

Cloning and mRNA expression of antioxidant enzymes in the Pacific oyster, *Crassostrea gigas* in response to cadmium exposure

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Abstract

Cadmium (Cd) is one of the most toxic heavy metal pollutants in the aquatic environment and can induce the formation of reactive oxygen species (ROS) that cause oxidative stress. In present study, we cloned catalase (CAT) and glutathione peroxidase (GPX) cDNA, and investigated its time- and dose-related effects of three Cd concentrations (0.01, 0.05 or 0.1 ppm) on mRNA levels of antioxidant enzymes (superoxide dismutase (SOD), CAT, GPX) in the gill and changes enzyme levels in the hemolymph of the Pacific oyster, *Crassostrea gigas*. The cDNA identified encoded proteins of 516 and 244 amino acids corresponding to CAT and GPX, respectively. BLAST analysis from other species indicated that the residues essential to the enzymatic function of CAT and GPX proteins of *C. gigas* are highly conserved. Cd treatment significantly increased antioxidant enzyme mRNA expression in the gill in a time- and dose-dependent manner. The mRNA expression at 0.1 ppm Cd concentration increased up to 3 days (CAT, GPX) or 7 days (SOD) and then decreased by 7 days (CAT, GPX) or 11 days (SOD). Aspartate aminotransferase, alanine aminotransferase and hydrogen peroxide (H₂O₂) concentrations levels increased significantly with exposure to 0.05 or 0.1 ppm Cd for 7 days. These results suggest that antioxidant enzymes play important roles in the physiological changes related to metabolism and cell protection that occur in Pacific oysters exposed to Cd.

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1. Introduction

Recently, heavy metal contamination has become prominent along the southern coast of Korea where the farming of bivalve mollusks takes place. The contamination is caused by aging farming facilities, aquatic wastes, sediments within the farmland, and abandoned copper mines. Cadmium (Cd) is a heavy metal pollutant in the aquatic environment that is highly toxic to humans, even at low doses (Benavides et al., 2005). Cd is released to and accumulated in not only the natural environment, but also the aquatic environment, through industrial activities such as mining, plating and metal refining (Choi et al., 2007).

Among aquatic organisms, bivalve mollusks, including oysters, have poor mobility in relation to species such as fish and crustaceans and are therefore wholly exposed to Cd concentrations in their habitats. Bivalve mollusks also feed via filtration through the gills, where a large amount of Cd can be absorbed from the environment and accumulated in the body (Philip, 1995). Excessive Cd accumulation in body tissues induces a variety of oxidative effects. Of these, a large amount of reactive oxygen species (ROS), e.g., superoxide radical (O⁻²), hydrogen peroxide (H₂O₂), hydroxyl radical (HO⁻), and singlet oxygen (¹O₂), are formed and promote oxidative stress (Stohs et al., 2000). Oxidative stress caused by ROS leads to lipid peroxidation, protein denaturation, and DNA damage to the constituents of a living body. It also changes and inhibits a variety of enzyme activation effects and causes cell damage and an imbalance in cells resulting in apoptosis (Choi et al., 2007). Accordingly, this causes numerous

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physiological problems such as the promotion of aging, reduction in disease resistance, and lowering of reproductive ability (Kim and Phyllis, 1998).

Living organisms have antioxidant defense systems for protection against oxidative stress caused by heavy metals and other toxic substances. The antioxidant defense system consists of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). These antioxidant enzymes play an important role in maintaining cellular homeostasis and antioxidant defense by removing ROS (Rudneva, 1999). SOD is one of the most representative defense elements against ROS. An important metalloenzyme, it is found in every oxygen-consuming living organism. SOD removes the superoxide radical through the process of dismutation to O_2 and H_2O_2 ($2O_2^- + H^+ \rightarrow H_2O_2 + O_2$) (Fridovich, 1975). The H_2O_2 produced from this process is reduced to H_2O and O_2 by CAT and GPX (Mruk et al., 2002). CAT is an oxidoreductase enzyme that breaks down two molecules of hydrogen peroxide to two molecules of water and oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$), therefore removing the toxicity of hydrogen peroxide (Kashiwagi et al., 1997). GPX is another important peroxidase enzyme for the detoxification of hydroperoxide. It catalyzes the reduction of hydroperoxide and hydrogen peroxide to water and oxygen (Swiergosz-Kowalewska et al., 2006). GPX is divided into two types: selenium-dependent GPX (Se-GPX) and selenium-independent GPX (non-Se-GPX). Se-GPX catalyzes the reduction of organic and inorganic peroxides, whereas non-Se-GPX only reduces organic peroxides (Almar et al., 1998).

The enzymatic antioxidant system that includes SOD, CAT, and GPX is enhanced as an adaptation or a compensatory reaction to ROS formation or toxic effects (Livingstone et al., 1990). The induction of such an enzymatic antioxidant system is receiving much attention as a biomarker that indicates oxidative stress in the environment. Using this system, studies to identify aquatic environmental contamination are being carried out in a variety of species, including bivalve mollusks.

