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Molecular cloning, expression, biochemical characteristics, and biomarker potential of theta class glutathione *S*-transferase (GST-T) from the polychaete *Neanthes succinea*

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Abstract

We cloned and sequenced the full-length cDNA of a theta class glutathione *S*-transferase (GST-T) from the polychaete *Neanthes succinea*. The open reading frame of *N. succinea* GST-T cDNA was 678 bp and encoded 226 amino acid residues. We generated recombinant *N. succinea* GST-T by expression in transformed *Escherichia coli* and studied the kinetic properties as well as the effects of inhibitors, pH, and temperature on *N. succinea* GST-T. GST-T expression was studied using real-time RT-PCR in response to exposure to the model oxidative stress-inducing agent, $CuCl_2$. Copper induced a concentration-dependant increase in the expression of GST-T. Moreover, polychaetes collected from a heavily contaminated lake near an industrial complex showed significantly higher levels of GST-T expression. Interestingly, the site-collected polychaetes with the highest GST-T mRNA expression levels also showed the highest metallothioneins levels. These results suggest that GST-T in polychaetes may have an antioxidant role and that *N. succinea* GST-T expression may be a useful biomarker for exposure to environmental contaminants such as copper. Our findings provide a better understanding of the biochemical characteristics of *N. succinea* GST-T, and elucidate the potential role of GST-T in heavy metal-induced oxidative stress and as a biomarker for environmental contamination.

Keywords: Polychaete; *Neanthes succinea*; Glutathione *S*-transferases; Heavy metals; Metallothioneins; Oxidative stress; Biomarker

1. Introduction

Glutathione *S*-transferases (GSTs) are a family of diverse multifunctional proteins that are primarily involved in xenobiotic detoxification. Upon exposure to prooxidant xenobiotics, GST genes are over-expressed in most organisms (Hayes et al., 2005). In mammals, cytosolic GSTs exist as a hetero- or homodimer subunits with molecular masses ranging from 23 to 28 kDa depending on the gene class and the organism (Sheehan et al., 2001; Frova, 2006). GSTs have been classified as alpha, mu, pi, and theta on the basis of 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). To date, novel classes of non-mammalian GSTs such as sigma, zeta, beta, delta, and epsilon have been isolated and characterized from bacteria, plants, insects, and fish (Sheehan et al., 2001). However, information about marine invertebrate GSTs is quite scanty (Contreras-Vergara et al., 2004). A few of the aquatic invertebrates in which GST has been studied include, bivalve mollusks (*Atactodea striata*) (Yang et al., 2003), gastropods (*Bulinus truncates*) (Abdalla et al., 2006), marine shrimp (*Litopenaeus vannamei*) (Contreras-Vergara et al., 2004), crayfish (*Macrobrachium vollenhovenii*) (Adewale and Afolayan, 2005), clams (*Raditapes decussates*) (Hoarau et al., 2002), squids (*Ommastrephes sloani pacificus*) (Tomarev et al., 1993), octopi (*Octopus vulgaris*) (Chiou et al., 1995), and blue mussels (*Mytilus edulis*) (Fitzpatrick et al., 1995). Most of these studies focused on purification and/or biochemical measurement of total GST or different GST isoforms using *in vivo* organisms. Therefore, molecular characterization of GSTs from

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marine organisms is needed for further application in genomics, proteomics, and biomarker studies.

Marine environments, particularly the Korean intertidal zones, have been affected by different pollutants derived primarily from industrial activities. One polluted site is Lake Shihwa, which was formerly an inner part of a small peninsula in the Yellow sea. Lake Shihwa is located near an industrial complex in the South Korean Peninsula and has an area of approximately 61 km^2. The topography of Lake Shihwa was recently changed to a man-made lake after an artificial dam was constructed. The surrounding area near Lake Shihwa has plenty of mud layers that are heavily polluted by the metal-plating industry and other heavy industrial units (Lee et al., 2001a,b; Li et al., 2004a,b; Koh et al., 2005; Rostkowski et al., 2006). Thus, a dominant bottom-dwelling species such as polychaete *Neanthes succinea* may be a pertinent organism for the discovery of biomarkers for sedimentary pollution. Currently, the use of molecular biomarkers, especially through biomarker gene expression, has evolved as a reliable approach for environmental biomonitoring (Regoli et al., 2002; Tanguy et al., 2002; Venturino et al., 2003; Galloway, 2006; Sarkar et al., 2006). Previously, glutathione *S*-transferase genes were determined to be potential biomarkers through our laboratory-based investigation of a marine copepod *Tigriopus japonicus* (Lee et al., 2006a).

The study of polychaete GST expression may be helpful for assessing the impact of pollutants, especially those with pro-oxidant potential. However, the molecular and biochemical information in polychaetes is limited. Moreover, as GSTs can be induced or inhibited in response to exposure to environmental toxicants, these molecules may be used as biomarkers to determine exposure in aquatic organisms (Van der Oost et al., 2003; Amado et al., 2006). Therefore, studies on different GSTs in dominant marine species such as *N. succinea* would prove very helpful.

In this study, we cloned the cDNA from theta class of GST (GST-T) genes in *N. succinea*, purified a recombinant GST-T, and analyzed its catalytic properties. In addition, we investigated GST-T mRNA expression patterns after exposure to a known oxidative stressor (copper). In order to make a preliminary assessment of the use of GST-T as a biomarker for exposure, we also studied GST-T mRNA expression in polychaete samples collected from polluted sites and compared these expression patterns with metallothionein (MT) levels.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents used in this study were molecular biology grade and were purchased from either Sigma-Aldrich Co. (St. Louis, MO, USA), Qiagen (USA), or Invitrogen (USA). All oligonucleotide synthesis and DNA nucleotide sequencing reactions were performed at the Bionics (Seoul, South Korea). The pCR® T7 TOPO TA expression kit was supplied by Invitrogen, the Ni^+-NTA column was obtained from Qiagen (Germany), and the prestained broad range protein molecular weight markers were from Fermentas (USA). The GST assay kit was purchased from Sigma. All bacterial strains and plasmid vectors used in this study were purchased from Invitrogen.

2.2. Polychaete

The polychaete *N. succinea* were maintained in an aquarium in the Department of Molecular and Environmental Bioscience in the Graduate School of Hanyang University in Seoul. The aquarium conditions were a water temperature of 20 °C, a photoperiod of 12-h light:12-h darkness, and a salinity of 33 ppt. The species identity was checked by stereomicroscopy and by the sequence analysis of mitochondrial DNA cytochrome oxidase 1 (CO1) as a life barcode.

