Fish and Shellfish Immunology 88 (2019) 364–374

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Cloning and functional characterization of rockfish peroxiredoxin 4 homolog with its innate immune responses

G.I. Godahewa, N.C.N. Perera, Hyukjae Kwon, Jehee Lee*

Department of Marine Life Sciences & Fish Vaccine Research Center, Jeju National University, Jeju Self-Governing Province, 63243, Republic of Korea

ARTICLE INFO

Keywords:
Antioxidant
Innate immunity
Peroxiredoxin 4
ROS

ABSTRACT

The fourth member of the typical 2-cysteine peroxiredoxin (Prx4) is a well-known antioxidant enzyme, which reduces different peroxides in their catalytic process. The present study reports the identification of the rockfish *Sebastes schlegelii* Prx4 (SsPrx4) at a genomic level, as well as the characterization of its structural and functional features. SsPrx4 harbors a complete ORF of 786 bp encoding a polypeptide (29 kDa) of 262 amino acids (aa) with an isoelectric point of 6.2. Thioredoxin 2 domain was prominent in the SsPrx4 sequence, which has a signal peptide (31 bp) at the N-terminus. Hence, the SsPrx4 may be functionally active in the cytoplasm of rockfish cells. Moreover, two VCP motifs and three catalytic triad residues (^{112}T, ^{115}C, ^{191}R) were identified in the SsPrx4 protein sequence. A peroxidatic cysteine ($^{115}C_P$) and resolving cysteines ($^{236}C_R$) were detected at the VCP motifs. The rockfish Prx4 genome consists of seven exons, which are similar to the architecture of other Prx4 orthologs. The deduced amino acid sequence of SsPrx4 shares a relatively high amino acid sequence identity (91.6%) and close evolutionary relationship with *Miichthys miiuy* and *Stegastes partitus* Prx4. The potential for scavenging extracellular H_2O_2 was evidenced by the purified recombinant SsPrx4 protein (rSsPrx4) *in vitro* system. Moreover, rSsPrx4 may protect the plasmid DNA in a metal-catalyzed oxidation system and catalyze the reduction of an insulin disulfide bond. Quantitative real-time PCR revealed that SsPrx4 mRNA was ubiquitously expressed in fourteen different tissues, with the highest expression observed in the liver followed by the ovary, and kidney tissues. Transcriptional modulations were observed in liver and spleen tissues of rockfish after injecting them with bacterial stimuli, including *Streptococcus iniae*, LPS, and a viral mimic of poly I:C. Together, the results suggest that SsPrx4 may play an important role in both the antioxidant and innate immune defense of black rockfish. These findings provide structural and functional insights into the SsPrx4 of the teleost.

1. Introduction

Many cellular activities generate reactive oxygen species (ROS) as an inevitable side-effect of aerobic life. They are radicals, ions, or molecules with a single unpaired electron, including superoxide ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), nitric oxide ($NO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) [1]. When this ROS overwhelms the cellular antioxidant capacity, oxidative stress may lead to cellular damage, including lipids, proteins, and DNA. Thus, maintaining a delicate balance between ROS and antioxidants is vital to prevent damage caused by oxidative stress. Antioxidants can be divided into two main categories, as enzymatic (superoxide dismutases, catalases, peroxiredoxins, and thioredoxins) and non-enzymatic (all-trans-retinol 2, ascorbic acids and alpha-tocopherol) antioxidants.

Among the enzymatic antioxidants, peroxiredoxins are a diverse family of enzymes that constitute a class of thiol-specific, non-selenium dependent proteins. They can eliminate peroxide with a conserved cysteine residue (Cys) known as a peroxidatic Cys, and mediate redox homeostasis [2]. The peroxiredoxin (Prx) family constitutes of six peroxiredoxin members peroxiredoxin 1 (Prx1)- peroxiredoxin 6 (Prx6). Peroxiredoxin 1-4 belong to the 2-Cys peroxiredoxin class. peroxiredoxin 5 is a member of the atypical 2-Cys peroxiredoxin, whereas Prx6 is the only Prx of the 1-Cys peroxiredoxin class. The primary function of Prx is to mediate oxidative stress. Their diversifying function has also been studied, including apoptosis, intracellular signal transduction, and H_2O_2-redox sensing [3,4].

