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mRNA expression of antioxidant enzymes and physiological responses in the Pacific oyster, *Crassostrea gigas*, exposed to an hypoxic environment

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Abstract

We measured the trends of oxygen (O_2) consumption by Pacific oysters (*Crassostrea gigas*) exposed to an hypoxic environment and compared mRNA expression of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). We also measured changes in osmolality and inorganic ion concentration in the haemolymph. O_2 consumption led to increased expression of SOD mRNA in gills and digestive glands up to 1 h, when it then decreased. CAT and GPX mRNA in gills and digestive glands tended to decrease. Haemolymph osmolality and K^+ level decreased with increasing water influx for O_2 uptake, and Ca^{2+} and Mg^{2+} levels increased through mobilization of calcium carbonate to provide bicarbonate to buffer against acidosis. Therefore, under hypoxic conditions, activities of these antioxidant enzymes seem to be key in minimizing tissue damage caused by reactive oxygen species.

Key words: CAT, GPX, hypoxia, osmolality, SOD

Introduction

Oxygen (O_2) is a very important element not only for terrestrial animals and plants, but also for aquatic flora and fauna. Hypoxia caused by red tides, high water temperatures and O_2-deficient water masses directly influences the behavioural, biochemical, and physiological responses of fishes (Wu 2002). Pacific oysters are sessile organisms that often inhabit tidal zones, and they have developed a control system to maintain themselves in hypoxic environments (David *et al.* 2005). They do this through the ability to lower their metabolic rate, enabling them to survive hypoxic conditions (Storey 1993). However, exposure to an hypoxic environment for a long time or the occurrence of severe anoxia acts as a stress factor in oysters, which leads to mass mortality (Boyd and Burnett 1999) and affects immune mechanisms (Macey *et al*. 2008).

In the course of O_2 metabolism, aerobic organisms produce potentially harmful reactive oxygen species (ROS). ROS, highly oxidative O_2 species that are generated in various metabolic processes as O_2 flows into the living body through respiration, are used in the process of oxidation but can attack tissues and damage cells. ROS include the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO).

ROS generated in the living body in a hypoxic environment are highly oxidative and bind with other substances. They attack cells or organ membranes and damage cellular functions (Ferraris *et al.* 2002). To protect cells from oxidative stress and prevent damage to cellular functions caused by ROS, body tissues contain antioxidant defence systems consisting of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (Chance *et al.* 1979; Wendel and Feuerstein 1981).

Antioxidant enzymes serve an important role in maintaining homeostasis of cells and cellular antioxidant defense by removing ROS (Rudneva 1999). SOD is one of the key defense elements against ROS. It is a metalloenzyme found in all living organisms that consume O_2. SOD removes superoxide radicals in the process of dismutation into O_2 and H_2O_2 ($2O_2^- + H^+ \rightarrow H_2O_2 + O_2$) (Fridovich 1975). H_2O_2 created through this process is broken down into H_2O and O_2 by CAT and GPX (Mruk *et al.* 2002). CAT, an oxidoreductase, removes toxic H_2O_2 by breaking down two molecules of H_2O_2 into two molecules of H_2O and O_2 ($2H_2O_2 \rightarrow 2H_2O + O_2$) (Kashiwagi *et al.* 1997). GPX is also an important peroxidase that removes toxic hydroperoxides, breaking them down into H_2O and O_2 (Świergosz-Kowalewska *et al.* 2006). Furthermore, GPX can largely be divided into selenium-dependent GPX (Se-GPX) and selenium-independent GPX (non-Se-GPX). Se-GPX promotes the breakdown of both organic and inorganic peroxides, while non-Se-GPX promotes organic peroxide breakdown (Almar *et al.* 1998).