2.3. cDNA cloning of glutathione S-transferase-T from a N. succinea cDNA library

N. succinea specimens ($n = 10$) were frozen in liquid nitrogen prior to total RNA extraction. Whole body tissues were first ground with a mortar and pestle and then homogenized in three volumes of TRIZOL (MRC, USA) with a tissue grinder. Total RNA was extracted according to the manufacturers' protocol. mRNA from total *N. succinea* RNA was purified using an mRNA purification kit (Invitrogen) according to the manufacturer's protocol. The unidirectional λcDNA library was made using a λZAPII cDNA library packaging kit (Stratagene, USA). The linker and adaptor with *Eco*RI and *Xho*I restriction enzyme sites were used for unidirectional cDNA cloning. Packaging and titration of primary *N. succinea* λ recombinant phage were performed by following the manufacturers' protocol. A small quantity (3–4 μL) of an *N. succinea* λZAPII cDNA library was subjected to an *in vitro* conversion to phagemid DNA with the help of a helper phage and an *E. coli* SOLR strain. Of the rescued colonies, 500 were randomly picked for culture in order to identify inserts. After cutting the inserts with *Eco*RI, the recombinant pBlusecript phagemid DNAs (over 1.0 kb) were sent for sequence analysis with an ABI 370 automated sequencer. The *N. succinea* expressed sequence tags (ESTs) were compared with the GenBank database to identify the closest homologues, and submitted to the *N. succinea* EST database (to date, not released to the public domain). One of the *N. succinea* ESTs was identified as a partial glutathione *S*-transferase theta class (GST-T) and was further characterized using 5′-random amplification of cDNA ends (5′-RACE).

2.4. Phylogenetic tree

We generated a phylogenetic tree by using Clustal X (Version 1.83) to align the GST-T gene from *N. succinea* with GST-T genes from diverse species including mammals, insects, fish, and amphibia at the level of the deduced amino acid sequence (Thompson et al., 1997). Gaps and missing data were completely excluded from the data analysis. This data matrix was analyzed with Maximum Parsimony (MP). A total of 100 bootstrap replicates were employed. After analysis, the consensus tree was constructed under bootstrap 50% majority-rule and then visualized with Tree View of PHYLIP (Page, 1996). The GST mu class

derived from the European house dust mite (*Dermatophagoides pteronyssinus*) was designated as outgroup.

2.5. Recombinant N. succinea GST-T

Prokaryotic expression plasmids were constructed according to the manufacturers' instructions. Briefly, the open-reading frame (ORF) region of *N. succinea* GST-T was amplified with *N. succinea* GST-T-F/R primers (Table 1) using an iCycler (Bio-Rad, USA). The amplified product was eluted from a 1% agarose gel using an elution kit (Qiagen) and was then directly inserted into the 6 × His tagged pCR®T7 NT-TOPO expression vector.

Expression of the recombinant *N. succinea* GST-T protein was achieved by transforming the *E. coli* strain BL21(DE3)pLysS with the *N. succinea* GST-T/pCR T7 NT-TOPO vector. Transformed cells were grown in 1L LB broth containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). The expression of recombinant *N. succinea* GST-T protein was induced by adding 1 mM isopropyl-l-β-D-thiogalactopyranoside (IPTG, Sigma). Bacterial cells were harvested after an 18 h incubation at 30 °C and directly analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel. Collected cells were resuspended in ice-cold 1 × homogenizing buffer (20 mM Tris/pH 7.9, 0.5 M NaCl, 5 mM imidazole, 10 mL per g of wet pellet weight) and sonicated (3 times, 10 min each) with a sonicator (Branson Co., USA). The homogenate was centrifuged at 20,000 × *g* at 4 °C for 20 min. The supernatant was used for protein purification and subsequent enzyme kinetics studies. A self-ligated pCR ®T7 TOPO TA expression vector was used as a negative control.

2.6. Purification of soluble N. succinea GST-T

The soluble recombinant *N. succinea* GST-T protein was purified using a 6 × His-tagged affinity column (10 mL Ni-NTA resin) in the LP system (Bio-Rad). The supernatant fraction was loaded onto the column and the column was thoroughly washed with 1 × wash buffer (20 mM Tris/pH 7.9, 60 mM imidazole, 0.5 M NaCl) for 30 min with a flow rate of 1 mL/min. Subsequently, recombinant *N. succinea* GST-T was eluted in 10 fractions with 1 × elution buffer (20 mM Tris/pH 7.9, 1 M imidazole, 0.5 M NaCl). The eluted fraction was dialyzed overnight in 1 × dialysis buffer (20 mM Tris/pH 7.9, 0.5 M NaCl, 5 mM imidazole, 0.5 mM EDTA, pH 8.0). The pooled fractions were analyzed on 12% SDS-PAGE gels and then visualized by western blotting. Protein concentration was measured using Bio-Rad protein assay reagents (Bio-Rad).

SDS-PAGE (12% gels) was carried out as described by Laemmli (1970). Electrophoresed proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell Co., USA) using a Mini Protean III Transblotting System (Bio-Rad). Following the 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 incubated with anti-His G-HRP antibody (1:5000) (Invitrogen) at room temperature for 3 h and then was washed three times with 0.1% TTBS for 60 min. Detection was accomplished with an ECL plus Western Blotting kit (Amersham, USA).

2.7. GST assay

The specific activity of the recombinant enzyme was measured with a spectrophotometer using 1 mM l-chloro-2,4-dinitrobenzene (CDNB) and 2 mM reduced glutathione (GSH) as substrates at 340 nm and at 25 °C using the GST kit as described in the manufacturers' instructions.

2.8. Kinetics study

Purified *N. succinea* GST-T activity was studied at various concentrations of CDNB (0.25–4 mM) and GSH (0.25–2.0 mM) as well as in a fixed concentration of GSH (2 mM) or CDNB (1 mM) using a commercial GST assay kit. The data were plotted as a Lineweaver–Burk plot to determine the value for K_M.

2.9. Effects of pH, temperature, and inhibitors on recombinant NS-GST-T

Effect of pH on the activity of the purified *N. succinea* GST-T isoenzyme was studied at 25 °C after the enzyme was equilibrated at 50 mM of the following buffers: acetate (pH 5), phosphate (pH 6.0 and 7.0), and Tris–HCl (pH 8.0 and 9.0) for 3 min. The remaining activity was then measured as described above. For temperature effects, the enzyme was incubated in PBS (0.1 M, pH 6.5) at temperatures ranging from 4 to 50 °C for 5 min and then the remaining enzyme activity was measured. Standardized concentrations of cibacron blue (0.0–10 μM), hematin (0.0–10 μM), and *N*-ethylmaleimide (NEM 0.0–1000 μM) were used in an inhibition assay according to the method described

Table 1
Primers used in this study

Gene	Oligo name	Sequences (5′ → 3′)	Purpose
NS-GST-T	F	ATG TCA AGA CTG AAG CTA TAC TTT G	ORF amplification
	R	TTA AAT CTT GGA TGC CAG GAA GTG	
NS-GST-T	F	TCG TAT CTT CAG TGG CAT TGT TTG	Real-time RT-PCR
	R	CGA TCC ACT GGT TTG TTC ATA GC	
NS-β-Actin	F	TGT GCT GTC CCT GTA CGC TTC	β-Actin cDNA amplification
	R	GCC GTG GTT GTG AAG GAG TAA C	

