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Effects of heavy metals on the expression of a zinc-inducible metallothionein-III gene and antioxidant enzyme activities in *Crassostrea gigas*

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Abstract Sequestration by metallothioneins and antioxidant defense are two kinds of important defense mechanisms employed by mollusks to minimize adverse effects caused by heavy metal contaminants in marine environment. In the present study, a novel metallothionein gene, *CgMT-III*, was cloned from *Crassostrea gigas*, consisting of eighteen conserved cysteine residues and encoding a MT III-like protein with two tandem β domains. The expression level of CgMT-III transcript induced by zinc was much higher than that induced by cadmium exposure. It suggested that CgMT-III was perhaps mainly involved in homeostatic control of zinc metabolism, which was distinct from previously identified MTs in *C. gigas*. Among the tested antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), SOD and GPx showed varying up-regulations in a tissue-specific manner, while CAT activities were inhibited in both gill and hepatopancreas from *C. gigas* exposed to heavy metals. It can be inferred that CgMT-III was mainly involved in zinc homeostasis, and CgMT-III gene together with CAT enzyme could be potential biomarkers to indicate heavy metal, especially zinc pollution in marine organisms.

Keywords *Crassostrea gigas* · Metallothionein · Cadmium · Zinc · Antioxidant enzyme

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Introduction

With the rapid development of modern industry, various contaminants including both inorganic and organic substances were discharged into marine environments. Among these contaminants, heavy metals can be accumulated by the aquatic organisms and cause many adverse effects on their physiological reactions (Dovzhenko et al. 2005; Li et al. 1967; Felten et al. 2008; Viselina and Luk'anova 2000; Sokolova et al. 2005). For example, cadmium (Cd) is a nonessential heavy metal contaminant that accompanies zinc mineral, and is released into the environment by mining, refining and plating processes (Choi et al. 2007). Cadmium can inactivate many important enzymes by competing the catalytic sites of other metals (Chang et al. 2009; Bouilly et al. 2006; Dhavale et al. 1988; Bandyopadhyay et al. 1997), and induce oxidative stresses which cause damages to many important biological molecules including lipid, protein, DNA in aquatic organisms (Dovzhenko et al. 2005; Chappie 1997). In addition, some trace essential metal, such as zinc, when the concentration surpasses the quantity demanded, could displace other trace metals and interfere with the normal metabolism pathways, which would result in

deleterious effects on fertilization, sexual maturity and growth of the organisms (Münzinger and Guarducci 1988; Murphy et al. 2011; Ballatori 2002). In some mineral refineries of Hulu Island in China, waste water was discharged into the Bohai Sea without strict disposal. Consequently, marine organisms were faced with pollution from both cadmium and zinc as their coexistence.

For marine organisms, various defense strategies such as sequestration, antioxidant defense enzymes, etc., are employed to minimize the harmful effects of heavy metal contaminants (Geret et al. 2002, 2003; Jo et al. 2008; Klaassen et al. 1999; Kovářová and Svobodová 2009; Sato and Bremner 1993; Manduzio et al. 2004). Metallothioneins (MTs) are a super-family of metal-binding proteins with low molecular weight (6–7 kDa) and involved in multiple important physiological activities, such as metal homeostasis, detoxification, and antioxidant protection (Cols et al. 1999; Marie et al. 2006; Olafson and Thompson 1974; Roesijadi 1992; Wang et al. 2009; Coyle et al. 2002; Atif et al. 2006; Nordberg and Nordberg 2009). Both excessive essential (copper, zinc, iron, etc.) and non-essential (cadmium, mercury, etc.) heavy metals can induce the expression of MTs, which is primarily regulated at transcriptional levels (Pulido et al. 1966; Amiard et al. 2006; Bado-Nilles et al. 2008; Palmiter 1987). Presently, MTs from marine organisms have been proposed as a useful biomarker for heavy metal contaminants in marine environment (Amiard et al. 2006; Savva and Li 2000; Kovářová and Svobodová 2009; Wang et al. 2009; Van der Broeck et al. 2010; Fang et al. 2009; Liu and Wang 2011).

According to the nomenclature of metallothioneins, MT proteins have been divided into three classes, fifteen families among the animal, plant, prokaryote and fungi kingdoms (Binz and Kagi 1999; Kojima 1991). Despite the diversity of amino acid sequences in MTs, they are commonly structurally analogical with high content of cysteine residues (up to 30 %) and lack of aromatic amino acids (Nordberg and Nordberg 2009; Boulanger et al. 1983). The conserved Cys residues constitute mainly two kinds of domains in MT proteins: α domain including 11 Cys in an –NCNCN– or –NCCNCC– array, and β domain containing 9 Cys in –NCNCN or –NCNNCN– motif (Cols et al. 1999; Boulanger et al. 1983; Furey et al. 1986).

