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Antioxidant Defenses and Physiological Changes in Olive Flounder (*Paralichthys olivaceus*) in Response to Oxidative Stress Induced by Elevated Water Temperature

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ABSTRACT We determined oxidative stress caused by thermal stress in olive flounder *Paralichthys olivaceus* based on the altered-mRNA expression and enzymatic activity of two key antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), along with monitoring of several other biomarkers. When the fish were exposed to acute thermal change (from 20°C to 25°C and 30°C), the expression and activity of both enzymes were significantly higher at elevated temperatures (25°C and 30°C) than at 20°C. Lipid peroxidation (LPO) was also higher at 25°C and 30°C than at 20°C. In addition, the plasma H_2O_2 concentration was significantly increased by thermal stress. Furthermore, we investigated changes due to thermal stress by measuring levels of plasma alanine aminotransferase (AlaAT) and aspartate aminotrasferase (AspAT). Both were significantly increased by thermal stress. As an immune indicator, the lysozyme concentration was lower at 30°C than at 20°C, indicating that thermal stress decreases immune function. Therefore, thermal stress could induce oxidative stress and suppress immune function and can cause physiological stress.

Key words : Thermal stress, SOD, CAT, H_2O_2, LPO, lysozyme

INTRODUCTION

Temperature is a major environmental factor that affects growth, survival, reproduction, and immune function in teleost fishes (Schreck *et al.*, 1989; Bly and Clem, 1992; Bowden, 2008). In particular, the acute changes of water temperature can cause stress in fish (Abele *et al.*, 1998), combined with an increase in oxygen consumption to maintain homeostasis (Hochachka and Somero, 1984; Bagnyukova *et al.*, 2007). Generally, when acute oxygen consumption increases, the influx of oxygen in aerobic cells is converted to reactive oxygen species (ROS), oxygen radicals (O_2^-), or hydrogen peroxide (H_2O_2) (Chance *et al.*, 1979), and increased tissue oxygen consumption entails elevated rates of ROS production in mitochondria (Boveris *et al.*, 1976). Therefore, water temperature changes, especially to high temperatures, potentially enhance ROS release (Halliwell and Gutteridge, 1989) and increased lipid peroxidation, and may affect cell viability by causing membrane damage and enzyme inactivity. Subsequently, cell senescence, 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, reduced disease resistance, and reduced reproductive ability (Kim and Phyllis, 1998; Pandey *et al.*, 2003).

However, complex antioxidant defense systems operate to maintain homeostasis in changing environments and protect the aerobic organisms against ROS and subsequent oxidative stress damage (Bagnyukova *et al.*, 2007). Antioxidant function may include ROS-scavenging or detoxifying enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione S-transferase (GST) as well as protein (e.g., metallothionein, MT) or non-protein (e.g., vitamin C and E) compounds (McFarland *et al.*, 1999). Antioxidant defense systems are found in the livers and kidneys of marine organisms (Basha and Usha, 2003), and they have antioxidant functions as follows. As phase 1 enzymes, SOD and CAT directly scavenge ROS; SOD removes O_2^- through the process of dismutation to O_2 and H_2O_2 ($2O_2^- + H^+ \rightarrow H_2O_2 + O_2$), and then H_2O_2 produced by SOD is sequentially reduced to H_2O and O_2 by CAT, in

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the end CAT is an oxidoreductase that breaks down two molecules of H_2O_2 into two molecules of H_2O and O_2 ($2H_2O_2 \rightarrow 2H_2O+O_2$), thereby counteracting the toxicity of H_2O_2 (Kashiwagi *et al.*, 1997).

Acute water temperature changes also affect the immune function and capacity of fish. For example, Wang *et al.* (2008) and Cheng *et al.* (2009) reported that lysozyme activity was reduced in sea cucumbers and orange-spotted grouper (*Epinephelus coioides*) exposed to high temperature.

Generally, increase of water temperature during summer season may often cause acute stress and even outbreak of mass mortality in marine organisms (Collazos *et al.*, 1995). In this study, we investigated the mRNA expression and/or enzymatic activities of the antioxidant enzymes SOD and CAT as well as the plasma H_2O_2 concentration and lipid peroxidation (LPO) in order to examine the oxidative stress in olive flounder (*Paralichthys olivaceus*) exposed to high temperatures. We also examined potential change of immune function during thermal stress based on the analysis of plasma lysozyme concentration, and determine plasma alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) concentrations to measure general stress levels arisen from the thermal changes.

