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Transcriptional up-regulation of disk abalone selenium dependent glutathione peroxidase by H_2O_2 oxidative stress and *Vibrio alginolyticus* bacterial infection

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Abstract Selenium dependent glutathione peroxidase (Se-GPx) belongs to the family of selenoprotein, which acts mainly as an antioxidant in the cellular defence system. We have identified Se-GPx full length cDNA from disk abalone (*Haliotis discus discus*) designated as AbSe-GPx. It has a characteristic codon at ^{223}TGA^{225} that corresponds to selenocysteine (Sec) amino acid as U_{75}. The full length cDNA consists of 675 bp, an open reading frame encoding 225 amino acids. Sequence characterization revealed that AbSe-GPx contains a characteristic GPx signature motif 2 (^{97}LGFPCNQF^{104}), an active site motif (^{183}WNFEKF^{188}) and essential residues for the enzymatic function. Additionally, the eukaryotic selenocysteine insertion sequence (SECIS) is conserved in the 3′ UTR. The AbSe-GPx amino acid sequence exhibited the highest level of identity (46%) with insect (*Ixodes scapularis*) GPx, and shares 41% with bivalve (*Unio tumidus*) Se-GPx. The RT-PCR analysis revealed that AbSe-GPx mRNA was expressed constitutively in gill, mantle, gonad, abductor muscle, digestive tract, and hemocytes in a tissue specific manner. AbSe-GPx mRNA expression was significantly up-regulated in gill and digestive tract tissues after H_2O_2 injection and *Vibrio alginolyticus* infection. However, AbSe-GPx expression was not up-regulated after Aroclor 1254 injection. These results indicate that AbSe-GPx mRNA is expressed at a basal level in abalone tissues, which can be

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up-regulated transcriptionally by H_2O_2 oxidative stress and *Vibrio alginolyticus* infection. Therefore, AbSe-GPx may be involved in a protective role against H_2O_2 oxidative stress and immune defence against bacterial infection.

Introduction

Production of reactive oxygen species (ROS), including superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), alkyl peroxides, singlet oxygen ($^1O_2$), and hydroxyl radicals (OH) in aerobic organisms is a common phenomenon. ROS play an important role in host defence mechanism against microbial infection. There are numerous studies about the immune related role of ROS, which contributes to the pathogenesis of several diseases [1,2]. Compared to vertebrates, invertebrates must rely on an efficient innate immune defence to protect them against invaders, since they do not possess an adaptive immune system with specific antibodies and antigen receptors [3]. In molluscs, defence against pathogens is covered mainly by a variety of innate immunity systems and components, including the use of RNA interference (RNAi), pattern-recognition receptors (PRRs), antibacterial peptides (AMPs), phagocytosis, encapsulation, respiratory burst, and melanization by pro-phenoloxidase activating system (proPO) and ROS production [4–6]. It has been proved that phagocytosis is one of the important immune defences in invertebrates, when the organism is infected by a microorganism. During the process of phagocytosis, the membrane bound enzymes and host NADPH-oxidase get activated, which leads to up-regulation of the glycolytic reactions with high oxygen consumption. Then, the molecular oxygen reduces to the $O_2^{\cdot-}$ anion, leading subsequently to the production of other forms of ROS to kill or inactivate foreign invaders [1,7]. However, accumulations of these ROS are very toxic to animals and cause serious cell damage. The extent of biological damage caused by excessive ROS depends largely on the antioxidant defences, which can be counteracted by non-enzymatic (ascorbic acid, β-carotene, glutathione, α-tocopherol) and enzymatic antioxidant systems, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and thioredoxin peroxidase (TPx) [1,2]. Glutathione peroxidase (GPx) is the generic name of the GPx family of enzymes, which have been described as six different multiple isozymes in mammals (Table 1) [8–13]. These GPxs are classified into two subgroups as selenium dependent glutathione peroxidase (Se-GPx) and non-selenium glutathione peroxidase (non-Se-GPx), based on the presence of Sec encoded by a TGA. Functionally, GPx family enzymes are detoxifying lipids and H_2O_2, which are produced during phagocytosis or physiological metabolisms. They catalyze the reduction of organic hydroperoxide (ROOH) and H_2O_2 to lipid alcohol and/or water by oxidizing glutathione, with the oxidized glutathione then being recycled by glutathione reductase [9,14,15].

At present, few molluscan GPxs have been identified and are available in GenBank, such as Se-GPx of bivalves *U. tumidus* [16], *Dreissena polymorpha* [17], *Corbicula fluminea* (ABQ24217), Pacific oyster, *Crassostrea gigas* (ABS19600) and non-SeGPx of *Aplysia californica* (AF510851). In marine molluscs, only one complete Se-GPx sequence has been identified from Pacific oyster. More importantly, there is no detailed description of Se-GPx from any of the marine molluscs to date. Abalone is a marine mollusc, considered as one of the most highly priced shellfish in worldwide aquaculture. However, abalone production has decreased gradually due to many reasons, including disease outbreaks and unfavorable environmental conditions [18,19]. These invertebrates are increasingly being brought into mariculture, where it is important to monitor immune function to minimize the stresses that could suppress their immunity. Recently, a link has been established in abalone between increased stress and decreased immune function, leading to higher rates of bacterial infections and mortality. However, our understanding of immune systems and stress responses in abalone is limited [18].

In this study, we isolated a cDNA clone, exhibiting homology to a known Se-GPx protein family (that also belongs to the selenoprotein family) and named as disk abalone selenium dependent glutathione peroxidase (AbSe-GPx). The AbSe-GPx sequence was characterized and compared with known GPx isozymes. Additionally, we established the tissue specific expression of AbSe-GPx by isolating total RNA from different abalone tissues and RT-PCR. Also, AbSe-GPx mRNA up-regulation was analyzed by oxidative stress and bacterial infection using Aroclor 1254, H_2O_2 and *V. alginolyticus*, respectively.

Table 1 Description of mammalian GPx isozymes

GPx	Name	Group	Cellular location	Reference
GPx-1	Cellular/cytosolic glutathione peroxidase	Se-dependent	Ubiquitously	[8]
GPx-2	Gastrointestinal glutathione peroxidase	Se-dependent	Gastrointestinal	[9]
GPx-3	Plasma/extra cellular glutathione peroxidase	Se-dependent	Kidney	[10]
GPx-4	Phospholipids hydroperoxide glutathione peroxidase	Se-dependent	Testis, kidney	[11]
GPx-5	Epididymal glutathione peroxidase	Se-independent	Epididymal tissue	[12]
GPx-6	Olfactory epithelium glutathione peroxidase	Se-independent/ Se-dependent	Embryo, olfactory epithelium	[13]

Materials and methods

Disk abalone cDNA library and isolation of AbSe-GPx full-length cDNA

An abalone cDNA library was constructed using mRNA isolated from a disk abalone digestive gland and cDNA library construction kit (Creator SMART, Clontech, USA). The cDNA library was normalized using a Trimmer-Direct cDNA normalization kit according to the manufacturer's protocol (Evrogen, Russia). After sequencing the 5' end of 6700 clones with M13 forward primer, the putative clone (cDNA27-A01) was identified and showed similarity to known Se-dependent GPx family proteins using the NCBI BLAST program . Then, the full-length cDNA sequence was obtained by 3' end internal sequencing of the clone using an oligo dT primer, a Big Dye Terminator sequencing kit, and ABI 3700 sequencer (Macrogen, Korea).