Studies on hypoxia acclimation by marine invertebrates have been conducted on shore crabs (Morris and Butler 1996), blue crabs (Defur *et al.* 1990), shrimps (Hagerman and Uglow 1982), prawns (Cheng *et al.* 2003), lobsters (McMahon *et al.* 1978), crayfish (McMahon *et al.* 1974), Asian clams (Byrne *et al.* 1991), and abalones (Cheng *et al.* 2004). However, no comprehensive study has investigated physiological modulation and molecular endocrinology in relation to O_2 consumption by Pacific oysters exposed to

hypoxia. Therefore, we measured their O_2 consumption patterns of and compared the mRNA expression of SOD, CAT, and GPX, as well as changes in osmolality and inorganic ions according to O_2 consumption.

Material and Methods

Experimental oysters

One-year-old *Crassostrea gigas* (average shell length: 112±10.7 mm; shell height: 31.1±5.4 mm; weight: 20.3±3.9 g), obtained from the oyster hatchery on Dae-bu Island, in Goseong (Gyeongnam, Korea), were placed in 40 L circulation filter tanks at a density of 40 oysters per tank. During the experimental period, the water temperature was kept at 20±0.5°C and the photoperiod was a 12L/12D cycle (Jo *et al*. 2008). Five oysters were used for each experimental or control group.

Measuring O_2 consumption and sampling

We repeated each experiment three times by promptly moving stabilized *C. gigas* into a respiration chamber inside a closed O_2 consumption measuring device (OxyGuard 6; OxyGuard International A/S, Birkerd, Denmark). Water temperature was maintained by a constant temperature water bath (JS-WBP-170RP; Johnsam Co., Bucheon, Korea). The oysters were not fed during the period of the experiment to minimize changes in respiration and O_2 consumption by digestive metabolism. The structure of the O_2 consumption measuring device is illustrated in Figure 1. The respiration chamber was constructed of transparent acrylic material to allow observation of shell movement and mortality of the oysters. To measure O_2 consumption, we used the Oxyguard 6 program, a multichannel dissolved O_2 measuring system. In addition, the level of dissolved O_2 was automatically measured every 10 min using a dissolved O_2 sensor attached to an acrylic tank, and data were transferred into a computer. O_2 consumption by the oysters was displayed as the average O_2 consumption per unit animal weight calculated on the basis of dissolved O_2 automatically measured during the experiment. Prior to commencing the experiment, we collected gills, digestive glands, and haemolymph of the oysters, after 1, 3, and 5 h from the closed acrylic tank. The collected samples were stored in a super low-temperature freezer at –80°C until RNA extraction.

Quantitative real-time Polymerase chain reaction (QPCR)

QPCR was conducted to determine the relative mRNA expression of antioxidant enzymes (SOD, CAT, and GPX) using total RNA extracted from the gills and digestive glands of control and hypoxia-treated oysters. With 2.5 μg of total RNA as a template, equal starting amounts of cDNA were synthesized using M-MLV reverse transcriptase (Bioneer). First-strand cDNA synthesis was conducted using oligo-$d(T)_{15}$ primer (Promega). Primers for QPCR were designed with reference to known SOD (AJ496219), CAT (EF687775), GPX (EF692639), and β-actin (AF026063) gene sequences of *Crassostrea gigas* as follows: cgSOD forward primer, 5'-CTC CTG GAA CAC CTG TGA CAT TG-3'; cgSOD reverse primer, 5'-GTG CCT CTC GTG ATC CTC TGG-3'; cgCAT forward primer, 5'-AAC TAC TTC GCT GAG GTG-3'; cgCAT reverse primer, 5'-GGT CTT GGC TTT GTA TGG-3'; cgGPX forward primer, 5'-GAC CGT GGA ACC AAT GGA CAT C-3'; cgGPX reverse primer, 5'-GTT GGA TTC GGA CAC AGA TAG GG-3'; cgβ-actin forward primer, 5'-TGG ATC GGT GGT TCC ATC CTT-3'; and cgβ-actin reverse primer, 5'-GGT CCA GAT TCG TCG TAC TCC-3'. QPCR amplification was conducted using a Bio-Rad MiniOpticon™ System (Bio-Rad, Hercules, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. QPCR was undertaken by denaturation at 95ºC for 5 min, followed by 40 cycles of denaturation at 95ºC for 20 s and annealing at 55ºC for 20 s. All primers used were shown to amplify only one size of template, melting at only one temperature. PCR products were also confirmed by sequencing. All analyses were based on the calculated threshold cycle time (CT) values of the PCR products. The CT was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR program, QPCR data were analysed with analysis software (Gene Expression Analysis for iCycler iQ® Real-Time PCR Detection System, Bio-Rad) to estimate transcript copy numbers for each sample. The stated mRNA expression levels refer to an *n*-fold difference relative to β-actin as the internal control.