MATERIALS AND METHODS

1. Experimental fish and conditions

Olive flounder (n=60; length, 10±0.5 cm; weight, 19.9±1.3 g) were obtained from a commercial fish farm (Hwanam fishery, Gijanggun, Busan, Korea) and allowed to acclimate to the experimental conditions for 2 weeks in three 300-L flow-through tank system. The water temperature and photoperiod were maintained at 20±1°C, and 12-h light : 12-h dark, respectively. And pH was 7.8~7.9 and ammonia was no detected (0 ppm) in water. The fish were fed a commercial feed formed as extruded pellet (jeilfeed company, kyoungnam, Korea) twice daily (09:00 and 17:00).

2. Treatment of high water temperature

The water temperature was increased by 1°C every day from 20°C to 30°C (1°C/day) using automatic temperature regulation system (Johnsam Co., Boocheon, Korea), we sampled at 25°C and 30°C after 5 days and 10 days started elevating temperature, respectively. The thermal experiment was performed with three replications per group, and five fish from each group [control group (20°C), experimental groups (25°C and 30°C)] 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 obtain liver samples, the fish were euthanized by spinal transection. Immediately after collection, the tissue samples were frozen in liquid nitrogen and stored at −80°C until used for total RNA extraction.

3. Quantitative real-time PCR (QPCR)

QPCR was conducted to determine the relative expression of SOD and CAT mRNA in the total RNA extracted from the liver using the Trizol method according to the manufacturer's instructions (Gibco/BRL, Grand Island, NY, USA). The concentration and purity of the RNA samples was determined by UV spectroscopy at 260 and 280 nm. Total RNA (2.5 μg) was reverse transcribed in a total volume of 20 μL using an oligo-d $(T)_{15}$ anchor primer and M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's instructions. Primers for QPCR were designed with reference to the known sequences of olive flounder as follows [GenBank 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′), 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′), β-actin forward primer (5′-GCA AGA GAG GTA TCC TGA CC-3′) and β-actin reverse primer (5′-CTC AGC TCG TTG TAG AAG G-3′). PCR amplification was conducted using an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. QPCR was performed as follows: denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 20 s, and annealing at 55°C for 20 s, and extension at 72°C for 20 s. As an internal control, experiments were duplicated with β-actin. All data were expressed as change with respect to the corresponding β-actin calculated cycle threshold (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal control (β-actin) was calculated [$\Delta\Delta Ct = 2^\wedge\text{-} (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$]. Also, to ensure that the primers amplified a specific product, we performed a melting curve, melting at only one temperature.

4. SOD and CAT activity analysis

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

Cayman Chemical (Ann Arbor, MI, USA).

SOD activity was assessed by using a tetrazolium salt for detecting 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 according to the manufacturer's instructions (Cayman Chemical, USA). Absorbance was read at 450 nm (Victor X3, PerkinElmer, USA). Each assay was performed in duplicate, and enzyme units were recorded as U/mL.

For CAT activity, the assay 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, which upon oxidation changes from colorless to purple. Absorbance was read at 540 nm (Victor X3, PerkinElmer, USA). Each assay was performed in duplicate, and CAT activity was expressed as nmole/min/mL.

5. 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). A lipid hydroperoxide assay kit (Cayman Chemical, USA) 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 chromagen. The hydroperoxide concentration was determined based on absorption at 500 nm (Victor X3, PerkinElmer, USA).

6. H_2O_2 assay

H_2O_2 concentrations were measured using the modified methods of Nouroozzadeh *et al.* (1994) and a Peroxidetect Kit (Sigma). Twenty microliters of olive flounder serum was added per well to flat-bottom 96-well microtiter plates. Plates were left at room temperature for 20 min to allow the serum to settle and adhere to the plate. A working color reagent was prepared by mixing 100 mL distilled water containing 100 mM sorbitol and 125 μM xylenol orange (Sigma) with 1 mL of 25 mM ferrous ammonium sulfate prepared in 2.5 M sulfuric acid (Sigma). Two hundred microliters 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 (Victor X3, PerkinElmer, USA) and the concentration of H_2O_2 was interpolated from a standard curve. Concentrations are expressed as nM/mL.

