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

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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

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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-----RPFGCEGQLEFRALLFIPRRAPLDLFENKKKKNNIKLY 350
cfHSP90 298:TQEEYGEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFVPRRAPFDLFENKKKKNNIKLY 357
haHSP90 300:TQEEYAEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFLPKRAPFDMFENKKKKNNIKLY 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.

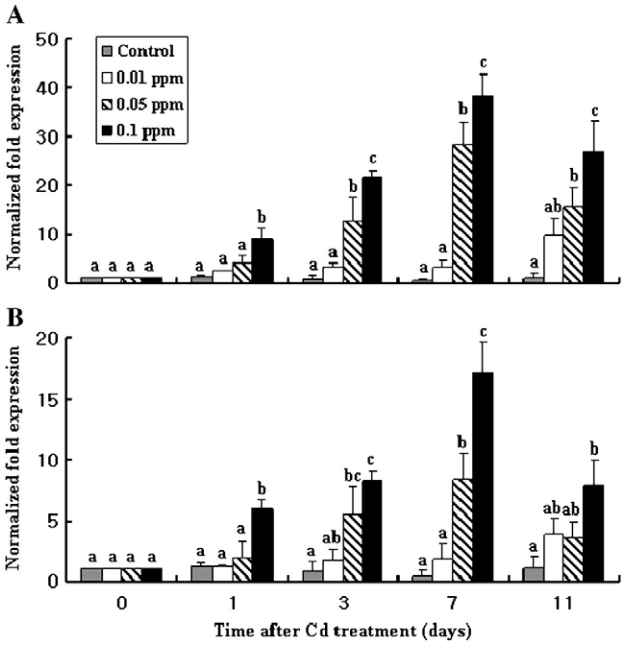

Fig. 2. Expression of HSP90 mRNA in response to cadmium treatment in the Pacific oyster (*Crassostrea gigas*) digestive gland (A) and gill (B). Oysters were treated with 0 (control), 0.01, 0.05, or 0.1 ppm Cd for 1, 3, 5, 7, or 11 days. The HSP90 mRNA expression levels relative to 28S rRNA levels were 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 ($n=4$).

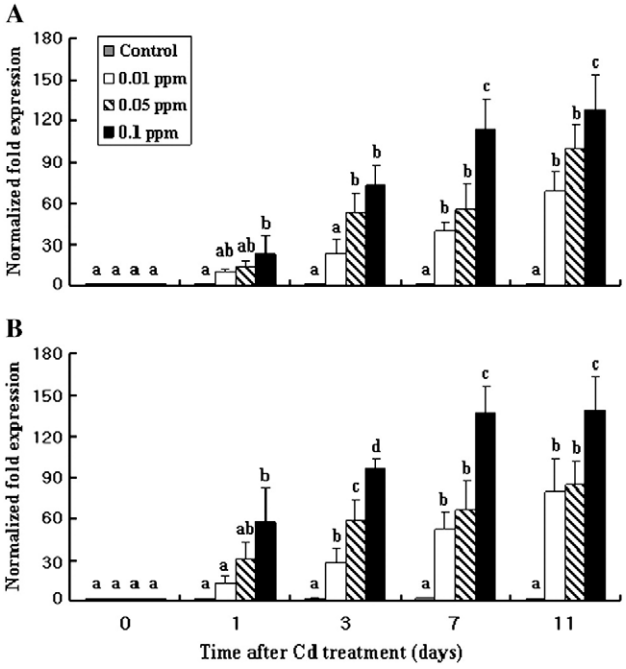

Fig. 3. Expression of MT mRNA in response to cadmium treatment in the Pacific oyster (*Crassostrea gigas*) digestive gland (A) and gill (B). Oysters were treated with 0 (control), 0.01, 0.05, or 0.1 ppm Cd for 1, 3, 5, 7, or 11 days. The MT mRNA expression levels relative to 28S rRNA levels were analyzed 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).

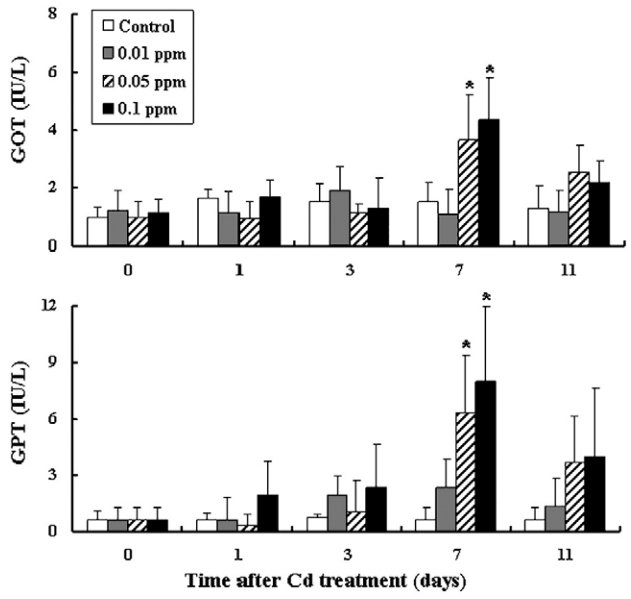

Fig. 4. Changes in the levels of the enzymes glutamate oxaloacetate transaminase (Aspartate aminotransferase) (GOT) and glutamate pyruvate transaminase (Alanine aminotransferase) (GPT) in the hemolymph of the Pacific oyster (*Crassostrea gigas*) with cadmium treatment. Oysters were treated with 0 (control), 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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