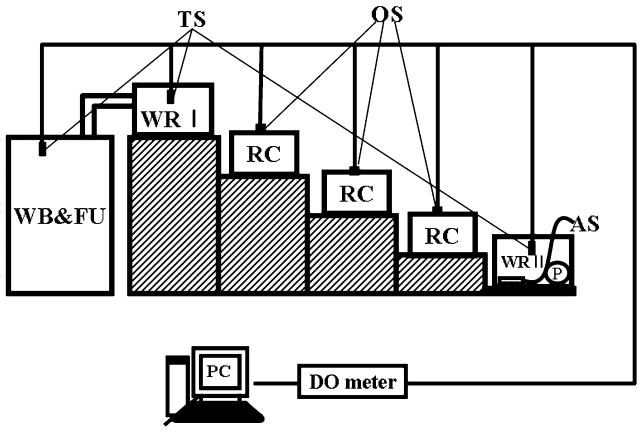

FIGURE 1. Schematic diagram of the oxygen (O_2) consumption measuring system used in the present study. AS, air supply; FU, filtering unit; OS, O_2 sensor; PC, personal computer; RC, respiratory chamber, TS, temperature sensor; WB; water bath; WR I and II, water reservoirs I and II, respectively.

Haemolymph analysis

Haemolymph was withdrawn from the pericardial cavity using a 3-mL syringe. The samples were centrifuged at 10,000×*g* (MICRO 17TR; Hanil, Seoul, Korea) and 4°C for 5 min, and the supernatant (after centrifugation) was stored at –80°C until analysis. Haemolymph osmolality was

measured using a vapour pressure osmometer (Vapro 5520; Wescor, Logan, UT, USA). K^+, Ca^{2+}, and Mg^{2+} were measured using a biochemistry autoanalyser (model 7180; Hitachi, Tokyo, Japan).

Statistical analysis

Treatment differences were tested using one-way analysis of variance (ANOVA) followed by the Tukey or least significant difference (LSD) test using the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA) at a significance level of P=0.05.

Results

O_2 consumption pattern

At the commencement of the experiment, the dissolved O_2 level was 5.4 mg L^{-1} in the closed respiration chamber. The level decreased to 4.3±0.5 mg L^{-1}, 1.8±0.15 mg L^{-1}, and 0.4±0.15 mg L^{-1} after 1, 3, and 5 h, respectively. The total length of time until the oysters consumed all of the O_2 was 6 h, with a trend of continuous decrease until approximately 350 min after commencing the experiment (Figure 2). The hourly dissolved O_2 consumption was 0.9 mg L^{-1} and O_2 consumption per hour per individual was 0.3 mg L^{-1}. Curve-fitting of the functional relationship between the time elapsed and the level of dissolved O_2 consumption using the least squares method gave a linear relationship. The regression between time and dissolved O_2 obtained through curve-fitting by the least squares method was dissolved O_2 level (y) = –0.1543 × x (time) + 4.9675 (Figure 2).

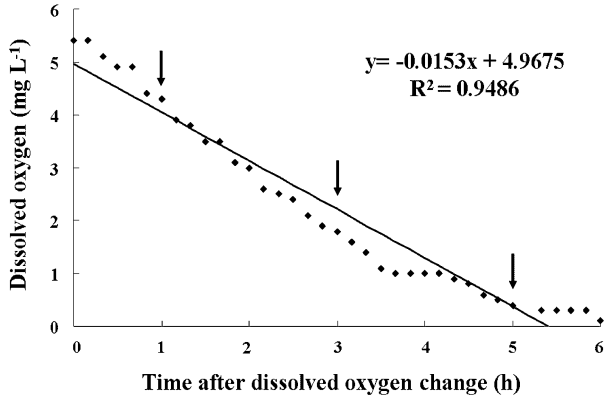

FIGURE 2. Linear relationship by least squares curve fitting for oxygen consumption of *Crassostrea gigas* vs. elapsed time. Samples were gathered at 1, 3, and 5 h after the start of treatment, and sample points are indicated by arrows.