7. Plasma parameters analysis

Plasma AlaAT and AspAT were measured with Biochemistry Autoanalyzer (model 7180; Hitachi, Tokyo, Japan). 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°C and absorbance at 530 nm was measured between 0.5 and 4.5 min with a spectrophotometer. A lysozyme activity unit was defined as the amount of enzyme producing a 0.001/min decrease in absorbance.

8. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One way ANOVA followed by Dunnett's post hoc test was used to compare the differences to 20°C group in the data ($P<0.05$).

RESULTS

1. Expression and activity of antioxidant enzymes

SOD mRNA expression was significantly increased

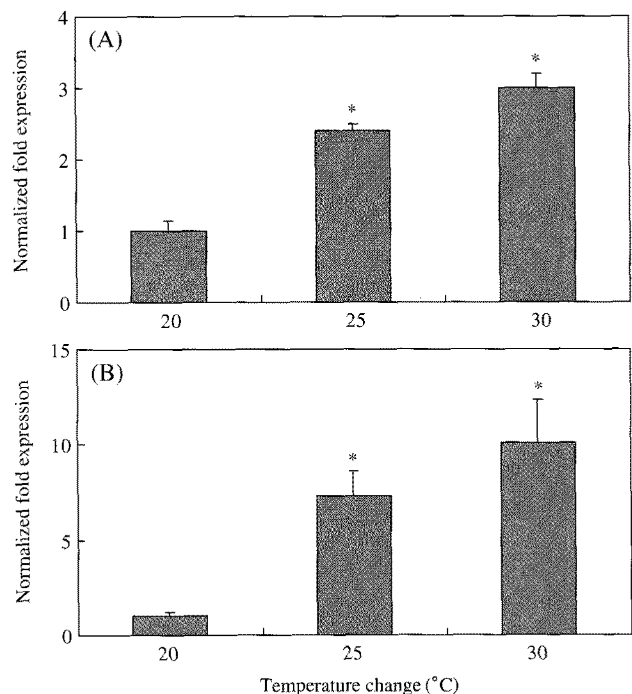

Fig. 1. Expression of SOD and CAT mRNA, as measured by quantitative real-time PCR, in olive flounder exposed to thermal stress (25°C and 30°C). Total liver RNA (2.5 μg) was reverse-transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin in the same sample. Asterisks indicate significant difference compared with 20°C ($P<0.05$). All values are means ± SD (n=5).

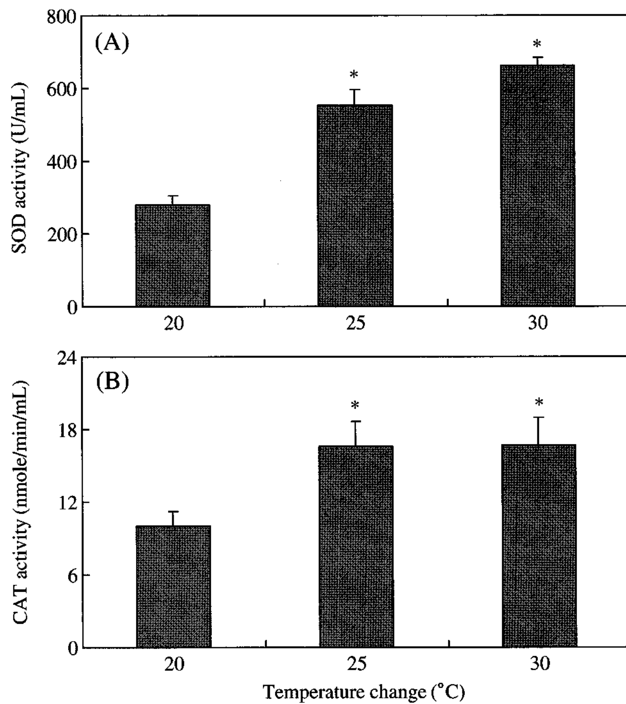

Fig. 2. Activity of SOD and CAT in olive flounder exposed to thermal stress (25°C and 30°C) by microplate reader. Asterisks indicate significant difference compared with 20°C ($P<0.05$). All values are means±SD ($n=5$).

