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ORIGINAL ARTICLE

Characterization and mRNA expression of Mn-SOD and physiological responses to stresses in the Pacific oyster *Crassostrea gigas*

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Abstract
Superoxide dismutases (SODs) are metalloenzymes that play an important role in mollusc immune defence systems by eliminating oxidative stress to reactive oxygen species. We investigated physiological changes in the Pacific oyster *Crassostrea gigas* caused by exposure to pollutants (cadmium, tributyltin) and acute water temperature change. We analysed mRNA expression of Mn-SOD in gills using a quantitative polymerase chain reaction (QPCR), and measured the glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and hydrogen peroxide (H_2O_2) levels in haemolymph based on time- and dose-related effects of pollutants and acute water temperature treatments. We cloned a cgMn-SOD full-length cDNA that included an open reading frame of 675 nucleotides that was predicted to encode proteins of 225 amino acids. BLAST analysis of other species indicated that residues essential to the enzymatic functions of Mn-SOD proteins are highly conserved. Levels of Mn-SOD mRNA expression, GOT and GPT were gradually increased and then decreased during the experimental periods. On the other hand, H_2O_2 levels increased continuously during the exposure periods. These results suggest that Mn-SOD plays an important role in the physiological changes related to metabolism and cell protection that occur in *C. gigas* when exposed to oxidative stress by pollutants and fluctuations in water temperature.

Key words: *Acute water temperature, cadmium, hydrogen peroxide, Mn-superoxide dismutase, TBT*

Introduction

Reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (HO^-), and singlet oxygen ($^1O_2$) are naturally generated in the course of respiratory metabolism of all organisms. Moreover, the generation of ROS in living organisms is promoted by external stimulants such as water temperature change, infection, and toxic substances including cadmium (Cd), tributyltin (TBT), and hydrocarbon (Anderson et al. 1997; Chen et al. 2007; Murugavel et al. 2007). In addition, excessive production of ROS induces oxidative stress and can therefore cause lipid peroxidation, protein denaturation, and DNA breakage. It also induces apoptosis by causing cellular damage and imbalance as well as denaturation or obstruction of various enzyme activities (Wang et al. 2004; Choi et al. 2007; Murugavel et al. 2007). Accordingly, a number of physiological defects have resulted in accelerated ageing, reduced resistance against diseases, and lowered fertility (Kim & Phyllis 1998).

Living organisms implement antioxidant defence systems to protect themselves from oxidative stress. These systems consist of enzymes (superoxide dismutase (SOD), catalase, and glutathione peroxidase) that play an important role in protecting cells and maintaining homeostasis by eliminating ROS (Rudneva 1999). SOD, one of the representative defence elements against ROS, removes ROS by dismutating O_2^- into O_2 and H_2O_2 ($2O_2^- + H^+ \rightarrow H_2O_2 + O_2$) (Fridovich 1975). Its structure and function have been reported in a variety of aerobic

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organisms including aquatic invertebrates (Fink & Scandalios 2002). As a metalloenzyme, SOD is generally classified into copper/zinc-SOD (Cu/Zn-SOD), manganese-SOD (Mn-SOD), and iron-SOD (Fe-SOD) based on the metals bonded to enzyme active sites in cells (Mruk et al. 2002). Cu/Zn- and Mn-SOD differ from one another in terms of structures and evolutionary perspectives. While Cu/Zn-SOD is expressed in cytoplasm, Mn-SOD is mostly expressed in mitochondria (Fukuhara et al. 2002; Zelko et al. 2002). To date, full-length Mn-SOD cDNA has only been reported in *Mizuhopecten yessoensis* (GenBank accession no. AB222783) among the Bivalvia. Partial cDNA has also been reported in three species: *Corbicula fluminea* (EF446611), *Hyriopsis schlegelii* (EU145730), and *Venerupis philippinarum* (EF520698). Boutet et al. (2004) reported the existence of Cu/Zn-SOD in the Pacific oyster *Crassostrea gigas* (AJ496219) following hydrocarbon exposure. However, no study has reported on Mn-SOD.

Studies on SOD as an oxidative stress marker are being conducted in various species to investigate the health of marine organisms (Gonzalez et al. 2005; Monari et al. 2005) and to identify aquatic environmental pollution (Bebianno et al. 2004; Zelck et al. 2005). Pacific oyster, the experimental target of this study, is a highly nutritious and profitable food that is farmed in various countries. However, Pacific oyster farms have recently been suffering from marine environmental pollution caused by wastewater, ageing of farming facilities, marine wastes, and marine trafficking. Acute water temperature change, such as temperatures over 30°C in summer and cold pools, is also an environmental problem. Pollutants and acute water temperature changes can exert stress to living organisms and can therefore negatively affect their health.

The purpose of this study was to examine physiological changes in *Crassostrea gigas* (Thunberg, 1793) caused by exposure to Cd, TBT, and acute water temperature changes. To accomplish this, we cloned Mn-SOD cDNA isolated from the gill tissue of *C. gigas*. We also analysed Mn-SOD mRNA expression levels and changes in H_2O_2 concentration as well as the production of SOD dismutation in the haemolymph. Changes in the level of glutamate oxalacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were also monitored.

Materials and methods

Experimental oysters

We used 1-year-old Pacific oysters (mean shell length: 112 ± 10.7 mm; height: 31.1 ± 5.4 mm; weight: 20.3 ± 3.9 g) obtained from the oyster hatchery on Daebu island in Goseong (Gyeongnam, Korea). These were placed in two 50 l circulating filter tanks in the laboratory at 50 oysters per tank. During the experimental period, the water temperature, salinity and dissolved oxygen were maintained at 20 ± 0.5°C, 35 ppt and 5.4 ± 0.2 mg l^{-1}, respectively, under a photoperiod of 12 h light/12 h dark, and no food was supplied.

