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Molecular cloning and thermal stress-induced expression of a pi-class glutathione S-transferase (GST) in the Antarctic bivalve *Laternula elliptica*

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ABSTRACT

Glutathione S-transferases (GSTs) are multifunctional phase II detoxification enzymes that catalyze the attachment of electrophilic substrates to glutathione. The pi-class GST cDNA (leGSTp) was cloned from the cold-adapted Antarctic bivalve *Laternula elliptica*. We used degenerated primers designed based on highly conserved regions of known mollusk GSTs to amplify the corresponding *L. elliptica* mRNA. Full-length cDNA was obtained by rapid amplification of cDNA ends (RACE). The full sequence of the GST cDNA was 1189 bp in length, with a 5′ untranslated region (UTR) of 74 bp, a 3′ UTR of 485 bp, and an open reading frame of 630 bp encoding 209 amino acid residues with an estimated molecular mass of 23.9 kDa and an estimated isoelectric point of 8.3. Quantitative RT-PCR confirmed basal expression of leGSTp, which was up-regulated upon heat treatment (10 °C for different time periods) by a factor of 2.3 (at 24 h) and 2.7 (at 48 h) in the digestive gland and gill tissues, respectively. The recombinant leGSTp expressed in *Escherichia coli* was purified by affinity chromatography and characterized. The purified leGSTp exhibited high activity towards the substrates ethacrynic acid (ECA) and 1-chloro-2,4-dinitrobenzene (CDNB). The recombinant leGSTp had a maximum activity at approximately pH 8.0, and its optimum temperature was 35 °C.

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1. Introduction

Glutathione S-transferases (GSTs) comprise a family of multifunctional phase II detoxification enzymes that are widely distributed in animals, plants, and microorganisms. They are found in the cell cytosol and other water-based components of living systems. GSTs play a key role in the cellular detoxification process by facilitating the attachment of tripeptide glutathione (GSH: γ-Glu-Cys-Gly) to a variety of potentially harmful electrophilic substrates, thereby preventing damage to the cell membrane and other macromolecules (Ketterer et al., 1983). Among their cellular functions, GSTs play an important role in the detoxification of reactive oxygen species (ROS) and the regulation of redox balance (Konings and Penninga, 1985; Siritantikorn et al., 2007). Furthermore, GSTs possess ligand binding activities and participate in stress-mediated cell signaling pathways as well as apoptosis (Cho et al., 2001; Wang et al., 2001).

Most GSTs are cytosolic or microsomal enzymes that form homodimers or heterodimers of subunits ranging from 23 to 28 kDa in molecular mass. They commonly possess two binding domains: a GSH binding site (G-site) and a substrate binding site (H-site) (Mannervik and Danielson, 1988; Rushmore and Pickett, 1993). The cytosolic GSTs have been grouped into several classes (alpha, mu, pi, theta, sigma, kappa, zeta, omega, and rho) based on primary structure, substrate specificity, and immunological reactivity (Mannervik et al., 1985; Meyer et al., 1991; Meyer and Thomas, 1995; Pemble et al., 1996; Board et al., 2000, 2001; Konishi et al., 2005). The pi-GSTs are generally the most prevalent in mammalian cells because of their involvement in drug metabolism and their ability to deactivate lipoperoxydation products (e.g., lipid hydroperoxide) and their derivatives. Members of the GST isozyme class possess an SH group that enables them to inactivate reactive oxygen species. In addition, several reports suggest that these GSTs exhibit ligand binding activity and act as inhibitors of Jun N-terminal kinase, which protects cells against oxidative stress-induced cell death (Monaco et al., 1999; Yin et al., 2000).

The bivalve *Laternula elliptica*, which is a stenothermal clam endemic to the Antarctic, is one of the most abundant macrobenthic species in the Antarctic coastal region. It has been recognized as an important sentinel species for monitoring changes in coastal ecosystems (Ahn et al., 1996; Choi et al., 2007; Park et al., 2007). Several studies have examined oxidative stress and antioxidant defense systems in marine mollusks, however, the characterization and expression of GSTs in response to oxidative stress directly induced by thermal stress remains poorly understood for bivalve mollusks. Previously our studies have suggested that GSH and GSH-related enzymes play a major role in a coordinated protection mechanism against potential toxicity from reactive oxygen species during thermal stress in *L. elliptica* (Park et al., 2008). Here, we report the cloning of a pi-class GST cDNA from *L. elliptica* and the relative pattern of leGSTp

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mRNA expression upon acute warming. Furthermore, the recombinant leGSTp was expressed in *Escherichia coli*, and it has been characterized.