Sequence characterization and phylogenetic analysis of AbSe-GPx

Sequence identities of AbSe-GPx with known GPx sequences were determined using NCBI BLAST analysis (http://www.ncbi.nlm.nih.gov). Pairwise and multiple sequence alignments were generated using a ClustalW 1.8 program [20]. A phylogenetic relationship was determined based on the deduced AbSe-GPx amino acid sequence using the neighbor-joining (NJ) method and MEGA 3.1 program [21]. Signal peptide was predicted through a SignalP worldwide P server (http://www.cbs.dtu.dk/). The Se-GPx signature motif 2 was determined by a motif scan program (http://myhits.isb-sib.ch/cgi-bin/motif_scan). The SECIS element and stem loop structure was predicted and generated by a SECISearch 2.19 program (http://genome.unl.edu/SECISearch.html).

Animals

Healthy disk abalone (*H. discus discus*), with an average weight of 50–60 g, were obtained from Fisheries Resources Research Institute (Jeju, Republic of Korea). They were maintained in flat-bottomed rectangular tanks (40 L) of aerated and sand-filtered seawater at 15–18 °C with fresh seaweed feeding materials. A maximum of 10 animals per tank were kept undisturbed for 1 week to acclimatize to their environment before starting the experiment.

Abalone tissues and hemolymph isolation

Abalone gill, mantle, gonad, abductor muscle, and digestive tract tissues were isolated from three healthy animals for the tissue specific AbSe-GPx expression analysis. The abalone hemolymph (2–3 mL per animal) was collected separately into sterilized syringes from three pericardial cavities of the abalone and was centrifuged immediately at 3000 × g for 10 min at 4 °C. The supernatant was removed and hemocytes were collected for RNA extraction. All the tissues and hemocytes were immediately snap-frozen in liquid nitrogen and stored at −70 °C.

Aroclor 1254 and H_2O_2 injection

Polychlorinated biphenyls (PCBs), such as Aroclor 1254, are a widespread aquatic contaminant and possible agents for developing oxidative stress in marine organisms. Antioxidant gene expression behavior due to PCB exposure has not been examined in abalone. Therefore, to investigate the AbSe-GPx mRNA expression, we have used two potential chemicals, namely Aroclor 1254 and H_2O_2. Abalone (n = 9) were injected intramuscularly with 200 μL (1 mg mL^{-1}) of Aroclor 1254 (Sigma, USA) dissolved in methanol, and three abalone were injected with the same volume of methanol as a control group. The gill and digestive tract tissues from the Aroclor injection group were isolated after 6, 12, and 24 h (n = 3 for each time frame), separately. Tissue samples were isolated from the methanol injected animals at 6 h post injection. In another chemical induction experiment, six abalone (n = 6) were injected intramuscularly with 50 μL (0.3 g mL^{-1}) of H_2O_2. Three healthy abalones were kept in the same conditions as a separate control group. The gill and digestive tract tissue samples were isolated from H_2O_2 injected animals at 3 and 6 h time frames (n = 3 for each time frame).

Bacterial challenge by *Vibrio alginolyticus*

Gram-negative *V. alginolyticus* bacteria were obtained from Korean Collection for Type Cultures (No: KCTC2472) for a bacterial challenge experiment. Briefly, *V. alginolyticus* cells were incubated in marine LB plate at 25 °C overnight. A single colony was selected to inoculate 4 mL of marine broth at 25 °C for 16 h, while shaken at 200 rpm. Then inoculated culture (1.5 mL) was centrifuged at 7000 × g for 5 min at 4 °C, when the cell density was 1.0 at 600 nm absorbance. The supernatant fluid was removed and the bacterial pellets were resuspended in phosphate-buffered saline (PBS) and used as bacterial stock for injection. Abalone (n = 9) were injected intramuscularly with 150 μL of *V. alginolyticus* bacteria. Three healthy abalone (n = 3) were injected with the same amount of PBS and kept separately as a control group. Abalone gill and digestive tract tissue samples were isolated 12, 24, and 48 h after the *V. alginolyticus* challenge and at 12 h post injection from the control group.

RNA extraction and cDNA synthesis

Total RNA was extracted from isolated tissues using Tri Reagent™ (Sigma, USA), according to the manufacturer's protocol. The RNA concentration was measured at 260 nm in a UV-spectrometer (BioRad, USA). Then, the purified RNA was diluted up to 1 μg μL^{-1} concentration and it was reconfirmed by visualizing gel band intensity after running the RNA on a 1.5% agarose gel. A sample of 2.5 μg RNA was used to synthesize cDNA from each tissue using a Cloned AMV first-strand cDNA synthesis kit (Invitrogen, USA). Briefly, RNA was incubated with 1 μL of 50 μM oligo$(dT)_{12-18}$ and 2 μL of 10 mM dNTP for 5 min at 65 °C. After incubation, 4 μL of 4× cDNA synthesis buffer, 1 μL of dithiothreitol (DTT, 0.1 M), 1 μL of RNaseOUT™ (40 U μL^{-1}), and 1 μL of cloned AMV reverse transcriptase (15 U μL^{-1}) were added and incubated for 1 h at 45 °C. The reaction was terminated by adjusting the temperature to 85 °C for 5 min. Finally, 1 μL

of RNase H was added to each cDNA sample, incubated at 37 °C for 20 min and the resulting cDNA was stored at −20 °C.

