 3
30 days
40
30
20
10
0
a a a
d
b b
c
a a
b
a a
b
a a
*
*
*
0 6 12 24 48
B
Normalized fold expression
Diet 1
Diet 2
Diet 3
60 days
40
30
20
10
0
a a a
b
a a
c
a a
b
a a
b
a a
*
*
*
*
0 6 12 24 48
Time after exposure (h)

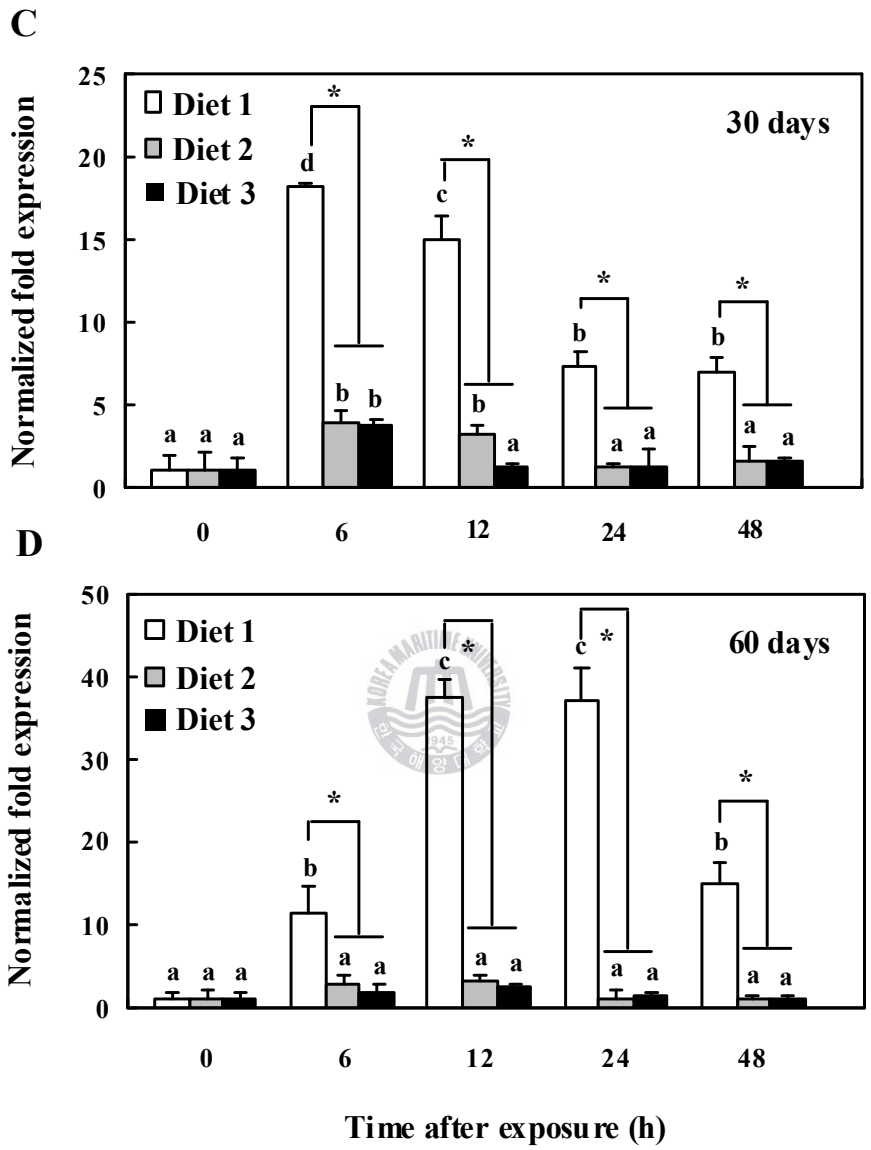
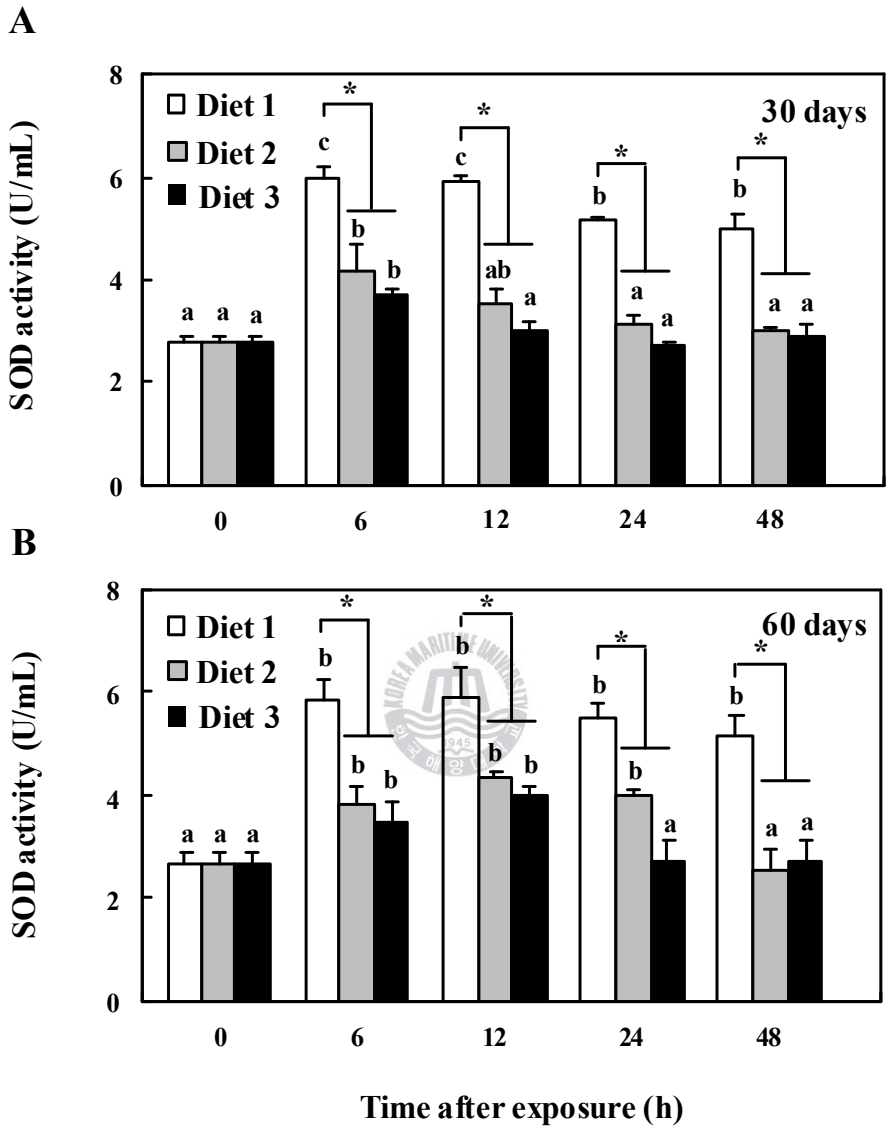

C
Normalized fold expression
Diet 1
Diet 2
Diet 3
30 days
0
5
10
15
20
25
0
6
12
24
48
D
Normalized fold expression
Diet 1
Diet 2
Diet 3
60 days
0
10
20
30
40
50
0
6
12
24
48
Time after exposure (h)

Fig. 2. Expression of SOD (A) and CAT (C) in olive flounder fed a diet containing quercetin for 30 days and SOD (B) and CAT (D) in olive flounder fed a diet containing quercetin for 60 days. The mRNA levels in the livers of olive flounder exposed to Cd (0, 6, 12, 24, and 48 h) determined using quantitative real-time PCR. First, 2.5 μg of total RNA prepared from the liver was reverse-transcribed and amplified using gene-specific primers. The results are expressed as normalized fold expressions with respect to β-actin levels for the same sample. Values with different superscripts indicate significant differences from controls for the same diet, and asterisks indicate significant differences between Diet 1 and Diets 2 and 3 ($P<0.05$). All values are means ± SD ($n=5$).

3.3 SOD and CAT activity

The effects of quercetin on Cd-induced antioxidant enzyme (SOD and CAT) activities are shown in Fig. 3. After feeding for 30 days, with Diet 1, the SOD activity was increased significantly at 12 h and then decreased, whereas the activity levels with Diets 2 and 3 were increased significantly at 6 and 12 h and then decreased, but they were lower than with Diet 1 (Fig. 3A); the CAT activity was increased at 12 h and maintained until 48 h, and the activity level with Diets 2 and 3 was increased significantly at 12 h and then decreased, but they were lower than with Diet 1 (Fig. 3C). After feeding for 60 days, with Diet 1, the SOD activity was increased significantly at 6 h and maintained until 48 h, and the activity levels with Diets 2 and 3 were increased significantly at 12 h and then decreased, but they were lower than with Diet 1 (Fig. 3B); the CAT activity increased gradually until 48 h, and the activity levels with Diet 2 and 3 were increased significantly at 12 h and then decreased, but they were lower than with Diet 1 (Fig. 3D).


