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Characterization of a 1-cysteine peroxiredoxin from big-belly seahorse (*Hippocampus abdominalis*); insights into host antioxidant defense, molecular profiling and its expressional response to septic conditions

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ABSTRACT

1-cysteine peroxiredoxin (Prx6) is an antioxidant enzyme that protects cells by detoxifying multiple peroxide species. This study aimed to describe molecular features, functional assessments and potential immune responses of Prx6 identified from the big-belly seahorse, *Hippocampus abdominalis* (HaPrx6). The complete ORF (666 bp) of *HaPrx6* encodes a polypeptide (24 kDa) of 222 amino acids, and harbors a prominent peroxiredoxin super-family domain, a peroxidatic catalytic center, and a peroxidatic cysteine. The deduced amino acid sequence of HaPrx6 shares a relatively high amino acid sequence similarity and close evolutionary relationship with *Oplegnathus fasciatus* Prx6. The purified recombinant HaPrx6 protein (rHaPrx6) was shown to protect plasmid DNA in the Metal Catalyzed Oxidation (MCO) assay and, together with 1,4-Dithiothreitol (DTT), protected human leukemia THP-1 cells from extracellular H_2O_2-mediated cell death. In addition, quantitative real-time PCR revealed that *HaPrx6* mRNA was constitutively expressed in 14 different tissues, with the highest expression observed in liver tissue. Inductive transcriptional responses were observed in liver and kidney tissues of fish after treating them with bacterial stimuli, including LPS, *Edwardsiella tarda*, and *Streptococcus iniae*. These results suggest that HaPrx6 may play an important role in the immune response of the big-belly seahorse against microbial infection. Collectively, these findings provide structural and functional insights into *HaPrx6*.

1. Introduction

Species of fish and shellfish are challenged by a variety of pathogenic organisms and hazardous substances in marine aquatic ecosystems. They are vulnerable to different oxidative stresses, which can be caused by i) biological components, such as pathogenic microorganisms and toxic algae; ii) physical components, such as UV radiation and high temperature; and iii) chemical factors, including heavy metal pollutants [1]. Reactive oxygen species (ROS) encompass a number of reactive molecules that are naturally produced as byproducts of aerobic metabolism in somatic cells [2], including superoxide anions ($O_2^{\bullet-}$), superoxide radicals (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\bullet$) [3]. These ROS are vital for various cellular functions, like cell proliferation, cell differentiation, maintaining intracellular signaling cascades [4–6], and eliciting immune responses [7,8]. However, excessive levels of free radicals may cause serious damage to host cells, including DNA strand breaks, lipid peroxidation, protein oxidation, and cell death [9]. To protect host cells from oxidative stress, aerobic cells have developed a potent antioxidant defense system comprised of molecular antioxidants, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (Gpx), and peroxiredoxin (Prx) enzymes.

Thiol-specific antioxidant enzymes of the Prx family can prevent cellular oxidative damage [10]. Those Prx family members contain the catalytically active cysteine (Cys) residues at N- or C-terminals, even though the N-terminal region is directly involved in peroxidase activity [11]. Based on the number of catalytically active Cys

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residues, Prxs are categorized into three main subgroups: typical 2-Cys Prxs (Prx1, Prx2, Prx3, Prx4), atypical 2-Cys Prx (Prx5), and 1-Cys Prx (Prx6). Among the six Prx subgroups, Prx 6 consists of a single catalytically active peroxidatic Cys, while the others contain two redox-active Cys residues [12]. Peroxiredoxins are crucial for the reduction of hydrogen peroxide, peroxynitrite, and organic hydroperoxides (ROOH) through their peroxidase activity ($ROOH + 2e^- \rightarrow ROH + H_2O$) [13,14]. In addition, Prxs are associated with host immune responses against viral and bacterial infections [12,15].

Prx6 is referred to as a "bifunctional" enzyme; it is a crucial participant in cellular redox reactions that protect cells against oxidative injury [12], and is also involved in phospholipid metabolism *via* its Ca^{2+}-independent phospholipase A_2 activity [16–18]. Prx6s are restricted to the cytosol, which enables them to function efficiently [19], however, the catalytic efficacies of the Prx family are comparatively lower than those of CAT or Gpx [20]. To date, several reports have described the functional and transcriptional responses of Prx6 derived from the kingdoms, Animalia and Plantae. However, comprehensive studies exploring structure and function at the sequence level or host immune responses elicited by Prx6 are limited to only a few fish species. Hence, further studies in other teleost species, like the big-belly seahorse, would broaden the understanding of Prx6 and its associated functions.

The big-belly seahorse (*Hippocampus abdominalis*) is an important aquaculture species that has been used as a traditional medicine in Asian countries like Korea, China, and Japan. Due to overexploitation and pathogenic attacks [21,22], the seahorse is categorized under appendix II of CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora). In addition, unfavorable environmental stress conditions lead to the suppression of immunity in aquatic animals, but an understanding of stress responses and immune mechanisms in the seahorse is currently lacking. Therefore, to broaden our understanding of the physiology and immunity of seahorses, the present study focused on the characterization of big-belly seahorse peroxiredoxin 6 (*HaPrx6*) in terms of its molecular sequence features and structure, the antioxidant activity of its recombinant protein, tissue-specific mRNA expression, and mRNA expression in response to bacterial challenges.