Cd and TBT treatments

The oysters were acclimated for 48 h in 300 l circulating filter tanks. After acclimation, 30 oysters were transferred to 50 l plastic aquaria filled with 1 μm filtered natural seawater (control; 40 l water) or Cd- and TBT-treated seawater (experimental groups; 40 l water). The whole quantity of water was exchanged daily during experimental period. For Cd treatments, Cd was added to the water as $CdCl_2 2.5H_2O$ (Kanto Chemical Co., Tokyo, Japan) to a dissolved Cd^{2+} concentration of 0.01, 0.05, or 0.1 ppm. For TBT treatments, TBT was added to the water as TBTO (Tributyltin (IV) oxide) (Riedel-de Haen, Seelze, Germany) to a dissolved TBT concentration of 5, 10, or 20 ppb. Oysters were exposed to treatments for 11 days; the water was changed daily and resupplied with the corresponding concentration of treatments. Haemolymph and tissues were sampled from randomly selected 5 oysters after 0, 1, 3, 7, and 11 days of treatments.

Acute water temperature treatments

Thirty oysters were directly transferred to 50 l plastic aquaria filled with 1 μm filtered seawater of 10°C (low water temperature), 20°C (control) and 30°C (high water temperature). Five oysters were randomly selected for haemolymph and tissue sampling from each experimental group at each of the following time period: 0, 1, 3, 6, 12, and 24 h. Immediately after collection, all tissues were frozen in liquid nitrogen and stored at −80°C until total RNA was extracted. No mortalities were observed in any of the experimental groups or the control group during the experimental period.

Haemolymph GOT and GPT analysis

Haemolymph was withdrawn from the pericardial cavity using a 3 ml syringe. The samples were centrifuged at 10,000 x*g* at 4°C for 5 min, and the supernatant was stored at −80°C until analysis. The activity of GOT and GPT was measured using Pureauto S AST (Daichi, Tokyo, Japan) and Pureauto S ALT (Daichi) kit by biochemistry autoanalyser

(model 7180; Hitachi, Tokyo, Japan) according to the manufacturer's instructions.

Hydrogen peroxide (H_2O_2) assays

H_2O_2 concentrations were measured using the modified methods of Nouroozzadeh et al. (1994) and a Peroxidetect kit (Sigma-Aldrich, St. Louis, MO, USA). Twenty microlitres of whole oyster haemolymph in marine anticoagulant (MAC; 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 50 mM EDTA, 0.45 M sodium chloride, pH 7.5) was added per well to flat bottom 96-well microtitre plates. Plates were left at room temperature for 20 min to allow haemocytes to settle and adhere. A working colour reagent was prepared by mixing 100 ml distilled water containing 100 mM sorbitol and 125 μM xylenol orange (Sigma-Aldrich) with 1 ml of 25 mM ferrous ammonium sulphate prepared in 2.5 M sulphuric acid (Sigma-Aldrich). Two hundred microlitres of this reagent were then added to each well and allowed to incubate at room temperature for 1 h. Absorbance was read at 560 nm and concentrations of H_2O_2 were interpolated from a standard curve. Concentrations are expressed as nM ml^{-1}.

Cloning of Mn-SOD cDNA

Mixed primers for Mn-SOD of *C. gigas* (cgMn-SOD) were designed using highly conserved regions of *M. yessoensis* (GenBank accession no. AB222783), *Haliotis discus discus* (DQ530210), and *Biomphalaria glabrata* (AY500813) Mn-SOD mRNA: Mn-SOD forward primer (5′-AAG CAY ACW YTG CCA GAY CT-3′) and Mn-SOD reverse primer (5′-TAD GCR TGY TCC CAS ACA TC-3′). Total RNA was extracted from various tissues (gill, digestive gland, intestine, mantle, and adductor muscle) of the oyster using the Trizol method, according to the manufacturer's instruction (Gibco/BRL, Grand Island, NY, USA). The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. Two and a half micrograms of total RNA were reverse transcribed in a total volume of 20 μl, using an oligo-$d(T)_{15}$ anchor and M-MLV reverse transcriptase (Bioneer, Seoul, Korea) according to the manufacturer's protocol. Polymerase chain reaction (PCR) amplification was performed using a 2X Taq Premix I (Solgent, Daejeon, Korea), according to the manufacturer's instructions. PCR was carried out as follows: initial denaturation at 95°C for 2 min; 40 cycles of denaturation at 95°C for 20 s, annealing at 54°C for 40 s and extension at 72°C for 1 min; followed by 7 min at 72°C for the final extension. The amplified PCR product was processed by electrophoresis on 1% agarose gels. The PCR product was purified and ligated into the pGEM-T Easy Vector (Promega, Madison, WI, USA). The colony formed by transformation was cultivated in DH5α, and plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Seoul, Korea) and EcoRI (Fermentas, Hanover, MD, USA). Based on the plasmid DNA, Mn-SOD partial cDNA sequence data were analysed using an ABI DNA Sequencer (Appleid Biosystems, Foster City, CA, USA).

For RACE reactions, total RNA was extracted from the gills of *Crassostrea gigas*. Using 3 μg of total RNA as template, 3′-RACE-ready cDNA and 5′-RACE-ready cDNA were generated using the protocols and reagents provided in the CapFishing Full-length cDNA Premix kit (Seegene, Seoul, Korea). Gene-specific primers were selected from the Mn-SOD partial cDNA of *C. gigas* obtained by PCR. For 3′-RACE, the 50 μl PCR reaction mixture contained 5 μl of 3′-RACE-ready cDNA, 1 μl of 10 μM 3′ target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μl of 10 μM 3′ RACE cgMn-SOD primer (5′-ACA AGG CAG CCC ACT CTT TGA GGG TTG-3′) and 25 μl of SeeAmp Taq Plus Master Mix (Seegene). PCR was carried out for 40 cycles at 94°C for 45 s for denaturation, 62°C for 45 s for primer annealing, and 72°C for 90 s for extension; followed by 5 min at 72°C for extension. For 5′-RACE, the 50 μl PCR reaction mixture contained 5′-RACE-ready cDNA, 5′ RACE cgMn-SOD primer (5′-CTG AGG GTC TCC CAG AAT ATG CTG TGG-3′), 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), and SeeAmp Taq Plus Master Mix at the same volumes as for 3′-RACE under the same PCR conditions. Transformation and sequencing were conducted using the same methods described above. The DNA and deduced amino acid sequences were analysed using GENETYX-WIN (Software Development, Tokyo, Japan) and the BLAST algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov/blast).

