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Developmental and Comparative Immunology 33 (2009) 980-990

Contents lists available at ScienceDirect



Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci



Gene cloning of a sigma class glutathione S-transferase from abalone (*Haliotis diversicolor*) and expression analysis upon bacterial challenge

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ARTICLE INFO

Article history: Received 1 December 2008 Received in revised form 9 April 2009 Accepted 25 April 2009 Available online 14 May 2009

Keywords: Haliotis diversicolor Glutathione S-transferase Sigma GST Differential gene expression Bacterial challenge PCR

ABSTRACT

Glutathione S-transferases (GSTs) are a multigene family of xenobiotic metabolizing phase II detoxification enzymes which take part in many pathological and physiological processes, and which can potentially be used as indicators and biomarkers for cancer diagnoses and organic or inorganic pollutant exposure. In this study, a full-length cDNA of a sigma class GST (abGSTsigma) (GenBank accession number EF546619) from variously colored abalone (Haliotis diversicolor) was identified. It was 1328 bp containing an open reading frame of 624 bp, encoding 208 amino acid residues with a predicted protein molecular weight of 23.67 kDa and an estimated pl of 5.67. Sequence analysis showed that the predicted protein sequence of abGSTsigma cDNA contained the conserved domain of the GST_N_Sigma_like (PSSM: cd03039) and GST_C_Sigma_like (PSSM: cd03192). Alignment analysis demonstrated that the abGSTsigma of H. diversicolor was in a branch position with other known class sigma GSTs from different organisms. The abGSTsigma mRNA was distributed in multiple tissues tested and was highly demonstrated in the gill and mantle of normal abalones. In bacteria-challenged abalone, the abGSTsigma gene was significantly expressed in the hemocytes, gill, mantle and digestive gland and the total GSTs enzyme and SOD were also induced in the four tissues. The increased activities of SOD and GSTs can result in the elimination of reactive oxygen species (ROS) indicating antioxidant activities involved. The preliminary work revealed that the sigma class glutathione S-transferase gene abGSTsigma, a phase II detoxification enzyme, had a positive response to bacterial challenge, and that will lead to an insightful study on elucidating the interactions between immune responses and biotransformation exerted by abGSTsigma.

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1. Introduction

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a wellcharacterized family of multifunctional isoenzymes that are ubiquitously distributed in bacteria, plants, and animals [1]. These isoenzymes represent about 1% of the total cellular proteins in

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eukaryotes and some prokaryotes, and belong to a group of phase II detoxification enzymes playing a crucial role in xenobiotic metabolism [2]. The primary function of GSTs is cellular defense against the toxicity induced by xenobiotic lipophilic compounds. GSTs also play a prominent role in many other physiological processes, including resisting oxidative stress [3], transporting endogenous hydrophobic compounds [2], catalyzing biosynthetic reactions [4] and acting as signaling molecules [5].

Previous studies found that GST expression levels are increased upon exposure to hydrogen peroxide and heavy metal [6], microcystin-LR [7], 4-nonylphenol [8], and endocrine-disrupting chemicals such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls and tributyltin [9]. Although no criteria were clearly established to classify GSTs from marine organisms, the activities and transcript levels of GSTs have been widely investigated in some aquatic species as biomarkers upon exposure to organic or inorganic pollutants. Genes or proteins of GSTs are identified in some marine mollusks, such as the squid (*Ommastrephes sloani pacificus*) [10], blue mussel (*Mytilus edulis*) [11,12],

Abbreviations: 3-MC, 3-methylcholanthrene; *abGSTsigma*, *H. diversicolor* sigma class GST; CD, conserved domain; CDD, Conserved Domain Database; CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferases; G-sites, GSH-binding sites; GlSTrs, partial cDNA of GST; HLS, hemocyte lysate suspension; H-sites, hydrophobic substrate binding sites; LPS, lipopolysaccharide; MW, molecular weight; NJ, neighbor-joining; ORF, open reading frame; qPCR, quantitative real-time PCR; PGDS, prostaglandin D₂ synthase; RACE, rapid amplification of cDNA ends; ROS, reactive oxygen species; RT-PCR, reverse transcription PCR; SDD, superoxide dismutase; SSH, suppression subtractive hybridization; sqRT-PCR, semi-quantitative reverse transcription PCR.

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⁰¹⁴⁵⁻³⁰⁵X/\$ – see front matter \circledcirc 2009 Elsevier Ltd. All rights reserved. doi:10.1016/j.dci.2009.04.003

quahog (*Mercinaria mercinaria*) [13], Mediterranean mussel (*Mytilus galloprovincialis*) [14], and disk abalone (*H. discus discus*) [9]. Previous study indicates that marine mollusks are widely used as indicator organisms in the marine environment [9].

In our previous study, one partial cDNA of GST (GISTrs) was screened from the suppression subtractive hybridization (SSH) cDNA library of hemocytes in variously colored abalone challenged with bacteria [15]. It is known that GSTs play a major role in cellular defense against chemically induced toxicity, and few studies so far concerning the response of marine mollusk GSTs against bacterial infection [16]. Thus, this present study aimed to elucidate the full-length sequence of GISTrs (*abGSTsigma*) and investigate the distribution of its transcripts in various tissues of normal abalones and the expression pattern in tissues of bacteriachallenged abalones. From this study we expect to provide new insight into the GST functions associated with immune processes other than xenobiotic metabolism.

2. Materials and methods

2.1. Experimental animals

Eighty live normal female *H. diversicolor*, averaging 55 ± 5 mm in shell length, obtained from the Zhangpu abalone farm in Fujian Province, were acclimated in the laboratory at a salinity of 3% for 7 days at 24 ± 1 °C seawater temperature, before experimentation. Animals were reared in 80 L PVC tanks containing 40 L natural seawater treated with sand filtration, kept on a natural daylight cycle and fed with the marine alga *Gracilaria tenuistipitata* during the acclimation and experimental period.

2.2. Bacterial challenge and preparation of different tissues

A mixed suspension of five bacterial strains including two Gram negative bacteria (*Escherichia coli* CGMCC 1.2389 and *Vibrio parahaemeolyticus* CGMCC 1.1615) and three Gram positive bacteria (*Staphylococcus aureus* CGMCC 1.89, *Micrococcus lysodeikticus* CGMCC 1.634 and *Staphylococcus epidermidis* CGMCC 1.2429), were prepared for the challenge experiments as described previously [15]. Abalones were each injected with a dose of 5×10^5 cfu in the front of the foot with 25 µL of stock bacterial suspension. Similarly, an equal volume of sterile saline solution (0.85% NaCl) was inoculated to each abalone as an unchallenged control. Three abalones at each challenge time point were arranged for the challenged and saline control groups, respectively. The abalones for each group were separately reared in individual tank under the same culture conditions. Meanwhile, three normal abalones were reared in an individual tank as a normal control group.

Sampling was performed at different time intervals (3, 6, 12, 24, 36, 48, 60, 72 and 84 h) after bacterial challenge. Hemolymph samples were collected by cutting the foot of each abalone from each group. Hemocytes were isolated by centrifugation at $800 \times g$, at 4 °C for 10 min and immediately kept in liquid nitrogen.

Samples from the gill, mantle, gonad, foot, epipodium, hypobranchial gland, radula, digestive gland, hemocytes, shell muscle, anus and kidney were separately collected from each individual abalone, and were individually frozen immediately in liquid nitrogen, and stored at -80 °C.

2.3. GST cDNA identification and generation of full-length cDNA

A cDNA clone, HDr3CJ41, with the homologous GST partial sequence was identified from the SSH cDNA library of hemocytes in *H. diversicolor* challenged with bacteria using BLASTp and BLASTx of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) [15].

The total RNAs were extracted from the hemocytes of the bacteria-challenged abalones using TRIZOL reagent following the manufacturer's instructions (Invitrogen) and were used for 5'- and 3'-rapid amplification of cDNA ends (RACE) cDNA synthesis with sense primers (3CJ41GST-S1 and 3CJ41GST-S2) and anti-sense primers (3CJ41GST-X1 and 3CJ41GST-X2) designed (Fig. 1) from the partial GST sequence (GISTrs) identified in the SSH cDNA library. The RACE reactions were performed using the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. 5'- and 3'-RACE products were purified from an agarose gel using a QiaQuick gel purification kit (Qiagen), ligated into the T/A cloning vector pMD18-T (TaKaRa) and transformed into E. coli X-Blue competent cells by heat shock. Three randomly selected clones were identified as positive clones using PCR and 1% agarose gel electrophoresis. The PCR reaction was performed using 1 µL bacterial culture, primers M13-47 and M13-48, and rTaq DNA polymerase (TaKaRa). The amplification conditions were: 3 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 55 °C (annealing temperature), 90 s at 72 °C; then 3 min at 72 °C for further extension. Selected positive clones were sequenced at least twice using ABI 3730 automated sequencers (Applied Biosystems, USA) at Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (China).

2.4. Sequence analysis

The cDNA and predicted protein sequences were analyzed by means of GeneTool1.0 Lite, DNAStar5.0 (to deduce the amino acid sequence, to predict protein molecular weight (MW) and p*I*, and to calculate the percentage identity with the Clustal W program of MegAlign). Homology searches were performed using BLASTn and BLASTp in NCBI. The CD-Search service was used to identify the conserved domains (CDs) present in predicted protein sequences against NCBI's Conserved Domain Database (CDD, http:// www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The deduced amino acid sequence of *abGSTsigma* was aligned with 30 known homologous proteins of GST class sigma obtained from GenBank using ClustalX1.83 software. The neighbor-joining (NJ) method was used to reconstruct a phylogenetic tree with 1000 bootstrap replicates by means of MAGA v4.0 software.