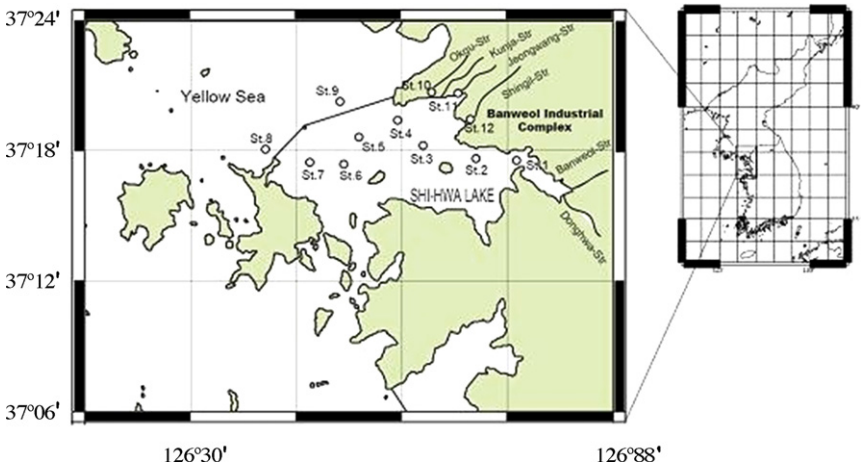

Fig. 1. Site map of Lake Shihwa, South Korea (St, sampling site).

by Tahir et al. (1985) which was adopted by Lee et al. (2006b).

2.10. Heavy metal exposure

Adult *N. succinea* ($n = 3$ per group) were exposed to 0, 12, 36, 72 μg/L of copper chloride for 96 h. In order to acclimatize the *N. succinea* before exposure, we kept the polychaetes in filtered seawater and the water was changed everyday for 6 days. During exposure to copper chloride, the polychaetes were maintained under the same conditions as described above but without food. The mRNA expression was studied using real time RT-PCR.

2.11. Field sampling for polychaetes and metal analysis

Surface sediment samples were collected by using a grab sampler at each site in Lake Shiwha during October 2005 (site map shown in Fig. 1). Polychaetes (*N. succinea*) were isolated by hand from the sediment without sieving and identified by microscopic observation. For metal analysis, freeze-dried samples of sediment and polychaete were digested with 65% HNO_3 and an acid mixture (30% HC1, 48% HF, and 65% HNO_3). Heavy metals of interest were analyzed using Inductively Coupled Plasma–Mass Spectroscopy (ICP-MS) and an inductively coupled plasma–atomic emission spectrometer (ICP-AES) at the Korea Basic Science Institute, Seoul.

2.12. Metallothionein

Metallothionein content in *N. succinea* was measured using the spectrophotometric method described by Viarengo et al. (1997). The polychaete samples were homogenized in three volumes of 0.5 M sucrose and 20 mM Tris–HCl (pH 8.6) with 0.006 mM leupeptin (Sigma) and 0.5 mM phenylmethylsulphonyl fluoride (Sigma) as antiproteolytic agents and 0.01% 2-mercaptoethanol (Sigma) as a reducing agent. The homogenates were then centrifuged at 30,000 × *g* for 20 min to obtain a supernatant that contained MT. The supernatant was treated with ethanol/chloroform as described by Kimura et al. (1979). In each sample, 1.05 mL of cold ethanol and 80 μl of chloroform were added to 1 mL aliquots of supernatant. The samples were centrifuged at 6000 × *g* for 10 min at 4 °C. The collected supernatants were combined with calf liver RNA (Sigma), and 37% HCl with three volumes of cold ethanol. The samples were kept at −20 °C for 1 h and centrifuged at 6000 × *g* for 10 min. The MT-containing pellet was washed with 87% ethanol and 1% chloroform in a homogenizing buffer in order to remove soluble thiol. After the washes, the pellet was centrifuged at 6000 × *g* for 10 min and dried under nitrogen gas. 5,5-Dithiobis-2-nitrobenzoic acid (DTNB, pH 8, Sigma) was added to the sample at room temperature and the absorbance was measured at 412 nm using a spectrophotometer (Varian, Cary 50). GSH was used as a standard.

2.13. mRNA expression study

The NS-GST-T-specific mRNA expression was studied in Cu-exposed and field collected polychaetes. Total RNA was isolated with TRIZOL® reagent (Invitrogen) from the adult *N. succinea* ($n = 3$) according to the manufacturers' instructions. To make single-stranded cDNA, 2 μg total RNA from each RNA sample was reverse transcribed to first strand cDNA using SuperScriptTM III reverse transcriptase (Invitrogen) and oligo $(dT)_{20}$ primers in a reaction volume of 20 μl. RT-PCR reaction conditions were as follows: 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 products, melting curve cycles were continued under the following conditions: 95 °C/1 min; 55 °C/1 min; 80 cycles of 55 °C/10 s with 0.5 °C increase per cycle using real time-RT-PCR F or R primers (Table 1). SYBR Green fluorescence was used for detecting specific PCR products (Molecular Probe Inc., USA). SYBR Green amplification and detection was performed with the MyiQ (Bio-Rad). The *N. succinea* β-actin gene (Table 1) was used as a reference to normalize the expression levels between samples. All experiments were performed in triplicate. The fold change for comparing the relative gene expression to controls was determined by the $2^{-\Delta\Delta CT}$ method (Giulietti et al., 2001).

Data were expressed as the means ± S.D. and were analyzed by an unpaired Student's *t*-test after normalization. Differences were considered significant at $P < 0.05$.

3. Results and discussion

3.1. N. succinea GST-T cDNA

The *N. succinea* GST-theta (NS-GST-T) cDNA was sequenced (Fig. 2), and deposited in GenBank (Accession No. EF394324) The full-length ORF region was 678 bp and encoded a 226 amino acid peptide. When we searched the conserved domain using the pfam database (Finn et al., 2006) we discovered that TJ-GST had a G-site (2–77 aa), which binds the tripeptide glutathione in the N-terminal region and an H-site (107–203 aa), which is a substrate binding site in the C-terminus. Multiple alignments of insect GST-T genes with *N. succinea* showed that the *N. succinea* and the insect GST-T genes shared some domains (Fig. 3) and implying that GST-T may be expressed in response to similar stresses. When the phylogenetic tree was constructed by the MP method, NS-GST-T was placed into the theta class of insect GST-T. Sea urchin (genus *Strongylocentrotus*) and other species (e.g., chicken, *Xenopus*, fish, human, and mouse) had polytomy patterns under 50% following major rules of consensus tree generation (Fig. 4). This suggests that the *N. succinea* NS-GST gene, which we characterized, belongs to the theta class of the GST gene family. Therefore, we named our cloned product the theta class of *N.*