Tissue expression and transcriptional up-regulation analysis of AbSe-GPx by RT-PCR

For a tissue specific expression profile, AbSe-GPx mRNA was assayed in different tissues, namely gill, mantle, gonad, abductor muscle, digestive tract, and hemocytes by RT-PCR. The AbSe-GPx (359-bp) fragment was amplified using gene-specific primers Se-GPx F1 and Se-GPx R1 (Table 2). Primers were obtained from Integrated DNA technologies (USA). All PCR per particular induction were carried out at the same time in a 25 μL reaction volume containing 2 μL of cDNA from each tissue, 2.5 μL of 10× TaKaRa Ex Taq buffer, 2.0 μL of 2.5 mM dNTP mix, 1.0 μL of each primer (20 pmol μL^{-1}), and 0.125 μL (5 U μL^{-1}) of TaKaRa Ex Taq DNA polymerase (TaKaRa, Japan). Preliminarily, PCR was optimized by performing different cycle numbers (n = 22, 24, 26, 28, and 30) to avoid the PCR saturation. After this optimization step, PCR with 28 and 30 cycles were selected for tissue expression profile and 26 cycles for transcriptional up-regulation analysis. Disk abalone ribosomal protein (GenBank: EF103427) was expressed by amplification of a 420-bp cDNA fragment, using ribosomal F2 and ribosomal R2 primers (Table 2) as an internal PCR control. The cycling protocol was: one cycle of 94 °C for 2 min; 22–30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; and one cycle of 72 °C for 5 min for the final extension. The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide. The gel images of PCR products were analyzed as integrated density values using Scion Image analysis software (Scion Image alpha 4.0.3.2, Scion Corporation, MD, USA). The AbSe-GPx expression level was calculated based on the mean of three RT-PCR assays. The AbSe-GPx expression was normalized and interpreted as a ratio with respect to the quantity of abalone ribosomal protein expression in corresponding tissues and time frames. The relative AbSe-GPx expression was calculated as a percentage (%) relative to hemocyte expression level for the tissue specific expression and respective control tissues for each induction experiment. All data represent means ± standard deviation and subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using SPSS 11.5 program. Differences were considered statistically significant at $p < 0.05$.

Results

Isolation and sequence characterization of AbSe-GPx cDNA

One EST clone (cDNA27-A01) was identified from the disk abalone digestive gland cDNA library that showed similarity to known GPx family proteins. It has a codon at ^{223}TGA^{225} encoding Sec amino acid as U_{75}, which is the main characteristic feature of the selanoprotein family. This full-length cDNA sequence was named as disk abalone selenium dependent glutathione peroxidase (AbSe-GPx) and deposited in the NCBI under accession number of EF103379. The nucleotide and deduced amino acid sequences are shown in Fig. 1. The 1473-bp full-length AbSe-GPx consisted of 675 bp, an open reading frame (ORF) encoding 225 amino acids. The AbSe-GPx protein has putative molecular mass of 25.0 kDa with a 5.8 isoelectric point (p*I*). Using the SignalP 3.0 program, the N-terminus of the AbSe-GPx was found to have 19 amino acids putative signal peptide, representing a cleavage site at 19–20 aa. Also, AbSe-GPx contains a characteristic GPx signature sequence motif 2, consisting of 8 amino acids at ^{97}LGFPCNQF^{104}. Also, it showed an extra active site motif located at ^{183}WNFEKF^{188} and glutamine-Gln (Q_{103},Q_{107}), tryptophan-Trp (W_{179},W_{183}) residues, which are responsible for fixation of Se. Additionally, AbSe-GPx showed two arginine residues at R_{124} and R_{195} for directing donor glutathione substrate towards the catalytic center. A potential *N*-glycosylation site ^{112}NATE^{115} was identified using the PROSITE program, which has been observed in other GPx amino acid sequences. Also, AbSe-GPx showed a 745-bp 3′ UTR ending with a polyadenylation (Poly A) tail. In the 3′ UTR, characteristic SECIS element (100 bp) was identified by SECISearch 2.19 program. The putative AATAAA polyadenylation signal was detected at 48 nucleotides upstream of the poly A tail.

ClustalW pairwise amino acid sequence alignment was performed to determine the identity percentage (%) of AbSe-GPx with other species Se-GPxs. The AbSe-GPx exhibited the highest level of identity (46%) with insect (*I. scapularis*) Se-GPx while, it shared 41% with fresh water bivalve (*U. tumidus*) Se-GPx. Sequence comparison of AbSe-GPx with other GPx isozymes showed that higher identities to GPx1 isozyme of bovine (44%), mouse (43%), rat (42%) and human (42%); however it showed lower identities to GPx2 of mouse (37%), rat (37%) human (36%) as well as GPx3, GPx4, GPx5, and GPx6 respective isozymes (Table 3). Additionally, pairwise amino acid comparison was conducted among 40 different GPxs isozymes. The identity range was varied from 26 to 46% between AbSe-GPx and different organisms GPxs. However, results showed that identities were below 50% with mollusc species GPxs such as *U. tumidus* (41%), *C. fluminea* (39%), Pacific oyster (38%) and *D. polymorpha* (36%). ClustalW multiple alignment results of AbSe-GPx with selected vertebrate and invertebrate Se-GPxs are shown in Fig. 2. The results showed that characteristic Sec (U_{75}) residue, ^{97}LGFPCNQF^{104} signature motif 2, ^{183}WNFEKF^{188} active site, functional residues of Gln (Q_{107}), Arg (R_{124}) and Trp (W_{183}) were aligned with all selected sequences. Therefore, sequence characterization

Table 2 A description of primers used in this study

Name	Target	Sequence
Se-GPx F1	RT-PCR amplification	AGCTAACGCCACCGAGATTCTCAA
Se-GPx R1	RT-PCR amplification	AGCTGTGTCGCTAGTGGTCATTGT
Ribosomal F2	Internal PCR control	GGGAAGTGTGGCGTGTCAAATACA
Ribosomal R2	Internal PCR control	TCCCTTCTTGGCGTTCTTCCTCTT