The Pacific oyster, *Crassostrea gigas*, is a kind of commercially important mollusk cultivated around the world. As sedentary, filter feeding, widely distributed marine bivalves, the oysters are regarded as suitable indicator animals to environmental pollution in many monitor programs, including the "Mussel Watch Program" initiated by the USA since 1986. Recently studies reported that oysters could accumulate heavy metal, especially zinc, to an extremely high level of body concentration (Cheung and Wang 2008). And the concentration of zinc in oysters is detected 10 times higher than that in mussels or clams (Pan and Wang 2012), which suggests that oysters are perhaps more suitable to indicate zinc pollution than other mollusk. To date, three kinds of MT genes have been cloned and functionally identified from *C. gigas* (Tanguy and Moraga 2001; Tanguy et al. 2001). In this study, a new kind of MT cDNA (designated as CgMT-III) was cloned from the digestive gland subtractive library of *C. gigas*, encoding a novel kind of MT protein that was different to previously identified MT proteins in *C. gigas*. The expression profiles of CgMT-III in hepatopancreas of *C. gigas* stressed by cadmium and zinc were investigated due to their high ecological risk to marine environment. Additionally, several important antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), were examined as well in order to investigate the potential roles of CgMT-III and antioxidant enzymes in response to heavy metal pollution.

Materials and methods

Animals, metal exposure and tissue collection

Adult *C. gigas* (averaging 52.3 g in total weight) were collected from an unpolluted aquaculture farm in Yantai. They were allowed to acclimate for 8 days in aerated, filtered seawater (19–21 °C, 32 psu, collected from pristine environment). A mixture of *Isochrysis galbana* and *Chlorella vulgaris Beij* was used to feed the oysters after the seawater was renewed daily.

After acclimatization, all the oysters were divided into four groups (control and three heavy metal-exposd groups), each containing three replicates. The first group received no challenge and was used as the control. The other three groups were exposed to 20 μg L^{-1} of Cd (as $CdCl_2$), 50 μg L^{-1} Zn (as $ZnSO_4$) and a mixture of Cd (20 μg L^{-1}) and Zn (50 μg L^{-1}), respectively. The concentrations of Cd and Zn used for the exposure experiment can be found in certain polluted sites along the coast of the Bohai Sea (Zhang 2001; Marie et al. 2006). Five oysters from each group were randomly sampled at 0, 24, 48, 72 h, respectively. The dissected tissues of gill and hepatopancrea were flash frozen in liquid nitrogen and stored at −80 °C prior to enzyme assay. A slice of hepatopancreas was subjected to RNA extraction immediately by using TRIzol® Reagent (Invitrogen, Cat. #15596-026).

cDNA library construction and EST analysis

A subtractive cDNA library was constructed from the digestive gland of Pacific oyster *C. gigas*, using a PCR-Select™ cDNA subtraction kit (BD Clontech, Cat. #

637401). Random sequencing of 200 randomly-selected positive clones yielded a fragment which was highly similar to previously identified metallothionein. This sequence was then selected for further cloning of the full-length cDNA of CgMT-III in *C. gigas*.

Cloning the full-length cDNA of CgMT-III gene

Two gene-specific primers, sense primer P1, P2 (Table 1) were designed based on the above fragment to clone the full-length cDNA of CgMT-III. A modified touch down-PCR (TD-PCR) was performed to amplify the 3′ end of *CgMT-III* in a Biometra Thermocycler using sense primer P1 and oligo dT (Table 1). The TD-PCR program was as following: 94 °C for 5 min, followed by 5 cycles of 5 s at 94 °C, 2 min at 64 °C, 25 cycles of 5 s at 94 °C for, 2 min at 62 °C. For the second round of PCR, 1 μL of 1:100 diluted PCR product was used as template, P2 and oligo dT were employed to carry out a semi-nested PCR. The PCR products were gel-purified and cloned into the pMD19-T simple vector (Takara Bio, Cat. #D104). After transformed into the competent cells of *E. coli* top10, the recombinants were screened by blue-white color selection in ampicillin-containing LB plates. Three positive clones were sequenced in both directions, and the resulting sequences were verified and subjected to cluster analysis. Primers P3 and P4 (located at 5′ and 3′ end of CgMT-III, respectively) were used to verify the clustered sequence.

