Thesis for the Degree of Master of Science

Molecular and Physiological Studies on the Effects of Waterborne Pollutants in Pacific Oyster, *Crassostrea gigas*

Yong Ki Choi

Department of Marine Bioscience and Environment

The Graduate School

Korea Maritime University

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Molecular and Physiological Studies on the Effects of Waterborne Pollutants in Pacific Oyster, *Crassostrea gigas*

Advisor: Prof. Cheol Young Choi

by

Yong Ki Choi

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Molecular and Physiological Studies on the Effects of Waterborne Pollutants in Pacific Oyster, *Crassostrea gigas*

A dissertation

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Yong Ki Choi

Certified by **Cheol Young Choi**
.....
Thesis Advisor

Accepted by **Sung Hwoan Cho**
.....
Chairman

In-Seok Park
.....
Member

Cheol Young Choi
.....
Member

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Contents

Page

List of Figures

참굴, *Crassostrea gigas*의 해양오염물질 노출에 따른 분자 생리학적 변화

최 용 기

한국해양대학교 대학원 해양생명환경학과

요 약

카드뮴은 급속한 산업 발달에 따라 복잡하고 다양한 형태로 수서 환경으로 유입되며, 유입된 카드뮴은 그 양이 소량일지라도 수서생물의 체내에 축적되어 먹이사슬을 통해 인간에게도 악영향을 초래한다. 본 연구는 카드뮴이 참굴, *Crassostrea gigas*의 생리적 변화에 미치는 영향을 조사하기 위하여, 참굴을 카드뮴 (0.01, 0.05, 0.1 ppm)에 노출시킨 후, heat shock protein 90 (HSP90), metallothionein (MT) 및 manganese- superoxide dismutase (Mn-SOD) mRNA의 발현과 헤모림프(hemolymph)내의 hydrogen peroxide (H_2O_2) 농도와 glutamate oxaloacetate trasaminase (GOT) 및 glutamate pyruvate transaminase (GPT) 분석을 실시하였다.

본 연구를 통하여, 참굴 아가미 조직으로부터 전장의 HSP90 cDNA (GenBank accession no. EF687776)와 Mn-SOD cDNA (EU420128)를 분리하였다. 참굴 HSP90 및 Mn-SOD cDNA는 현재 보고된 패류 종과 각각 84%, 62% 이상의 상동성을 나타내었다. 또한 각 단백질의 특이적인 요소와 잘 보존된 영역을 포함

하고 있었다.

참굴 HSP90과 MT 및 Mn-SOD mRNA 발현은 카드뮴 처리 농도가 증가함에 따라 mRNA 발현량이 유의적으로 증가하였다. 이는 참굴 체내에서 카드뮴이 독성 스트레스로 작용하였고 이에 세포보호 및 항상성 유지를 위해 HSP90, MT 및 Mn-SOD mRNA의 발현이 유도되었다고 판단된다. 또한 본 실험에서 0.05 ppm과 0.1 ppm 농도에서는 HSP90과 Mn-SOD mRNA 발현이 7일째까지 발현의 증가하다가 이후 11일째 감소하는 경향을 나타내었다. 이는 카드뮴의 강한 독성에 의해 생체 대사능력의 감소로, 지속적으로 축적된 카드뮴이 과도한 활성산소종(reactive oxygen species, ROS)를 발생시켜, 이로 인한 강한 산화 스트레스에 의해 생체대사능력이 감소하고 이에 따라 생체 방어를 위한 HSP90 및 Mn-SOD mRNA의 발현량도 감소하는 것으로 사료된다. MT mRNA 발현은 카드뮴의 농도와 노출시간에 따라 발현량이 유의적으로 증가하였다. MT는 카드뮴의 생체내 축적 정도를 나타내는 biomarker로, 중금속 농도에 비례하여 상대적으로 증가하는 것으로 알려져 있다. MT mRNA의 발현변화는 노출시간과 농도에 비례하여 참굴 체내에 카드뮴이 축적되었음을 뒷받침 해준다.

참굴의 헤모림프 내의 Hydrogen peroxide (H_2O_2) 농도의 변화 분석한 결과, 카드뮴의 농도가 높을수록, 시간이 경과할수록 증가하는 경향을 나타냈다. 이는 체내 카드뮴의 축적에 의해 산화작용이 유도되었으며 이 과정에서 다량의 ROS가 생성되었음 알 수 있다. 참굴 헤모림프 내의 효소성분인 GOT 및 GPT의 농도는 0.1 ppm 7일째에 유의하게 증가하였다. 이는 카드뮴에 의한 조직손상에 의한 결과로 볼 수 있으며, GOT, GPT 활성이 7일째 유의한 증가는 HSP90과 Mn-SOD mRNA의 발현이 감소하는 시점과 일치하여, 과도한 스트레스에 의한 조직손상의 결과로 추측되어진다.

또한 참굴의 tributyltin (TBT) 노출 실험에서도, Mn-SOD 및 헤모림프 내의 H_2O_2농도와 GOT, GPT 활성이 카드뮴의 노출과 매우 유사한 결과를 나타내었다. TBT는 주석을 포함하고 있는 유기화합물로 해양부착생물방지제 속에 포함되는 강한 독성을 가진 환경오염물질로서 카드뮴과 같이 체내에 독성물질로 작

용하여, ROS 생성 및 mRNA 발현 유도에 유사한 메커니즘을 나타내는 것을 알 수 있었다.

결론적으로, 카드뮴의 노출에 의하여 체내에 ROS가 발생하여 산화스트레스를 유도하였고, 이에 스트레스 및 독성 제거와 항상성 유지를 위해 HSP90, MT, Mn-SOD mRNA의 발현이 증가하였다. 또한 카드뮴에 과다하게 노출되면 생체는 활성을 잃게 되어, 산화스트레스에 대한 방어능력의 감소로 조직의 손상을 가져오게 된다. 따라서, 본 연구의 결과는 HSP90, MT 및 Mn-SOD가 카드뮴 독성으로부터의 세포보호와 항상성유지를 위하여 발현됨을 시사하며, mRNA발현 및 헤모림프 내의 H_2O_2농도와 GOT, GPT 활성은 참굴의 스트레스 정도를 판단할 수 있는 생리적 지표로서의 가능성이 기대된다.

I. General Introduction

The oyster, often referred to as the "milk of the sea" is a favorite food item in countries renowned for the longevity of their citizens. Nutritionally, oyster is a good source of glycogen, taurine and essential amino acids, as well as vitamins and minerals. Species such as Pacific oyster, *Crassostrea gigas*, Japanese oyster, *C. nippona* and flat oyster, *Ostrea denselamellosa*, are farmed extensively along the Korean coastline. Pacific oyster in particular is a species of significant industrial and economical scale, and very important to Korea both as a substantial protein supply for the nation and as an economical export. Currently, Pacific oyster agriculture is mainly being conducted in the waters near Tongyeong and Yeosu of the South Sea, which are designated as clean waters by the Food and Drug Administration of the United States.

However, recently, heavy metal contamination has become prominent along the southern coast of Korea where the farming of Pacific oyster takes place. The contamination is caused by aging farming facilities, aquatic wastes, sediments within the farmland and abandoned copper mines. Aquatic organisms, oysters are then exposed to these contaminants within the aquatic environment, and even very small amounts of harmful heavy metals such as lead, cadmium (Cd) and mercury (Hg) cause toxicity in living bodies. These toxins accumulate within the bodies of aquatic organisms and can cause adverse effects for humans through the food chain (Kobayashi, 1971; Rainbow and White, 1989). Particularly, Cd is a nonessential element and is

potentially highly toxic to humans, animals and plants, even at low doses (Benavides et al., 2005). Cd is released into aquatic environments from industrial sources involved in, for example, mining, ore refining and plating processes, as well as from natural sources such as rocks and soils (Choi et al., 2007). It has physiologically adverse effects on the growth, reproduction and osmoregulation of fish (Kim et al., 2004). Itai-itai disease, which is Cd toxicosis caused by Cd in wastewater discharged from lead and zinc refineries in Japan 50 years ago, is a representative case that illustrates the harmfulness of Cd (Kobayashi, 1971). Cd accumulates within the bodies of organisms and alters and degrades processes of enzyme activation (Sastry and Subhadra, 1982). It also causes cell damage and can result in cell death (Benavides et al., 2005). In addition, Cd induces oxidization and thus generates reactive oxygen species (ROS) that promote oxidative stress (Stohs et al., 2000). Living organisms, however, possess physiological mechanisms to defend against toxicity and stress and to maintain homeostasis, including the expression of heat shock proteins (HSPs), which are representative stress-defense proteins, the metal-binding protein metallothionein (MT) and antioxidant defense systems to protect themselves from oxidative stress. HSP90, stress-defense proteins that are highly expressed in response to stress caused by changes in environmental factors such as temperature, heavy metal concentrations and active oxygen concentration, protect the structure and function of cells from stress and play an important role in maintaining cellular homeostasis. MT is a protein that has a high cysteine content, a low molecular weight of ~7 kDa, and a high affinity for metals. It binds with

metals and both regulates the homeostasis of essential trace metals such as copper and zinc and takes part in counteracting the toxic effects of heavy metals such as Cd, Hg and silver. And, antioxidant defense 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 is metalloenzyme and one of the representative defense 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).