Levels of SOD, CAT, and CAT transcripts

SOD mRNA expression in the gills (about 4.6-fold increase than control group) and digestive glands (about 1.8-fold increase than control group) of the oysters tended to increase rapidly, after 1 h exposed to hypoxia, and then decreased. CAT and GPX mRNA expression displayed a significant decrease as time elapsed (P=0.05) (Figure 3).

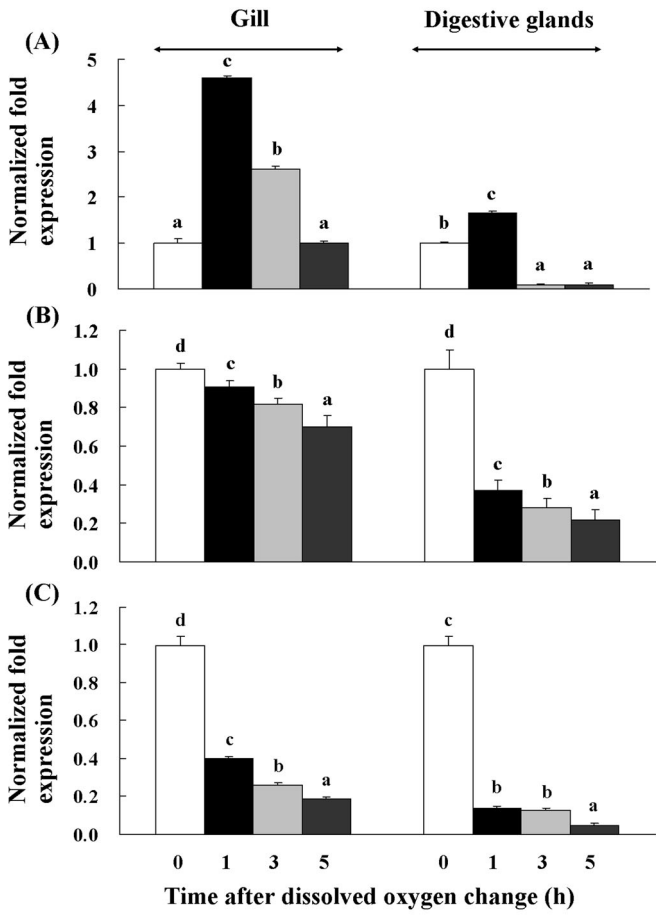

FIGURE 3. Time-related effect on superoxide dismutase (SOD, A), catalase (CAT, B), and glutathione peroxidase (GPX, C) mRNA levels in gills of *Crassostrea gigas* during hypoxia adaptation. The values are percentage increases relative to the control value. The expression level of each sample was normalized with respect to the β-actin mRNA signal, and expressed as a relative value. Values with dissimilar letters are significantly different (P=0.05) from each other. Values are means±SD of three experiments. Each experimental group consisted of five oysters.

Haemolymph osmolality and ion concentration

Osmolality in the haemolymph of the oysters exposed to hypoxia decreased from 988±8.86 mOsm kg^{-1} at the beginning of the experiment to 946.3±5.86 mOsm kg^{-1} after 5 h. The concentration of K^+ decreased from 13.0±1.65 mmol L^{-1} to 10.1±0.62 mmol L^{-1}. However, Ca^{2+} increased from 75.0±3.0 mmol L^{-1} to 95.5±6.06 mmol L^{-1} and Mg^{2+} increased from 55.0±3.77 mmol L^{-1} to 81.5±2.29 mmol L^{-1} (P=0.05) (Figure 4).

