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Glutathione S-transferase as a biomarker in the Antarctic bivalve *Laternula elliptica* after exposure to the polychlorinated biphenyl mixture Aroclor 1254

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ABSTRACT

Glutathione S-transferases (GSTs) are a family of multifunctional enzymes involved in cellular detoxification that catalyze the attachment of electrophilic substrates to glutathione. Two classes of GSTs related to the rho and sigma classes of enzymes in Antarctic bivalves have been cloned from *Laternula elliptica*. The full-length cDNA of rho class GST (leGSTr) is 1530 bp in length and contains an open reading frame (ORF) of 672 bp encoding 223 amino acid residues. The deduced amino acid sequences of this gene have 41% and 40% identity to rho class GSTs from *Ctenopharyngodon idella* and *Pleuronectes platessa*, respectively. The sigma class GST (leGSTs) cDNA, however, is 1127 bp in length and contains an ORF of 696 bp encoding 231 amino acid residues. The deduced amino acid sequences share only 22% identity with sigma class GST from *Xenopus laevis*. The transcriptional expression of leGSTr, leGSTs, and leGSTp cloned in our previous study were examined using real-time polymerase chain reaction in response to exposure to a polychlorinated biphenyl (PCB) mixture. The expressions of these three GST transcripts were rapidly upregulated, although they showed different expression levels and patterns within each isoform. Moreover, leGSTs was the most upregulated in the gill and digestive gland in response to PCB exposure. The recombinant GSTs were highly expressed in transformed *Escherichia coli*, and their kinetic properties were studied with various substrates. As a result, the three classes of GSTs were found to have diverse biological functions and were responsible for different enzymatic features.

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1. Introduction

Polychlorinated biphenyls (PCBs) are major persistent organic pollutants (POPs) and are ubiquitous toxic compounds in both aquatic and terrestrial environments. Despite the remote location, PCBs were reported in Antarctica in the 1960s and 1970s (Risebrough et al., 1968; Risebrough et al., 1976). Increasing levels of PCBs in various environmental matrices in Antarctica, as a result of long-distance transport in the atmosphere, precipitation, and in cold condensation, are a growing concern (Wania and Mackay, 1993).

Exposure to environments contaminated with POPs leads to adaptive biochemical responses, and thus the response of some biotransformation enzymes may be considered potent bioindicators of aquatic pollution (Payne et al., 1987). Among the possible biomarkers currently under investigation, glutathione-S-transferases (GSTs; EC 2.5.1.18) are most interesting as biomarkers of exposure to environmental pollutants. Changes in GST activity and transcriptional induction in response to PCB exposure have been investigated in several aquatic mollusks (Stien et al., 1998; Goldberg and Bertine, 2000; Le Pennec and Le Pennec, 2003; Boutet et al., 2004; Hoarau et al., 2006). GSTs are a well established gene family that comprise major members of phase II detoxification mechanisms (Eaton and Bammler, 1999). They play a key role in the cellular detoxification process by facilitating attachment of glutathione to a variety of potentially harmful electrophilic substrates, including hydrocarbons, organochlorine insecticides, and PCBs (Ketterer et al., 1983; Willett et al., 2000). GSTs have been found in virtually all living species examined, including animals, plants, and microorganisms (Pearson, 2005). They are mainly present in the cytosol as soluble enzymes, although some membrane-bound and mitochondrial isoforms have been identified (DeJong et al., 1988). GSTs have been grouped into at least 15 classes (alpha, beta, delta, epsilon, kappa, lambda, mu, omega, phi, pi, sigma, tau, theta, zeta, and rho) on the basis of their primary and tertiary structure, substrate/inhibitor specificity, and immunological cross-reactivity (Hayes et al., 2005; Konishi et al., 2005). They commonly possess two binding domains. The N-terminal domain contains a GSH-binding site (G-site) that is well conserved among different classes of GSTs, and the C-terminal domain contains a hydrophobic substrate binding site (H-site) that varies widely in different classes, resulting in different substrate specificities (Mannervik and Danielson, 1988; Ivarsson et al., 2003).

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Bivalve mollusks filter large amounts of water for their nutritional and respiratory needs, so they bioaccumulate environmental pollutants and have been widely used as indicator species in numerous marine environmental assessments. The Antarctic bivalve *Laternula elliptica* (King and Broderip), a key species in the Antarctic food chain, is a sentinel organism for environmental monitoring in Antarctic shallow waters by virtue of its wide distribution, high population density, large body, long life span, and high metal-accumulating capacity (Ahn et al., 1996). The presence of xenobiotic compounds such as PCBs can threaten Antarctic biota because PCBs accumulate in the tissues of these organisms (Kumar et al., 2002; Goerke et al., 2004); the potential toxicities in key species of Antarctic ecosystems, however, have not been extensively evaluated as their detoxifying systems are not yet fully understood.

In a previous study, we reported the cloning and characterization of the pi class GST from the Antarctic bivalve *L. elliptica* (Kim et al., 2008). Here, we have characterized the complete cDNA sequence of two more GSTs from *L. elliptica*, each belonging to a different class, rho and sigma. In addition, we overexpressed three GST isoenzymes of rho and sigma class GST in this study, and pi class GST from our previous study, in *Escherichia coli* and characterized the biochemical properties of these recombinant GSTs. We also investigated the response of mRNA expression of the rho, sigma, and pi class GSTs to PCB exposure and their potential use as biomarkers of PCB exposure.

2. Materials and methods

2.1. Organisms and PCB 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. For the exposure experiments, 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 2 days, *L. elliptica* were transferred to 40-L temperature-controlled aquarium filled with filtered (<0.2 μm) natural seawater (control) or PCBs-treated seawater. For the PCBs treatment, one group was treated with a final concentration of a 10 μg L^{-1} PCB mixture, Aroclor 1254, in 1 mL methanol carrier, and the other group was used as control and treated with 1 mL methanol without Aroclor 1254. Each aquarium contained 14 or 15 animals and the water was exchanged every 48 h. The samples were not fed during the experiment. Seawater was aerated and water temperature was maintained at 1.0 ± 0.1 °C throughout the experimental period. After 0, 6, 12, 24 and 48 h, three specimens were randomly selected and removed from each aquaria (i.e. $n = 3$ per treatment and control) for total RNA isolation and it used for quantitative real-time RT-PCR.