2.5. Analysis of gene expression pattern of abGSTsigma in H. diversicolor using semi-quantitative reverse transcription PCR and quantitative real-time PCR

In order to investigate the tissue distribution of GST transcripts, total RNA was extracted from various tissues of normal abalones, including the gill, mantle, gonad, foot, epipodium, hypobranchial gland, radula, digestive gland, hemocytes, shell muscle, anus and kidney tissues. In addition, to investigate the inducibility of GST transcripts in some tissues of *H. diversicolor* challenged with bacteria, total RNAs of the gill, mantle, digestive gland and hemocytes during the time course of bacteria-challenge were separately isolated from each tissue using TRIZOL reagent (Invitrogen). Complementary DNA was synthesized using the PrimeScript[™] RT reagent kit (TaKaRa) following the manufacturer's instructions.

Total RNA of 50 ng for each sample of normal abalones was used as a template of semi-quantitative reverse transcription PCR (sqRT-PCR) to analyze the differential transcription of *abGSTsigma* from 12 tissues of normal abalones, using two specific primers 3CJ41GST-S1 and 3CJ41GST-X1. PCR reactions were performed in a final volume of 25 μ L containing 0.375 μ L dNTP mix (10 mM), 0.625 U Ex Taq (TaKaRa), 2.5 μ L 10× Ex Taq buffer (Mg²⁺ plus), and 2.5 pmol of each gene-specific primer. The amplification conditions were: 1 min at 94 °C; 26 cycles of 30 s at 94 °C, 40 s at 60 °C

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Fig. 1. Complementary DNA and predicted amino acid sequences of *abGSTsigma* from *H. diversicolor*. The polyadenylation signal is underlined and the stop codon is indicated by double asterisks (**). Primers for 5'-, 3'-RACE and analysis of gene differential expression binding sites are shown with arrows (5' to 3'). The organization of the predicted conserved domains (CD) using the CD-Search service are framed including GST_N_sigma_like CD and GST_C_sigma_like CD. "#": GSH-binding sites (G-site) in N_terminal. "*": sites of substrate binding pocket (H-site) in C_terminal. "-": interacting interface sites of N_terminal domain with C_terminal domain. "+": dimer interface sites (GenBank accession number EF546619).

(annealing temperature), 40 s at 72 °C; then 7 min at 72 °C for further extension. The PCR product of 3 μ L which was mixed with equal volumes from three abalones in the same condition was subjected to 1% agarose gel electrophoresis stained with ethidium bromide to evaluate expression levels in comparison with the internal standard housekeeping gene actin (GenBank accession number EF587284), which was obtained by sqRT-PCR with a pair of specific primers 4CJ11actin-S1: 5'-ACGGGTATTGTTCTT-GACTCTGGTG-3' and 4CJ11actin-X1: 5'-TTTCTCCTTGATGTCCCT-GACGATT-3' [15]. The intensities of amplified fragments were measured by Scion Image. Relative quantification of the GST expression level was calculated using the intensity value ratio of the target gene dividing actin gene.

Differentially expressed *abGSTsigma* transcripts after bacterial challenge were investigated by quantitative real-time PCR (qPCR), which was performed using the 7500 Real Time PCR System (Applied Biosystems). Total RNA was separately extracted from each tissue (gill, mantle, digestive gland and hemocytes) of the challenged, unchallenged control and normal abalones using TRIZOL reagent (Invitrogen). Two micrograms of total RNA from each group (n = 3) were separately reverse transcribed in a final

volume of 40 µL using a PrimeScript[™] RT reagent kit (Perfect Real Time) (TaKaRa) following the manufacturer's instructions. Real-time PCR was performed in a reaction mixture of 20 µL containing cDNA obtained from 10 ng of total RNA reverse transcribed, 5 pmol of each gene-specific primer (3CJ41GST-S3 and 3CJ41GST-X3) and 10 µL of Power SYBR Green PCR Master Mix (Applied Biosystems, UK). The standard cycling conditions were 95 °C for 10 min (initial polymerase activation), followed by 40 cycles of 95 °C for 15 s (denaturation), 60 °C for 25 s (annealing), and 72 °C for 40 s (extending and fluorescence data collection). The PCR specificity was checked with a heat dissociation protocol from 60 to 95 °C. Data of raw relative quantification were calculated using the 7500 system SDS software version 1.3.1.21, using the housekeeping actin gene (specific primers 4CJ11actin-S2: 5'-ACCACGGGTATTGTTCTTGAC-3' and 4CJ11actin-X2: 5'-CGGTGGTGGTGAAGGAGTAAC-3') as the internal standard and normal group data as the calibrator. Statistical analysis of differences was done using SPSS 13.0 by one-way analysis of variance (ANOVA). Student's 't'-test was used to determine the differences between the challenged and saline control groups (p < 0.05).

2.6. Sample collection, tissue preparation and GST and SOD enzyme assays

Tissue samples were collected from each abalone of the challenged, saline control and normal groups (n = 3). Hemolymph was collected from individual abalone in a pre-chilled tube and the hemocytes were isolated by centrifugation at $800 \times g$, $4 \degree C$ for 10 min. The hemocytes suspended in PBS (100 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM EDTA and a few crystals of phenylmethylsulfonyl fluoride, a protease inhibitor) were subjected to cell disruption by sonication (20 kHz, 50 W, $3 \times 20 \text{ s}$) in an ultrasonicator (Scientz JY92-II, Ning Bo Xinzi) and the resultant homogenates were centrifuged ($12,000 \times g$, 30 min, $4 \degree C$). Other tissue samples (gill, mantle and digestive gland) were individually homogenized (1:10, w/v) in PBS and then centrifuged for 30 min at $12,000 \times g$ ($4 \degree C$). An aliquot of the resulting supernatant from each sample was used for determination of GST activity.

Glutathione S-transferase activity was assayed by the method of Habig et al. [17]. Briefly, the reaction was performed in a final volume of 1 mL containing 100 mM phosphate buffer of pH 6.5; 10 mM GSH reduced; 10 mM 1-chloro-2,4-dinitrobenzene (CDNB) and an appropriate amount of enzyme. Before addition of substrate, the enzyme mixture was incubated for 10 min at 37 °C and the reaction was initiated by addition of CDNB, and absorbance at 340 nm was monitored at 25 °C for 5 min. The changes in absorbance per minute were converted into moles of the substrate conjugated/min/mg protein using the molar extinction coefficient: ε 340 = 9.6 mM⁻¹ cm⁻¹ for CDNB.

Superoxide dismutase (SOD) activity was measured as the degree of inhibition of auto-oxidation of pyrogallol at an alkaline pH by the method of Marklund and Marklund [18]. One unit of SOD activity is defined as the amount of enzyme (per protein milligram) that inhibits the oxidation reaction by 50% of maximal inhibition.

Results are reported as mean \pm S.D. of three observations per group and the data were subjected to one-way analysis of variance (one-way ANOVA) followed by Student's 't'-test. Differences between saline control and bacterial challenge groups were considered significant at p < 0.05.

3. Results and discussion

3.1. Sequence analysis of the H. diversicolor abGSTsigma cDNA

The complete cDNA *abGSTsigma* (GenBank accession number EF546619) is 1328 bp, containing an open reading frame (ORF) of 624 bp with a coding capacity of 208 amino acid residues. The 3' non-coding region is composed of 637 bp with a polyadenylation signal appearing at position 1286 nt and a poly(A) tail at position 618 nt downstream of stop codon TGA (Fig. 1). The predicted signal peptide cleavage site of its deduced protein was not found using the Signal P 3.0 Server (http://www.cbs.dtu.dk/services/SignalP), thus *abGSTsigma* might be a cytosolic glutathione S-transferase. The predicted MW of this protein was 23.67 kDa and its estimated *pl* was 5.67. The cytosolic GSTs in most organisms are all dimeric with subunit molecular masses from 21 to 29 kDa [19]. Our predicted MW of *abGSTsigma* was in accordance with a previous report that a sigma class GSTs show subunit MWs with an average of 23 kDa [1].

The complete cDNA sequence of *H. diversicolor abGSTsigma* was first reported in this study and showed less similarity to known sequences based on the BLASTn results. However, the predicted amino acid sequence of *abGSTsigma* was found to be homologous with the proteins of GST class sigma when analyzed by BLASTp with a creditable expectation value (*E*-value $\leq 10^{-3}$). On a search of the CD using the CD-Search service against NCBI's CDD, the

predicted protein sequence of abGSTsigma cDNA was matched to CDs of the GST_N_Sigma_like (PSSM: cd03039) and GST_C_Sigma_like (PSSM: cd03192), containing a G-site (from Tyr⁴ to Ala⁷³) which binds the GSH in the N-terminal region and an H-site (from Leu⁸³ to Val¹⁹⁰) which is a substrate binding site in the C-terminal (Fig. 1). The sigma class GSTs rely on a Tyr (Y) residue for GSH stabilization in the G-site [1], which was also found in the sequence of abGSTsigma (Fig. 1). However, the similarity in the homologous proteins of the GST class sigma was relatively low, with a maximum identity of 38.7% in comparison with the GST-sigma of the African clawed frog (Xenopus laevis, AAM82563). The identity of abGSTsigma with each of eight class sigma GSTs from other organisms was at least 35%, which was shown as 36.6% identical with barber pole worm (Haemonchus contortus, AAF81283), 35.1% identical with red fire ant (Solenopsis invicta, ABA39530), 37.6% with X. laevis (NP_001079730), 35.9% and 35.7% with Caenorhabditis elegans (NP_494883 and NP_496357), 35.0% with African malaria mosquito (Anopheles gambiae, P46428), 37.6% with jewel wasp (Nasonia vitripennis, XP_001600977), and 36.9% with pig roundworm (Ascaris suum, P46436). The identities with class sigma GSTs of three mollusks, including disk abalone (*H. discus discus*, ABF67507), Pacific oyster (Crassostrea gigas, CAE11863) and squid (O. sloani pacificus, AAA92066) were 30.8%, 33.0% and 32.0%, respectively. The glutathione-dependent prostaglandin D₂ synthases (PGDSs) of chicken (Gallus gallus, O73888), rat (Rattus norvegicus, O35543), and human (Homo sapiens, O60760) are members of the sigma class GST [4,20,21], and their identities with abGSTsigma were 33.2%, 27.6%, and 28.1% respectively.