A
B
SOD activity (U/mL)
Diet 1
Diet 2
Diet 3
30 days
60 days
0
2
4
6
8
0
6
12
24
48
Time after exposure (h)
*
a
b
c
ab

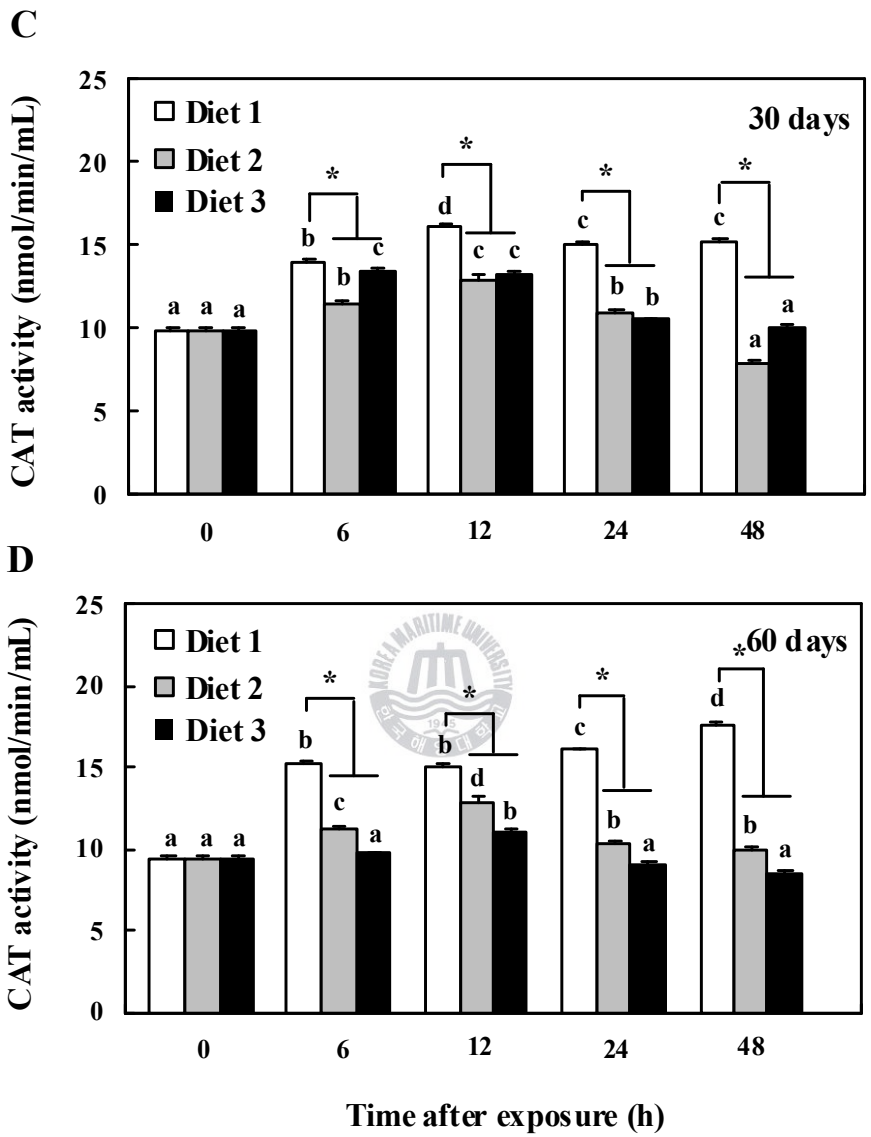

C
CAT activity (nmol/min/mL)
Diet 1
Diet 2
Diet 3
30 days
D
60 days
Time after exposure (h)

Fig. 3. Activity of SOD (A) and CAT (C) in olive flounder fed a diet containing quercetin for 30 days and SOD (B) and CAT (D) in olive flounder fed a diet containing quercetin for 60 days. These activities in livers of olive flounder exposed to Cd (0, 6, 12, 24, and 48 h) determined using a microplate reader. Values with different superscripts indicate significant differences from controls for the same diet, and asterisks indicate significant differences between Diet 1 and Diets 2 and 3 (P<0.05). All values are means ± SD (n=5).

3.4 H_2O_2 assay

After feeding for 30 days, with Diet 1, the plasma H_2O_2 concentration increased significantly at 6 h and then decreased, and H_2O_2 concentrations with Diet 2 and 3 were increased significantly at 12 h and then decreased to initial levels, but they were lower than with Diet 1. After feeding for 60 days, with Diet 1, the plasma H_2O_2 concentration increased significantly until 24 h and then decreased, and the H_2O_2 concentrations with Diet 2 and 3 were lower than with Diet 1 and not significantly different from the initial levels (Fig. 4).

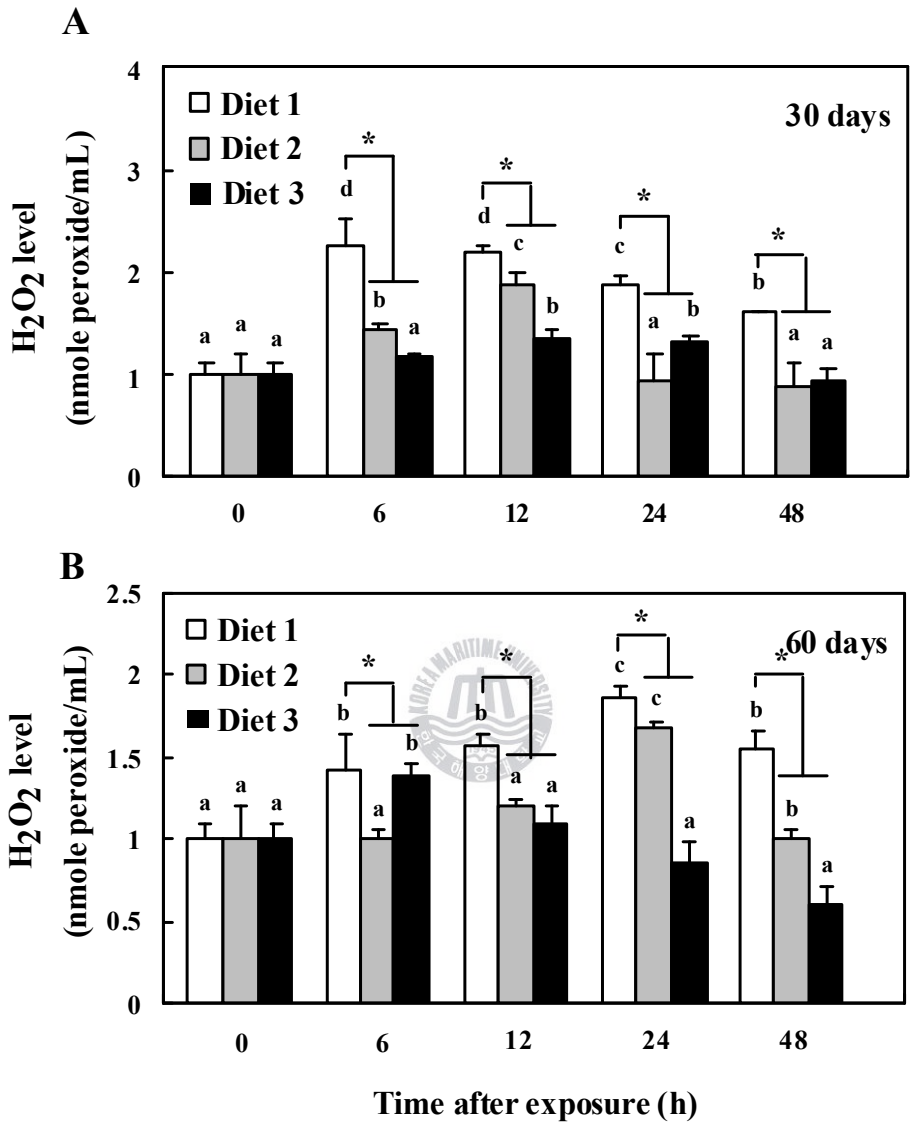

A
B
Diet 1
Diet 2
Diet 3
30 days
60 days
H2O2 level
(nmole peroxide/mL)
Time after exposure (h)
0
6
12
24
48

Fig. 4. H_2O_2 concentrations in plasma of olive flounder fed for 30 or 60 days diets containing quercetin 0.25 or 0.5%, after 6, 12, 24, and 48 h exposure to Cd. Values with different letters indicate significant differences from controls for the same diet, and asterisks indicate significant differences between Diet 1 and Diets 2 and 3 (P<0.05). All values are means ± SD (n=5).

3.5 LPO assay

After feeding for 30 days, with Diet 1, the LPO level increased significantly until 48 h, and the LPO levels with Diets 2 and 3 were not significantly different from the initial levels, but they were lower than with Diet 1. After feeding for 60 days, with Diet 1, the LPO level increased significantly at 24 h; with Diet 2, the LPO level increased significantly at 6 h and was maintained until 48 h; and with Diet 3, no significant difference was observed compared with the initial levels. The levels with Diets 2 and 3 were lower than with Diet 1 (Fig. 5).

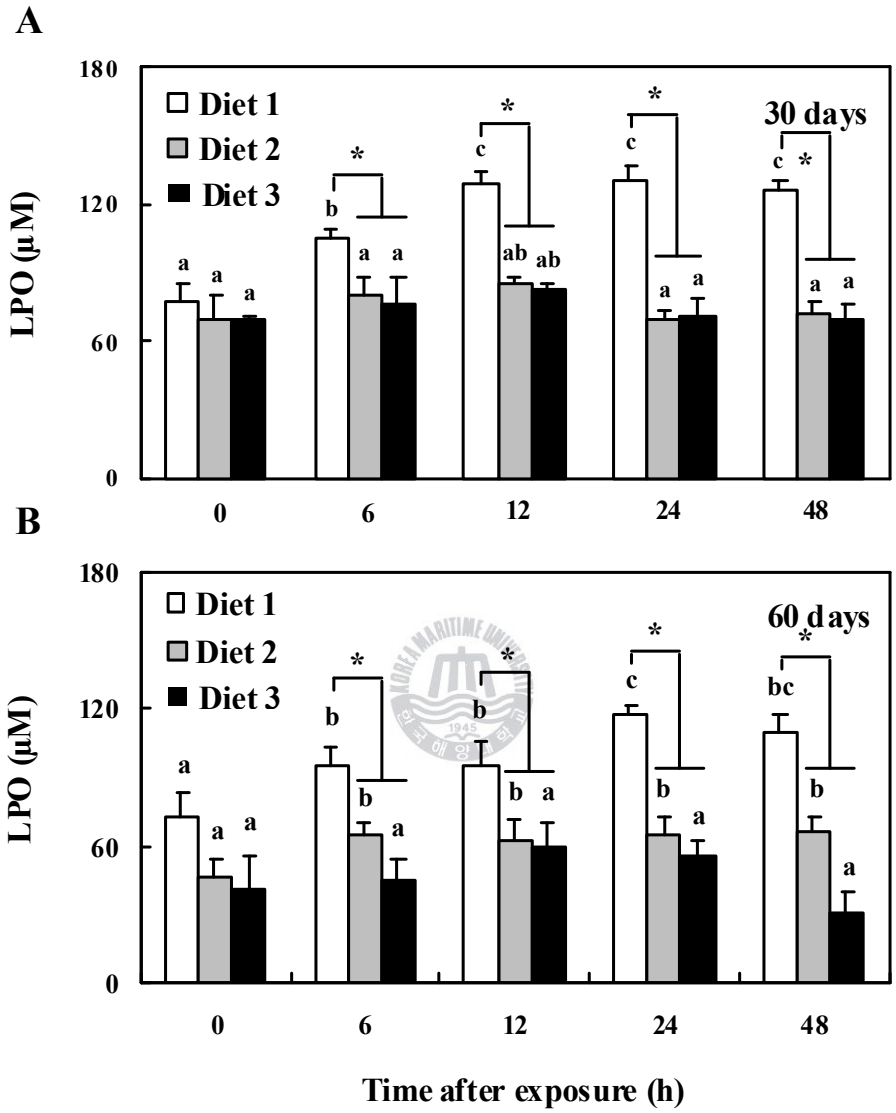

A
B
LPO (μM)
180
120
60
0
Diet 1
Diet 2
Diet 3
30 days
60 days
0
6
12
24
48
Time after exposure (h)

Fig. 5. Lipid peroxidation (LPO) in the livers of olive flounder fed for 30 or 60 days diets containing quercetin 0.25 or 0.5%, after 6, 12, 24, and 48 h exposure to Cd. Values with different letters indicate significant differences from controls for the same diet, and asterisks indicate significant differences between Diet 1 and Diets 2 and 3 (P<0.05). All values are means ± SD (n=5).