2.2. GST cDNA cloning

Total RNA was isolated using the TRIzol procedure (Invitrogen, Frederick, MD, USA) and precipitated in ethanol. Single-stranded cDNA was synthesized from 2 μg total RNA in a final volume of 20 μl containing 50 μg mL^{-1} 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 for 90 min at 42 °C and terminated by heating at 95 °C for 5 min. The partial cDNA sequences of rho and sigma class GSTs were obtained from a prepared cDNA library (Park et al., 2008). To obtain a full-length cDNA of the two GST genes, 3′- and 5′-ends were amplified using the Capfishing full-length cDNA kit (Seegene, Seoul, Korea) according to the manufacturer's instructions, with each of the gene-specific primers (5′-RACE primer: 5′-ACA CGG AGG CGA ACC CGA GC-3′, 3′-RACE primer: 5′-CAA TGC CAC CAC ACT GGG CAG A-3′ for leGSTr and 5′-RACE primer: 5′-CGA CTG GTG GCA TCA TGA CAG GGT A-3′, 3′-RACE primer: 5′-GGC GCA CAG AGA CAG GAT AGC-3 for leGSTs). The reverse transcription (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 (PerkinElmer, Foster City, CA, 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

Specific primers of two classes of GST for RT-PCR were designed from the above results. The primers for amplifying leGSTr were forward primer: 5′-GTG CCA ACC TTC ACA GAT GG-3′, reverse primer: 5′-CGG ATG GTC GTA TCA CTT GG-3′; for leGSTs, forward primer: 5′-GCG TGG CAG ACA GCA TTA TT-3′, reverse primer: 5′-CTA GAC CGT ATT TTC CCG CC-3′; for leGSTp, forward primer: 5′-CCC GGA TGT TTG AAA GAG TT-3′, reverse primer: 5′-GTT GCC ATT AAT CTG CAT TT-3′. Equal amounts of RNA (as determined spectrophotometrically) from the various tissues (gonad, digestive gland, mantle, gill, and intestine) of clams were used for RT-PCR. RT-PCR was performed using 2× ExTaq Premix 1 (Takara, Tokyo, Japan). As a control, *L. elliptica* β-actin cDNA (GenBank accession number EF198331) was amplified with the forward primer, 5′-GGT CGT ACC ACA GGT AT TGT-3′, and reverse primer, 5′-CAT CAG GTA GTC GGT CAA AT-3′, for each RT reaction product. The PCR products were visualized on a UV-transilluminator after electrophoresis on a 1.5% agarose gel containing ethidium bromide.

2.4. Quantification of GSTs mRNA expression by real-time quantitative RT-PCR

The mRNA levels of newly identified rho-GST and sigma-GST, and of previous identified pi-GST, were measured by real-time quantitative RT-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 primers used in RT-PCR, and 12.5 μL of Brilliant II SYBR Green QPCR mix (Stratagene, La Jolla, CA, USA). 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 cDNA of *L. elliptica* was used as a reference to normalize the expression levels between samples. The GST transcripts expression of treated samples were compared to it of control samples with collected same cultured times. All experiments were repeated at least three times in each specimen. The relative gene expression fold-change was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All the data represent means ± standard deviation and were subjected to one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range test, using SPSS version 12.0. Differences were considered statistically significant at $p < 0.05$.

2.5. Expression and purification of recombinant GSTs

The complete coding regions of two GST genes were amplified with each gene-specific forward and reverse primer. The primers for amplifying leGSTr were forward primer: 5′-CAT ATG GCC ACC ACC AGC AAA CC-3′ (*NdeI* site is underlined), reverse primer: 5′-CTC GAG CTA GCA GAG GTC AAG AAG A-3′(*XhoI* site is underlined); for leGSTs, forward primer: 5′-CAT ATG GCA GGA ACA GTA CAA G-3′ (*NdeI* site is underlined), reverse primer: 5′-CTC GAG TTA CAT AAA GCT GTC GCC A-3′(*XhoI* site is underlined). The PCR products were cloned into the pCR2.1-TOPO cloning vector (Invitrogen), and were subsequently cloned into the pET28b expression vector (Novagen, Madison, WI, USA), which facilitates $(His)_6$ affinity tag introduction. The identity of the insert was verified by sequencing. *E. coli* BL21(DE3)pLysE-competent cells (Invitrogen) were used as a bacterial expression host.

A

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agatctaccgatacgctagaccagtaaacatctagctataacgtaaactccagtgttaaa 60
ATGGCCACCACCAGCAAACCGTTCGTATATTGGGGCTCGGGTTCGCCTCCGTGTTGGAAG 120
 M  A  T  T  S  K  P  F  V  Y  W  G  S  G  S  P  P  C  W  K

GTGCTGCTTGTATTGCAGGAGAAGAAGATCGATTATGATGAGAAGATTATATCCTTTTCC 180
 V  L  L  V  L  Q  E  K  K  I  D  Y  D  E  K  I  I  S  F  S

AAAAAGGAACATAAATCAGAAGAGATTTTGGAGCTGAACCCGCGTGGACAGGTGCCAACC 240
 K  K  E  H  K  S  E  E  I  L  E  L  N  P  R  G  Q  V  P  T

TTCACAGATGGTGATGTGGTTGTCAACGAATCAACCGCAATCTGCATGTACCTGGAGGAG 300
 F  T  D  G  D  V  V  V  N  E  S  T  A  I  C  M  Y  L  E  E

AAGTACCCGAAGGTTCCACTGTTCCCAAGTGATACGACCATCCGGGCAAAGGTCTACCAG 360
 K  Y  P  K  V  P  L  F  P  S  D  T  T  I  R  A  K  V  Y  Q

CGCATGTTTGAGACCAGCAACATCTCCACAAATGTTATGGAATTTGTTCAATACAAGATG 420
 R  M  F  E  T  S  N  I  S  T  N  V  M  E  F  V  Q  Y  K  M

AAGAACAAGGACTCAATAGACCAGGTACTGCTGAAGGAGAAAAAAGATAAGGCCCATGTT 480
 K  N  K  D  S  I  D  Q  V  L  L  K  E  K  K  D  K  A  H  V