Interestingly, mammalian peroxiredoxin 4 (Prx4) is the only known secretory form located in both intracellular and extracellular spaces [5]. Prior research generally confirms that Prx4 is an efficient H_2O_2 scavenger, which is primarily involved in the removal of ER-generated

* Corresponding author. Marine Molecular Genetics Lab, Department of Marine Life Sciences, Jeju National University, Jeju Self-Governing Province, 63243, Republic of Korea.
E-mail address: jehee@jejunu.ac.kr (J. Lee).

https://doi.org/10.1016/j.fsi.2019.03.010
Received 18 November 2018; Received in revised form 1 March 2019; Accepted 4 March 2019
Available online 07 March 2019
1050-4648/

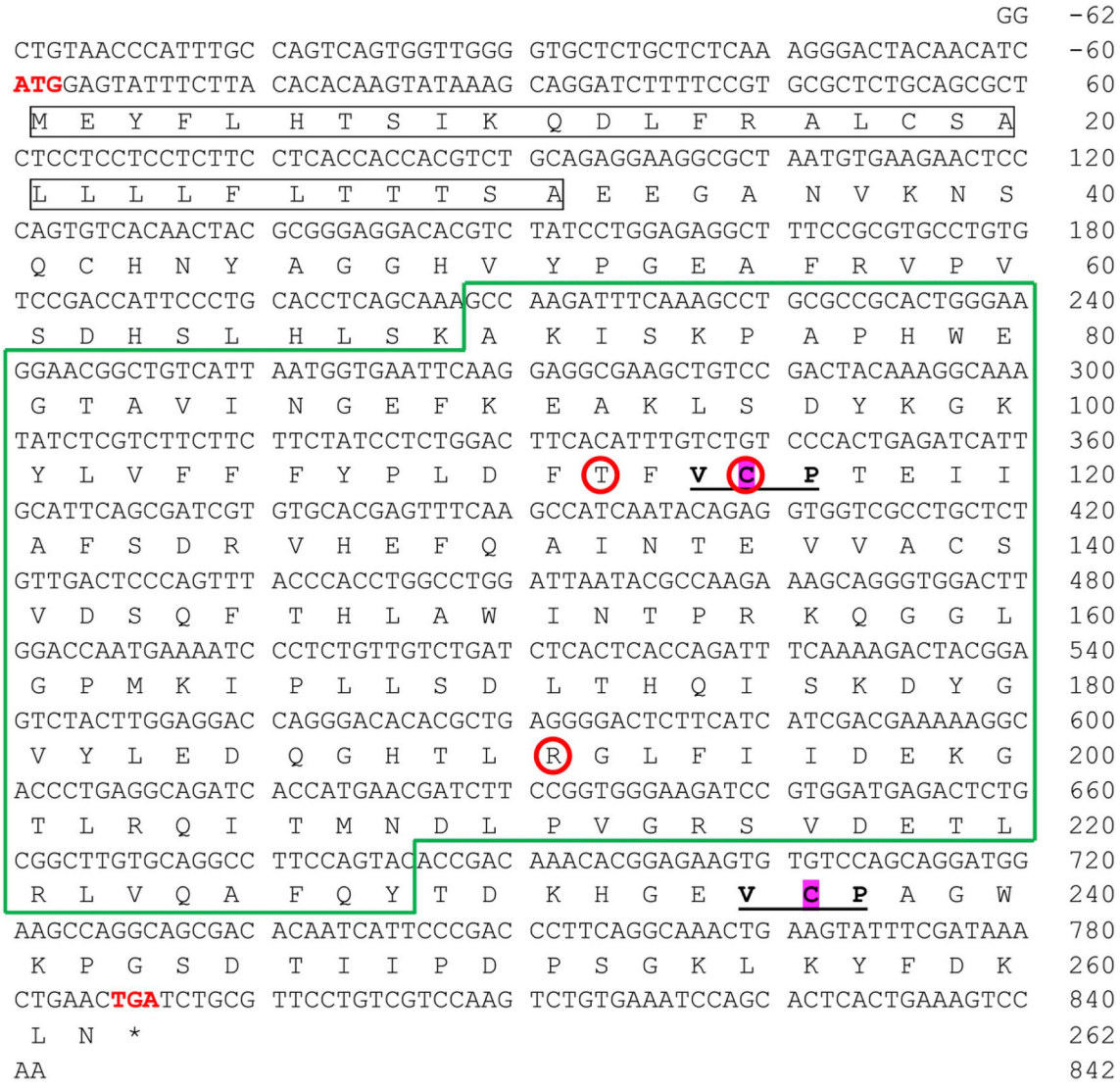

Fig. 1. The nucleotide and amino acid sequences of SsPrx4 cDNA and proteins are transcribed below the CDS. Start codon (ATG) and stop codon (TAA; *) are marked in red and boldface. The signal peptide is boxed. The thioredoxin 2 domain is marked in green lines. Conserved peroxidatic cysteine (CP) residue and resolving cysteine (CR) residue are shaded in magenta. The VCP motifs are in boldface and underlined boxed. Catalytic triad amino acids are in red circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

H_2O_2 [3]. It constitutes a redox-active cysteine, which forms the cysteine sulfenic acid (SOH) in the process of scavenging peroxides [3]. Additionally, Prx4 is involved in the reactivation of oxidized phosphatases [5,6] and possesses a regulatory role in NF-κB epidermal growth factor and p53. Moreover, the role of Prx4 has been extensively studied in inflammatory diseases, including in diabetes mellitus and atherosclerosis [7].