4. Discussion

To understand the antioxidant effect of quercetin on oxidative stress induced by Cd in olive flounder, we investigated the effects on lysozyme, which is related to immune activity and growth (Qinghui et al., 2004), T_3, an enhancing hormone related to the inhibition of stress (Ahmed et al., 2008), and the expression and activity of the antioxidant enzymes SOD and CAT.

We fed the fish diets containing 0% (Diet 1), 0.25% (Diet 2), or 0.5% (Diet 3) quercetin for 30 or 60 days and then measured the lysozyme activity as an immune index and T_3 concentration. The plasma lysozyme activity and T_3 concentration with Diets 2 and 3 were significantly higher than with Diet 1 after feeding for 30 or 60 days (Fig. 1). These results concur with those of Eo and Lee (2008), who reported that vitamin C improved the immune responses and disease resistance by increasing plasma lysozyme activity in Tiger puffer (*Takifugu rubripes*) fed vitamin C for 8 weeks, and with Zheng et al. (2009), who observed higher lysozyme activity in channel catfish (*Ictalurus punctatus*) fed oregano as an antioxidant. In addition, we observed higher T_3 concentrations in olive flounder fed quercetin, indicating that quercetin has a metabolic function such as enhancing growth or inhibiting stress.

Our study confirmed that quercetin had an immune effect and improved

physiological activity by increasing lysozyme activity and the T_3 concentration with Diets 2 and 3. Based on these results, we measured the expression and activity of antioxidant enzymes, the H_2O_2 concentration, and LPO levels as measures of oxidative stress in fish fed Diets 1, 2, and 3 that were exposed to Cd to examine whether quercetin reduces oxidative stress and has antioxidant effects.

Generally, environment contaminants induce oxidative stress by generating ROS in organisms. ROS tend to oxidize cell and organ membranes, causing cell damage (Ferraris et al., 2002). Many recent studies have examined the effects of antioxidants against oxidative stress induced by toxic materials. For example, Jayaraj et al. (2007) investigated the effects of pretreatment with three flavonoids (silybin, quercetin, and morin) on the effects of microcystins. Many studies of a variety antioxidants have been reported, including vitamin C (Qinghui et al., 2004; Weng et al., 2007), lycopene (Al-Jassabi, 2005), and tea polyphenols (Xu et al., 2007). In fish, although many studies have examined how supplementation with vitamin C (Trenzado et al., 2009), vitamin E (Prieto et al., 2008), selenium (Atencio et al., 2009), and glutamine (Chen et al., 2009) affect the antioxidant system, little is known about the antioxidant effects of supplemental quercetin. Accordingly, we examined the antioxidant effects of quercetin by measuring the expression and activity of the antioxidant enzymes SOD and CAT in response to oxidative stress induced by Cd in olive flounder fed quercetin.

The flavonoid quercetin is a natural antioxidant that can stop redox reactions by chelating ROS generated by toxic materials like Cd, and it inhibits LPO on the cell membrane. The phenoxy radical generated combines with another ROS and then inhibits the production of ROS (Frankle et al., 1993).

The expression of SOD and CAT mRNA with Diets 2 and 3 was significantly lower than with Diet 1 (Fig. 2). Similarly, the SOD and CAT activities were increased significantly in all groups exposed to Cd, but the activities with Diets 2 and 3 were significantly lower than with Diet 1 (Fig. 3). These results are similar to those of Jayaraj et al. (2007), who reported that the CAT activity in mice exposed to microcystin after feeding with quercetin was significantly lower than in controls, suggesting that quercetin, with its strong antioxidant activity, protects hepatic cells from toxic materials that induce oxidative stress. In addition, the activities of SOD and CAT in a freshwater fish, the matrinxã (*Brycon cephalus*), exposed to methyl parathion after feeding selenium were significantly lower than in controls, indicating that selenium is an antioxidant that scavenges the ROS induced by methyl parathion (Monteiro et al., 2009). Combined with the results of previous studies, the fact that the expression and activity of antioxidant enzymes in the fish fed Diets 2 and 3 were lower than in those fed Diet 1 indicates that quercetin has antioxidant activity by scavenging the ROS induced by Cd directly. In addition, the H_2O_2 concentrations with Diets 2 and 3 were significantly lower than with Diet 1 (Fig. 4), also indicating that quercetin

scavenges ROS overproduced in the olive flounder.

The oxidative stress caused by ROS generates LPO and damages cells (Valavanidis et al., 2006). In this study, the LPO levels with Diets 2 and 3 were significantly lower than with Diet 1. These results agree with Hiratsuka et al. (2008), who reported that LPO levels were reduced in mice fed docosahexaenoic acid (DHA).

In conclusion, quercetin increases lysozyme activity enhancing immune ability and T_3 concentrations to maintain hormone homeostasis in olive flounder. In addition, the mRNA expression and activity of the antioxidant enzymes SOD and CAT and the H_2O_2 concentrations in fish fed Diets 2 and 3 were significantly lower with Diet 1, indicating that quercetin scavenges the ROS induced by Cd to enhance antioxidant effects. Hence, we postulate that quercetin has beneficial immune effects and antioxidant ability. Additional studies should examine the effects of various antioxidants on environmental stress factors and oxidative stress.

III. Experiment 2

The effects of quercetin on physiological characteristics and oxidative stress resistance in the olive flounder, *Paralichthys olivaceus*

Abstract

We investigated the effect of quercetin on the plasma cholesterol level and the effects of quercetin pretreatment (Diet 1, 0%; Diet 2, 0.25%; and Diet 3, 0.5% quercetin) for 30 and 60 days on oxidative stress induced by hypoosmotic conditions (17.5, 8.75, and 4 psu) in the olive flounder. Total cholesterol levels were lower with Diets 2 and 3 than with Diet 1, leading us to hypothesize that quercetin removed low-density lipoproteins from circulation and thereby reduced total cholesterol. To understand the antioxidant role of quercetin, we measured the mRNA expression and activities of superoxide dismutase (SOD) and catalase (CAT) and the H_2O_2 concentration in quercetin-treated flounder exposed to osmotic stress The H_2O_2 concentration and the SOD and CAT expression and activity levels were lower in flounder fed with Diets 2 and 3 than with Diet 1, suggesting that quercetin directly scavenges reactive oxygen species to reduce oxidative stress. Furthermore, the plasma lysozyme activity and osmolality were higher with Diets 2 and 3 than with Diet 1, indicating that quercetin increases immune function and helps to maintain physiological homeostasis. Plasma cortisol was lower with Diets 2 and 3 than with Diet 1, suggesting the quercetin protects against stress. These

results indicate that quercetin has hypocholesterolemic and antioxidant effects, and increases immune function, and acts to maintain physiological homeostasis.
Keywords: Quercetin, Antioxidant, ROS, Salinity, Cholesterol, Lysozyme

1. Introduction

Environmental salinity is an important factor affecting metabolism, growth, survival, osmolality, and immune function in fish (Britoa et al., 2000). Specifically, changes in salinity can generate physiological stress via changes in hormones, energy metabolism, and ion status in marine organisms (Barton and Iwama, 1991). Furthermore, stress induced by salinity changes has been associated with enhanced reactive oxygen species (ROS) generation, which may seriously affect immune function and lead to oxidative stress (Fisher and Newell, 1986). Sea cucumbers (*Apostichopus japonicus*) exposed to hypoosmotic conditions showed decreased lysozyme activity, suggesting that stress induced by salinity changes can suppress immunity (Wang et al., 2008). ROS, including superoxide, hydrogen peroxide (H_2O_2), hydroxyl radicals, and singlet oxygen, are produced naturally during oxidative metabolism (Roch, 1999). Overproduction of ROS in response to environmental stress can lead to increased lipid peroxidation and may affect cell viability by causing membrane damage and enzyme inactivity. Subsequently, cell senescence and apoptosis, and the oxidation of nucleic acids and proteins may be accelerated. The resultant DNA damage may provoke a variety of physiological disorders such as accelerated aging and reduced disease resistance and reproductive ability (Kim and Phyllis, 1998; Pandey et al., 2003).

Complex antioxidant defense systems maintain homeostasis and protect aerobic organisms against ROS and the subsequent damage of oxidative stress. Antioxidants may be enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, and glutathione S-transferase, or compounds such as metallothionein, quercetin (Jayaraj et al., 2007), vitamin C, and vitamin E (α-tocopherol) (McFarland et al., 1999). Antioxidant defense systems are found in the livers and kidneys of marine organisms (Basha and Rani, 2003; Hansen et al., 2006).

This study investigated the effects of the natural antioxidant quercetin, which is almost ubiquitous in plants. Quercetin is a polyphenolic flavonoid compound with strong antioxidant capabilities. Broccoli and apples contain 7 to 110 mg/kg of quercetin, and onions contain 284 to 486 mg/kg (Scalbert and Williamson, 2000). Quercetin chelates ROS induced by lipid peroxidation and metal ions, provides H^+ ions to prevent lipid peroxidation in the cell membrane, and scavenges free radicals. Furthermore, quercetin converts ROS to energy and reduces metal concentrations to protect cell membranes (Bors and Saran, 1987). Previous studies have investigated antioxidant systems via xenobiotic exposure after natural antioxidant materials have been supplied (Pinho et al., 2005; Weng et al., 2007; Prieto et al., 2008; Chen et al., 2009). However, studies on environmental stress (e.g., changes in salinity) and antioxidant systems in fish have not been performed.

Here, we used olive flounder fed a diet containing quercetin for 30 or 60 days as an experimental model for studying the effects of quercetin on the plasma cholesterol level and on oxidative stress induced by hypoosmotic conditions. We measured the plasma total cholesterol concentration to determine the ability of quercetin to reduce plasma cholesterol, and the mRNA expression and activities of the antioxidant enzymes SOD and CAT, the plasma H_2O_2 concentration, lysozyme activity, plasma cortisol concentration, and plasma osmolality to elucidate the effects of quercetin on the oxidative stress induced by changes in salinity.