Multiple alignment and phylogenetic analysis

Searches for nucleotide and protein sequence similarities were conducted with BLAST algorithm at the National Center for Biotechnology Information (Altschul et al. 1997).

Table 1 Primers used in the present research

Primer name	Sequence (5′–3′)
Gene cloning	
P1 (forward)	ACCCTTGCGGATGCACGGAG
P2 (forward)	TCTTCTCCTGCATCATCTTTTG
Verifying primers	
P3 (forward)	CACCTCCAAAATGCCAATC
P4 (reverse)	CTGTTTCTATTCTTTCACAAGCA
RT-PCR primers	
P5 (forward)	ATGTAATTGCGGCGAAACCT
P6 (reverse)	TTCACAAGCAAATCTTCTCCTG
β-actin primers	
P7 (forward)	GCCCTGGACTTCGAACAA
P8 (reverse)	CGTTGCCAATGGTGATGA
cDNA synthesis	
Oligo (dT)	$CTCGAGATCGATGCGGCCGCT_{17}$

Multiple alignment of CgMT-III was performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). Phylogenetic tree was constructed with MEGA program (version 3.1) based on amino acid sequences alignment by using the neighbor-joining method with 1,000 replication in bootstrap test.

RNA extraction and quantification analysis of CgMT-III mRNA expression

Total RNA was extracted by using TRizol® Reagent (Invitrogen, Cat. #15596-026). Two microgram of RQI DNase-treated total RNA was used for cDNA synthesis with the M-MLV reverse transcriptase (Promega, Cat. # 9PIM170).

The fluorescent real-time quantitative PCR was performed on an ABI 7500 Real-Time Detection System (Applied Biosystems) to investigate the mRNA expression of CgMT-III gene. Gene-specific primers P5, P6 for CgMT-III (Table 1) were used to amplify a product of 192 bp. A pair of oyster *β*-actin primers, P7 and P8 (Table 1) were used to amplify a 100 bp fragment to verify the successful transcription and as an internal control to calibrate the cDNA template for corresponding samples (Farcy et al. 2009). The PCR amplifications were carried out in triplicate in a total volume of 25.0 μL containing 12.5 μL of 2× SYBR Green Master Mix (Applied Biosystems, Cat. # 4385617), 4.0 μL of 1:20 diluted cDNA, l.0 μL of each primer, 6.5 μL of PCR-grade water. The PCR program was 50 °C for 2 min and 95 °C for 5 min, followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C. The expression level of CgMT-III were analyzed by the comparative CT ($2^{-\Delta\Delta CT}$) method as previously described (Livak and Schmittgen 2001; Cong et al. 2009).

Enzyme assays

Gills and hepatopancreas were homogenized in liquid nitrogen, and enzymatic activities of SOD, GPx, CAT and total protein concentrations were assayed according to the manufacturer's protocols (Jiancheng, Nanjing, China, Cat. # A001-1, A005, A007, A045-3, respectively). All the enzyme activities were expressed in term of units per mg of protein (U/mg), where one unit represented the change in absorbance spectra per milligram protein in 1 min.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's test was used to compare the significance among the biological data from control and three heavy metal-exposed groups at each time point ($n = 5$). All the data were given in terms of means ± standard deviation (SD). A p value $p < 0.05$ is considered statistically significant.

Results

cDNA cloning and analysis of the CgMT-III

A 349 bp nucleotide sequence representing the full-length cDNA of CgMT-III was deposited in GenBank under accession number JF781299. The full-length cDNA consisted of a 5′ untranslated region (UTR) of 28 bp, a 3′ UTR of 132 bp and an open reading frame of 189 bp encoding a polypeptide of 63 amino acids (Fig. 1a) with an estimated molecular mass of 6.39 kDa and theoretical isoelectric point of 4.075. The deduced amino acid sequence of CgMT-III contained 18 Cys residues, and no aromatic residue was found.

Multiple alignment and phylogenetic analysis

BLAST analysis revealed that CgMT-III matched closely with previously reported MT family members. For example, CgMT-III exhibited 69.4 % identity to *Crassostrea virginica* MT IIIA, 67.7 % to *C. virginica* MT and *C. virginica* MT IIIC, 66.1 % to *C. virginica* MT IIIB, while less identity to other MTs in *C. gigas* (≤34 %). Eighteen Cys residues in CgMT-III were totally conserved in all the selected MT proteins (Fig. 1b). Six –NCNCN– and two –NCNCNNC– patterns, which could be divided into two clusters with each containing nine Cys residues, appeared in the amino acid sequence of CgMT-III. Based on the distribution of Cys residues, CgMT-III exhibited the typical characteristics of β-domain in MT protein (Otvos and Armitage 1980).

