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ScienceDirect
Chemosphere 69 (2007) 893–902
CHEMOSPHERE
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Sequence, biochemical characteristics and expression of a novel Sigma-class of glutathione *S*-transferase from the intertidal copepod, *Tigriopus japonicus* with a possible role in antioxidant defense

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Received 6 March 2007; received in revised form 18 May 2007; accepted 26 May 2007
Available online 19 July 2007

Abstract

Glutathione *S*-transferases (GSTs) play a major role in detoxification of xenobiotics and antioxidant defense. Here we report full-length cDNA sequence of a novel Sigma-class of GST (GST-S) from the intertidal copepod *Tigriopus japonicus*. The full sequence was of 1136 bp in length containing an open reading frame (ORF) of 651 bp that encoded 217 amino acid residues. The recombinant *Tigriopus* GST-S was highly expressed in transformed *Escherichia coli.* Kinetic properties and effects of pH, temperature and chemical inhibitors on *Tigriopus* GST-S were also studied. The expression of GST-S was studied using real-time RT-PCR in response to exposure to two oxidative stresses-inducing agents, viz., hydrogen peroxide (H_2O_2) and heavy metals (copper, manganese). It was observed that H_2O_2 (2 mM) exposure down-regulated its expression at the initial stage but there was recovery and up-regulation shortly afterwards. In case of heavy metal exposure there was concentration-dependent increase in *Tigriopus* GST-S gene expression up to 24 h. These results suggest that *Tigriopus* GST-S expression is modulated by prooxidant chemicals and it may play a role against oxidative stress. A majority of other GST isoforms is known to play an important role in antioxidant defense. This study provides a preliminary insight into the possible antioxidant role for Sigma-class of GST in *T. japonicus*.

Keywords: *Tigriopus japonicus*; Glutathione *S*-transferases; Recombinant glutathione S-transferase-sigma; Marine pollution; Oxidative stress; Antioxidant defense

1. Introduction

Glutathione *S*-transferases, GSTs (EC 2.5.1.18) are a family of diverse multifunctional proteins mainly involved in detoxification of xenobiotics and antioxidant defense (Frova, 2006). They also play a role in the biosynthesis of prostaglandins (Hayes et al., 2005). In most of the organisms, GST expression is modulated in response to exposure to prooxidant xenobiotics. Overall, it is believed that the induction of GSTs is an evolutionary conserved response of the organisms towards exposure to prooxidants (Armstrong, 1997; Hayes et al., 2005; Masella et al., 2005). The cytosolic GSTs exist as heterodimers or homodimers of the subunits with a molecular mass ranging from 23 to 28 kDa. The mammalian GSTs have been classified as Alpha, Mu, Pi, and Theta based on the N-terminal amino acid sequence similarity, substrate specificity and immunological cross-reactivity (Hayes and Pulford, 1995; Pemble et al., 1996; Sheehan et al., 2001; Frova, 2006). Some novel classes of non-mammalian GSTs such as Sigma, Zeta and

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doi:10.1016/j.chemosphere.2007.05.087

Beta have been isolated and characterized mainly from bacteria, plants, insects, and fish (Sheehan et al., 2001; Frova, 2006). Although mammalian and to some extent insect novel classes of GSTs have been studied in detail, information on these GSTs from other species is limited (Contreras-Vergara et al., 2004; Frova, 2006).

Most of the studies on GSTs have focused on purification and biochemical characterization of various isoforms (Tomarev et al., 1993; Chiou et al., 1995; Fitzpatrick et al., 1995; Hoarau et al., 2002; Yang et al., 2003; Contreras-Vergara et al., 2004; Adewale and Afolayan, 2005; Abdalla et al., 2006). Besides the four major classes of GSTs (Alpha, Theta, Mu and Pi), novel GST isoforms have been studied in great detail mainly in insects (Sawicki et al., 2003; Lumjuan et al., 2005; Valles et al., 2006; Yamamoto et al., 2007). In insects, study of GSTs is motivated by the perception that they have a role in insecticide resistance (Ranson et al., 2001). However, information on molecular characterization of novel GSTs in marine species, in general, and copepods, in particular, is not available.

Compared to terrestrial environment, marine environment has enormous genetic diversity and information about the gene structure and function of a conserved protein such as GST in ecologically- and phylogenetically-relevant species may provide useful information. Additionally, study of stress responses in marine organisms has become an interesting area of research in recent years (Dahlhoff, 2004; Lesser, 2006; Moore et al., 2006). We have studied expression, sequences and kinetics characteristics of some xenobiotic detoxification enzymes (GST and glutathione reductase, GR, Lee et al., 2006a; Seo et al., 2006b) and stress response protein (*Hsp 20*, Seo et al., 2006a) in an ecologically-relevant marine species, *Tigriopus japonicus* (an intertidal Harpacticoid copepod). These studies have encouraged us to explore the detoxification and antioxidant strategies adopted by this species, which inhabits a stressful habitat. *T. japonicus* is a small organism; adults generally are of about 1.0 mm in length. It inhabits shallow supratidal rock pools that have infrequent tidal inundation. Therefore, *T. japonicus* experiences extreme environmental conditions including temperature and salinity fluctuations (Raisuddin et al., in press).