Discussion

In this study, *Crassostrea gigas* began to experience hypoxia inside the closed respiration chamber as soon as they began consuming O_2.

During the first hour of exposure to hypoxia, the oysters began consuming O_2 inside the closed respiration chamber (dissolved O_2 level: 4.3 mg L^{-1}); SOD mRNA expression in gill tissues increased and then decreased. However, CAT and

GPX mRNA expression displayed a trend of continuous decrease from the start of the experiment (Figure 3). This result is in accordance with previous studies of *L. littorea* (Pannumzio and Storey, 1998) and common carp *Cyprinus carpio* (Lushchak *et al*. 2005), both of which reported that activity of CAT and GPX were reduced under hypoxia condition. Therefore, it is possible that SOD mRNA was increased to convert O_2^- into H_2O_2 and O_2 during the first hour following exposure to hypoxia, while reducing mRNA expression of CAT and GPX under hypoxia. This may reflect a general metabolic suppression response to hypoxia. Romero *et al.* (2007) reported that as a result of exposing stone crabs *Paralomis granulosa* (Jacquinot, 1847) to a dry atmosphere of 6°C for 24 h, SOD mRNA expression rapidly increased in haemolymph for 3 h and then decreased, but CAT mRNA expression displayed a continuously decreasing trend. Pannunzio and Storey (1998) reported that GPX activity decreased in the hepatopancreas of the gastropod *Littorina littorea* (Linnaeus, 1758) exposed to anoxia. In conclusion, a hypoxic environment reduces ROS generation, therefore leading to a decrease in activities of antioxidant enzymes (Hermes-Lima *et al.* 2001). The results of Hermes-Lima *et al.* (2001) correspond to those of this study, that is, the expression of antioxidant genes decreased in response to exposure to hypoxia once the oysters begin to consume O_2. However, the pattern of SOD, CAT, and GPX mRNA expression in digestive glands was similar to the pattern displayed in gill tissues. Larade and Storey (2002) reported that various protein syntheses decreased in digestive glands 30 min after exposing *L. littorea* to anoxia. These results support our findings and show that a rapid decrease in anti-oxidation-related mRNA expression takes place in the digestive gland tissues of *C. gigas* under hypoxic conditions.

In this study, higher levels of SOD, CAT, and GPX mRNA expression were found in gill tissues than in digestive gland tissues when the oysters were exposed to hypoxia. The gill tissue comprises the primary interface between the haemolymph or cytoplasm and the external environment. Tissues of digestive glands, however, take part in energy metabolism such as food digestion, absorption, and discharge (David *et al.* 2005). As a direct absorption route for seawater, gill tissues have a wide contact area with seawater and are known to be directly influenced by their environment (Hosoi *et al.* 2007). The reason for higher mRNA levels in gills may be that because they function to absorb dissolved O_2, greater levels of oxidative stress following exposure to a hypoxic environment take place in the gills than in digestive glands.

We also measured changes in osmolality, K^+, Ca^{2+}, and Mg^{2+} ions in haemolymph when the oysters were exposed to hypoxia. While osmolality and the K^+ ion continuously decreased, Ca^{2+} and Mg^{2+} displayed a pattern of continuous increase (P=0.05) (Figure 4). Cheng *et al.* (2003) measured dissolved O_2 and exposure time to hypoxia using the giant prawn *Macrobrachium rosenbergii* (De Man, 1879) and found a pattern of decrease in osmolality and K^+ in the haemolymph as the level of dissolved O_2 decreased. This is similar to the results of the present study. In a hypoxic environment, organisms increase ventilation with seawater, and by enlarging the surface area of the gills to extract O_2 from the seawater, O_2 absorption is enhanced (Cheng *et al.* 2003). Such behavioural responses may have increased the inflow of water and therefore led to a reduction in osmolality and K^+ concentration in our experiments.