GAACTGGGGCACTGGGAGAATTATCTAAAACAGACCGGAGGTTTTGTTGCAACAAAGGAG 540
 E  L  G  H  W  E  N  Y  L  K  Q  T  G  G  F  V  A  T  K  E

TTCACAATGGCTGACGTATTTTTCTTCCCAATGGTCGCACTCATCGTACGCCAGGGCGCA 600
 F  T  M  A  D  V  F  F  F  P  M  V  A  L  I  V  R  Q  G  A

AATCTGAAAGACAGCTACCCCAACATATTTAAATATTACAACATGATGATGGATCGACCC 660
 N  L  K  D  S  Y  P  N  I  F  K  Y  Y  N  M  M  M  D  R  P

ACCATTGTGAAGACAATGCCACCACACTGGGCAGAATCTGACAGTCCTGGAAATCTTCTT 720
 T  I  V  K  T  M  P  P  H  W  A  E  S  D  S  P  G  N  L  L

GACCTCTGCTAGtcagcaccaagcaaatagttcggcaccatgggaagcctccatagaggc 780
 D  L  C  *

aactcaatctgtctgtatttgtgaccaaccacgcaaaaattaaaaatctctattagggat 840
agggtaaaaaaaaaatatgctataattaaaaagagtaaggtcttgactgtctgttttaaaa 900
tgtattccaaatgacgttttcagctgtgtgtttgccggaaagtttgggtttcattacccc 960
acccaccctactgattacatgtaacatgaaactccagaacaaacaaacttttctaagtga 1020
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tctcgtgtgtacaaatattattatttcaagcaatttttgtttgtgcacttgaaaaaaaatcat 1320
acagtattgagttctagcgcatttggtgccagtattccagacaggccggcaagtattgaaa 1440
taaaggccgcctttatcattccataaacttattgaaaacaaaacgtgtaacctcttacta 1440
gtgttactagaagtgccatacatgttgggatcaaactgtataaaaggttgaccaaataaa 1500
ctcgtccaaaaaaaaaaaaaaaaaaaaaaa 1530
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B

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L. elliptica  MATTSKPFVYWGSGSPPCWKVLLVLQEKKID-YDEKIISFSKKEHKSEEILKLNPRGQVP 59
D. rerio      MAQN--MLLYWGTGSPPCWRLMIALEEKQLQGYKHKLLSFDKKEHQSPEVKALNPRAQLP 58
H. molitrix   MAQN--MMLYWGSGSPPCWRVMIALEEKLLQGYKHKLLSFDKKEHQGAEVKALNPRVQLP 58
C. idella     MAQN--MMLYWGSGSPPCWRVMIALEEKKLQGYKHKLLSFDKKEHQSAEVKALNPRAQLP 58
P. platessa   MAKD--MTLLWGSGSPPCWRVMIVLEEKNLQAYNSKLLSFEKGEHKSAEVMSMNPRGQLP 58

L. elliptica  TFTDGDVVVNESTAICMYLEEKYP--KVPLFPSDTTIRAKVYQRMFETSNISTNVMEFVQ 117
D. rerio      TFKHGEIVVNESFAACLYLESVFKSQGTRLIPDNPAEMALVYQRMFETENLQQKMYEVAF 118
H. molitrix   TFKHGDLIVNESFAACLYLESAFKSQGTRLIPDDPAEQALVYQRMFETNNLQQKMYDVAF 118
C. idella     TFKHGDLIVNESFAACLYLESALKSQGTRLIPDDPAEQALVYQRMFETNNLQQKMYDVAF 118
P. platessa   SFKHGSKVLNESYAACMYLESQFKSQGNKLIPDCPAEQAMMYQRMFEGLTLAQKMADVIY 118

L. elliptica  YKMKNKDS-IDQVLLKEKKDKAHVELGHWENYLKQTG--GFVATKEFTMADVFFFPMVAL 174
D. rerio      YDWLVPEGERLESALKRNKEKLIEELKLWEGYLEKMGKGSYLAGKNFSMADVVCFPVIAY 178
H. molitrix   YEWYIPEGERHESALKRNKENLVTELKLWDGYLEKMGKGSYLAGKNFTMADVVCFPVVAY 178
C. idella     YKWHVPEGEQQESALKRNKENLVTELKLWDGYLEKMGKGSYLAGKNFTMADVVCFPIIAF 178
P. platessa   YSWKVPEAERHDSAVKRNKENLSTELKLWEEYLQKTS-GSFVAGKSFSLADVSVFPGVAY 177

L. elliptica  IVRQGANLKDSYPNIFKYYNMMMDRPTIVKTMPPHWAESDSPGNLLDLC 223
D. rerio      FPRLQCP-KERCPRLMEYYEMVKDRPSIKASWPPEWLEKPVGEDILKSL 226
H. molitrix   FPRLHCP-KEKCPRLMEYYEMVKDRPSIKASWPLEWLEDPVGQDTLKNL 226
C. idella     FPRLHCP-KEQCSRLMEYYEMVKDRPSIKASWPPHWLENPVGPDTLKNL 226
P. platessa   LFRFGLT-EERYPQLTAYYNSLKERPSIKASWPPTWLESPQGQDMLKDV 225
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C

Percent similarity

		1	2	3	4	5
L. elliptica	1		57	56	58	58
D. rerio	2	40		93	93	77
H. molitrix	3	40	85		96	78
C. idella	4	41	85	92		78
P. platessa	5	40	63	64	62	

Percent identity

The transformants were cultivated in 250 mL LB (Luria–Bertani) medium at 37 °C in the presence of 50 μg mL^{-1} ampicillin until the *A* at 600 nm reached about 0.6. At this point, the temperature was changed to 20 °C, and expression of the His-tagged proteins was induced by adding 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 ice-cold phosphate-buffered saline (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 (Amersham Biosciences, Piscataway, NJ, USA). 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 Bradford method using bovine serum albumin as a standard (Bradford, 1976).

