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Cloning of a novel glutathione S-transferase 3 (*GST3*) gene and expression analysis in pearl oyster, *Pinctada martensii*

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ABSTRACT

Microsomal glutathione S-transferase (MGST) functions in cellular defense against xenobiotics and provides protection against the action of lipid hydroperoxides produced as a consequence of oxidative stress. In this study, a full-length cDNA encoding MGST3 (referred to as PmMGST3) was identified from the pearl oyster, *Pinctada martensii* by a combination of expressed sequence tag (EST) analysis and rapid amplification of cDNA ends (RACE). The full-length cDNA of *PmMGST3* is 971 bp and contains a 5′ UTR of 39 bp, a 3′ UTR of 491 bp with a canonical polyadenylation signal sequence (AATAAA), and an open reading frame (ORF) of 447 bp encoding a polypeptide of 146 residues. The deduced polypeptide contains a conserved motif ($FNCx_1QRx_2H$) characteristic of the MGST3 subfamily. The *PmMGST3* transcript could be detected in all tissues tested, with highest transcript level seen in hepatopancreas. Cadmium treatment significantly increased *PmMGST3* mRNA levels in gill and hepatopancreas, while bacterial challenge initially depressed mRNA levels and then increased its level in haemocytes, gill and hepatopancreas in a time-dependent manner. In an assay using cumene hydroperoxide as a substrate, we demonstrated that PmMGST3 possesses glutathione-dependent peroxidase activity. These results suggest that *PmMGST3* plays an important role in cellular defense against oxidative stress caused by cadmium and bacteria.

1. Introduction

Microsomal glutathione S-transferases (MGSTs) are members of the MAPEG (Membrane Associated Protein in Eicosanoid and Glutathione metabolism) protein family [1,2] and they play critical roles in detoxification of xenobiotics and metabolites produced as a consequence of oxidative stress [3]. In rat, MGSTs possess glutathione - dependent peroxidase activity towards lipid hydroperoxides [4] and specific hydrophobic substrates [5]. Human MGSTs are located in membranes of the endoplasmic reticulum, suggesting that they play a role in the protection of membranes against lipid peroxide generated as a consequence of oxidative stress [6]. In addition to roles in detoxication of xenobiotics and antioxidant defense against oxidative stress, some members of MGSTs are also involved in redox regulation and are associated with apoptosis or cancer development [7] and have pro-inflammatory activities in human [3].

MGST proteins have been identified in various species, including four MGSTs in mammals [2,8]. MGST1 was originally identified in rat liver microsomes in 1982 [9] and the enzyme is different from cytosolic GST (cGST) in subunit molecular mass and primary sequence [10]. MGST2 was identified and characterized in 1996 from human [11] and MGST3 and MGST1-L1 were identified a year later [7,12]. MGST1, MGST2 and MGST3 proteins each have glutathione transferase as well as glutathione peroxidase activities. However, as opposed to MGST1 and MGST2, human MGST3 cannot catalyze the conjugation of GSH to 1-chloro-2, 4-dinitrobenzene (CDNB) [3]. Both MGST2 and MGST3 are able to catalyze the conjugation of glutathione with leukotriene A4, an important mediator of inflammation in human [13]. In non-mammals, *MGSTs* have been cloned from *Drosophila*, pufferfish (*Takifugu obscurus*), European flounder (*Platichthys flesus*), common carp (*Cyprinus carpio L*), and *Xenopus* [14]. In addition, homologues to MGST have also been identified in plants, fungi, and bacteria [3,8]. To the best of our knowledge, no *MGST* except two pearl oyster ESTs (GenBank accession no. FG591726 and FG597209) has been reported to date.

Pearl oyster, *Pinctada martensii*, is a commercially important aquatic shellfish for pearl production in China, Japan, and Southeast Asia. Both heavy metals and pathogens in seawater can cause oxidative stress in pearl oysters which, in turn, can lead to a decline in pearl production. Thus, the cloning of genes involved in oxidative

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stress responses and the study of their expression patterns could provide insights into both antioxidant mechanisms in mollusks generally and disease control in cultured pearl oyster.

In the present study, we cloned a full-length *PmMGST3* cDNA, examined the distribution of the *PmMGST3* transcript in different adult tissues, and investigated the temporal expression patterns of *PmMGST3* in pearl oysters challenged with *Vibrio alginolyticus* or after exposure to cadmium. The glutathione-dependent peroxidase activity of recombinant PmMGST3 fused to maltose binding protein (MBP) was also measured.

2. Materials and methods

2.1. Cloning of the full-length cDNA of PmMGST3 and phylogenetic analysis

By functional annotation of pearl oyster EST sequences obtained from NCBI GenBank using Blast2GO software, EST sequences (GenBank accession no. FG591726 and FG597209) that shared high similarity to vertebrate *MGST3s* were obtained. Full-length cDNA sequence of *PmMGST3* was further obtained using RT-PCR followed by 5′ and 3′ rapid amplification of cDNA ends (RACE) based on the known sequence (GenBank accession no. FG591726). AMV reverse transcriptase (Promega) was used to generate cDNA from 1 μg of total RNA prepared from gill tissue using SV Total RNA Isolation System (Promega) as recommended by manufacturer. 5′ RACE and 3′ RACE were carried out using SMART RACE cDNA Amplification Kit (Clontech) according to manufacturer's manual with the following modification: to enhance the specificity of the PCR, the adaptor primer (Universal Primer A Mix) was omitted for the first five cycles of PCR. Gene specific primers for 5′ RACE (5P1 and 5P2, Table 1) and 3′ RACE (3P1 and 3P2, Table 1) were designed according to the EST sequence (FG591726).