2. Materials and methods

2.1. Organisms and heat exposure experiments

Specimens of *L. elliptica* (shell length ~80 mm) were hand-collected by SCUBA divers from depths of 20 to 30 m in Marian Cove, near King Sejong Station, on the northern Antarctic Peninsula (62°13′ S, 58°47′ W) in January 2006. The samples were divided into two groups and acclimated under conditions equivalent to those of the field temperature (~1.0 °C) with a constant air supply. After acclimation for two days, one group was transferred to water at 10±1 °C, which is the thermal limit of survival (Peck et al., 2002), to induce acute heat stress, and the other group was kept at 1.0±1 °C as a control with well-aerated natural seawater and without feeding. The samples were collected from each group, thermally stressed and non-thermally stressed, for the various exposure time points.

2.2. GST cDNA cloning

Total RNA was isolated using the TRIzol procedure (Invitrogen) and precipitated in ethanol. Single-strand cDNA was synthesized from 2 μg total RNA in a final volume of 20 μL containing 50 pM oligo(dT_{20}), 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 50 mM DTT, 0.75 U RNasin, 0.2 mM of each dNTP, and 1 U Superscript III™ reverse transcriptase (Invitrogen). Reactions were incubated at 42 °C for 90 min and terminated by heating at 95 °C for 5 min. Oligonucleotides used in this study are listed in Table 1. The partial cDNA clone of the pi-class GST was obtained by reverse transcriptase PCR (RT-PCR) with degenerated primers (GSTF and GSTR). To get a full-length cDNA of the GST, 3′ and 5′-end were amplified using Capfishing full-length cDNA kit (Seegene, Seoul, Korea) according to manufacturers' instruction with each of gene-specific forward and reverse primer (5′GST and 3′ GST). The RT-PCR and RACE products, cloned in pCR2.1-TOPO, were sequenced from both the 5′ and 3′ ends using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, USA) and an automated fluorescence sequencer. Complete sequences were analyzed by searching for similarities using the BLASTX search program of the National Center for Biotechnology Information (NCBI) GenBank.

2.3. Tissue expression analysis

Equal amounts of RNA (as determined spectrophotometrically) from the various tissues (gonad, digestive gland, mantle, gill, and intestine) of thermal-treated clams were used for reverse transcription. RT-PCR was performed using 2× ExTaq Premix 1 (Takara, Tokyo, Japan) with gene specific primers, RTGSTF and RTGSTR. As a control, *L. elliptica* β-actin (GenBank accession number EF198331) was amplified for each RT reaction product. The PCR products were visualised on a UV-transilluminator after electrophoresis on a 1.5% agarose gel that contained ethidium bromide (0.5 μg/μL).

Table 1
Primers used

GSTF[a]	5′-ASMMTSATCTAYMMWAACTATGA-3′
GSTR[a]	5′-YTAYTGTTTDCCRTTKCCRTT-3′
5′GST	5′-TCTTGACGAATTCCTCCTTTCCTGC-3′
3′GST	5′-ATTTCCAAACCATTGCTGATCGACC-3′
RTGSTF	5′-GGATTGACCTACAAAGAAAT-3′
RTGSTR	5′-CCACGCTGTCGTTGACCATA-3′
QGSTF	5′-CCCGGATGTTTGAAAGAGTT-3′
QGSTR	5′-GTTGCCATTAATCTGCATTT-3′
EGSTF	5′-CATATGACAAACTACGAGCTTGAA-3′
EGSTR	5′-CTCGAGTTATTGTTTTCCGTTGCCATTA-3′
QActF	5′-GGTCGTACCACAGGTATTGT-3′
QActR	5′-CATCAGGTAGTCGGTCAAAT-3′

[a] D, A/G/T; K, G/T; M, A/C; S, C/G; R, A/G; W, A/T; Y, C/T.

2.4. Quantification of leGSTp mRNA expression by Real-Time PCR

The mRNA levels of leGSTp were measured by semi-quantitative Real-Time PCR. PCR amplifications were performed in 25-μL reactions that contained cDNA generated from 2 μg of the original RNA template, 0.2 μM each of the gene-specific forward and reverse primers (QGSTF and QGSTR), and 12.5 μL of Brilliant II SYBR Green QPCR mix (Stratagene). The amplified signals were monitored continuously with the Mx3000P QPCR System (Stratagene), and the amplification protocol was as follows: an initial 15 s of denaturation and enzyme activation at 95 °C, followed by 45 cycles at 95 °C for 5 s, 52 °C for 15 s, and 72 °C for 15 s. The β-actin gene of *L. elliptica* was used as a reference to normalise the expression levels between samples. All data were relatively calculated and expressed for β-actin, to compensate for any difference in reverse transcriptase efficiency. All experiments were repeated in triplicate. The relative gene expression fold-change was determined by the 2-ddCt method (Livak and Schmittgen, 2001). All data are expressed as the mean±SD and were analyzed by an unpaired Student's *t*-test after normalisation. Differences were considered statistically significant at $P<0.05$.