Since GSTs are important components of detoxification pathways, their role in overall stress mitigation in *T. japonicus* may offer insight into the adaptive and defense responses of this species. Moreover, since GSTs can be induced or inhibited in response to exposure to environmental toxicants, they have also been used as biomarkers of exposure in aquatic organisms (van der Oost et al., 2003; Amado et al., 2006). In this study, we report cloning of cDNA of a novel Sigma-class of GST gene from *T. japonicus* (*TJ-GST-S*), purification of the recombinant TJ-GST-S protein, and some of its biochemical properties. In addition, we investigated the expression of GST-S in response to exposure to some known prooxidant agents to find out if it has any role in antioxidant defense in *T. japonicus*.

2. Material and methods

2.1. Chemicals and reagents

pCR® T7 TOPO-TA expression kits were supplied by Invitrogen (USA). Ni^{+}-NTA column was obtained from Qiagen (Germany) and pre-stained broad range protein molecular weight markers were from Fermentas (USA). GST assay kit was purchased from Sigma-Aldrich Co. (USA). All other chemicals were of molecular biology grade procured from Sigma unless otherwise stated.

2.2. Copepods

Copepods (*T. japonicus*) were maintained under the following conditions: water temperature – 20 °C, photoperiod – 12 h/12 h light/darkness, salinity – 33 ppt., pH – 7.9 and dissolved oxygen (DO) – 5.24 mg/l. Phytoplanktons *Tetraselmis suecica* and *Isochrysis galbana* were fed as food to copepods. Copepods were acclimatized for two weeks before their use in various experiments.

2.3. cDNA sequence and phylogenetic tree

The *T. japonicus* GST-S cDNA was sequenced by ABI370 sequencer. Phylogenetic placement of *T. japonicus* GST-S gene was studied using Neighbor-Joining method after aligning the sequence with ClustalX ver 1.83. GST-Mu classes of the European house dust mite (*Dermatophagoides pteronyssinus*) and the Pacific white shrimp (*Litopenaeus vannamei*) were designated as outgroups.

2.4. Total RNA extraction and reverse transcription

Total RNA was isolated with TRIZOL® reagent (Invitrogen, Scotland) from adult *T. japonicus* ($n = \sim 100$) according to the manufacturers' instructions. Single-strand cDNA was synthesized from 2 μg of total RNA using oligo $(dT)_{20}$ primer for reverse transcription in 20 μl reaction mix using the SuperScript™ III RT kit (Invitrogen).

2.5. Overexpression and purification of recombinant *T. japonicus GST-S*

Prokaryotic expression plasmids were constructed according to the manufacturers' instructions as described by Lee et al. (2006a). Briefly, the open-reading frame (ORF) region of TJ-GST-S was amplified with primer TJ-GST-S-pro-F/R (Table 1) using iCycler (Bio-Rad, USA). The amplified product was eluted from agarose gel and inserted into expression vector, 6× His tagged pCR®T7 NT-TOPO. *E. coli* strain BL21(DE3)pLysS was transformed by TJ-GST-S/pCR T7 NT-TOPO vector. Transformed cells were grown in LB broth containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). The expression of recombinant TJ-GST-S protein was induced by adding isopropyl-1-β-D-thiogalactopyranoside

Table 1
Detail of primers used in the study

Gene	Oligo name	Sequences (5′–>3′)	Remarks
TJ-GST-S	RT-F	ATGACTGGATTCGGATTTGGAC	TJ-GST-S cDNA amplification
	RT-R	GGCGTTTGGTCACATATTCGG	
TJ-β-actin	RT-F	ATGGTGTCACCCACACTGTGCC	β-actin cDNA amplification
	RT-R	TGTGGTGGTGAAGCTGTAGCCTC	

(IPTG, Sigma) at a final concentration of 1 mM. Bacterial cells were harvested after 18 h incubation at 30 °C and analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE (Laemmli (1970). The self-ligated pCR®T7 TOPO TA expression vector was used as a negative control in all the experiments. The purification of soluble recombinant TJ-GST-S protein was carried out using 6x His-tagged affinity column (15 ml Ni^{+}-NTA resin) in the LP system (Bio-Rad). The eluted fraction was dialyzed overnight and pooled fractions were analyzed by SDS-PAGE and Western blotting. Protein concentration was determined with Bio-Rad protein assay reagents (Bio-Rad).

2.6. Western blotting

The denatured samples were boiled for 5 min, and separated by SDS-PAGE. Electrophoresed proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell. Co., USA) using a Mini Protean III transblotting system (Bio-Rad). Following transfer of proteins to membrane, the membrane was blocked with 5% bovine serum albumin (BSA) in 0.1% TTBS (200 mM Tris/pH 7.0, 1.37 M NaCl, 1% Tween-20) for 1 h at room temperature. The membrane was then incubated with anti-His G-HRP antibody (1:5000) (Invitrogen) at room temperature for 3 h, rinsed and washed three times with 0.1% TTBS for 60 min. ECL-plus Western Blotting kit (Amersham, USA) was used for detection.

2.7. GST assay

The GST-S specific activity was measured using GST assay kit according to the manufacturers' instructions by a spectrophotometer with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 2 mM glutathione-reduced (GSH) as substrates at 340 nm and 25 °C.