2. Materials and methods

2.1. Experimental fish and conditions

Olive flounder (n = 800; length, 10 ± 0.5 cm; weight, 19.9 ± 1.3 g) were obtained from a commercial fish farm and were allowed to acclimate to the experimental conditions for 2 weeks in nine 300-L circulation filter tanks in the laboratory. During the experiments, the water temperature and photoperiod were maintained at 20 ± 1 °C and 12-h light:12-h dark, respectively. The fish were fed a commercial feed twice daily (09:00 and 17:00). In all, 800 flounders were randomly chosen, distributed among the nine 300-L flow-through tanks, and divided into three experimental groups (Diets 1, 2, and 3).

2.2. Experimental diets

In the three experimental diets, fish meal, dehulled soybean, and corn gluten meal were used as protein sources, and wheat flour and fish oil-salmon were used as carbohydrate and lipid sources, respectively. The diet ingredients (Table 1) were mixed well with water, at a 3:1 ratio of ingredients to water, and then pelletized, dried at room temperature, and stored at −20°C until needed. In the experimental diets, the crude protein content ranged from 55.1 to 56.0%, the crude lipid content was 9.1 to 9.7%, and the estimated energy content was 4.1 kcal/g. The experimental

diets also contained 0% (Diet 1), 0.25% (Diet 2), or 0.5% (Diet 3) quercetin at the expense of 0, 0.25, or 0.5% wheat flour, respectively (Table 1). The quercetin used in this study was extracted from discarded onion peels on a chromatographic column (Dongsun Industrial, Gyunggi-do, Korea); the product was sterilized, and its quality was assured.

2.3. Plasma total cholesterol analysis

Cholesterol ester in plasma was hydrolysised to cholesterol and fatty acid by Cholesterol Esterase (CE), and Cholesterol was oxidized to generate hydrogen peroxide by Cholesterol Oxidase (CHO). Hydrogen peroxide provoked a condensation reaction by peroxidase (POD) under 4-AAP and phenol, and then we measured cholesterol amount through measuring absorbance of chelate dye which was formated by condensation reaction (HBi, Korea).

2.4. Osmotic stress produced by changes of salinity

The experimental fish were kept in seawater (35 psu) for 24 h and then transferred sequentially to tanks with salinities of 17.5, 8.75, and 4 psu, which were produced by adding underground water. The fish were maintained at each salinity for 24 h. Four fish from each group (Diets 1, 2, and 3) were randomly selected for blood and tissue sampling. The fish were anesthetized with 200 mg/L tricaine methanesulfonate (MS-222, Sigma, St.

Louis, MO, USA) prior to blood collection. Blood was collected from the caudal vasculature using a 3-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4 °C; 10,000 rpm; 5 min) and stored at −80 °C until analysis. To collect liver tissue samples, the fish were euthanized by spinal transection. Immediately after collection, the samples were frozen in liquid nitrogen and stored at −80 °C until total RNA was extracted for analysis.

2.5. Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression of SOD and CAT mRNA in total RNA extracted from the liver. Primers for QPCR were designed with reference to the known sequences of olive flounder as follows (accession no. EF681883 (SOD); GQ229479 (CAT); EU090804 (β-actin)): SOD forward primer (5'-CGT TGG AGA CCT GGG GAA TGT G-3'), SOD reverse primer (5'-ATC GTC AGC CTT CTC GTG GAT C-3'), β-actin forward primer (5'-AAA TGG GAA CCG CTG CCT C-3'), β-actin reverse primer (5'-TTC CTT CTG CAT ACG GTC AG-3'), CAT forward primer (5'-GGC TGA GAA GTT CCA GTT CAA TCC-3') and CAT reverse primer (5'-CTC CAC CTC TGC AAA GTA GTT GAC-3'). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, USA) and iQ™ SYBR Green Supermix (Bio-Rad, USA) according to the manufacturer's instructions.

QPCR was performed as follows: denaturation at 95 ºC for 5 min; 40 cycles of denaturation at 95 ºC for 20 s, annealing at 55 ºC for 20 s. As an internal control, experiments were duplicated with β-actin, and all data were expressed as change with respect to corresponding β-actin calculated threshold cycle (CT) levels.

2.6. SOD and CAT activity analysis

The tissues were homogenized in ice-cold 0.1M phosphate-buffered saline (PBS, pH 7.4) containing 1 mM EDTA. The homogenates were centrifuged at 10,000 rpm for 15 min at 4 ºC, and removed supernants and then remaining sample for analyses. SOD and CAT activities were determined using commercial kits supplied by Cayman Chemical (USA).

SOD activity is assessed by using a tetrazolium salt for detection of superoxide radicals generated by xanthine oxydase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Absorbance read at 450 nm. Each assay was performed in duplicate, and enzyme units were recorded as U/mg protein.

For CAT activity, the method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen

(Wheeler et al., 1990). Purpald specifically forms a bicyclic hetero cycle with aldehydes, which upon oxidation changes from colorless to a purple color. Absorbance read at 540 nm. Each assay was performed in duplicate, and CAT activity was expressed in nmol of formaldehyde/min/mg protein.

2.7. H_2O_2 assay

H_2O_2 concentrations measured using the modified methods of Nouroozzadeh et al. (1994) and a Peroxidetect kit (Sigma, USA). 20 μL of olive flounder serum was added per well to flat bottom 96-well microtitre plates. Plates were left at room temperature for 20 min to allow serum to settle and adhere. A working color reagent was prepared by mixing 100 ml distilled water containing 100 mM sorbitol and 125 μM xylenol orange (Sigma, USA) with 1 mL of 25 mM ferrous ammonium sulphate prepared in 2.5 M sulphuric acid (Sigma-Aldrich). 200 μL of this reagent was then added to each well and allowed to incubate at room temperature for 1 h. Absorbance was read at 560 nm and concentration of H_2O_2 were interpolated from a standard curve. Concentrations are expressed as nM/ml.

2.8. Plasma lysozyme activity analysis

To determine the lysozyme activity of olive flounder, plasma (50 μL) was added to 950 μL of a suspension of *Micrococcus lysodeikticus* (0.2 mg/mL) in a 0.05 M sodium phosphate buffer (pH 6.2) .The reactions were carried

out at 25 ℃ and absorbance at 530 nm was measured between 0.5 and 4.5 min by a spectrophotometer. A lysozyme activity unit was defined as the amount of enzyme producing a 0.001/min decrease in absorbance.

2.9. Plasma osmolality and cortisol analysis

Plasma osmolality was measured with a vapor pressure osmometer (Vapro 5520; Wescor Inc., Logan, UT, USA) and plasma cortisol was analyzed using a radioimmunoassay kit (Diagnostic Systems Laboratories, Atlanta, GA, USA).

2.10. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One way ANOVA followed by post hoc Duncan's multiple range test was used to compare the differences in the data ($P<0.05$).

3. Results

3.1. Plasma total cholesterol

Total cholesterol levels in flounder fed Diets 2 and 3, which contained quercetin, for 30 and 60 days were significantly lower than those in flounder fed Diet 1, which did not contain quercetin. In addition, total cholesterol levels in flounder fed Diets 2 and 3 were significantly lower after 60 days compared with 30 days (Fig. 6).

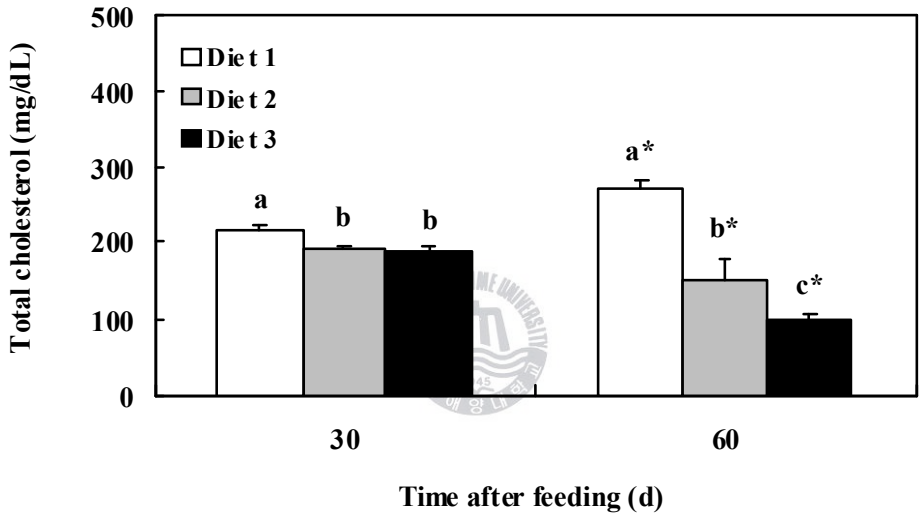
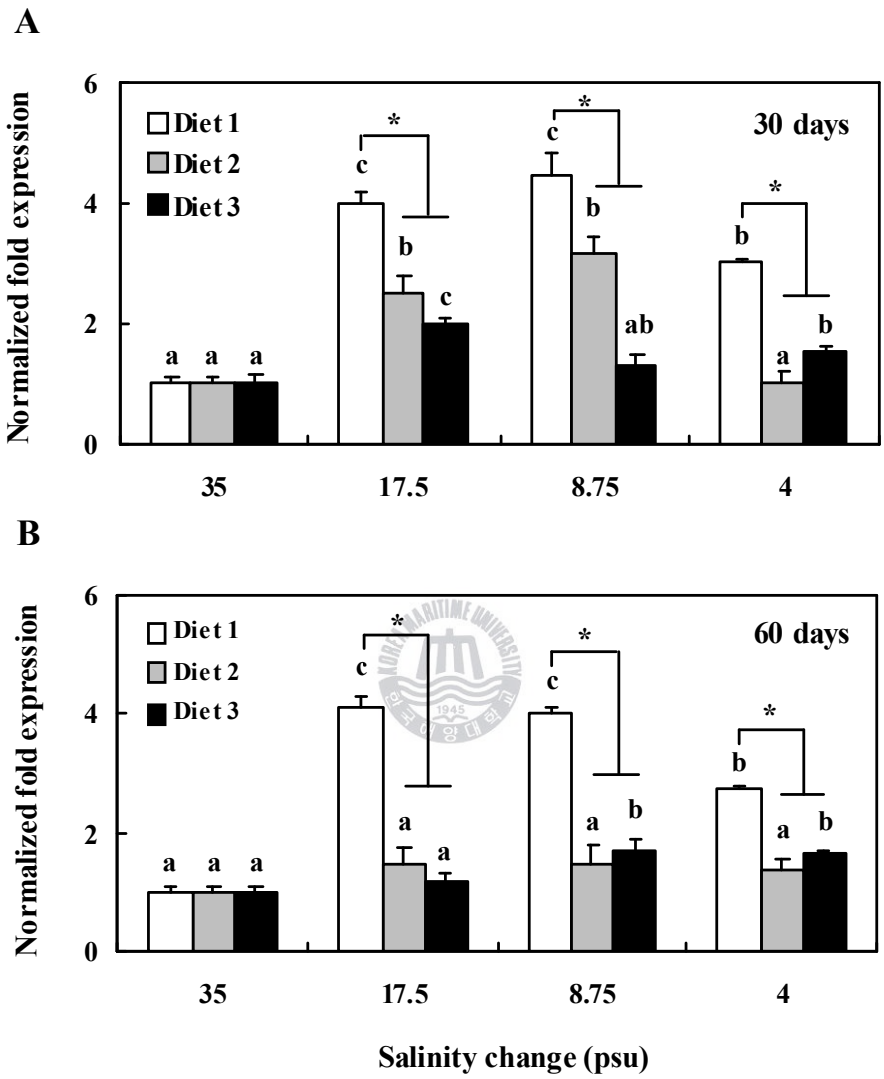