2.5. Expression and purification of recombinant GST

The complete coding region of leGSTp gene was amplified by polymerase chain reaction (PCR) with each of gene-specific forward and reverse primer (EGSTF and EGSTR). The PCR products were cloned into pCR2.1-TOPO cloning vector (Invitrogen), and these genes ware subsequently cloned into the pET19b expression vector (Novagen), which facilitates $(His)_{10}$ affinity tag introduction, utilizing the engineered restriction sites. The identity of the insert was verified by sequencing, and the plasmid was designated pET19b/leGST in *E. coli*. *E. coli* BL21(DE3)pLysE-competent cells (invitrogen) were used as a bacterial expression host. The transformants were cultivated in 250 mL of LB (Luria-Bertani) medium at 37 °C in the presence of 50 μg mL^{-1} ampicillin until the *A* at 600 nm reached approx. 0.6. At this point, the temperature was changed to 20 °C, and expression of the His-tagged proteins was induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG), followed by another 15 h shaking of the culture. The cells were collected by centrifugation at 6000*g* for 15 min at 4 °C. The cell pellet was resuspended in 20 mL of ice-cold PBS buffer and lysed by sonication on ice. The cell debris was removed by centrifugation at 12,000*g* for 20 min, and the supernatant was loaded onto a Ni–NTA resin column (Amarsham Biosciences). The column was washed with 50 mM PBS (pH 8.0) containing 20 mM imidazole and then eluted with 50 mM PBS (pH 8.0) containing 500 mM imidazole. Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard (Bradford, 1976). The purity of eluted samples was analyzed by SDS/PAGE.

2.6. SDS/PAGE and Western blot analysis

SDS/PAGE was performed by the method of Laemmli (1970). Migration of the proteins was determined in 12% polyacrylamide gel, and stained with Coomassie brilliant blue R-250. For Western-blot (immunoblot) analysis, proteins were transferred to a PVDF membrane (Immobilon PVDF membrane, Millipore) by semi-dried electrophoretic transfer as described by Towbin et al. (1979). The blotted PVDF membrane was incubated in 4% skim milk in PBS at room temperature for 2 h. After the washing process with PBS

containing 0.1% Tween 20, the membrane was incubated with anti-His G-HSP antibody (Invitrogen) diluted 1:5000 at 25 °C for 1 h. ECL-plus Western blotting kit (Amersham Biosciences) was used for detection.

2.7. Enzymatic activity assay

Recombinant leGSTp catalytic activities were analyzed with four alternative substrates. All activity measurements were performed at 25 °C with a double beam UV-1800 spectrophotometer (Shimadzu). Recombinant leGSTp catalytic activity was measured using 1-chlor-2,4-dinitbenzene (CDNB), 4-nitrophenyl acetate (4-NPA) and ethacrynic acid (ECA) by monitoring the change in the absorbance at 340, 400 and 270 nm, respectively, in the presence of 0.1 M sodium phosphate buffer (pH 6.5) and 2 mM GSH by the method of Habig et al. (1974).

Activity with 7-chloro-4-nitrobenzo-2-oxo-1,3-diazole (NBD-Cl) was carried out in the presence of 0.1 M sodium acetate buffer (pH 5.0), 2 mM GSH and 1 mM NBD-Cl by monitoring the change in the absorbance at 419 as described by Ricci et al. (1994). Activities were calculated using absorptivities of 9.6 mM^{-1} cm^{-1}, 5.0 mM^{-1} cm^{-1}, 18.3 mM^{-1} cm^{-1} and 14.5 mM^{-1} cm^{-1} for CDNB, ECA, 4-NPA and NBD-Cl, respectively (Habig et al., 1974; Ricci et al., 1994). The optimum pH and temperature for the recombinant leGSTp were evaluated by a spectrophotometric assay using a CDNB substrate at different pH and temperatures, respectively.

3. Results

3.1. Cloning and sequence analysis of the leGSTp cDNA

The cDNA of an *L. elliptica* GST gene was amplified using the degenerated primers GSTF and GSTR (Table 1), which were based on highly conserved regions of known mollusk GST sequences. The complete sequence coding for *L. elliptica* was obtained by 3′ and 5′ RACE-PCR. The cloned sequence showed high similarity to a mammalian pi-class GST. The *L. elliptica* pi-class GST (denoted leGSTp) cDNA was 1189 bp in length, with a 5′ untranslated region (UTR) of 74 bp, a 3′ UTR of 485 bp containing a consensus polyadenylation site (AATAAA), and an open reading frame (ORF) of 630 bp encoding 209 amino acid residues (Fig. 1). The complete cDNA of the leGSTp has been deposited in GenBank database (accession number EU131183). The theoretical molecular weight of leGSTp based on the deduced amino acid sequence was calculated to be 23.9 kDa with an isoelectronic point (*p*I) of 8.3. Fig. 2 shows an alignment of these

