Short communication

cDNA cloning and mRNA expression of four glutathione S-transferase (GST) genes from *Mytilus galloprovincialis*

Chunyan Wang a,b, Jianmin Zhao b, Changkao Mu a,*, Qing Wang b, Huifeng Wu b, Chunlin Wang a,*

**A C T I V E I N F O**

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**A B S T R A C T**

Glutathione S-transferases (GSTs) are phase II enzymes involved in the regulation of redox homeostasis and innate immune responses against bacterial infection, which also play important roles in the detoxification of xenobiotics. In this study, we reported four genes of the GST family (named MgGSTz, MgGSTS1, MgGSTS2, and MgGSTS3, respectively) from *Mytilus galloprovincialis*. MgGSTz, MgGSTS1, MgGSTS2, and MgGSTS3 consisted of open reading frame (ORF) of 648 bp, 612 bp, 621 bp and 609 bp respectively, which encoded proteins of 216, 204, 207 and 203 amino acids residues, respectively. Sequence analysis showed that the predicted protein sequence of MgGSTs contained the conserved domain of the GST_N and GST_C. Alignment analysis indicated that the MgGSTs were divided into two types, one was of alpha GST, and the others were of sigma class. Tissue distribution study revealed that MgGSTz, MgGSTS2, MgGSTS3 transcripts were highly expressed in hemocytes, while MgGSTS1 mRNA was most abundantly expressed in hepatopancreas. After bacterial challenge, the expression level of these MgGSTs in hemocytes were all significantly higher than that of the control group. These results suggested that MgGSTs might play important roles in the modulation of immune response in *M. galloprovincialis*.

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**1. Introduction**

In marine environments, bivalves are commercially and ecologically important as food and non-food resources. Because of their benthic and sedentary mode of life, they are easily exposed to biotic and abiotic stresses, such as various pathogens and pollutants [1]. Reactive oxygen species (ROS) such as hydrogen peroxide (H2O2) and superoxide anion (O2−) are usually generated in response to these stresses [2,3]. Organisms have evolved different strategies to cope with the negative physiological effects of ROS by a group of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and some non-enzymatic antioxidant molecules [1,4].

Among the antioxidant enzymes, glutathione S-transferase (GST, EC 2.5.1.18) is a multifunctional dimeric protein involved in cellular detoxification of reactive electrophilic compounds, and protecting tissues against oxidative damage [5,6]. Currently, at least 15 different classes of GSTs (alpha, beta, delta, epsilon, kappa, lambda, mu, omega, phi, pi, sigma, tau, theta, zeta, and rho) have been reported in numerous organisms based upon their substrate specificity, antibody cross-reactivity and sensitivity to inhibitors [5,7].

*Mytilus galloprovincialis* is widely distributed around the coastal area, and is susceptible to suffering serious problems such as bacterial infection and pollution exposure [8]. However, most mussels could survive in this environment owing to their strong immune system and detoxification ability [9]. So, it is interesting to investigate the resistance mechanisms of mussels to pathogenic infection and oxidative damage. In this study, four GSTs genes from *M. galloprovincialis* were identified and characterized. To further characterize the roles of MgGSTSs in innate immune responses, the expression patterns of these four MgGSTs were also investigated after the mussels were challenged with *Listonella anguillarum*.

**2. Materials and methods**

2.1. Animal culture and *L. anguillarum* challenge

Adult mussels *M. galloprovincialis* about 4 cm in shell length were purchased from a local farm and acclimatized for 10 days...
at 18–20 °C. After acclimation, the mussels were divided into six 50L-tanks, each containing 50 individuals. During the whole experiment, mussels were fed with Isochrysis galbana and Platymonas helgolandica, with sea water (32 psu) totally changed daily.

The mussels were injected into adductor muscle with 50 μL of live L. anguillarum (final concentration of 10⁷ CFU mL⁻¹) suspended in phosphate buffered saline (PBS, pH 7.4) or the same volume of PBS (control group). For L. anguillarum challenge experiment, one tank was served as control. The other six injected mussels were randomly sampled at 12 h, 24 h, 48 h and 96 h after bacterial challenge. The hemolymph was collected from the adductor muscle using a syringe and centrifuged (2000 g for 10 min at 4 °C) to harvest the hemocytes. The gills, adductor muscle, mantle, gonad, hemocytes and hepatopancreas from the control were sampled to determine the tissue distribution of MgGSTs, MgGSTS1, MgGSTS2, MgGSTS3 transcripts, and hemocytes from the control and infected groups were sampled to determine the expression levels of MgGSTS, MgGSTS1, MgGSTS2, MgGSTS3 transcripts after bacteria stimulation.