Based on the alignment analysis of the deduced protein sequences between abGSTsigma and 30 other GSTs with GST sigma CD including three PGDSs (GST class sigma), it was found that the N-terminal region of all the GSTs compared was highly homologous and conserved, while the C-terminal was relatively diverse (Fig. 2). Each GST is known to contain a G-site binding the GSH substrate in its N-terminal and an H-site binding the xenobiotic compounds in its C-terminal [22,23]. All of the members of the highly diverse GST super family are capable of binding the tripeptide GSH, thus it is suggested that the structural features of the G-site might share a highly conserved amino acid sequence. The H-site binding the xenobiotic compounds is the main structure accounting for speciality and activity of GSTs and it has few homologous amino acid sequences with that of the G-site. In general, the similarity of interclass protein sequences is rarely greater than 35% in the H-site region [1]. Xenobiotic compounds have various features, however the most important seems to be the possession of a carbon to carbon double bond bordering the electron-withdrawing group, which can be obtained through a phase I detoxification enzyme (cytochrome P450) if the compounds lack them intrinsically [24].

An NJ phylogenetic tree of abGSTsigma amino acid sequence with 30 GSTs containing GST sigma CD and 26 other class GSTs was reconstructed using MEGA v4.0 software. Three plant phi class GSTs of Arabidopsis thaliana (P42761) and Nicotiana tabacum (P46440 and BAA01394) were rooted to build the phylogenetic tree. It was shown that abGSTsigma of H. diversicolor was in a branch position with other known sigma class GSTs from different organisms, such as human, rat, African clawed frog, disk abalone, Pacific oyster, and Tigriopus japonicus (Fig. 3). GSTs are a multifunctional enzyme family, and the classification of the GST super family has been evolving to include cytosolic, microsomal and mitochondrial components [25]. Microsomal GSTs are less characterized and four classes of I-IV are identified [26]. The unique kappa class GSTs which belong to the mitochondrial GST isoenzymes have been investigated in mammalian species. This class has a very high peroxidase activity and their amino acid sequences are quite distinct from the other class cytosolic GSTs