2.8. Kinetics study and effect of pH and inhibitors

The activity of the purified TJ-GST-S was studied at various concentrations of CDNB (0.25–4.0 mM) and GSH (0.25–2.0 mM) with a fixed concentration of GSH (2.0 mM) or CDNB (1.0 mM), respectively, for each substrate. The enzyme activity was measured using the kit as described above. The data were plotted as a Lineweaver–Burk plot to determine the K_m. The effect of pH on the activity of purified TJ-GST-S was analyzed at 25 °C after the enzyme was equilibrated in 50 mM of the following buffer for 3 min: acetate (pH 5), phosphate (pH 6.0 and 7.0) and Tris–HCl (pH 8.0 and 9.0). The remaining activity was then measured using 1 mM CDNB and 2 mM GSH. Cibacron blue (0.0–10 μM), hematin (0.00–10 μM) and *N*-ethylmaleimide (0.0–1000 μM) were used as inhibitors and the activity was measured as per the methods described previously (Tahir et al., 1985; Lee et al., 2006b).

2.9. Disk assay

Disk assay was performed using a modified procedure of Park et al. (2005) to compare the relative efficacy of transformed *E. coli* with TOPO-NT containing TJ-GST-S cDNA and bacteria with only TOPO-NT vector (as a negative control). Whatman filter-paper disks (3-mm diameter) were placed onto the agar plate inoculated with *E. coli* cultured for 4 h at 25 °C after induction with 0.5 mM IPTG. After blotting, 3 or 6 μl of hydrogen peroxide (H_2O_2) were dispensed onto the each disk. Plates were incubated overnight at 37 °C and zone of clearance was recorded.

2.10. Exposure to H_2O_2 and heavy metals

Two different classes of chemical agents with oxidative stress-inducing potential (H_2O_2 and heavy metals) were used to study the expression pattern of *GST-S* gene. Adult *T. japonicus* ($n = \sim 100$) were exposed to standardized concentration of H_2O_2 (2 mM) for different time intervals (0, 10, 20, 30, 60, 120 and 180 min). Equal numbers of copepods were exposed to heavy metals (10 and 50 μg/l $CuSO_4 \cdot 5H_2O$ or 50 and 100 μg/l $MnSO_4 \cdot H_2O$) for 0, 6, 12, 24, 48 h. During exposure, copepods were maintained in the same condition as described above but without food. mRNA expression was studied using real time RT-PCR.

2.11. Real-time RT-PCR

From each sample, 2 μg total RNAs were reverse transcribed to first strand cDNA by using SuperScript™ III reverse transcriptase (Invitrogen) in the reaction volume of 20 μl. Reaction conditions were as following condition: 94 °C/4 min; 40 cycles of 94 °C/30 s, 55 °C/30 s, 72 °C/30 s; and 72 °C/10 min. To confirm the amplification of specific product, cycles for melt curve were continued at following condition: 95 °C/1 min, 55 °C/1 min, 80 cycles

of 55 °C /10 s with 0.5 °C increase per cycle. SYBR Green (Molecular Probes, Invitrogen) was used as fluorescence for detection of the specific PCR product using MyiQ (Bio-Rad). *T. japonicus* β-actin gene was used as a reference to normalize the expression levels between the samples. All the experiments were performed in triplicate. Fold change for the gene expression relative to controls was determined by the $2^{-\Delta\Delta\ CT}$ method of Giulietti et al. (2001).

All the data expressed as the means ± SD were analyzed by unpaired Student's *t*-test after normalization. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Tigriopus japonicus GST-S cDNA

The cDNA of *T. japonicus* GST gene was sequenced (Fig. 1a), and deposited to GenBank (Accession No. DQ088365). The full length of ORF region was 651 bp encoding peptide of 217 amino acid residues. In order to get more information of *TJ-GST* gene, we carried out the 5′- and 3′-RACE (Rapid Amplification of cDNA Ends) according to conventional method. On search of the

