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Short communication

Two variants of selenium-dependent glutathione peroxidase from the disk abalone *Haliotis discus discus*: Molecular characterization and immune responses to bacterial and viral stresses

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ABSTRACT

Glutathione peroxidase (GPx) is an essential member of the antioxidant systems of living organisms and may be involved in immune defense against pathogenic invasion. In the current study, two selenium-dependent glutathione peroxidases (*AbSeGPx*s) that shared 54.3% identity were identified from the disk abalone *Haliotis discus discus*. The open reading frames (ORFs) of *AbSeGPx-a* and *AbSeGPx-b* coded for 222 and 220 amino acids, respectively, with a characteristic selenocysteine residue encoded by an opal stop codon (TGA). The conserved selenocysteine insertion sequence (SECIS) element was predicted in the 3′ untranslated region (UTR) of both isoforms, and they were found to form two stem-loop structures. Amino acid comparison and phylogenetic studies revealed that the AbSeGPxs were closely related to those in other mollusk species and were evolutionarily distinct from those of other taxonomic groups. The SYBR Green qPCR was employed in investigating the transcripts of *AbSeGPx*s. The expression of *AbSeGPx*s mRNA was examined in different embryonic developmental stages and differential expression patterns for *AbSeGPx-a* and *AbSeGPx-b* were noted. Meanwhile, the highest expression of *AbSeGPx*s was detected in the hepatopancreas of healthy adult animals. Next, transcriptional levels were profiled in hemocytes of adults to determine the immune responses of *AbSeGPx*s to microbial infections. The results revealed the significant up-regulation of *AbSeGPx-a* in a time-dependent manner after bacterial (*Listeria monocytogenes* and *Vibrio parahaemolyticus*) and viral (viral hemorrhagic septicemia virus) infections. Consequently, these findings indicate that *AbSeGPx-a* and *AbSeGPx-b* might be involved in the embryonic development of disk abalone and the regulation of immune defense system of adult animals.

1. Introduction

Reactive oxygen species (ROS), generated during aerobic respiration and substrate oxidation, are involved in number of biological processes including stimulation of signal transduction, mediation of cell apoptosis, and defense against invading microbial pathogens [1,2]. Excessive ROS accumulation causes detrimental effects to cells by oxidative damage to proteins, lipids, and nucleic acids. Consequently, maintaining ROS at an optimum level is crucial for the survival of aerobic organisms. In order to protect against damage induced by the oxidative stress occurring with excessive ROS, aerobic organisms have developed enzymatic [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] and non-enzymatic [ascorbic acid, β-carotene, glutathione (GSH), α-tocopherol, etc.] antioxidant defense systems [3,4].

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Glutathione peroxidases (EC.1.11.1.9) are a family of endogenous antioxidant enzymes that play a key role in mediating the antioxidant defense reaction by catalyzing the reduction of hydrogen peroxide and organic hydroperoxides to water and oxygen using GSH as an electron donor [5]. Based on the presence of selenocysteine (SeC), which is encoded by an opal TGA codon [6], GPxs are divided into two sub-families including selenium-dependent glutathione peroxidase (SeGPx) and non-selenium glutathione peroxidase (non-SeGPx). Four SeGPxs have been identified in different organs and cellular compartments in mammals: cellular GPx (GPx1), gastrointestinal GPx (GPx2), extracellular GPx (GPx3), and phospholipid hydroperoxide GPx (GPx4) [7,8].

In comparison with vertebrates, only a few studies have been reported on the characterization of mollusk SeGPxs. It has been demonstrated that marine mollusk species including *Mizuhopecten yessoensis* [9], *Chlamys farreri* [10], *Venerupis philippinarum* [11], and *Haliotis discus discus* [12] express *SeGPx* mRNA in response to bacterial infections. In addition, the antioxidant effect of SeGPxs after toxic chemical exposure has been reported in *V. philippinarum* [11], *Mytilus galloprovincialis* [13], and *H. discus discus* [12]. However, there are very few reports on immune responses of mollusk SeGPxs to virus infections.

The disk abalone, *H. discus discus*, is a commercially important marine gastropod species in Korean aquaculture. In the past decades, high mortality and poor growth have occurred as a result of environmental contamination mainly with chemicals and pathogens [14–16]. As with other invertebrates, disk abalones have to rely exclusively on their innate immunity because they do not possess an adaptive immune system. Consequently, knowledge about innate immune defense mechanisms of this species will support the health management and disease control in abalone aquaculture. In the present study, two distinct variants of SeGPx were identified from the disk abalone transcriptome database, and the level of mRNA expression in different stages of embryonic development, basal transcription in adult animals, and temporal mRNA expression of adults in response to bacterial and viral infections were investigated.