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acagtactagtgtactttagataaagaggacacgagcagtagcggatagcaagactgacg 60
caattttaaacacaATGACAAACTACGAGCTTGAATATTTCCCTGTTAGAGGGCGAGGGG 120
                M   T   N   Y   E   L   E   Y   F   P   V   R   G   R   G
AGGCAATTCGACTTTTGTTGTCCGACAGTGGATTGACCTACAAAGAAATCAATGTTCTTT 180
E   A   I   R   L   L   L   S   D   S   G   L   T   Y   K   E   I   N   V   L
CCCCTGACCCGAGCGCCTGGGCAAATAAGTTTAAACCGCGTATGCCTTTTGGTCAGTGCC 240
S   P   D   P   S   A   W   A   N   K   F   K   P   R   M   P   F   G   Q   C
CTATGTTTCGGGATGGGGATTTTGAACTGGTTCAGTCCAACGCCATCTTGCGCTATCTCG 300
P   M   F   R   D   G   D   F   E   L   V   Q   S   N   A   I   L   R   Y   L
GTAGAAAACACGGCCTGGTAGGAAACTCGAACGAGGACTTGTCAAAGGCCGATATGGTCA 360
G   R   K   H   G   L   V   G   N   S   N   E   D   L   S   K   A   D   M   V
ACGACAGCGTGGAGGACTTGCGTTCTGAATACGTCCGATTTTATCTATCAAAATTACGATG 420
N   D   S   V   E   D   L   R   S   E   Y   V   R   F   I   Y   Q   N   Y   D
CAGGAAAGGAGGAATTCGTCAAGAAACTGCCAGAAAAACTCAAACCTTTTGAGAAATTTC 480
A   G   K   E   E   F   V   K   K   L   P   E   K   L   K   P   F   E   K   F
TGGCCAATGGAAAAAGCACGTTTGTGTTGGATAAGATCACATTTGTGGACTACAACTTAT 540
L   A   N   G   K   S   T   F   V   L   D   K   I   T   F   V   D   Y   N   L
TCGACCTTCTCGACATCCTGTCTGTCTTGTCACCCGGATGTTTGAAAGAGTTTCCGGTTC 600
F   D   L   L   D   I   L   S   V   L   S   P   G   C   L   K   E   F   P   V
TTGCGAAATATTTCCAAACCATTGCTGACCGACCAGGGATCAAGGAAAGACGCAGCTCGG 660
L   A   K   Y   F   Q   T   I   A   D   R   P   G   I   K   E   R   R   S   S
AAGCTTGCAAAAAAATGCAGATTAATGGCAACGGAAAACAATAAacagttccatgtggag 720
E   A   C   K   K   M   Q   I   N   G   N   G   K   Q   *
tatatcgacattaaacagttccatgtggagtatatcgacattaaacagttccatgtggag 780
tatatcgacattaaacagttccatgtggagtatatcgacattaaacggattcttaaattg 840
ttagctttcctcttggatatggatatttttacggaacaaatatatattttaaatccagca 900
tcatgccaagtactagtattatcttgaaaacagtaacaagtgtaaagcgagcttgtgtta 960
atgtataaaaagtatcaagtttgttatcaaaatatattcacactgagacaaagcaaatgt 1020
aaatcaaatccatcttttgtctgtttcaaacggttagactataccctgcagactatttaa 1080
cggacgtacgtatatgtcttgaactgatgtactatgcataatcaagaataaagatatcgt 1140
ataaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa 1189
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Fig. 1. Nucleotide and deduced amino acid sequences of the leGSTp cDNA from *Laternula elliptica*. The start and stop codons are underlined, and the consensus polyadenylation signal (AATAAA) is double underlined. The 3′ and 5′ UTR regions are in lower case. Positions of the probes used in semi-quantitative RT-PCR to amplify the 119-bp fragment are shown in gray.