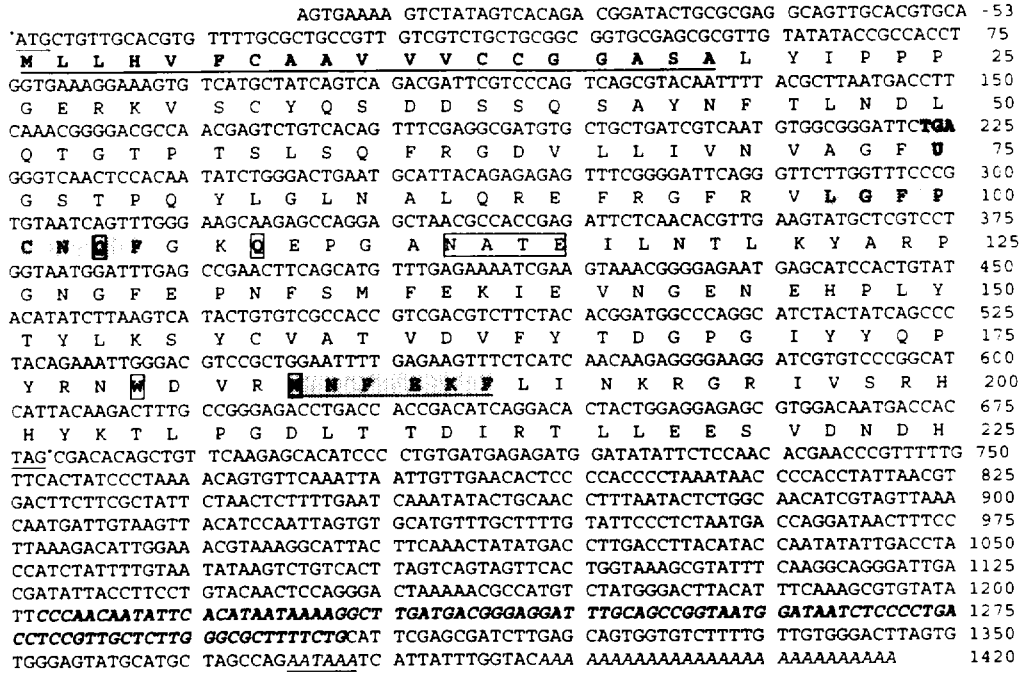

Figure 1 Full length nucleotide and complete amino acid sequences of disk abalone Se-GPx cDNA. The Se-GPx nucleotide sequence has the accession number EF103379 in the GenBank database. The start (ATG) and stop (TAG) codons are underlined with an asterisk (*) on the top. The codon ^{223}TGA^{225} and selenocysteine (U_{75}) are bold shaded. The predicted signal peptide is bold underlined. The Se-GPx signature motif 2 (^{97}LGFPCNQF^{104}) is shaded. The active site motif at ^{183}WNFEKF^{188} is shaded underlined. Putative *N*-glycosylation residues are in a box. Catalytically important residues, Gln (Q_{103}, Q_{107}) and Trp (W_{179}, W_{183}) are bold in a box. The predicted SECIS element in the 3′ UTR is in bold italic. The polyadenylation signal (AATAAA) is underlined in italics and the poly(A) tail is in italic at the end.

results revealed that AbSe-GPx encodes a full-length nucleotide sequence with the main characteristic motifs and functional amino acid residues of the Se-GPx protein family.

Comparison of SECIS element and stem loop structure of AbSe-GPx

The nucleotide sequence of the AbSe-GPx was analyzed with SECISearch 2.19 for occurrence of stem-loop structure resembling SECIS element. The analysis results showed that a 100-bp SECIS element was present in the AbSe-GPx 3′ UTR (Fig. 1), which formed a stem loop secondary structure in the mRNA. Predicted secondary structure of the AbSe-GPx SECIS element, along with those of the mammalian consensus SECIS element and human GPx SECIS described by Kryukov et al. [13] are shown in Fig 3. According to Fig. 3, eukaryotic SECIS elements are composed of two helixes separated by an internal loop, a SECIS core structure, a quartet located at the base of the helix 2, and an apical loop. The quartet is formed by four non-Watson–Crick interacting base pairs, and is the main functional site of the stem loop structure [13,22]. It was predicted that the abalone SECIS element consisted of AUGAC in the 5′ stem, CA in the apical loop, and the CUGAC in the 3′ stem (Fig. 3C).

Table 3 Identity percentage (%) comparison of AbSe-GPx with known GPx isozymes

Species	GPx isozyme					
	GPx1	GPx2	GPx3	GPx4	GPx5	GPx6
Human	42	36	39	29	38	43
Mouse	43	37	39	29	38	38
Rat	42	37	39	26	38	39
Bovine	44	–	38	28	36	–

Dashes (–) represents unavailable sequences.

Phylogenetic analysis of AbSe-GPx

To determine the position of AbSe-GPx gene in evolution, 37 representative Se-GPx and non Se-GPx sequences were analyzed to construct a phylogenetic tree using a bacteria (*Nitrobacter winogradskyi*) Se-GPx as an out-group. Based on the phylogenetic analysis, mammalian GPx proteins can be classified into six subclusters, named as GPx1, GPx2, GPx3, GPx4, GPx5, and GPx6 (Fig. 4). However, the mammalian GPx4 subcluster was positioned as an independent cluster without any direct link to other mammalian GPx subclusters. Interestingly, GPx1 and GPx2 subclusters were diverted from one main branch of vertebrate origin, whereas GPx3, GPx5, and GPx6 subclusters were diverted from the opposite direction of that origin. The invertebrate GPx protein family was scattered without forming a clearly defined invertebrate GPx cluster. However, some molluscs

Figure 2 ClustalW multiple comparison of disk abalone (*H. discus discus*) Se-GPx amino acid sequence with vertebrate and invertebrate Se-GPx sequences. Residues shaded are completely conserved across all species aligned with AbSe-GPx sequence. The GPx signature motif 2 elements are bold in a box with the name on the top. Active site is bold underlined. The GenBank accession numbers of these sequences are the same as the numbers used in Fig. 4.

and nematodes shared one single subcluster. The new abalone Se-GPx appeared to fit more closely into insect (*I. scapularis*) GPx, which was diverted from mollusc species GPx subcluster. As expected, abalone Se-GPx was connected to the origin of the main invertebrate branch origin. It was observed that some invertebrates, such as white shrimp and fruit fly, were diverged from mammalian GPx1 and GPx4 subclusters, respectively, as previously reported [23]. Therefore, phylogenetic analysis provides evidence that the AbSe-GPx has been derived from a common ancestor with other Se-GPx family proteins, but it extends further following the identification of new Se-GPx family genes.

Tissue specific mRNA expression of AbSe-GPx

The tissue specific mRNA expression was determined by conducting RT-PCR with AbSe-GPx gene specific primers. The AbSe-GPx bands were normalized and interpreted as a ratio with respect to the quantity of abalone ribosomal protein expression in corresponding tissues. Constitutive AbSe-GPx mRNA expression was detected in gill, mantle, abductor muscle, digestive tract tissues, and hemocytes obtained from healthy abalone (Fig. 5a).We also examined the PCR saturation level by conducting different PCR cycles, and our results showed that AbSe-GPx amplification could saturate in 30 cycles in certain tissues, such as mantle, gonad, mussel, and digestive tract (Fig. 5a(A2)). Relative AbSe-GPx expression was calculated based on the lowest expression observed in hemocytes, which is shown as 100% in Fig. 5b. The relative AbSe-GPx mRNA expression was significantly higher in all selected tissues other than the hemocytes ($p < 0.05$), showing tissue specific variation. The highest relative AbSe-GPx mRNA expression was detected in abalone digestive tract tissue.