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EF546619	M-PSYKYTYPNLRARGEIPRLILKTAGADFEDNRVEFSEWPALKEQTPFGQLPFIEIDGKPFPESMSISRLLAAEFGVAG	79
XP_001600977	M-PSYKVTYFNIKGLGEPIRFILSQAGVDFVDDRVESADWPKIKPTTPFGQMPVLEVDGKKINQTNAICRYLAKQYGLAG	79
AAM82563	M-PSYKLIYPNLEGGEILRYLFSYSNIDFEDRYVEFADWPALKPTIPYGQLPVWEIDGVIYNQBLAIGRYLAKKAGLTG	79
AAP79878	M-PTYKLTYFNVAGLGESLRYMLHHCGIKFEDVRVEFDDWPKLKPNMPMGOMPILEIDGKIYHOSRAIGRYIAKKGNLYG	79
ABA39530	M-PTYKLTYFNVTGLGESLRYMLHHCGIKFEDVRVEFDDWPKLKPNMPMGQMPILEIDGKIYHQSRAIGRYIAKKGNLYG	79
XP_001605470	M-PTYKLSYLNVTGLGEPLRFLLSYGGADFEDNRINFEDWPKQKPKMPMEQVPILEFDGKIYHQTRAIGRFLAKKYKLYG	79
NP_497121	M-PTYKLTYPNSRFVAEPARILPHLAGVPFEDIRIIHGDGWEQIKDKTPFGQVPVLNINGFEIPOSTAIIRVLATKFGVAG	81
ABV44730	M-PNYNTYFNYAALABPLAFIGAIGELELLAYGGTEPLLAYSKE - WPILKS - MPROMPVLEVDGRYNGSISMAKIAANVGLYG	79
P46428	M-PDYKVYYFNVKALGEPLRFLLSYGNLPFDDVRITREEWPALKPTMPMGOMPVLEVDGKKVHOSVAMSRYLANOVGLAG	79
XP_001661869	M-PDYKVYYFNVKALGEPLRFLLSYGNLPFDDIRITREEWPALKPTMPMGQMPVLEVDGKRVHQSLAMCRYVAKQIGLAG	79
XP_001661871	M - PDYKVYYPNVKALGEPLRFLLSYGNLPFDDIRITREE - WPALKPT - MPMGOMPVLSVDGKKVHQSVAMSRVLARQVGLAG	79
BAD26698	M-PUVEFIEFVEALGESUCHLIAFGUEFEINKISSENWEFKEAEFFUEFUEFUEFUEFUEFUEFUEFUEFUEFUEFUEFUEFU	79
XP 001608225	M-PYYKLTYFPVKALGEPIRFLFSYGGVDFVDDRFDQADWPKIKPTTPFGQVPILEVDGKKVCQSTAICRYLAKOFGLAG	79
- P46436	M-PQYKLTYFDIRGLGEGARLIFHQAGVKFEDNRLKREDWPALKPKTPFGQLPLLEVDGEVLAQSAAIYRYLGRQFGLAG	79
AAA92066	M-PKWTLHYFPLMGRAELCRFVLAAHGEEFTDRVVEMADWPNLKATMYSNAMPVLDIDGTTMSOSMCIARHLAREFGLDG	79
073888	M-PNYKLTYPNLKGKABICKYLFAYAGIKYEDHKLEGADWEYKKPTIPFGKVPILEVDGVIIHOSLAIAKYLAKESGLAG	79
060760	M-PNYKLTYFNMRGRAEIIRYIFAYLDIOYEDHRIEOADWPEIKSTLPFGKIPILEVDGLTLHGSLAIARYLTNNTDLAG	79
AAY89316	MAPSVKLVYFPLRGRAELIRLILEAKGISYQDETIPSEK WGDKKAS MPFRSLPVLYWVGEEIGQSLTIARSVAKKAGLAG	80
XP_969146	MAPAYKLTYFDGRGLAETSRFIMKYGGIDFEDCRIKRED - WPQIKSK - YPFGQLPVLEHNGKTVNQSHSIARYLAKQVKLAG	80
NP 494883	MV-HYKVSYPPIRGAGEIARQILAYAGODFEDNRIPKEEWPAVKPSTPFGQLPLLEVDGKVLAOSHAIARYLARGOPGING	79
NP 494902	MV-HYKLSYFPIRAGEVIRVIAGOSFBURISIBB-WAAVFI-PFGUEDUVGKVLGGGHAISRFLARGAGING	79
NP 496357	MV-SYKLTYFNGRGAGEVSRQIFAYAGQQYEDNRVTQEQWPALKETCAAPFGQLPFLEVDGKKLAQSHAIARFLAREFKLNG	81
AAF81283	MV-HYKLTYFNGRGAAEIIRQVFVLAGQDYEDVRLTHEEWPKHKASMPFGQLPVLEVDGKQLPQSVAIVRYLARKFGYAG	79
CAE11863	MA-SYRLHYPDVRGRGEIVRMLFKLAQAEFGDIRVTQGEWTDVKHDTPTGELPVLEVGEKQLTGSLTIARVLAREFGLAG	79
ABE67507	MS-TIKLIIFNIFGLAEFIRFLLAQSGIRFEDKRIDIEEWFAIRSEMFLGQVFLEIDGKVIIGKAISKLIARKNNFIG MS-TYKLIVLOFGLAEISULFALAGOVEDVDFKREDWPAVENK-VIGGTULEVDGKOVGGMAIASFLAFFGFHG	79
EF546619	ETALDRLRADVLTEQTRSMKEGIYKYFFEGDAKIKAEAQKNFEEKIRPKYLKTWEEAAKENTFGSGHLVGKALTIADLA	158
XP_001600977	ANDWENLEIDATVDTIHDLRAKIGAYYYETNAEAKAEKEK-AAKELVPFYVERLDEQVKKNGGYFVGGKLTWADLL	154
AAM82563	KSELDEIRVDALIDTIDDFFS-KFPWMDT	147
AAP79878	SDELEAMEIDATIDSMDDIROALSTYYWEODPAFKA-KLKETAFEKLPFYLDKFEAOVKKNGGYFVGGKLSWADFL	154
ABA39530	SDELEAMEIDATIDSMDDIRQALSTYYWEQDPAFKA-KLKETAFEKLPFYLDKFEAQVKKNGGYFVGGKLSWADFL	154
XP_001605470	NDELODLEIDLNVDDVEDWRTNFSRFFREADETQKA-KLKAFALEKTPFYLGKFEERVKKNGGYFVAGKLSWADVH	154
NP_497121	KTPEEOAWADAICDOVRDPMAVPFKDIVVAKRVGKTEEEVSQLINEGFVPGRDAVFKIITRILEKNKSGFLVGDGITPADIV	163
ABV44730 ABV60329	SDAWEDGIDIVUDIINDFRUKIAVVSIEDDUVERKLVILINEVIPFILEKLDSIAKENKEHFALGKLWADLY	155
P46428	ADDWENLMIDTVVDTVNDFRLKIAIVAYEP DDMVKEKKMVTLNNEVIPFYLTKLNVIAKEN NGHLVLGKPTWADVY	155
XP_001661869	SDPVEELQIDAIVDTINDFRLKIAIVAYEPDDMVKEKKMITLTNEVIPFYLTKLNVIAKENNGHLVLGKPTWADVY	155
XP_001661871	ADDWENLMIDTVVDTINDFRLKIAVVSYEPDDDVKEKKUVLNSEVIPFYLEKLDDIARDNNGHMANGKLTWADMY	155
NP_001036994	AND EASPEIDONVERINDIKASAASVHEEKDEAVAAKKAELEETKIPFFFEKLNEIDIKANGHIALGKLTWGDFV	155
XP 001608225	KDDWEALEIDAAVDTIHDLRAKIAAHHYENNATAKAEKLA-AAKELVPFYVQRLDEOVKRNGGYLVGGKLSWADIV	154
- P46436	KTPMEEAOVDSTEDOFKDEMAELRPCFRVI AGFEEOKEKVI.KEVAVPARDKHI.PLI.EKFLAKS - GSEVMVGKSVTWADI.V	
		159
AAA92066	KTSLEKYRVDEITETLODIFNDVVKIKFAPEAAK-EAVQQNYEKSCKRLAPFLEGLLVSNGGGDGFFVGNSMTLADLH	159 156
AAA92066 073888	KTSLEKNRVDEITETLODIFNDVVKIKFAP EAAK-EAVOONVEKSCKRLAPFLEGLLVSNGGDGFFVGNSMTLADLH OTPVEOALADAIVDTIDDFMM-LFFMAEKNOVKEKAFNDILTNKAPELIKDLTFLOVKWEVGKSVUWADPV VTFLOOVUNUNUNTDFDWS-LFDMAEMOVVEFKAFNDILTNAPELIKDLTFL	159 156 152
AAA92066 073888 035543 060760	KTSLEKNRVDEITETLODIFNDVVKIKFAPEAAK-EAVOONVEKSCKRLAPFLEGLLVSNGGGDGFFVGNSMILADLH OTPVEQALADAIVDTIDDFM-FFPMAEKNOVKEKAFNDILTNKAPELLKOLDTFLGDKKWFVGKSVUMADFY KTELEOCOVDAVVDTLDDFMS-FFPMAEKNOUKEKFTFNDLLTRQAPHLKDLDTYLGGKEWFIGNVVWADFY NTEMEOCHVDAIVDTLDDFMS-FFPMAEKOVKEOMFNELTYNAPHLMODLDTYLGGREWIIGNSVWADFY	159 156 152 152 152
AAA92066 073888 035543 060760 AAY89316	KTSLEKEVDEITETLODIPNDVVKIKFAPBAR-EAVQONYEKSCKELAPELGLUSNGGGGFFVGNNTLADLH QTPVEQALADAIVDTIDDFN-LFFMAEN	159 156 152 152 152 152
AAA92066 073888 035543 060760 AAY89316 XP_969146	KTSLEK¥RVDEITETLODIFNDVVKIKFAPEAAK-EAVQQNYEKSCKRLAPFLEGLLVSNGGGDGFFVGNSMTLADLH QTPVEQALADAIVDTIDDFM-LFFMAENOVKEKAFNDIITNKAPELKDLDTFLGDKWFFVGKSVTWADFY KTELEQCQVDAVVDTLDDFMS-LFFMAENOVKEKFNDLITRAPHLKDLDTYLGDKEWFIGNYVTWADFY NTEMEQCRVDAIVDTLDDFMS-CFFMAEN	159 156 152 152 152 156 156
AAA92066 073888 035543 060760 AAX89316 XP_969146 NP 494883	KTSLEKNRVDEITETLODIFNDVVKIKFAPEAAK-EAVQQNYEKSCKRLAPFLEGLLVSNGGDGFFVGNSMTLADLH QTPVEQALADAIVDTIDDFM-LFFWAEKNOVKEKARNDIITNKAPELLKDLTFLGOKKWFVGKSVTWADFY KTELEQCVDAVVDTLDDFMS-LFFWAEKNOLKERFFNDLITROAPHLLKDLTYLGGREWLIGNVTWADFY NTEMEQCHVDAIVDTLDDFMS-CFFWAEKK	159 156 152 152 152 156 156 156
AAA92066 073888 035543 060760 AAY89316 XP_969146 NP 494883 NP_494884 NP_494984	KTSLEKNRVDEITETLODIFNDVVKIKFAPEAAK-EAVOONVEKSCKRLAPFLEGLLVSNGGDGFFVGNSMTLADLH QTPVEQALADAIVDTIDDFM-LFFWAEKNOVKERAFNDIITNKAPELKDLITFLGOKKWFVGKSVTWADFY KTELEQCUDAVVDTLDDFMS-CFFWAEKN	159 156 152 152 152 156 156 159 159
AAA92066 073888 035543 AAX89316 XP_969146 NP_494883 NP_494884 NP_494902 NP_496357	KTSLEK KRVDEITETLODIFNDUVKIKFAPEAAK-EAVQQNYEKSCKRLAPFLEGLLVSNGGGDGFFVGNSMTLADLH QTFVEQALADAIVDTIDDFMS-LFFMAENOVKERAFNDIITNKAPELKDLDTFLGDKWFFVGKSVTMAPFY KTELEQCVDAVVDTIDDFMS-LFFMAENOVKERAFNDIITNKAPFLKDLDTYLGREWTIGNYVWAPFY NTEMEQCHVDAIVDTIDDFMS-GFFMAENSTKSEATQAFHILKDLDTYLGREWTIGNSVTWAPFY NDEFQARADAIVDTVADFFKLFFKIKD	159 156 152 152 156 156 159 159 159 159
AAA92066 O73888 O35543 O60760 AAX89316 NP_49483 NP_49483 NP_494884 NP_494802 NP_496357 AAF81283	KTSLEKKRVDEITETLODIFNDUVKIKFAPEAAK-EAVQQNYEKSCKRLAPFLEGLUSNGGGDGFFVGNSMTLADLH (TFVEQALADAIVDTIDDFMS-LFFMAENOVKEKAFNDIITNKAPELKDLDTFLGDKWFFVGKSVTWADFY KTELEQCVDAVVDTLDDFMS-LFFMAENOVKEQMFNELLTYNAPHLKDLDTYLGREWLIGNSVTWADFY NTDEQARADAIVDTVANLFFKLFELKKKD	159 156 152 152 152 156 156 159 159 159 161
AAA92066 O73888 O35543 O60760 AAY89316 XP 969146 NP 494883 NP_494884 NP_494902 NP 496357 AĀF81283 CAE11863	KTSLEKKRVDEITETLODIFNDUVKIKFAPEAAK-EAVQQNYEKSCKRLAPFLEGLLVSNGGGDGFFVGNSMTLADLH (TFVEQALADAIVDTIDDFM-LFFMAENOVKEKAFNDIITNAPELLKDLITFLGKKWFVGKSVTWADFY KTELEQCVDAVVDTLDDFMS-LFFMAENOVKEKAFNDIITNAPHLKDLITYLGKEWFVGKSVTWADFY NNDIEQARADAIVDTLDDFMS-CFFMAENOVKEGMFNELITYNAPHLKDDLITYLGKEWFIGNYUWADFY NNDIEQARADAIVDTVANLFTKLFEIKNKDESTKSEAIQAFLNTELSGILDISKNLKNRGG-KFFTGSKLIGNSUTWADFY NNDIEQARADAIVDTVANLFTKLFEIKNKDBETKSEAIQAFLNTELSGILDISKNLKNRGG-KFFTGSKLIGNSUTWADFY KCAWEEAQVNSIADOFKDILNEVRFYFNG	159 156 152 152 152 156 159 159 159 161 159
AAA92066 O73888 O35543 O60760 NP 969146 NP 49489316 NP 494883 NP 494883 NP 494884 NP 494902 NP 494357 AĀF81283 CAE11863 XP 624682 AĀF67507	KTSLEK KRVDEITETLODIPNDVKIKFAPEAAK-EAVQQNYEKSCKRLAPFLEGLUSNGGGOGFFVGNSMTLADLH CTVVGALADAITUDTIDDFMS-LFFMAEN	159 156 152 152 152 156 156 159 159 161 159 156 156
AAA92066 O73888 O35543 O60760 XP 969146 NP 49489316 XP 494883 NP_494884 NP_494902 NP 494357 AAF81283 CAE11863 XP 624682 AEF67507	KTSLEK KRVDEITETLOD IFNDUVKIKFAPBAR-EAVQQNYEKSCKRLAPFLEGLUSNGGGDGFFVGNSMTLADLH QTPVGALADAIVDTIDDFMS-LFFMAENOVKERAFNDIITNKAPELKDLDTFLGDKKWFVGKSVTMAPFY KTELEQCVDAVVDTIDDFMS-LFFMAENOVKERAFNDIITNKAPELKDLDTYLGDKEWFIGNYVTWAPFY NTENGQCHVDAIVDTIDDFMS-GFFMAEN	159 156 152 152 152 156 159 159 159 159 156 154 156