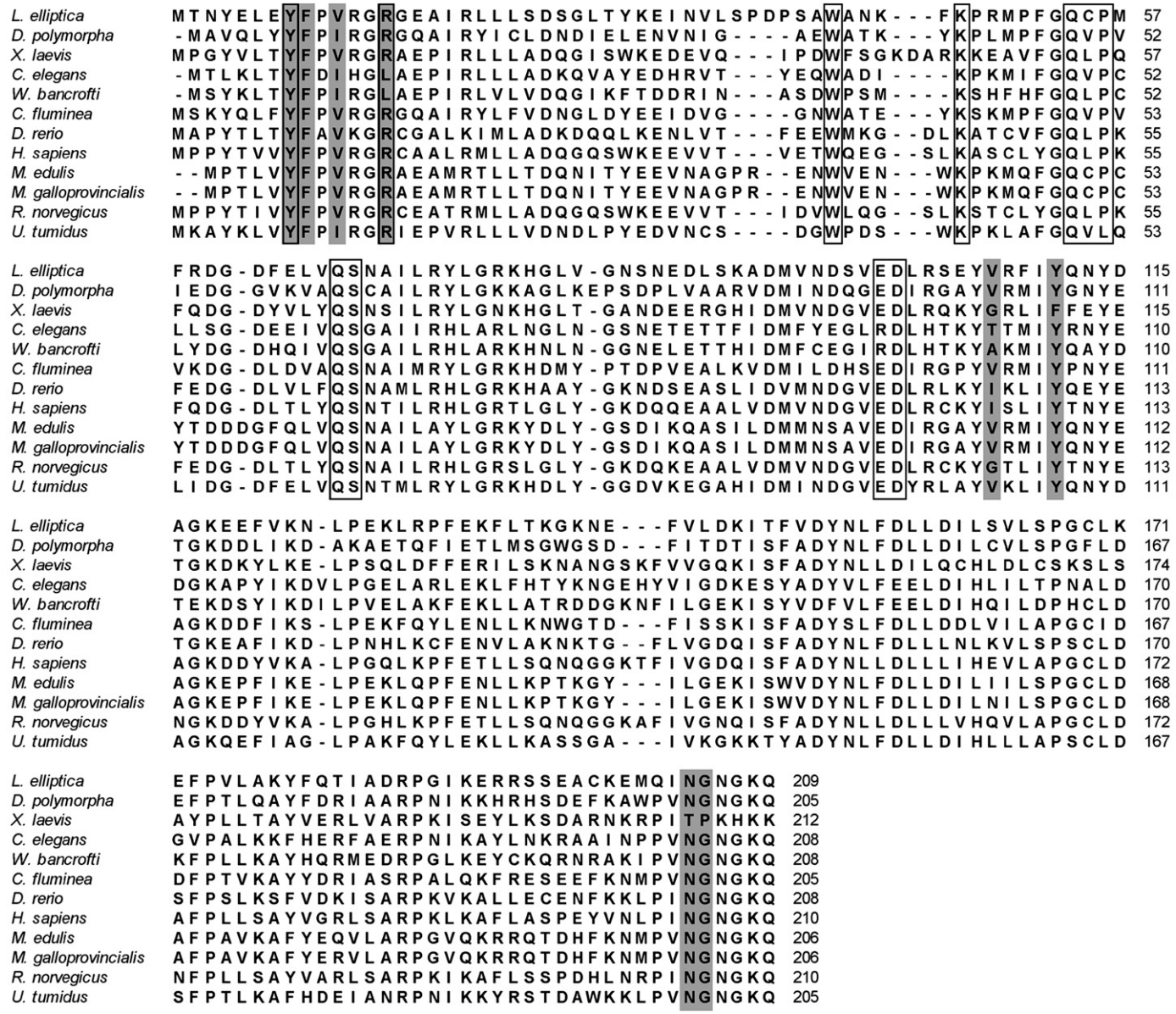

Fig. 2. Multiple alignment of the deduced amino acid sequence of leGSTp with the sequences of other pi class GSTs from different organisms. Boxes and shading indicate amino acids defining the G-site (GSH binding) and H-site (substrate binding), respectively. The GenBank accession numbers for the sequences are as follows: *Dreissena polymorpha*, ABP73387; *Xenopus laevis*, NP_001082252; *Caenorhabditis elegans*, NP_499006; *Wuchereria bancrofti*, AAO45827; *Corbicula fluminea*, AAX20374; *Danio rerio*, NP_571809; *Homo sapiens*, AAC13869; *Mytilus edulis*, AAS60226; *Mytilus galloprovincialis*, AAM91994; *Rattus norvegicus*, NP_036709 and *Unio tumidus*, AAX20373.

sequences with several invertebrates and vertebrates pi-class GST sequences using CLUSTAL X 1.83 program. The obtained leGSTp showed 46–54% identity with other pi-class GST. It was most similar to proteins found in *Mytilus galloprovincialis* and *M. edulis*. To reveal the molecular phylogenetic position of the cloned leGSTp, a phylogenetic tree was constructed by neighbor-joining method from a distance matrix, calculated with the Mega 4 software. As shown in Fig. 3, leGSTp was grouped in pi-class GST cluster.

3.2. Tissue distribution of leGSTp mRNA

To confirm the expression of leGSTp gene at the transcriptional level, RT-PCR analysis was performed using mRNA prepared from various tissues. leGSTp mRNAs was expressed in all the gill, digestive gland, gonad, mantle, and intestinal tissues (Fig. 4).

3.3. Quantification of leGSTp mRNA expression after temperature treatment

To clarify the induction of GST transcript in response to an external temperature stimulus, *L. elliptica* was exposed to a temperature of 10 °C for up to 2 days, while the control was maintained at 1 °C. The induction of GST mRNAs was analyzed by semi-quantitative Real-Time PCR. We used β-actin as an internal standard and for normalisation to verify successful transcription and to calibrate the cDNA template in corresponding samples. As shown in Fig. 5, the level of leGSTp increased up to 2.3-fold at 24 h in digestive gland and 2.7-fold at 48 h in gill during exposure to high-temperature conditions compared to the control. The expression of the leGSTp mRNA reached the highest level at 24 h in the digestive gland, where it maintained a relatively high level thereafter. The same general trend was observed in gill tissue, except that the mRNA level continuously increased throughout the experimental period.