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-14                                                    GCTAATTAGCAATC
  1  ATGTCAAGACTGAAGCTATACTTTGATCTGATGTCTCAACCTTCCCGGGCTGTCTGGATC
     M  S  R  L  K  L  Y  F  D  L  M  S  Q  P  S  R  A  V  W  I
 61  TTTCTCAAAGCAACAGGAATTCCCTTCGAGGAGAAGCCAGTTGCTCTCAGAAAAGGAGAA
     F  L  K  A  T  G  I  P  F  E  E  K  P  V  A  L  R  K  G  E
121  CATCAAACCGAAGAATTTGCAAAAATAAATCCTTTCCAACTTGTCCCTGTGATTGATGAT
     H  Q  T  E  E  F  A  K  I  N  P  F  Q  L  V  P  V  I  D  D
181  GGGGGATTCGTATTATATGAGAGTCTTTCAATTTGCAAATACCTTGCCAAAAGTCGAAAC
     G  G  F  V  L  Y  E  S  L  S  I  C  K  Y  L  A  K  S  R  N
241  TTAGCTGACCATTGGTACCCATCTGAATTAAAACATCGAGCAAGAATTGAATCGTATCTT
     L  A  D  H  W  Y  P  S  E  L  K  H  R  A  R  I  E  S  Y  L
301  CAGTGGCATTGTTTGATGGTCAGACTTTTCGCTAGTCAAGTGTTTAGAATTCAGGTGATT
     Q  W  H  C  L  M  V  R  L  F  A  S  Q  V  F  R  I  Q  V  I
361  GAGCCAAGAGCTATGAACAAACCAGTGGATCGCCAGAGGCTGGCCAAGTATGAAAACATC
     E  P  R  A  M  N  K  P  V  D  R  Q  R  L  A  K  Y  E  N  I
421  CTCTCCGTTGTCCTGGACAGTTTTGAAACAGTGTGGCTGAAGGACACACCTTACATTTGC
     L  S  V  V  L  D  S  F  E  T  V  W  L  K  D  T  P  Y  I  C
481  AGTAATGAGATCTCCATTGCTGATGTGGCTTGCATCTGTGAACTCATGCAGGTGTATGCT
     S  N  E  I  S  I  A  D  V  A  C  I  C  E  L  M  Q  V  Y  A
541  GTGGACTATCCTCTGTGGGAGGATCGACCCAAATTGGAGGCTTGGTCAAAAAGAGTGAGG
     V  D  Y  P  L  W  E  D  R  P  K  L  E  A  W  S  K  R  V  R
601  GAACGTCTCAACCCCCATTACGACCAGGCCAACTTCATGGTGGATAAAGTGAGAAATCAC
     E  R  L  N  P  H  Y  D  Q  A  N  F  M  V  D  K  V  R  N  H
661  TTCCTGGCATCCAAGATTTAACTGCATCACTCAAACAACTGTATTACAAGAGATAATGTA
     F  L  A  S  K  I  *
721  TCCAATTAGTAACATAGTGCATTTGTCAGAGTTTGGCAGAGTTTTGATTGTCCACAGGCA
781  GAGAAAGTAATATTAATAGGCGAAAGACTCTAAATCACTTCTAAAATAAACAGTGATAAA
841  ACTTGTGTGTTATTTGAATGGGCAATGTAAATGATTAAATCAGTGAAATGAGGTCAAATA
901  AGTGATAAACTGAAAGGTTGGCAGCAGTAAAGTTATCTAAGTTAAATGAATCAATGTCCT
961  AAGGCTTGCAGGCCCTCTTACAAAAAAAAATCATTAGTGGTAAAAGGCTCTCAATTAAAA
1021 ACGTGTGTAATATGGCCACAAGGCCCACAGATATGATGTAAAAAAAAAATTGAAATCACT
1081 GAAACAAGGTTATGAGATCAAAGAAGTGGATATCAAAACATCTTTTTTTTTTGGACGAAAA
1141 GTTAAGTTAAAACACATTATTGTCATGAGGATTGCTGGCCCTTTTCCAAAATTAACCAAA
1201 AATATTGATAGTAGGTTATAGCCGCATGATTAGGCTCAAAGTATGAAACGAGGTTATGCG
1261 GTAATACTTGCCATTCAAAATGTCTATTATAGCTGCATGATTAGGCTCAAAGTATGAAAC
1321 GAGGTTATGCGGTAATACTTGCCATTCAAAATGTCTATTATAGCTGCATGATTAGGCTCA
1381 AAGTATGAAACGAGGTTATGCGGTAATACTTGCCATTCAAAATGTCTATTATAGCTGCAT
1441 GATTAGGCTCAAAGTATGAAACGAGGTTATGCGGTAATACTTGCCATTCAAAATGTCTAT
1501 TATAGCTGCATGATTAGGCTCAAAGTATAATAATATGTCTGTAATGTCCTATATAGTCCA
1561 GAATTTACTCTAGCAACTGACACAAAGTGTTGAGTCGAAAATGTATACAATAGGTTAGGA
1621 CTTGTAATTTAGTGAGATACAAATATATATCTAATGCATATATATCTACAAAACAATCCC
1681 GGTAAATTGGCTACTATTGTTACCAGATTGAACTGTCAATAACTACATAAAAATTACATT
1741 CTGTAAGTTATTATACCATTTGGCCGTTCAATGAAACGATATGTAATTACTTGGCTATTT
1801 GATGAAAGCATAATGCCTGGGCCAACCAAAGTGAAGATAATGGTATGCAGTTTTGGCCCC
1861 AAAACTTTAAAGAATACCTTTCAGAATTTAGCAATTCCTGCTCCATTAATTTCGACCAAT
1921 GAATTATGACGTCCATGCTCTAAATAATGGAGAAAAAATTGTCTTGACTATTAAAAATAT
1981 TTTCTAATGTTACTGACAGCACATTCTGGTGGCATATAGTGCCCAAAATTAAGTGATCAT
2041 GAACTATATCATAGAACTATATCATAGCGTTATCATACTCATATCCTGTATGAACATATA
2101 ATTTGACTAGTGTCTTTTTGTGCGCATATCTACTACAGAGGACACTATACGTCCAGTAAT
2161 CGGTTAAGTTAAAATTTTGAGTATTCATAATGATTCATTCATATGCATAATATGGTTATT
2221 AGTCCCCAGTGCATGCCAAGAGTGAGTGAAAATGATCTAAAAACACTTTCCATGATTGAT
2281 CGCAGAAAGTTTTGAAAAAATTGCTTGATGACCATTCCAACTGAAAGGCTCCCAGAAAGT
2341 AGAGTTTGTGCGACTTGGAAAGAACTTGTCTTGGAGGGCTGCACTTCCCATACCTTTTAC
2401 ATAGTTATCAGTATTAACTTTAACAATTAATATATTTCAATTTTCATATTCTCTTGTGTT
2461 GAATAATTATTGACTGCCTGTGTGCATTTGTCACATGGACACCAATTAGTACAGTATTTT
2521 ATATAGTATTGCGTTTGTAAAATGAGATGTGCTAATGGCATAATTTGTTGTCAAAAAGAA
2581 AGGCTGTGAATTAAGAATAAGACATATATATCATTAACGAGGTGCACATTTTGTGTGAAT
2641 ATCTATGAACTAAGGCTGTGAATTTTGTAATGTTTTGTAAAATTTTCAACACAACCAAAA
2701 ATTGTGATTTACAGAATTTTGTAATTATATTGTATATTTGTTTTGTATCGAGATATTGAT
2761 TGTTATTGACAATTTTTAGAGGCATAAACTGTGTGATTATAATATTATAAACTATTAAAT
2821 GTTTAATCATTAAAAAAAAAAAAAAAAAA
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Fig. 2. Nucleotides and deduced amino acid sequences of the glutathione *S*-transferase gene from *Neanthes succinea*.