In this paper, to determine the effects of Cd on organisms, time- and dose-related effects on mRNA levels of HSP90, MT and Manganese-SOD were investigated in the gill and digestive gland and, changes enzyme (glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT)) levels and H_2O_2 concentration in the hemolymph of Pacific oyster *C. gigas*.

II. Experiment 1

Cadmium Affects the Expression of Heat Shock Protein 90 and Metallothionein mRNA in Pacific Oyster, *Crassostrea gigas*

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Cadmium affects the expression of heat shock protein 90 and metallothionein mRNA in the Pacific oyster, *Crassostrea gigas*

Yong Ki Choi, Pil Gue Jo, Cheol Young Choi *

Division of Marine Environment & Bioscience, Korea Maritime University, Busan, 606-791, South Korea

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Abstract

Cadmium (Cd) is a widespread nonessential heavy metal that enters the aquatic environment as a result of natural processes and human activities such as wastewater production, agriculture, and mining. To determine the effects of Cd on organisms, we investigated its time- and dose-related effects on mRNA levels of heat shock protein 90 (HSP90) and metallothionein (MT) in the gill and digestive gland and changes enzyme levels in the hemolymph of the Pacific oyster *Crassostrea gigas*. Full-length HSP90 cDNA was isolated from *C. gigas* by rapid amplification of cDNA end (RACE) techniques and found to contain 2154 nucleotides, including an open reading frame, and was predicted to encode a protein of 717 amino acids. BLAST analysis indicated that the HSP90 gene of *C. gigas* shared high homology with known HSP90 genes of other mollusks. The expression of HSP90 mRNA increased significantly with exposure to 0.01 ppm Cd for 11 days or 0.05 or 0.1 ppm Cd for 7 days. The expression of MT mRNA increased significantly with exposure to 0.01, 0.05, or 0.1 ppm Cd for 11 days. Glutamate oxaloacetate and glutamate pyruvate levels increased significantly with exposure to 0.05 or 0.1 ppm Cd for 7 days. These results indicate that HSP90 and MT play important roles in the physiological changes related to metabolism and cell protection that occur in Pacific oysters exposed to Cd.

Keywords: Cadmium; Gene expression; Hemolymph; HSP90; MT; Pacific oyster

1. Introduction

The industrial development of recent times has resulted in the continuous discharge of various organic and inorganic materials, chemical substances, and heavy metals contained in domestic and industrial sewage into aquatic environments. Aquatic organisms are then exposed to these contaminants within the aquatic environment, and even very small amounts of harmful heavy metals such as lead, cadmium (Cd), and mercury cause toxicity in living bodies. These toxins accumulate within the bodies of aquatic organisms and can cause adverse effects for humans through the food chain (Kobayashi, 1971; Rainbow and White, 1989).

Cd is a nonessential element and is potentially highly toxic to humans, animals, and plants, even at low doses (Benavides et al., 2005). Cd is released into aquatic environments from industrial sources involved in, for example, mining, ore refining, and plating processes, as well as from natural sources such as rocks and soils (Choi et al., 2007). It has physiologically adverse effects on the growth, reproduction, and osmoregulation of fish (Kim et al., 2004). Itai-itai disease, which is Cd toxicosis caused by Cd in wastewater discharged from lead and zinc refineries in Japan 50 years ago, is a representative case that illustrates the harmfulness of Cd (Kobayashi, 1971).

Cd accumulates within the bodies of organisms and alters and degrades processes of enzyme activation (Sastry and Subhadra, 1982). It also causes cell damage and can result in cell death (Benavides et al., 2005). In addition, Cd induces oxidization and hus generates reactive oxygen species (ROS) that promote oxidative stress (Stohs et al., 2000). Organisms, however, possess physiological mechanisms to defend against toxicity and stress and to maintain homeostasis, including the expression of heat shock proteins (HSPs), which are representative stress-defense proteins, and the metal-binding protein metallothionein (MT).

HSPs, stress-defense proteins that are highly expressed in response to stress caused by changes in environmental factors such as temperature, heavy metal concentrations, active oxygen

* Corresponding author. Tel.: +82 51 410 4756; fax: +82 51 404 3988.
E-mail address: choic@hhu.ac.kr (C.Y. Choi).

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concentration, and salinity (Beckmann et al., 1990), protect the structure and function of cells from stress and play an important role in maintaining cellular homeostasis (Sanders, 1993; Ackerman and Iwama, 2001). HSPs are divided into several groups based on their molecular mass and functional aspects: molecular mass ≤ 60, 70, 90, and 110 kDa (Gao et al., 2007). The abundant HSP90 of the 90-kDa HSP family comprises 1–2% of cellular proteins. It occurs in small quantities in normal cells that are not exposed to stress and binds to protein kinases, steroid receptors, actin, tubulin, and other substances in the cells to maintain proteins and deliver signals among cells (Pratt, 1997; Csermely et al., 1998). HSP90 is also induced by stressors such as changes in water temperature, salinity, active oxygen concentration, heavy metal concentrations, and organic and inorganic chemical substances (Choi et al., 2006; Gao et al., 2007). Studies of HSPs in relation to stress and immune responses to heavy metals have been conducted for variety of vertebrates and mollusks. In bivalves, HSP studies have been conducted on mussels *Mytilus edulis*, *Mytilus galloprovincialis*, *Dreissena polymorpha*, oysters, *Crassostrea gigas*, *Crassostrea angulata*, *Ostea edulis*, and abalone *Haliotis rufescens* (Gao et al., 2007). However, most HSP studies have focused on HSP70, and HSP90 has received less attention (Gao et al., 2007).

MT is a protein that has a high cysteine content, a low molecular weight of ~7 kDa, and a high affinity for metals. It binds with metals and both regulates the homeostasis of essential trace metals such as copper and zinc and takes part in counteracting the toxic effects of heavy metals such as Cd, mercury, and silver (Viarengo et al., 1999). Many recent studies have examined the use of MT as an index to evaluate heavy metal contamination in aquatic environments (Woo et al., 2006; Choi et al., 2007).

Among aquatic organisms, bivalve mollusks, including oysters, display little mobility compared to other species such as fish and crustaceans. Heavy metals are absorbed and accumulated in the tissue of bivalve mollusks as they filter large amounts of seawater through the gills and ingest the filtered organic substances (Philip, 1995; Engel, 1999). Heavy metal contamination is becoming prominent in areas of Korea and East Asia in which the farming of bivalve mollusks occurs due to the aging of farming facilities and the presence of aquatic wastes, sediments below the farming areas, and abandoned copper mines. Studies are necessary to identify the relationship between heavy metals and stress responses in bivalve species. Therefore, we analyzed changes in the properties of the enzymes glutamate oxaloacetate transaminase (Aspartate aminotransferase — GOT) and glutamate pyruvate transaminase (Alanine aminotransferase — GPT) in the hemolymph, as well as changes in the patterns of HSP90 and MT mRNA expression, to identify the extent of stress caused by the exposure of the Pacific oyster, *C. gigas* to Cd.

2. Materials and methods

2.1. Experimental oysters

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

2.2. $CdCl_2$ treatments and sampling

After acclimatization for 48 h in the tanks, 25 oysters were transferred to 25-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. Gill and digestive gland tissues were randomly sampled from three oysters after 0, 1, 3, 7, and 11 days of treatment 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 × *g*, 4 °C, for 5 min and the supernatant, after centrifugation stored at −80 °C until analysis. Activities of GOT and GPT were measured using a biochemistry autoanalyzer (model 7180; Hitachi, Tokyo, Japan).

2.4. Rapid amplification of complementary DNA 3′ and 5′ ends (3′ and 5′ RACE) of HSP90

For RACE reactions, total RNA was extracted using a Trizol kit (Gibco/BRL, Grand Island, NY, USA) from the gill of Pacific oysters. Reverse transcription was performed using M-MLV reverse transcriptase (Bioneer, Daejeon, Korea). Using 3 μg of total RNA as the 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 Pacific oyster HSP90 partial cDNA (GenBank accession no. CB617443). 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 gene-specific primer (5′-TCC ACA ACA ACT CTG TCT GCA ACC AAG-3′), and 25 μL of SeeAmp Taq Plus Master Mix (Seegene). Polymerase chain reaction (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. The PCR product was amplified, cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA), and sequenced. For 5′-RACE, the 50 μL PCR reaction mixture contained 5′-

RACE-ready cDNA, 5′ gene-specific primer (5′-CAG GCT GAG ATT GCT CAG TTG ATG AGC-3′), 5′ target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), and SeeAmp Taq Plus Master Mix, as for 3′-RACE, under the same PCR conditions. The PCR product was amplified, cloned into pGEM-T Easy Vector, and sequenced. The DNA and deduced amino acid sequences were analyzed using GENETYX-WIN (Software Development, Tokyo, Japan).