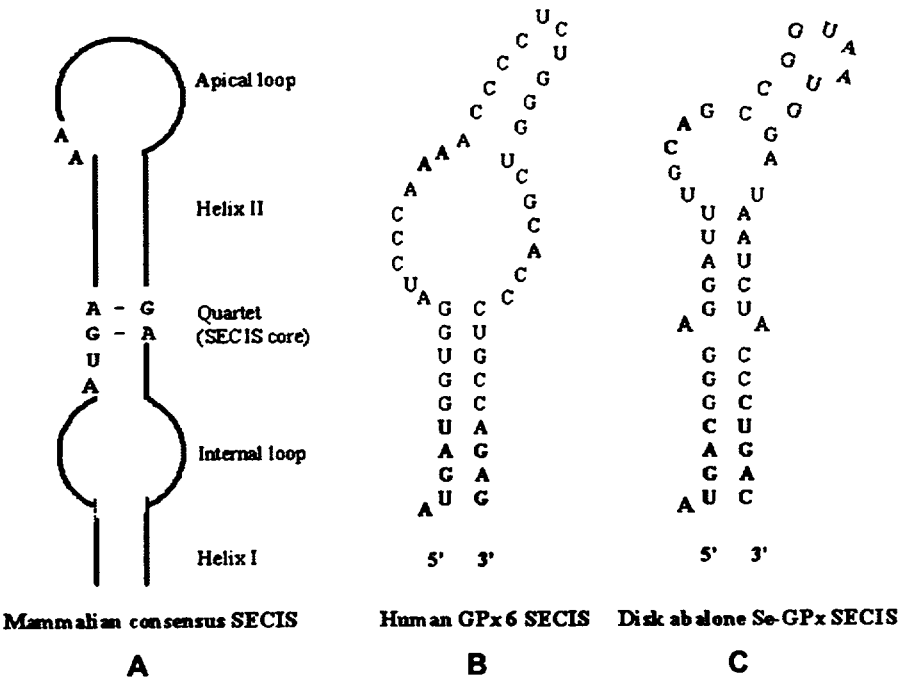

Figure 3 Consensus mammalian SECIS and predicted secondary structure in SECIS of human GPx6 according to Kryukov et al. [13] and predicted AbSe-GPx. Only the upper portions of SECIS elements are shown. Conserved nucleotides in the SECIS core (Quartet) and apical loop are shown in bold face. (A) Mammalian consensus SECIS; (B) human GPx6; (C) disk abalone Se-GPx.

Transcriptional up-regulation of AbSe-GPx by Aroclor 1254, H_2O_2 and *V. alginolyticus*

To determine the transcriptional up-regulation of AbSe-GPx against oxidative stress and bacterial infection, we challenged the abalone with Aroclor 1254, H_2O_2 and *V. alginolyticus*, respectively. For Scion image densitometric analysis, expression results of 26 and 22 PCR cycles were selected for AbSe-GPx and ribosomal protein expression, respectively. The relative AbSe-GPx mRNA was calculated as a percentage (%) based on the gill and digestive expression, which is normalized to ribosomal protein expression. First, AbSe-GPx transcriptional up-regulation was analyzed after the Aroclor 1254 treatment. The results showed that relative AbSe-GPx expression was significantly ($p < 0.05$) decreased in both gill and digestive tract by Aroclor 1254 injection (0.2 mg per animal) during 24 h post injection compared to the methanol injected group (Fig. 6). Transcriptional up-regulation of AbSe-GPx mRNA after H_2O_2 injection is shown in Fig. 7. The relative AbSe-GPx transcripts were significantly ($p < 0.05$) increased in gill and digestive tract compared to control group at 3 and 6 h (Fig. 7b), indicating that the AbSe-GPx is up-regulated after H_2O_2 treatment. The up-regulation continued significantly ($p < 0.05$) higher at 6 h in both tissues, however lower than the relative expression at 3 h. Additionally, the Se-GPx up-regulation level was higher in gill than in the digestive tract.

To assess the induction of AbSe-GPx gene on bacterial infection, abalone were infected by intramuscular injection of *V. alginolyticus*. The result showed that the relative mRNA level was significantly ($p < 0.05$) increased in gill and digestive tract after 12 h infection compared to PBS injected (control) group (Fig. 8). Furthermore, up-regulation was significantly ($p < 0.05$) higher than control up to 48 h post infection; however, it was decreased at 48 h compared to 24 h (Fig. 8b). Additionally, the level of up-regulation was higher in the digestive tract than in the gill. Also, no abalone motility occurred due to *V. alginolyticus* infection during the experimental period.

Discussion

In this study, we have characterized the Se-GPx cDNA sequence from disk abalone with tissue specific mRNA expression and transcriptional up-regulation after H_2O_2 oxidative stress and *V. alginolyticus* infection. One of the objectives of this study was to determine the expression profile and transcriptional up-regulation responses of AbSe-GPx, which may be applicable to the development of stress-immune interactions and defence strategies in abalone-like invertebrates. There has been an extension of the genetic code to include the codon TGA as the 21st codon specifying the presence of the Sec in the polypeptide structure of selenoproteins [24]. The AbSe-GPx contains a ^{223}TGA^{225} codon corresponding to Sec (U_{75}) residue, suggesting that abalone GPx is a selenium dependent protein and could be considered as a member of a selenoprotein family. Most Se-GPxs have amino acids numbering in the range of 200–250; for example, mouse GPx1, 201; human GPx1, 203; bovine GPx1, 205; bivalve (*C. fluminea*), 211; bivalve (*U. tumidus*) 232; bivalve (*D. polymorpha*) 243; Pacific oyster (*C. gigas*) 244. Therefore, AbSe-GPx contains 225 amino acids with the predicted molecular mass of 25 kDa, which is consistent with most molluscs and