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 polyvinylidene fluoride (PVDF) membrane (Immobilon PVDF membrane; Millipore, Billerica, MA, USA) by semidry electrophoretic transfer as described by Towbin et al. (Towbin et al., 1979). The blotted PVDF membrane was incubated in 4% skimmed 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 GST catalytic activities were analyzed with four alternative substrates. All activity measurements were performed at 25 °C with a double beam UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Recombinant GST catalytic activity was measured using 1-chlor-2,4-dinitrobenzene (CDNB), 4-nitrophenyl acetate (4-NPA), and 2-(2,3-dichloro-4-[2-methylene-butyryl]phenoxy)acetic acid (ethacrynic acid, ECA) by monitoring the change in absorbance at 340, 400, and 270 nm, respectively, in the presence of 0.1 M sodium phosphate buffer (pH 6.5) and 2 mM glutathione (GSH) by the method of Habig et al. (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 absorbance at 419 nm 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 K_m and V_{max} values for GSH (0.1–1.0 mM) were determined with 1 mM CDNB, whereas those for CDNB (0.1–1.0 mM) were determined with 1 mM GSH. Lineweaver–Burk plots were used to determine the parameters. The optimum pH and optimum temperature for the recombinant GST were evaluated by a spectrophotometric assay using a CDNB substrate at different pH and temperature, respectively.

3. Results and discussion

3.1. Cloning and sequence analysis of L. elliptica full-length rho and sigma class GST cDNA

Based on the EST sequences, two sets of specific primers were designed to clone the full-length cDNA sequences of two GST genes. The rho class GST (denoted leGSTr) cDNA was 1530 bp in length, with a 5′-untranslated region (UTR) of 60 bp, a 3′-UTR of 798 bp, and an open reading frame (ORF) of 672 bp encoding 223 amino acid residues with an estimated molecular mass of 25.8 kDa and an estimated isoelectric point of 6.1 (Fig. 1A). The sigma class GST (denoted leGSTs) cDNA was 1127 bp in length, with a 5′-UTR of 81 bp, a 3′-UTR of 350 bp, and an ORF of 696 bp, encoding 231 amino acid residues with an estimated molecular mass of 25.9 kDa and an estimated isoelectric point of 6.3 (Fig. 2A). The leGSTr has two polyadenylation sites (AATAA) as indicated by the presence of multiple transcripts encoding one protein postulated differential use (Caizzi et al., 1990), and these multiple polyadenylation sites have been reported in some other genes, including *Crassostrea gigas* pi-GST (Leff et al., 1986; Boutet et al., 2004). The leGSTr and leGSTs sequences showed three and five ATTTA motifs in their 3′-UTR, respectively. These ATTTA consensus sequence elements mediate RNA instability (Sachs, 1993) and suggest functional properties in maintaining higher levels of GST transcripts.

Sequence comparison between GST isoforms in this species showed that leGSTr and leGSTs had 17.7% identity at the amino acid level, and only 16.1% and 26.3% amino acid identities with leGSTp, respectively. A multiple alignment of the leGSTr protein with other rho class GSTs and a multiple alignment of the leGSTs protein with other sigma class GSTs are shown in Fig. 1B and C, respectively. leGSTr showed high identities with *Ctenopharyngodon idella* (41%) and *Pleuronectes platessa* (40%) (Fig. 1C). leGSTs showed high identity with sigma class GST of *Xenopus laevis* (22%) (Fig. 2C).

The leGSTr encoded by the cDNA has a GST G-site domain at residues 9–78 and a GST H-site domain at residues 98–197, and the leGSTs has a GST G-site domain at residues 10–81 and a GST H-site domain at residues 110–207 in the Conserved Domain Database in NCBI (Marchler-Bauer et al., 2005). These two distinct domains suggest that the GST N-terminal domain is responsible for differential GSH-binding ability and that the C-terminal domain modulates specificities for alternate electrophilic substrates (Mannervik and Danielson, 1988). While seven amino acid residues (Tyr^{10}, Pro^{17}, Gln^{57}, Val^{58}, Pro^{59}, Glu^{70}, and Ser^{71}) were suggested to be signature residues of the G-site domain and seven signature residues were found in the H-site (Ser^{106}, Thr^{110}, Asn^{111}, Glu^{114}, Phe^{115}, Met^{171}, and Leu^{174}) in leGSTr (Dirr et al., 1994; Ji et al., 1997), the G-site, where five amino acid residues (Tyr^{10}, Gln^{57}, Pro^{59}, Glu^{70}, and Ser^{71}) in all sequences were aligned identically, appeared to be more conserved than the H-site (Fig. 1B). The leGSTs also showed highly conserved signature residues of the G-site with Tyr^{14}, Arg^{21}, Met^{59}, Pro^{60}, Pro^{61}, Gln^{72}, and Thr^{73}, whereas the H-site had variable feature residues (Fig. 2B). To reveal the molecular phylogenetic position of the two cloned GSTs, a phylogenetic tree was constructed by the neighbor-joining method from a distance matrix. As shown in Fig. 3, the two cloned GSTs of *L. elliptica* are grouped in rho and sigma class GST clusters. The evolutionary relationships of cytosolic GST isozymes has been still controversial (Blanchette et al., 2007). The first accounts of the evolution of the GST subfamily suggested that theta class GSTs

Fig. 1. cDNA and deduced protein sequences of the rho class glutathione S-transferase of *Laternula elliptica* (accession number in GenBank: FJ615307). (A) The nucleotide and deduced protein sequences of leGSTr cDNA. The start and stop codons are underlined and an asterisk marks the termination site. The consensus polyadenylation signal (AATAAA) is double-underlined and ATTTA motifs are enclosed in boxes. (B) Alignment of the amino acid sequence of leGSTr with known rho class GST. Identical amino acids in all sequences are highlighted in dark gray and conserved residues are highlighted in light gray. The feature amino acid residues for the G-site (▲) and H-site (●) are indicated. GenBank accession numbers for rho class GST sequences are as follows: *Danio rerio*, NP_001038525; *Hypophthalmichthys molitrix*, ABV24478; *Ctenopharyngodon idella*, ABV24479, and *Pleuronectes platessa*, CAA45293. (C) Pairwise identities and similarities of the deduced amino acid sequence in leGSTr with other rho class GSTs.