Total cholesterol (mg/dL)
500
400
300
200
100
0
Diet 1
Diet 2
Diet 3
a
b
b
a*
b*
c*
30
60
Time after feeding (d)

Fig. 6. Total cholesterol in plasma of olive flounder fed during 30 and 60 days with diet containing quercetin, 0, 0.25 and 0.5%. Values with different letters indicate significantly different from Diet 1, and asterisk indicates significantly different between 30 days and 60 days in the same concentration of diet (P<0.05). All values are means ± SD (n=5).

3.2. SOD and CAT mRNA expression

Using a quantitative polymerase chain reaction assay, we examined changes in SOD and CAT mRNA expression in flounder that were fed the experimental diets and then exposed to osmotic stress. After 30 days of feeding with Diet 1, SOD mRNA expression was significantly increased in the fish at 17.5 and 8.75 psu, followed by a decrease at 4 psu. The SOD mRNA level was lower with Diets 2 and 3 than with Diet 1 (Fig. 7A). CAT mRNA expression increased gradually with changes in osmotic pressure, and similar to SOD mRNA expression, the CAT mRNA level was lower with Diets 2 and 3 than with Diet 1 (Fig. 7C). After 60 days of feeding with Diet 1, the SOD mRNA expression level was significantly increased at 17.5 psu, followed by decreases at 8.75 and 4 psu, and the SOD mRNA level was lower with Diets 2 and 3 than with Diet 1 (Fig. 7B). CAT mRNA expression was increased at 8.75 psu and then decreased at 4 psu with Diet 1. CAT mRNA levels were lower with Diets 2 and 3 than with Diet 1 (Fig. 7D).


A
Normalized fold expression
Diet 1
Diet 2
Diet 3
30 days
35
17.5
8.75
4
B
Normalized fold expression
Diet 1
Diet 2
Diet 3
60 days
35
17.5
8.75
4
Salinity change (psu)

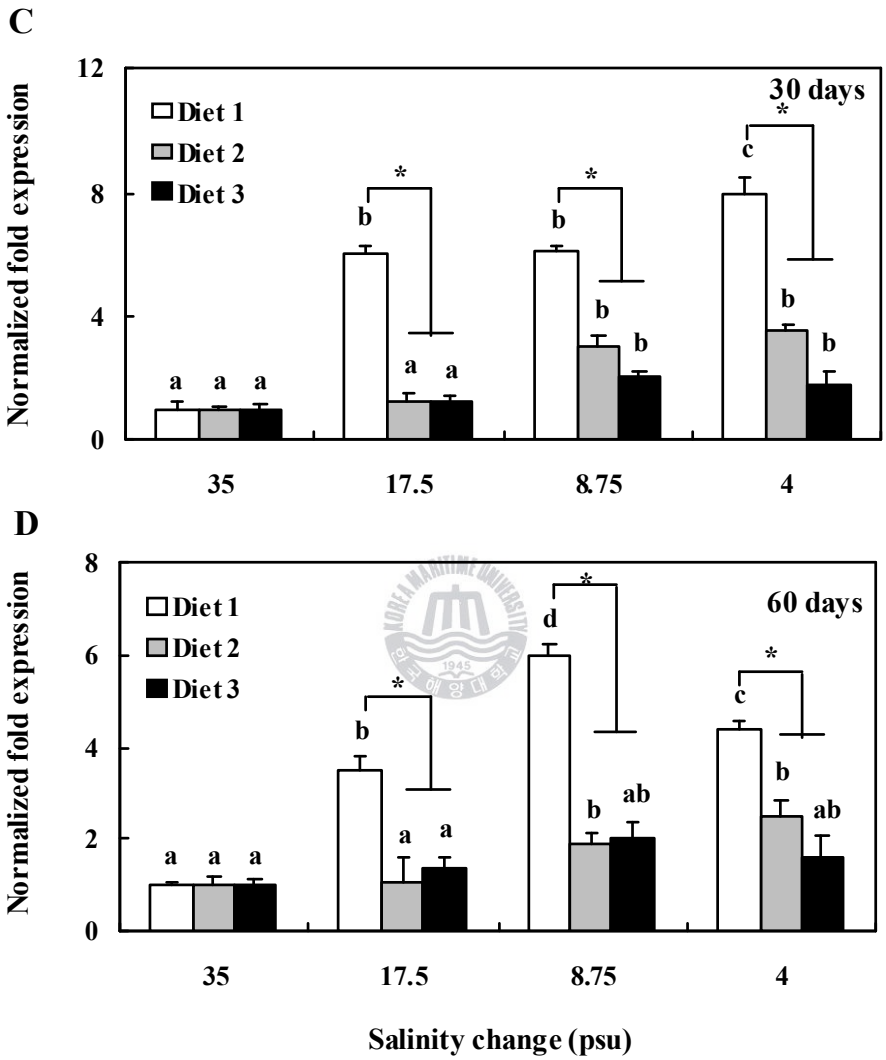
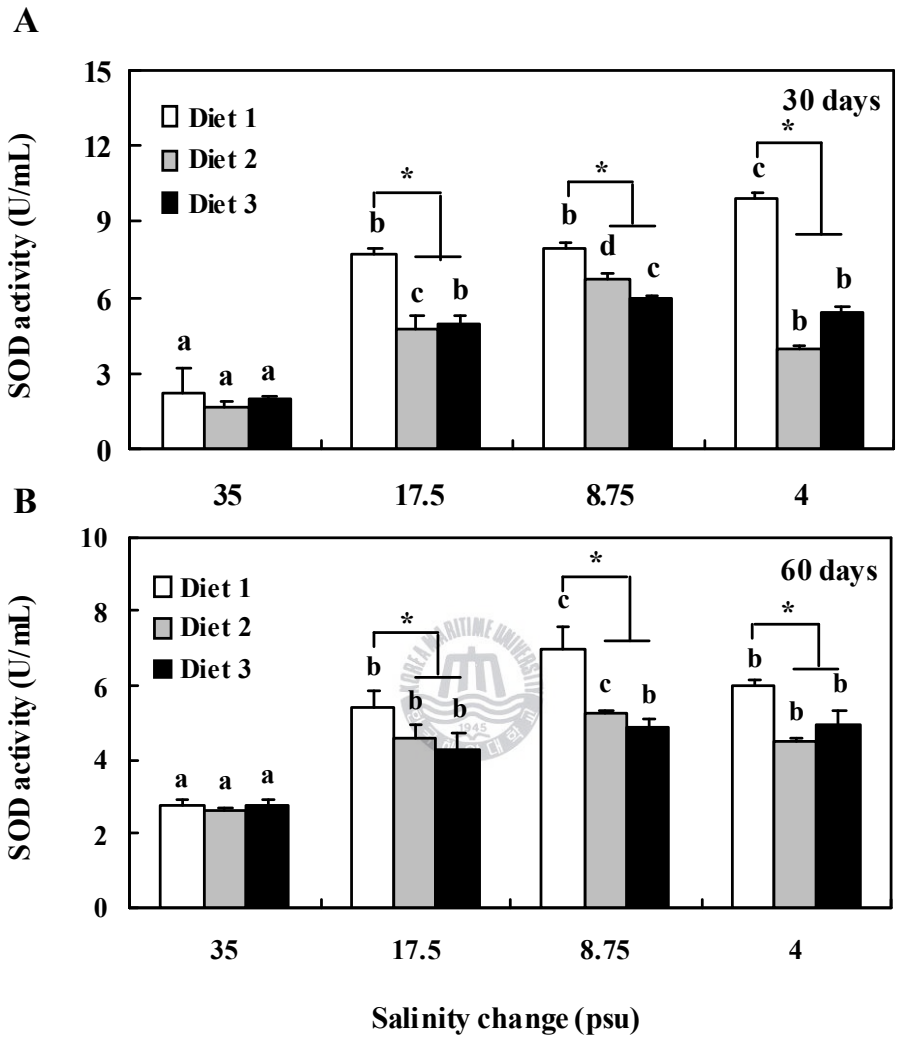

C
Normalized fold expression
12
8
4
0
Diet 1
Diet 2
Diet 3
30 days
a a a
b a a
b b b
c b b
35
17.5
8.75
4
D
Normalized fold expression
8
6
4
2
0
Diet 1
Diet 2
Diet 3
60 days
a a a
b a a
d b ab
c b ab
35
17.5
8.75
4
Salinity change (psu)

Fig. 7. Expression of SOD (A), CAT (C) in olive flounder fed diet contained quercetin during 30 days and SOD (B), CAT (D) in olive flounder fed diet contained quercetin during 60 days. These mRNA in liver of olive flounder under osmotic stress (17.5, 8.75 and 4 psu) by quantitative real-time PCR. 2.5 μg of total RNA prepared from the liver was reverse-transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expressions with respect to β-actin levels for the same sample. Values with different superscripts indicate significantly different from control in the same concentration of diet and asterisk indicates significantly different between Diet 1 and Diet 2, 3 (P<0.05). All values are means ± SD (n=5).