2.2. Cloning the full-length cDNAs of MgGSTs

Total RNA from different tissues was extracted by using Trizol Reagent (Invitrogen, USA). One microgram of total RNA was subjected to cDNA synthesis with M-MLV reverse transcriptase (Promega) as previously described [10]. The 3’ ends of MgGSTs was obtained by rapid amplification of cDNA ends (RACE) using the 3’-Full RECA Core Set Ver.2.0 (20 RT Reactions) Kit (TaKaRa, Japan) according to manufacturer’s recommendations. The primers used for cloning the full-length cDNAs of MgGSTS were listed in Table 1. The resultant PCR products were cloned into pMD18-T vector (Takara, Japan), and then transformed into Escherichia coli Top10F' competent cells. For sequence confirmation, three positive clones for each MgGST were sequenced on an ABI3730 Automated Sequencer (Applied Biosystem, USA).

2.3. Sequence analysis

The cDNA and amino acid sequences of MgGSTS were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast) and the Expert Protein Analysis System (http://www.expasy.org/). The “GST_N_alpha-like”, “GST_C_alpha-like”, “GST_N_sigma-like” and “GST_C_sigma-like” were predicted using the CD-Search in NCBI’s CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple alignments were performed with the ClustalW multiple alignment program (http://www.ebi.ac.uk/clustalw) and multiple alignment show program (http://www.biosoft.net/sms/index.html). A phylogenic NJ tree was constructed with the neighbor-joining (NJ) method embedded in Mega 4 software package [11]. To derive the confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times.

2.4. Tissue distribution and temporal expression of MgGSTS mRNA in hemocytes after L. anguillarum challenge

Tissue distribution of MgGSTS and their responses to L. anguillarum challenge were measured by QRT-PCR in Applied Biosystem 7500 System. The primers used for real-time RT-PCR were listed in Table 1. Each assay was performed in triplicate with β-actin mRNA as the internal control. The reaction component and thermal profile were conducted as previously described [10]. All data were presented as means ± S.E., and subjected to one-way analysis of variance (one-way ANOVA). In all cases, P-value less than 0.05 were considered statistically significant.

3. Results

3.1. Sequence and phylogenetic analysis

Four nucleotide sequences of 842 bp, 1047 bp, 729 bp and 785 bp representing the complete cDNA sequence of MgGSTS (Fig. 1A), MgGSTS1 (Fig. 1B), MgGSTS2 (Fig. 1C), and MgGSTS3 (Fig. 1D), respectively, were obtained by overlapping EST with the fragments amplified by RACE. The deduced amino acid sequences of MgGSTS, MgGSTS1, MgGSTS2, and MgGSTS3 were shown in the corresponding nucleotide acid sequence in Fig. 1. Based on the deduced amino acid identities and phylogenetic analysis with other GSTs, the four MgGSTs were categorized into two classes, sigma type (MgGSTS1, MgGSTS2 and MgGSTS3, GenBank accession number JX485636, JX485637, JX485738, respectively) and alpha type (MgGSTS, GenBank accession number JX485635). Among these GSTs, MgGSTS had the highest molecular weight (24.9 kDa) and MgGSTS3 possessed the lowest molecular weight (22.8 kDa). MgGSTS2 and MgGSTS3 contained a molecular mass 23.6 kDa and 23.3 kDa respectively. The putative MgGSTS1, MgGSTS1, MgGSTS2 and MgGSTS3 proteins were calculated to have a theoretical molecular mass 23.3 kDa respectively. MgGSTS1, MgGSTS2 and MgGSTS3 possessed the lowest molecular weight (22.8 kDa).

The predicted protein sequence of MgGSTS contained the conserved domain of GST_N_alpha-like (from Tyr² to Arg⁵³) and GST_C_alpha-like (from Ile⁶⁹ to Asp¹⁸⁸). Similarly, the GST_N_sigma-like and GST_C_sigma-like domains were also identified in the three sigma type MgGSTS, respectively. A consensus polyadenylation site (AAATAAA) were located downstream of the translation termination codon in MgGSTS, MgGSTS1 and MgGSTS2.