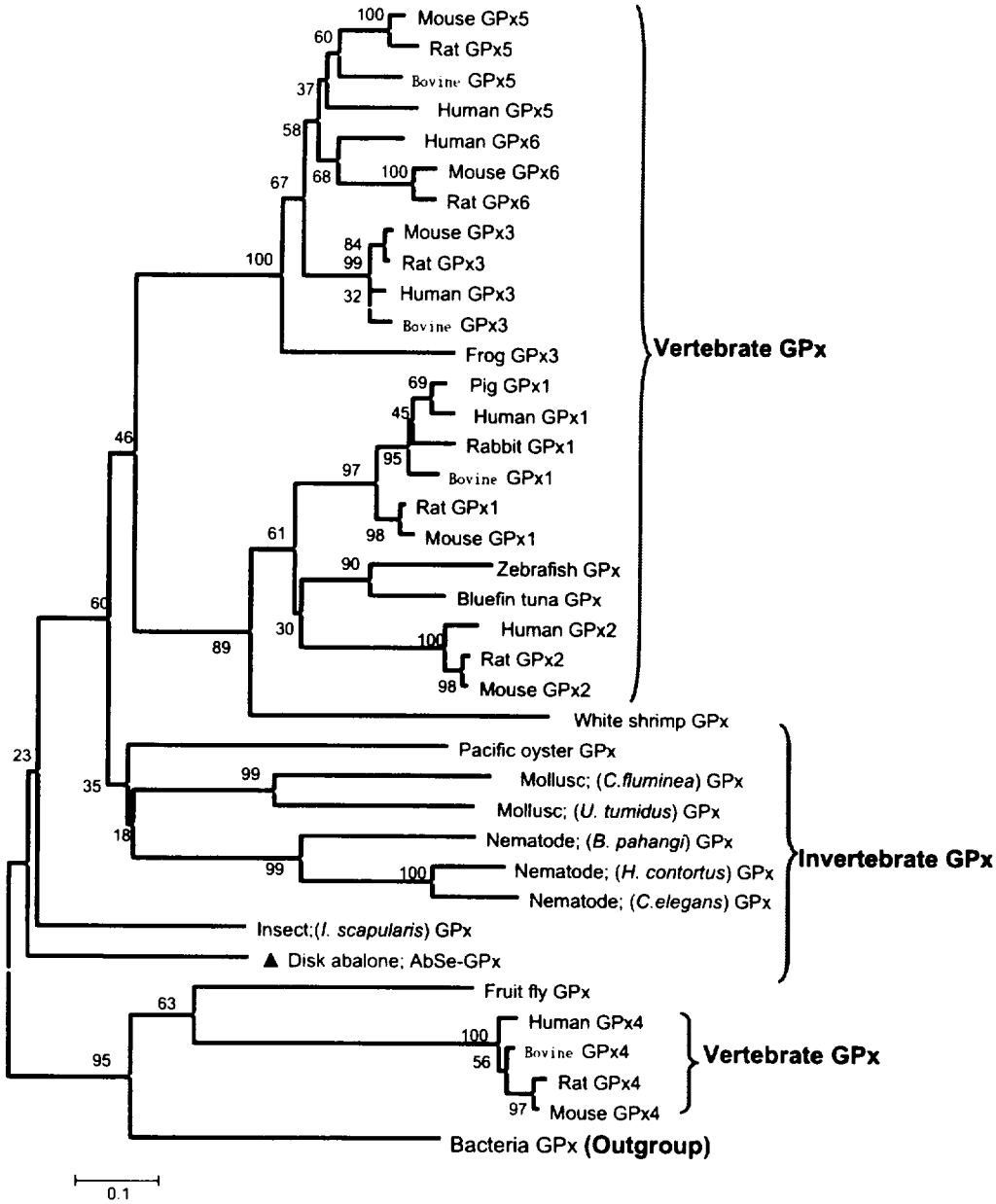

Figure 4 Phylogenetic analysis of disk abalone Se-GPx. The tree is based on an alignment corresponding with 37 representative complete GPx sequences using ClustalW and MEGA (3.1). The numbers at the branches show bootstrap majority consensus values on 1000 replicates. Bacteria (*Nitrobacter winogradskyi*) GPx, YP_319244 was used as an out-group. The GenBank accession numbers for the GPx sequence designations as follows. Disk abalone (*H. discus discus*), EF103379; insect (*Ixodes scapularis*), AAY66814; fruit fly (*Drosophila melanogaster*), NM_139550; nematode (*Caenorhabditis elegans*), NP_509616; nematode (*Haemonchus contortus*), AAT28332; nematode (*Brugia pahangi*), CAA48882; mollusc (*Unio tumidus*), ABH10623; mollusc (*Corbicula fluminea*), ABQ24217; Pacific oyster (*Crassostrea gigas*), ABS19600; white shrimp (*Litopenaeus vannamei*), AY973252; zebrafish (*Danio rerio*), AAO86703; bluefin tuna (*Thunnus maccoyii*), ABO38817; frog (*Xenopus tropicalis*), GPx3, NP_988961; rat (*Rattus norvegicus*) GPx1, NM_030826; GPx2, NM_183403; GPx3, NM_022525; GPx4, NM_017165; GPx5, CAA44274; GPx6, NP_671694; mouse (*Mus musculus*) GPx1, NM_008160; GPx2, BC054848; GPx3, NP_032187; GPx4, NM_001037741; GPx5, NP_034473; GPx6, NP_663426; rabbit (*Oryctolagus cuniculus*) GPx1, NP_001078913; bovine (*Bos taurus*) GPx1, NM_174076; GPx3, NP_776502; GPx4, NM_174770; GPx5, AAY58231; pig (*Sus scrofa*) GPx1, NP_999366; human, (*Homo sapiens*), GPx1, NP_000572; GPx2, NM_002083; GPx3, NM_002084; GPx4, NM_001039848; GPx5, NP_001500; GPx6 NP_874360.

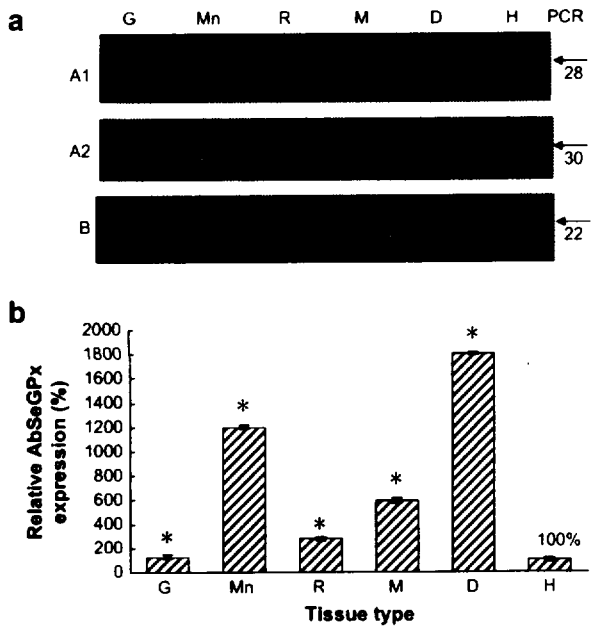

Figure 5 Tissue specific expression of AbSe-GPx mRNA and relative expression percentage. (a) Agarose gel electrophoresis of AbSe-GPx; (A1) 28 PCR cycles, (A2) 30 PCR cycles, and (B) corresponding ribosomal protein expression in 22 PCR cycles. G, gill; Mn, mantle; R, gonad; M, abductor muscle; D, digestive tract; and H, hemocytes. (b) Relative AbSe-GPx mRNA expression percentage (%) in different tissues by densitometric analysis. The expression levels are means of three RT-PCR assays, which are normalized by ribosomal protein expression. Relative expression percentage (%) was calculated relative to the expression recorded in hemocytes as shown 100%. Bars represent the means ± SD and significance difference of the specific tissues was denoted as (*) at $p < 0.05$ level compared to hemocytes.