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cgHSP90   1:MPEPE-H-MEEGEVETFAFQAEIAQLMSLIINTFYSNKEIFLRELISNASDALDKIRYES 58
cfHSP90   1:MPEPEGQAMEDGEVETFAFQAGIAQLMSLIINTFYSNKEIFLRELISNCSDALDKIRYES 60
haHSP90   1:MPEPQEAQMDEGEVETFAFQAEIAQLMSLIINTFYSNKEIFLRELISNSSDALDKIRYES 60
            ****    *  ********** ************************** ***********

cgHSP90  59:LTDPSKLDSGKDLEIRIVPDKESKTLTIMDTGIGMTKADLVNNLGTIAKSGTKAFMEALQ 118
cfHSP90  61:LTDPSKLDSGKELEIKIVPNKDDNTLSIMDTGIGMTKADLVNNLGTIARSGTKAFMEALQ 120
haHSP90  61:LTDPSKLDASKDLQIRIVPDKESKTLIIEDSGIGMTKADLVNNLGTIAKSGTKAFMEALQ 120
            ********  * * * *** *   ** * * ***************** ***********

cgHSP90 119:AGADISMIGQFGVGFYSAYLVADRVVVETKHNDDEQYIWESSAGGSFTVKTCSENTIGRG 178
cfHSP90 121:AGADISMIGQFGVGFYSAYLVADRVVVETKNNDDEHYIWESSAGGSFTVRS-GDGSFILG 179
haHSP90 121:AGADISMIGQFGVGFYSAYLVAERVVVESKHNDDEQYIWESSAGGSFTIRSSNDPTLPRG 180
            ********************** ***** * **** ************          *

cgHSP90 179:TKITLFLKEDQTEYLEERRIKEVVKKHSQFIGYPIKLLVEKERDKEVSDDEEEEEKKEED 238
cfHSP90 180:TRITLHMKEDQAEYLEEKKVKEIVKKHSQFIGYPIKLQVEKERDVEVSDDEEEEEKKEED 239
haHSP90 181:TRITLYMKEDQAEYLEERRIKEIVKKHSQFIGYPIKLMVEKERDKEVSDDEEDEKKEDEE 240
            * ***  **** *****   ** ************** ****** ******* * *  *

cgHSP90 239:KAEEK-E-EDKPKVEDLDEDEEDDSKSKD-KKKKKIKEKYTEDEELNKTKPIWTRNPDDI 295
cfHSP90 240:KDAEKSE-DDKPKVEDLDDEDDDEDKSKD-KKKKKIKGKYIEDEELNKTKPIWTRNPDDI 297
haHSP90 241:KKEDEEENEDKPKVEDLD-EDEDEDKSKDKKKKKKIKEKYTEDEELNKTKPLWTRNADDI 299
            *     *  **********    *  **** ******* ** ********** **** ***

cgHSP90 296:TQEEYGEFYKSLTNDWE-----RPFGCEGQLEFRALLFIPRRAPLDLFENKKKNNIKLY 350
cfHSP90 298:TQEEYGEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFVPRRAPFDLFENKKKNNIKLY 357
haHSP90 300:TQEEYAEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFLPKRAPFDMFENKKKNNIKLY 359
            ***** ***********        *  *********** * *** * **************

cgHSP90 351:VRRVFIMDNCEELIPEYLNFARGVVDSEDLPLNISREMLQQSKILKVIRKNLVKKCIELI 410
cfHSP90 358:VRRVFIMDNCNEVIPEYLNFVRGVVDSEDLPLNISREMLQQSKILKVIRKNLVKKCMELF 417
haHSP90 360:VRRVFIMDNCEDLIPEYLNFVRGVVDSEDLPLNISREMLQQSKILKVIRKNLVKKCMELF 419
            **********   ******* ********************************** **

cgHSP90 411:EDLTEDKDNYKKFYEQFAKNLKLGIHEDSTNRKKLADFLRYYSSQSGDEMTSLKDYVSRM 470
cfHSP90 418:DDIAEDKENYKKFYEQFAKNLKLGIHEDTTNRKKIADFLRYHTSQSGDEMTSFKEYVSRM 477
haHSP90 420:EDLTEDKDNFKKFYEQFSKNLKLGIHEDSTNRKKLSELLRYYTSQSGDEMTSLKDYVSRM 479
             *  *** * ******* ********** *****    ***  ********* * *****

cgHSP90 471:KENQKSIYYITGESREVVQSSAFVERVKKRGMEVIYMVDPIDEYAVQQLKEYDGKPLVNV 530
cfHSP90 478:KENQKSIYYITGESREVVQSSAFVENVKKRGIEVIYMVDPIDEYAVQQLKEYEGKTLVSV 537
haHSP90 480:KENQKSIYYITGESRDSVQNSAFVERVKKRGFEVVYMTDPIDEYCVQQLKEYDGKTLVCV 539
            ***************  ** ***** ***** ** ** ****** ******* ** ** *

cgHSP90 531:TKEGLELPEDEEERKRFEEAEAEYEGLCKVMKDILDKKVEKVVVSNRLVTSPCCIVTSQY 590
cfHSP90 538:TKEGLELPEDEEEKKRFEEATAEYEGLCKVVKEILDKKVEKVTVSNRLVTSPCCIVTSQY 597
haHSP90 540:TKEGLELPEDEEEKKKLEEAKAQFEGLCKVMKEILDKKVEKVVVSNRLVTSPCCIVTSQY 599
            ************* *  *** *  ****** * ********* ******************

cgHSP90 591:GWSANMERIMKAQALRDSSTMGYMAAKKHLEINPDHSIIKSLKDKAEADKNDKSVKDLVM 650
cfHSP90 598:GWSANMERIMKAQALRDSSTMGCMAAKKHLEINPDHAIIKSLKEKAGLDKNDKSVKDLVL 657
haHSP90 600:GWSANMERIMKAQALRDTSTMGYMAAKKHLEINPDHPIVKTLKEKADADKNDKAVKDLCM 659
            ***************** **** ************* * * ** **  ***** ****

cgHSP90 651:LLFETSLLASGFSLEEPGTHASRIHRMIKLGLGIDEDE--TPETQEPVTEDMPPLEGDED 708
cfHSP90 658:LLFETSMLASGFSLEEPGTHANRIHRMIKLGLGIDDDDSGAPETSDENVEEPPPLEGDED 717
haHSP90 660:LLFETSLLASGFSLEDPTSHANRIHRMIKLGLGIDEDDIPAESATESGTDEMPPLEGDED 719
            ****** ******** *  ** ************* *               ********

cgHSP90 709:DASRMEEVD                                                    717
cfHSP90 718:DASRMEEVD                                                    726
haHSP90 720:DASRMEEVD                                                    728
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Fig. 1. Multiple alignment of the HSP90 gene of the Pacific oyster (*Crassostrea gigas*, cgHSP90, GenBank accession no. EF687776), Zhikong scallop (*Chlamys farreri*, cfHSP90, AY362761), and abalone (*Haliotis asinina*, haHSP90, EF621884). Shaded regions indicate the five HSP90 family signature sequences and the consensus sequence MEEVD located at the C terminus. Identical amino acids are indicated by asterisks.

2.5. Quantitative real-time PCR (QPCR)

QPCR was conducted to determine the relative expression of HSP90 and MT mRNA. Total RNA was extracted from the gill and digestive gland of control and Cd-treated oysters. Reverse transcription were conducted using M-MLV reverse transcriptase (Bioneer), and cDNA was synthesized using 1 μg of the extracted total RNA according to the manufacturer's instructions. Primers for QPCR were designed with reference to known HSP90 (in this paper) and MT (AJ243263) gene sequences of the Pacific oyster as follows: HSP90 forward primer, 5′-GGT GAA TGT TAC CAA GGA AGG-3′; HSP90 reverse primer, 5′-GTT ACG ATA CAG CAA GGA GAT G-3′; MT forward primer, 5′-TTG GAG GAC AAG AGG AGA AAC ATC-3′; MT reverse primer, 5′-GGA CAC GAA TCA GAG CAG ACA C-3′; 28S ribosomal RNA (28Sr) forward primer, 5′-AAA CAC GGA CCA AGG AGT CT-3′; and 28Sr reverse primer, 5′-AGG CTG CCT TCA CTT TCA TT-3′.