Protein sequences were aligned using Clustal W 1.81 [15] and similarity was shaded with GeneDoc version 2.6.002 [16]. The GST-MAPEG domain was determined through Search for Conserved Domains within a protein sequence in NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). A phylogenetic tree was constructed by the Neighbor-Joining (NJ) Method with MEGA version 4.1 [17], and tested for reliability over 1000 bootstrap replicates. The phylogenetic relationship was further analyzed using Maximum Parsimony method with MEGA version 4.1 [17].

2.2. Animals treatment and expression analyses of PmGST3 mRNA

Pearl oysters (shell length 4.5–5.5 cm, 2 years old) were purchased from a culture farm in Zhanjiang, Guangdong Province, China, and maintained at 20–22 °C in 400 L of filtered and well-aerated seawater (salinity, 32‰) for one week before processing.

The pearl oyster bacterial challenge experiment was carried out as described by Zhang [18] with some modifications. 100 acclimated pearl oysters were randomly transferred into 2 plastic tanks (50 per tank) filled with 400 L of seawater (salinity, 32‰). The bacteria strain for challenge experiment was cultured on LB plates at 30 °C overnight, ant then a single colony was inoculated in 5 ml of LB broth at 30 °C for 12 h. The resultant culture was centrifuged at 6000 g, 4 °C for 10 min, and resuspended in PBS (pH 7.4, OD600 = 0.4). In the challenged group, 50 μl of live *V. alginolyticus* resuspended in PBS (pH 7.4, OD600 = 0.4) was injected into adductor muscle of pearl oysters, and then the pearl oysters were cultured in a static renewal condition. Untreated pearl oysters and pearl oysters injected with 50 μl PBS (pH 7.4) were used as the blank and control groups, respectively. The seawater was changed daily. No mortality and obviously tissue infection was observed in the challenge, blank and control group throughout the experiment. Haemocytes, gill and hepatopancreas from three individuals were randomly collected at 0, 3, 6, 12, 24, 36, 48, and 72 h post-challenge from three pearl oysters, and stored at −80 °C.

Challenge with cadmium was carried out according to Kim et al. [19] and Choi et al. [20] with some modifications. After acclimatization for 7 day, 30 pearl oysters were transferred to plastic tank filled with 400 L of well-aerated and filtered seawater or Cd-containing seawater. For Cd treatments, animals were exposed through tank water in a static renewal culture condition, where cadmium chloride ($CdCl_2 \cdot 2.5H_2O$) was added to the seawater with dissolved Cd^{2+} concentration of 0.1 ppm. Cd concentration was selected according to reports of Cd contamination events occurred in China [21] and previously studies in *Crassostra gigas* [20,22]. The pearl oysters were exposed to the treatment for 5 day, and the seawater or seawater containing Cd with a final concentration of 0.1 ppm was changed daily. No mortality was observed in the test or control group throughout the experiment. Gill and hepatopancreas tissues from three individuals were randomly collected from three pearl oysters at 0, 1, 2, 3, 4, and 5 d post-exposure and stored at −80 °C for later use.

To determine the temporal expression patterns of *PmMGST3* at mRNA level in gill and hepatopancreas tissues from pearl oyster exposed to cadmium and that in haemolymph, gill and hepatopancreas tissues from pearl oysters challenged with *V. alginolyticus*, quantitative real-time PCR (qRT-PCR) was performed with SYBR® PrimeScript™ RT-PCR Kit (Perfect Real Time) (TaKaRa) as recommended by the manufacturer in Rotor-Gene 3000 (Qiagen, Germany) using the primers qMGSTF and qMGSTR (Table 1). Total RNA from haemocytes was isolated as described above. cDNA was prepared by reverse-transcribing total RNA (300–500 ng) of adult pearl oyster tissues with an oligo $(dT)_{16}$ primer and M-MLV reverse transcriptase (Clontech) according to manufacturer's instruction. Thermal cycling conditions for qRT-PCR were as follows: 95 °C for 1 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s, 72 °C for 20 s, 80 °C for 20 s. Amplification of *P. martensii* β-actin RNA was used as internal control to normalize the expression levels between the samples [18]. Dissociation curves were run after the qRT-PCR to identify the specificity of PCR products. The relative amplification efficiency of the primers was calculated using serial dilutions of the sample [23]. Fold change at mRNA level was assessed based on the relative amplification efficiency of the primers used in the study with REST 2009 software [24]. At each time point, three individuals were analyzed ($n = 3$). For each sample, the test and control reactions were run in triplicate.

To analyze the tissue distribution of *PmMGST3* mRNA, cDNA was prepared by reverse-transcribing the total RNA (500 ng) from haemocytes, gill, hepatopancreas, adductor muscle, mantle, gonad,

Table 1
Primers used in this study.

Primer	Sequence(5′→3′)
For 5′ and 3′ RACE	
5P1	CTAACAATGTCAGGAGCCCAATG
5P2	GTAACCAAAACTTCCCCATCTTC
3P1	TACAGGCATCGCTACATCGTTT
3P2	GAAGTTTGAAGTCCCTTACCCCA
For qRT-PCR	
qMGSTF	CGGAGCAGGTTCTTTGTATTATGC
qMGSTR	GTCCTTGATGATTTCCAGTAGTTTGAG
β-actinF	CGGTACCACCATGTTCTCAG
β-actinR	GACCGGATTCATCGTATTCC
For plasmid construction	
ORFF (BamH I)[a]	AGCTGGATCCATGGGTAATCTTTCCAAAGTAGC
ORFR (Sal I)[a]	AGCTGTCGACTTAGTCCTTGATGATTTCCAGTAGT

[a] Restriction sites for BamH I and Sal I are underlined.