AAA92066 O73888 O35543 O60760 XP_969146 NP 494884 NP_494884 NP_494884 NP_494884 NP_494884 NP_494884 CAE11863 XP 624682 AEF67507 EF546619	KTSLEKKRVDEITETLODIFNDUVKIKFAPEAAK-EAVQQNYEKSCKRLAPFLEGLLVSNGGGDGFFVGNSMTLADLH (TFVEQALADAIVDTIDDFM-LFFMAENOVKEKAFNDIITNKAPELKDLDTFLGOKEWFVGKSVTWADFY KTELEQCVDAVVDTIDDFMS-LFFMAENOVKEKAFNDIITNKAPELKDLDTYLGOKEWFVGKSVTWADFY NTEMQCRVDAIVDTLDDFMS-GFFMAEN	159 156 152 152 152 156 159 159 159 159 159 159 154 156
AAA92066 073888 035543 060760 AAY89316 NP_494883 NP_494883 NP_494883 NP_494883 NP_494883 NP_494883 NP_494883 NP_494883 NP_494883 NP_494883 NP_494883 NP_494883 CAEL1863 XP_624682 ABF67507 <u>EF546619</u> XP_001600977 <u>ABF875619</u>	KTSLEKKFVDEITETLODIFNDUVKIKFAPBAAK-EAVQQNYEKSCKFLAPLEGLUSNGGGGFFVGNSMILADLH QTPVEQALADAIVDTLDDFNS-LFFMAEN	159 156 152 152 152 156 159 159 159 159 159 159 159 154 156 154 156
AAA920c6 O73888 O35543 O60760 AAY89316 NP_49483 NP_494883 NP_494884 NP_494902 NP_494357 AĀF81283 CAE11863 XP 624682 XP 624682 EF546619 XP_001600977 AAM82563 NP 001079730	KTSLEK KRVDEITETLODIFNDUVKIKFAPEAAK-EAVQQNYEKSCKRLAPFLEGLUSNGGGDGFFVGNSMTLADLH CTPVGALADAIVDTIDDFMS.LFPMAEN	159 156 152 152 152 156 159 159 159 159 159 159 159 154 156 154 156 154 156 154 154
AAA92066 O73888 O35543 O60760 XP 969146 NP 49489316 XP 494883 NF 494883 NF 494883 NF 494902 NF 494357 AAF81283 CAE11863 XP 624682 AEF67507 <u>EF546619</u> XP_001600977 AAM82563 NP_001079730	KTSLEKKRVDEITETLODIFNDUVKIKFAPEAAK-EAVQQNYEKSCKRLAPFLEGLUSNGGGDGFFVGNSMTLADLH (TFVEQALADAIVDTIDDFMS.FFMAEN	159 156 152 152 152 156 156 159 159 159 159 159 159 156 154 156 202 202 202 197 202
AAA92066 073888 035543 060760 AAY89316 XP_969146 NP_494883 NP_494883 NP_494883 NP_494883 NP_494883 NP_494883 NP_494883 NP_494883 NP_494883 CAE11863 XP_624682 ABF67507 <u>EF546619</u> XP_001609977 AAM82563 NP_001079730 AAP787878 ABA39530	KTSLEKK RVDEITETLOD IFNDUVKIKFAP EAAK - EAVQQNYEKSCKRLAPFLEGLUSNGGGDGFFVGNSMTLADLH QTPVGALADAIVDTIDDFMS.FPMAEN OVKEKARNDILTNKAPELLKULDTFL GDKWFFVGKSVTWAPFY KTELEQCUVAUVDTLDDFMS.FPMAEN OVKEKARNDILTNKAPELLKULDTFL GDKWFFVGKSVTWAPFY NTEMQCRUDAVVDTLDDFMS.FPMAEN OVKEKARNDILTNKAPELLKULDTFL GDKWFFVGKSVTWAPFY NTEMQCRUDAVVDTLDDFMS.FPMAEN OVKECMRNELLTYNAPHLKULDTTL GREWLIGNSVTWAPFY NDEQADAIVDTVANLFYKLFKKNO STKSEATOAFLNTELSGILDISENLLKNRGG- CREWENGENKKYGKSVTWAPFY NDEWENLEIDAIVDTFNDLRLKIVAFFEQ DEKKKTILENLNKDVFPQYLTFFEIVKKN- -KGYFALGRLTWAPY NDWENLEIDAIVDTFNDLRLKIVAFFEQ DEKKKTILENLNKDVFPQYLTFFEIVKKN- -KGYFALGRLTWAPY KCAWEEAQVNSIADQFKDINEWEPYWWVK-MGFAEGDLDALAKDVFLPGKKHYGFINFLKAS- -SGSFLIGDSUTVODL KCAWEEAQVNSIADQFKDINDURSYNUK-MGFAEGDLDALAKDVFLPGKHYGFINFLKAS- -SGSFLIGDSUTVODL KCAWEEAQVNSIADQFKDINDURSYNUK-MGFAEGDADALKNDFLPNFKKNOCFTNFLKAS- -SGSFLIGDSUTVODL KTAMEEAQVNSIADQFKDINDURSYNUK-MGFAEGDEDADALKNDFLPNFKKNOCFTNFLKAS- -SGSFLIGDSUTVODL KSAWEEAVUSIADQFKDINDURSYNUK-MGFAEGDEDADALKNDFLPNFKKNOCFTNFLKAS- -SGSFLIGDSUTVODL KSAWEEAVUSIADQFKDINDURSYNUK-MGFAEGDEDADALKNDFLPNFKKNOCFTNFLKAS- -SGSFLIGDSUTVODL STDVEVSLADQFKDINDIRSYNUK-MGFAEGDEDADALKNDFULPAFEKYGELVNSLKAS- SGSFLIGDSUTVODL S	159 156 152 152 152 156 156 159 159 159 159 159 159 159 156 154 156 154 156 154 156 154 156 154 156 154 156 159 159 159 159 159 159 159 159 159 159
AAA92066 O73888 O35543 O60760 AAY89316 XP 969146 NP 494883 NP 494883 NP 494883 NP 494883 NP 494883 NP 494883 NP 49483 XP 624682 AAF67507 <u>EF546619</u> XP_001600977 AAM82563 XP 001600977 AAP79878 ABA35530 XP_001605470	KTSLEKKRVDEITETLODIFNDUVKIKFAPEAAK-EAVQQNYEKSCKRLAPFLEGLUSNGGGDGFFVGNSMTLADLH CTVUGALADAIVDTIDDEMS_LFPMAEN	159 156 152 152 152 156 156 159 159 159 159 159 159 159 159 156 154 156 202 - 202 - 202 - 202 - 202 - 202 - 202 - 202
AAA920c6 O73888 O35543 O60760 AAY89316 XP 969146 NP 494883 NP 494883 NP 494803 NP 494357 AĀF81283 CAE11863 XP 624682 ABF67507 EF546619 XP_001600977 AAM82563 NP 001079730 AAP79878 ABA3957 ABA3957 AAP79878 ABA3957 ABA3957 AAP79878 ABA3957 ABA3957 AAP79878 ABA3957 ABA3977 ABA3977 ABA3977 ABA39777 ABA397777 ABA397777777777777777	KTSLEK KRVDEITETLODIFNDUVKIKFAPBAAK-EAVQQNYEKSCKRLAPFLEGLUSNGGGDGFFVGNSMTLADLH CTFVEQALADAIVDTIDDFMS.FFMAEN	159 152 152 152 156 159 159 159 159 159 159 159 159 159 202
AAA92066 O73888 O35543 O60760 XP 969146 NP 49489316 XP 969146 NP 494883 NP 494883 NP 494883 NP 494902 NP 494357 AAF81283 CAE11863 XP 624682 AEF67507 <u>EF546619</u> XP-00160997 AAM82563 NP 001079730 AA79878 ABA39530 XP 00165470 NP 497121 AEV44736 ABV60329	KTSLEKKRVDEITETLODIFNDUVKIKFAPEAAK-EAVQQNYEKSCKRLAPFLEGLUSNGGDGFFVGNSMTLADLH (TFVEQALADAIVDTIDDFM-LFFMAEN	159 159 152 152 152 156 159 159 159 159 159 159 159 156 154 156 154 156 202 202 202 202 202 202 203 203 203 203
AAA92066 O73888 O35543 O60760 AAY89316 XP 969146 NP 494883 NP 494883 NP 494883 NP 494883 NP 494883 NP 494883 NP 494892 NP 49623 CAE11863 XP 624682 ABF67507 <u>EF546619</u> XP_001600977 ABA39530 XP_001605470 NP 497121 ABV4736 ABV60329 P 46428	KTSLEKKFVDEITETLODIFNDUVKIKFAPBAAK-EAVQQNYEKSCKRLAPLEGLUVSNGGGDGFFVGNSMTADDH QTPVGALADATUDTIDDEM-LFFMAEN	159 152 152 152 156 159 159 159 159 159 159 159 159 159 159 202
AAA920c6 O73888 O35543 O60760 AAY89316 NP_969146 NP_494883 NP_494883 NP_494882 NP_494902 NP_494357 AĀF81283 CAE11863 XP 624682 AĒF67507 EF546619 XP_001600977 AAM82563 NP_0101079730 NP_0101079730 NP_0101079730 XP_001605470 NP_497121 AĒV4736 ABV60329 P46428 XP_001661869	KTSLEKKRVDEITETLODIFNDUVKIKFAPEAAK-EAVQQNYEKSCKRLAPFLEGLUSNGGGDGFFVGNSMTLADLH CTPVEQALADAIVDTIDDFM-LFFMAEN	$\begin{array}{c} 159\\ 156\\ 152\\ 152\\ 152\\ 152\\ 156\\ 159\\ 159\\ 159\\ 159\\ 159\\ 159\\ 159\\ 159$
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Fig. 2. Comparison of the amino acid sequence deduced from *H. diversicolor abGSTsigma* cDNA with some similar protein sequences of GST class sigma or the like. The aminoacid sequence deduced from *abGSTsigma* of variously colored abalone (*H. diversicolor*, GenBank accession number <u>EF546619</u>) is underlined. The conserved G-site of Tyr (Y) is indicated with the second asterisk present in the N_terminal of *abGSTsigma*. The aminoacid sequence of *abGSTsigma* in the N_terminal is relatively conserved. Other similar proteins are predicted and obtained from GenBank, as follows: jewel wasp GST class-sigma and GST isoform 2 (*Nasonia vitripennis*, XP_00160977, XP_001608225 and XP_001605470); African clawed frog GST sigma class (*Xenopus laevis*, AAM82563 and NP_001079730); red fire ant GSTS1 (*Solenopsis invicta*, AAP79878 and ABA39530); *Caenorhabditis elegans* GST-31, GST-7, GST-8, GST-30, and GST-5 (NP_497121, NP_494883, NP_494884, NP_494902, and NP_496357); *Phlebotomus papatasi* GST-1ike (ABV44736); *Lutzomyia longipalpis* GST1 (ABV60329); African malaria mosquito GST class-sigma (*Anopheles gambiae*, P46428); *Stegomyia aegypti* GST (*Aedes aegypti*, XP_001661869 and XP_001661871); domestic silkworm GST sigma (*Ommastrephes sloani pacificus*, AAA92066); chick PGDS or GST class sigma (*Callus gallus*, 073888); rat PGDS or GST class sigma (*Rattus norvegicus*, 035543); human PGDS or GST class sigma (*Homo sapiens*, 060760); *Tigriopus japonicus* sigma class GST (*AAY89316*); red flour beetel GST-like (*Tribolium castaneum*, XP_969146); barber pole worm GST (*Haemonchus contortus*, AAF81283); Pacific oyster sigma class GST (*Crassostrea gigas*, CAE11863); honey bee GSTS1 isoform A-like (*Apis mellifera*, XP_624682); and disk abalone GST isoform 2 (*Haliotis discus discus*, ABF67507).