Phylogenetic analysis of Mn-SOD

Phylogenetic analysis was performed on the amino acid sequences from full-length Mn-SOD cDNA from various vertebrates and molluscs. Amino acid sequence data were aligned using the BioEdit Software (Hall 1999). The sequences compared to the cgMn-SOD (GenBank accession no. EU420128) were as follows: Mn-SOD of *Mizuhopecten yessoensis* (AB222783), *Haliotis discus discus* (DQ530210), *Biomphalaria glabrata* (AY500813), *Danio rerio* (NM_199976), *Xenopus laevis* (NM_001090499),

Bos taurus (BT020988) and *Homo sapiens* (M36693). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei 1987) and analysed using Mega 3.1 software package (Center for Evolutionary Functional Genomics, Tempe, AZ, USA). The degree of support for internal branches was inferred using bootstrapping (1000 replicates) analysis.

Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was conducted to determine the relative expression of cgMn-SOD in various oyster tissues. Total RNA was extracted from the gill, digestive gland, intestine, mantle, and adductor muscle of experimental (Cd, TBT, and acute water temperature treatments) oysters. The extraction of total RNA and synthesis of cDNA was conducted as mentioned above. Primers for RT-PCR were designed with reference to cgMn-SOD cDNA (GenBank accession no. EU420128) by PCR and cgβ-actin (AF026063) gene sequences of Pacific oyster as follows: cgMn-SOD forward primer, 5′-AAG CAC ACC TTA CCA GAT CT-3′; cgMn-SOD reverse primer, 5′-ATA GCG TGC TCC CAG ACA TC-3′; cgβ-actin forward primer, 5′-GAC TTC GAA CAA GAG ATG-3′; and cgβ-actin reverse primer, 5′-GAT ATC GAC ATC ACA TTT C-3′. Polymerase chain reaction (PCR) amplification was performed using a 2X Taq Premix I (Solgent, Daejeon, Korea), according to the manufacturer's instructions. PCR was carried out as follows: initial denaturation at 95°C for 2 min; 35 (cgMn-SOD) and 25 (cgβ-actin) cycles of denaturation at 95°C for 20 s, annealing at 54°C (cgMn-SOD and cgβ-actin) for 40 s and extension at 72°C for 1 min; followed by 7 min at 72°C for the final extension. The cgβ-actin was amplified in each PCR reaction as a loading control. The PCR products from different cycles of amplification were visualized on a UV-transilluminator after electrophoresis on 1% agarose gel containing ethidium bromide (0.5 μg μl^{-1}). The signal intensity was quantified using the Gel-Doc System and Gelpro 3.1 software (KBT, Incheon, Korea). The cycle numbers that generate half-maximal amplification were used for subsequent quantitative analysis of gene expression, and they are 35 cycles for Mn-SOD and 25 cycles for β-actin.

Quantitative real-time PCR (QPCR)

QPCR was conducted to determine the relative mRNA expression of Mn-SOD. Total RNA was extracted from the gill of experimental (Cd, TBT, and acute water temperature treatments) oysters. The extraction of total RNA and synthesis of cDNA were conducted as mentioned above. Primers for QPCR were designed with reference to known Mn-SOD (GenBank accession no. EU420128) and cgβ-actin (AF026063) gene sequences of the *Crassostrea gigas* as follows: cgMn-SOD forward primer, 5′-GAC CTG CCC TAT GAC TAC AAT GC-3′; cgMn-SOD reverse primer, 5′-TCT GGT GAT GTT TGC TGT GAT 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 manufacturers' instructions. QPCR was undertaken by denaturation at 95°C for 5 min; followed by 40 cycles of denaturation at 95°C for 20 s and annealing at 55°C for 20 s. To ensure that the primers amplified a specific product, we performed a melt curve (data not shown), as well as analysed the PCR product size using capillary electrophoresis (Agilent Technologies, Santa Clara, CA, USA). All primers used were shown to amplify only one size of template, melting at only one temperature. PCR products were also confirmed by sequencing (data not shown). QPCR was conducted to determine the expression of Mn-SOD mRNA relative to β-actin mRNA using total RNA extracted from the gills of the control and experimental oysters ($n=5$). Each experimental group was run in triplicate to ensure consistency. As an internal control, experiments were duplicated with β-actin, and all data were expressed as the change with respect to the corresponding β-actin Ct levels. All analyses were based on the 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 programme, QPCR data from three replicate samples were analysed with analysis software of the system (Bio-Rad) to estimate transcript copy numbers for each sample. The efficiencies of the reactions were determined by performing the QPCR. The efficiencies were found to be as follows: β-actin = 97.3%, Mn-SOD = 95.2%. Also, to ensure that the primers amplified a specific product, we performed a melt curve, melting at only one temperature. The mRNA expression levels stood for an *n*-fold difference relative to β-actin as the internal control.

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

Identification of Mn-SOD cDNA

RT-PCR was used to generate a 496-base pair fragment of Mn-SOD using total RNA extracted from *Crassostrea gigas*. A PCR-based cloning strategy (RT-PCR followed by 3′ and 5′ RACE) was used to clone a full-length cDNA encoding a putative Mn-SOD from the gill tissue of *C. gigas*. cgMn-SOD full-length cDNA contained 965 nucleotides including an open reading frame of 675 nucleotides that was predicted to encode a protein of 225 amino acids. The cDNA sequence of the cgMn-SOD gene was deposited in NCBI/GenBank under accession number EU420128.

The predicted amino acid sequence of cgMn-SOD was compared to those deduced from known Mn-SOD cDNAs of other vertebrates and molluscs (Figure 1). The cgMn-SOD had high amino acid sequence identity with other molluscs: 63% identity to *Haliotis discus discus* Mn-SOD (GenBank accession no. DQ530210) and 62% identity to *Mizuhopecten yessoensis* (AB222783) and *Biomphalaria glabrata* (AY500813) Mn-SODs. cgMn-SOD was also similar to Mn-SODs from vertebrates: 60% identity to *Xenopus laevis* (NM_001090499), *Bos taurus* (BT020988), and *Homo sapiens* (M36693) Mn-SODs, and 58% to *Danio rerio* Mn-SOD (NM_199976). The Mn-SODs showed four conserved manganese-binding sites responsible for coordinating the metal (His^{53}, His^{101}, Asp^{187}, and His^{191}) and characteristic signatures (DVWEHAYY; residues 187–194) among all species (Figure 1).

