Many environmental stress such as cadmium (Cd) increase reactive oxygen species (ROS) and provoke oxidative stress in organisms (Storey, 1996). ROS include superoxide ($\text{O}_2^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$), the hydroxyl radical ($\text{HO}^-$), and singlet oxygen ($\text{1O}_2$) (Kinnula et al., 1995). Overproduction of ROS by Cd can increase lipid peroxidation (LPO), the oxidation of nucleic acid and proteins, and DNA damage.

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 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 (Pinho et al., 2005; Weng et al., 2007; Prieto et al., 2008) 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), mouse (Mus musculus) (Weng et al., 2007), and tilapia (Oreochromis niloticus) (Prieto et al., 2008) 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$_2$O$_2$ treatment occurred via the activity of the antioxidant enzymes SOD and CAT.

In 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). It can stop redox reactions by chelating ROS generated by toxic materials like Cd, and it inhibits LPO on the cell membrane. The pheoxy radical generated combines with another ROS and then inhibits the production of ROS (Frankle et al., 1993).

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.

Antioxidant improved immune effect as well as antioxidant ability, Qinghui et al. (2004) reported beneficial effects of vitamin C on immunological parameters, such as lysozyme, in the Japanese seabass (Lateolabrax japonicus). Increased immune ability was demonstrated by the increased lysozyme (Ortuno et al., 1999; Ai et al., 2004). Many antioxidants are reported to enhance immune capacity as well as antioxidant capacity.

Therefore, this study determined the effects of quercetin on enhancing growth and immune ability by measuring the plasma lysozyme activity. 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 H2O2 concentration, and LPO as an oxidative stress parameter.

**MATERIALS AND METHODS**

**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 and experimental diets 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.

**Extraction of quercetin**

The extraction of quercetin was carried out according to the methods of Velioglu and Mazza (1991) and Kang et al. (1998), with modification. The dried onion peels were blended with methanol in a Waring blender for 5 min and filtered through Whatman No. 1 filter paper, and then the filtrate was concentrated using a rotary evaporator at 40°C. The residue was washed with ether in a separatory funnel to remove lipids and other fat soluble materials. The onion extract was fractionated in the order of ethylacetate to purified glucosides type of quercetin, and then we examined quercetin content level by HPLC.

**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 pelleted. 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.

Experimental diets using purified quercetin were made by jeilfeed company (Kyoungnam, Korea), and were coated for protecting soluble nutritions include quercetin in the water. Experimental diets were in the water for a while, quercetin content level was not significantly different from the content of original experimental diets. 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).

**Plasma lysozyme activity**

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.

**Cd exposure**

The experimental fish were exposed to CdCl2·2.5H2O (Kanto Chemical, Tokyo, Japan) dissolved in water to a Cd2+ 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 after fed Diet 1, 2 and 3 for 30 and 60 days respectively. 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
Table 1. Ingredients and nutrient composition of the experimental diets

<table>
<thead>
<tr>
<th>Ingredient (%)</th>
<th>Diet 1 (0%)</th>
<th>Diet 2 (0.25%)</th>
<th>Diet 3 (0.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal¹ (ML)</td>
<td>45.00</td>
<td>45.00</td>
<td>45.00</td>
</tr>
<tr>
<td>Corn gluten ML</td>
<td>15.00</td>
<td>15.00</td>
<td>15.00</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>19.90</td>
<td>19.65</td>
<td>19.40</td>
</tr>
<tr>
<td>Soybean ML</td>
<td>10.00</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Fish oil - salmon</td>
<td>2.50</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>Squid ML</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Krill ML</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Lecithin</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Mono-calcium</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Choline</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>VITAMIX²</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>MINEMIX³</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>QUERCETIN</td>
<td>0.00</td>
<td>0.25</td>
<td>0.50</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

¹ Imported from Chile.
² 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.
³ Mineral premix contained the following ingredients (g/kg mix): MgSO4.7H2O, 80.0; NaH2PO4.2H2O, 370.0; KCl, 130.0; Ferric citrate, 40.0; ZnSO4.7H2O, 20.0; Ca-laectate, 356.5; CuCl, 0.2; AlCl3.6H2O, 0.15; KI, 0.15; Na2SeO3, 0.01; MnSO4.4H2O, 2.0; CoCl2.6H2O, 1.0.