Sebastes schlegelii (black rockfish) fish farming is a rapidly growing and economically profitable industry in South Korea. However, production loss due to the extensive production and prevalence of infectious diseases has drastically increased with time. In order to develop proper disease prevention measures, investigation of its innate immunity and related defense mechanisms is essential. Hence, here we describe the rockfish peroxiredoxin 4 (SsPrx4) sequence characteristics, genomic structure, recombinant protein purification, and its differential biochemical properties. The tissue-specific gene expression and immune responses of SsPrx4 were measured prior to and after the immune challenge experiment.

2. Methodology

2.1. Black rockfish database construction

The black rockfish cDNA and gDNA databases were assembled prior to the present study. First, the cDNA database was created through the 454 GS-FLX sequencing technique as described in Perera et al., 2016 [8]. Second, the gDNA database was constructed through a *de novo* genome assembly method, as demonstrated in Godahewa et al., 2018 [9]. Third, putative SsPrx4 coding cDNA contig and gDNA contig was obtained from the constructed cDNA and gDNA databases, respectively (Accession No: MK160257).

2.2. Bioinformatics analysis

Isolated SsPrx4 cDNA contig was assessed and compared with the Prx4 ortholog sequences, available from the NCBI database. Putative open reading frame (ORF), predicted molecular weight, and the theoretical isoelectric point were obtained through the DNAssist program (ver. 2.2). Different bioinformatics tools including the conserved domain search program at NCBI, Motif Scan, SMART, and ExPASy