Among such studies are those of the antioxidant system in relation to Cd contamination in a variety of fish species such as tilapia, *Oreochromis mossambicus* (Almeida et al., 2002) and *Oreochromis mossambicus* (Siraj Basha and Usha Rani, 2003), brown trout, *Salmo trutta* (Hansen et al., 2007), sea bass, *Dicentrarchus labrax* (Romeo et al., 2000) and rock bream, *Oplegnathus fasciatus* (Cho et al., 2006). In bivalve mollusks, studies are limited to mussels such as *Bathymodiolus azoricus* (Company et al., 2004), *Perna perna* (Almeida et al., 2004) and *Mytilus galloprovincialis* (Viarengo, 1990). Thus far, no examination of oysters has been made. Also, most studies of antioxidant enzymes have examined the relation to the activation of antioxidant enzymes (Brouwer et al., 1998; Geret et al., 2002); however, there are insufficient studies of the mRNA expression of antioxidant enzymes at the cellular level.

Therefore, we cloned and sequenced CAT and GPX cDNA, and analyzed changes in enzymatic properties (i.e., aspartate aminotransferase, AspAT; alanine aminotransferase, AlaAT) and H_2O_2 concentrations in hemolymph, as well as changes in the patterns of antioxidant enzymes (SOD, CAT and GPX) mRNA

expression, to identify the extent of oxidative stress caused by Cd to Pacific oyster.

2. Materials and methods

2.1. Experimental oysters

We used 1-year-old Pacific oysters (*Crassostrea gigas*) (average shell length: 112 ± 10.7 mm; height: 31.1 ± 5.4 mm; mass: 20.3 ± 3.9 g) obtained from the oyster hatchery on Daebu Island in Goseong (Gyeongnam, Korea). Oysters were placed in 50-L circulating filter tanks in the laboratory at 50 specimens per tank. During the experimental period, the water temperature and salinity were maintained at 20 ± 1 °C and 35 ppt, respectively, under a photoperiod of 12 h light/12 h dark, and no food was supplied.

2.2. $CdCl_2$ treatments and sampling

After acclimatization for 48 h in the tanks, 30 oysters were transferred to 30-L plastic aquaria filled with filtered natural seawater (control) or Cd-treated seawater. For Cd treatments, Cd was added to the water as $CdCl_2 \cdot 2.5H_2O$ (Kanto Chemical Co., Tokyo, Japan) to a dissolved Cd^{2+} concentration of 0.01, 0.05 or 0.1 ppm. Oysters were exposed to treatments for 11 days; the water was changed daily and resupplied with the corresponding concentration of Cd. No mortality was observed in either the Cd treatment or control group during the experimental period. The tissues (i.e., gill, digestive gland, intestine, mantle and adductor muscle) were sampled from randomly selected five oysters after 0, 1, 3, 7 and 11 days of treatments and stored at -80 °C until the extraction of total RNA.

2.3. Hemolymph analysis

Hemolymph was withdrawn from the pericardial cavity using a 3-mL syringe. The samples were centrifuged at $10,000 \times g$ at 4 °C for 5 min, and the supernatant was stored at -80 °C. The activities of AspAT (EC 2.6.1.1) and AlaAT (EC 2.6.2.1) were measured using Pureauto S AST (Daichi, Tokyo, Japan) and Pureauto S ALT (Daichi) kit by biochemistry autoanalyzer (model 7180; Hitachi, Tokyo, Japan), according to the manufacturer's instructions.

2.4. Hydrogen peroxide 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 NaCl, 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 color reagent was prepared by mixing 100 mL distilled water containing 100 mM sorbitol and 125 μ M

xylene 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 was then added to each well and allowed to incubate at room temperature for 1 h. Absorbance was read at 560 nm and concentrations of H₂O₂ were interpolated from a standard curve. Concentrations are expressed as nM/mL.

2.5. Identification of CAT and GPX cDNA

Mixed primers for CAT were designed using highly conserved regions of *Mytilus edulis* (GenBank accession no. AY580271), *Mytilus californianus* (AY580259), and *Nucula proxima* (AY580231) CAT mRNA: CAT forward primer (5'-GAT CCT ATG YTG TTY CCM AGC-3') and CAT reverse primer (5'-AGA AGG CKATYT GTT CYA CYT C-3'). Mixed primers for GPX were designed using *Unio tumidus* (DQ830766), *Xenopus tropicalis* (NM_203630), and *Ixodes scapularis* (DQ066177) GPX mRNA: GPX forward primer (5'-AAY GTR GCS ACM TAC TGA GG-3') and GPX reverse primer (5'-CAG AAA CTT YTC RAA GTT CCA-3'). Total RNA was extracted from the gill of Pacific oyster using a Trizol kit (Gibco/BRL, Grand Island, NY, USA). With 2.5 µg of total RNA as a template, cDNA were synthesized using M-MLV reverse transcriptase (Bioneer, Seoul, Korea). First-strand cDNA synthesis was conducted at 42 °C for 1 h using oligo-d(T)₁₅ primer (Promega, Madison, WI, USA). Polymerase chain reaction (PCR) amplification was performed using a 2X Taq Premix I (Solgent, Daejeon, Korea), according to the manufacturer's instructions. 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). 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, CAT and GPX partial cDNA sequence data were analyzed using an ABI DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.6. Rapid amplification of complementary DNA 3' and 5' ends (3' and 5' RACE) of CAT and GPX