2. Materials and methods

2.1. Identification of cDNA sequences

A disk abalone transcriptome database was constructed by employing the Roche 454 platform and GS-FLX™ technology (DNA Link, Inc.) as previously described [17]. Shotgun sequencing reads were assembled into contigs by the *de novo* assembling technique. Two contigs that are homologous to the known SeGPx were identified from the transcriptome database after analyzing the expressed sequence tags (ESTs) by applying the BLAST algorithm and designated as *AbSeGPx-a* and *AbSeGPx-b*.

2.2. Bioinformatics analysis

The cDNA and deduced amino acid sequence of AbSeGPxs were analyzed with the DNAssist 2.2 program. The signal peptide and its cleavage site were predicted by means of the SignalP 4.1 online tool (http://www.cbs.dtu.dk/services/SignalP/). The NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) was used to predict the potential N-glycosylation sites in AbSeGPx proteins. Fundamental domains and characteristic active sites were analyzed using the NCBI conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the MotifScan program (http://myhits.isb-sib.ch/cgi-bin/motif_scan) in the ExPASy tools. The selenocysteine insertion sequence (SECIS) element and its secondary structure were predicted by the SECISearch 2.19 program (http://genome.unl.edu/SECISearch.html). The representative GPx sequences from other vertebrates and invertebrates were obtained from the NCBI database. Percentages of identity and similarity between AbSeGPxs and other homologues were calculated using the MatGAT program [18] with default parameters. Multiple sequence alignments were performed with the ClustalW program in BioEdit 7.1 software. A neighbor-joining (NJ) phylogenetic tree was constructed using the MEGA version 5.05 software, and 5000 bootstrap trials were performed to determine the confidence value of the taxa.

2.3. Collection of different embryonic developmental stages

Disk abalone (*H. discus discus*) egg and sperm were collected from the Ocean and Fisheries Research Institute, Jeju. They were allowed to fertilize in filtered sea water at 18 °C and different stages of embryonic development were obtained: egg, sixteen cell stage [3 h post fertilization (pf)], morula (4 h 30 min pf), gastrula (6 hpf), trochophore (16 hpf), and three time points of veliger stage (early; 24 hpf, middle; 36 hpf and late; 48 hpf). Before the collection of samples, the developmental synchronies were monitored using light microscope to ensure that over 75% of the larvae in each sample were at the same stage. Each of the collected samples were washed with cold PBS and stored at −70 °C.

2.4. Animal collection, maintenance, and immune challenges

Adult disk abalones (~50 g), were purchased from a commercial abalone farm (Youngsoo) in Jeju Island, Republic of Korea. They were acclimatized in a recirculating seawater system at a salinity of 34 ± 0.6‰ and a temperature of 20 ± 1 °C in the laboratory for one week before the experiment. Animals were fed with fresh marine seaweed (*Undaria pinnatifida*) during the acclimatization period.

To analyze the tissue expression pattern, different tissues including digestive tract, gills, hemolymph, gonads, muscles, mantle, and hepatopancreas were collected from four disk abalones. For the immune challenge experiments abalones were infected with two bacterial species (*Listeria monocytogenes* and *Vibrio parahaemolyticus*) and a viral strain, viral hemorrhagic septicemia virus (VHSV), as previously reported [17]. Saline (0.9% NaCl) was used to suspend the bacterial and viral strains and was injected into a group of abalone that served as negative controls. Four disk abalones were randomly sampled from each challenged group at different time points (3, 6, 12, 24, 48, 72, 96, and 120 h) and hemocytes were isolated. All the tissues isolated from unchallenged and challenged abalones were immediately frozen in liquid nitrogen and stored at −70 °C until use.

2.5. Complementary DNA synthesis and quantification of AbSeGPx transcripts

Total RNA was extracted from different developmental stages and adult tissues using Tri Reagent™ (Sigma) according to the manufacturer's protocol. The concentration of total RNA was determined by measuring the absorbance at 260 nm and the purity was assessed based on the absorbance ratio of A_{260}/A_{280}. An aliquot (2.5 μg) of total RNA was reverse transcribed by using PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa, Japan) following the manufacturer's recommendations. Subsequently, cDNA was diluted 40 fold and stored in −20 °C.

The Real Time System TP800 Thermal Cycler Dice™ (TaKaRa, Japan) was used to determine the level of transcripts of *AbSeGPxs* in healthy and immune-challenged abalones. Gene-specific primers for *AbSeGPx-a* and *AbSeGPx-b* (Supplementary Table 1) were designed and the SYBR Green quantitative real-time PCR (qPCR)