Prx4			*S. schlegelii*	*M. miiuy*	*S. partitus*	*S. aurata*	*F. heteroclitus*	*N. coriiceps*	*C. variegatus*	*P. olivaceus*	*P. reticulata*	*P. formosa*	*T. rubripes*	*B. pectinirostris*	*H. comes*	*X. laevis*	*H. sapiens*	*R. norvegicus*	*S. scrofa domesticus*	*M. musculus*	*B. taurus*
Organism	**Accession Number**		**Identity%**																		
Sebastes schlegelii	MK160257	Similarity%		91.6	91.6	91.3	91.2	91.2	90.8	90.6	90.5	90.1	88.9	88.5	88.2	79.9	77.5	76.9	76.8	76.6	75.2
Miichthys miiuy	AGT56737		95.4		91.2	90.5	90.8	87.7	90.8	89.8	90.8	90.0	88.9	88.2	87.7	81.3	76.8	75.8	76.1	75.5	74.5
Stegastes partitus	XP_008274477		95.4	96.6		91.6	92.0	88.1	91.2	91.3	90.4	90.4	88.1	88.9	89.3	80.1	77.9	76.2	77.2	75.9	75.9
Sparus aurata	ADI78067		95.1	95.4	95.1		91.3	87.1	89.7	89.4	90.9	90.9	89.0	87.5	88.2	78.6	77.1	74.5	75.6	75.0	74.0
Fundulus heteroclitus	XP_012710784		94.7	95.4	96.6	95.1		87.7	96.2	89.0	96.6	96.6	89.3	88.5	87.4	80.1	77.9	76.6	77.6	75.9	76.6
Notothenia coriiceps	XP_010793385		96.2	94.6	94.3	93.2	92.7		88.1	86.7	87.0	87.4	86.6	88.5	87.0	78.3	76.8	74.7	75.4	74.5	73.8
Cyprinodon variegatus	XP_015260134		94.3	95.4	96.2	93.9	97.3	93.5		88.7	95.0	95.0	89.4	88.9	88.1	79.8	76.8	76.9	76.5	76.6	76.6
Paralichthys olivaceus	XP_019941327		93.9	93.2	93.9	93.2	93.2	91.3	92.8		87.9	87.9	87.5	87.1	86.4	81.2	79.3	77.3	77.9	77.4	77.0
Poecilia reticulata	XP_008425236		94.7	95.4	95.8	95.1	98.1	92.3	96.6	93.2		99.2	88.9	87.8	87.7	79.4	77.1	75.5	76.5	75.2	74.8
Poecilia formosa	XP_007557304		94.3	95.0	95.4	94.7	97.7	92.3	96.2	92.8	99.6		88.5	87.8	87.4	79.0	77.5	75.1	76.8	74.8	75.2
Takifugu rubripes	XP_011609892		92.4	92.3	92.3	92.4	93.5	91.2	95.0	92.0	92.3	92.3		86.6	88.9	79.9	77.1	75.8	76.5	76.3	76.0
Boleophthalmus pectinirostris	XP_020778774		93.9	95.4	94.3	92.4	92.7	94.6	92.3	92.8	92.7	92.3	91.6		87.0	82.0	76.2	75.9	75.2	75.9	74.5
Hippocampus comes	XP_019729044		93.5	94.6	93.9	93.9	93.1	95.3	92.7	90.9	92.3	92.3	92.7	91.9		78.7	77.5	76.6	76.8	76.3	75.2
Xenopus laevis	AEM44541		86.1	87.6	86.5	88.0	87.3	85.8	86.5	89.1	86.5	86.5	86.9	87.3	85.4		81.7	79.9	81.1	80.7	79.1
Homo sapiens	EAW98996		85.2	85.6	85.6	85.6	86.0	85.6	84.1	86.7	84.9	84.9	86.3	84.5	86.0	89.7		89.1	92.6	89.1	90.9
Rattus norvegicus	AAH59122		85.0	84.6	85.3	86.1	85.0	85.0	85.0	84.6	83.5	83.5	84.6	83.9	84.6	86.1	93.0		90.1	97.1	87.7
Sus scrofa domesticus	JAG69260		84.2	84.6	84.9	87.1	85.3	84.6	83.8	84.9	85.7	85.7	84.6	83.8	85.7	87.5	94.5	93.4		90.5	94.9
Mus musculus	AAH19578		85.0	85.4	84.3	86.5	85.8	85.4	84.7	85.4	85.4	85.4	84.7	83.6	84.3	85.8	92.7	98.2	93.4		87.7
Bos taurus	AAG53660		82.8	82.1	83.6	84.7	84.3	83.6	83.9	83.6	83.9	83.9	84.3	83.6	83.9	85.8	93.8	93.4	97.1	93.4	

Fig. 2. Pairwise sequence alignment of the SsPrx4 protein sequence with other Prx4 ortholog protein sequences. Sequence identity percentage and similarity percentage are denoted in brown and green, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

PROSITE were used to explore the characteristic domains and motifs of the SsPrx4 sequence. Sequence identity and similarity between Prx4 orthologs were assessed with MatGAT software. Multiple sequence alignment of the Prx4 orthologs was assessed with the Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) tool. The phylogenetic tree was drawn with MEGA (ver. 7.0) software following the Neighbor-Joining (NJ) method. The SsPrx4 protein folding pattern was predicted using the Fold index© online tool (http://bip.weizmann.ac.il/fldbin/findex).

The isolated SsPrx4 gDNA contig was assessed using the Splign genomic alignment online tool (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi) along with its cDNA sequence. Then, the exon-intron boundaries of SsPrx4 were determined. Other Prx6 ortholog genomic sequences and their exon-intron boundaries were obtained from the Ensemble database (http://asia.ensembl.org/index.html). The GeneMapper (v2.5) software was used to visualize all the genomic arrangements in the present study.