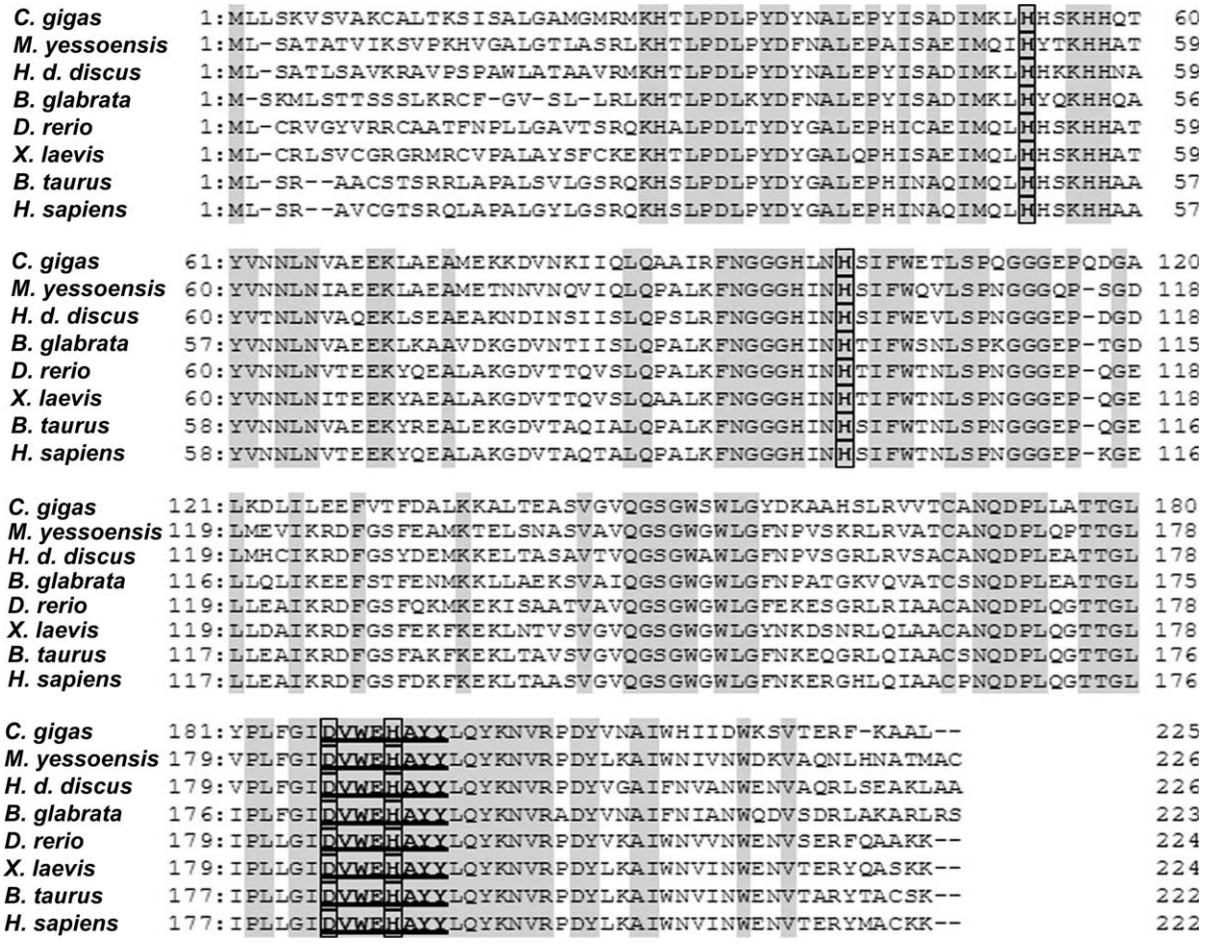

Figure 1. Multiple alignment of the Mn-SOD gene of *Crassostrea gigas* (GenBank accession no. EU420128), *Mizuhopecten yessoensis* (AB222783), *Haliotis discus discus* (DQ530210), *Biomphalaria glabrata* (AY500813), *Danio rerio* (NM_199976), *Xenopus laevis* (NM_001090499), *Bos taurus* (BT020988), and *Homo sapiens* (M36693). The Mn-SOD signature sequence (DVWEHAYY) is bold and underlined. Four conserved amino acids responsible for manganese binding are bold and boxed. Identical amino acids among the different species are indicated by shaded regions.

A phylogenetic tree was constructed to further analyse the evolutionary relationship of Mn-SOD sequences among various vertebrates and molluscs. Phylogenetic analysis indicated that cgMn-SOD clustered closely with the Mn-SOD of *H. discus discus* (Figure 2).

Hydrogen peroxide (H_2O_2) concentrations

Haemolymph H_2O_2 concentrations significantly increased following Cd, TBT, and water temperature change treatments in a dose- and time-dependent manner (Figure 3). H_2O_2 concentrations increased with time and reached the highest level after 11 days with exposure to 0.01 (4 ± 0.3 nM ml^{-1}), 0.05 (6 ± 0.6 nM ml^{-1}), and 0.1 ppm Cd (10.5 ± 0.8 nM ml^{-1}) from an initial level of 2.4 ± 0.3 nM ml^{-1}. The maximal response of H_2O_2 concentrations was observed at the highest dose of Cd tested (0.1 ppm) (Figure 3A). In TBT treatments, H_2O_2 concentrations increased significantly after 11 days with exposure to 5 (3.9 ± 0.3 nM ml^{-1}), 10 (5.2 ± 0.2 nM ml^{-1}), and 20 ppb TBT (8.5 ± 0.5 nM ml^{-1}) from an initial concentration of 2.4 ± 0.3 nM ml^{-1} (Figure 3B). In acute water temperature treatments, H_2O_2 concentrations increased significantly by 3 h at 10°C (4.1 ± 0.5 nM ml^{-1}), 6 h at 30°C (6.1 ± 0.2 nM ml^{-1}), and then decreased to the level observed in the control (Figure 3C).

