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Antioxidative properties and structural features of atypical 2-Cys peroxiredoxin from *Sebastes schlegelii*

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ABSTRACT

Atypical 2-Cys peroxiredoxin (Prx5) is an antioxidant protein that exerts its antioxidant function by detoxifying different reactive oxygen species (ROS). Here, we identified mitochondrial Prx5 from rockfish (SsPrx5) and described its specific structural and functional characteristics. The open reading frame (ORF) of SsPrx5 (570 bp) was translated into a 190-amino acid polypeptide that contained a mitochondrial targeting sequence (MTS), thioredoxin 2 domain, two Prx-specific signature motifs, and three conserved cysteine residues. Sequence comparison indicated that the SsPrx5 protein sequence shared greatest identity with teleost orthologs, where the phylogenetic results showed an evolutionary position within the fish Prx5. The coding sequence of SsPrx5 was scattered in six exons as found in other vertebrates. Additionally, the potent antioxidant functions of recombinantly expressed SsPrx5 protein was demonstrated by insulin reduction and extracellular H_2O_2 scavenging both *in vitro* and *in vivo*. Quantitative real time PCR (qPCR) detected ubiquitous mRNA expression of *SsPrx5* in healthy rockfish tissues, with remarkable expression observed in gill, liver, and reproductive tissues. Prompt transcription of *SsPrx5* was shown in the immune-stimulated gill and liver tissues against *Streptococcus iniae* and lipopolysaccharide injection. Taken together, present results suggest the indispensable role of SsPrx5 in the rockfish antioxidant defense system against oxidative stresses and its role in maintaining redox balance upon pathogen invasion.

1. Introduction

Antioxidants are essential in aerobic organisms in order to interact with the free radicals *via* maintaining redox signaling, and prevent cell damage caused by oxidative stresses. Peroxiredoxin (Prx) is a highly conserved superfamily of selenium-free and heme-free peroxidases (Bryk et al., 2000) which is well-categorized as an antioxidant. Prxs catalyze the reduction of hydrogen peroxides, alkyl hydro-peroxides, and peroxynitrites (Chae et al., 1994; Flohe et al., 1999; Wood et al., 2003b). They also have been recognized as therapeutic tools because of their role in specific pathophysiological conditions, including different types of cancers (Deighton et al., 2014; Jo et al., 2013; Ummanni et al., 2012; Wang et al., 2013). Additionally, Prxs are involved in a variety of signaling pathways, including NF-κB, JAK/STAT, and MAPK kinase pathways (Brigelius-Flohe and Flohe, 2011; Kim et al., 2015; Liu et al., 2012). The roles of Prxs in regulating inflammation through NF-κB activation have been extensively studied (Kim et al., 2013a, 2013b).

Depending on the number of conserved cysteine (Cys) residues and their location, Prxs can be divided into three subclasses: typical 2-Cys Prx, atypical 2-Cys Prx, and 1-Cys Prx (Bryk et al., 2000; Kang et al., 1998). Typical 2-Cys Prxs are the largest class of Prxs, and are known to act as obligate homodimers (Hirotsu et al., 1999; Wood et al., 2002). Prx1-4 belonging to typical 2-Cys Prxs. Atypical 2-Cys Prx (Prx5) is the second group of Prxs that shares the same catalytic mechanism for reducing peroxides as typical 2-Cys Prxs. However, the atypical 2-Cys Prx is functionally monomeric (Declercq et al., 2001; Seo et al., 2000). 1-Cys Prxs (Prx6) are the third class of the Prx family, possessing only one conserved peroxidatic cysteine for its peroxidation function (Choi et al., 1998). To date, six Prx isoforms have been identified in species including mammals (Chae et al., 1999) and fish (Perez-Sanchez et al., 2011). As

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for the cellular localization of peroxiredoxins, Prx1 and 2 can be found in the cytosol, and Prx4 and Prx6 are located in the plasma and the plasma membrane, respectively (Fujii and Ikeda, 2002). Prx3 and Prx5 are localized in the mitochondria (Knoops et al., 1999; Wood et al., 2003b).

Prx5 is the final isoform identified in the Prx superfamily (Knoops et al., 2011), which is also known as PRDX5, ACRI, PMP20, and AOEB166. Prx5 function is different from other Prxs due to the unique properties of its enzymatic mechanism (Knoops et al., 2011). Prx5 exhibits high affinity toward organic peroxides and has a wide cellular distribution (Lu et al., 2006), as it can be mostly found in the mitochondria, followed by cytosol, peroxisomes, and nucleus. Prx5 interacts with the peroxisome receptor 1, which produces the mitochondrial form of Prx5 (Park et al., 2016). Thiol dependent Prx5 is reduced by thioredoxin or thioredoxin-like proteins, and is therefore commonly known as thioredoxin peroxidase (Seo et al., 2000). Differential expression patterns of Prx5 in response to different pathogenic infections and oxidative stresses reveal its putative significance in redox signaling (Knoops et al., 2011; Li et al., 2011; Perez-Sanchez et al., 2011). It has also been shown that Prx5 overexpression reduces apoptosis in human cells (Kropotov et al., 2006; Yuan et al., 2004). Additionally, H_2O_2 or tertbutylhydroperoxide caused cell death and DNA damage was reduced in Prx5 expressed Chinese hamster cells (Banmeyer et al., 2004).