For RACE reactions, total RNA was extracted from the gills of Pacific oyster. Using 3 µg of total RNA as template, 5'-RACE-ready cDNA and 3'-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 CAT and GPX partial cDNA of the Pacific oyster 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-3'), 1 µL of 10 µM 3' RACE gene-specific primer (3' RACE cgCAT primer: 5'-GCG ACC TAC TGA GGT TTC ACC TAC CAG-3', 3' RACE cgGPX primer: 5'-CGA GGT CGG GAA GAT GGT GCT GAA CAG-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' gene-specific primer (5' RACE cgCAT primer: 5'-CTG GTA GGT GAA ACC TCA GTA GGT CGC-3', 5' RACE cgGPX primer: 5'-CTA TAA CCG TCC GGT GTG CCT CGG TCT-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 analyzed using GENETYX-WIN (Software Development, Tokyo, Japan) and the BLAST algorithm at the NCBI website (<http://www.ncbi.nlm.nih.gov/blast>).

2.7. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was conducted to determine the relative expression of SOD, CAT, and GPX mRNA and 28S ribosomal RNA (28Sr) in various oyster tissues. 2.5 µg of total RNA extracted from the gill, digestive gland, intestine, mantle and adductor muscle of Cd-treated oysters were reverse transcribed with M-MLV reverse transcriptase (Bioneer) and oligo-d(T)₁₅ primer (Promega). Primers for RT-PCR were designed with reference to known SOD (GenBank accession no. AJ496219), CAT (EF687775), GPX (EF692639) and 28S ribosomal RNA (28Sr) (Z29546) gene sequences of the Pacific oyster as follows: cgSOD forward primer, 5'-ATG TCATCT GCT CTG AAG GC-3'; cgSOD reverse primer, 5'-TGG TGA TAC CGA TCA CTC CA-3'; cgCAT forward primer, 5'-GAC CCC ATC CTG TTC CCC AGC-3'; cgCAT reverse primer, 5'-AGA AGG CGATCT GTT CCA CCT C-3'; cgGPX forward primer, 5'-AAC GTA GCG ACC TAC TGA GG-3'; cgGPX reverse primer, 5'-AAG AAA CTT CTC GAA GTT CCA-3'; cg28Sr forward primer, 5'-TGC TCT GGA CTG TCC TAG GA-3'; and cg28Sr reverse primer, 5'-ACC GAT TCG CCA CTG ACC AT-3'. Polymerase chain reaction (PCR) amplification was performed using a 2X Taq Premix I (Solgent, Daejeon, Korea), according to the manufacturer's instructions. The 28Sr 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). 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 30 cycles for SOD, CAT and GPX, 25 cycles for 28Sr.

2.8. Quantitative real-time PCR (QPCR)

QPCR was conducted to determine the relative mRNA expression of antioxidant enzymes (SOD, CAT, and GPX) using total RNA extracted from the gills of control and Cd-treated oysters. With 2.5 µg of total RNA as a template, cDNA were