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Fig. 3. Phylogenetic analysis of deduced amino acid sequences from *H. diversicolor abGSTsigma* and other GST cDNAs obtained from GenBank of the NCBI using MEGA v4.0. *H. diversicolor abGSTsigma* is underlined. Each class name of GST is marked. Neighbor-joining phylogenetic tree rooted with three plant GST including thale cress GST class (*Arabidopsis thaliana*, P42761), common tobacco GST and GST class-phi (*Nicotiana tabacum*, BAA01394 and P46440). Numbers next to the branches indicate bootstrap value of each internal branch in the phylogenetic tree nodes from 1000 replicates.

[25], which is shown in the NJ phylogenetic tree of *abGSTsigma* (Fig. 3). The classes of cytosolic GSTs are identified as alpha, mu, pi, sigma, theta, zeta and omega in mammals. Except for the sigma and omega classes, the rest are subdivided into subclasses [25]. So far, there are no clearly established criteria to classify GSTs from marine organisms [1]. In marine GSTs, the sigma class GSTs of the squid (*O. sloani pacificus*, AH003423) and *T. japonicus* (AAY89316) show little homology with other class GSTs [6,10]. Comparison of the sequence data indicates the unique nature of the GSTs from the sigma class, suggesting that it might play a role in physiological processes beyond its detoxification functions [1].

3.2. Differential tissue-specific expression of the abGSTsigma gene in normal abalones

Tissue-specific expression of *abGSTsigma* was analyzed using sqRT-PCR in multiple tissues of normal *H. diversicolor*, including both outer (gill, mantle, foot, epipodium, hypobranchial gland and anus) and inner (gonad, radula, digestive gland, hemocytes, shell muscle and kidney) tissues. Actin was used as an internal standard gene and the expression level of *abGSTsigma* in hemocytes was used as a calibrator contrasting with other tissues. The *abGSTsigma* cDNA fragment amplified from these tissues was 348 bp in length

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Fig. 4. Distribution of *abGSTsigma* mRNA transcripts in 12 tissues of normal variously colored abalone analyzed using RT-PCR. M, mantle; Gi, gill; HG, hypobranchial gland; K, kidney; F, foot; SM, shell muscle; A, anus; H, hemocytes; E, epipodium; R, radula; Go, gonad; DG, digestive gland.

(Fig. 4). As observed, the *abGSTsigma* gene expression was distributed in all tissues tested but the amount of transcripts in each tissue was very different. The high amount of *abGSTsigma* transcripts was demonstrated in the gill and mantle, and a relatively higher transcription level observed in hypobranchial gland, epipodium and kidney. In contrast, the *abGSTsigma* gene was low expressed in the other tissues tested, especially in hemocytes.

GSTs, as the most important components of xenobiotic metabolic enzymes of detoxification, are expressed with many patterns. Members of the GST super family exhibit different primary structures, enzyme properties, and physiological functions [27]. Each of the GST classes might have different distributions in tissues. To date, much research has investigated the tissue distribution of GSTs and analyzed the pathological and physiological functions of GSTs. Their expressions in marine organisms are generally abundant in the gill, digestive gland, gonad and mantle to protect organisms against the cytotoxic and or genotoxic effects of xenobiotics [8,9,28-30]. In shrimp GST class mu is expressed in the hepatopancreas and gills [28] and in disk abalone a high level of GST class mu (HdGSTM1) transcripts was observed in the gills and gonad, which may have a role in protecting tissues and gametocytes against endogenous and exogenous stress [9]. In addition, GST class sigma (XIGSTS1-1) of X. laevis is largely present in various amphibian tissues detected by Western blotting, including the liver, lung, heart, kidney and ovary. It is also indicated that XIGSTS1-1 might play an important role in protection against the toxicity of xenobiotics [30]. In our study, a high level of *abGSTsigma* transcripts was shown in the gill, mantle, hypobranchial gland, epipodium and kidney tissues, but was at a low level in the gonad and digestive gland of normal variously colored abalone. These results were somewhat different from the earlier studies, suggesting that abGSTsigma might be involved in some functions differing from the other class GSTs.

3.3. The expression pattern of abGSTsigma gene in tissues of H. diversicolor during the time course of bacterial challenge

Based on the results obtained by RT-PCR analysis, four tissues (the gill, mantle, digestive gland and hemocytes) of variously colored abalone were selected and the *abGSTsigma* gene expression pattern was evaluated in the four tissues after bacterial challenge using qPCR. Actin was used as an internal control gene. Saline solution was run in parallel at each time point of bacterial challenge in order to monitor the possible influence of saline solution on *abGSTsigma* expression. The mRNA level of *abGSTsigma* in each corresponding tissue of normal abalones was used as a calibrator to analyze the expression of *abGSTsigma* induced by bacteria.

As shown in Fig. 5, it was observed that the *abGSTsigma* gene was significantly expressed in tissues tested in comparison with the saline control after bacterial challenge. The *abGSTsigma* gene expression was induced not only in the outer tissues (gill and mantle) but also in the inner tissues (hemocytes and digestive gland). The gene expression pattern of abGSTsigma showed a similar trend among the four tissues. Two highest expression peaks of the abGSTsigma gene were observed during the period of bacterial challenge from 3 to 84 h, that is, the *abGSTsigma* gene expression was increased at 3 h after challenge in the hemocytes (Fig. 5A), gill (Fig. 5B), mantle (Fig. 5C) and at 12 h in the digestive gland (Fig. 5D), then the expression was induced to a markedly higher level in the four tissues at 24 h, and this we thought to be the first peak of gene expression. The highest amount of abGSTsigma transcripts in hemocytes, gill, mantle and digestive gland after 24 h of bacterial challenge showed approximately 45fold, 25-fold, 15-fold and 25-fold increases compared to the normal group. It is very interesting to note that *abGSTsigma* gene expression returned to a lower level in the gill, mantle and hemocytes after 36 h postchallenge, and even more sharply down to a normal level in the digestive gland. After 48 h of bacterial challenge, the abGSTsigma mRNA level again increased and the second expression peak was observed around 60-72 h in the mantle, digestive gland and gill, while in the hemocytes the second highest expression level appeared around 48-60 h. Similarly as observed in gill and digestive gland at the time point of 36 h bacterial challenge, the *abGSTsigma* gene returned to a much lower level at 84 h after its highest peak. The expression level of the abGSTsigma gene in the hemocytes and mantle at the second peak was similar to those at the first peak, whereas in the digestive gland the expression level at the second peak around 60-72 h drastically increased and was nearly 80 times higher than at the first peak. In addition, it was also observed that the *abGSTsigma* gene could be induced to express when inoculated with saline solution, but the total expression level was much lower than that induced with bacterial challenge during the time course from 3 to 72 h with the exception of the gill at 24 h and the digestive gland at 3 and 60 h. Moreover, the gene expression induced with saline stimulation appeared higher than that induced by bacterial challenge at 84 h in all four tissues. Tissue-specific expression in normal abalones showed that the *abGSTsigma* transcripts were demonstrably higher in the gill and mantle than in the hemocytes and digestive gland (Fig. 4). Whereas, the abGSTsigma gene expression pattern, as described above postbacterial challenge, demonstrated that the inner tissues such as the hemocytes and digestive gland seemed to be more sensitive to the bacterial challenge, especially in the digestive gland as shown in Fig. 5 in which the *abGSTsigma* gene expression was highly increased to around 1960-fold the normal control at 60-72 h postchallenge. The reason may be related to the status of this animal. As is known, the aquatic environment is easily polluted with environmental xenobiotic compounds. GSTs are important phase II xenobiotic metabolism enzymes and induction of this type of enzyme would be a positive response of hosts protecting themselves from damage by chemically induced toxicity. In addition, there is a diversity of microorganisms existing in the marine environment and the outer tissues (gill and mantle) could also directly contact the environment in which some microorganisms already exist, thus potentially stimulating abGSTsigma gene expression in the gill and mantle, while the hemocytes and digestive gland are inner tissues which do not directly contact the environment and thus a lower response might be observed in normal animals. However under the conditions of bacterial challenge, the inner tissues are also H.-L. Ren et al./Developmental and Comparative Immunology 33 (2009) 980-990