PCR amplification was conducted using a Bio-Rad Mini-Opticon™ System (Bio-Rad, Hercules, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. QPCR was carried 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. All data were based on the calculated threshold cycle time (CT) levels. The values based on normalization of individual samples to 28S rRNA and then comparison to control group.

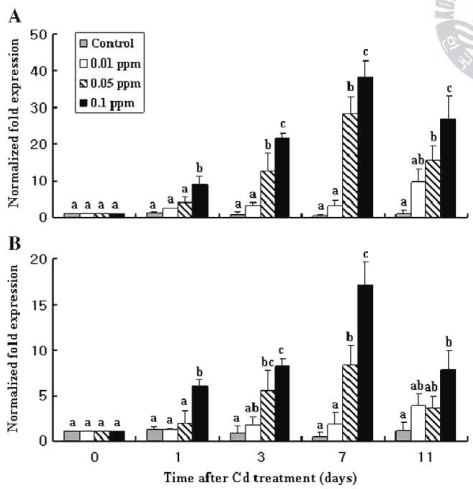

Fig. 2. Expression of HSP90 mRNA in response to cadmium treatment in the Pacific oyster (*Crassostrea gigas*) digestive gland (A) and gill (B). Oysters were treated with 0 (control), 0.01, 0.05, or 0.1 ppm Cd for 1, 3, 5, 7, or 11 days. The HSP90 mRNA expression levels relative to 28S rRNA levels were analyzed using real-time PCR. Different lowercase letters indicate significant differences ($P<0.05$) among Cd concentrations within sampling times. Values indicate the mean±SD ($n=4$).

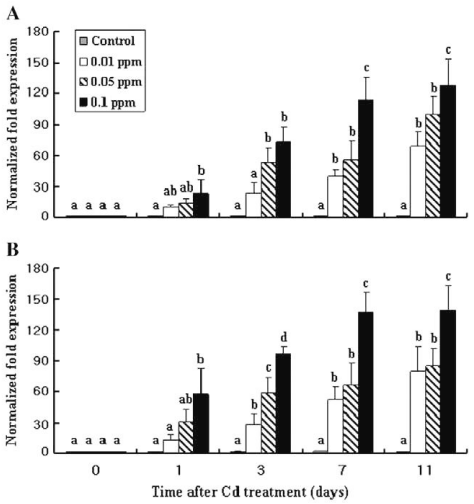

Fig. 3. Expression of MT mRNA in response to cadmium treatment in the Pacific oyster (*Crassostrea gigas*) digestive gland (A) and gill (B). Oysters were treated with 0 (control), 0.01, 0.05, or 0.1 ppm Cd for 1, 3, 5, 7, or 11 days. The MT mRNA expression levels relative to 28S rRNA levels were analyzed using real-time PCR. Different lowercase letters indicate significant differences ($P<0.05$) among Cd concentrations within sampling times. Values indicate the mean±SD ($n=4$).

2.6. 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 $P<0.05$.

3. Results

3.1. Identification of HSP90 cDNA

A PCR-based cloning strategy (RT-PCR followed by 3′ and 5′ RACE) was used to clone cDNA encoding a putative HSP90 from the gill of *C. gigas*. HSP90 full-length cDNA contained 2154 nucleotides, including an open reading frame (ORF), and was predicted to encode a protein of 717 amino acids. The sequence contained the HSP90 protein family signature (NKEIFLRELISN[A/C/S]SDALDKIR, LGTIA[K/R]SGT, IGQFGVGFYSAYLVA[E/D], IKLYVRRVFI, GVVDSEDLPL NISRE) and had the consensus sequence MEEVD at the C terminus (Fig. 1). The cDNA sequence of the Pacific oyster HSP90 gene was deposited in NCBI/GenBank under accession number EF687776. The deduced amino acid sequence of the Pacific oyster HSP90 was compared to those deduced from known HSP90 cDNA of other mollusk species (Fig. 1). Pacific oyster HSP90 had an 85 and 84% amino acid sequence similarity with Zhikong scallop, *Chlamys farreri* (AY362761) and abalone, *Haliotis asinina* (EF621884), respectively (Fig. 1).

3.2. HSP90 mRNA expression levels

Cd treatment significantly increased HSP90 mRNA expression in the gill and digestive gland in a dose- and time-dependent manner (Fig. 2). The maximal response was observed at the highest dose of Cd tested (0.1 ppm). HSP90 mRNA expression increased with time and reached the highest level at day 11 with exposure to 0.01 ppm Cd, whereas it increased until day 7 and then decreased with exposure 0.05 or 0.1 ppm Cd. The HSP90 mRNA level was maximal on day 7 in the gill (17 times greater than the control; $P<0.05$) and digestive gland (38 times greater than the control; $P<0.05$) for the highest dose of Cd tested (0.1 ppm).

3.3. MT mRNA expression levels

Cd treatment significantly increased MT mRNA expression in the gill and digestive gland in a dose- and time-dependent manner (Fig. 3). The maximal response was observed at the highest dose of Cd tested (0.1 ppm). MT mRNA expression increased rapidly until day 11 for all treatment groups. The MT mRNA level was maximal in gill (139 times greater than the control; $P<0.05$) and digestive gland (128 times greater than the control; $P<0.05$) for the highest dose of Cd tested (0.1 ppm).

3.4. Hemolymph analysis

The levels of GOT and GPT in the hemolymph increased significantly by day 7 at Cd concentrations of 0.05 and 0.1 ppm (Fig. 4).

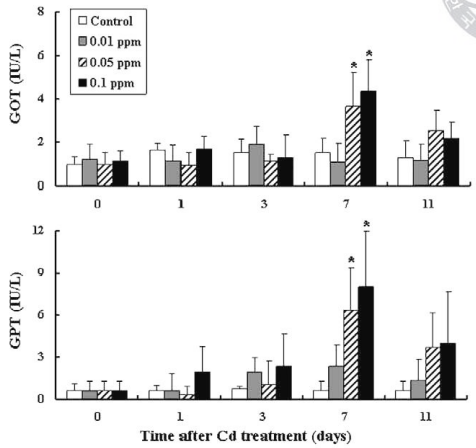

Fig. 4. Changes in the levels of the enzymes glutamate oxaloacetate transaminase (Aspartate aminotransferase) (GOT) and glutamate pyruvate transaminase (Alanine aminotransferase) (GPT) in the hemolymph of the Pacific oyster (*Crassostrea gigas*) with cadmium treatment. Oysters were treated with 0 (control), 0.01, 0.05, or 0.1 ppm Cd for 1, 3, 5, 7, or 11 days. Asterisks indicate a significant difference from the control ($P<0.05$). Values indicate the mean±SD ($n=4$).

4. Discussion

Rapid industrial development has caused the release of diverse and complex forms of Cd into aquatic environments. Heavy metals that enter aquatic environments accumulate within the bodies of aquatic organisms, and even small quantities cause adverse effects to humans through the food chain. Therefore, we investigated the physiological changes that occur in Pacific oyster after exposure to Cd by analyzing the properties of hemolymph and the expression patterns of MT and HSP90 mRNA. We isolated the complete HSP90 cDNA (GenBank accession no. EF687776; 2154 bp) from the gill of Pacific oysters. The only other reported HSP90 sequences of bivalve species belong to Zhikong scallop, *C. farreri* (GenBank accession no. AY362761) and abalone, *H. asinina* (EF621884). The HSP90 of the Pacific oyster, *C. gigas* (cgHSP90) displayed high homology with those of the Zhikong scallop and abalone, at 85 and 84%, respectively. In addition, it contained five signal peptides that are well conserved in the HSP90 family: NKEIFLRELISN(A/C/S)SDALDKIR, LGTIA(K/R)SGT, IGQFGVGFYSAYLVA(E/D), IKLYVRRVFI, and GVVDSE DLPLNISRE (Gupta, 1995; Gao et al., 2007). Furthermore, the consensus sequence MEEVD located at the 3′ terminus (Gupta, 1995; Gao et al., 2007) was identified in cgHSP90 at amino acid residues 713–717. Scheufler et al. (2000) reported that the amino acid sequence EEVD located at the 3′ terminus is the most well conserved area; moreover, it is the part that is involved in the binding of HSP70 and HSP90 and is regulated as a typical characteristic of HSP90. The cgHSP90 is similar to the HSP90 family in its general features and contains all of the recognized consensus sequences and/or signal peptides.

The level of cgHSP90 mRNA expression increased with increases in the dose and duration of exposure to Cd. This is consistent with previous findings in which HSP90 mRNA expression increased with dose and duration in scallops exposed to 0.05, 0.1, or 0.2 ppm Cd for 20 days (Gao et al., 2007) and with duration in carp exposed to 10 ppm Cd for 96 h (Hermesz et al., 2001). These results were produced because cadmium acted as toxic stress, and HSP90 expression is induced to maintain homeostasis and protect the cells (Sanders, 1993; Ackerman and Iwama, 2001).