Tissue distribution of Mn-SOD

The expression of cgMn-SOD mRNA in various tissues from experimental (Cd, TBT, and acute water temperature treatments) oysters measured by RT-PCR (Figure 4). The cgMn-SOD mRNA was detected in all tissues tested and was highly expressed in gill. In contrast, digestive gland, intestine, mantle, and adductor muscle had low expression.

mRNA expression levels of Mn-SOD to $CdCl_2$ treatments

Cd treatments significantly increased Mn-SOD mRNA expression in gill tissue in a dose- and time-dependent manner (Figure 5A). The maximal response was observed at the highest dose of 0.1 ppm Cd tested. Mn-SOD mRNA expression increased over time, reaching the highest level after 11 days with exposure to 0.01 and 0.05 ppm. At 0.1 ppm, levels significantly increased for 7 days and then subsequently decreased. The Mn-SOD mRNA level was highest on 7 days (100 times greater than the control, $P < 0.05$) at 0.1 ppm.

mRNA expression levels of Mn-SOD to TBT treatments

TBT treatments significantly increased Mn-SOD mRNA expression in gill tissue in a dose- and time-dependent manner (Figure 5B). No significant effect of TBT was observed at 5 ppb. The maximum response was observed at the highest dose tested (20 ppb). Mn-SOD mRNA expression increased over time and reached the highest level after 11 days with exposure to 10 ppb. At 20 ppb, it significantly increased for 7 days and then decreased afterwards. The Mn-SOD mRNA level was highest on 7 days (51 times greater than the control, $P < 0.05$) at 20 ppb.

mRNA expression levels of Mn-SOD to acute water temperature treatments

Response of Mn-SOD mRNA expression in gill tissue in acute water temperature treatments are indicated

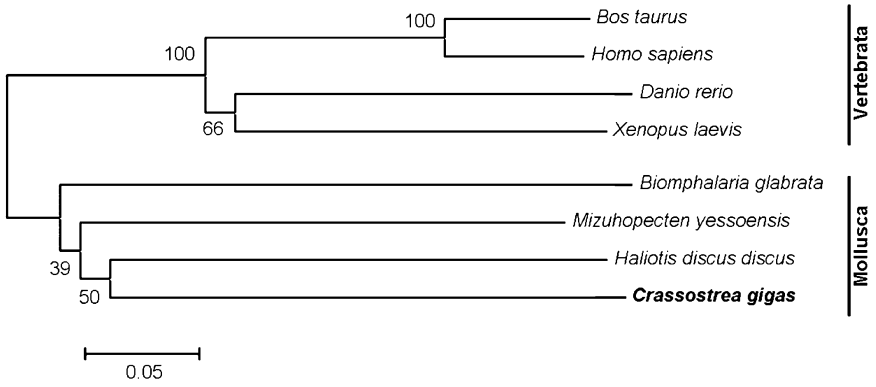

Figure 2. Phylogenetic tree based on amino acid alignments for Mn-SOD in vertebrates and mollusks. Bootstrap values (%) are indicated (1000 replicates). The score between two protein sequences, which is a measure of the relative phylogenetic relationship, is represented by the horizontal distance (i.e. a shorter distance indicates a higher degree of relatedness). The Mn-SOD sequences are *Crassostrea gigas* (GenBank accession no. EU420128), *Mizuhopecten yessoensis* (AB222783), *Haliotis discus discus* (DQ530210), *Biomphalaria glabrata* (AY500813), *Danio rerio* (NM_199976), *Xenopus laevis* (NM_001090499), *Bos taurus* (BT020988), and *Homo sapiens* (M36693). The scale bar indicates the evolutionary distance between groups.

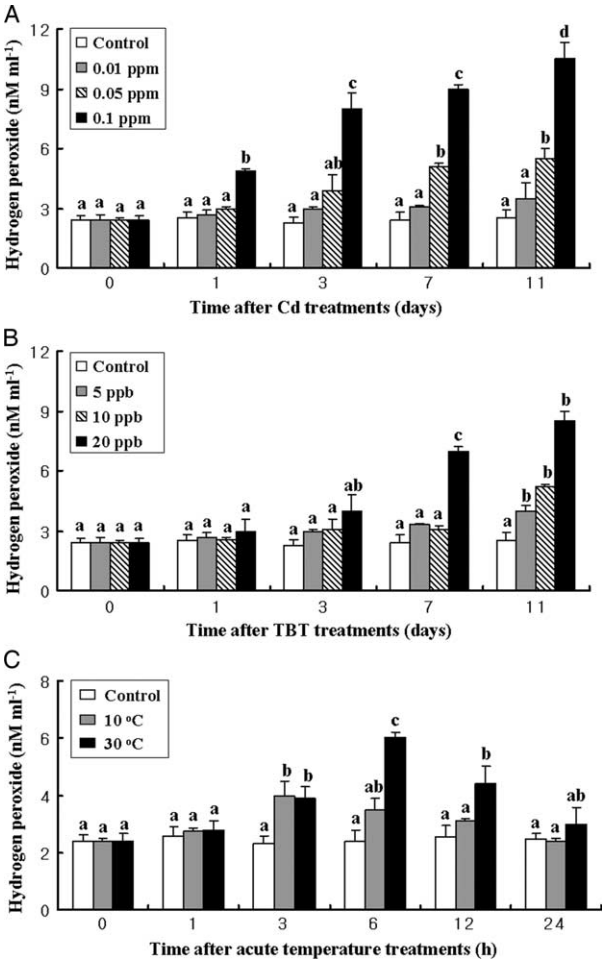

Figure 3. H_2O_2 concentrations in the haemolymph of *Crassostrea gigas* by (A) Cd treatments (0 [control], 0.01, 0.05, or 0.1 ppm Cd for 1, 3, 7, or 11 days), (B) TBT treatments (0 [control], 5, 10, or 20 ppb TBT for 1, 3, 7, or 11 days), and (C) acute water temperature treatments (20°C [control], 10°C [low], or 30°C [high] for 1, 3, 6, 12, or 24 h). Lowercase letters indicate significant differences ($P<0.05$) among sampling times. Values indicate the mean $\pm$SD ($n=5$).

in Figure 5C. No significant response of Mn-SOD mRNA expression was observed at 20°C (control). Mn-SOD mRNA expression at 10°C (low temperature) increased over time, reaching the highest level after 3 h and then decreased. Mn-SOD mRNA level was maximal at 3 h (6.3 times greater than the control, $P<0.05$) at 10°C. Mn-SOD mRNA expression at 30°C (high temperature) increased over time, reached the highest level after 6 h, and then decreased. Mn-SOD mRNA level was maximal at 6 h (7.5 times greater than the control, $P<0.05$) for 30°C.