3.3. SOD and CAT activities

To further examine the effect of quercetin on antioxidant activity in the flounder, the osmotic stress-induced activities of the antioxidant enzymes SOD and CAT were measured in flounder fed the three experimental diets (Fig. 8). After 30 days of feeding with Diet 1, SOD activity was significantly increased with decreases in osmotic pressure until 4 psu. SOD activities with Diets 2 and 3 were significantly increased, but remained lower than those with Diet 1 (Fig. 8A). With Diets 2 and 3, CAT activity levels were increased at 17.5 psu and maintained until 4 psu, but all levels were lower than those with Diet 1 (Fig. 8C). After 60 days of feeding with Diet 1, SOD activity was significantly increased at 17.5 and 8.75 psu, followed by a decrease at 4 psu. SOD activities with Diets 2 and 3 were significantly increased, but remained lower than those with Diet 1 (Fig. 8B). CAT activity was increased at 17.5 and 8.75 psu, after which it decreased at 4 psu with Diets 2 and 3, all levels were lower than those with Diet 1 (Fig. 8D).


A
SOD activity (U/mL)
15
12
9
6
3
0
Diet 1
Diet 2
Diet 3
30 days
a a a
b c b
b d c
c b b
*
*
*
B
SOD activity (U/mL)
10
8
6
4
2
0
Diet 1
Diet 2
Diet 3
60 days
a a a
b b b
c c b
b b b
*
*
*
35
17.5
8.75
4
Salinity change (psu)

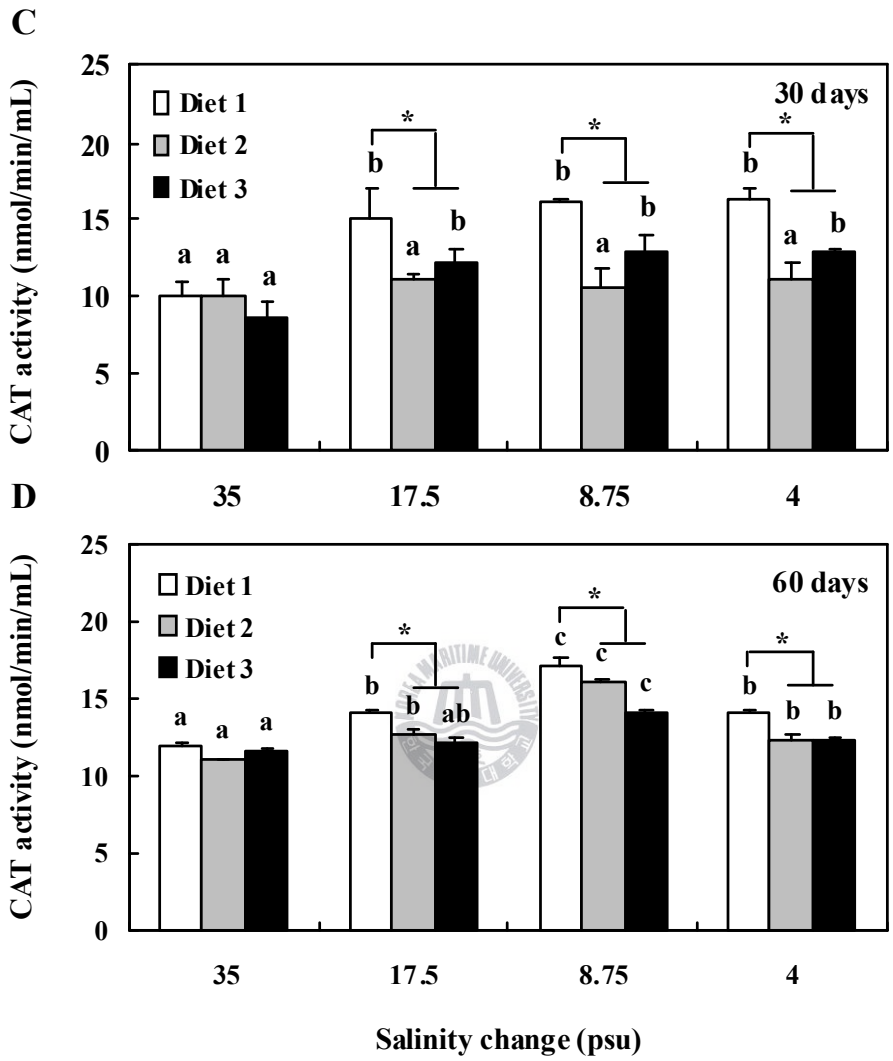

C
CAT activity (nmol/min/mL)
Diet 1
Diet 2
Diet 3
30 days
D
60 days
35
17.5
8.75
4
Salinity change (psu)

Fig. 8. Activity of SOD (A), CAT (C) in olive flounder fed diet contained quercetin during 30 days and SOD (B), CAT (D) in olive flounder fed diet contained quercetin during 60 days. These acitivities in liver of olive flounder under osmotic stress (17.5, 8.75 and 4 psu) by microplate reader. Values with different superscripts indicate significantly different from control in the same concentration of diet and asterisk indicates significantly different between Diet 1 and Diet 2, 3 (P<0.05). All values are means ± SD (n=5).

3.4. H_2O_2 concentration

To explore the ability of quercetin to scavenge ROS, the plasma H_2O_2 concentration was determined in osmotically stressed flounder. After both 30 and 60 days, the plasma H_2O_2 concentration in the flounder was significantly increased at 17.5 and 8.75 psu with Diet 1, followed by a decrease at 4 psu. Although significantly increased at 17.5 and 8.75 psu, the H_2O_2 concentrations with Diets 2 and 3 were lower than those with Diet 1 after both 30 and 60 days (Fig. 9).

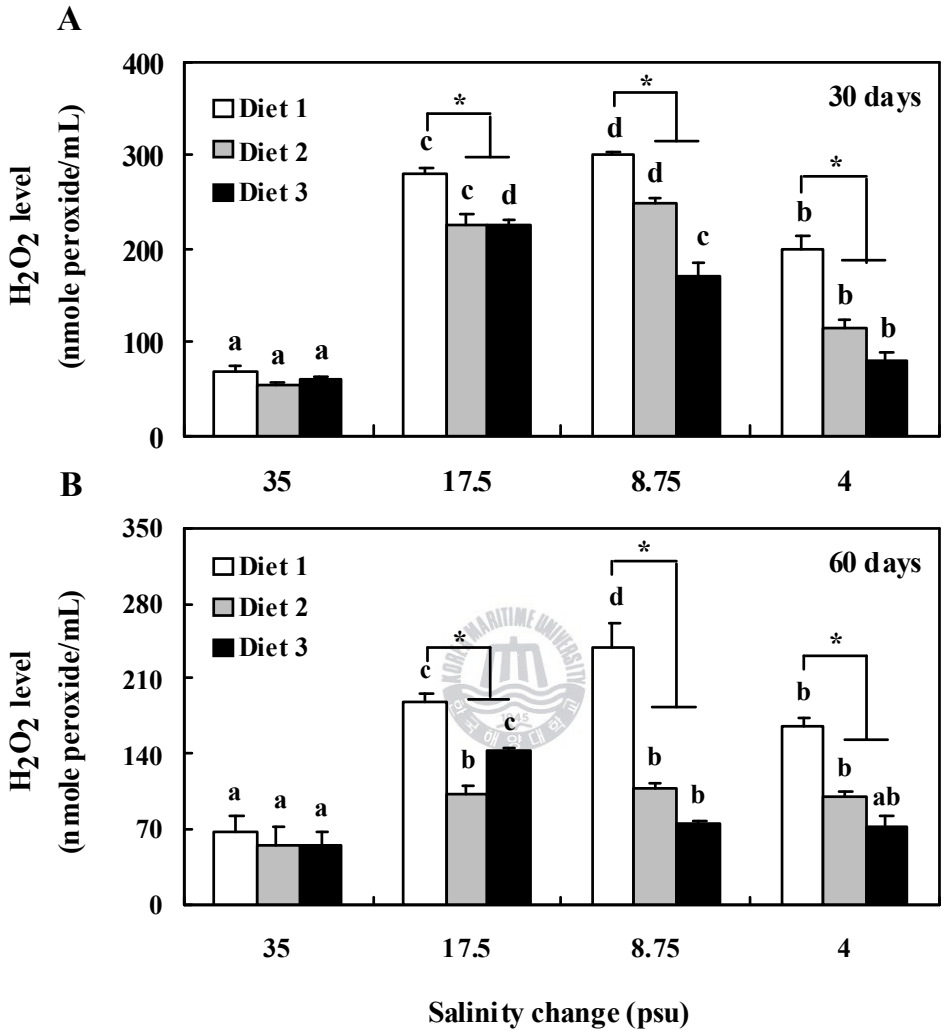

A
400
300
200
100
0
H2O2 level
(nmole peroxide/mL)
Diet 1
Diet 2
Diet 3
30 days
a a a
c c d
d d c
b b b
*
*
*
B
350
280
210
140
70
0
60 days
a a a
c b c
d b b
b b ab
35
17.5
8.75
4
Salinity change (psu)

Fig. 9. H_2O_2 concentrations in plasma of olive flounder fed during 30 (A) and 60 days (B) with diet containing quercetin, 0.25 and 0.5%, after under osmotic stress (17.5, 8.75 and 4 psu). Values with different letters indicate significantly different from control in the same concentration of diet and asterisk indicates significantly different between Diet 1 and Diet 2, 3 (P<0.05). All values are means ± SD (n=5).

3.5. Plasma lysozyme activity

As an indicator of immune function, the plasma lysozyme activity was measured. After both 30 and 60 days of feeding with Diet 1, the plasma lysozyme activity in the flounder was significantly decreased with decreases in salinity. Plasma lysozyme activities were also significantly decreased with Diets 2 and 3 after both 30 and 60 days, but always remained higher than those with Diet 1 (Fig. 10).