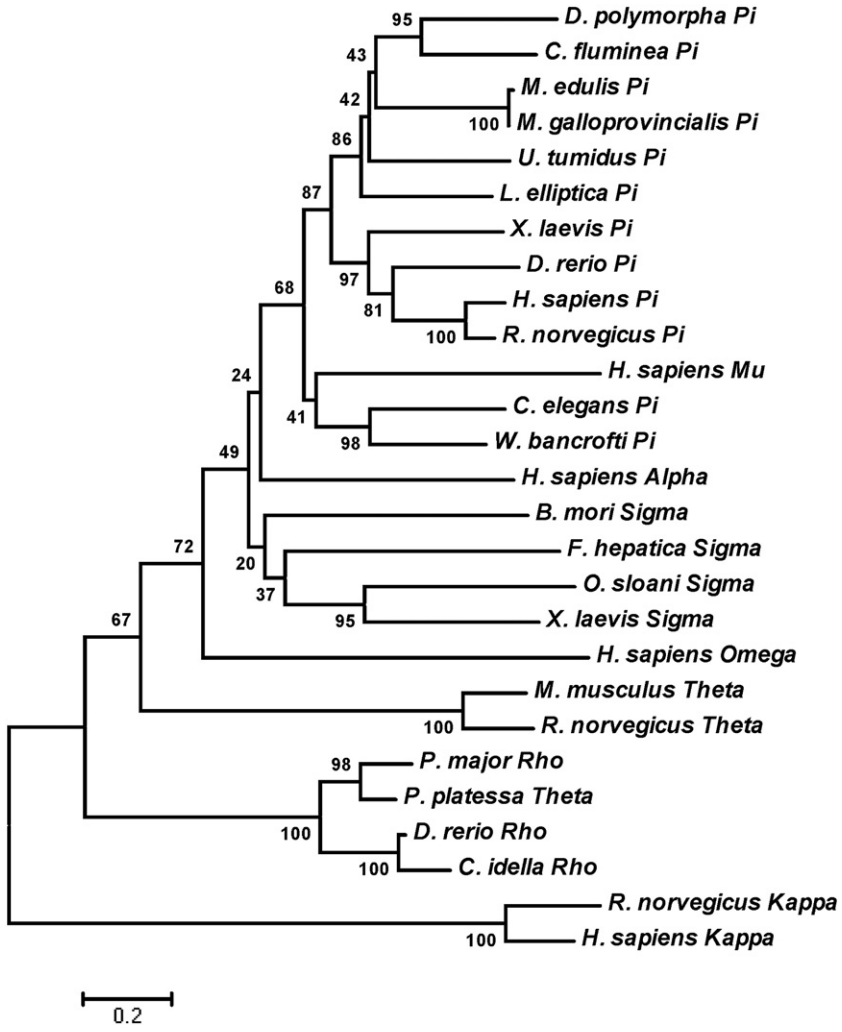

Fig. 3. Phylogenetic analysis of the leGSTp protein compared with 15 characterized GSTs of other species. Numbers at each branch indicate the percentage of times a node was supported in 1000 bootstrap pseudoreplications by the Neighbor-Joining method. The GenBank accession numbers for the sequences are as follows: *Homo sapiens* Alpha, NM145740; *Fasciola hepatica* Sigma, ABI79450; *Ommastrephes sloani* Sigma, P46088; *Xenopus laevis* Sigma, AAM82563; *Bombyx mori* Sigma, NP_001036994; *Ctenopharyngodon idella* Rho, ABV24479; *Homo sapiens* Omega, NP_004823; *Mus musculus* Theta, NP_598755; *Rattus norvegicus* Theta, NP_445745; *Danio rerio* Rho, NP_001038525; *Pagrus major* Rho, BAD98442; *Pleuronectes platessa* Theta, CAA45293; *Rattus norvegicus* Kappa, NP_852036; and *Homo sapiens* Kappa, AAS01180. The GenBank accession numbers of the pi class GSTs are as in Fig. 2.