A phylogenetic tree was constructed based on the amino acid sequences of selected MTs with the neighbor-joining method. MT proteins from *C. gigas* (CAB85588, CAC 48045, CAB64869) were first clustered together into a subbranch which clustered with MTs from *B. thermophilus*, and then clustered with another two MT proteins from *C. gigas* (CAC82788, CAK22381). However, CgMT-III was clustered with MT proteins from the eastern oyster *C. virginica* (AAQ23919, AAQ23917, AAQ23918, AF506978), and fell into the MT III subgroup (Fig. 1c).

Quantitative analysis of CgMT-III gene expression after heavy metal exposure

In the Cd-exposed group, significant enhancement of CgMT-III expression occurred at 48 h compared with the control (7.9-fold, $p < 0.05$), while no significant difference was observed at other time points. For the Zn-exposed oysters, significant increments of CgMT-III expression were observed at 24 and 72 h (6.3-fold, 235.8-fold, $p < 0.05$) as compared to the control. However, for the oysters in Cd + Zn-exposed group, no significant difference in the expression of CgMT-III transcript was observed compared with that of the control group throughout the sampling time points (Fig. 2).

Effect of Cd and Zn exposures on enzyme activities

Antioxidant enzymes including SOD, GPx, and CAT were examined in gill and hepatopancrea tissues. For SOD activities in gills, there was no significant difference between each treatment during the exposure period (Fig. 3a). However, significant up-regulation ($p < 0.05$) of SOD activity in hepatopancreas of the Zn-exposed oysters was observed at 24 h post exposure. Slight increments of SOD activities were found in the Cd- and Cd + Zn-exposed treatments; nevertheless, no significant difference existed between each heavy metal-exposed group and the control (Fig. 3b).

As for GPx activities in gills, Zn exposure significantly induced the up-regulation ($p < 0.05$) at 72 h. Significant increments were also recorded in Cd + Zn-exposed group in comparison to that of the control group at 24 and 72 h ($p < 0.05$) (Fig. 4a). However, no significant difference in GPx activity was observed in the Cd-exposed group compared with that of the control group. Similarly, significant increment occurred in hepatopancreas of oysters exposed to Cd + Zn at 72 h ($p < 0.05$). However, no significant changes in GPx activity were observed from either Cd- or Zn-exposed group.

Contrary to the up-regulation trends of SOD and GPx activities, inhibition of CAT activities was observed in both gills and hepatopancreas (Fig. 5). In gills, significant decrease in CAT activity was detected in Cd- and Cd + Zn-exposed groups ($p < 0.05$) at 24 h, and in the Zn- and Cd + Zn-exposed groups ($p < 0.05$) at 48 h. As time progressed on, CAT activities in gills of all treatments recovered to the control level at 72 h. In hepatopancreas, significant down-regulations were observed in Cd- and Zn-exposed groups at 24 h ($p < 0.05$). However, there was no significant difference between the exposed and control groups at other time points, though significant decrement occurred in CAT activity of the Zn-exposed group compared with those of the Cd- and Cd + Zn-exposed groups at 72 h.

Discussion

Due to the rapid industrial development, heavy metal contaminants, such as cadmium, have been increasingly aggravated in the marine and coastal environments. Mollusks (mussel, oyster, clam, etc.) have been used as sentinel organisms for heavy metal monitoring in neritic regions (Dumbauld et al. 2009; Geret et al. 2003; Liang et al. 2004). Presently, MTs and antioxidant enzymes are well acknowledged as important biomarkers to heavy metal