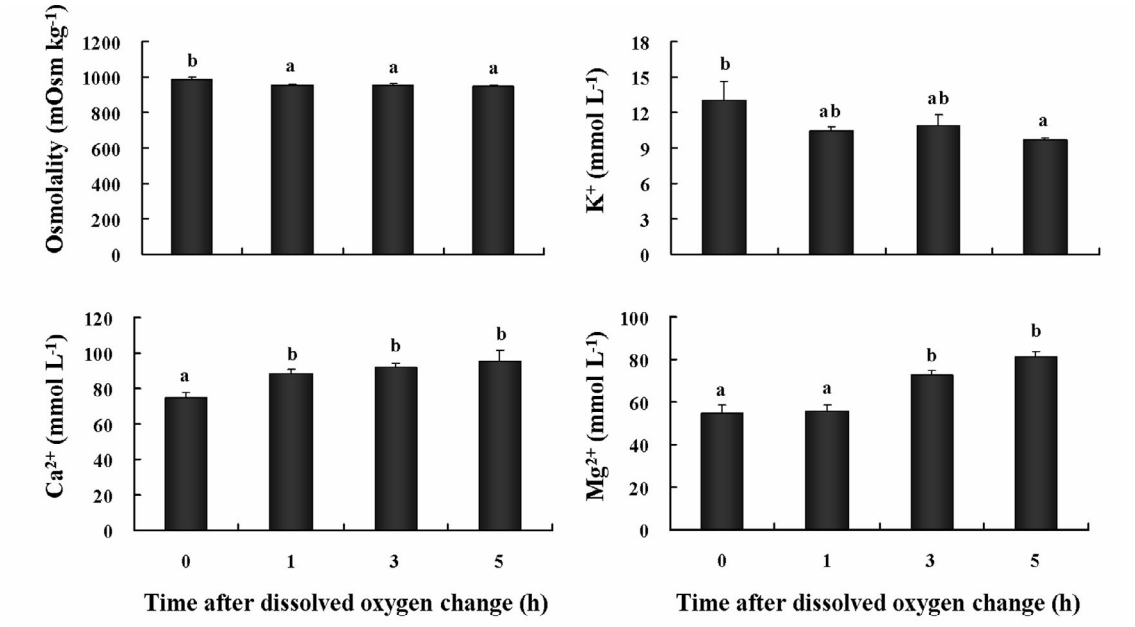

FIGURE 4. Osmolality, K^+, Ca^{2+}, and Mg^{2+} in the haemolymph of *Crassostrea gigas* during hypoxia adaptation. Values with dissimilar letters are significantly different (P=0.05) from each other. Values are means±SD of three experiments. Each experimental group consisted of five oysters.

The levels of Ca^{2+} and Mg^{2+} in haemolymph increase not only in purple shore crabs *Leptograpsus variegatus* (Fabricius, 1793) (Morris and Butler 1996), but also in Asian freshwater clams *Corbicula fluminea* (Muller, 1774) (Byrne *et al*. 1991) exposed to hypoxia, similar to our results with *C. gigas*. Byrne *et al.* (1991) reported that the calcium carbonate in the shell was used to supply bicarbonate as a way of buffering acidosis in the body as the haemolymph became acidified under hypoxia due to a decrease in pH. We hypothesise that the increase in haemolymph Ca^{2+} in *C. gigas* observed in this study was also caused by this mechanism.

In summary, we observed the expression of antioxidant enzymes, as well as changes in osmolality and inorganic ions in *C. gigas* exposed to a hypoxic environment. Hypoxia occurred as the *C. gigas* consumed O_2 within the closed respiration chamber. After 1 h the level of dissolved O_2 in the seawater had decreased to 4.3 mg L^{-1}. SOD mRNA expression increased up to 1 h of exposure in this environment and then decreased. CAT and GPX mRNA expression continuously decreased from the time of initial exposure. This may reflect a general metabolic suppression response to hypoxia that may affect both protein synthesis and xenobiotic processing (Pannumzio and Storey 1998; Lushchak et al. 2005). These results can be used as a basic marker system to identify physiological responses that occur at the time of exposure to hypoxia in Pacific oysters, and probably also in other species of bivalves.

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