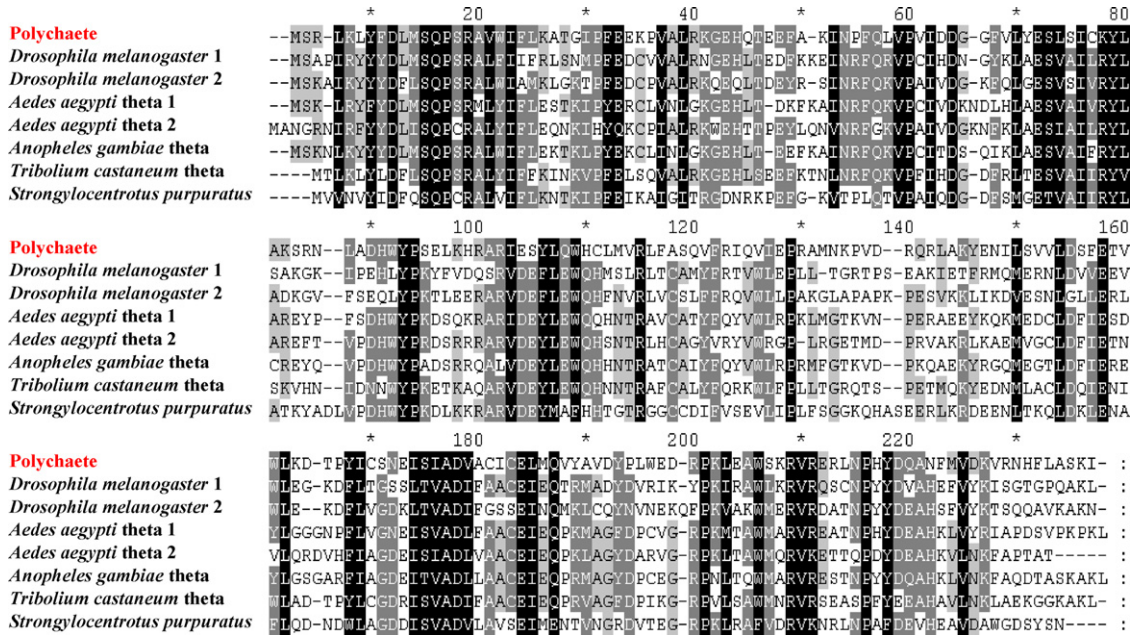

Fig. 3. Multiple alignment of *Neanthes succinea* GST-T with other insect GST-T and sea urchin GST-T genes. Mosquito (*Aedes aegypti* thetal, AY819711; theta2, AY819712), malaria mosquito (*Anopheles gambiae* theta2, XM_316865), fruit fly1 (*Drosophila melanogaster*, NM_167706), fruit fly2 (*Drosophila melanogaster*, NM_136665), red flour beetle (*Tribolium castaneum* theta, XM_965303), and purple urchin (*Strongylocentrotus purpuratus* thetal, XM_775123).

succinea glutathione *S*-transferase (abbreviated as NS-GST-T). However, other annelid species do not have information that pertains to the GST-T gene and detailed therefore placement classification of the NS-GST-T gene in annelids is not possible. We are also characterizing other xenobiotic detoxification enzyme genes such as superoxide dismutase (SOD) along with mining a massive number of ESTs from this species (unpublished data). These studies will certainly offer an understanding of the detoxification strategy adopted by *N. succinea*, which lives in marine sediment that is often laced with toxic chemicals.

3.2. Characteristics of the recombinant NS-GST-T protein

The SDS-PAGE analysis showed each protein as a single band with a molecular size of approximately 30.0 kDa that includes the co-expressed six histidines that are 3.5 kDa total in size (Fig. 5B). These results are in good agreement with the calculated molecular weight of 26.66 kDa for NS-GST-T protein. The theoretical *pI* of *N. succinea* GST-T is 8.52. This molecular weight is approximately the same size that was reported for the silkworm (*Bombyx mori*) rGSTTl gene, (24 kDa) by Yamamoto et al. (2005) and for the fish, *Rivulus marmoratus* (30 kDa) (Lee et al., 2006b).

The purification yield of recombinant NS-GST-T was 26.43% of the total activity collected after Ni-NTA column. The specific activity of this recombinant protein towards CDNB was 6.082 ± 0.05 (μmol/min/mg). Whereas crude lysate of the recombinant theta class GST (rGSTTl) of silkworm has been reported to have a specific activity of 4.3 μmol/min/mg (Yamamoto et al., 2005), the fish *R. marmoratus* protein has a rather high specific activity (9.94 ± 0.17 μmol/mg/min) toward CDNB (Lee et al., 2005).

Enzyme kinetics studies revealed that when the concentration of GSH was fixed, the V_{max} and K_M values for recombinant NS-GST-T were 12.78 ± 1.12 (μmol/mg/min) and $3.11 + 0.07$ (mM), respectively. Furthermore, when the CDNB concentration was fixed, the V_{max} and K_M values were $14.92 + 0.82$ (μmol/mg/min) and $2.50 + 0.31$ (mM), respectively (Table 2). In the case of the GSTT1 silkworm gene, Yamamoto et al. (2005) reported the V_{max} and K_M values to be $5.31 + 0.58$ and $0.48 + 0.08$, respectively. These workers also studied kinetics parameters with some other substrates, such as ethacynic acid (ECA), 4-hydroxynonenol (4-HNE), and 4-nitrophenyl acetate (4-NAP). Among these substrates, 4-NPA showed the highest values for both parameters. This demonstrates that GST-T kinetics values vary by species and substrate. Although theta class GST has been reported in other insect species (Singh et al., 2000; Corona and Robinson, 2006), detailed data are not available on the biochemical characteristics of theta class GST in these species.

The recombinant NS-GST-T had an optimum activity at 37 °C but 80–85% of the activity could be retained in temperature range from 0 to 50 °C. rGSTTl from the silkworm has also shown sufficient activity up to 50 °C (Yamamoto et al., 2005). GSTs in general show significant activities in broad temperature ranges. Besides our observations in self-fertilizing fish *R.*

Table 2
Kinetic parameters of NS-GST-T with two different substrates, CDNB and GSH

Substrate	K_m (mM)	V_{max} (μmol/mg/min)
CDNB	2.50 ± 0.31	14.9 ± 0.82
GSH	3.11 ± 0.27	12.78 ± 1.12