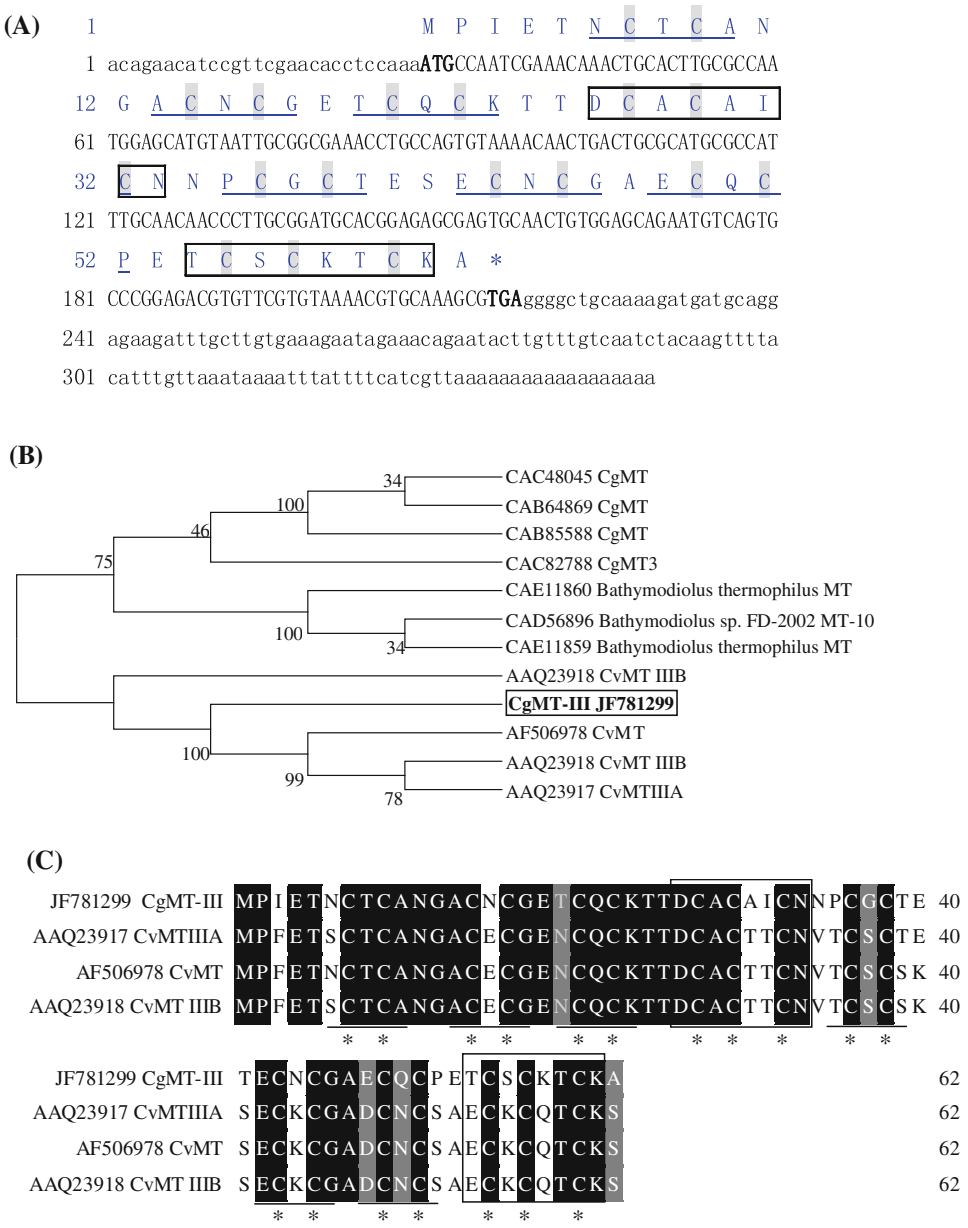

Fig. 1 **a** Nucleotide sequence of CgMT-III cDNA from *C. gigas* and its deduced amino acid sequence. The start and stop codens are in *bold*. Conserved Cys residues are *shaded*. Residues containing Cys in –NCNCN– pattern are *underlined* and those in –NCNNCN– pattern are *boxed*. **b** Multiple alignment of selected MT proteins and the deduced amino acid sequence of CgMT-III. The selected sequences include *Crassostrea virginica* MT IIIA (AAQ23917), *C. virginica* MT IIIC (AAM90258), *C. virginica* MT IIIB (AAQ23918). Identical residues are shaded *dark gray* and similar residues are shaded *light gray*. The conserved Cys residues were indicated with "*asterisk*". Cys residues in –NCNCN– patterns are *underlined* and those in –NCNNCN– pattern are *boxed*. **c** A phylogenetic tree constructed based on the selected MTs. One thousand bootstrap trials were run by using the neighbor-joining algorithm embedded in MEGA program version 3.1. The number associated with each internal branch was the local bootstrap probability, which was an indicator of confidence

contaminants (Ivanina et al. 2008; Baraj et al. 2011; Choi et al. 2008).