Haemolymph analysis

Levels of GOT and GPT in the haemolymph are shown in Figure 6. In Cd treatments, GOT and GPT levels increased significantly by 7 days at concentrations of 0.05 (GOT: 3.7 ± 1.5 IU l^{-1}; GPT: 6.3 ± 3.1 IU l^{-1}) and 0.1 ppm (GOT: 4.3 ± 1.4 IU l^{-1}; GPT: 8.0 ± 3.0 IU l^{-1}) (Figure 6A). In TBT treatments, GOT levels increased significantly by 3 (11.3 ± 3.5 IU l^{-1}) and 7 days (17.3 ± 4.8 IU l^{-1}), and GPT levels increased significantly by 7 days (18.0 ± 5.0 IU l^{-1}) at 20 ppb (Figure 6B). In acute water temperature treatments, levels of GOT increased significantly by 3 (8.5 ± 2.0 IU l^{-1}) and 6 h (7.9 ± 3.5 IU l^{-1}), and the levels of GPT increased significantly by 1 (6.8 ± 1.6 IU l^{-1}) and 3 h (7.6 ± 2.4 IU l^{-1}) at 10°C (low temperature). GOT levels also increased significantly by 6 h (11.4 ± 2.5 IU l^{-1}), whereas no significant change in GPT levels was observed at 30°C (high temperature) (Figure 6C).

Discussion

To date, full-length Mn-SOD cDNA has been reported in only three species of mollusc: *Mizuhopecten yessoensis* (GenBank accession no. AB222783), *Haliotis discus discus* (DQ530210), and *Biomphalaria glabrata* (AY500813). In our study, we analysed Mn-SOD mRNA expression, H_2O_2, GOT and GPT levels of the Pacific oyster *Crassostrea gigas* in order to investigate the effects of Cd and TBT exposure and acute water temperature change on the physiological responses of this organism. Using the BLAST algorithm (Blastp) of the National Center

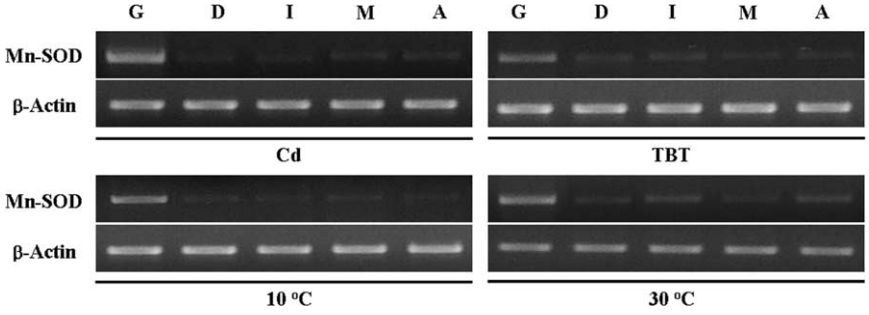

Figure 4. Tissue-specific expression of Mn-SOD mRNA in various tissues (gill, G; digestive gland, D; intestine, I; mantle, M; adductor muscle, A) from the Pacific oyster, *Crassostrea gigas* by Cd treatments (A), TBT treatments (B), and acute water temperature treatments (C) 10°C, (D) 30°C. Amplification of β-Actin was used as an internal control.

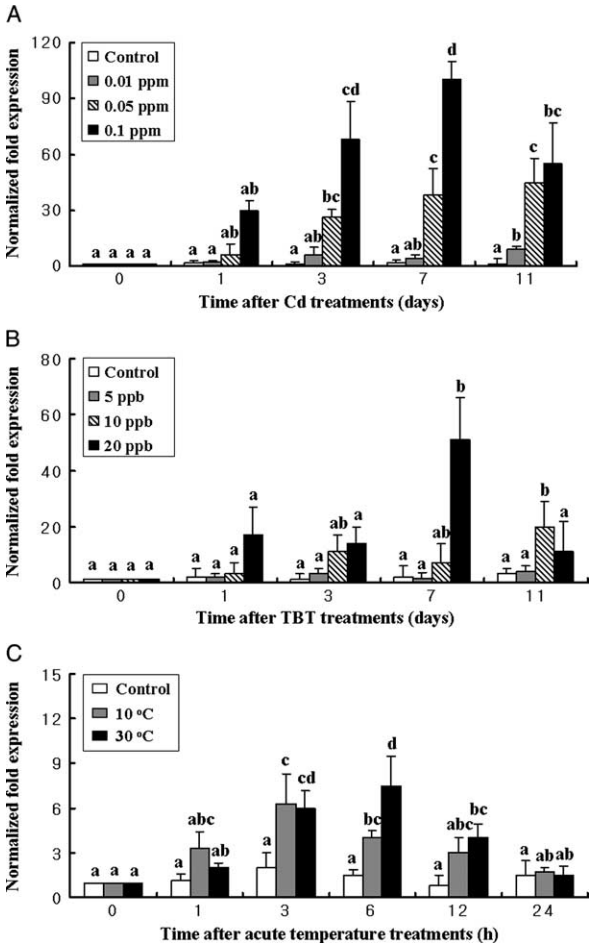

Figure 5. Response of Mn-SOD mRNA expression in *Crassostrea gigas* by (A) Cd treatments, (B) TBT treatments, and (C) acute water temperature treatments. cgMn-SOD mRNA expression levels relative to β-Actin levels were analysed using real-time PCR. Lowercase letters indicate significant differences ($P<0.05$) among sampling times. Values indicate the mean ± SD ($n=5$).