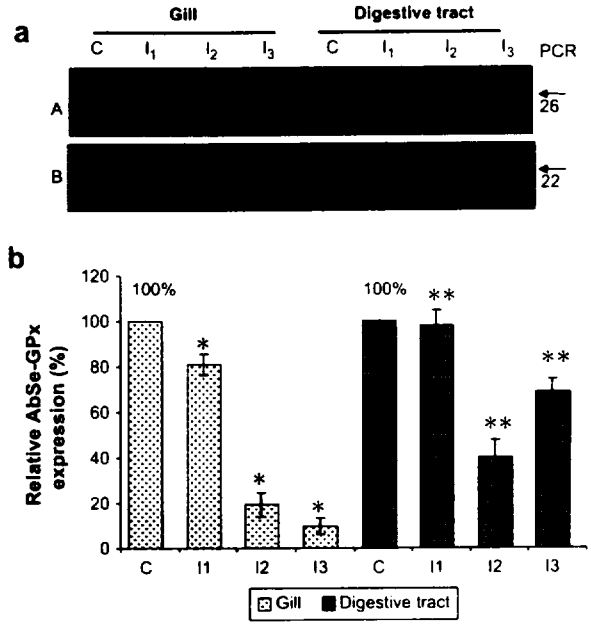

Figure 6 AbSe-GPx up-regulation by Aroclor 1254 oxidative stress. (a) Time course analysis of AbSe-GPx mRNA expression in abalone gill, and digestive tract tissues named top of the respective lanes. C, control (methanol injected); I_1, 6 h post injection; I_2, 12 h post injection; I_3, 24 h post injection of Aroclor 1254. (A) AbSe-GPx expression 26 PCR cycles. (B) Corresponding ribosomal protein expression in 22 PCR cycles. (b) Relative AbSe-GPx expression percentage (%) induced by Aroclor 1254 using densitometric analysis. The expression levels are means of three RT-PCR assays, which are normalized by ribosomal protein expression. Relative expression percentage (%) was calculated relative to the expression recorded in each control tissue of gill and digestive tract as shown 100%. Bars represent the means ± SD and significance differences of the relative expression percentage (%) in gill and digestive were denoted as (*) and (**), respectively, at $p < 0.05$ level compared to respective control.

vertebrates. The deduced amino acid sequence displays two important motifs, namely the Se-GPx signature 2 at ^{97}LGFPCNQF^{104} and the active site at ^{183}WNFEKF^{188}, which contain Gln(Q) and Trp(W) described as important residues for GPx catalytic activity [25]. In previous studies, important amino acids have been identified for different functional roles of Se-GPx. Two residues, Gln_{106} and Trp_{179}, are involved in the fixation of selenium [26]. Also, two arginine residues, Arg_{123} and Arg_{195}, contribute to the electrostatic architecture that directs the glutathione donor substrate toward the catalytic center [27]. Similarly, Gln_{107}, Trp_{179}, Arg_{124}, and Arg_{195} amino acid residues exists in the AbSe-GPx sequence, suggesting they may be involved in its functional and biochemical activities. The structural stability of glutathione peroxidase depends on the three loop structures: the first extending from Asn_{67} to Tyr_{78}, the second from Leu_{96} to Gln_{106}, and the third from Trp_{179} to Phe_{181} [11]. The conservation of Asn_{70}, Tyr_{81}, Leu_{97}, Gln_{107}, Trp_{179}, and Phe_{185} are observed in the abalone Se-GPx sequence, and those may be used to form similar loop structures.

Ursini et al. [11] pointed out that although phylogenetic analysis of GPx family proteins has been conducted, the order of the divergence of each isozyme from a common ancestor has not been clarified. In the present analysis, extensive numbers of GPx isozymes have been included for covering the vertebrates and invertebrates to get better insight into the GPx evolution. The tree showed that six GPx isozymes clustered separately, while GPx4 diverged first. The early divergence and isolated cluster position of GPx4 could be supported by the functional explanation described by Arthur [14], as GPx4 has been shown to have different substrate specificity compared with other GPx family proteins. It also explained that early divergence might be advantageous for the enzyme to acquire specific functions. Similarly, AbSe-GPx has shown early divergence compared to all other mollusc species, showing a need to continue with further research on closely related mollusc species.

Expression of Sec containing proteins requires the presence of a *cis*-acting mRNA structure, called SECIS element, which directs Sec insertion at UGA codon. In bacteria, this structure is located in the coding region immediately downstream of the Sec-encoding UGA codon,

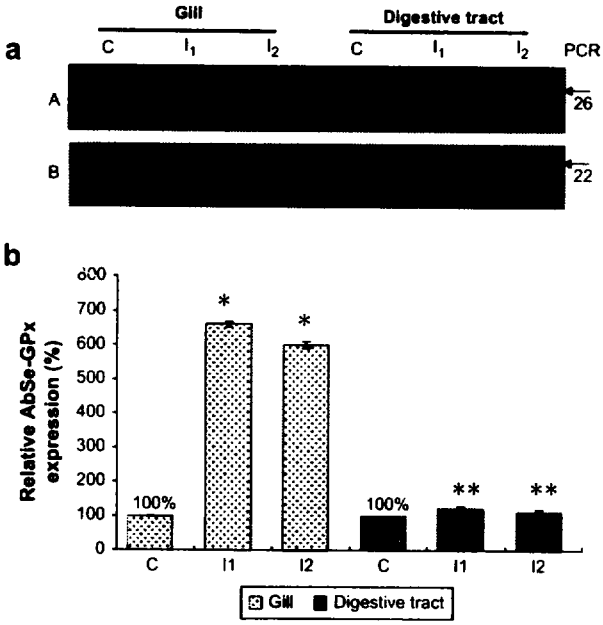

Figure 7 AbSe-GPx up-regulation by H_2O_2 oxidative stress. (a) Time course analysis of AbSe-GPx mRNA expression in abalone gill, and digestive tract tissues named top of the respective lanes. C, control (un-induced); I_1, 3 h post injection; I_2, 6 h post injection of H_2O_2. (A) AbSe-GPx expression in 26 PCR cycles. (B) Corresponding ribosomal protein expression in 22 PCR cycles. (b) Relative AbSe-GPx expression percentage (%) induced by H_2O_2 using densitometric analysis. The expression levels are means of three RT-PCR assays, which are normalized by ribosomal protein expression. Relative expression percentage (%) was calculated relative to the expression recorded in each control tissue of gill and digestive tract as shown 100%. Bars represent the means ± SD and significance differences of the relative expression percentage (%) in gill and digestive tract were denoted as (*) and (**), respectively, at $p < 0.05$ level compared to respective control.