In the present study, the cDNA encoding CgMT-III gene was cloned from the Pacific oyster *C. gigas*. The 18 conserved Cys residues in the deduced amino acid sequence of CgMT-III constitute two tandem β domains, and showed high identities with CvMT III isoforms. It suggested that CgMT-III was probably a new member of the MT-III protein, distinct from previously identified MT proteins in *C. gigas*. It has been suggested that MT proteins containing two β domains mainly play a housekeeping function in metal-regulation of physiological metals (Cols et al. 1999; Narula et al. 1995). Zinc is an important essential metal for the organisms (Klaassen et al. 1999), however, excessive zinc can cause toxicity to the marine organisms (Münzinger and Guarducci 1988). Fortunately, MTs have the capacity to bind excessive zinc and facilitate the host to keep a normal concentration of internal zinc (Klaassen et al. 1999). Hepatopancreas of mollusks are often used as the target tissue in ecotoxicology due to the strong detoxification function (Le Pennec and Le Pennec 2001). In this study, the quantitative PCR data indicated that CgMT-III gene in hepatopancreas was induced significantly by zinc and slightly by cadmium. It was suggested that CgMT-III was mainly involved in zinc homeostasis and kept the oysters from toxic effect of excessive zinc. In addition, cadmium is a non-essential heavy metal, which has adverse effects on the physiological responses of mollusks

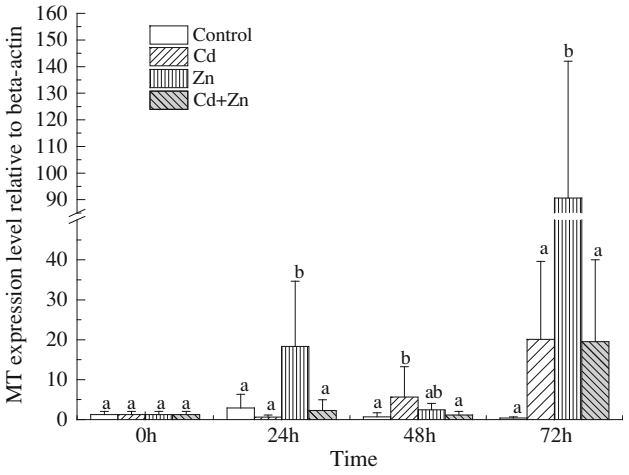

Fig. 2 Temporal mRNA expression profiles of CgMT-III gene after exposure to Cd, Zn, Cd and Zn mixture

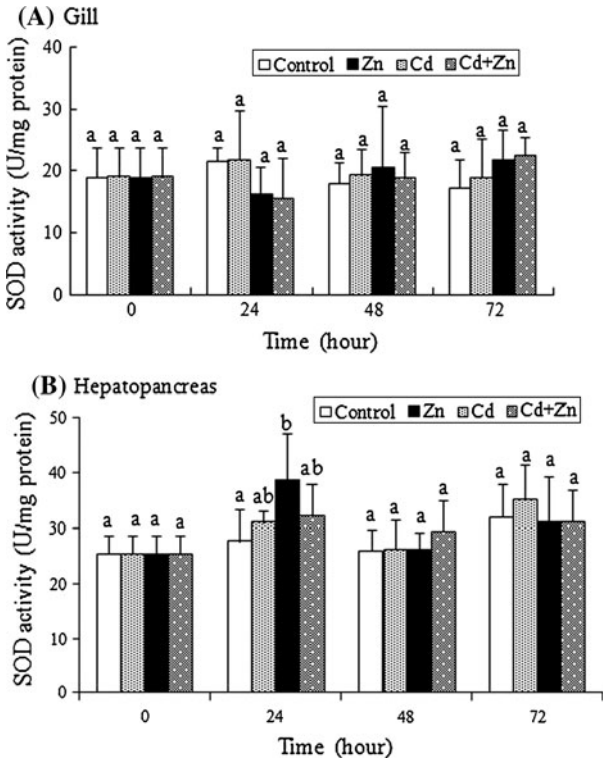

Fig. 3 SOD activities in tissues of gills (**a**) and hepatopancreas (**b**) of oysters after exposure to Cd, Zn, Cd and Zn mixture. Data were expressed as mean ± SD (n = 5). *Different letters* denote values that are significantly different ($p < 0.05$) and the columns that share a *same letter* are not significantly different ($p > 0.05$)