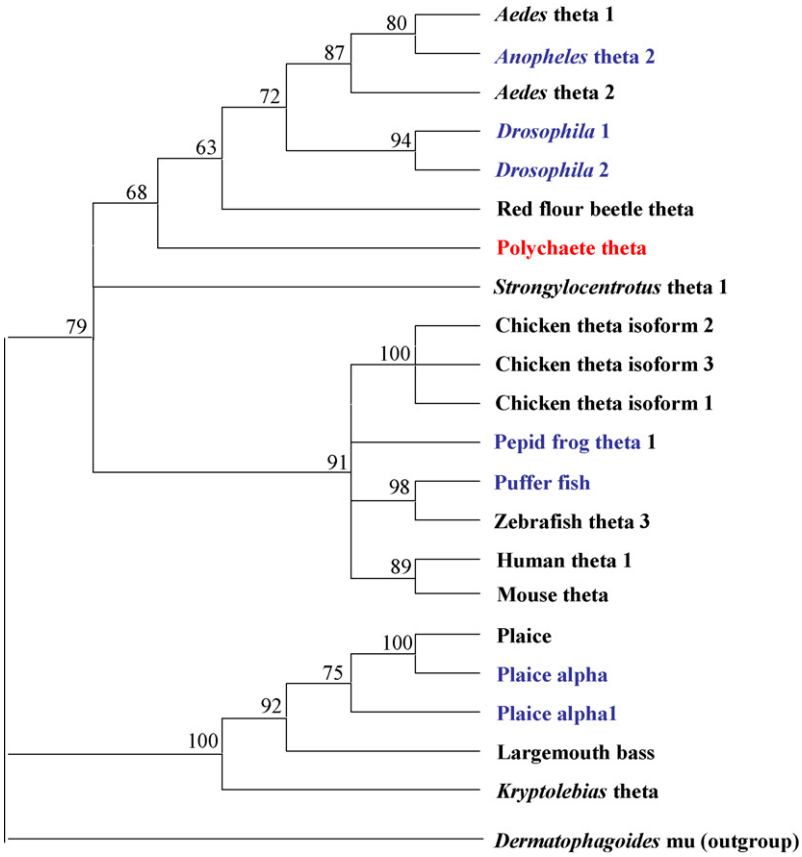

Fig. 4. Phylogenetic tree of the *Neanthes succinea* GST-T gene along with GST-T genes from other species. The GST mu class of the European house dust mite was designated as the outgroup. Bootstrap supporting values were on the node. Mosquito (*Aedes aegypti* theta1, AY819711; theta2, AY819712), malaria mosquito (*Anopheles gambiae* theta2, XM_316865), fruit fly1 (*Drosophila melanogaster*, NM_167706), fruit fly2 (*Drosophila melanogaster*, NM_136665), red flour beetle (*Tribolium castaneum* theta, XM_965303), purple urchin (*Strongylocentrotus purpuratus* thetal, XM_775123), chicken (*Gallus gallus* thetal isoform 1, XM_001231970; isoform2, XM_001231999; isoform3, XM_001232034), pepid frog (*Xenopus tropicalis* thetal, NM_001006810), puffer fish (*Tetraodon nigroviridis* CAAE01015007), zebrafish (*Danio rerio* theta3, XM_687335), human (*Homo sapiens* thetal, NM_000853), mouse (*Mus musculus* theta2, X98056), plaice (*Pleuronectes platessa* X63761; alpha, X95199; alphal, X95199), largemouth bass (*Micropterus salmoides*, AY335905), Kryptolebias (*Kryptolebias marmoratus* theta, DQ525602), and house dust mite (*Dermatophagoides pteronyssinus* mu, AY825939).

marmoratus (Lee et al., 2006b), which showed GST-T activity in a broad temperature range, Cailleaud et al. (2006) recently demonstrated GST (total) activity in copepod *Eurytemora affinis* in temperatures as low as 4 °C. This suggests that NS-GST-T as well as other GST isoforms are expressed and may be involved in detoxification and antioxidants over a broad temperature range.

We also studied the functional pH ranges for NS-GST-T. The recombinant NS-GST-T had an optimum pH range around 9.0 (Fig. 6). Likewise, silkworm rGSTTl shows optimum activity at pH 8 (Yamamoto et al., 2005).

NS-GST-T was sensitive to cibacron blue, while hematin and *N*-ethylmaleimade were not effective in inhibiting the activity of recombinant NS-GST-T in the concentration range used in this study (Fig. 7). Although no study is available for direct comparison, in one study of GST-sigma from webworm (*Hyphantria cunea*), Yamamoto et al. (2007) used insecticides as inhibitors. We previously studied the inhibition of GST-T in *R. marmoratus* (Lee et al., 2006b) using the three inhibitors and observed that NEM was the most effective in inhibiting enzyme activity. This implies that polychaete GST-T may have different mechanisms of inhibition compared to GST-Ts in fish.

3.3. Heavy metal exposure and NS-GST-T mRNA expression

Copper exposure caused an up-regulation of NS-GST-T expression. This indicates that NS-GST-T may be a factor as antioxidant on heavy metal exposure, since copper induces oxidative stress by its involvement in redox-cycling (Stohs and Bagchi, 1995; Valko et al., 2005). A Cu exposure of 72 μg/L for 96 h significantly induced NS-GST-T mRNA expression. However, the expression magnitude was low at lower Cu concentrations (12 to 36 μg/L) at 96 h (Fig. 8). This indicates that NS-GST-T gene induction is concentration- and time-dependent. This kind of expression pattern modulation was confirmed for manganese exposures using other stress related genes such as manganese superoxide dismutase and copper/zinc superoxide dismutase in this species (data not shown). Taken together, we suggest that NS-GST-T may be involved in a defense mechanism that protects against oxidative stress and is induced by exposure to metals and other agents.