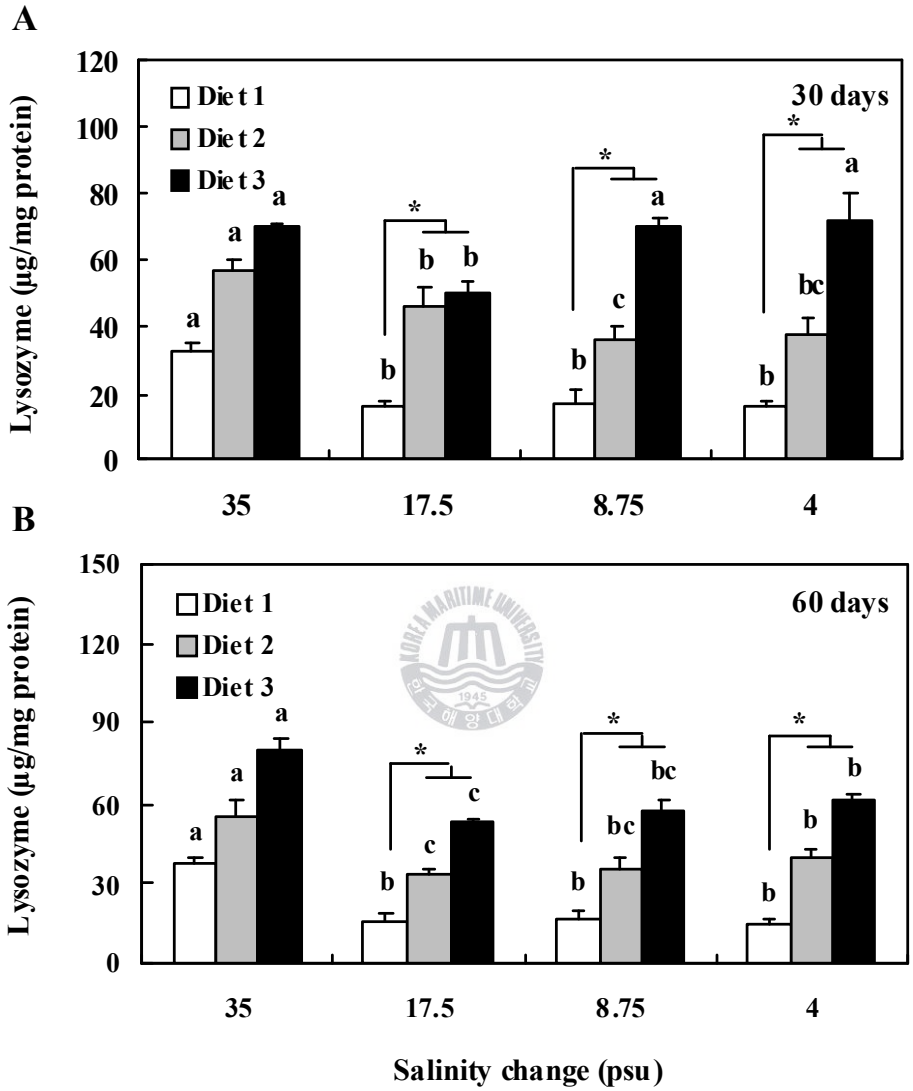

A
Lysozyme (μg/mg protein)
120
100
80
60
40
20
0
Diet 1
Diet 2
Diet 3
30 days
a
a
a
b
b
b
b
c
a
b
bc
a
*
*
*
35
17.5
8.75
4
B
Lysozyme (μg/mg protein)
150
120
90
60
30
0
Diet 1
Diet 2
Diet 3
60 days
a
a
a
b
c
c
b
bc
bc
b
b
b
*
*
*
35
17.5
8.75
4
Salinity change (psu)

Fig. 10. Lysozyme in the plasma of olive flounder fed during 30 (A) and 60 days (B) with diet containing quercetin, 0.25 and 0.5%, after under osmotic stress (17.5, 8.75 and 4 psu). Values with different letters indicate significantly different from control in the same concentration of diet and asterisk indicates significantly different between Diet 1 and Diet 2, 3 (P<0.05). All values are means ± SD (n=5).

3.6. Plasma cortisol

As a measure of oxidative stress, the plasma cortisol levels were determined in the flounder. After both 30 and 60 days of feeding with Diet 1, the plasma cortisol concentration was increased significantly in the flounder at 17.5 psu and decreased with further decreases in salinity. Plasma cortisol concentrations with Diets 2 and 3, although significantly increased at 17.5 psu, were lower than those with Diet 1 after both 30 and 60 days (Fig. 11).

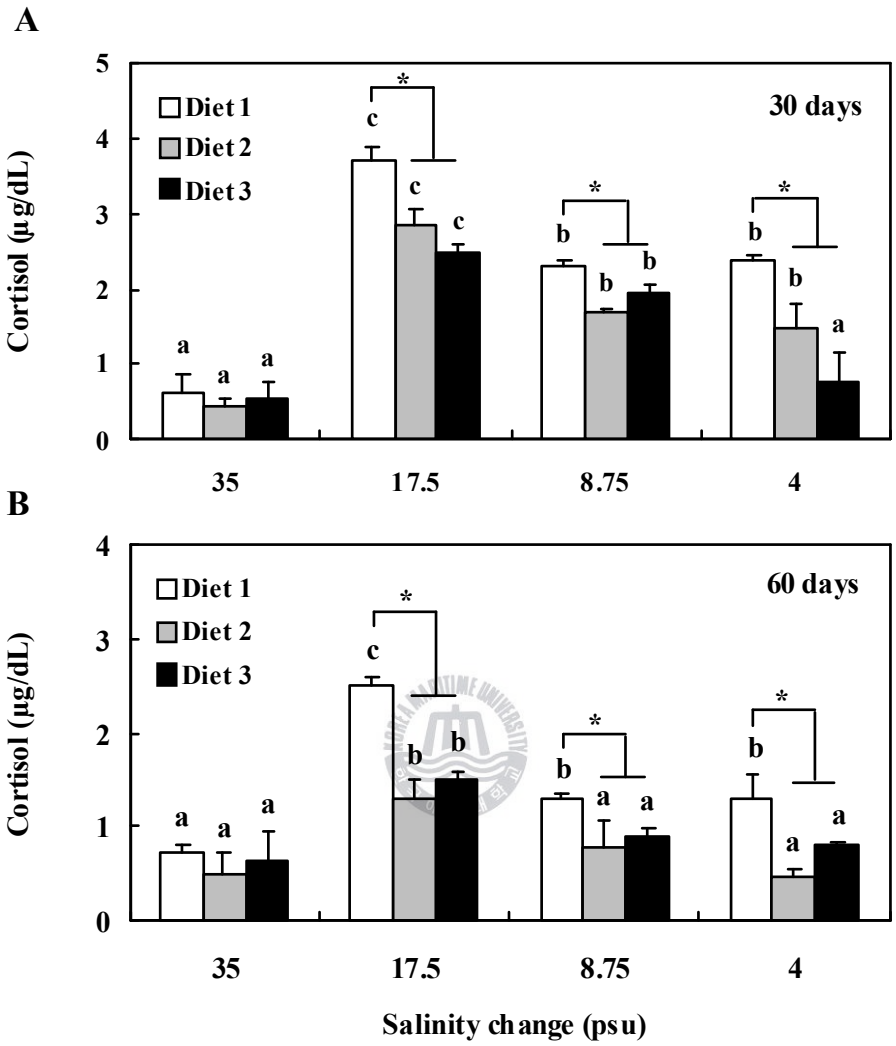

A
Cortisol (μg/dL)
Diet 1
Diet 2
Diet 3
30 days
35
17.5
8.75
4
B
60 days
Salinity change (psu)

Fig. 11. Cortisol in the plasma of olive flounder fed during 30 (A) and 60 days (B) with diet containing quercetin, 0.25 and 0.5%, after under osmotic stress (17.5, 8.75 and 4 psu). Values with different letters indicate significantly different from control in the same concentration of diet and asterisk indicates significantly different between Diet 1 and Diet 2, 3 (P<0.05). All values are means ± SD (n=5).

3.7. Plasma osmolality

After both 30 and 60 days of feeding with Diet 1, the plasma osmolality level in the flounder significantly decreased with decreases in environmental osmotic pressure. Plasma osmolality levels with Diets 2 and 3 were significantly decreased, but were higher than those with Diet 1 (Fig. 12).

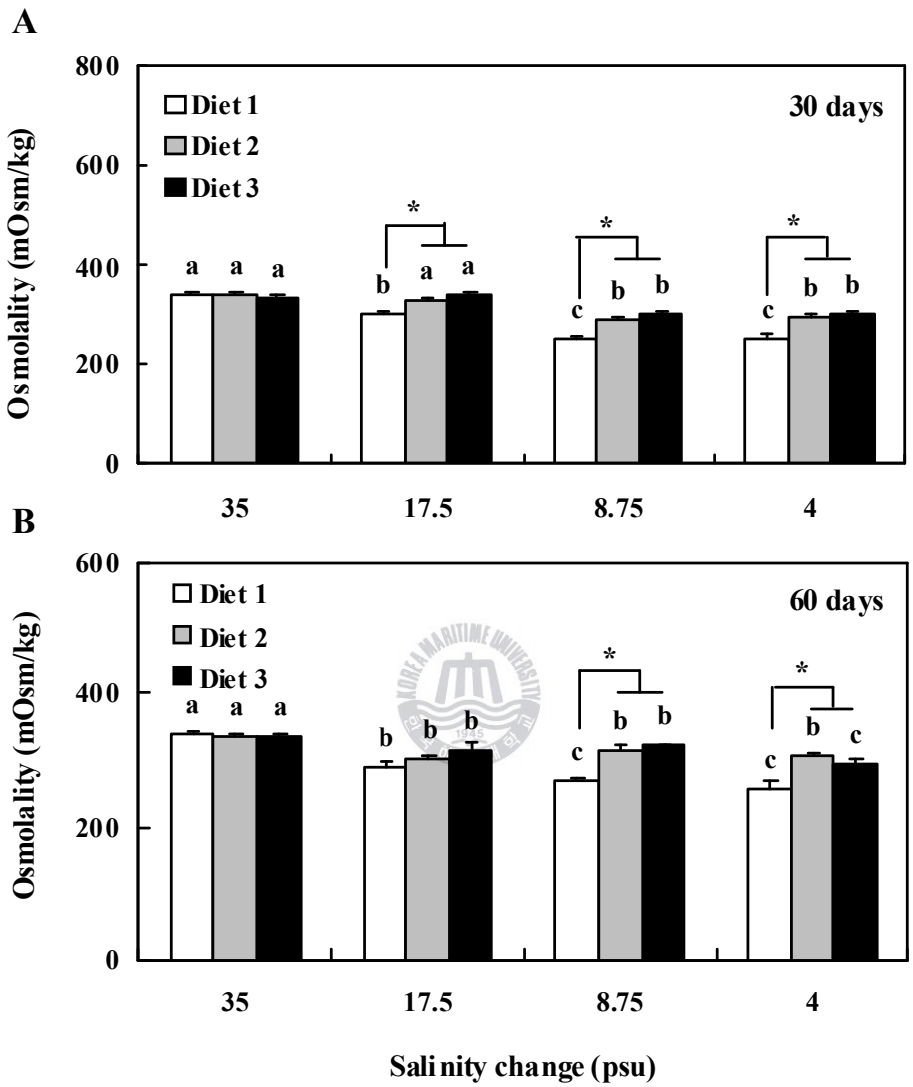

A
800
600
400
200
0
Osmolality (mOsm/kg)
Diet 1
Diet 2
Diet 3
30 days
a a a
b a a
c b b
c b b
*
*
*
35
17.5
8.75
4
B
600
400
200
0
Osmolality (mOsm/kg)
Diet 1
Diet 2
Diet 3
60 days
a a a
b b b
c b b
c b c
*
*
35
17.5
8.75
4
Salinity change (psu)

Fig. 12. Osmolality in the plasma of olive flounder fed during 30 (A) and 60 days (B) with diet containing quercetin, 0.25 and 0.5%, after under osmotic stress (17.5, 8.75 and 4 psu). Values with different letters indicate significantly different from control in the same concentration of diet and asterisk indicates significantly different between Diet 1 and Diet 2, 3 (P<0.05). All values are means ± SD (n=5).