Fig. 5. Inducibility of *abGSTsigma* mRNA in hemocytes (A), gill (B), mantle (C) and digestive gland (D) of *H. diversicolor* during the time course of bacterial challenge analyzed using quantitative real-time PCR. "N": the normal groups. Statistical analysis of differences between the saline control and the bacterial challenged groups was done by one-way analysis of variance (ANOVA) using SPSS 13.0 software. An asterisk indicates that the difference is statistically significant at p < 0.05 levels.

stimulated by invading bacteria and thus the higher *abGSTsigma* gene expression detected in the present study.

GSTs are versatile functional enzymes. Sigma class GSTs play a crucial role in xenobiotic metabolism in the same way as other classes of GSTs. T. japonicus GST class sigma (GST-S) is up-regulated in response to exposure to two oxidative stress inducing agents, hydrogen peroxide (H₂O₂) and heavy metals (copper and manganese), suggesting that Tigriopus GST-S expression modulated by prooxidant chemicals may play a role against oxidative stress [6]. In a recent study three sigma GSTs (HdGSTS1, HdGSTS2, and HdGSTS3) of disk abalone are reported and it was found that HdGSTS1 exhibits a proper inducibility by pollutants but the others reveal a minor role in the response of pollutants [31]. In our study, we found abGSTsigma expressions in hemocytes, gill, mantle, and digestive gland of H. diversicolor injected with bacteria, and the upregulated abGSTsigma was induced with bacterial infection. The present data may support the hypotheses that the abGSTsigma had relationships with the immune event of bacterial infection and that the GST members of the multiple function enzyme super family might play a role in the immune functions of organisms in addition to xenobiotic metabolism. As a result, some putative factors such as reactive oxygen species brought forward from respiratory burst in the process of phagocytosis would be considered to be associated with the up-regulation of abGSTsigma in abalones challenged with bacteria besides other factors such as the metabolites produced by live bacteria, the endotoxin (such as lipopolysaccharide (LPS)) released from dead bacteria, and antibacterial immune-related substances synthesized. Previous studies show that there is an increase in oxygen consumption and then an increase in the production of reactive oxygen species (ROS) (which is the so-called respiratory burst) occurred during phagocytosis in animals [32]. The generation of ROS leading to oxidative damage will be interdicted by the antioxidant system in which several enzymes (superoxide dismutase, catalase and glutathione peroxidase) are subsequently induced as antioxidant defenses [33]. In the present study, the SOD activities were increased in the gill, mantle, hemocytes and digestive gland, at different challenge time points in the four tissues tested (Fig. 7). This result indicated that SOD could be induced upon bacterial challenge and its activity increase may account for the respiratory burst produced by bacterial invasion.

3.4. The induction of GST and SOD enzymes in tissues after bacterial challenge

The induction of the total GST enzyme known to be related to oxidative stress and antioxidant defense [6] was also investigated in the above mentioned tissues in order to evaluate whether the *abGSTsigma* gene and its related enzyme are correspondingly induced to express with the bacterial challenge. Total GST activity in each of the tissues was significantly higher when challenged with bacteria in comparison with that of the saline injected and the normal control. In the gill the highest activity was observed after 6 h of bacterial challenge and the increase in GST activity was statistically significant (p < 0.05) during the period of challenge except at 48 and 72 h (Fig. 6b). A similar increasing trend was observed for the mantle after bacterial challenge and the increase in GST activity was statistically significant (p < 0.05), but no

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Fig. 6. Induction of the total GST enzyme in HLS (a), gill (b), mantle (c) and digestive gland (d) of abalone at different times during the course of bacterial challenge. "n": the normal groups. Student's 't'-test was used to determine the differences between the challenged and saline control groups. An asterisk indicates that the difference is statistically significant at p < 0.05 levels.

significant expression was detectable at 48, 60 and 72 h (Fig. 6c). In the digestive gland the highly GST activity was observed after 6 and 36 h of bacterial challenge and the increase in GST activity was statistically significant (p < 0.05) during all the time except 72 h of bacterial challenge (Fig. 6d). Although the HLS did not show significant increase for 3, 24, 48 and 72 h of bacterial challenge, the increase in GST activity after 6, 12, 36, 60 and 84 h of bacterial challenge also showed statistical significance (p < 0.05) when compared to the control (Fig. 6a). Moreover, the saline-injected groups showed significant increase only at 6 h in the gill and mantle and there was no significant increase detected in any of the tissues examined. Saline can induce homologous GST gene expression in that salinity changes induce alpha class GST mRNA up-regulation in the liver of the olive flounder (*Paralichthys olivaceus*) and increases GST activity in Mediterranean mussels (*M. galloprovincialis*) [34]. In the present study SOD activity in hemocytes and digestive gland of abalone was significantly induced before 36 h of postbacterial challenge (Fig. 7) and these results were more corresponding to those of total GST enzyme in the same tissue upon bacterial challenge (Fig. 6). It was also observed that the SOD activity in the gill and mantle significantly increased until 60 h of postbacterial challenge (Fig. 7), which was relatively corresponding to the total GST enzyme in the gill and mantle (Fig. 6). Induction of SOD and GSTs enzyme activities due to a bacteria *Galleria mellonella* infection was described in a recent publication [16]. Our results may be in agreement with Dubovskiy et al. [16] study and indicated that induction of SOD and GSTs enzyme may lead to the elimination of ROS generated due to bacterial challenge.



Fig. 7. Induction of SOD activities in HLS (a), gill (b), mantle (c) and digestive gland (d) of abalone at different times during the course of bacterial challenge. "*n*": the normal groups. Student's '*t*-test was used to determine the differences between the challenged and saline control groups. An asterisk indicates that the difference is statistically significant at *p* < 0.05 levels.

Interestingly, we found that the activities of SOD and GSTs showed somewhat difference between the inner tissues (hemocytes and digestive gland) and the outer tissues (gill and mantle). The induction of two enzyme activities is relatively corresponding to *abGSTsigma* gene expression at the first peak, but in later stage (after 36 h of postbacterial challenge in hemocytes and digestive gland) these enzyme activities did not show significant induction whereas *abGSTsigma* gene expression was highly increased to a second peak after 48 h of postbacterial challenge. We noted that the activity of SOD and total GSTs enzyme was increased in the outer tissues (gill and mantle) even after 60 or 84 h of postbacterial challenge. Combined with the *abGSTsigma* gene expression we assumed whether the outer tissues are more prone to responding to the bacterial infection than the inner tissues, which is left to be studied further.

In our study, SOD and total GSTs enzyme were significantly induced in the gill, mantle, digestive gland and hemocytes of bacteria-challenged abalone. The significant induction of SOD and total GSTs activity further supported the presumption that the *abGSTsigma* gene expression in the study responding to the bacterial challenge might be associated with the antioxidant defense reaction. It is commonly known that a higher GST activity can imply a greater detoxification capacity [35,36], and so it can be postulated from our present study that *abGSTsigma* was not only involved in antioxidant defenses as a biotransformation enzyme to detoxify the damage in the digestive gland produced by bacteria, but was also probably involved in the direct response against bacteria invading the gill, mantle and hemocytes.

Interactions of immune systems and biotransformation have been demonstrated to be phylogenetically conserved from fish to mammals. In many cases, fish exposure to xenobiotics might be magnified with bacterial infection. The subsequent activation of the immune system can suppress detoxification activities and lead to increased mortality of fish [37]. In the present study, *abGSTsigma* was not down-regulated in *H. diversicolor* following injection with bacteria, differing from GST expression in the liver, spleen and head kidney of fish [38]. Knowledge concerning the immune defense and stress responses in abalone is scare and whether the sigma class GST of abalone plays an important role in the process of immune defense after bacterial challenge still waits to be further characterized.

4. Concluding remarks

A full-length cDNA sequence encoding an abGSTsigma has been characterized in this study, and abGSTsigma transcripts were widely distributed in various tissues tested of normal abalones. After bacterial challenge, the abGSTsigma gene and total GSTs enzyme were significantly expressed in the hemocytes, gill, mantle and digestive gland. The induction of SOD and GSTs resulted in the elimination of reactive oxygen species (ROS) indicating antioxidant activities involved in the innate immune defense. The present study indicated that the sigma class glutathione S-transferase abGSTsigma, a phase II detoxification enzyme, had a positive response to bacterial infection both in the outer and inner tissues of abalones. The findings from this study will provide new insights into the functions of GSTs and may lead to in depth studies concerning the potential interactions between immune reactions and biotransformation exerted by abGSTsigma.