The deduced amino acid sequences of MgGSTS showed high similarities to counterparts of other species (Fig. 2). For example, three sigma type GST identified from M. galloprovincialis shared high similarity with sigma GST from Haliotis discus discus...
Chlamys farreri (ACF25904.1), Branchiostoma floridae (XP_002605352.1), Rudites philippinarum (AEW46326.1) and Haliotis diversicolor (ABV01122.2), while MgGSTa shared high identity of 48% with H. discus discus GSTa (ABO26598.1), 45% identity with B. floridae GSTa (XP_002590291.1) and 44% with Perinereis nuntia GSTa (AEI70672.1).

To evaluate the molecular evolutionary relationships of GSTs, we constructed an un-rooted phylogenetic tree using the NJ method. According to the phylogenetic tree (Fig. 3), MgGSTs1, MgGSTs2 and MgGSTs3 clustered with the mollusc sigma type GSTs, while MgGSTa formed its own clades with alpha type GSTs from other organisms.
Fig. 2. Multiple alignment of MgGSTα(A) and MgGSTβ(B) with corresponding counterparts deposited in GenBank. The black shadow region indicates positions where all sequences share the same amino acid residue. Gaps are indicated by dashes to improve the alignment. The species and the GenBank accession no. are as follows: Haliotis discus discus (ABO26598.1), Perinereis nuntia (AEI70672.1), Branchiostoma floridae (XP_002590293.1), Solen grandis (AEW434511), Chlamys farreri (ACF259041).
Glutathione S-transferases are well-characterized family of multifunctional isoenzymes that are ubiquitously distributed in bacteria, plants, and animals [12]. GSTs are a family of proteins which play important roles in the oxidative stress responses and the detoxification pathways [13]. Blast analysis revealed that MgGSTs shared relatively high homology with those from other organisms. The GST classes relied on their G-site homology and mechanisms and H-site homology [12]. Using the CD-Search in NCBI's CDD, the predicted protein sequence of MgGSTs cDNA was matched to the GST_N_alpha_like and GST_C_alpha_like, containing an H-site which is a substrate binding site in the C-terminal [14]. The longer alpha C-terminal also forms a a-helix, which is thought to be important to dimer stabilization and affects both the GSH-binding rate and the ionization state of the catalytically essential residue Tyr [15,16]. The sigma class GSTs are lacking in both components of the ball-and-socket interface, and rely on a Tyr residue for GSH stabilization in the G-site [12], which was also found in the sequence of MgGSTS1, MgGSTS2 and MgGSTS3. The G-site (responsible for tripeptide GSH binding) and H-site (accounting for the binding of xenobiotic compounds) are highly conserved in the four MgGSTs, respectively. So the four MgGSTs were categorized into two classes, sigma type (MgGSTS1, MgGSTS2 and MgGSTS3) and alpha type (MgGSTS). On the basis of all these typical description, MgGSTs, MgGSTS1, MgGSTS2 and MgGSTS3 were proposed to be new members of the GSTs family.

As compared to vertebrate GSTs, there is a few information about the expression pattern of different classes of GSTs in mollusks so far. Ren et al. [14] reported that a high level of abGSTs transcripts was shown in the gill of H. diversicolor. Zhang et al. [7] suggested that the differences in tissue distribution of various GST isoforms were associated with differential susceptibility to antioxidant damage. In the present study, tissue distribution of MgGSTs revealed that they were generally abundantly expressed in hemocytes and hepatopancreas. Studies also showed that MgGSTS, MgGSTS2 and MgGSTS3 transcripts were dominantly expressed in hemocytes, whereas MgGSTS1 exhibited the highest expression level in the tissue of hepatopancreas, and trace transcript of MgGSTS2 was detected in the gills. Members of the GST super family exhibit different primary structures, enzyme properties, and physiological functions [17]. These results suggested that each of the GST classes might have different distributions in tissues, and perhaps involved in some physiological functions in the basal metabolism of M. galloprovincialis.

Molluscs rely highly on their innate immunity, and hemocyte-mediated phagocytosis is one of the main arms of their innate-defense strategies [2]. The killing of pathogens by hemocytes is usually accompanied by a sudden release of ROS through the respiratory burst to clear the invading microorganism [18,19]. However, the homeostasis of redox balance in cells would be disrupted because of ROS generation. In the present study, the abundance of MgGSTs mRNA were up-regulated in the mass and post bacterial challenge. Significant increase of MgGSTS2 and MgGSTS3 expression level (3.5-fold and 4.2-fold higher than that of the control, P < 0.05, respectively) were detected at 48 h post infection, while the maximum expression level of MgGSTS1 and MgGSTS2 were detected at 12 h and 24 h after infection (5.2-fold and 36.1-fold compared with the control, P < 0.05, respectively). As time progressed, the expression of MgGSTS1, MgGSTS3 and MgGSTS mRNA nearly returned to the baseline level at 96 h post-challenge (compared with the control, P > 0.05, respectively). At 12 h post-challenge, the expression level of MgGSTS2 mRNA was higher than that of the others (MgGSTS1, MgGSTS1, and MgGSTS3).