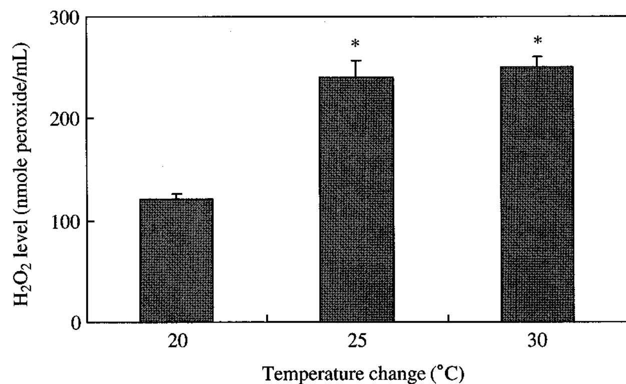

Fig. 3. Plasma H_2O_2 concentrations in olive flounder exposed to thermal stress (25°C and 30°C). Asterisks indicate significant difference compared with 20°C ($P<0.05$). All values are means±SD ($n=5$).

about 2.4- and 3-fold in fish from the 25°C and 30°C groups respectively as relative to those from 20°C group, and CAT mRNA expression was significantly increased about 7.3- and 10.1-fold at 25°C and 30°C respectively than at 20°C (Fig. 1). SOD activity was significantly increased ($P<0.05$) about 1.9- and 2.3- fold at 25°C and 30°C respectively than at 20°C. CAT activity was significantly increased ($P<0.05$) about 1.6-fold at 25°C and 30°C than at 20°C (Fig. 2).

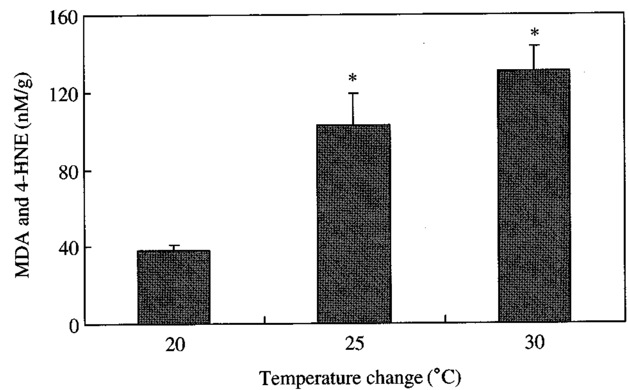

Fig. 4. MDA and 4-HNE concentrations in olive flounder exposed to thermal stress (25°C and 30°C). Asterisks indicate significant difference compared with 20°C ($P<0.05$). All values are means±SD ($n=5$).

Table 1. Plasma AlaAT, AspAT and lysozyme activity in olive flounder exposed to thermal stress

Ambient	AlaAT (IU/L)	AspAT (IU/L)	Lysozyme activity (μg/mg protein)
20°C	13.0±3.0	1.3±0.2	14.0±3.0
25°C	50.0±8.0*	7.0±1.0*	17.0±2.4
30°C	49.0±2.2*	6.0±1.2*	6.0±1.1*

Asterisks indicate significant difference compared with 20°C group ($P<0.05$). All values are means±SD ($n=5$).

2. H_2O_2 concentration

The plasma H_2O_2 concentration was significantly increased about 1.9-fold in fish from 25°C water than in fish from 20°C and was maintained at that level at 30°C (Fig. 3).

3. LPO assay

LPO (expressed as MDA and 4-HNE compounds) increased significantly as the temperature increased about 2.6-(25°C) and 3.4-fold (30°C), respectively (Fig. 4).

4. Plasma parameters

Plasma AlaAT levels were 13±3 IU/L in the 20°C group, but they increased significantly (50.1±8 IU/L) in the 25°C group. Plasma AspAT levels were 1.3±0.2 IU/L in the 20°C group, but they significantly increased (7±1 IU/L) in the 25°C group and the level was maintained in the 30°C group (Table 1). However, plasma lysozyme activity increased slightly at 25°C and then decreased about 2.3-fold at 30°C than at 20°C (Table 1).