In contrast, only weak HSP90 mRNA expression was observed in the control group, which did not receive Cd exposure. Lai et al. (1984) reported that HSP90 is abundant at 1–2% of the cellular proteins within tissues in a normal unstressed state, HSP90 is deduced to have basic physiological roles, regardless of exposure to stress.

The level of HSP90 mRNA expression increased up to day 7 at doses of 0.05 and 0.1 ppm Cd and then decreased by day 11. This may have occurred due to two main reasons. First, the decrease might have occurred because of a decrease in the metabolic capacity of the organism caused by the strong toxicity of Cd. Zhang et al. (2004) reported that whereas defense mechanisms function under weak oxidative stress, organisms cannot achieve appropriate levels of metabolic function under strong oxidative stress. Strong oxidative stress is generated when Cd accumulates in the tissues beyond a certain threshold

concentration. Therefore, the metabolic capacity and HSP90 mRNA expression level would decrease accordingly. Second, HSP90 expression might decrease due to the accommodation of the toxin. Exposure to toxic substances results in the expression of a variety of enzymes and proteins such as cytochrome *P450* (CYP450), flavin-containing monooxygenase (FMO), monoamine oxidase (MAO), glutathione-S transferase (GST), superoxide dismutase (SOD), and catalase (CAT), in addition to HSPs, for defense against toxicity (Boutet et al., 2004). Pacific oysters exposed to organic chemicals (i.e., polycyclic aromatic hydrocarbons) had increased levels of HSP70 mRNA expression up to day 7 after which they decreased, and CYP450, FMO, GST, and SOD mRNA expression also increased with HSP70 mRNA expression (Boutet et al., 2004). Exposure to Cd resulted in similar dose- and time-dependent patterns of HSP70 mRNA expression (Moraga et al., 2005). These are similar to our results for HSP90 mRNA. Exposure to xenobiotics such as oil, pesticides, and organic substances similarly increased the expression of HSP70 and HSP90 mRNA (Bagchi et al., 1996; Snyder et al., 2001; Boutet et al., 2004). Thus, the mechanisms of HSP70 and HSP90 expression in response to toxins appear to be similar. We can deduce that HSP90 expression decreases because the effects of heavy metals have been accommodated and the cells are protected by the actions of enzymes and proteins. However, enzymes decreased after 7 days, which can be interpreted as a reduction in the metabolic capacity of the organism because of strong toxicity.

We did not perform the experiment for cDNA sequence of cgMT. cgMT were reported in GenBank accession no. AJ243263. The level of MT mRNA expression increased significantly in response to Cd exposure in a dose- and time-dependent manner. This indicates that Cd accumulated within the oysters in proportion to the time and dose of exposure. The degree of Cd accumulation in *C. gigas* can only be determined directly using MT mRNA expression. MT binds with metals for their removal from cells and protects cells from oxidative stress. MT mRNA expression increases in proportion to the heavy metal dose (Butler and Roesijadi, 2001; Choi et al., 2007). MT is being widely researched as a biomarker for the degree of Cd accumulation in living organisms (Unger and Roesijadi, 1993; Rebeblo et al., 2003).

Also, HSP90 and MT mRNA expression of digestive gland and gill displayed very similar tendency of expression, therefore no difference was observed in terms of expression tendency per organ.

The levels of GOT and GPT in the hemolymph of Pacific oysters increased significantly by day 7. In bivalve species such as the Pacific oyster, proteins, carbohydrates, salts, ions, and hemocytes in hemolymph flow from the pericardial cavity to the tissues to provide defense mechanisms in the form of phagocytosis and encapsulation by blood cells (Gagnaire et al., 2006). In *C. gigas*, changes in the constituents of the hemolymph occur in response to pathogenic agents, heavy metal contamination, and changes in various environmental factors (His et al., 1996; Xue and Tristan, 2000). Increases in GOT and GPT activities are generally used to diagnose tissue damage caused by environmental contaminants (Sakamoto and Yone, 1978; Casillas et al., 1982). Although limited data are available on enzyme activation in bivalve species in relation to pollutants, we deduced that the increase in protein levels in the hemolymph of *C. gigas* exposed to Cd was caused by the inflow of cells affected by tissue damage (Oruc and Uner, 1998; Pickwell and Steinert, 1988). In several species of fish exposed to Cd, GOT and GPT activities increased with the increase Cd dose over time (Vaglio and Landriscina, 1999; de la Torre et al., 1999). Therefore, increases in GOT and GPT activities in the hemolymph of *C. gigas* can be regarded as resulting from tissue damage by Cd; the significant increase on day 7 likely occurred because tissue damage caused by Cd increased, and HSP90 and MT mRNA expression increased accordingly to protect the damaged cells.

HSP90 and MT mRNA expression increased in the gill and digestive gland of *C. gigas* exposed to Cd. This was because of an increase in stress and tissue damage caused by Cd. In other words, this result indicates that expression of mRNA expression and GOT and GPT activities in the hemolymph take place in order to maintain homeostasis and to protect cell from cadmium toxicity. Therefore, it might be possible to use HSP90 and MT as indices to determine the degree of Cd contamination in *C. gigas*. Additional studies of other heavy metals and oxidative stress are required to determine the mechanisms used to protect living tissues from heavy metal toxicity.

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III. Experiment 2

Characterization and mRNA Expression of Manganese-Superoxide Dismutase and Physiological Responses to Cadmium and Tributyltin in Pacific Oyster *Crassostrea gigas*

Abstract

Superoxide dismutases (SODs) are metalloenzymes that play an important role in mollusk immune defense systems by eliminating oxidative stress to reactive oxygen species. Physiological changes were investigated in Pacific oyster *Crassostrea gigas* caused by exposure to pollutants (cadmium and tributyltin). mRNA expression of manganeses-SOD (Mn-SOD) were analyzed 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 hemolymph based on time- and dose-related effects of pollutants and acute water temperature treatments. *C. gigas* Mn-SOD full-length cDNA (965 nucleotides) were cloned 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 GOT gradually increased and then subsequently 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.

Keywords: Cadmium, Hemolymph, Hydrogen peroxide, Mn-superoxide dismutase, TBT

1. 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 aging, reduced resistance against diseases, and lowered fertility (Kim and Phyllis, 1998).

Living organisms implement antioxidant defense 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 defense 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 has been reported in a variety of aerobic organisms including aquatic invertebrates (Fink and 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 giant scallop, *Mizuhopecten yessoensis* (GenBank accession no. AB222783) among the Bivalvia. Partial cDNA has also been reported in three species: marsh clam, *Corbicula fluminea* (EF446611), pearl mussel, *Hyriopsis schlegelii* (EU145730) and Manila clams, *Venerupis philippinarum* (EF520698). Boutet et al. (2004) reported the existence of Cu/Zn-SOD in 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 *C. gigas*, 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, aging of farming facilities, marine wastes and marine trafficking. Pollutants can exert stress to living organisms and can therefore negatively affect their health.

The purpose of this study was to examine physiological changes in *C. gigas* caused by exposure to Cd and TBT. To accomplish this, I cloned Mn-SOD cDNA isolated from the gill tissue of *C. gigas*. I also analyzed Mn-SOD mRNA expression levels and changes in H_2O_2 concentration as well as the production of SOD dismutation in the hemolymph. Changes in the level of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were also monitored.

2. Materials and methods

2.1. Experimental oysters

One-year-old Pacific oysters (average shell length: 112 ± 10.7 mm; height: 31.1 ± 5.4 mm; weight: 20.3 ± 3.9 g) were obtained from the oyster hatchery on Daebu island in Goseong (Gyeongnam, Korea). These were placed in two 200 l circulating filter tanks in the laboratory at 200 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, respectively, under a photoperiod of 12 h light/12 h dark, and no food was supplied.

2.2. 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 $CdCl_2 \cdot 2.5H_2O$ (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. Hemolymphs and tissues were sampled from randomly selected five oysters after 0, 1, 3, 7 and 11 days of treatments. Immediately after collection, all tissues were frozen in liquid nitrogen and stored at -80°C until total RNA was extracted. Oyster's mortality was not observed in all experimental groups or control group during the experimental period.

2.3. Hemolymph GOT and GPT analysis

Hemolymph was withdrawn from the pericardial cavity using a 3 ml syringe. The samples were centrifuged at 10,000×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 autoanalyzer (model 7180; Hitachi, Tokyo, Japan) according to the manufacturer's instructions.

2.4. 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). 20 μl of whole oyster hemolymphs 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 hemocytes to settle and adhere. A working color reagent was prepared by mixing 100 ml distilled water containing 100 mM sorbitol and 125 μM xylenol orange (Sigma-Aldrich) with 1 ml of 25 mM ferrous ammonium sulphate prepared in 2.5 M sulphuric acid (Sigma-Aldrich). 200 μl of this reagent was then added to each well and allowed to incubate at room temperature for 1 h. Absorbance was read at 560 nm and concentrations of H_2O_2 were interpolated from a standard curve. Concentrations are expressed as nM/ml.