<i>C. gigas</i>	1:M-STRDKATEQLNEFK-LSHA-TPEQCTTGTGAPIGLKTATMTAGPLGVLVQDFVFNDE	57
<i>C. farreri</i>	1:M-ANRDKATNQLLEEF--KKAQSKADVLTTGTGAPVGTKTATLTAGPRGPVLIQDFTFTDE	57
<i>H. D. discus</i>	1:M-ATRDKASEQLNEF--SKGQKPDVLTGTGAPVGRKTATMTVGPVLLQDFVFTDE	57
<i>D. rerio</i>	1:MADREKSTDMKLWKEGRGSRPDVLTGAGVPIGDKLNAMTAGPRGPLLVDVVFTE	60
<i>O. fasciatus</i>	1:MADNRGKATDMKMTWKENRSSQRPDVLTGAGHPVGDKLNLTQTAGPRGPLLVDVVFTE	60
<i>R. rugosa</i>	1:MADRREKSADQMKLWESRANQKPDVLTGGGNPVSDKLNLLTVGPRGPLLVDVVFTE	60
<i>R. norvegicus</i>	1:MADSRDPASDQMKQWKEQRAPQKPDVLTGGGNPIGDKLNIMTAGPRGPLLVDVVFTE	60
<i>H. sapiens</i>	1:MADSRDPASDQMHWKEQRAAQKADVLTGAGNPVGDKLNVIIVGPRGPLLVDVVFTE	60
	* *	
<i>C. gigas</i>	58:MAH <u>FDRERIPERVVHAKGAGA</u> FGYFECTHDISKYTKAKPFESVGGKTPVGVRFSTVGGES	117
<i>C. farreri</i>	58:MAH <u>FNRERIPERVVHAKGGGA</u> FGYFEVTHDITKYCKAKPFVGVGKTPVGIKRFSTVGGES	117
<i>H. D. discus</i>	58:MAH <u>FNRERIPERVVHAKGAGA</u> FGYLEITHDITKYCKAKVFERVGGKTPLAIRFSTVGGEK	117
<i>D. rerio</i>	61:MAH <u>FDRERIPERVVHAKGAGA</u> FGYFEVTHDITRYSKAKVFEHVGGKTPIAVRFSTVAGEA	120
<i>O. fasciatus</i>	61:MAH <u>FDRERIPERVVHAKGAGA</u> FGYFEVTHDITRYSKAKVFEHVGGKTPIAVRFSTVAGES	120
<i>R. rugosa</i>	61:MAH <u>FDRERIPERVVHAKGAGA</u> FGYFEVTHDITRYSKAKVFEFIGKRTPIAVRFSTVAGEA	120
<i>R. norvegicus</i>	61:MAH <u>FDRERIPERVVHAKGAGA</u> FGYFEVTHDITRYSKAKVFEHIGKRTPIAVRFSTVAGES	120
<i>H. sapiens</i>	61:MAH <u>FDRERIPERVVHAKGAGA</u> FGYFEVTHDITKYSKAKVFEHIGKRTPIAVRFSTVAGES	120
	**** *	
<i>C. gigas</i>	118:GSADTARDPRGFVAVKMYTEDGNWDIVGNNNTPIFFIRDPIILFSPFIHTQKRNPRTLKDPD	177
<i>C. farreri</i>	118:GSADSARDPRGFVAVKMYTEDGNWDVVGNNNTPIFFIRDPMPLFPNFIHTQKRNPQTHLKDPD	177
<i>H. D. discus</i>	118:GSADTARDPRGFAIKFYTEDGNWDLVGNNNTPIFFIRDPMPLFPNFIHTQKRNPQTHLKDPD	177
<i>D. rerio</i>	121:GSSDTRDPRGFVAVKMYTEDGNWDLTGNNNTPIFFIRDVTLFSPFIHQSQRNPQTHLKDPD	180
<i>O. fasciatus</i>	121:GSADTARDPRGFVAVKMYTEEGNWDLTGNNNTPIFFIRDALLFSPFIHQSQRNPQTHMKDPD	180
<i>R. rugosa</i>	121:GSADTARDPRGFVAVKMYTEDGNWDLTGNNNTPIFFIRDAMLFSPFIHQSQRNPQTHMKDPD	180
<i>R. norvegicus</i>	121:GSADTARDPRGFVAVKMYTEDGNWDLVGNNTPIFFIRDAMLFSPFIHQSQRNPQTHLKDPD	180
<i>H. sapiens</i>	121:GSADTARDPRGFVAVKMYTEDGNWDLVGNNTPIFFIRDPIILFSPFIHQSQRNPQTHLKDPD	180
	** *	
<i>C. gigas</i>	178:MFWDFISLRPETHQVSFLFSRDRGTPDGYRRMNGYGSHTFKLVNKDDKPVCFKHFHKTQ	237
<i>C. farreri</i>	178:MFWDFISLRPETHQVSFLFSRDRGTPNGFRKMNGYGSHTFKMVNKEGKPVYCKFHFKTDQ	237
<i>H. D. discus</i>	178:MFWDFITLRPETHQVAFLFSNRGTPDGYRHMNGYGSHTFKMVNAKGEVYCKFHFKTNQ	237
<i>D. rerio</i>	181:MVWDFWLSLRPESLHQVSFLFSRDRGIPDGYRHMNGYGSHTFKLVNAQQGQPVYCKFHFKTNQ	240
<i>O. fasciatus</i>	181:MVWDFWLSLRPESLHQVSFLFSRDRGLPDGYRHMNGYGSHTFKLVNAAGERFYCKFHFKTDQ	240
<i>R. rugosa</i>	181:MVWDFWALRPESLHQVSFLFSRDRGIPDGHHRMNGYGSHTFKLVNAKDEPIYCKFHFKTDQ	240
<i>R. norvegicus</i>	181:MVWDFWLSLRPESLHQVTFLFSRDRGIPDGHHRMNGYGSHTFKLVNANGEAVYCKFHFKTDQ	240
<i>H. sapiens</i>	181:MVWDFWLSLRPESLHQVSFLFSRDRGIPDGHHRMNGYGSHTFKLVNANGEAVYCKFHFKTKGQ	240
	* *	
<i>C. gigas</i>	238:GIQNLSAAEANRLSAEDPDYAIRDLYNINIEDGKYPSTLKIQIMTFEQAEEKYKWNPFDLT	297
<i>C. farreri</i>	238:GIKNLMADQAAELSKNDPDYAIRDLFNALISEGDFPSWSLFIQVMTFEEAEKFKYNPFDLT	297
<i>H. D. discus</i>	238:GIKNLTGAQADKLASVDPDYATRDLYNALIAEGKYPWSLFIQVMNVKDAEKLKWNPFDLT	297
<i>D. rerio</i>	241:GIKNI PV E EADRLAATDPDYSIRDLYNALIANGNFSPWTFYIQVMTFEQAENKWNPFDLT	300
<i>O. fasciatus</i>	241:GIKNLPVEEADRLASTNPDYAIGDLFNALIANGNCPSWTFYIQIMTFEQAEEKFRPNPFDLT	300
<i>R. rugosa</i>	241:GIRNLTVEEANRLSAEDPDYIGDLYEAIANGNYPWTFYIQVMTFEQAERYFPNPFDLT	300
<i>R. norvegicus</i>	241:GIKNLPVEEAGRLAQEDPDYGLRDLFNALIASGNYPWTFYIQVMTFEAEATFPNPFDLT	300
<i>H. sapiens</i>	241:GIKNLSVEDAARLSQEDPDYIGIRDLFNALATGKDPSWTFYIQVMTFNQAETFPNPFDLT	300
	** *	
<i>C. gigas</i>	298:KVWSQKDYPLIEVGKMLNRPNNYFAEVEQIAFSPAHIIPGVEASPKMLQGR <u>LFYSYSD</u>	357
<i>C. farreri</i>	298:KVWPHGEYPLIPVGRMVLNRPNNYFAEVEQIAFSPAHIIPGVEASPKMLQGR <u>LFYSYSD</u>	357
<i>H. D. discus</i>	298:KVWPHGEYPLIPVGRMVLNRPNNYFAEVEQIAFSPAHIIPGVEASPKMLQGR <u>LFYSYSD</u>	357
<i>D. rerio</i>	301:KVWSHKEFPLIPVGRFVLRNRPNNYFAEVEQLAFDPSNMPPGIEPSPDKMLQGR <u>LFYSYSD</u>	360
<i>O. fasciatus</i>	301:KIWSHKEYPLIPVGRMVLNRPNNYFAEVEQLAFDPSNMPPGIEPSPDKMLQGR <u>LFYSYSD</u>	360
<i>R. rugosa</i>	301:KIWSHKEYPLIPVGRMVLNRPNNYFAEVEQIAFDPSPNMPPGIEPSPDKMLQGR <u>LFYSYSD</u>	360
<i>R. norvegicus</i>	301:KVWPHKDYPLIPVGRMVLNRPNNYFAEVEQMAFDPSNMPPGIEPSPDKMLQGR <u>LFAYSPD</u>	360
<i>H. sapiens</i>	301:RVWPHKDYPLIPVGRMVLNRPNNYFAEVEQIAFDPSPNMPPGIEASPDKMLQGR <u>LFAYSPD</u>	360
	* *	
<i>C. gigas</i>	358: <u>THR</u> RRLGANYLQIPVNCYPYKAKTFHYQRDGPQCVNDNQGGAPNYFPNSFSGPMDNVPV-C	416
<i>C. farreri</i>	358: <u>THR</u> RRLGNSYLQAVNCFNTKAKNYQRDGPQCVNDNQGNAPNYFPNSFSGPQDNKQFLE	417
<i>H. D. discus</i>	358: <u>THR</u> RRLGNSYLQLPVNCYPYNTRLSNYQRDGPQCVNDNQGGAPNYFPNSFSGPQEEESKEME	417
<i>D. rerio</i>	361: <u>THR</u> RRLGANYLQLPVNCYPYRTRVANYQRDGPQCMHDNQGGAPNYFPNSFAPDVQPRFLE	420