DISCUSSION

In this study, we measured plasma H_2O_2 and LPO lev-

els, as well as the expression and activity of the antioxidant enzymes SOD and CAT to understand the oxidative stress and mechanism in olive flounder exposed to the high temperature environment. We also examined lysozyme-related immune function, AlaAT and AspAT to investigate physiological changes induced by oxidative stress.

The expression and activity of antioxidant enzymes increased in olive flounder exposed to high temperature of 25°C and 30°C (Figs. 1 and 2). These results are in agreement with a previous report that found increased SOD, GPX, and GST activity in goldfish exposed to high temperatures from 3°C to 23°C (Bagnyukova *et al.*, 2007). Also, SOD activity increased when goldfish were exposed to 35°C water (Lushchak and Bagnyukova, 2006a), as well as in mice exposed to a high temperature (38°C; Djordjević *et al.*, 2004), indicating possibly its key role in protection against ROS produced at high temperature (Lushchak and Bagnyukova, 2006a). Furthermore, in this study, to examine oxidative stress induced by thermal stress, we investigated H_2O_2 in plasma, and found that the plasma H_2O_2 concentration was significantly increased in fish from 25°C and 30°C water than in fish from 20°C water (Fig. 3). When the Antarctic intertidal limpet (*Nacella concinna*) is exposed to a temperature change of more than 10°C, its plasma H_2O_2 concentration increases (Abele *et al.*, 1998). An acute water temperature change induces stress (Abele *et al.*, 1998) and increases oxygen consumption, which leads to an influx of oxygen into cells. Oxygen then is converted to ROS, O_2^-, and H_2O_2, and increased oxygen consumption occurs in tissues (Boveris *et al.*, 1976). ROS increase the risk of oxidative damage (Halliwell and Gutteridge, 1989). In addition, Harari *et al.* (1989) demonstrated that thermal stress accelerates the oxidation of polyamine in cells to generate ROS, so oxidative stress induced by ROS is related to the antioxidant response (Abele *et al.*, 1998; Liu *et al.*, 2007). Therefore, stimulated mRNA expression and enzymatic activity of the antioxidant enzymes with elevated water temperature would be tightly associated with the enhanced oxidative stress in olive flounder.

Moreover ROS production enhances LPO through lipid damage, as well as increasing the expression and activity of antioxidant enzymes (Bagnyukova *et al.*, 2007). In this study, we found the LPO increased as the temperature increased (Fig. 4). This result is in agreement with Lushchak and Bagnyukova (2006a, b), who found that products of LPO, lipid peroxides (LOOH) and thiobarbituric acid-reactive substances (TBARS), increase quickly in goldfish tissues due to oxidative stress induced by thermal stress (35°C), and An and Choi (2010) reported that LPO increased in ark shell (*Scapharca broughtonii*) exposed to high temperature, indicating oxidative stress induced tissue damage. In addition, Chien and Hwang (2001) reported that malondialdehyde (MDA) increased in thornfish (*Terapon jarbua*) exposed to 36°C water. Also, Parihar *et al.* (1996) showed increased levels of LPO which, taken together with the SOD changes indicate increased ROS generation and oxidative stress in the liver of catfish (*Heteropneustes fossilis*) exposed to high temperature, and the results indicate an increase in oxidative stress of catfish exposed to high temperature.