2.5. Identification of Mn-SOD cDNA

Mixed primers for Mn-SOD of *C. gigas* Mn-SOD were designed using highly conserved regions of *M. yessoensis* (GenBank accession no. AB222783), disk abalone, *Haliotis discus discus* (DQ530210) and snail, *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 were 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). 2.5 μg of total RNA was 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. 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 analyzed using an ABI DNA Sequencer (Appleid Biosystems, Foster city, CA, USA).

2.6. Rapid amplification of complementary DNA of Mn-SOD

For RACE reactions, total RNA was extracted from the gills of *C. 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 Mn-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 Mn-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 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. Phylogenetic analysis of Mn-SOD

Phylogenetic analysis was performed on the amino acid sequences from full-length Mn-SOD cDNA from various vertebrates and mollusks. Amino acid sequence data were aligned using the BioEdit Software (Hall, 1999). The sequences compared to the *C. gigas* Mn-SOD (GenBank accession no. EU420128) were as follows: Mn-SOD of *M. yessoensis* (AB222783), *H. discus discus* (DQ530210), *B. glabrata* (AY500813), zebrafish, *Danio rerio* (NM_199976), frog, *Xenopus laevis* (NM_001090499), cow, *Bos taurus*

(BT020988) and human *Homo sapiens* (M36693). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) and analyzed 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 (1,000 replicates) analysis.

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

RT-PCR was conducted to determine the relative expression of *C. gigas* Mn-SOD in various oyster tissues. Total RNA were 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 were conducted as mentioned above. Primers for RT-PCR were designed with reference to *C. gigas* Mn-SOD cDNA (GenBank accession no. EU420128) by PCR and *C. gigas* β-Actin (AF026063) gene sequences of Pacific oyster as follows: Mn-SOD forward primer, 5`-AAG CAC ACC TTA CCA GAT CT-3`; Mn-SOD reverse primer, 5`-ATA GCG TGC TCC CAG ACA TC-3`; β-Actin forward primer, 5`-GAC TTC GAA CAA GAG ATG-3`; and β-Actin reverse primer, 5`-GAT ATC GAC ATC ACA TTT C-3`. PCR amplification was performed using a 2X Taq Premix I (Solgent, Daejeon, Korea), according to the manufacturer's instructions. The β-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). 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.

2.9. Quantitative real-time PCR (QPCR)

QPCR was conducted to determine the relative mRNA expression of Mn-SOD. Total RNA were extracted from the gill of experimental (Cd and TBT 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 β-Actin (AF026063) gene sequences of the *C. gigas* as follows: Mn-SOD forward primer, 5`-GAC CTG CCC TAT GAC TAC AAT GC-3`; Mn-SOD reverse primer, 5`-TCT GGT GAT GTT TGC TGT GAT GG-3`; β-Actin forward primer, 5`-TGG ATC GGT GGT TCC ATC CTT-3`; and β-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, A melt curve (data not shown) were performed, as well as analyzed 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 program, QPCR data from three replicate samples were analyzed 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, a melt curve were performed melting at only one temperature. The mRNA expression levels stood for an n-fold difference

relative to β-actin as the internal control.

2.10. 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 $P<0.05$.

3. Results

3.1. Identification of Mn-SOD cDNA

RT-PCR was used to generate a 496-base pair fragment of Mn-SOD using total RNA extracted from *C. 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*. Mn-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 *C. gigas* Mn-SOD gene was deposited in NCBI/GenBank under accession number EU420128.

The predicted amino acid sequence of Mn-SOD was compared to those deduced from known Mn-SOD cDNAs of other vertebrates and mollusks (Fig. 5). The Mn-SOD cDNA had high amino acid sequence identity with other mollusks: 63% identity to *H. discus discus* Mn-SOD (GenBank accession no. DQ530210) and 62% identity to *M. yessoensis* (AB222783) and *B. glabrata* (AY500813) Mn-SODs. *C. gigas* Mn-SOD was also similar to Mn-SODs from vertebrates: 60% identity to *X. laevis* (NM_001090499), *B. taurus* (BT020988) and *H. sapiens* (M36693) Mn-SODs, and 58% to *D. 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 (Fig. 5).

A phylogenetic tree was constructed to further analyze the evolutionary relationship of Mn-SOD sequences among various vertebrates and mollusks. Phylogenetic analysis indicated that *C. gigas* Mn-SOD clustered closely with the Mn-SOD of *H. discus discus* (Fig. 6).

3.2. Hydrogen peroxide (H_2O_2) concentrations

Hemolymph H_2O_2 concentrations significantly increased following Cd and TBT treatments in a dose- and time-dependent manner (Fig. 7). H_2O_2 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 an initial level of 2.4 ± 0.3 nM/ml. The maximal response of H_2O_2 concentrations was observed at the highest dose of Cd tested (0.1 ppm) (Fig. 7A). In TBT treatments, H_2O_2 concentrations increased significantly after 11 days with exposure to 5 (3.9 ± 0.3 nM/ml), 10 (5.2 ± 0.2 nM/ml) and 20 ppb TBT (8.5 ± 0.5 nM/ml) from an initial concentration of 2.4 ± 0.3 nM/ml (Fig. 7B).

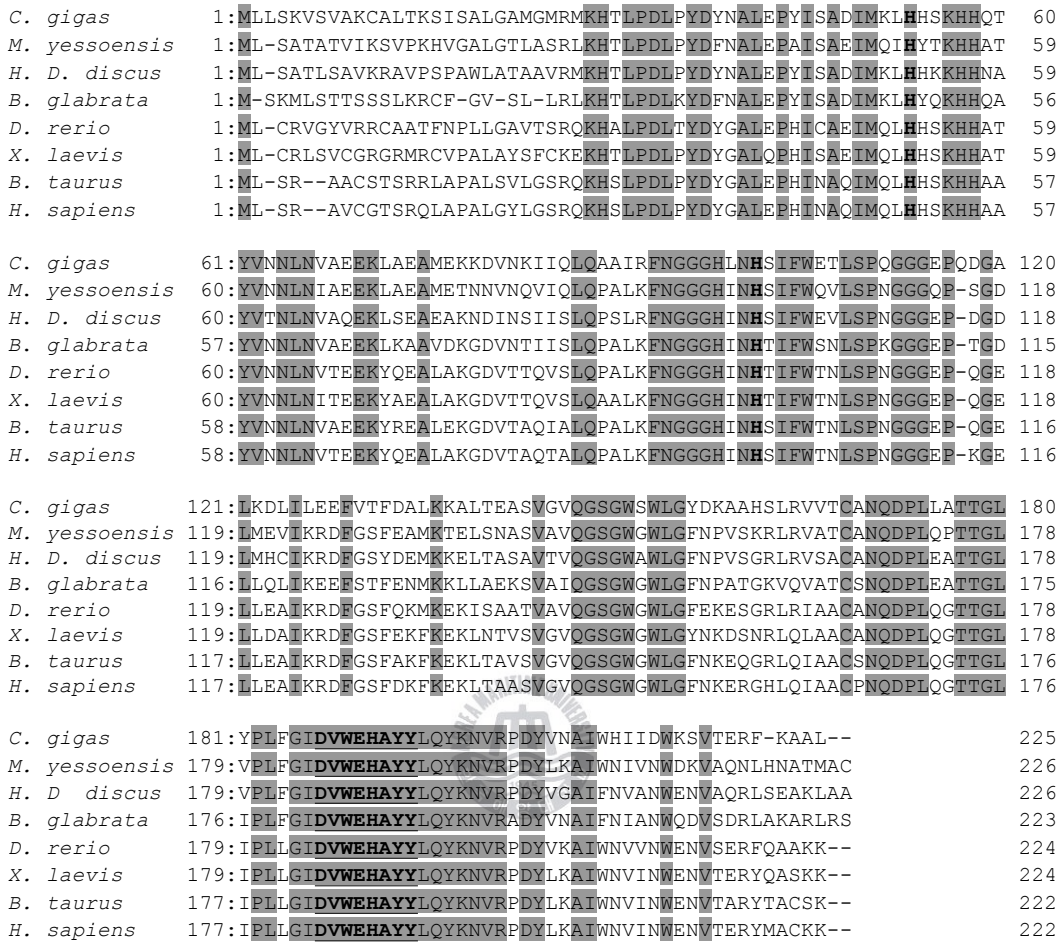

Fig. 5. Multiple alignment of the Mn-SOD gene of *Crassostrea gigas* (GenBank accession no. EU420128), *Mizuhopecten yessoensis* (AB222783), *Haliotis discus discus* (DQ530210), *Biomphalaria glabrata* (AY500813), *Danio rerio* (NM_199976), *Xenopus laevis* (NM_001090499), *Bos taurus* (BT020988) and *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.