Fig. 1. Multiple alignment of the CAT gene of *Crassostrea gigas* (GenBank accession no. EF687775), *Chlamys farreri* (DQ862859), *Haliotis discus discus* (DQ530211), *Danio rerio* (NM_130912), *Rana rugosa* (AB031872), *Oplegnathus fasciatus* (AY734528), *Rattus norvegicus* (NM_012520) and *Homo sapiens* (AY028632). The catalase proximal heme ligand signature (RLFSYNDTH) is bold and double lined. The proximal active site signature (FDRERIPERVVHAKGAGA) is bold and underlined. The conserved catalytic amino acids are boxed. Identical amino acids among the different sites are indicated by asterisks.

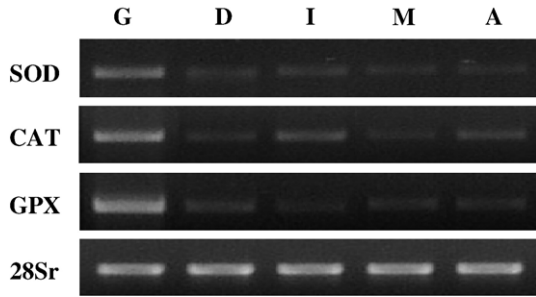


Fig. 3. Tissue-specific expression of SOD, CAT and GPX mRNA in various tissues (gill, G; digestive gland, D; intestine, I; mantle, M; adductor muscle, A) from the Pacific oyster, *Crassostrea gigas* by RT-PCR. Amplification of 28S ribosomal RNA was used as an internal control.

of 0.05 (AspAT, 3.7 ± 1.5 IU/L; AlaAT, 6.3 ± 3.1 IU/L) and 0.1 ppm (AspAT, 4.3 ± 1.4 IU/L; AlaAT, 8.0 ± 3.0 IU/L) (Table 1).