(Sokolova et al. 2005; Viselina and Luk'anova 2000; Wu et al. 2010). The slight increment of CgMT-III implied that CgMT-III was perhaps involved in cadmium detoxification as well. Moreover, mixture of zinc and cadmium exposure had no significant effect on the expression of CgMT-III, indicating that Zn and Cd probably performed antagonistic

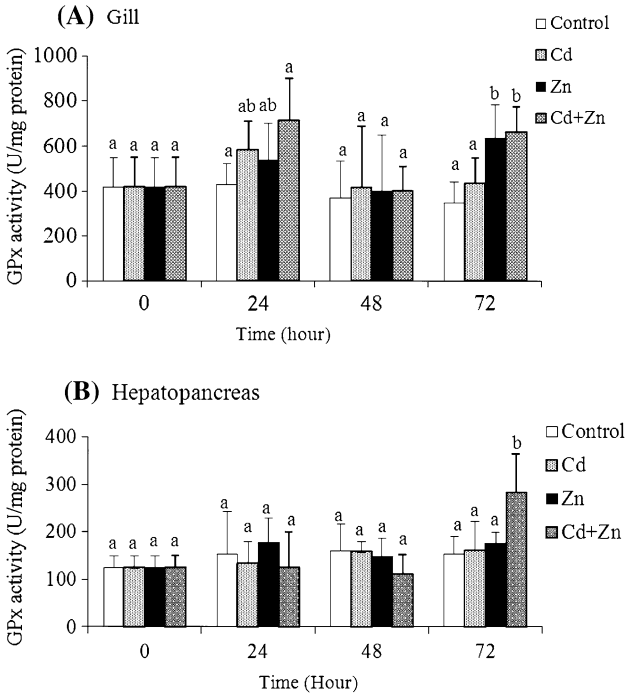

Fig. 4 GPx activities in tissues of gills (**a**) and hepatopancreas (**b**) of oysters after exposure to Cd, Zn, Cd and Zn mixture. Data were expressed as mean ± SD (n = 5). *Different letters* denote values that are significantly different ($p < 0.05$) and the columns that share a *same letter* are not significantly different ($p > 0.05$)

effects in *C. gigas* because of the chemical similarity between them (Daka and Hawkins 2006). It was consistent with previous studies that zinc was a cadmium antagonist in mollusks (Daka and Hawkins 2006; Hemelraad et al. 1987).

So far, several MT genes have been isolated from *C. gigas*. It was reported that mRNA expressions of CgMT1 and CgMT2 were both increased significantly (from 7- to 11-fold) upon cadmium stress, while remained no change towards zinc stress. However, CgMT3 exhibited a very low inducibility to zinc or cadmium stress compared with CgMT1 and CgMT2, which was presumed that CgMT3 had little physiological functions under metal exposure or expressed in some particular developmental stages in the Pacific oysters (Marie et al. 2006). Compared with CgMT1, CgMT2 and CgMT3, it can be inferred that CgMT-III was mainly responsible for the regulation of zinc homeostasis rather than other MT genes in *C. gigas*. Therefore, CgMT-III could be used as the potential biomarker for zinc pollution in *C. gigas*.

Besides sequestration by MT proteins, a series of antioxidant enzymes are also involved in the detoxification by eliminating reactive oxygen species (ROS) frequently produced by excessive heavy metal contaminant in mollusks (Jo et al. 2008; Geret et al. 2002, 2003; Manduzio