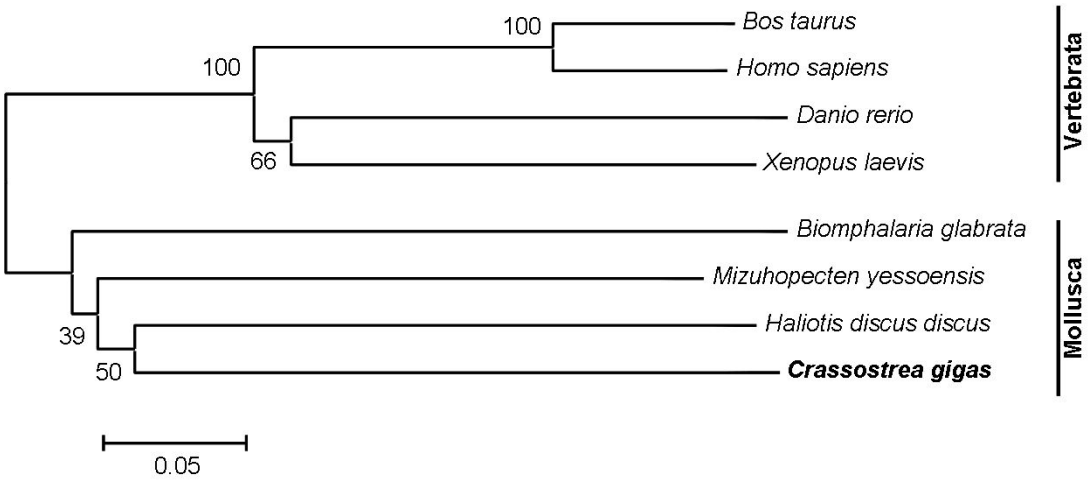

Fig. 6. Phylogenetic tree based on amino acid alignments for Mn-SOD in vertebrates and mollusks. Bootstrap values (%) are indicated (1,000 replicates). The score between two protein sequences, which is a measure of the relative phylogenetic relationship, is represented by the horizontal distance (i.e., 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.

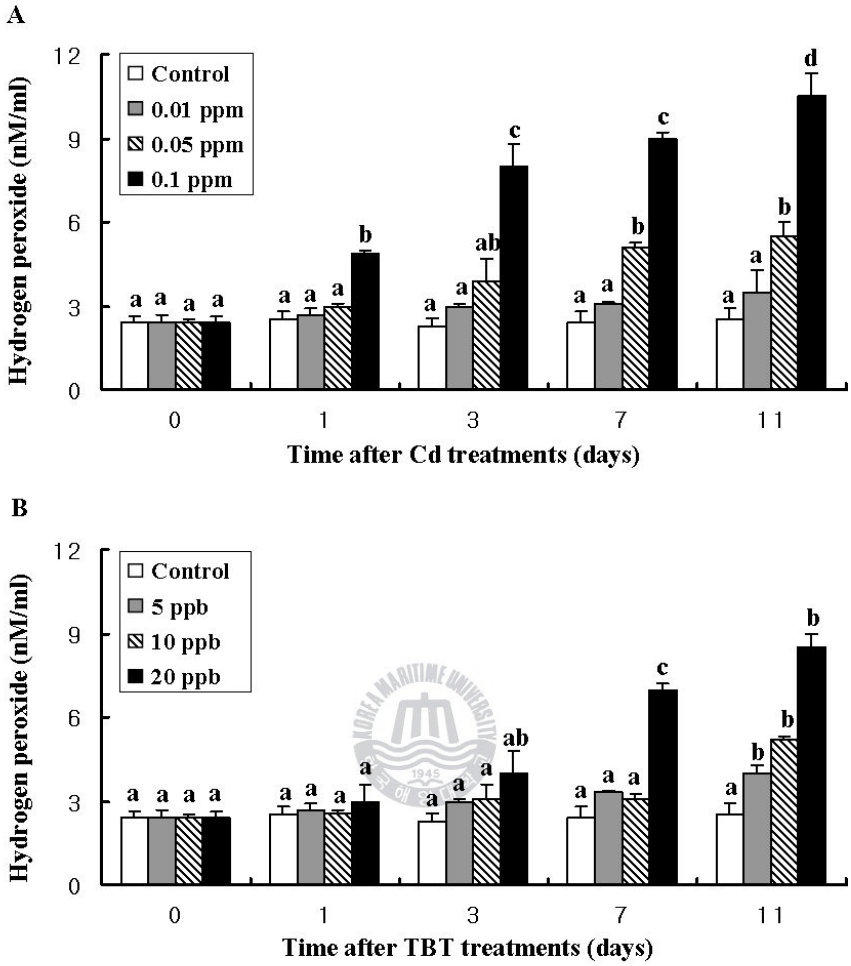

Fig. 7. H_2O_2 concentrations in the hemolymph of *Crassostrea gigas* by (A) Cd treatments (0 [control], 0.01, 0.05 or 0.1 ppm Cd for 1, 3, 7 or 11 days), (B) TBT treatments (0 [control], 5, 10 or 20 ppb TBT for 1, 3, 7 or 11 days). Lowercase letters indicate significant differences (P<0.05) among sampling times. Values indicate the mean±SD (n = 5).

3.3. Tissue distribution of Mn-SOD

The expression of Mn-SOD mRNA in various tissues from experimental (Cd and TBT treatments) oysters measured by RT-PCR (Fig. 8). The Mn-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.

3.4. 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 (Fig. 9A). 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.

3.5. 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 (Fig. 9B). 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.

3.6. Hemolymph analysis

Levels of GOT and GPT in the hemolymph are shown in Fig. 4 and Fig. 10. In Cd treatments, GOT and GPT levels increased significantly by 7 days at concentrations of 0.05 (GOT: 3.7 ± 1.5 IU/l; GPT: 6.3 ± 3.1 IU/l) and 0.1 ppm (GOT: 4.3 ± 1.4 IU/l; GPT: 8.0 ± 3.0 IU/l) (Fig. 4). In TBT treatments, GOT levels increased significantly by 3 (11.3 ± 3.5 IU/l) and 7 days (17.3 ± 4.8 IU/l), and GPT levels increased significantly by 7 days (18.0 ± 5.0 IU/l) at 20 ppb (Fig. 10).

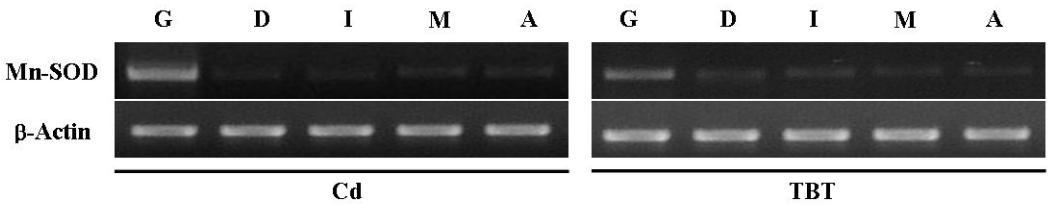

Fig. 8. Tissue-specific expression of Mn-SOD mRNA in various tissues (gill, G; digestive gland, D; intestine, I; mantle, M; adductor muscle, A) from Pacific oyster, *Crassostrea gigas* by Cd treatments, TBT treatments. Amplification of β-Actin was used as an internal control.