Generally, AlaAT and AspAT are amino transfer enzymes and their blood concentrations are a general index of liver function in vertebrates. These enzymes can be used to evaluate the stress response caused by temperature change, low oxygen, pH, ammonia, or heavy metals (Pan *et al.*, 2003), and many studies have measured AlaAT and AspAT to examine stress levels (Vaglio and Landriscina, 1999; Choi *et al.*, 2007, 2008a, b). In this study, plasma AlaAT levels increased during experimental period (Table 1), suggesting that hepatocytes are damaged and increase stress levels due to the temperature increase, leading to a decrease in liver function. Also, lysozyme, a lysosomal enzyme implicated in the inflammatory process, is a nonspecific humoral factor that acts under stressful conditions such as acute temperature change (Eo and Lee, 2008). It is released by leukocytes and plays an important role in antimicrobial activity (Eo and Lee, 2008). However, stress induced by acute environmental change suppresses the immune system's ability to inhibit lysozyme activity (Wang *et al.*, 2008). Lysozyme activity decreased when sea cucumbers were transferred from 12°C to 32°C (Wang *et al.*, 2008), and when orange-spotted grouper (*Epinephelus coioides*) were transferred from 27°C to 35°C (Cheng *et al.*, 2009). Therefore, the stressor causes secretion of cortisol by the interrenal gland (Pickering and Pottinger, 1989; Harris and Bird, 2000), cortisol suppresses phagocytosis by decreasing phagocyte production (Harris and Bird, 2000), and resistance against bacterial pathogens is reduced (Maule *et al.*, 1989). In this study, as an indicator of immune function, plasma lysozyme activity was measured, plasma lysozyme activity in was significantly decreased at 30°C than at 20°C (Table 1). This result is in agreement with An and Choi (2010), who found that lysozyme activity decreased in ark shell exposed to high temperature, suggesting that acute temperature changes suppress immune function and reduce resistance against infection. In particular, lysozyme activity increased slightly at 25°C and then decreased at 30°C. We suggest that lysozyme activity increased to enhance immune function against temperature change stress up to 25°C, but lysozyme activity was inhibited by the 30°C environment (Wang *et al.*, 2008). So the increasing temperature in short period induced acute stress and affects immune function in olive flounder by reduced lysozyme activity.

In conclusion, in the present study, the expression and activity of the antioxidant enzymes (SOD and CAT) in olive flounder exposed to high temperature environments

were increased, and induced oxidative stress. These results indicate that antioxidant enzymes operated against thermal stress by increasing plasma H_2O_2 concentrations and LPO levels in olive flounder exposed to high temperature environments. Additionally, thermal stress decreased lysozyme activity and then led to suppressed immune ability. Furthermore, these results, along with the changes of antioxidant enzymes (expression and activity of SOD and CAT) and H_2O_2, LPO and lysozyme generated by thermal stress can provide basic data on antioxidant mechanism by thermal stress in fish.

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고수온 환경에 의해 유도된 산화 스트레스에 대한 넙치의 항산화 작용과 생리적 변화

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요 약 : 고수온 환경 (25°C와 30°C)에 노출시킨 넙치의 산화 스트레스 정도를 알아보기 위하여 넙치의 간 조직에서 항산화 효소 [superoxide dismutase (SOD)와 catalase (CAT)] mRNA의 발현량 및 그 활성을 측정한 결과, 20°C 대조구보다 25°C와 30°C 실험구에서 증가하는 경향을 보였다. 또한 지질 과산화 지표로 사용되는 lipid peroxidation (LPO)을 측정한 결과, 25°C와 30°C 실험구에서 증가하는 경향을 나타내었다. LPO의 증가는 SOD 및 CAT의 증가와 밀접한 관련이 있으며, 체내의 H_2O_2 농도 또한 25°C와 30°C 실험구에서 증가하는 것으로 보아 고수온 환경이 넙치의 산화 스트레스를 유발하고 있는 것으로 사료된다. 고수온 환경에 노출시킨 넙치의 혈중 alanine aminotransferase (AlaAT)와 aspartate aminotransferase (AspAT) 값을 측정한 결과, AlaAT와 AspAT 모두 유의적으로 증가하는 경향을 보였다. 또한 면역 지표로 사용되는 lysozyme 활성도가 20°C 대조구보다 30°C 실험구에서 유의적으로 낮은 값을 나타낸 점으로 보아, 고수온 환경에 노출된 넙치에서는 간 세포의 손상뿐만 아니라 면역력 또한 저해되고 있는 것으로 사료된다. 고수온 환경에 노출시킨 넙치에서 항산화 효소인 SOD와 CAT mRNA 발현량 및 활성이 증가하였을 뿐만 아니라 활성산소와 LPO 값 또한 증가된 점으로 보아, 고수온 환경은 넙치의 체내에서 산화 스트레스를 유발시키는 동시에 면역 기능을 저해시키고 있는 것으로 사료된다.

찾아보기 낱말 : 항산화, 산화 스트레스, H_2O_2, LPO, lysozyme