2.3. Molecular cloning and recombinant protein expression

The ORF of the SsPrx4 gene was cloned into the pMAL-c5x vector. Briefly, gene-specific primers (F: GAGAGAcatatgCACACAAGTATAAAG CAGGATCTTTTCCGTGC and R: GAGAGAggatccTCAGTTCAGTTTATC GAAATACTTCAGTTTGCCTG) were used to amplify the target fragment using liver cDNA as a template. The thermal cycle profile was as follows: a single cycle at 94 °C for 4 min, 30 cycles at 95 °C for 30 s, 59 °C for 30 s, 72 °C for 50 s, and a single cycle at 72 °C for 10 min. Both insertors and plasmids were subjected to restriction digestion with NdeI and BamHI enzymes. Recombinant plasmids were created by ligating the digested cDNA fragment and plasmids using Mighty Mix (TaKaRa, Japan). Recombinant vectors were then transformed into DH5α competent cells and their sequence was verified (Macrogen, Korea). Subsequently, positive recombinant plasmids were transformed into ER2523 competent cells and expressed the protein (rSsPrx4) at 20 °C for 10 h by adding 0.3 mM IPTG. The rSsPrx4 proteins were purified with the pMAL Protein Fusion and Purification system following the vendor's protocol (New England Biolabs, USA). Protein concentrations were determined through a Bradford assay [10] and purity was assessed by 12% SDS-PAGE.

2.4. MTT assay

The MTT assay was completed as reported in our previous study with slight modifications [9]. Cell culture was carried out from the human leukemia THP-1 cell stocks. Cells were cultured in RPMI 1640 medium, which was supplemented by 100 mg/mL streptomycin, 100 U/mL penicillin, and 10% FBS. Cell concentrations of 1×10^5 cells/mL were incubated with 0-, 25-, 50-, 75-, and 100 μg/mL of rSsPrx4 for 45 min in the presence of 1 mM of DTT. 100 μg/mL rMBP was used as a control treatment. Samples were then subjected to extracellular oxidative stress by adding 400 μmol of H_2O_2. The percentage of viable cells was determined after a 24 h incubation period from the triplicate samples.

2.5. Flow cytometry

Similarly, the 1×10^5 cells/mL THP-1 cells were treated with 0, 50, 75, and 100 μg/mL rSsrx4 or 100 μg/mL rMBP for 30 min with DTT, followed by 400 μmol of H_2O_2 for 24 h. To determine the extracellular H_2O_2 scavenging activity of rSsPrx4, intracellular ROS levels were determined through flow cytometry. Finally, treated cells were stained with H_2DCFDA and analyzed using a BD FACScalibur flow cytometer (BD Biosciences, USA). Obtained data were examined through CellQuest Pro software (BD Biosciences). Average ROS generated cell numbers were illustrated from the triplicated experiments.

2.6. MCO assay

The metal-catalyzed oxidase (MCO) assay was carried out to determine the supercoiled DNA protection of rSsPrx4. The 4 mM DTT, 30 μM $FeCl_3$, and H_2O were mixed with 6.25-, 12.5-, 25-, 50-, and 100 μg/mL rSsPrx4 and incubated for 45 min at 37 °C. pUC19 (1 μg) supercoiled DNA was then added and further incubated for 15 min at 37 °C. As the control protein, 100 μg/mL rMBP was used. All the samples were PCR purified (Bioneer, Korea) to terminate the reaction and assessed via gel electrophoresis.