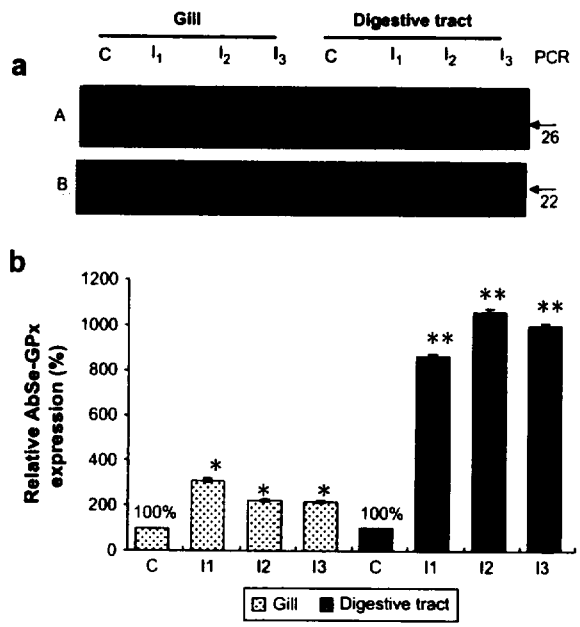

Figure 8 AbSe-GPx up-regulation by *V. alginolyticus* bacteria infection. (a) Time course analysis of AbSe-GPx mRNA expression in abalone gill, and digestive tract tissues named top of the respective lanes. C, control (PBS injected); I_1, 12 h post injection; I_2, 24 h post injection; I_3, 48 h post injection of *V. alginolyticus*. (A) AbSe-GPx expression in 26 PCR cycles. (B) Corresponding ribosomal protein expression in 22 PCR cycles as positive control. (b) Relative AbSe-GPx expression percentage (%) induced by *V. alginolyticus* bacteria infection using densitometric analysis. The expression levels are means of three RT-PCR assays, which are normalized by ribosomal protein expression. Relative expression percentage (%) was calculated relative to the expression recorded in each control tissue of gill and digestive tract as shown 100%. Bars represent the means ± SD and the significance differences of the relative expression percentage (%) in gill and digestive tract were denoted as (*) and (**), respectively, at $p < 0.05$ level compared to respective control.

whereas in eukaryotes a completely different SECIS element has evolved in the 3′ UTR [28]. SECIS element and related stem loop structures have been identified in human, bovine, rat, and mouse GPxs [13,29]. We also observed SECIS element in the 3′ UTR with AUGAC, CA, and CUGAC conserve regions in the predicted stem loop structure of AbSe-GPx. Therefore, having SECIS element with conserve stem loop structure in the abalone Se-GPx may show similar Sec incorporation, which is required to be confirmed after functional assay. Thereby, abalone SECIS is the first described SECIS element in the mollusc to date.

Tissue specific expression varies with the type of GPx isozymes. GPx1 is known to distribute ubiquitously in various tissues; however, expression of other GPx isozymes is more tissue specific than GPx1 [14,30]. Liu et al. [23] showed wide distribution of GPx mRNA in gill, muscle, hepatopancreas, digestive tract, and hemocytes of white shrimp (*L. vannamei*), and its role as an antioxidant. Similarly, we observed diversified tissue expression profile in abalone, expressing Se-GPx mRNA in all six selected tissues. Therefore, higher identity to known GPx1 isozymes and a diversified tissue expression profile indicate that the cloned abalone Se-GPx may be a GPx1 isoform. Previous studies on tissue expression analysis of fresh water bivalves *U. tumidus* and *D. polymorpha* showed that Se-GPx mRNA was expressed at higher level in the digestive gland than gill [16,17]. In the present study, the highest relative expression level of AbSe-GPx in the digestive tract is similar to those previous results.

AbSe-GPx transcriptional regulation was analyzed in gill and digestive tract tissues after oxidative stress induced by Aroclor 1254, H_2O_2, and bacterial infection by *V. alginolyticus*. The polychlorinated biphenyls (PCBs) such as Aroclor 1254 are environmental contaminants often considered as stress agents responsible for biological and physiological alterations in living species. They are widely using for expression studies of antioxidant genes like Se-GPx to evaluate the toxicity status of the marine environment. However, our results showed that Aroclor 1254 injection (0.2 mg per animal) was not able to switch on the transcriptional up-regulation

of AbSe-GPx in gill and digestive tract tissues during the 48 h. Similarly, bivalve *U. tumidus* showed no induction of Se-GPx expression in the digestive gland and excretory system after a 72 h exposure to Aroclor 1254 at different concentrations from 2.5 to 50 mg kg^{-1} fresh weight [16]. Catalase and GPx are the most important antioxidants for detoxifying H_2O_2, thereby preventing the generation of hydroxyl radical by the Fenton reaction. Dash and group [31] showed that a higher concentration of H_2O_2 (9.79 mM) induced GPx mRNA transcripts as compared to lower concentration of H_2O_2 (0.979 mM) during a 1 h exposure of fresh water *Hydra vulgaris*. Therefore, we used H_2O_2 as a potential oxidative stress agent for Se-GPx mRNA up-regulation analysis. As expected, AbSe-GPx mRNA level is up-regulated after H_2O_2 induction in gill and digestive tissues. Additionally, up-regulation at 3 h post injection could be considered as an immediate response to H_2O_2 since H_2O_2 easily can penetrate into cellular structures or tissues.

The Gram-negative bacterium *V. alginolyticus* treatment was conducted in order to evaluate the relationship between bacterial infection and antioxidant response in abalone. In a bacteria challenged experiment, AbSe-GPx transcript was up-regulated significantly at 12 h in the gill and digestive tract. Similarly, in shrimp (*L. vannamei*), the GPx transcription was increased significantly at 12 h after *V. alginolyticus* infection [23]. The enhancement of AbSe-GPx is considered as a antioxidant defence against excess ROS after infection with bacteria, suggesting that bacterial challenged abalone would generate a mass of ROS, which need to be detoxified by Se-GPx protein translated by induced extra Se-GPx transcripts mRNA.

Michiels et al. [32] has shown that the presence of Se-GPx, SOD, and catalase all are important to the normal functioning of cells, and these three enzymes work cooperatively. Taking this argument further into invertebrate animals like abalone, we have identified and characterized different antioxidant genes, such as Mn-SOD [33], catalase (DQ821496), Cu,Zn-SOD (DQ821492), thioredoxin peroxidase isoform 1 (EF103376), thioredoxin peroxidase isoform 2 (EF103376), thioredoxin 2 (EF103378), and peroxiredoxin 6 (EF103356) from our disk abalone. Therefore, it seems obvious that expression and transcriptional up-regulation patterns are highly interrelated with antioxidant enzymes in different tissues in a single species. Identification of this Se-GPx in abalone will therefore expand our research further into more integrated expression studies with all antioxidant genes in disk abalone.

In conclusion, we identified and characterized the Se-GPx full-length cDNA from the marine mollusc disk abalone (*H. discus discus*). Constitutive mRNA expression was observed in tissue specific manner, suggesting that disk abalone Se-GPx may play an important role as an antioxidant and in innate immune responses at a basal level with varying capacities. Results revealed that AbSe-GPx transcription can be induced by H_2O_2 and *V. alginolyticus*. Hence, the expression profiles of Se-GPx mRNA in abalone may have potential use as molecular biomarkers for assessing stress, heavy metal toxicity, specific microbial infection, and the quality of the aquatic environment. Additionally, description of AbSe-GPx tissue specific expression profiles will be an important step toward future functional analysis of the selenoprotein family.

Acknowledgements

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