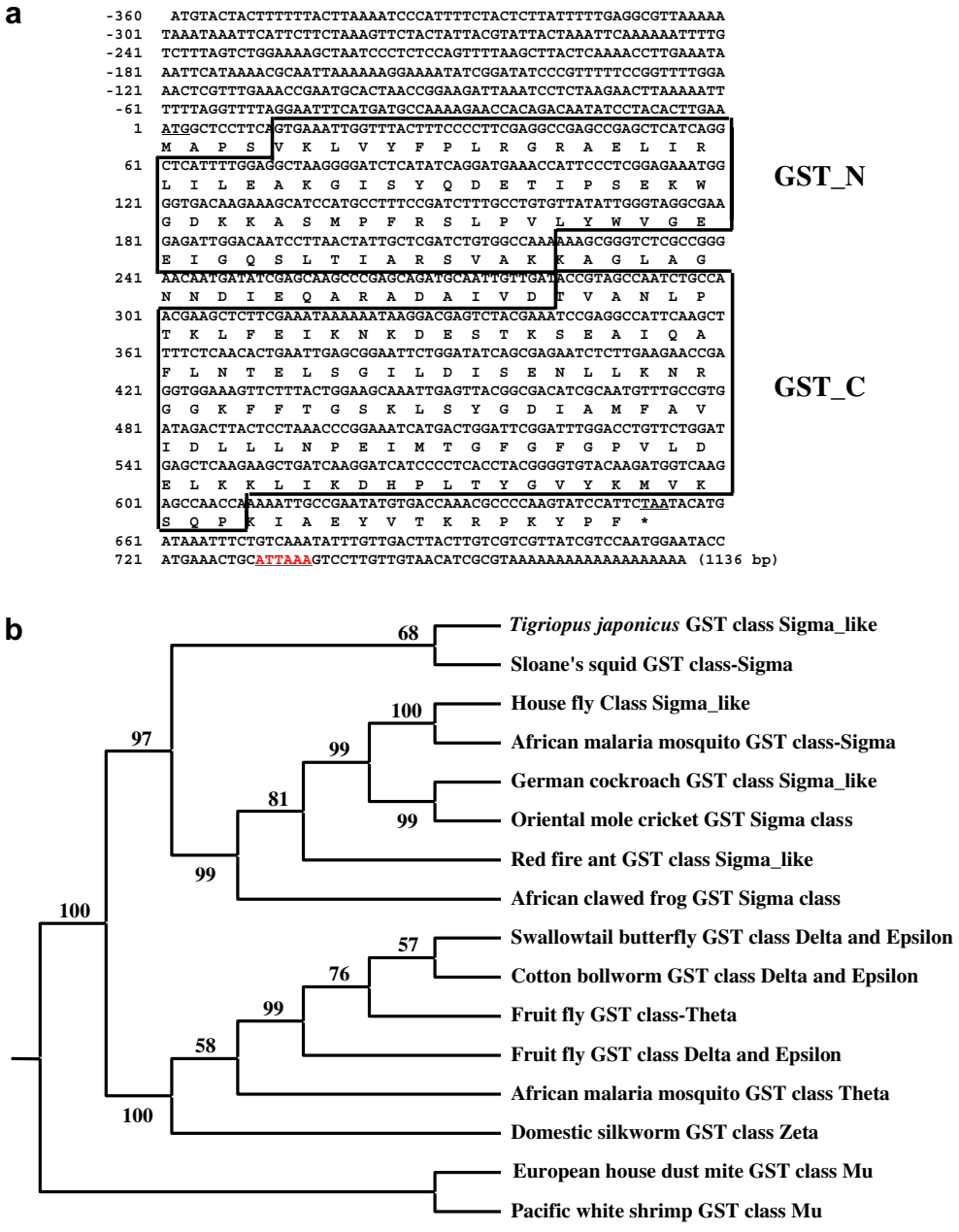

Fig. 1. (a) Nucleotide and deduced amino acid sequences of the glutathione *S*-transferase gene from *Tigriopus japonicus*. The start codon and stop codon are shown in bold and underlined. The boxes show the conserved GST_*N* terminal (5–74 aa) and GST_*C* terminal domain (95–203 aa). (b) Phylogenetic tree of *Tigriopus japonicus GST-S* gene along with *GST* genes of other species constructed by Neighbor-Joining method. GST Mu classes of the European house dust mite and the Pacific white shrimp were designated as outgroups.

conserved domain using pfam database, it was found that TJ-GST had a G-site (4–74 aa) which binds the tripeptide GSH in the *N*-terminal region and an H-site (85–186 aa) which is a substrate binding site in the C-terminal. BLAST search showed that *TJ-GST* gene was close to Sigma-class GST of squid (P46088) (Tomarev et al., 1993) and *Xenopus* (U02616) (Carletti et al., 2003). However, the degree of similarity was low; 31% and 28% with for squid and *Xenopus*, respectively. When the phylogenetic tree was constructed by Neighbor-Joining method, it was observed that the TJ-GST was placed into Sigma-class of squid GST gene along with other GST Sigma-class (Fig. 1b). The sequence of GST-C domain of *TJ-GST* gene was classified as a class Sigma-like family. This indicates a proximity to Sigma-class of GST, and then we named this gene as Sigma-class of *T. japonicus* glutathione *S*-transferase (*TJ-GST-S*).

3.2. Characterization of the recombinant TJ-GST-S

The column chromatography profile of recombinant TJ-GST-S is shown in Fig. 2a. The SDS-PAGE analysis showed each protein as a single band with a molecular mass of approximately 27.5 kDa, containing co-expressed part, six of histidine, of 3.5 kDa (Fig. 2b and c). The yield of purification of recombinant TJ-GST-S was 44.79% of the total activity collected after Ni^{+}-NTA column. Specific activity toward CDNB of this recombinant protein was 0.30 ± 0.01 (μmol/mg/min). Enzyme kinetics