for Biotechnology Information, cgMn-SOD isolated in this study displayed homology of 62% (*H. discus discus*) or higher with that of invertebrates and also showed a homology of 58% (*Danio rerio*) or higher with Mn-SOD of vertebrates (Figure 1). Through multiple alignment analysis, we observed several characteristic elements among all species including cgMn-SOD. The cgMn-SOD signature sequence (DVWEHAYY; residues 187–194) and four conserved manganese-binding sites (His^{53}, His^{101}, Asp^{187}, and His^{191}) responsible for coordinating the metal were confirmed (Jackson & Cooper 1998) (Figure 1). High homology and conserved sites among species support the presumption that cgMn-SOD belongs to the Mn-SOD family and that it performs the functions and roles of Mn-SOD. cgMn-SOD was also phylogenetically closest to the *H. discus discus* Mn-SOD (Figure 2). Following our analysis using BioEdit software, we confirmed the molluscs (*C. gigas*, *H. discus discus*, *M. yessoensis*, and *B. glabrata*) and vertebrates (*D. rerio*, *Xenopus laevis*, *Bos taurus*, and *Homo sapiens*) as two separate groups of Mn-SOD and Mn-SOD of *C. gigas* was genetically the closest to that of *H. discus discus*.

We compared the expression of Mn-SOD mRNA in various tissues of experimental (Cd, TBT and acute water temperature treatments) oysters using RT-PCR (Figure 4). Mn-SOD mRNA expression was observed in all tissues. In particular, high levels of expression were observed in gill tissues. The gill tissue of Pacific oysters has an ample contact area with the surrounding environment. As the direct primary absorption route of environmental substances, the gills are largely influenced by external environments. Furthermore, they take in necessary food and oxygen, and discharge unnecessary residues through inflow/outflow of seawater (Legeay et al. 2005). Kim et al. (2007) reported high Mn-SOD mRNA expression levels in the gill tissues of abalone *H. discus discus* when treated with heavy metals and high water temperature. Therefore, we used gill tissue of *C. gigas* to compare Mn-SOD mRNA expression levels after exposure to Cd and TBT as well as to acute water temperature change.

Mn-SOD mRNA expression in gill tissue by exposure to Cd and TBT significantly increased with exposure time and concentration. Cd is a toxic heavy metal that exerts a high level of toxicity to living organisms even at low doses (Benavides et al. 2005). TBT, an organic compound containing tin, is an environmental pollutant of high toxicity that is contained in anti-marine biological adhesion agents (Lee & Lee 2003). Excessive Cd and TBT exposure induces various oxidative reactions and, in the process, can generate a large quantity of ROS that promote oxidative stress (Stohs et al. 2000). We found that Mn-SOD mRNA was expressed in order to eliminate oxidative stress caused by ROS. Funes et al. (2005) reported that SOD activity was higher in the oyster *Crassostrea angulata* and the mussel *Mytilus galloprovincialis* from regions of high heavy metal concentration than that of oysters and mussels from other areas. Our results confirm SOD mRNA expression induced in Pacific oysters due to oxidative stress by exposure to organic compounds (hydrocarbons) such as TBT, as reported by Boutet et al. (2004).

Interestingly, Mn-SOD mRNA expression significantly increased jointly with H_2O_2 concentrations until 7 days of exposure to 0.1 ppm Cd and 20 ppb TBT. However, while H_2O_2 concentration continued to increase, Mn-SOD mRNA expression decreased after 7 days of exposure. This indicated that extreme oxidative stress was induced after 7 days of exposure to pollutants. A decrease in Mn-SOD mRNA expression as the time of exposure elapsed