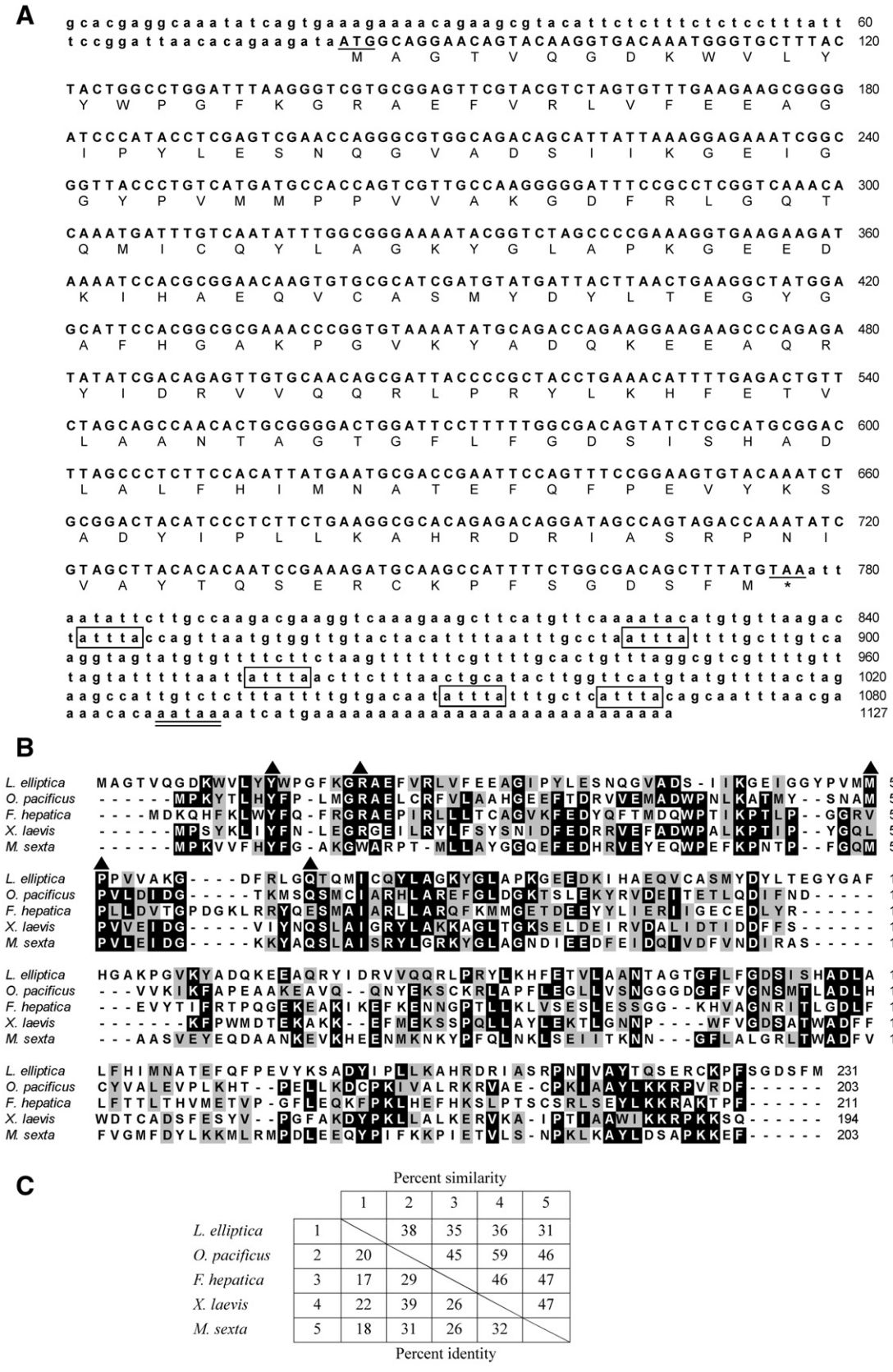

Fig. 2. cDNA and deduced protein sequences of the sigma class glutathione S-transferase of *Laternula elliptica* (accession number in GenBank: FJ615308). (A) The nucleotide and deduced protein sequences of leGSTs cDNA. The start and stop codons are underlined and an asterisk marks the termination site. The consensus polyadenylation signal (AATAAA) is double-underlined, and ATTTA motifs are enclosed in boxes. (B) Alignment of the amino acid sequence of leGSTr with known rho class GST. Identical amino acids in all sequences are highlighted in dark gray and conserved residues are highlighted in light gray. The feature amino acid residues for the G-site (▲) are indicated. GenBank accession numbers for the sigma class GST sequences are as follows: *Ommastrephes sloani pacificus*, P46088; *Fasciola hepatica*, ABI79450; *Xenopus laevis*, AAM82563 and *Manduca sexta*, AAA92881. (C) Pairwise identities and similarities of the deduced amino acid sequence in leGSTs with other sigma class GSTs.