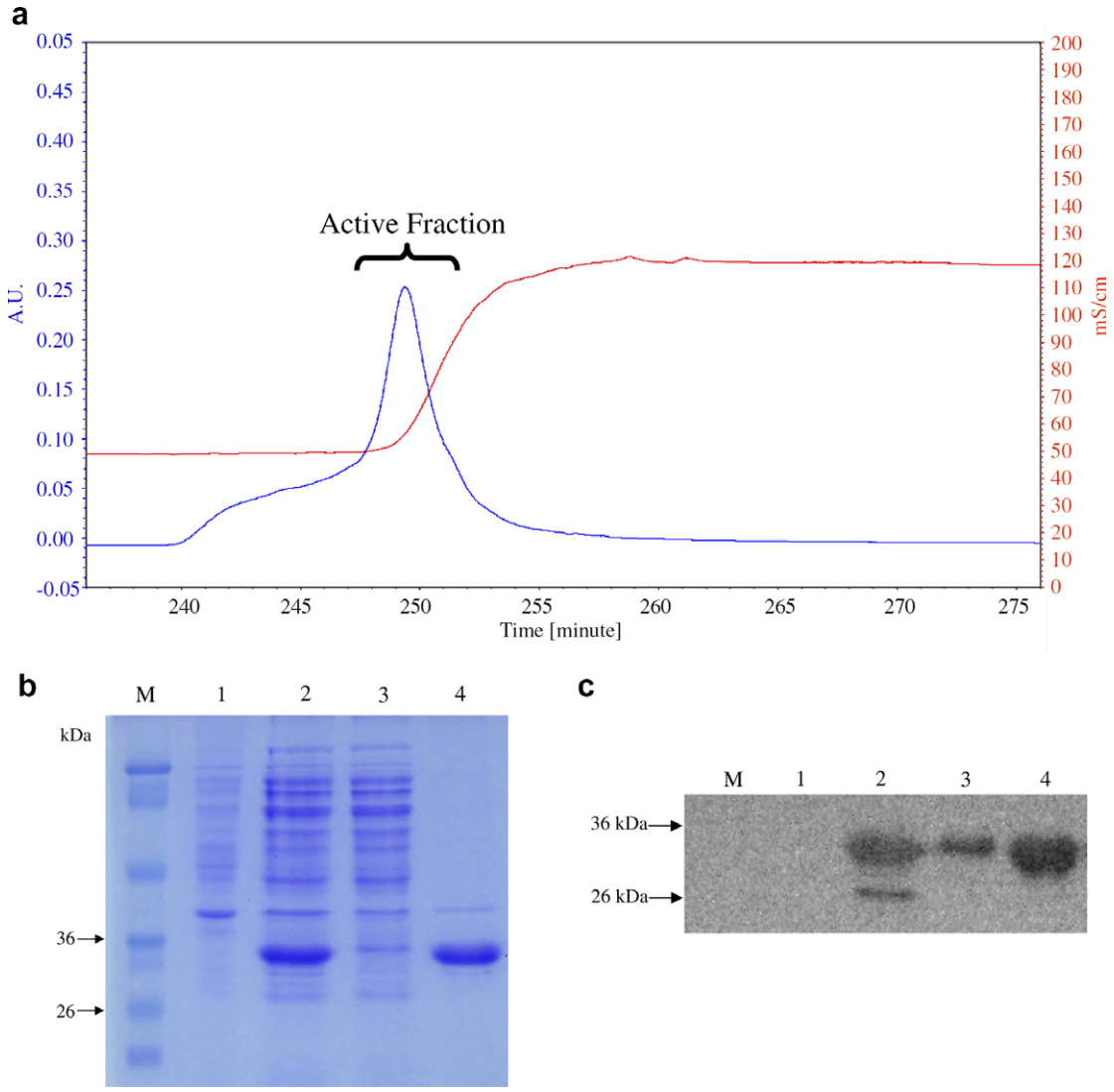

Fig. 2. Purified recombinant *T. japonicus* GST-S. (a) LP-chromatography with Ni^{+}-NTA column. Active fraction indicates purified soluble *Tigriopus* GST-S, (b) SDS-PAGE of purified recombinant *Tigriopus japonicus* GST-S, (c) Western blotting with anti-HisG antibody. M. Prestained protein size markers, 1. vector control, 2. total crude extract, 3. total soluble fraction, 4. purified *Tigriopus japonicus* GST-S.

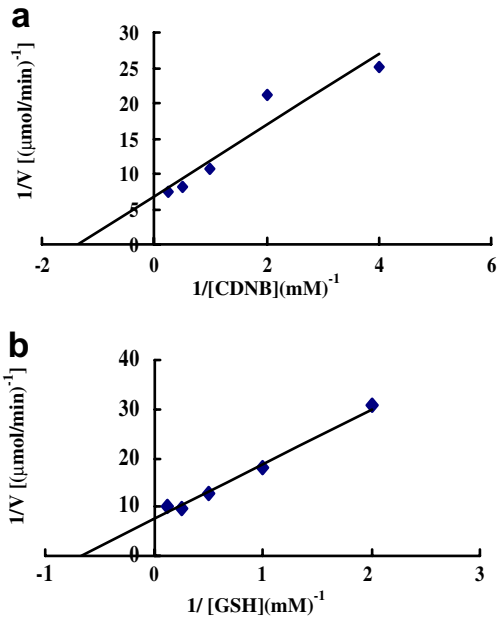

Fig. 3. Lineweaver–Burk plot and kinetic parameters of recombinant *Tigriopus japonicus* GST-S. a. Effect of varied concentrations of CDNB (0.25–4 mM) as substrate in presence of 2 mM GSH. b. Effect of varied concentrations of GSH (0.25–2 mM) in presence of 1 mM CDNB as substrate. The reaction was recorded as μmol/min/mg protein. Values are means of three independent replicates.