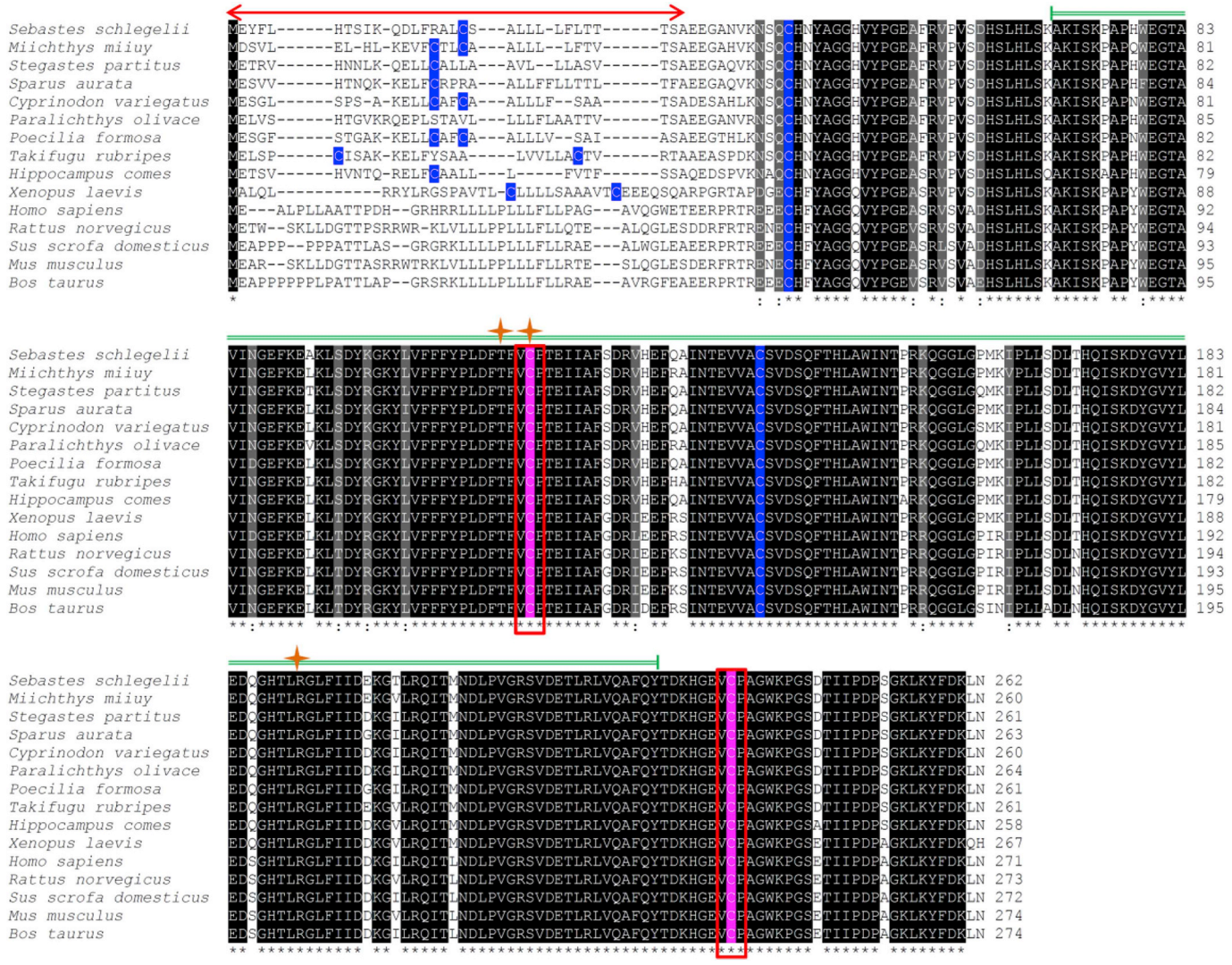

Fig. 3. Multiple sequence alignment of the SsPrx4 protein sequence with other Prx4 ortholog protein sequences. Identical residues among the Prx4 counterparts are shaded black (*) while similar residues are shaded grey (:). Conserved VCP motifs are in red boxes. Conserved peroxidatic cysteine (CP) and resolving cysteine (CR) residues are shaded in magenta. Other cysteine residues are shaded in blue. The thioredoxin 2 domain is marked in green lines. Catalytic triad amino acids are marked with stars. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.7. Oxidoreductase assay

In order to determine the oxidoreductase activity of the rSsPrx4, an insulin disulfide reduction assay was carried out. Here, 2 mg/mL insulin was mixed with 100 or 200 μg/mL rSsPrx4 supplemented with 4 mM EDTA (pH 7.0). Again, 200 μg/mL rMBP was used in a control treatment. All the treatments began by adding 2 mM DTT. Absorbencies were then measured at 650 nm (25 °C) and mean results from triplicates were plotted against the incubation time.

2.8. Challenge experiment and qPCR analysis

Uninfected rockfish (200 ± 10 g) were selected and adapted to Jeju National University laboratory aquariums (400 L, 22 ± 1 °C). Five fish were randomly selected and anesthetized (100 mg/mL tricaine methanesulfonate), and samples of blood, gill, liver, ovary, spleen, head kidney, kidney, intestine, muscle, skin, stomach, heart, testis, and brain tissues were collected.