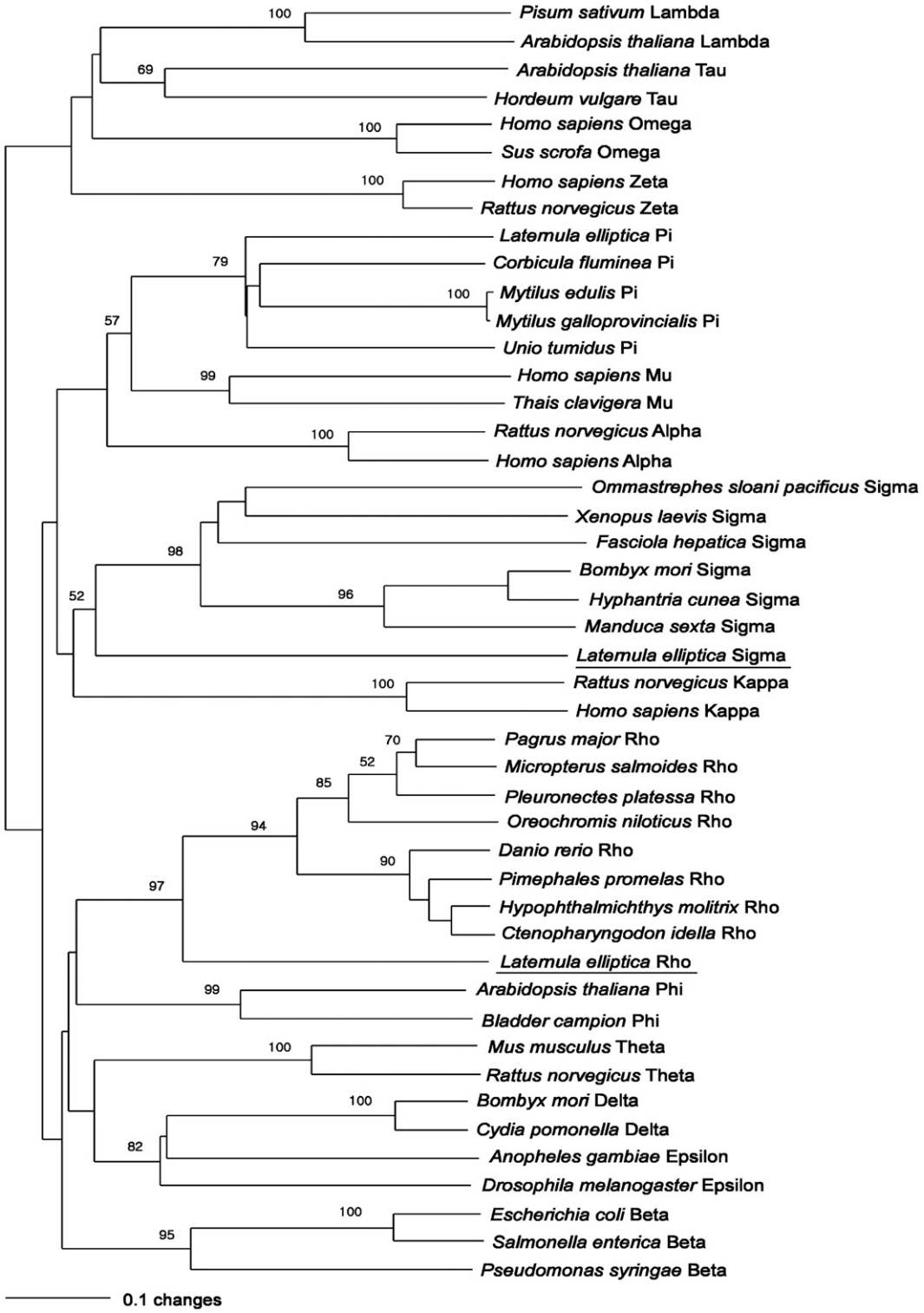

Fig. 3. Phylogenetic analysis of the two glutathione S-transferase proteins compared to other species. Numbers at each branch indicate the percentage of the times a node was supported in 1000 bootstrap pseudoreplications by the neighbor-joining method. GenBank accession numbers for the sequences are as follows: *Corbicula fluminea* Pi, AAX20374; *Mytilus edulis* Pi, AAS60226; *M. galloprovincialis* Pi, AAM91994; *Unio tumidus* Pi, AAX20373; *Homo sapiens* Alpha, NM145740; *Rattus norvegicus* Alpha, AAF37739; *Escherichia coli* Beta, ACB02841; *Salmonella enterica* Beta, YP_001588096; *Pseudomonas syringae* Beta, NP_790768; *Bombyx mori* Delta, NP_001036974; *Cydia pomonella* Delta, ACJ23087; *Drosophila melanogaster* Epsilon, NP_611323; *Anopheles gambiae* Epsilon, XP_319969; *Arabidopsis thaliana* Phi, NP_192161; *Bladder campion* Phi, AAA33931; *A. thaliana* Tau, NP_180506; *Hordeum vulgare* Tau, BAE44477; *H. sapiens* Zeta, NP_001504; *Rattus norvegicus* Zeta, NP_001102915; *Arabidopsis thaliana* Lambda, AC007651; *R. norvegicus* prostaglandin D2 synthase, NP_113832; *Mus musculus* prostaglandin D2 synthase, NP_062328; *Hyphantria cunea* Sigma, BAE16754; *Bombyx mori* Sigma, NP_001036994; *Pimephales promelas* Rho, AAF78081; *Oreochromis niloticus* Rho, ABV24480; *Micropterus salmoides* Rho, AAQ91198; *H. sapiens* Omega, NP_004823; *Sus scrofa* Omega, NP_999215; *Mus musculus* Theta, NP_598755; *R. norvegicus* Theta, NP_445745; *Pagrus major* Rho, BAD98442; *H. sapiens* Mu, NM_000850; *Thais clavigera* Mu, ACD13785; *R. norvegicus* Kappa, NP_852036 and *H. sapiens* Kappa, AAS01180.

were the ancient progenitor, considering their ubiquitous presence from aerobic bacteria to higher eukaryotes according to sequence comparisons (Buetler and Eaton, 1992). The sigma class GSTs were suggested to have diverged from the mammalian alpha/mu/pi group and to have evolved distinct functional properties, such as prostaglandin synthesis (Sheehan et al., 2001), but Blanchette et al. (2007) proposed pi/sigma intermediate split off early into pi and sigma, after then rho/theta split off into rho and theta from alpha/mu/rho/theta intermediate. Comparing of the sequence data, a sigma class GST was only 17% to 22% identities to selected other sigma class GSTs (Fig. 2C), which might be indicated the unique nature of this GST. A rho class GST was originally identified by Leaver and coworkers (Leaver et al., 1993) in plaice, and fish-type rho class GST is suggested to have occurred at least 500 million years ago (Fan et al., 2007). The rho class GST is mostly known from fish, and this is the first report of a rho class GST identified in an invertebrate.

3.2. Characterization of the recombinant leGSTp

An expression vector including the entire ORF of leGSTr and leGSTs with a 5′ additional His tag was constructed and transformed into *E. coli*. Recombinant GSTs were purified on a Ni-NTA resin column, and purified leGSTr and leGSTs with the His tag yielded a single band of about 25 kDa for each enzyme on SDS-PAGE gels after Coomassie blue staining (Fig. 4A). The effect of temperature and pH for leGSTr and leGSTs are shown in Fig. 4B and C, respectively. The optimum pH for the recombinant leGSTr is about pH 9.5, and the leGSTr showed less than 20% of maximum activity below pH 6.0. The optimum temperature for enzymatic activity was about 25 °C with CDNB as substrate. In contrast, the optimum pH for the recombinant leGSTs was about pH 10.0, and the optimum temperature for enzymatic activity was about 30 °C with CDNB as substrate.