Table 2
Effect of inhibitors on the enzyme activity of purified TJ-GST-S protein

Inhibitor	Concentration (μM)	Enzyme activity (% control)
Cibacron blue	1	85.79 ± 7.12
	10	78.83 ± 1.2
Hematin	1	84.32 ± 0.5
	10	45.16 ± 6.10
N-ethylmaleimide	10	102.33 ± 0.43
	100	78.79 ± 0.72

Enzyme activity was determined using CDNB (1-chloro-2,4 dinitrobenzene) as a substrate. All experiments were repeated at least three times and results represent means ± S.D.

study revealed that when the concentration of GSH was fixed, V_{max} and K_M values of recombinant TJ-GST-S were 0.15 ± 0.01 (μmol/mg/min) and 0.75 ± 0.07 (mM), respectively, and in the case of fixed CDNB concentration the same values were 0.13 ± 0.003 (μmol/mg/min) and 0.31 ± 0.01 (mM), respectively (Fig. 3). The recombinant TJ-GST-S showed optimum pH range between pH 7.5 and 8.0. However, it showed appreciable activity at pH 9. Inhibition study showed that the recombinant TJ-GST-S was sensitive to hematin, while Cibacron blue and *N*-ethylmaleimide did not significantly inhibit its activity in the concentration ranges used in this study (Table 2).

3.3. Disk assay result

The disk with TJ-GST-S expressed in *E. coli* showed higher viability in presence of H_2O_2 than that observed in control plates as determined by the area of plaque formed after overnight incubation at 37 °C (Fig. 4).

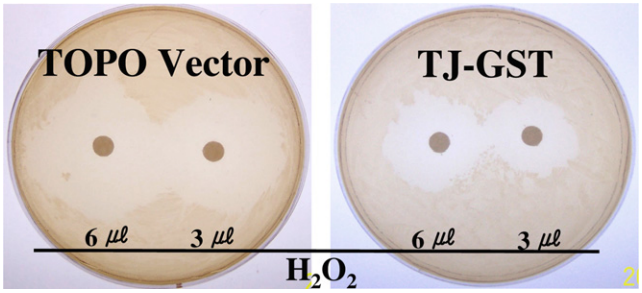

Fig. 4. Comparison of antioxidant activity between *Escherichia coli* transformed with plasmid TOPO NT containing *Tigriopus japonicus* glutathione *S*-transferase cDNA (TOPO-TJ-GST-S) and TOPO NT Vector (TOPO vector) as control. Whatman filter-paper disks (3-mm diameter) were placed on top of an agar plate inoculated with *E. coli* cultured for 4 h at 25 °C with 0.5 mM IPTG. After adding 6 or 3 μl H_2O_2 onto the each disk, cells were grown overnight at 37 °C.

3.4. TJ-GST-S mRNA expression in response to chemical exposure

In response to H_2O_2 exposure TJ-GST-S mRNA expression was first decreased, then gradually increased 120 min after exposure and reached a highest level at 180 min (Fig. 5). Both the heavy metals caused modulation of TJ-GST-S expression with a slightly different pattern. Cu exposure significantly induced TJ-GST-S mRNA expression at the both concentrations (10 and 50 μg/l) from 6 to 48 h. However, magnitude of expression was low at lower concentration (10 μg/l) (Fig. 6a). Almost a similar pattern of expression was observed following exposure to Mn at 6–24 h (Fig. 6b). Expression subsided at 48 h and in case of Mn the relative expression at 48 h was significantly lower ($P < 0.05$) than that observed at 0 h.

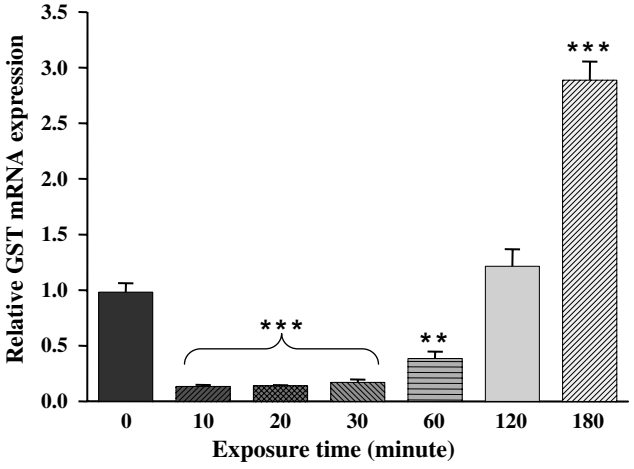

Fig. 5. Relative TJ-GST-S mRNA expression in *Tigriopus japonicus* exposed to 2 mM H_2O_2. Expression was studied using real-time RT-PCR. Significant differences over 0 h are indicated by $^{**}P < 0.1$ and $^{***}P < 0.001$.