Acknowledgements

This work was supported by a grant (2007AA091406) from the National High Technology Research, Development Program of China (863 Program) and MEL Young Scientist Visiting Scholarship (MEL0905). We thank Professor John Hodgkiss for assisting in the preparation of this manuscript.

References

- Blanchette B, Feng X, Singh BR. Marine glutathione S-transferases. Mar Biotechnol 2007;9:513–42.
- [2] Salinas AE, Wong MG. Glutathione S-transferases—a review. Curr Med 1999;6:279–309.
- [3] Ding YC, Hawkes N, Meredith J, Eggleston P, Hemingway J, Ranson H. Characterization of the promoters of Epsilon glutathione transferases in the mosquito Anopheles gambiae and their response to oxidative stress. Biochem J 2005;387:879–88.
- [4] Jowsey IR, Thomson AM, Flanagan JU, Murdock PR, Moore GBT, Meyer DJ, et al. Mammalian class Sigma glutathione S-transferases: catalytic properties and tissue-specific expression of human and rat GSH-dependent prostaglandin D-2 synthases. Biochem J 2001;359:507–16.
- [5] Cho SG, Lee YH, Park HS, Ryoo K, Kang KW, Park J, et al. Glutathione Stransferase mu modulates the stress-activated signals by suppressing apoptosis signal-regulating kinase 1. J Biol Chem 2001;276:12749–55.
- [6] Lee YM, Lee KW, Park H, Park HG, Raisuddin S, Ahn IY, et al. Sequence, biochemical characteristics and expression of a novel Sigma-class of glutathione S-transferase from the intertidal copepod, *Tigriopus japonicus* with a possible role in antioxidant defense. Chemosphere 2007;69:893–902.
- [7] Wang L, Liang XF, Liao WQ, Lei LM, Han BP. Structural and functional characterization of microcystin detoxification-related liver genes in a phytoplanktivorous fish, Nile tilapia (*Oreochromis niloticus*). Comp Biochem Physiol C 2006;144:216–27.
- [8] Lee YM, Chang SY, Jung SO, Kweon HS, Lee JS. Cloning and expression of alpha class glutathione S-transferase gene from the small hermaphroditic fish *Rivulus marmoratus* (*Cyprinodontiformes, Rivulidae*). Mar Pollut Bull 2005;51: 776–83.
- [9] Wan Q, Whang I, Lee J. Molecular characterization of mu class glutathione-Stransferase from disk abalone (*Haliotis discus discus*), a potential biomarker of endocrine-disrupting chemicals. Comp Biochem Physiol B 2008;150:187–99.
- [10] Tomarev SI, Zinovieva RD, Guo K, Piatigorsky J. Squid glutathione S-transferase. Relationships with other glutathione S-transferases and S-crystallins of cephalopods. J Biol Chem 1993;268:4534–42.
- [11] Fitzpatrick PJ, Krag TO, Hojrup P, Sheehan D. Characterization of a glutathione S-transferase and a related glutathione-binding protein from gill of the blue mussel, *Mytilus edulis*. Biochem J 1995;305:145–50.
 [12] Yang HL, Zeng QY, Li EQ, Zhu SG, Zhou XW. Molecular cloning, expression and
- [12] Yang HL, Zeng QY, Li EQ, Zhu SG, Zhou XW. Molecular cloning, expression and characterization of glutathione S-transferase from *Mytilus edulis*. Comp Biochem Physiol B 2004;139:175–82.
- [13] Blanchette BN, Singh BR. Purification and characterization of the glutathione-S-transferases from the northern quahog *Mercinaria mercinaria*. Mar Biotechnol 1999;1:74–80.
- [14] Hoarau P, Damiens G, Romeo M, Gnassia-Barelli M, Bebianno MJ. Cloning and expression of a GST-pi gene in *Mytilus galloprovincialis*. Attempt to use the GST-pi transcript as a biomarker of pollution. Comp Biochem Physiol C 2006;143:196–203.
- [15] Wang KJ, Ren HL, Xu DD, Cai L, Yang M. Identification of the up-regulated expression genes in hemocytes of variously colored abalone (*Haliotis diversicolor* Reeve, 1846) challenged with bacteria. Dev Comp Immunol 2008;32: 1326–47.
- [16] Dubovskiy IM, Martemyanov VV, Vorontsova YL, Rantala MJ, Gryzanova EV, Glupov VV. Effect of bacterial infection on antioxidant activity and lipid peroxidation in the midgut of *Galleria mellonella* L. larvae (Lepidoptera, Pyralidae). Comp Biochem Physiol C Toxicol Pharmacol 2008;148:1–5.
- [17] Habig WH, Pabst MJ, Jacoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 1974;249:7130–9.
- [18] Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974;47:469–74.
- [19] Mannervik B, Danielson UH. Glutathione transferases-structure and catalytic activity. CRC Crit Rev Biochem 1988;23:283-337.
- [20] Thomson AM, Meyer DJ, Hayes JD. Sequence, catalytic properties and expression of chicken glutathione-dependent prostaglandin D2 synthase, a novel class Sigma glutathione S-transferase. Biochem J 1998;333:317–25.
- [21] Kanaoka Y, Ago H, Inagaki E, Nanayama T, Miyano M, Kikuno R, et al. Cloning and crystal structure of hematopoietic prostaglandin D synthase. Cell 1997;90:1085–95.
- [22] Armstrong RN. Structure, catalytic mechanism, and evolution of the glutathione transferases. Chem Res Toxicol 1997;10:2–18.
- [23] Hayes JD, Flanagan JU, Jowsey IR. Glutathione transferases. Annu Rev Pharmacol Toxicol 2005;45:51–88.
- [24] Oost vander R, Goksoyr A, Celander M, Heida H, Vermeulen NPE. Biomonitoring of aquatic pollution with feral eel (*Anguilla anguilla*). II. Biomarkers: pollution-induced biochemical responses. Aquat Toxicol 1996;36:189–222.
- [25] Torres-Rivera A, Landa A. Glutathione transferases from parasites: a biochemical view. Acta Trop 2008;105:99–112.
- [26] Frova C. Glutathione transferases in the genomics era: new insights and perspectives. Biomol Eng 2006;23:149–69.
- [27] Ivarsson Y, Mackey AJ, Edalat M, Pearson WR, Mannervik B. Identification of residues in glutathione transferase capable of driving functional diversification

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in evolution. A novel approach to protein redesign. J Biol Chem 2003;278: 8733-8.

- [28] Contreras-Vergara CA, Harris-Valle C, Sotelo-Mundo RR, Yepiz-Plascencia G. A mu-class glutathione S-transferase from the marine shrimp *Litopenaeus vannamei*: molecular cloning and active-site structural modeling. J Biochem Mol Toxicol 2004;18:245–52.
- [29] Doi AM, Pham RT, Hughes EM, Barber DS, Gallagher EP. Molecular cloning and characterization of a glutathione S-transferase from largemouth bass (*Micropterus salmoides*) liver that is involved in the detoxification of 4-hydroxynonenal. Biochem Pharmacol 2004;67:2129–39.
- [30] Carletti E, De Luca A, Urbani A, Sacchetta P, Di Ilio C. Sigma-class glutathione transferase from *Xenopus laevis*: molecular cloning, expression, and sitedirected mutagenesis. Arch Biochem Biophys 2003;419:214–21.
- [31] Wan Q, Whang I, Lee J. Molecular cloning and characterization of three sigma glutathione S-transferases from disk abalone (*Haliotis discus discus*). Comp Biochem Physiol B 2008;151:257–67.
- [32] Hooper C, Day R, Slocombe R, Handlinger J, Benkendorff K. Stress and immune responses in abalone: limitations in current knowledge and investigative methods based on other models. Fish Shellfish Immunol 2007;22:363–79.

- [33] Gutteridge JM, Halliwell B. Free radicals and antioxidants in the year 2000. A historical look to the future. Ann N Y Acad Sci 2000;899:136–47.
- [34] Choi CY, An KW, An MI. Molecular characterization and mRNA expression of glutathione peroxidase and glutathione S-transferase during osmotic stress in olive flounder (*Paralichthys olivaceus*). Comp Biochem Physiol A 2008;149: 330–7.
- [35] Pflugmacher S, Wiegand C, Oberemm A, Beattie KA, Krause E, Codd GA, et al. Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatoxin microcystin-LR: the first step of detoxication. Biochim Biophys Acta 1998;1425:527–33.
- [36] Beattie KA, Ressler J, Wiegand C, Krause E, Codd GA, Steinberg CEW, et al. Comparative effects and metabolism of two microcystins and nodularin in the brine shrimp *Artemia salina*. Aquat Toxicol 2003;62:219–26.
- [37] Reynaud S, Raveton M, Ravanel P. Interactions between immune and biotransformation systems in fish: a review. Aquat Toxicol 2008;87:139-45.
- [38] Chambras C, Marionnet D, Taysse L, Deschaux P, Moreau J, Bosgiraud C. Xenobiotic-metabolizing enzymes in carp (*Cyprinus carpio*) liver, spleen, and head kidney following experimental *Listeria monocytogenes* infection. J Toxicol Environ Health Part A 1999;56:205–19.

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