The catalytic specificity of the three GST isoforms was explored using a range of model GST substrates. Table 1 shows the substrate specificity of the recombinant leGSTr, leGSTs, and leGSTp with various substrates. These GSTs show a broad substrate specificity spectrum for the examined substrates CDNB, NDB-Cl, ECA, and 4-NPA. The specific activity of leGSTr and leGSTp for ECA is about three times higher than that for CDNB because ECA is the prototype substrate for pi class GSTs (Yang et al., 2004). The specific activity of leGSTs showed highest activity for NBD-Cl and a similar substrate spectrum to the mammalian alpha class GSTs (Ricci et al., 1994), suggesting that sigma class diverged from alpha class GSTs (Sheehan et al., 2001). Although all GSTs participate in oxidative stress defense mechanisms through the GSH conjugation reaction with by-products of xenobiotics such as organic hydroperoxides, activated alkenes, epoxides, and quinones, these variable substrate specificities of each GST may be related to their biological function.

3.3. Tissue distribution and quantification of GST expression after PCB exposure

The expressions of the three GST isoforms from five tissues of *L. elliptica* were investigated at the transcriptional level with RT-PCR. β-Actin was used as an internal control gene. Three GSTs transcripts were detected in all examined tissues, gill, digestive gland, gonad, mantle, and intestine (Fig. 5); however, significant variation occurred in the expression levels of the different tissues. Although the major function of GST is detoxification, these three class GSTs of *L. elliptica* showed ubiquitous transcripts in all examined tissues of untreated samples, suggesting they might play a role in maintenance of normal cellular functions.

PCBs are highly durable and are among the most common xenobiotic compounds accumulated within the Antarctic biota through long-range transport and bioaccumulation (Goerke et al., 2004). Exposure of biota to environments contaminated with pollutants such as PCBs leads to

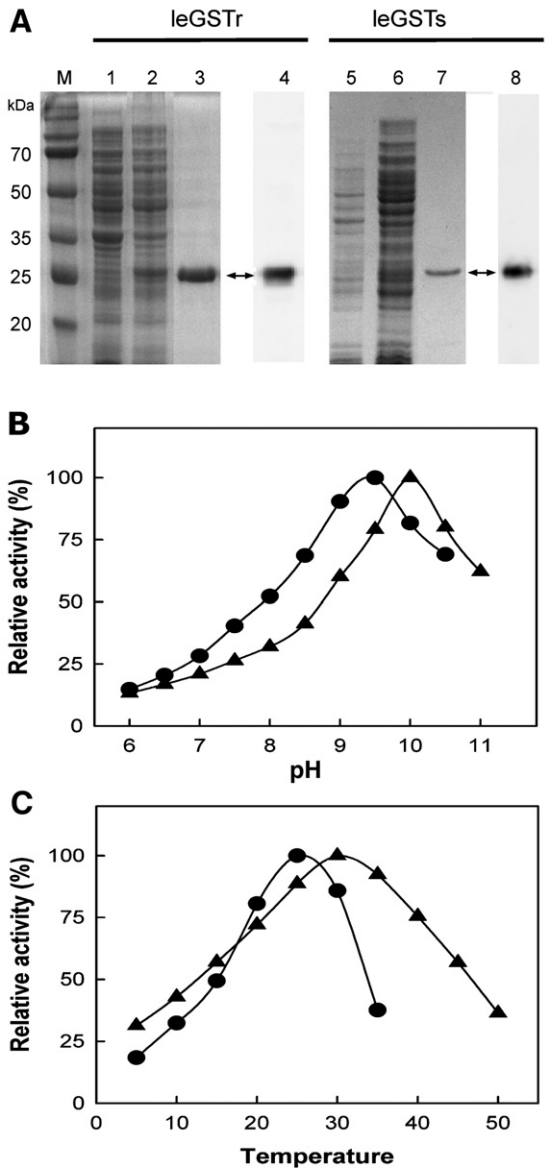

Fig. 4. (A) SDS-PAGE and Western blot analysis of expression and purification of the recombinant leGSTr and leGSTs. M, molecular mass markers with the sizes shown on the left in kDa; lanes 1–4 for leGSTr (lane 1, control before induction; lane 2, total soluble extract; lane 3, purified recombinant leGSTr; lane 4, Western blotting with anti-His antibody); lanes 5–8 for leGSTs ((lane 5, control before induction; lane 6, total soluble extract; lane 7, purified recombinant leGSTr; lane 8, Western blotting with anti-His antibody). (B) Effect of pH on purified recombinant leGSTr (●) and leGSTs (▲) activity. Activity was measured at 25 °C at various pH with 2 mM GSH and 2 mM CDNB. The buffer used was 0.1 M sodium phosphate buffer pH 6–7.5, 0.1 M Tris–HCl buffer at pH 7.5–9.5, and 0.1 M borax–NaOH buffer at pH 9.5–11. (C) Effect of temperature on purified recombinant leGSTr (●) and leGSTs (▲) activity. Activity was measured at 25 °C and various other temperatures with 2 mM GSH and 2 mM CDNB at pH 7.0.

biochemical responses in a combination of phase I and phase II detoxification systems that suggest their use as bioindicators of pollutants. One of most important phase II detoxification enzymes is GST, and the overall GST activity in mussels from contaminated areas was significantly higher than from uncontaminated areas (Hoarau et al., 2006). Also, several studies have described GST activity and induction of GST transcripts in response to PCB exposure (Machala et al., 1998; Perez Lopez et al., 2002; Doyen et al., 2005), but few studies related to specific classes of GSTs have been reported. In our study, mRNA levels of three GSTs (rho, sigma, and pi class) from gills and digestive glands were investigated after exposure to a 10 ug L^{-1} PCB commercial mixture (Aroclor 1254) using semiquantitative RT-PCR. We used β-actin as an

Table 1
Kinetic parameters of recombinant GSTs from *Laternula elliptica*.