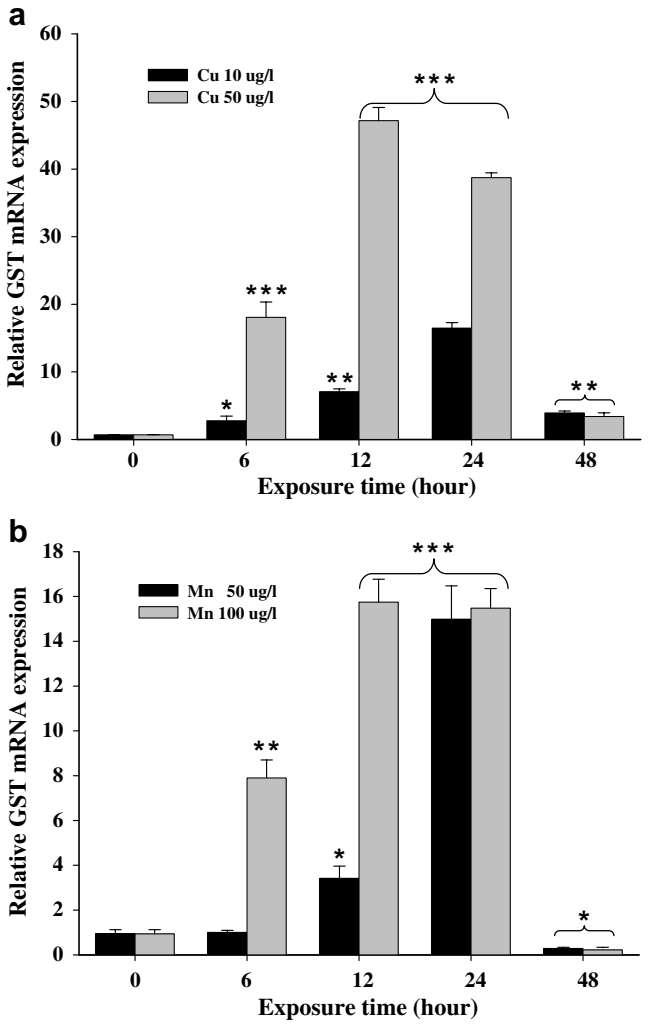

Fig. 6. Relative TJ-GST-S mRNA expression in *Tigriopus japonicus* exposed to heavy metals. a. Copper and b. manganese for 0–48 h. Expression was studied using real-time RT-PCR. Significant differences over 0 h are indicated by $^{*}P < 0.5$, $^{**}P < 0.1$ and $^{***}P < 0.001$.

4. Discussion

This is the first report of cloning and sequence analysis of a novel Sigma-class of GST from *T. japonicus*. In fact, no study has been reported on this class of GST from the whole copepoda. Although several functions are ascribed to GSTs, their main function is to catalyze the conjugation of the glutathione with compounds containing an electrophilic centre, and to form more soluble, and nontoxic peptide derivatives which can be readily excreted form the system (Frova, 2006). Initially, novel classes of GSTs including Sigma were described in non-mammalian species, but now several mammalian novel GST isoforms have been described (Sheehan et al., 2001). The BLAST search showed that *TJ-GST-S* gene was close to Sigma-class GST of squid (P46088) (Tomarev et al., 1993) and *Xenopus* (U02616) (Carletti et al., 2003). However, the degree of similarity was low; 31% and 28% with squid and *Xenopus laevis*, respectively.

Sigma-class of GST has been described in a number of insects and it has been suggested that it plays a role in detoxification of xenobiotics or indirectly in the resistance to insecticides (Rauch and Nauen, 2004; Yamamoto et al., 2007). In mammals and chicken, GST-Sigma has been described to be associated with GSH-dependant prostaglandin D_2 synthase (Thomson et al., 1998; Jowsey et al., 2001). Pennelli et al. (2000) purified a Sigma- class of GST from the secretion of granular glands in dorsal part of *Xenopus laeivis* and showed that it has a high sequence similarity with GSH-dependant prostaglandin D_2 synthase. These finding demonstrate a diverse functional role for GSTs. More studies are required to establish such a role for TJ-GST-S in *T. japonicus*. In our study, we found that *T. japonicus* GST-S has a possible antioxidant function. Disk assay showed that TJ-GST-S expressed in *E. coli* enabled the bacteria to survive more efficiently than the bacteria with control vector in presence of H_2O_2. Role of GSTs in resistance against H_2O_2-induced toxicity is well known in different *in vitro* models including bacteria (Fiander and Schneider, 1999; Singhal et al., 1999; Yang et al., 2001; Rao and Shaha, 2000; Kanai et al., 2006).

The SDS-PAGE analysis showed that the molecular mass of *T. japonicus* GST-S was approximately 27.5 kDa. This is in good agreement with calculated molecular weight of 24.13 kDa of TJ-GST-S protein. This molecular weight showed similar pattern with squid GST-S (Tomarev et al., 1993). Specific activity toward CDNB of the recombinant protein was 0.30 ± 0.01 (μmol/mg/min). The recombinant Sigma-class *Xenopus* GST protein has been reported to possess high specific activity (14.7 μmol/mg/min) toward CDNB (Carletti et al., 2003). Also, mutant forms of *Xenopus* GST-S in which a single conserved amino acid sequence was changed, showed higher or lower specific activity toward CDNB than wild form depending on the conformational change (Carletti et al., 2003). Therefore, in this study the difference of CDNB-binding activity between TJ-GST-S and *Xenopus* GST-S is supported by the fact that the GST-S of the two species show a low similarity of amino acid sequences. Amino acid sequence comparison (including *N*- and *C*-terminal sequences) of GST-S with previously reported GST of *T. japonicus* (Lee et al., 2006a) revealed that there were some differences between the sequences. This may result is different substrate specificities and inhibitor sensitivity. However, these kinetic properties are often overlapped between the GST classes (Sheehan et al., 2001). Both the isoforms showed good activity with CDNB which is a non-specific substrate for GSTs and has invariably been used in almost all the studies involving biochemical characterization of GSTs. In case of insects, Yamamoto et al. (2007) reported that CDNB was a favourable substrate for Sigma-class GST of fall webworm *Hyphantria cunea*.