The 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 g 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/ml.

The method for CAT activity is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H2O2. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-mercaptop-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 as nmol/min/ml.

H2O2 assay

H2O2 concentrations were 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 H2O2 was interpolated from a standard curve. Concentrations are expressed as nmol peroxide/ml.

LPO assay

LPO is quantified by measuring malondialdehyde...
(MDA) and 4-hydroxynonenal (4-HNE), the degradation products of polyunsaturated fatty acids (PUFAs) hydroperoxides (Esterbauer et al., 1991). 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.

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).

RESULTS
Quercetin contents
The amount of quercetin (glucosides type) from onion by HPLC was about 1 g/kg onion (0.1%), and then formulated to contain three concentrations (0, 0.25, and 0.5%) of extracted quercetin by replacing wheat flour in experimental diets.

Growth performance
The weight gain of flounder fed Diet 3, which contained quercetin, for 30 and 60 days were significantly higher than those in flounder fed Diet 1, which did not contain quercetin. This result indicated that high concentration (Diet 3) of quercetin is very effective than low concentration (Diet 2) in growth (Figure 1).

Plasma lysozyme activity
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 (Figure 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 (Figure 3A); 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 (Figure 3C). 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 (Figure 3B); 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 (Figure 3D).

SOD and CAT activity
The effects of quercetin on Cd-induced antioxidant enzyme (SOD and CAT) activities are shown in Figure 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 (Figure 4A); the CAT activity

Figure 1. Weight gain of olive flounder fed experimental diets containing quercetin. Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after feeding. The numbers indicate significant differences from the initial weight (within the same diet) (p<0.05). All values are means±SD (n = 5).

Figure 2. Lysozyme activity in plasma of olive flounder fed diets containing 0, 0.25, and 0.5% quercetin for 30 and 60 days. Values with alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after feeding. The numbers indicate significant differences from the 30 days (within the same diet) (p<0.05). All values are means±SD (n = 5).
amplified using gene-specific primers. The results are expressed as normalized fold expressions with respect to 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 alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after exposure. The numbers indicate a significant difference from the control (within the same diet) (p<0.05). All values are means±SD (n = 5).

**DISCUSSION**

To understand the antioxidant effect of quercetin on oxidative stress induced by Cd in olive flounder, we investigated the effects on lysozyme, and the expression and activity of SOD and CAT.

The weight gain of flounder fed Diet 3 for 30 and 60 days were significantly higher than those in flounder fed Diet 1 and 2 (Figure 1). This result indicated that high concentration (Diet 3) of quercetin is very effective than...
low concentration (Diet 2) in growth. Antioxidant, vitamin C could enhance the growth in fresh shrimp (Penaeus chinensis) (Wang and Li, 1996), African catfish (Clarias gariepinus) (Merchie et al., 1997a) and carp (Cyprinus carpio) (Gouillou-Coustans et al., 1998).

The plasma lysozyme activity with Diets 2 and 3 were significantly higher than with Diet 1 after feeding for 30 or 60 days (Figure 2). 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.

The expression of SOD and CAT mRNA with Diets 2 and 3 was significantly lower than with Diet 1 (Figure 3). 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 (Figure 4). 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 matrixx (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 H2O2 concentrations with Diets 2 and 3 were significantly lower than with Diet 1 (Figure 5), 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 (Figure 6). These results agree with Hiratsuka et al. (2008), who reported that LPO levels were reduced in mice fed docosahexaenoic acid (DHA), indicating that antioxidant can protect biomembranes and lipid

Figure 4. 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 alphabets indicate significant differences between Diet 1, 2 and 3 within the same time after exposure. The numbers indicate a significant difference from the control (within the same diet) (p<0.05). All values are means ±SD (n = 5).
In conclusion, quercetin increases lysozyme activity enhancing immune ability in olive flounder. In addition, the mRNA expression and activity of the antioxidant enzymes SOD and CAT and the H$_2$O$_2$ 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 confirmed that quercetin was a strong antioxidant material in this study. Additional studies should examine the effects of various antioxidants on environmental stress factors and oxidative stress.

REFERENCES


Eo, J. and K. J. Lee. 2008. Effect of dietary ascorbic acid on growth and non-specific immune responses of tiger puffer,