2. Methodology

2.1. H. abdominalis transcriptomic database construction

A seahorse transcriptomic database was constructed by the 454 GS-FLX™ sequencing technique. Briefly, total RNA from blood, liver, kidney, gill, and spleen tissues of 18 seahorses was extracted and purified with an RNeasy Mini kit (Qiagen, USA), followed by assessment of quality and quantity using an Agilent 2100 Bioanalyzer (Agilent Technologies, Canada). In order to prepare the GS-FLX 454 shotgun database, RNA was fragmented into an average size of 1147 bp using the Titanium system (Roche 454 Life Science, USA). Sequencing was performed on half of a picotiter plate on a Roche 454 GS-FLX™ DNA platform by Macrogen Corp (Korea). The raw 454 reads were trimmed to remove adaptor and low-quality sequences, and *de novo* assembled into contigs using GS Assembler (Roche 454 Life Science, USA) with default parameters set.

2.2. HaPrx6 cDNA sequence identification

A putative *HaPrx6* cDNA contig (Accession number: KX228392) that showed homology with known Prx6 counterparts was identified and isolated from the seahorse transcriptomic database. The *HaPrx6* sequence was affirmed through homology screening by the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi), available from the National Center for Biotechnology Information (NCBI).

2.3. Bioinformatics profiling

HaPrx6 cDNA was subjected to DNAssist version 2.2 to obtain the putative coding sequence (CDS) and to derive the corresponding protein sequence. The functional domain search was accomplished using the conserved domain search program (CDD; http://www.ncbi.nlm.nih.gov/cdd) at NCBI. Conserved cysteine residues were predicted using the Cys finder (http://clavius.bc.edu/~clotelab/DiANNA/). Potential *N*-linked glycosylation sites were predicted *via* the NetNGlyc web server (http://www.cbs.dtu.dk/services/NetNGlyc/). Pairwise amino acid identity and similarity between homologues were evaluated by the MatGAT program [23]. Multiple sequence alignment was performed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The phylogenetic tree was constructed using the neighbor-joining (NJ) method available in the Molecular Evolutionary Genetics Analysis (MEGA v 5.0) program [24], with bootstrap values calculated for 5000 replications to estimate the robustness of internal branches. The folding pattern for the tertiary arrangement of the HaPrx6 protein sequence was predicted using the FoldIndex© (http://bip.weizmann.ac.il/fldbin/findex) online bioinformatics tool [25].

2.4. Cloning of HaPrx6 CDS

The cDNA fragment encoding the CDS of the *HaPrx6* gene was cloned into the pMAL-c5X vector (New England Biolabs, USA) after carrying out a restriction digestion at *Nde* I and *Bam*H I sites (Supplementary Table 1) using corresponding enzymes. Briefly, PCR was performed for a 50 μL reaction containing 50 ng of liver cDNA, 5 μL of 10 × *Ex Taq* Buffer, 4 μL of 2.5 mM dNTPs, 25 pmol of each primer, and 5 units (U) of *Ex Taq* polymerase (TaKaRa, Japan). The PCR cyclic conditions were as follows: an initial denaturation at 95 °C for 5 min, 30 cycles of amplification at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. The restriction digested *HaPrx6* cDNA fragment and the pMAL-c5X vector were gel purified using an Accuprep™ gel purification kit (Bioneer, Korea). Overnight ligation was then continued at 4 °C using the Mighty Mix DNA Ligation Kit (TaKaRa, Japan). Subsequently, the recombinant construct was transformed into *Escherichia coli* DH5α competent cells, and positive clones were confirmed by restriction digestion followed by sequence verification (Macrogen, Korea). Finally, the sequence verified recombinant construct was transformed into *E. coli* ER2523 (New England BioLabs, UK) competent cells for protein expression.

2.5. Overexpression and purification of recombinant HaPrx6 (rHaPrx6)

rHaPrx6 fusion protein expression and purification were carried out as described in our previous study [12], following the instructions for the pMAL Protein Fusion and Purification System (New England Biolabs, USA). Recombinant maltose binding protein (rMBP) was also expressed and purified under the same conditions. rHaPrx6 and rMBP protein concentrations were determined by the Bradford assay [26]. Purified protein samples were further analyzed on a 12% SDS-PAGE gel along with a protein marker (Enzynomics, Korea) in order to examine the degree of rHaPrx6 protein induction, purity, and integrity. The SDS-PAGE gel was stained with 0.05% Coomassie blue R-250 and subjected to a standard de-staining procedure.

2.6. Metal-catalyzed oxidation (MCO) assay

The MCO assay was conducted to assess the DNA protection