4. Discussion

Fig. 3. Phylogenetic tree constructed by neighbor-joining method based on the sequences of GSTs from different animals. The numbers at the forks indicate the bootstrap values. The sequences were as follows: GST-alpha: Haliotis discus discus (AB202598.1), Ictalurus punctatus (NP_001387531), Anolis carolinensis (XP_003215482), Branchiostoma florbica (XP_002509291); GST-Sigma: Solen grandis (AEV43451), Chlamys fareri (ACF25904), Raditapes philippinarum (AD443171), Haliotis diversicolor (ABV011222). Crossasteria gigas (CAE118631).

3.2. Tissue-specific distribution of MgGSTs mRNA

The tissue-specific expression of MgGSTs transcripts was determined by quantitative RT-PCR. It was found that MgGSTs mRNA was expressed in all tissues examined. As shown in Fig. 4, the highest expression level of MgGSTS, MgGSTS2 and MgGSTS3 mRNA was detected in hemocytes. However, MgGSTS2 transcript was found to be most abundantly expressed in the tissue of hepatopancreas, and trace transcript was detected in the mantle and gills.

3.3. Temporal expression patterns of MgGSTs mRNA in hemocytes after bacterial challenge

QRT-PCR was employed to quantify the MgGSTs expression profiles after bacterial challenge with β-actin as internal control. For MgGSTs and β-actin genes, only one peak at the corresponding melting temperature was observed in the dissociation curve analysis, indicating that the PCR was specifically amplified (data not shown). During the whole experimental period, no notable change (P > 0.05) of MgGSTs mRNA expression was observed for the control treatment. As shown in Fig. 5, the effect of L. anguillarum on M. galloprovincialis innate immunity was detected as soon as 12 h after exposure. The expression level of MgGSTS1, MgGSTS2, MgGSTS3 and MgGSTS1 in hemocytes were all significantly up-regulated at different time intervals post bacterial challenge. Significant increase of MgGSTS2 and MgGSTS3 expression level (3.5-fold and 4.2-fold higher than that of the control, P < 0.05, respectively) were detected at 48 h post infection, while the maximum expression level of MgGSTS1 and MgGSTS2 were detected at 12 h and 24 h after infection (5.2-fold and 36.1-fold compared with the control, P < 0.05, respectively). As time progressed, the expression of MgGSTS1, MgGSTS3 and MgGSTS mRNA nearly returned to the baseline level at 96 h post-challenge (compared with the control, P > 0.05, respectively). At 12 h post-challenge, the expression level of MgGSTS2 mRNA was higher than that of the others (MgGSTS1, MgGSTS1, and MgGSTS3).
MgGSTS2 might play a leading role in immunization against pathogen infection, and the four MgGSTs isoforms exerted their function in a manner of synergy.

In conclusion, the full-length cDNA encoding four genes of the GST family (MgGSTα, MgGSTS1, MgGSTS2, and MgGSTS3) were isolated from *M. galloprovincialis*. All MgGSTs mRNA were constitutively expressed in the tested tissues, and were significantly up-regulated in hemocytes after bacterial challenge. These results suggested the involvement of MgGSTs in the defense response of *M. galloprovincialis* against bacterial infections.

**Fig. 4.** MgGSTα, MgGSTS1, MgGSTS2, and MgGSTS3 mRNA expression level in different tissues of adult clams detected by real-time PCR. The transcript level MgGSTs in hemocytes, gills, mantles, muscle, gonad, and hepatopancreas is normalized to that of adductor muscles, respectively. The results are shown as mean ± S.E. (*n* = 4), and bars with different letters are significantly different (*P* < 0.05).

**Fig. 5.** Temporal expression profile of MgGSTα, MgGSTS1, MgGSTS2, and MgGSTS3 mRNA in hemocytes after bacterial challenge measured by quantitative real-time PCR. The mRNA expression level is calculated relative to actin expression and shown as mean ± S.E. (*n* = 4). Data in the same exposure time with different letters are significantly different (*P* < 0.05).
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