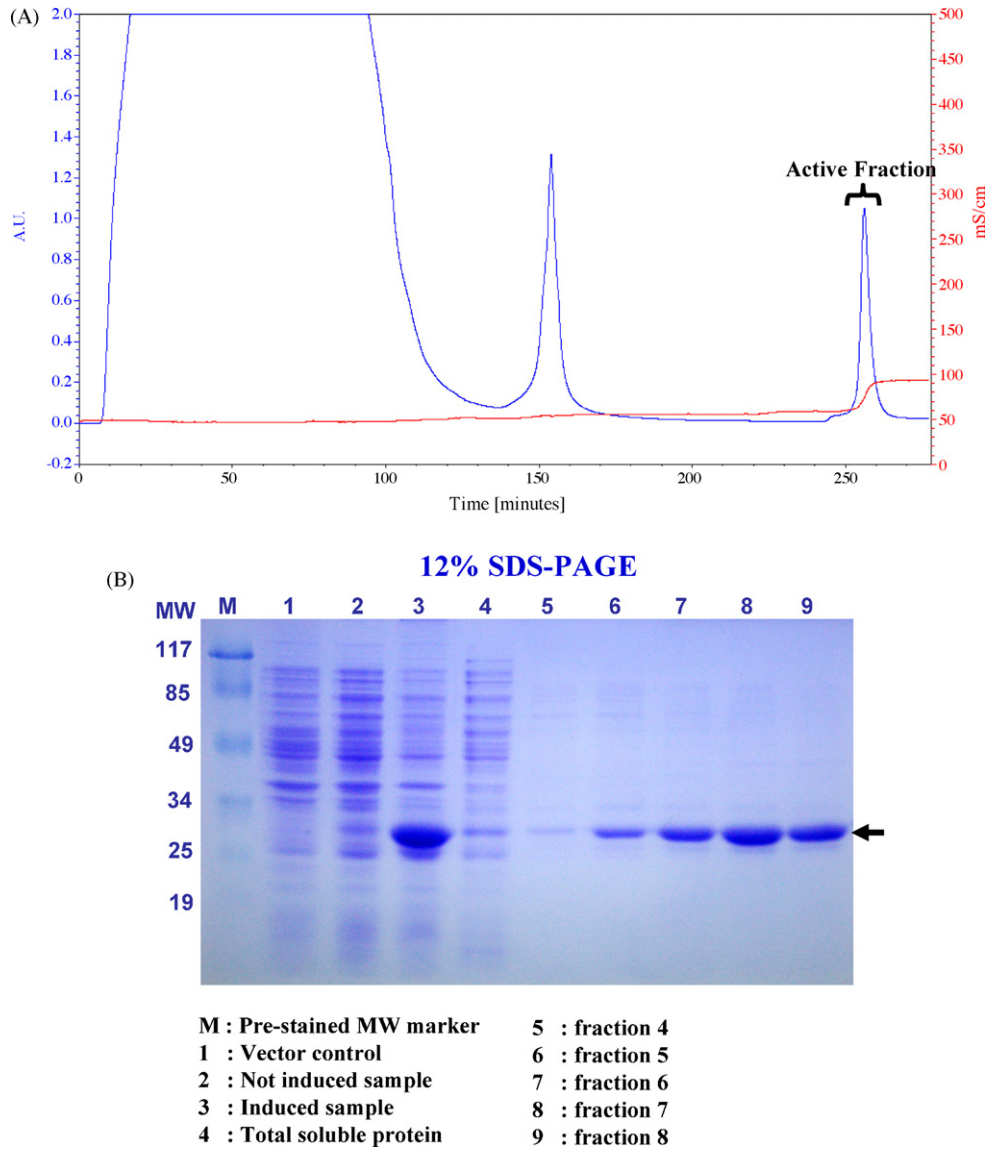

Fig. 5. A. His-tagged chromatography profile of recombinant *Neanthes succinea* GST-T on an affinity column within the Bio-Rad LP system. B. SDS-PAGE of purified recombinant GST-T. M. Prestained protein size marker, 1. Vector control, 2. Sample with no induction, 3. Induced sample, 4. Total soluble protein, 5. Fraction #4, 6. Fraction #5, 7. Fraction #6, 8. Fraction #7, and 9. Fraction #8.

3.4. Physico-chemical characteristics and heavy metal concentrations at sampling sites and in polychaete samples

Lake Shihwa is surrounded by the Banweol industrial complex (Fig. 1) where several copper pipe plants as well as zinc and manganese refinery plants are actively operating. The physico-chemical characteristics and heavy metal content profile of three randomly selected sampling sites are shown in Table 3. Although dissolved oxygen (DO) levels were within the acceptable range, all three sites showed significant levels of toxic heavy metals (arsenic, cadmium, copper, lead, zinc, etc.) in sediment samples. Polychaetes samples also showed significant levels of these metals. Although zinc and manganese were recorded in high quantities, copper levels were also high and a comparison showed that copper levels were greater than arsenic, cadmium, and lead.

3.5. NS-GST-T mRNA expression patterns and metallothioneins in field-collected polychaete samples

When the NS-GST-T expression pattern was measured by real-time RT-PCR, polychaetes from site 3 (St. 3, Fig. 9A) showed high expression compared to two other sites (St. 2 and St. 6) from the Banweol industrial complex. However, when we compared the copper contamination levels in the sediment and plychaete metal levels from various sites, St. 6 had the highest levels in the sediment and St. 2 had the highest levels in the polychaete samples. The MT contents of three sites were 35.243 μg/g (St. 2), 64.909 μg/g (St. 3), and 43.028 μg/g (St. 6) (Fig. 9B). Thus, polychaete at St. 3 showed the highest MT levels. Interestingly, this finding is correlated with the GST-T mRNA expression. MT induction, like GST, is regarded as a stress response of aquatic organisms to polluted sites. Increased

Table 3
Physico-chemical properties and metal content of sediment and polychaete samples collected from three sites at Lake Shihwa

Sampling station	St. 2	St. 3	St. 6
Water			
pH	7.76	8.03	7.85
Salinity (bottom) ppt	25	26.3	28.1
DO (bottom) mg/L	5.32	5.52	7.84
Sediment metal (mg/kg)			
Cu	69.27 (0.68)	40.59	87.27 (3.05)
Cd	0.56 (0.005)	0.36 (0.009)	0.65 (0.011)
Pb	41.86 (0.17)	30.94	54.88 (0.08)
Zn	273.2 (50.9)	234.1 (100.6)	226.2 (7.07)
Mn	323 (9.89)	314 (5.66)	380 (2.82)
As	7.42 (0.57)	5.33 (0.05)	5.23 (0.04)
Polychaete metal (μg/g)			
Cu	80.65 (2.71)	36.48 (0.43)	35.42 (2.59)
Cd	0.85 (0.33)	0.77 (0.80)	0.49 (0.09)
Pb	11.28 (7.1)	5.73 (4.4)	7.57 (0.47)
Zn	613.9 (80.7)	487.6 (145.2)	609.1 (224.7)
Mn	1280 (636.5)	222.3 (255.1)	365.7 (49.6)
As	6.29 (0.31)	6.41 (0.67)	3.02 (0.40)
Sediment grain size (%) (0–6 cm)			
Sand (%)	72	70	86
Silt (%)	28	30	14
Mean grain size (phi)	4.1	4.3	2.9

MT levels have been observed in organisms, including polychaetes, from sites polluted by metalloids or organic pollutants (Perez et al., 2004; Mosleh et al., 2005; Damiens et al., 2006). MT induction has also been proposed as a biomarker for heavy metal exposure (Amiard et al., 2006; Monserrat et al., 2006; Quiros et al., 2007) and the role of MT as an antioxidant has also been documented (Amiard et al., 2006; Atif et al., 2006). The polychaete samples collected from St. 3 apparently have experienced more stress from heavy metals as well as from other organic chemicals capable of inducting oxidative stress (Lee et al., 2001a,b; Li et al., 2004a,b; Koh et al., 2005; Rostkowski et al., 2006).

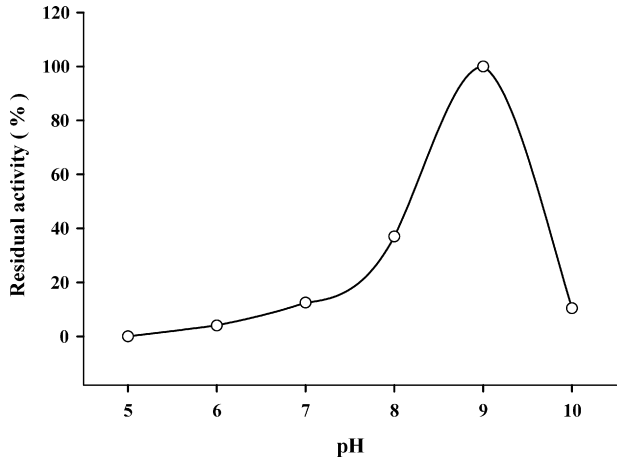

Fig. 6. Effect of pH on recombinant *Neanthes succinea* GST-T activity. The enzyme was incubated in 50 mM of the following buffers: acetate (pH 5) and Tris–HCl (pH 6 and 9) for 3 min. The residual activity was assayed with 1 mM CDNB and 2 mM GSH as substrates at 25 °C. Values are the means of three replicates.