4. Discussion

The effects of quercetin on the plasma cholesterol level and on oxidative stress induced by hypoosmotic conditions were investigated in the olive flounder. Our results demonstrated lower cholesterol levels, increased SOD and CAT mRNA levels and activities, lower H_2O_2 concentrations, higher lysozyme activity, lower cortisol concentrations, and decreased plasma osmolality in olive flounder fed diets containing quercetin compared with flounder fed a diet containing no quercetin.

After 30 and 60 days, the plasma cholesterol concentrations in the flounder fed Diets 2 and 3, which contained quercetin, were significantly lower than those in flounder fed Diet 1, which did not contain quercetin (Fig. 6). This result is consistent with data from Hayek et al. (1997), who reported that quercetin bound to low-density lipoprotein to suppress its oxidation and lower plasma cholesterol concentrations in mice fed quercetin for 6 weeks. Fuhrman et al. (2000) observed lower plasma cholesterol concentrations in mice fed the antioxidant ginger for 10 weeks. Taken together, these results demonstrate a hypocholesterolemic effect of quercetin.

To examine whether quercetin also has antioxidant and oxidative stress-reducing effects in olive flounder, the flounder were fed diets with (Diets 2 and 3) and without (Diet 1) quercetin for 30 and 60 days, and were then exposed to decreasing environmental osmotic pressures. We

measured the expression and activity of the antioxidant enzymes SOD and CAT, the plasma H_2O_2 concentration, plasma lysozyme activity, plasma cortisol level, and plasma osmolality at each salinity level.

Although the SOD and CAT mRNA expression levels and activities were increased significantly in all diet groups under hypoosmotic conditions, the expression levels (Fig. 7) and activities (Fig. 8) of both enzymes were significantly lower in the fish fed Diets 2 and 3 compared with Diet 1. These results indicate that quercetin possesses antioxidant activity and scavenges the ROS induced by changes in salinity. The H_2O_2 concentrations were also significantly lower with Diets 2 and 3 than with Diet 1 (Fig. 9), further indicating that quercetin scavenges the ROS produced during osmotic stress in the olive flounder. Chien et al. (2003) reported that SOD activity in tiger prawn (*Penaeus monodon*) exposed to hypoosmotic conditions after treatment (feeding) with astaxanthin was significantly lower than the control activity, suggesting that astaxanthin suppresses singlet oxygen activity. Additionally, CAT activity in mice exposed to microcystin after treatment with quercetin was significantly lower than control activity (Jayaraj et al., 2007). Collectively, these results show that strong antioxidants such as quercetin reduce oxidative stress induced by salinity changes and toxic materials.

Acute salinity changes are thought to suppress immunity (Britoa et al., 2000). Wang et al. (2008) reported that lysozyme activity was significantly

decreased in sea cucumbers (*Apostichopus japonicus*) upon exposure to hypoosmotic conditions, indicating an osmotic stress-induced reduction of immune function. In the present study, the plasma lysozyme activity in olive flounder exposed to hypoosmotic conditions was significantly higher in the flounder fed Diets 2 and 3 compared with Diet 1 (Fig. 10). Flavonoids such as quercetin have an excellent probability of inhibiting viruses and improving immune function (Brinkworth et al., 1992), but few studies have reported changes in immune function and lysozyme activity after antioxidant treatment. In one study, the plasma lysozyme activity was increased significantly in tiger puffer (*Takifugu rubripes*) after vitamin C treatment for 8 weeks, suggesting that vitamin C enhanced the immune ability (Eo and Lee, 2008). Zheng et al. (2009) observed that treatment with the antioxidant oregano significantly increased the plasma lysozyme activity in channel catfish. Consistent with these results, quercetin enhanced the immune ability in olive flounder in the present study. As acute salinity changes cause physiological stress in fish, we measured the plasma cortisol level as an indicator of stress due to salinity changes. Although the plasma cortisol concentrations were significantly increased in all of the diet groups, the flounder that were fed Diets 2 and 3 had significantly lower cortisol levels compared with the flounder that were fed Diet 1 (Fig. 11). Kawabata et al. (2009) reported biological effects of quercetin on the hypothalamic-pituitary-adrenal axis (a major component of the stress

response), leading to reduced stress. Belo et al. (2005) found that the plasma cortisol concentrations in Pacú (*Piaractus mesopotamicus*) maintained at high stocking density were significantly lower after treatment with vitamin E, compared with the controls. These results suggest that antioxidant preparations containing vitamin E can protect organisms from stress. Therefore, because these previous results are consistent with our results, we suggest that the antioxidant quercetin protects organisms against environmental stress.

The plasma osmolality in marine fish tends to decrease after exposure to hypoosmotic conditions (Sampaio and Bianchini, 2002). In the present study, the plasma osmolality in olive flounder exposed to hypoosmotic conditions was decreased significantly in all diet groups, but the osmolality was higher after treatment with quercetin (Diets 2 and 3) for 30 and 60 days, compared with Diet 1 (Fig. 12). There have been few studies on plasma osmolality changes with salinity stress after treatment with antioxidants. We hypothesize that quercetin affects the plasma osmolality by reducing the stress induced by hypoosmotic conditions.

In conclusion, we examined the hypocholesterolemic and antioxidant effects of quercetin in olive flounder. The expression and activities of SOD and CAT and the plasma H_2O_2 concentration were significantly lower in flounder receiving quercetin (Diets 1 and 2) compared with those fed Diet 1, indicating that quercetin has antioxidant effects and scavenges ROS

produced during the stress induced by acute changes in salinity. The antioxidant effects observed for Diets 2 and 3 were maintained for 30 and 60 days. Quercetin was also associated with increased lysozyme activity, which suggests that quercetin improves immune function against external stress. Additional studies should examine the effects of various antioxidants against environmental stress factors, from a molecular biological and physiological perspective.

V. Conclusion

In this study, to investigated the antioxidant efficacy of quercetin (0% Diet 1, 0.25% Diet 2, and 0.5% Diet 3) pretreatment for 30 and 60 days in cadmium (Cd) toxicity or exposed to osmotic stress in the olive flounder. First, we measured plasma lysozyme activity, T_3 concentration to understand the immune effects and hormone regulation of quercetin. The lysozyme activity and T_3 concentration with Diets 2 and 3 were higher than with Diet 1. Therefore, we postulated that quercetin increased the immune ability and maintained hormone homeostasis in the olive flounder. Also, we measured total cholesterol levels which were lower with Diets 2 and 3 than with Diet 1, leading us to hypothesize that quercetin removed low-density lipoproteins from circulation and thereby reduced total cholesterol.

Based on these results, with Diets 2 and 3, the expression and activity of antioxidant enzymes (SOD and CAT) and the H_2O_2 concentration were lower than with Diet 1. In addition, the LPO levels were lower than with Diet 1, which protected the cell membrane.

Therefore, quercetin increases lysozyme activity enhancing immune ability and T_3 concentrations to maintain hormone homeostasis, and reduced total cholesterol levels to removed low-density lipoproteins from circulation in olive flounder. Results in this study indicating that quercetin has antioxidant effects and scavenges ROS produced during the stress induced by Cd and acute changes in salinity.

VI. Acknowledgements

학부 2학년때 분자생물학이라는 학문에 관심을 가지고 들어와 어느 덧 4년이라는 시간이 흘러 그동안의 연구가 결실을 맺게 되었습니다. 그래서 좋은 결과를 얻게 해준 고마운 분들게 감사의 인사를 드리고자 합니다. 먼저 분자생물학에 대한 깊은 지식과 나아갈 길을 제시해주시고, 따뜻한 조언을 많이 해주신 최철영 지도교수님께 진심으로 감사의 인사를 드립니다. 그리고 전공 학문 지도에 아낌없이 학문적 가르침을 전해주신 조성환 교수님과 박인석 교수님께도 진심으로 감사드립니다. 학부 동안 학문의 기초를 전해주신 강효진, 노일, 서영완, 이호진, 안종웅, 임선영, 이경은 교수님께도 감사의 말씀을 전하고자 합니다.

그리고 해양분자생물공학 연구실에서 늘 가족처럼 같이 생활하고 학부때부터 지금까지 실험, 논문 작성과 고민 상담에 큰 도움을 주신 광욱오빠와 항상 격려해주고 제가 많이 의지하고 있는 예쁜 나나언니, 힘이 되어주었던 동기 명인오빠, 대학원 동기 수정이, 주리, 화정언니, 동아리선배이자 과선배로 많은 조언을 해주신 찬흠오빠, 룸메이트 민정언니에게 감사의 인사를 전합니다. 또한, 실험할 때 가장 도움을 많이 줘서 너무 고마운 지혜, 대학원 생활에 큰 힘이 되어준 친구들 혜민이, 은혜, 진화, 해진, 사랑하는 정화언니, 오랜 친구들인 애리, 현지, 진옥이에게도 고마움을 전합니다.

마지막으로 못난 저를 가장 사랑해주시고 이해해주시는 할머니, 아빠, 엄마 그리고 군대에서 고생하고 있을 멋진 동생 주호, 항상 티격태격하지만 나의 영원한 소울메이트 양고, 저에게 많은 관심과 애정을 가져주신 모든 분들에게 다시 한번 감사의 인사를 전합니다. 앞으로 이 분들의 기대에 어긋나지 않도록 성실하고 참된 사람이 되도록 하겠습니다.

신현숙 올림

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