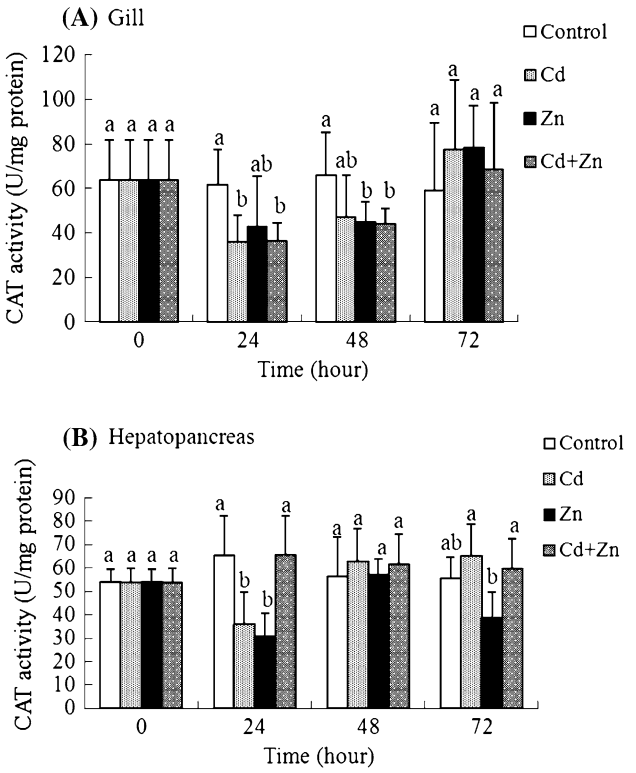

Fig. 5 CAT activities in tissues of gills (**a**) and hepatopancreas (**b**) of oysters after exposure to Cd, Zn, Cd and Zn mixture. Data were expressed as mean ± SD (n = 5). *Different letters* denote values that are significantly different ($p < 0.05$) and the columns that share a *same letter* are not significantly different ($p > 0.05$)

et al. 2004). Among the antioxidant enzymes, SOD, CAT and GPx play important roles in scavenging free radicals (Valavanidis et al. 2006). SOD catalyzes the dismutation of superoxide anion into hydrogen peroxide, which is in turn reduced by CAT into water and molecular oxygen. GPx neutralizes peroxides including hydroperoxides and organic peroxides into water or stable alcohols (Geret et al. 2003). In this study, a transient increment was observed in SOD activity of hepatopancreas in Zn-stressed group, while no significant change occurred in other stressed groups. It was suggested that SOD activity was more sensitive to zinc rather than cadmium exposure. Similar results were also reported in other organisms previously (Olin et al. 1995; Chakraborty et al. 2007). Perhaps it could be explained that zinc was an essential component of copper/zinc SOD, and addition of external zinc was convenient to stabilize copper/zinc SOD and enhance SOD enzyme activity (Kajihara et al. 1988; Jing et al. 2007). For GPx activity, significant up-regulations were observed in gills of the Zn-stressed group at the end of the experiment. Several studies have found that low concentration of zinc could enhance GPx activity in many marine organisms, such as mussels and trout, which was similar to the present result (Franco et al. 2006). Although the single exposure of cadmium exerted no significant effect on GPx activity, the combined exposure of cadmium and zinc increased GPx activity in both gills and hepatopancreas. It was probably ascribed to the positive influence of zinc, because it was known that exposure to cadmium impacted little on zinc effect on GPx activity (Rainbow 1997; Langston and Bebianno 1998; Daka and Hawkins 2006; Jihen et al. 2011). However, unlike to the up-regulation of SOD and GPx, inhibited CAT activities were observed in either gills or hepatopancreas tissues of the stressed oysters. The similar phenomenon was also reported in nemertean *Cephalothrix hongkongiensis* and oysters towards heavy metal exposure (Wu et al. 2010; Andersen et al. 2006). In a word, SOD and GPx activities exhibited tissue specific up-regulation towards zinc or mixed stresses of cadmium and zinc, however, CAT in both gills and hepatopancreas showed inhibited activities in a more sensitive manner. Previous studies have reported various results about the heavy metal toxicity on mollusks. For example, cadmium/copper caused no change in ROS production in mussel (Goméz-Mandikute and Cajaraville 2003). But sodium arsenite could inhibit the activities of CAT and induce oxidative stress in the gills of *Lamellidens marginalis* (Chakraborty et al. 2010). Whatever the antioxidant enzyme activities were up- or down-regulated, the heavy metal contamination exerted adverse effect on some immune parameters of mollusks, and the responding capability depended on the concentration of the metal (Girón-Pérez 2010). Similarly, the variations of the antioxidant enzymes activity in the present study revealed that 20 μg L^{-1} of cadmium or 50 μg L^{-1} of zinc affected the antioxidant system of Pacific oysters, and the related antioxidant enzyme could be used as sensitive biomarker to indicate the heavy metal contamination.

In conclusion, a novel kind of zinc-inducible metallothionein gene has been cloned from *C. gigas* and designated as CgMT-III. The expression of CgMT-III transcript appeared to be more sensitive to zinc exposure than cadmium, and was presumed to be mainly involved in homeostatic control of zinc metabolism. Among the tested antioxidant enzymes, SOD and GPx showed varying increased activities to zinc or cadmium exposure, while CAT activities of the heavy metal stressed groups decreased sensitively compared with that of control group. Therefore, it can be inferred that CgMT-III gene was a potential biomarker for zinc pollution and CAT was suitable to be used as an enzyme biomarker to present the adverse effect of heavy metal in oysters.

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