3.4. Characterization of the recombinant leGSTp

An expression vector including the entire open reading frame of leGSTp and a 3′ additional tag of pET19b was construct and transformed into *E. coli*. Recombinant GST was purified by Ni–NTA resin column, and the purified leGSTp with the His tag yielded a single band of ~25 kDa on SDS-PAGE gel after Coomassie blue staining (Fig. 6). The optimum pH for the recombinant leGSTp is about pH 8.0, and the optimum temperature of enzymatic activity was about 35 °C with CDNB as substrate (Fig. 7). Table 2 shows the specific activities for the recombinant leGSTp with various substrates. The recombinant

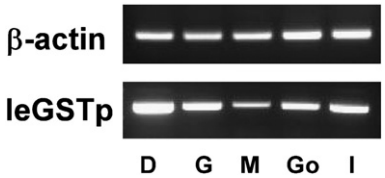

Fig. 4. Tissue-specific expression of leGSTp mRNAs in various tissues from *L. elliptica*, as assessed by RT-PCR. The β-actin RNA is used as an internal control. Go, gonad; D, digestive gland; M, mantle; G, gill; I, intestine.

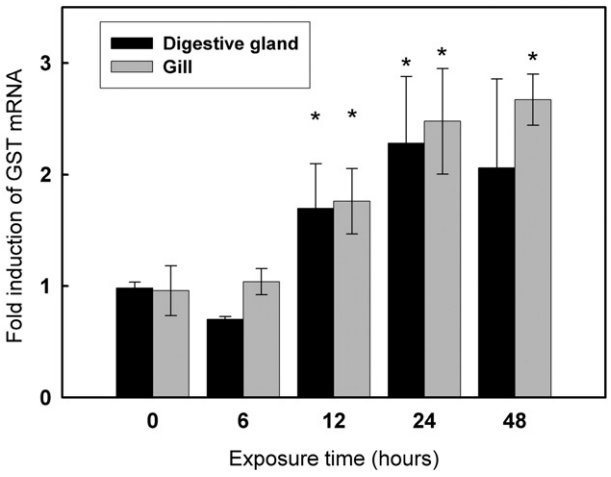

Fig. 5. The expression levels of leGSTp mRNA at different time points after heat treatment. Transcript levels for all samples were assessed by semi-quantitative RT-PCR with SYBR Green, and leGSTp expression was measured relative to β-actin expression. Values are presented as means±SD of the relative variations (fold induction) between each treatment and control sample; asterisks above the bars indicate statistically significant differences from the control sample (*$P<0.05$).

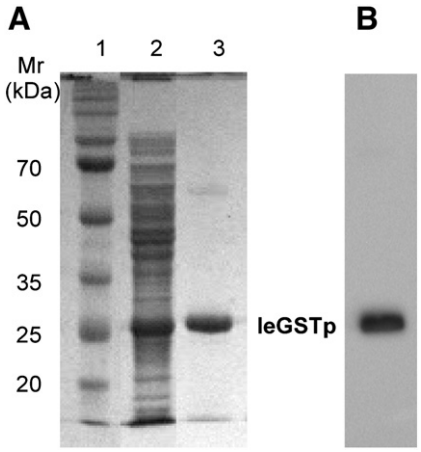

Fig. 6. SDS-PAGE (A) and Western blotting analysis (B) of the expression and purification of the recombinant meGST. Lane 1, molecular mass markers with the sizes shown on the left in kDa; lane 2, total crude extract; lane 3, the purified recombinant leGSTp.

leGSTp had the highest specific activity for NBD-Cl and lower specific activities for 4-NPA.

4. Discussion

Temperature is one of the most important factors influencing physiological disorder. This is especially true for Antarctic organisms, which are thought to have evolved unique adaptive mechanisms to cope with extremely cold environments over several million years and for which thermal stress is considered a major challenge to survival. Many studies have shown that exposure to thermal stress results in the accumulation of ROS (Rajagopal et al., 2005; Verlecar et al., 2007). The extent to which ROS induce biological damage depends on the effectiveness of defenses such as antioxidant enzymes and low molecular weight antioxidant compounds.

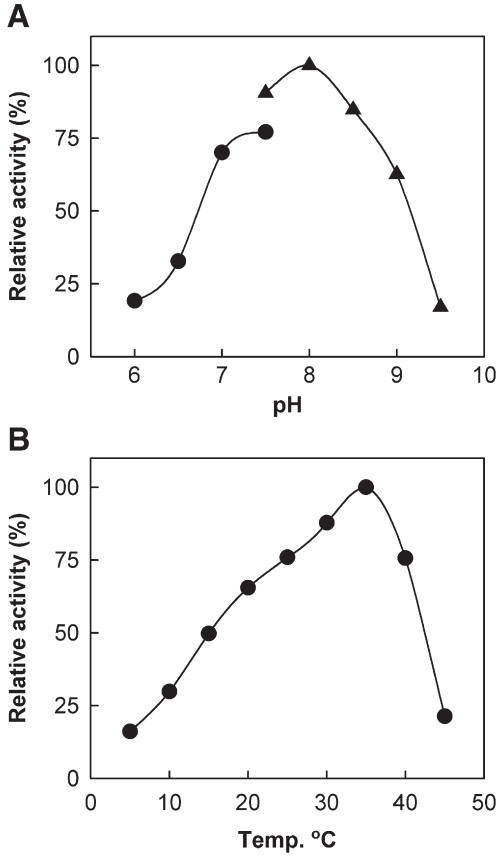

Fig. 7. Effect of pH and temperature on the purified recombinant leGSTp activity. (A) Effect of pH on leGSTp activity. The substrates were GSH and CDNB. Different symbols represent different buffers used. (●) 0.1 M potassium phosphate, pH 6.0–7.5; (▲) 0.1 M Tris–HCl, pH 7.5–9.5. (B) Effect of temperature on leGSTp activity.