H₂O₂ concentrations significantly increased in the hemolymph in a time- and dose-dependent manner of Cd treatment (Fig. 4). H₂O₂ concentrations increased with time and reached the highest level after 11 days with exposure to 0.01 (4.0 ± 0.3 nM/mL), 0.05 (6.0 ± 0.6 nM/mL) and 0.1 ppm Cd (10.5 ± 0.8 nM/mL) from 2.4 ± 0.3 nM/mL at the beginning of the trial. The maximal response of H₂O₂ concentrations was observed at the highest dose of Cd tested (0.1 ppm).

3.5. The mRNA expression levels of antioxidant enzymes

Cd treatment significantly increased antioxidant enzyme mRNA expression in the gill in a dose-and time-dependent manner (Fig. 5). The maximal response was observed at the highest dose of Cd tested (0.1 ppm). SOD mRNA expression increased with time and reached the highest level after 11 days with exposure to 0.01 and 0.05 ppm Cd. It significantly increased until 7 days and then decreased with exposure to 0.1 ppm Cd. The SOD mRNA level was maximal on 7 days (91 times higher than the control; $P < 0.05$) for 0.1 ppm Cd. Also, CAT and GPX mRNA expression in 0.01 and 0.05 ppm Cd significantly increased with time and reached the highest level at 11 days, whereas CAT and GPX mRNA increased until 3 days then decreased with exposure to 0.1 ppm Cd. The CAT

Table 1
Changes in AspAT and AlaAT in the Pacific oyster, *Crassostrea gigas*, hemolymph exposed to cadmium for 11 days

Parameter	Cd concentration	Duration of Cd treatment (days)				
		Control	1	3	7	11
AspAT (IU/L)	0 (control)	1.0±0.4	1.6±0.4	1.5±0.6	1.5±0.6	1.3±0.8
	0.01 ppm	1.2±0.7	1.2±0.7	1.9±0.8	1.1±0.9	1.2±0.7
	0.05 ppm	1.0±0.5	0.9±0.6	1.2±0.3	3.7±1.5*	2.5±1.0
	0.1 ppm	1.2±0.4	1.7±0.6	1.3±1.0	4.3±1.4*	2.2±0.8
AlaAT (IU/L)	0 (control)	0.6±0.5	0.7±0.3	0.7±0.2	0.7±0.6	0.7±0.6
	0.01 ppm	0.7±0.6	0.7±1.2	2.0±1.0	2.3±1.5	1.3±1.5
	0.05 ppm	0.7±0.6	0.3±0.6	1.0±1.7	6.3±3.1*	3.7±2.5
	0.1 ppm	0.9±0.1	1.7±1.2	2.0±1.0	8.0±3.0*	4.0±3.6

AspAT, aspartate aminotransferase; AlaAT, alanine aminotransferase.
*Asterisk indicates a significant difference from the control ($P < 0.05$). Values indicate the mean±SD ($n = 5$).

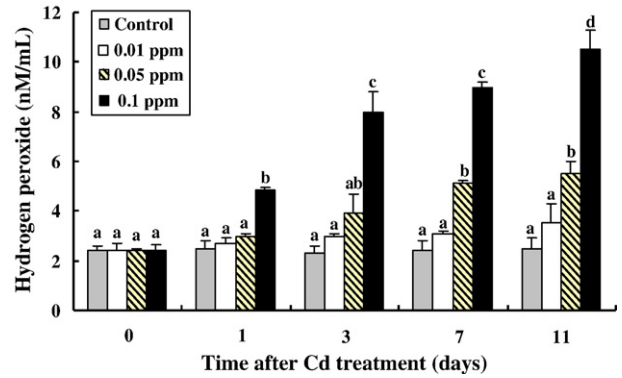


Fig. 4. Effect of Cd exposure on the hydrogen peroxide (H₂O₂) concentrations in hemolymph of *Crassostrea gigas* at the different concentration. Oysters were treated with 0 (control), 0.01, 0.05 or 0.1 ppm Cd for 1, 3, 7 or 11 days (mean±SD, $n = 5$). Different lowercase letters indicate significant differences ($P < 0.05$) among times after treatments within Cd concentrations.

and GPX mRNA level was maximal on 3 days (CAT, 19 times higher than the control; GPX, 8 times higher than the control; $P < 0.05$) for the highest dose of Cd tested (0.1 ppm).