Correlating the mRNA expression levels and copper content is difficult for sediment and biological samples on the basis of this preliminary study. The interactive role of other metals, metallothionein in polychaetes, and organic pollutants at the study sites cannot be ignored (Lee et al., 2001a,b; Li et al., 2004a,b). In a study on the Mediterranean polychaete species (*Sabella spallanzanii*), Bocchetti et al. (2004) also observed that there were significant variations between the levels of heavy metals (Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) and antioxidant levels (including GST) in samples collected from different sites. These studies were unable to form a clear cut correlation between the two sets of data. In another study involving polychaete species (*Neris diversicolor*) from the Cadiz Bay (SW Spain), Perez et al. (2004) used a suite of biomarkers including the neurotoxicity biomarker acetylcholine esterase (AChE) and GST activities. Of all the biomarkers, AChE showed a more consistent response. Nevertheless, two oxidative stress biomarkers (catalase, CAT

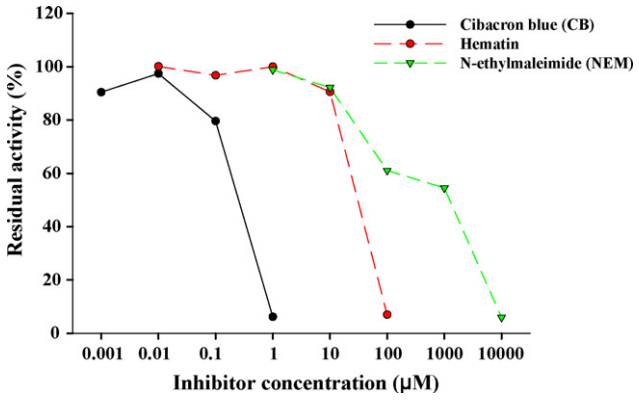

Fig. 7. Effect of various inhibitors on the activity of NS-GST-T protein.

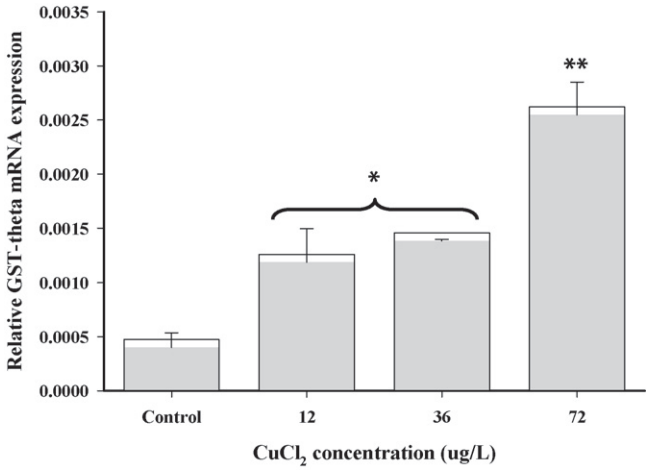

Fig. 8. Expression of *Neanthes succinea* GST-T after exposure to copper chloride (12–72 μg/L) for 96 h. Significant differences over control values are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$.

and GST) also showed an enhanced activity response in the samples collected from the polluted site. Polychaete MT content from polluted sites was also observed to be higher than a less polluted site. These authors suggested that because it is a sediment feeder, polychaete might provide better information on sediment-bound contaminants.

In a study involving a comparison of oxidative stress responses, including GST activity, in two populations of *Laeonereis acuta* (Polychaeta, Nereididae), Geracitano et al. (2004) observed that during chronic exposure, the polychaete population from the polluted site showed enhanced activities for GST, CAT, and SOD. Arsenic is also reported to interfere with antioxidant mechanisms, including GST from *L. acuta* (Ventura-Lima et al., 2007). All these cited studies have made use of GST (total) biochemical activity measurements, while no specific isoforms or sensitive approaches, such as gene expression studies, have been employed. However, all of these studies suggest that the polychaete species has a potential use in assessing the impact of marine sediment-bound pollutants. The use of expression studies for GST or other such biomarkers may refine

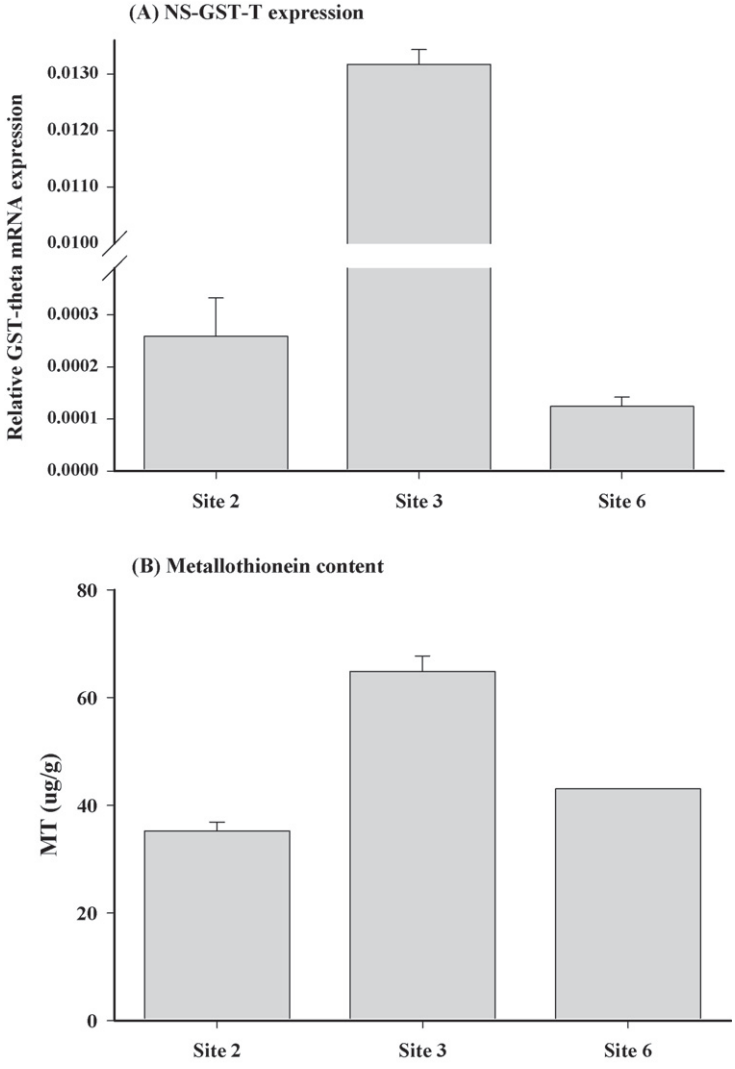

Fig. 9. (A) Expression of NS-GST-T in field-collected *Neanthes succinea*, (B) Metallothionein content in field-collected polychaetes samples.

the application of these genes in biomarker-based biomonitoring of polluted sites. However, more studies may be needed for confounding factors that influence the gene expression.

We conclude that GST-T of polycahaete *N. succinea* has a role in antioxidant defense because GST-T expression is modulated in response to exposure to a prooxidant (copper). Our findings may facilitate a better understanding of comparative detoxification mechanisms for polychaete and other marine organisms. These findings also suggest that GST-T from polychaetes may be used as a surrogate biomarker for exposure to marine sediment pollutants.

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