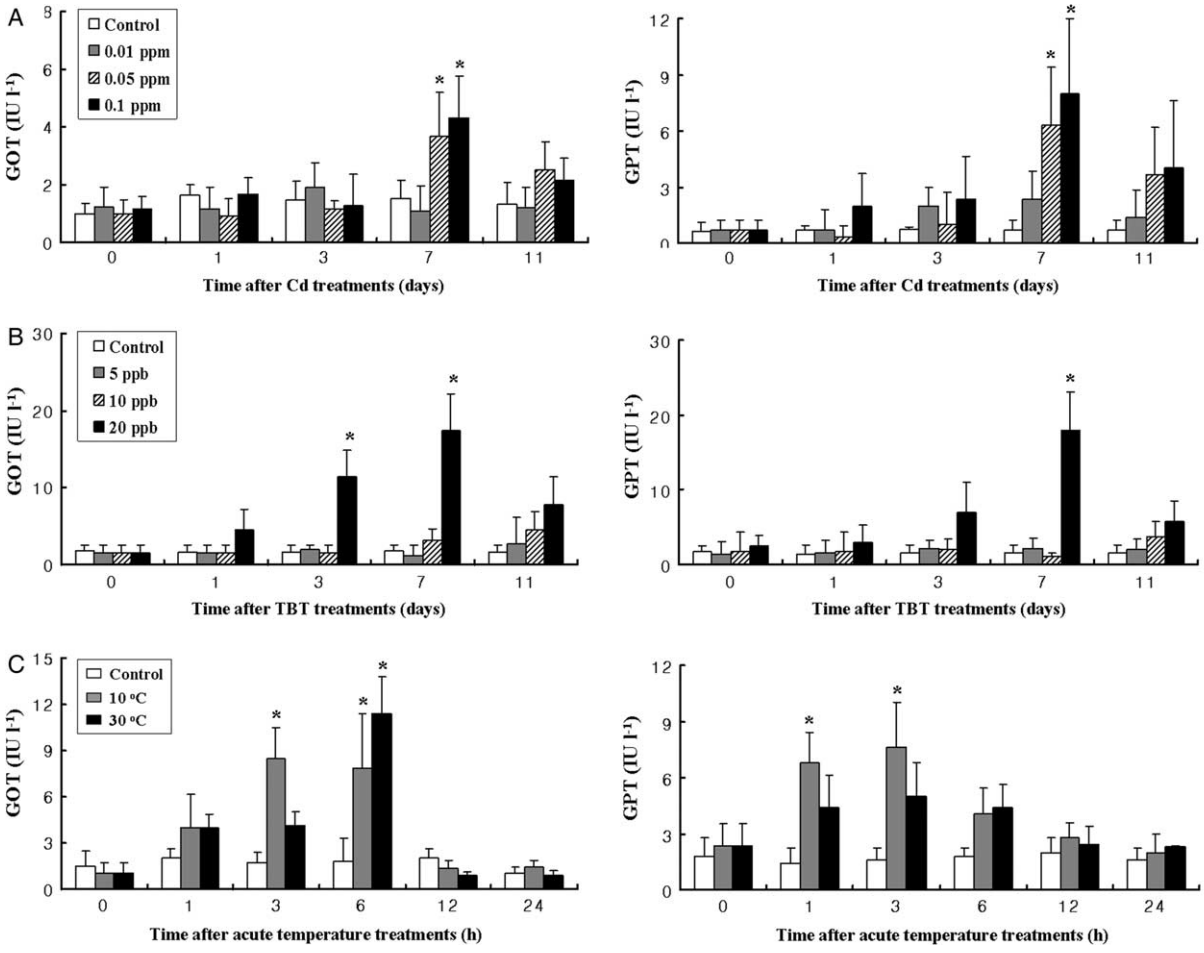

Figure 6. Change in the levels of the enzymes GOT and GPT in the haemolymph of *Crassostrea gigas* with (A) Cd treatments, (B) TBT treatments, and (C) acute water temperature treatments. Asterisks indicate a significant difference from the control ($P<0.05$). Values indicate the mean $\pm$SD ($n=5$).

is presumed to be the result of a lowered metabolic capacity due to the excessive generation of ROS that surpasses the controllable range of the antioxidant system. Zhang et al. (2004) reported that the metabolic function of a defence mechanism in an organism may be lost when placed in an environment of weak oxidative stress and subsequently exposed to strong oxidative stress. This supports the data obtained in our study.

ROS induce oxidative stress that can exert detrimental effects on organisms such as membrane damage, DNA breakage, lipid peroxidation, enzyme inhibition, amino acid oxidation, and apoptosis (Wang et al. 2004; Choi et al. 2007; Murugavel et al. 2007). Also, we measured H_2O_2 levels in order to identify the degree of oxidative stress by ROS generation after exposure to water temperature change and pollutants (Cd and TBT) in *C. gigas*. H_2O_2 levels in *C. gigas* displayed a tendency to increase as the concentration of pollutants and water temperature increased and as time elapsed (Figure 3). This result suggests that ROS generation was induced by Cd and TBT exposure. A decrease in enzymatic and non-enzymatic free radical scavengers caused by pollutants may contribute to the shift in the balance of free-radical metabolism toward H_2O_2 accumulation (Cho & Seo 2005). SOD dismutates a superoxide radical (O_2^-) into O_2 and H_2O_2 ($2O_2^- + H^+ \rightarrow H_2O_2 + O_2$) and, thus, releases ROS. Therefore, we found that ROS were generated due to acute water temperature change and toxicity of pollutants (Cd and TBT), and identified the degree of oxidative stress in *C. gigas* and its possible correlation with SOD by measuring the level of H_2O_2 production.

Water temperature acts as an important physical element for poikilothermic Pacific oysters. In our study, we moved *C. gigas* acclimated to 20°C, immediately to 10°C and 30°C sea water. As a result, we observed an increase in H_2O_2 concentration with a significant increase in Mn-SOD mRNA expression at 3 h of exposure to 10°C and at 6 h of exposure to 30°C. Bagnyukova et al. (2007) and Chen et al. (2007) reported in their studies on goldfish and scallops, respectively, that acute water temperature change generated ROS and, therefore, induced oxidative stress. Accordingly, increases of

H_2O_2 concentration in *C. gigas* haemolymph indicate that ROS was induced as a result of acute water temperature change and that Mn-SOD mRNA was expressed to eliminate H_2O_2 as a defence mechanism. Mn-SOD mRNA expression and H_2O_2 concentration also started to decrease slowly after 3 h of exposure to 10°C and 6 h of exposure to 30°C. This is a result of the antioxidant system in eliminating ROS generated by acute water temperature change and can be interpreted as the process of acclimation to the environment as the time of exposure elapsed. This result corroborates studies conducted on the stress of acute water temperature change and SOD to abalone (Kim et al. 2005, 2007).

The defence mechanism in molluscs, such as Pacific oysters, is triggered from the outside as blood cells in haemolymph, as well as proteins, carbohydrates, salt, and ions, are passed from the heart sac into tissues and are discharged (Gagnaire et al. 2006). Changes in constituting properties of haemolymph can occur due to various pollutants and environmental factors (His et al. 1996; Xue & Tristan 2000). Among the constituting properties of haemolymph, an increase in GOT and GPT activation is generally caused by inflow to the haemolymph of cells separated due to tissue damage by environmental contaminants (Casillas et al. 1982). As we measured GOT and GPT activities, significant increases of GOT and GPT were observed on the seventh day of exposure to 0.05 and 0.1 ppm Cd and 7 days of exposure to 20 ppb TBT. In the acute water temperature treatment, significant increases were observed by 3 h of exposure to 10°C and 6 h of exposure to 30°C. This result was similar to the report by Cho et al. (2001) and Choi et al. (2008) that showed GOT activity increased in Pacific oysters when exposed to pollutants. Therefore, it is deduced that the significant increase of GOT and GPT activities in haemolymph of Pacific oysters was due to tissue damage by pollutants and acute water temperature change (Pickwell & Steinert 1988).

In this study, we found that cell damage was caused by extreme oxidative stress induced by a large quantity of ROS generation in *C. gigas* when exposed to Cd and TBT as well as to acute water temperature change. The expression of Mn-SOD mRNA as an antioxidant mechanism increased in order to protect cells from oxidative stress and to help eliminate the oxidative stress. Therefore, Mn-SOD mRNA expression, haemolymph H_2O_2 concentration, and GOT and GPT activities can be used as physiological markers to determine the degree of stress in Pacific oysters. Studies of Mn-SOD expression and oxidative stress have rarely been reported, and further studies are needed.

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