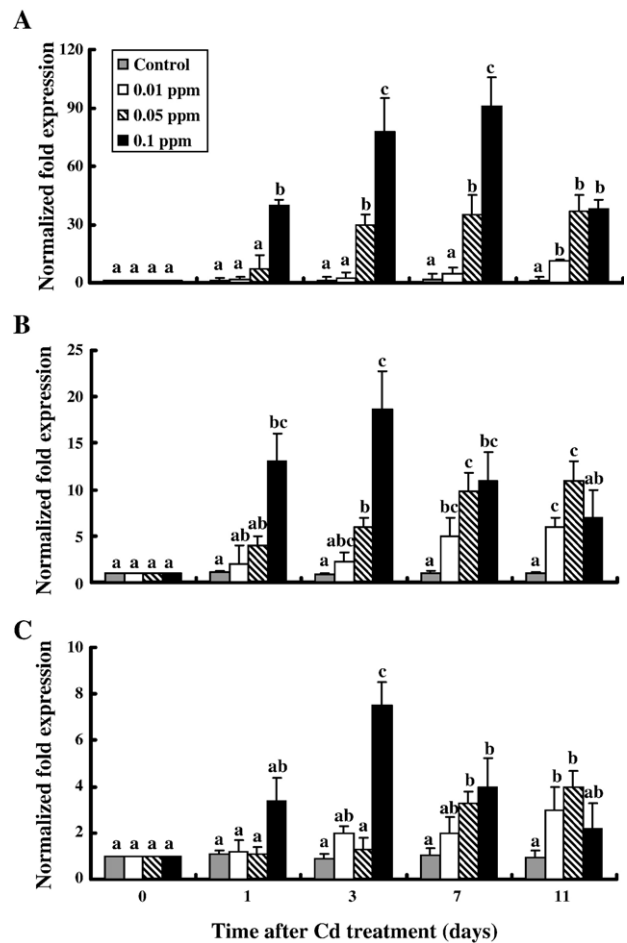


Fig. 5. Expression of antioxidant enzymes mRNA in response to cadmium treatment in the Pacific oyster, *Crassostrea gigas*; (A) SOD, (B) CAT and (C) GPX. Oysters were treated with 0 (control), 0.01, 0.05, or 0.1 ppm Cd for 1, 3, 7, or 11 days. The antioxidant enzymes mRNA expression levels relative to 28S rRNA levels were analyzed using real-time PCR. Different lowercase letters indicate significant differences ($P < 0.05$) among times after treatments within Cd concentrations. Values indicate the mean±SD ($n = 5$).

4. Discussion

Industrial development has caused the release of Cd into the aquatic environment through a variety of routes and in a variety of forms. Cd accumulates within a living body and causes oxidative stress. The living body protects itself against oxidative stress using antioxidant enzymes. We examined oxidative stress reactions in Pacific oyster exposed to Cd through the analysis of hemolymph properties and the expression of antioxidant enzymes.

We cloned the full-length CAT cDNA (GenBank accession no. EF687775) and GPX cDNA (EF692639) of Pacific oyster. The only other full-length CAT cDNA of mollusks belong to the two species *C. farreri* (DQ862859) and *H. discus discus* (DQ530211). The homology of CAT of Pacific oyster was high, at 74% and 72% with *C. farreri* and *H. discus discus*, respectively. Also, there was homology of $\geq 65\%$ with CAT of vertebrates.

As a result of multiple alignments using the cloned CAT, several characteristic elements were identified: His⁷², Asn¹⁴⁵, and Tyr³⁵⁵ were conserved catalytic amino acids of CAT in all species, including Pacific oyster (Tavares-Sanchez et al., 2004). The conserved sites of RLFSYNDTH (residues 351–359), which is the catalase proximal heme ligand signature sequence, and of FDRERIPERVVHAKGAGA (residues 61–68), which is the proximal active site signature, were also identified (Tavares-Sanchez et al., 2004) (Fig. 1). Therefore, the high homology and two conserved characteristic sites of CAT suggest that CAT of Pacific oyster is a member of the CAT family.

In full-length GPX cDNA of Pacific oyster, a characteristic nonsense TGA codon (position 259, 80th amino acid) was identified (Forstrom et al., 1978) (Fig. 2). This codon is a selenocysteine, which was first discovered in mouse. The selenocysteine is the activation site of the enzyme (Chambers et al., 1986). Also, in GPX of Pacific oyster, Glu¹¹² and Trp¹⁸⁷, which are catalytic residues that interact with selenocysteine, were conserved (Ursini et al., 1995). These results suggest that GPX of Pacific oyster is a Se-GPX gene (Se-GPX). In mollusks, full-length Se-GPX has been reported in only two species: *U. tumidus* (DQ830766) and *D. polymorpha* (EF194204). The comparison of GPX homology between mollusks and vertebrates using the GPX cloned here indicated a low level of homology ($\leq 33\%$). However, the catalytic residues Glu, Trp and selenocysteine were conserved in Se-GPX of all species.

We compared the expression of SOD, CAT and GPX mRNA in various tissues of Pacific oyster exposed to Cd using RT-PCR (Fig. 3). SOD, CAT and GPX mRNA expression was observed in all tissues. In particular, high levels of expression were observed in gill tissues. Gills are the primary and direct absorption route for toxic substances. The highest SOD, CAT and GPX expression occurred in gills because gills have a large area of contact with the surrounding environment and are subject to high levels of Cd accumulation (Legeay et al., 2005). No previous study has examined the expression of antioxidant genes among tissues in mollusks; however, higher expression in the liver of fish than in other organs such as gills and kidneys has been found (Cho et al., 2006), indicating that there are

differences among species in terms of expression patterns among tissues.