Table 2
Specific activity of recombinant leGSTp

Substrate	Specific activity	[substrate][a]
	μmol · min^{-1} · mg^{-1}	mM
CDNB	3.07	2.0
NBD-Cl	1.44	1.0
ECA	8.93	0.5
4-NPA	0.76	0.2

[a] GSH concentration of 2 mM was fixed.

GST expression has been studied in many biological systems, and GST is thought to be a major antioxidant enzyme (Lee et al., 2006; Bebianno et al., 2007; Cailleaud et al., 2007). Given to the expected sensitivity of cold-adapted species to heat-induced ROS, we cloned a GST cDNA from the Antarctic bivalve *L. elliptica*, and analyzed the transcription of the leGSTp gene. To date, only few complete GST cDNAs have been identified in mollusk, and little is known about GSTs in Antarctic bivalves (Yang et al., 2004; Doyen et al., 2005; Hoarau et al., 2006; Blanchette et al., 2007). We cloned a pi-class GST cDNA encoding a 24-kDa GST subunit. GSTs have two ligand binding sites per monomer. The G-site is highly specific for glutathione binding and contains 11 highly conserved amino acids: Tyr^{7}, Arg^{13}, Trp^{38}, Lys^{46}, Gln^{53}, Leu^{54}, Pro^{55}, Gln^{66}, Ser^{67}, Glu^{98}, and Asp^{99} (alignment positions in leGSTp 8, 14, 42, 50, 57, 58, 59, 71, 72, 104, and 105, respectively) (Reinemer et al., 1992; Dirr et al., 1994). The H-site is the binding site for electrophilic substrates. It contains eight conserved residues: Tyr^{7}, Phe^{8}, Val^{10}, Arg^{13}, Val^{104}, Tyr^{108}, Asn^{204}, and Gly^{205} (alignment positions in leGSTp 8, 9, 11, 14, 111, 115, 211, and 212, respectively) (Ji et al., 1997). All of these characteristic amino acids were found to be conserved in leGSTp, with the exception of Cys^{55} in the G-site, which was substituted for Leu (Fig. 2). This substitution has also been reported in *M. edulis* and *M. galloprovincialis* (Yang et al., 2004; Hoarau et al., 2006). In comparison with mammalian pi GSTs, leGSTp has a Met instead of Cys^{47} at alignment position 50 like as many known mussel pi GSTs, in which this Cys is thought to play an important role in the correct spatial arrangement of the G-site, leading to increased G-site flexibility and decreased GSH affinity (Lo Bello et al., 1995).

Temperature is a fundamental factor influencing the physiological state, and thermal stress is known to significantly affect ROS production (Abele et al., 1998, 2001). GSTs are thought to be involved in the response against external and cellular toxins, including those induced by oxidative stress. The antioxidant enzyme activity induced by thermal stress in *L. elliptica* has been studied previously, and GST was shown to be the enzyme most sensitive to thermal stress among studied antioxidant enzymes in this species. Specifically, GST activity increased 3.4- and 4.2-fold in the digestive gland and gill tissues, respectively (Park et al., 2008). By using mRNA expression analysis of this study, we confirmed the role of leGSTp in the antioxidant response to thermal stress. The leGSTp transcripts were expressed at similar levels in the digestive gland and gill (about 10% lower in gill, data not shown). The expression level of leGSTp significantly increased after 12 h of temperature challenge in both tissues and continuously increased for up to 48 h of exposure. Given that this enzyme is involved in numerous protective detoxification reactions such as the maintenance of cellular redox status using GSH, its induction becomes highly essential under stress conditions. Temperature-induced increases in GSH levels may allow bivalves to maintain physiological status under conditions of induced oxidative stress

(Verlecar et al., 2007). Thus, an instant increase in GSH and GSH-related enzyme levels would serve as a major defense mechanism against oxidative stress in *L. elliptica*, and our data indicate that leGSTp has a role in antioxidant defense-related thermal tolerance.

This is the first report of a pi-class GST isoform in the Antarctic bivalve *L. elliptica*. GSTs have received extensive attention as part of a suite of stress indices because their expression is highly variable in the presence or absence of stimuli. Our findings suggest that leGSTp is constitutively expressed and the mRNA level increases in response to thermal stress and therefore may play an important role in thermal tolerance. These findings may contribute to an understanding of adaptation and evolutionary processes of Antarctic marine species in response to their extremely harsh environments.

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