	Substrate	Specific activity uM·min^{-1}·mg^{-1}	K_m^{GSH} (mM)	V_{max}^{GSH} (mM·min^{-1}·mg^{-1})	K_m^{S} (mM)	V_{max}^{S} (mM·min^{-1}·mg^{-1})
leGSTr	CDNB	12.06	2.348	0.031	2.862	0.178
	NBD-Cl	14.40	0.458	0.017	4.041	0.245
	ECA	34.08	1.260	0.027	0.287	0.027
	4-NPA	4.49	N.D.	N.D.	41.197	2.913
leGSTs	CDNB	3.12	1.590	0.040	2.463	0.034
	NBD-Cl	15.28	8.353	0.211	3.190	0.143
	ECA	7.51	4.601	0.019	5.872	0.041
	4-NPA	2.60	N.D.	N.D.	50.746	2.438
leGSTp	CDNB	3.07	5.660	0.039	9.472	0.089
	NBD-Cl	1.44	3.983	0.013	8.580	0.048
	ECA	8.93	6.446	0.136	2.764	0.064
	4-NPA	0.76	0.094	0.007	107.055	1.255

internal standard, as well as for normalization to verify successful transcription and calibrate the cDNA templates in corresponding samples. As shown in Fig. 6, the high levels of GST transcripts observed in both tissues of this bivalve also showed strong differential expression between different GST isoforms. Analysis of GST expression in the digestive gland gave the highest level of leGSTr and leGSTp mRNA of 6.4-fold and 4.5-fold, respectively, compared to the control after 24 h exposure, while leGSTs mRNA continuously increased up to 48 h (Fig. 6A). In gills, expression of the three GST isoforms gave a slightly different pattern in the digestive gland; all three GSTs mRNA levels were dramatically upregulated after 6 h exposure. The highest expression levels of leGSTr and leGSTp increased 4.0-fold and 7.4-fold, respectively, after 6 h exposure, and gradually decreased thereafter. leGSTs mRNA, however, was strongly induced after PCB exposure for 6 h (up to 6.2-fold compared to the control) and continuously increased for up to 48 h of exposure (Fig. 6B). The differences in expression patterns between leGSTs and lsGETp/leGSTr indicate that these isoenzymes may play different physiological roles during the detoxification process in *L. elliptica*. The GSTs mRNA responses to PCB exposure demonstrated organ specificity, and their different expression patterns in gills and the digestive gland were highly significant. A short-term exposure to PCBs could cause acute oxidative stress and cytotoxicity to the gill, which is in direct contact with the surrounding environment, whereas in the digestive gland, where various enzymes can metabolize PCBs, excess concentrations can accumulate because the gland acts as a storage organ reflecting long-term PCB exposure (Bebianno et al., 2007).

4. Conclusions

Glutathione S-transferase activities and transcriptional levels have been measured in many aquatic species exposed to organic or inorganic contaminants, but few complete GST cDNAs have been identified in mollusks and little is known about GSTs in Antarctic bivalves. We cloned two novel GST genes, rho and sigma class, from the Antarctic bivalve *L. elliptica*, which were expressed in *E. coli*, and the recombinant GSTs were characterized. The three classes of GSTs showed different substrate specificity and biochemical properties that may allow the bivalve to cope with different detoxification reactions through different biological functions. We also investigated the response of mRNA expression of the pi, rho, and sigma class GSTs to PCB exposure to examine their potential use as biomarkers. All three GST transcripts of *L. elliptica* were constitutively upregulated after PCB exposure, but the expression levels and patterns varied in each GST isoform. These findings suggest that in *L. elliptica*, GSTs play an important role in detoxification mechanisms but have different physiological roles related to their biological function during the detoxification process.

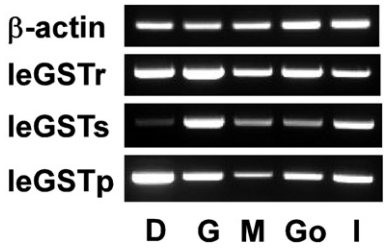

Fig. 5. Tissue-specific expression of leGSTr, leGSTs, and leGSTp mRNAs in various tissues of *Laternula elliptica* as assessed by RT-PCR. β-actin RNA was used as an internal control. Go, gonad; D, digestive gland; M, mantle; G, gill; I, intestine.

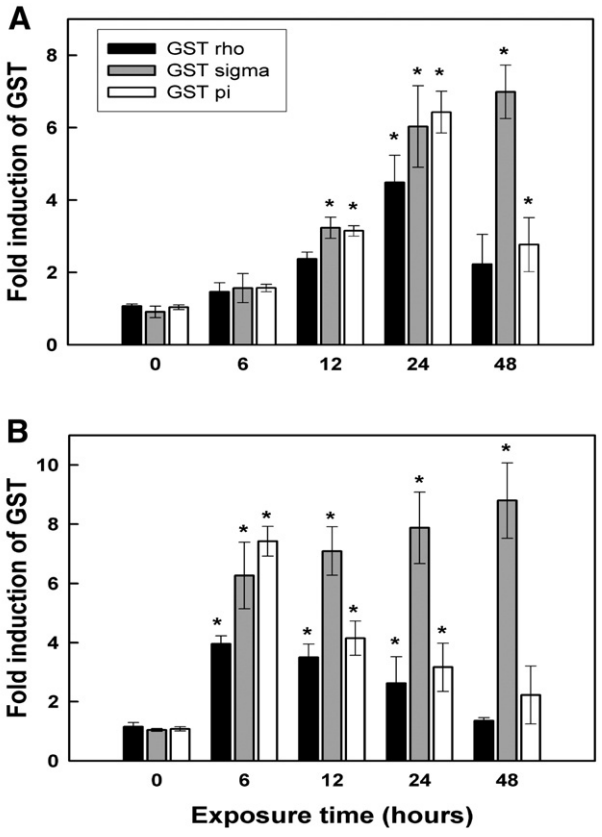

Fig. 6. Expression levels of three glutathione S-transferase mRNAs at different time points after exposure to the polychlorinated biphenyl mixture Aroclor 1254 (10 ug L^{-1}) in digestive gland (A) and gills (B). Transcript levels for all samples were assessed by semiquantitative RT-PCR with SYBR Green, and GST expression was measured relative to β-actin expression. Values are presented as means ± SD of the relative variations (fold induction) between each treatment and a control sample; asterisks above the bars indicate statistically significant differences compared to the control (*P<0.05).

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