The recombinant TJ-GST-S showed optimum pH range between pH 7.5 and 8.0. But it showed appreciable activity at pH 9. GSTs in general show activity in a broad range of temperature. For example, GST-S isoform of *H. cunea*

retained significant activity up to 50 °C (Yamamoto et al., 2007). GST-S of *H. cunea* also showed optimum pH around 8.0 but more than 50% activity was observed up to pH 10.0 (Yamamoto et al., 2007). This demonstrates that GST-S would be active in a broad range of pH enabling it to perform its functions efficiently in varying environmental conditions. Inhibition studies showed that the recombinant TJ-GST-S was sensitive to hematin, while Cibacron blue and *N*-ethylmaleimide were not effective in inhibiting the enzyme activity. GSTs show differential inhibition towards inhibitors. Thomson et al. (1998) reported that GST-S of chicken with GSH-dependent prostaglandin G_2 synthase activity was most sensitive to cibacron blue. Wu et al. (2006) studied effect of two inhibitors, Cibacron blue and albendazole on a cytosolic GST from human liver fluke (*Clonorchis sinensis*) and observed that Cibacron blue was more effective as an inhibitor of enzyme activity than albendazole. We have previously observed that GST-α from fish *Rivulus marmoratus* was highly sensitive to inhibition by Cibacron blue and GST-θ by hematin (Lee et al., 2006b). However, both the isoforms showed almost similar sensitivity to *N*-ethylmaleimide. This demonstrates that there is a great variation in terms of substrates as well as inhibitors of GSTs signifying their diverse biological roles.

The role of *T. japonicus* GST-S in antioxidant defense was further confirmed by *in vivo* studies involving mRNA expression in response to two different classes of oxidative stress-inducing agents, viz., H_2O_2 and heavy metals (Cu and Mn). In case of H_2O_2, TJ-GST-S mRNA expression was first decreased, then gradually increased 2 h after exposure and reached a highest level at 3 h. This gives an idea that *TJ-GST-S* gene may be induced after exposure to H_2O_2 for defense of cellular damage. As discussed earlier H_2O_2 is a model compound for oxidative stress used *in vitro* and *in vivo*. Amongst the heavy metals, copper is known to induce oxidative stress mainly by its involvement in redox-cycling (Stohs and Bagchi, 1995; Valko et al., 2005). Both the heavy metals caused modulation of expression of TJ-GST-S with slightly different patterns. Cu exposure significantly induced TJ-GST-S mRNA expression at the both the concentrations (10 and 50 μg/l). However, magnitude of expression was low at lower concentration (10 μg/l). This indicates that TJ-GST-S gene expression was concentration-dependant. At 48 h level of mRNA expression in case of Cu was decreased but still significantly higher than that observed at 0 h. However, in case of Mn the expression level at 48 h was lower than that observed at 0 h. This indicates that the different levels of critical concentrations are required for induction or suppression of TJ-GST-S mRNA expression. Nevertheless, these findings clearly demonstrate that *TJ-GST-S* expression is modulated by the oxidative stress-inducing agents and imply that this enzyme may be involved in defense mechanism against chemically-induced oxidative stress. Previously, we showed that expression of an isoform of GST from *T. japonicus* is modulated by endocrine-disrupting chemicals, EDCs (4,4, *O*′-octylphenol, 4,4-*O*′-P and polychlorinated biphenyls, PCB) (Lee et al., 2006a). While 4,4-*O*′-P caused upregulation, PCB exposure down-regulated the GST expression. EDCs also act by inducing oxidative stress (Choi and Lee, 2004; Iso et al., 2006). Therefore, modulatory effect of these chemicals on GST expression in *T. japonicus* demonstrates that GSTs have a role in antioxidant defense in this organism.

This is the first report on a novel GST isoform (GST-Sigma) of marine copepod *T. japonicus* with a possible role in antioxidant defense. Besides detoxification and antioxidant roles, GSTs perform multiple functions (e.g., GSH-dependant prostaglandin D_2 synthase) (Vararattanavech and Ketterman, 2003). It may be interesting to study such other roles of GST-S in *T. japonicus*. These findings would also provide a better understanding of comparative detoxification mechanisms between copepod and other marine organisms.

Acknowledgements

This work was supported by a National Research Lab (NRL) Grant (R0A-2006-000-10155-0) of Korea Science and Engineering Foundation (KOSEF) funded to Jae-Seong Lee, and also supported by a grant (Monitoring on Environmental Changes at the Korean Arctic and Antarctic Stations; PE07040) from the Korea Polar Research Institute funded to In-Young Ahn. S. Raisuddin is a Brain Pool fellow funded by the Korean Federation of Science and Technology Societies (KOFST).

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