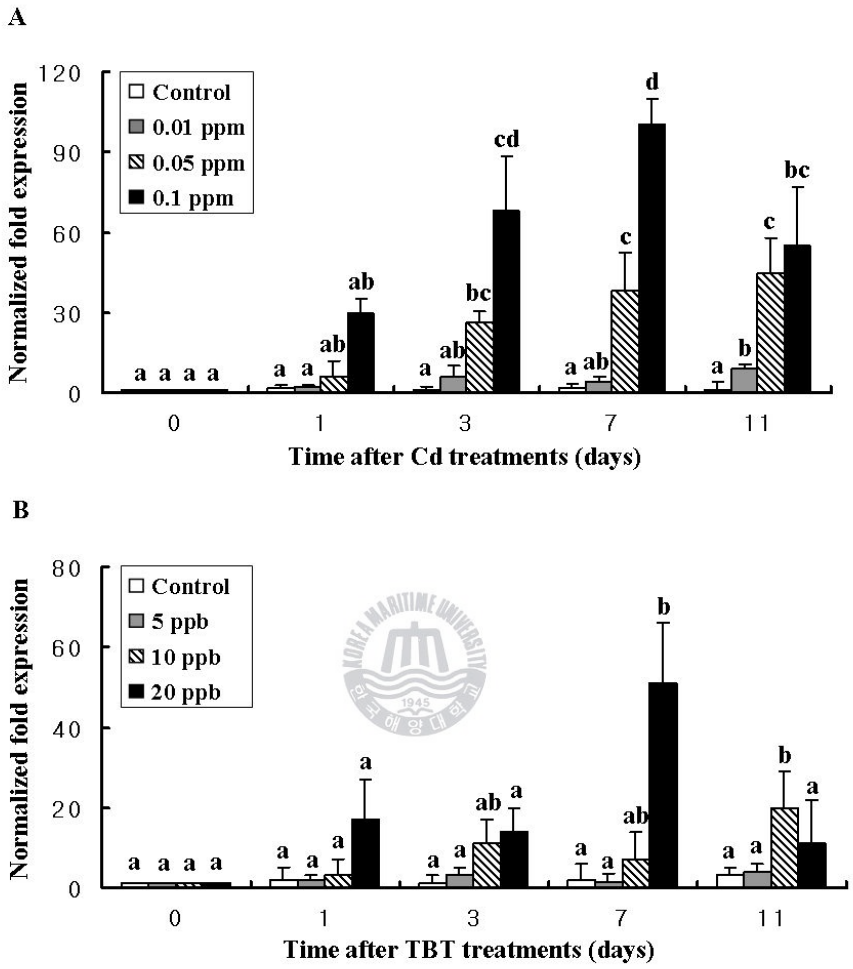

Fig. 9. Response of Mn-SOD mRNA expression in *Crassostrea gigas* by (A) Cd treatments, (B) TBT treatments. *C. gigas* Mn-SOD mRNA expression levels relative to β-Actin levels were analyzed using real-time PCR. Lowercase letters indicate significant differences (P<0.05) among sampling times. Values indicate the mean±SD (n = 5).

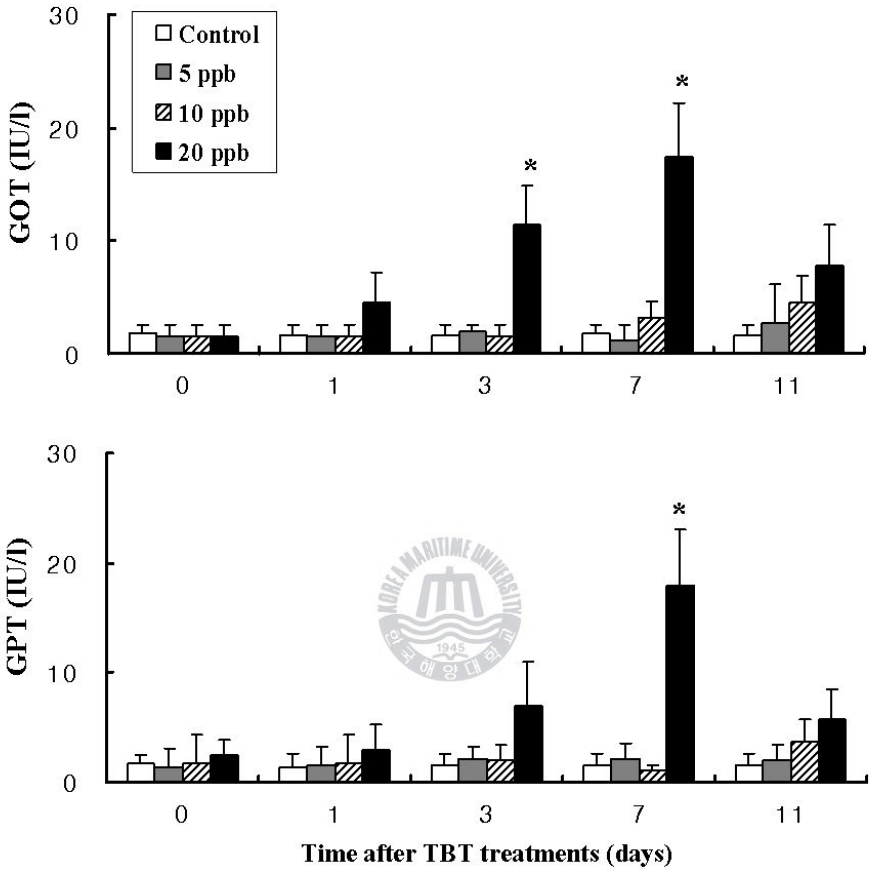

Fig. 10. Changes in the levels of the enzymes glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) in the hemolymph of Pacific oyster, *Crassostrea gigas* with TBT treatments. Oysters were treated with 0 (control), 5, 10 or 20 ppm TBT for 1, 3, 5, 7 or 11 days. Asterisks indicate a significant difference from the control ($P<0.05$). Values indicate the mean±SD (n = 5).

4. Discussion

To date, full-length Mn-SOD cDNA has been reported in only three species of mollusk: *M. yessoensis* (GenBank accession no. AB222783), *H. discus discus* (DQ530210) and *B. glabrata* (AY500813). In our study, Mn-SOD mRNA expression were analyzed H_2O_2 concentrations, GOT and GPT levels of Pacific oyster *C. gigas* in order to investigate the effect of Cd and TBT exposure on the physiological responses of this organism. Using the BLAST algorithm of the NCBI, *C. gigas* Mn-SOD isolated in this study displayed homology of 62% or higher with that of invertebrates. It also showed a homology of 58% or higher with Mn-SOD of vertebrates (Fig. 5). Through multiple alignment analysis, Several characteristic elements were observed among all species including *C. gigas* Mn-SOD. The Mn-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 and Cooper, 1998) (Fig. 5). High homology and conserved sites among species support the presumption that *C. gigas* Mn-SOD belongs to the Mn-SOD family and that it performs the functions and roles of Mn-SOD. *C. gigas* Mn-SOD was also phylogenetically closest to the *H. discus discus* Mn-SOD (Fig. 6). Following our analysis using BioEdit software, the mollusks (*C. gigas*, *H. discus discus*, *M. yessoensis* and *B. glabrata*) and vertebrates (*D. rerio, X.*

laevis, B. taurus and *H. sapiens*) were confirmed as two separate groups of Mn-SOD and Mn-SOD of *C. gigas* was genetically the closest to that of *H. discus discus*.

The expression of Mn-SOD mRNA were compared in various tissues of experimental (Cd and TBT treatments) oysters using RT-PCR (Fig. 8). 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. Therefore,gill tissue of *C. gigas* were used to compare Mn-SOD mRNA expression levels after exposure to Cd and TBT. 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 and 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). 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 on 7 days of exposure to pollutants. A decrease in Mn-SOD mRNA expression as the time of exposure elapsed 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 defense 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). Excessive Cd and TBT accumulation induces various oxidative reactions and, in the process, can generate a large quantity of ROS that promote oxidative stress (Stohs et al., 2000). In our study, ROS were generated due to toxicity of pollutants (i.e., Cd and TBT) by measuring the level of H_2O_2 production.

H_2O_2 concentrations in *C. gigas* displayed a tendency to increase as the concentration of pollutants increased and as time elapsed (Fig. 7). 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 and 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, ROS were generated due to toxicity of pollutants, 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.

The defense mechanism in mollusks, such as Pacific oysters, is triggered from the outside as blood cells in hemolymph, 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

hemolymph can occur due to various pollutants and environmental factors (His et al., 1996; Xue and Tristan, 2000). Among the constituting properties of hemolymph, an increase in GOT and GPT activation is generally caused by inflow to the hemolymph of cells separated due to tissue damage by environmental contaminants (Casillas et al., 1982). As GOT and GPT activities were measured, 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. This result was similar to the report by Cho et al. (2001) and Choi et al. (2008) that showed GOT activity increased when exposed to pollutants. Therefore, it is deduced that the significant increase of GOT and GPT activities in hemolymph of Pacific oysters was due to tissue damage by pollutants (Pickwell and Steinert, 1988).

In this study, 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. 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, hemolymph H_2O_2 concentration, GOT and GPT activities can be used as physiological markers to determine the degree of stress in Pacific oysters. Because study of Mn-SOD expression and oxidative stress was hardly reported, need more studies about this.

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IV. Conclusion

A large quantity of ROS generation caused by Cd toxicity accumulated in Pacific oyster *Crassostrea gigas* exposed to Cd. Excessive production of ROS induces oxidative stress and cellular damage and imbalance as well as denaturation or obstruction of enzyme activities.

The expression of HSP90, MT and Mn-SOD mRNA increased in order to maintain homeostasis, to protect cells from oxidative stress and to help eliminate the oxidative stress. Considering the increase in GOT and GPT in the hemolymph, cell damage occurred because of the loss of detoxification ability in Pacific oyster due to excess oxidative stress caused by the accumulation of Cd beyond a specific tolerance, were deduced.

Therefore, HSP90, MT and Mn-SOD mRNA expression, hemolymph H_2O_2 concentration and GOT and GPT activities can be used as physiological markers to determine for oxidative stress by heavy metals such as Cd contamination in Pacific oyster.

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해양분자생물공학연구실 N-217호에서

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