In addition, liver and spleen tissues were sampled from five immune-challenged fish at 0-, 3-, 6-, 12-, 24-, 48-, and 72 h post injection (p.i.). For the immune challenge, 200 μL of 1 × PBS, lipopolysaccharide (LPS; 1.25 μg/μL), *Streptococcus iniae* (1×10^5 CFU/μL), and poly I:C (1.5 μg/μL, Sigma, USA) were intraperitoneally injected. All the animal experiments were reviewed and accepted by the Animal Care and Use Committee of Jeju National University.

As reported in our previous study, total RNA was extracted from the above-mentioned tissues [8]. First stand cDNAs were then synthesized from 2.5 μg of extracted RNA, following the steps mentioned in Godahewa et al., 2014 [11]. Finally, 40-fold diluted cDNAs were used in a quantitative real-time polymerase chain reaction (qPCR) analysis.

The relative mRNA expression of SsPrx4 in different tissues and immune-challenged tissues were examined through qPCR using gene-specific primers (F: ACTGGGAAGGAACGGCTGTCATTA and R: AGCA GGCGACCACCTCTGTATT) in a real-time thermal cycler (TP950, TaKaRa, Japan). Relative expression levels of SsPrx4 were calculated using the $2^{(-\Delta\Delta C(T))}$ method [12]. The housekeeping gene elongation factor 1α (SsEF1α, GenBank ID: KF430623) (F: AACCTGACCACTGAG GTGAAGTCTG and R: TCCTTGACGGACACGTTCTTGATGTT) was used