H₂O₂ concentrations significantly increased in hemolymph of *C. gigas* as the Cd concentration and exposure time increased (Fig. 4). There is evidence that exposure to Cd increases the formation of ROS and many recent studies relative to these results were reported (Wang et al., 2004; Choi et al., 2007; Murugavel et al., 2007; Soares et al., 2008). This ROS induced by Cd toxicity in organisms was widely known to promote oxidative stress, which caused membrane damage, DNA breakage, lipid peroxidation, enzyme inhibition, amino acid oxidation and apoptosis.

SOD, CAT and GPX mRNA expression occurred to protect Pacific oyster from oxidative stress caused by Cd exposure. The expression increased as the Cd concentration and exposure time increased (Fig. 5). Funes et al. (2005) reported that CAT, SOD, and GPX activation was higher in mollusks (oysters, *Crassostrea angulata*; mussels, *M. galloprovincialis*) from areas that had severe heavy metal contamination than from areas without contamination. Other studies reported the induction of antioxidant genes in fish exposed to Cd, e.g., brown trout, *Salmo trutta* (Hansen et al., 2007), and sea bass, *Dicentrarchus labrax* (Romeo et al., 2000). Therefore, the expression of the antioxidant genes SOD, CAT and GPX mRNA by exposure to Cd suggests that ROS were induced by Cd in Pacific oyster, and that the antioxidant system was enhanced to remove the ROS by Cd exposure. Our results demonstrated an increasing trend in SOD, CAT and GPX mRNA expression at 0.01 ppm and 0.05 ppm Cd over time (Fig. 5). However, at 0.1 ppm Cd, SOD mRNA expression increased significantly from 3 to 7 days of exposure and then decreased. CAT and GPX mRNA expression increased up to 3 days and then decreased. H₂O₂ concentration increased up to 11 days. Therefore, we deduce that extreme oxidative stress was induced by ROS generation with Cd contamination in Pacific oyster exposed to 0.1 ppm Cd after 3 days. Also, the decrease in antioxidant enzyme mRNA expression after a specific period of time may have been caused by the lowering of the metabolic capacity of the living body caused by the strong toxicity of Cd. Zhang et al. (2004) reported that although defense mechanisms are activated under weak oxidative stress, a living body loses appropriate metabolic functions under strong oxidative stress. We found that antioxidant gene expression decreased after 3 days of exposure to 0.1 ppm Cd because excess accumulation of Cd beyond a certain level in a living body causes strong oxidative stress that dramatically lowers the metabolic capacity, resulting in a decrease in antioxidant enzyme mRNA expression. Also, CAT and GPX mRNA expression rapidly decreased whereas SOD mRNA expression did not after 7 days of exposure to 0.1 ppm Cd. This indicates that although SOD converts the superoxide radical to H₂O₂ and O₂, a decrease in CAT and GPX to break down the converted H₂O₂ into O₂ and H₂O leads to the failure to completely remove active oxygen (H₂O₂) that is harmful to a living body.

The analysis of the hemolymph of Pacific oyster exposed to Cd indicated no significant difference in the concentration of AspAT and AlaAT at 0.01 ppm Cd. However, at 0.05 ppm and

0.1 ppm, AspAT and AlaAT levels significantly increased up to 7 days of exposure and then decreased. In Pacific oyster, results have been reported on changes in the hemolymph constituents caused by heavy metal contamination, various environmental factors and pathogenic agents (His et al., 1996; Xue and Tristan, 2000). Among the changes in hemolymph constituents, an increase in AspAT and AlaAT activation is generally caused by inflow to the hemolymph of cells separated due to tissue damage by environmental contaminants. Choi et al. (2008) reported an increase in AspAT and AlaAT activation in Pacific oyster exposed to Cd. There are reports for several fish species of an increase in AspAT and AlaAT activation with Cd concentration over time (e.g., *Sparus aurata*, Vaglio and Landricina, 1999; *Cyprinus carpio*, de la Torre et al., 2000). Therefore, a significant increase in AspAT and AlaAT activation in the hemolymph of Pacific oyster by 7 days of exposure to Cd was induced as a result of tissue damage by Cd.

CAT and GPX mRNA expression significantly decreased together with an increase in AspAT, AlaAT and H₂O₂ concentration within the hemolymph of Pacific oyster by 7 days of exposure to 0.1 ppm Cd. This may have been the result of tissue damage by residual active oxygen if GPX and CAT failed to completely remove the hydrogen peroxide (H₂O₂) generated by SOD due to a decrease in the defensive capacity of the antioxidant systems as a result of excess oxidative stress exerted by Cd.

In conclusion, oxidative stress caused by Cd toxicity accumulated in Pacific oyster exposed to Cd. To counter this stress, antioxidant enzyme mRNA expression increased in the gill tissue. Considering the increase in AspAT and AlaAT in the hemolymph, we deduced that cell damage occurred because of the loss of detoxification ability by the secondary antioxidant system of CAT and GPX in Pacific oyster due to excess oxidative stress caused by the accumulation of Cd beyond a specific tolerance. Therefore, it is possible that the antioxidant genes SOD, CAT and GPX mRNA could be used as physiological index for oxidative stress by heavy metals such as Cd contamination in the Pacific oyster.

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