However, collective investigations of genomic structure, mRNA expression and distinct functional aspects of Prx5 in teleost species were not sufficient. Here, we identified the Prx5 homolog in Korean black rockfish, *Sebastes schlegelii* (SsPrx5) and characterized this protein with bioinformatics tools to decipher its structural features that may be important for its peroxidase function. Evolutionary relationships were analyzed *via* phylogenetic tree constructions and exploring the genomic structural features. Further, we cloned the SsPrx5 coding sequence and carried out functional assays to corroborate its role in the antioxidant defense system of rockfish. Therefore, the present study aimed to validate the potential oxidoreductase and peroxidase activities in both *in vitro* and *in vivo* and expand the current knowledge on teleostean Prx5 orthologs.

2. Methodology

2.1. cDNA and gDNA database construction

The black rockfish transcriptomic database was constructed as explained in our previous study (Perera et al., 2016) following the 454 GS-FLX sequencing technique (Droege and Hill, 2008). The black rockfish genomic database was constructed by *de novo* genome assembly method. In brief, Illumina paired-end (PE) and mate-pair (MP) library preparation protocols were used to prepare sequencing libraries and they were subjected to size selection for Illumina MiSeq and NextSeq sequencing techniques. Further, long non-fragmented DNA libraries were prepared using Pacbio manufacturer protocols (Pacific Biosciences, CA, USA). Finally, the transcriptomes were mapped to *Oplegnathus fasciatus* and curated consensus genes were subjected to functional annotation using reference databases.

2.2. Molecular characterization

First, a putative *SsPrx5* coding cDNA contig was isolated from the rockfish transcriptomic database and was compared with known Prx5 orthologs available from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/; NCBI). The open reading frame (ORF) and putative SsPrx5 amino acid sequence were derived from the DNAssist program (ver. 2.2). The conserved domain search program (CDD; http://www.ncbi.nlm.nih.gov/cdd) at NCBI and ExPASy PROSITE (http://prosite.expasy.org/) was used to delineate the domain architecture of SsPrx5. The SsPrx5 amino acid sequence was subjected to the Prot-Param tool in ExPASy (http://web.expasy.org/protparam/) to determine the molecular mass and isoelectric point. Subcellular location and mitochondrial targeting sequence (MTS) were predicted using TargetP (http://www.cbs.dtu.dk/services/TargetP/) and MITOPROT (http://ihg.gsf.de/ihg/mitoprot.html) servers. EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) and Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) tools were used to derive the pairwise and multiple sequence alignment of Prx5 orthologs, respectively. The phylogenetic tree was reconstructed through the Neighbor-Joining (NJ) method at MEGA (ver. 5.0). The predicted 3D structure of SsPrx5 was derived using the SWISS-MODEL protein modeling server (http://swissmodel.expasy.org/) and visualized using PyMOL v1.5 software.

2.3. Genomic analysis

Complete cDNA and gDNA sequences of SsPrx5 were aligned with the Splign genomic alignment tool (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi) and the exon-intron boundaries of SsPrx5 were determined. The Ensemble genome browser database (http://asia.ensembl.org/index.html) was mined to obtain the other Prx5 genomic structures for comparison with the SsPrx5 genomic structure. Then, all the genomic arrangements were visualized using GeneMapper (v2.5).

2.4. Cloning and protein expression

The *SsPrx5* gene-coding cDNA fragment, except the MTS sequence, was amplified using gene-specific primers (Forward; GAGAGAcatatgATGCCGATTAAGGTTGGTGAACGTCTC and Reverse; GAGAGAgaattcCTACAGCTCAGACAGAACATTGGAAGCC) from the liver cDNA. PCR cycles were as follows: initial incubation at 94 °C for 4 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The pMAL-c5x cloning plasmid and the DNA fragments were restriction digested and ligated together using Mighty Mix (TaKaRa, Japan). Recombinant plasmids were transformed into *Escherichia coli* DH5α competent cells and cloning efficiency was confirmed by restriction digestion of the positive clones followed by sequence verification (Macrogen, Korea).

Sequence-verified plasmids were again transformed into ER2523 (NEB Express) competent cells for protein expression. Cells were cultured at 37 °C until the OD_{600} reached approximately 0.5; then, the culture was induced for 10 h at 20 °C by adding 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG). Pelleted cells (centrifuged at 4000 × *g*, 20 min, 4 °C) were re-suspended in column buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl) and protein was purified following the pMAL Protein Fusion and Purification protocol (New England Biolabs, USA). To determine the protein concentration, eluted fractions of recombinant SsPrx5 (rSsPrx5) and maltose-binding protein (rMBP) were subjected to a Bradford assay (Bradford, 1976). The proteins were run using 12% SDS-PAGE and the success of protein expression and purification was determined.

2.5. Insulin reduction

An insulin reduction assay was conducted as described by Wang et al. (2015) with slight alterations. Briefly, 25, 50, 100, or 200 μg/mL rSsPrx5, 200 μg/mL rMBP, 4 mM EDTA (pH 7.0), and 2 mg/mL insulin were used for the different treatments. Solutions were brought to 250 μL with phosphate buffered saline (PBS) and 2 mM