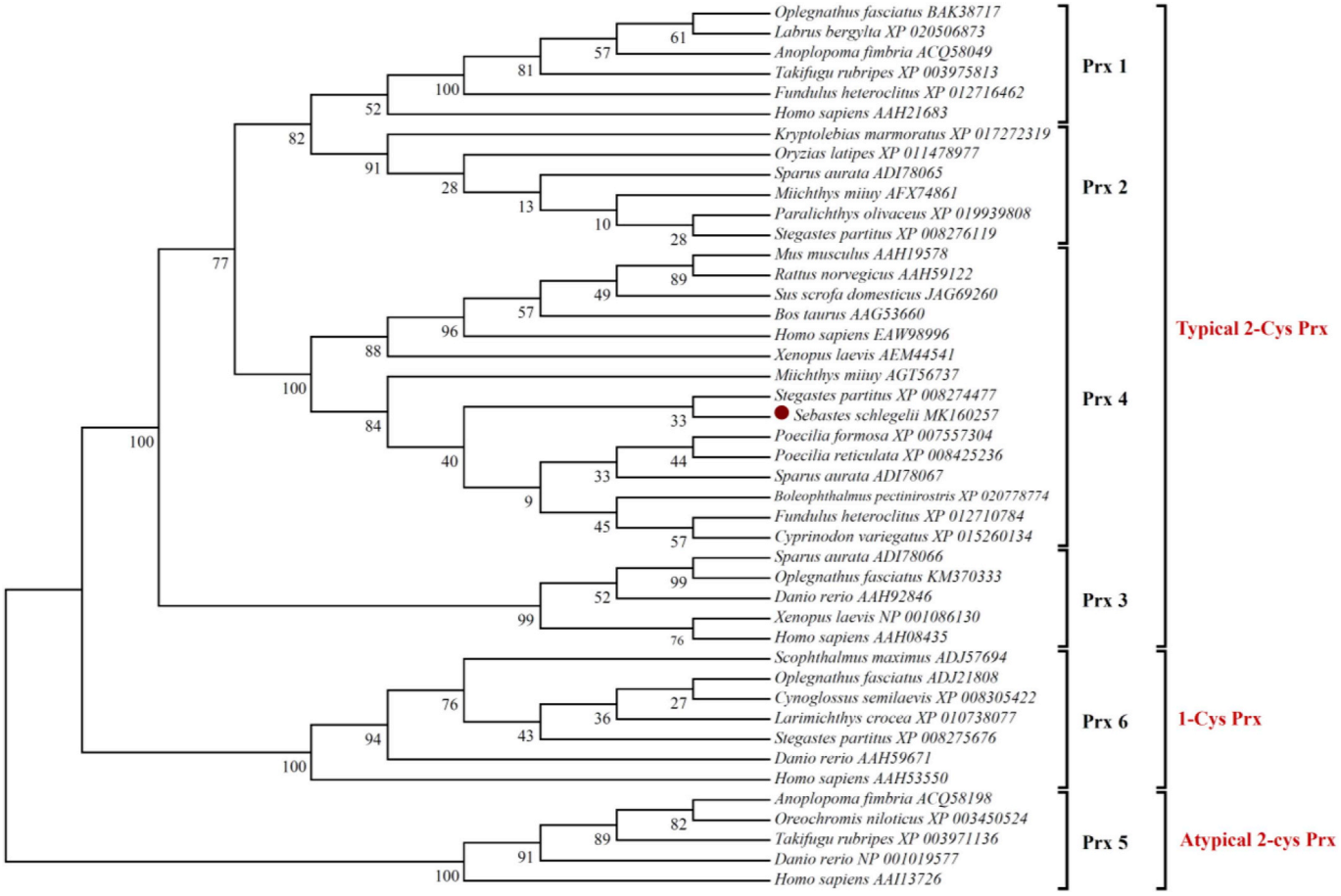

Fig. 4. Phylogenetic analysis of SsPrx4 and its evolutionary relationship with other Prx sub-families. The numbers at the branches state the bootstrap value percent after 5000 replicates. The accession number of each sequence is presented next to the organism. Sub-clusters are denoted within brackets with the names beside them.

as an internal reference. All the data were illustrated as mean ± standard deviation from triplicates. The qPCR assay data were analyzed through a two-tailed un-paired *t*-test using the GraphPad Software.

3. Results and discussion

3.1. Sequence analysis, alignment, and homology analysis

A rockfish cDNA database was previously constructed [8] and the BLASTX comparison of expressed sequence tags (ESTs) yielded a Prx4 homolog, which we designated as SsPrx4. As illustrated in Fig. 1, the SsPrx4 cDNA sequence was 904 bp constituting a 5′ UTR of 62 bp and a 3′ UTR of 53 bp. The coding sequence of 786 bp is translatable to a protein of 262 amino acids. SsPrx4 has a predicted molecular mass of 29 kDa and a 6.2 isoelectric point (pI). Domain analysis of deduced amino acid sequences of SsPrx4 revealed a signal peptide, suggesting that it may functionally activate outside of the cells [13]. Therefore, SsPrx4 is a secretory protein. In addition, the thioredoxin 2 domain and the conserved two cysteine residues were predicted as potential sites. Moreover, two VCP motifs were found through domain analysis and it is suggested that they are important for determining the substrate of its enzymatic activity [14]. Additionally, the catalytic triad amino acids have been predicted. They are important to modulate the structural switches in 2-Cys peroxiredoxins [15].

Pairwise sequence analysis (Fig. 2) revealed that SsPrx4 shared the highest identity (91.6%) and similarity (95.4%) value with *Miichthys miiuy* and *Stegastes partitus* counterparts. Interestingly, SsPrx4 shared > 85% identity with its teleosts Prx4, and > 75% identity with its mammalian counterparts. Similar high identity was observed with swimming crab Prx4 and teleost Prx4 [16]. According to the multiple sequences alignment (Fig. 3), numerous residues at the thioredoxin 2 domain were highly conserved, revealing the functional conservation of SsPrx4. However, the amino acids positioned in the N-terminus of teleosts Prx4 were not consistent with those of higher vertebrates, suggesting some insertion of the amino acids through the evolutionary process of higher vertebrates from lower vertebrates. Besides, some Prx4 orthologs may not consist of a signal peptide [17]. Interestingly, the cysteine residues and the VCP motifs are well-conserved among all the taxonomic groups considered in this study, suggesting that the catalytic function of the Prx4 may persist throughout evolution.

3.2. Evolutionary relationship

To determine the evolutionary relationships of SsPrx4, we have reconstructed a phylogenetic tree including several counterparts from different taxonomic groups available in the NCBI data base (Fig. 4). Interestingly, three major clades were observed, including the typical 2-Cys peroxiredoxins, 1-Cys peroxiredoxins, and atypical 2-Cys peroxiredoxins separately. The SsPrx4 was positioned in the teleost cluster and exhibited closer evolutionary proximity with teleosts Prx4. Therefore, it

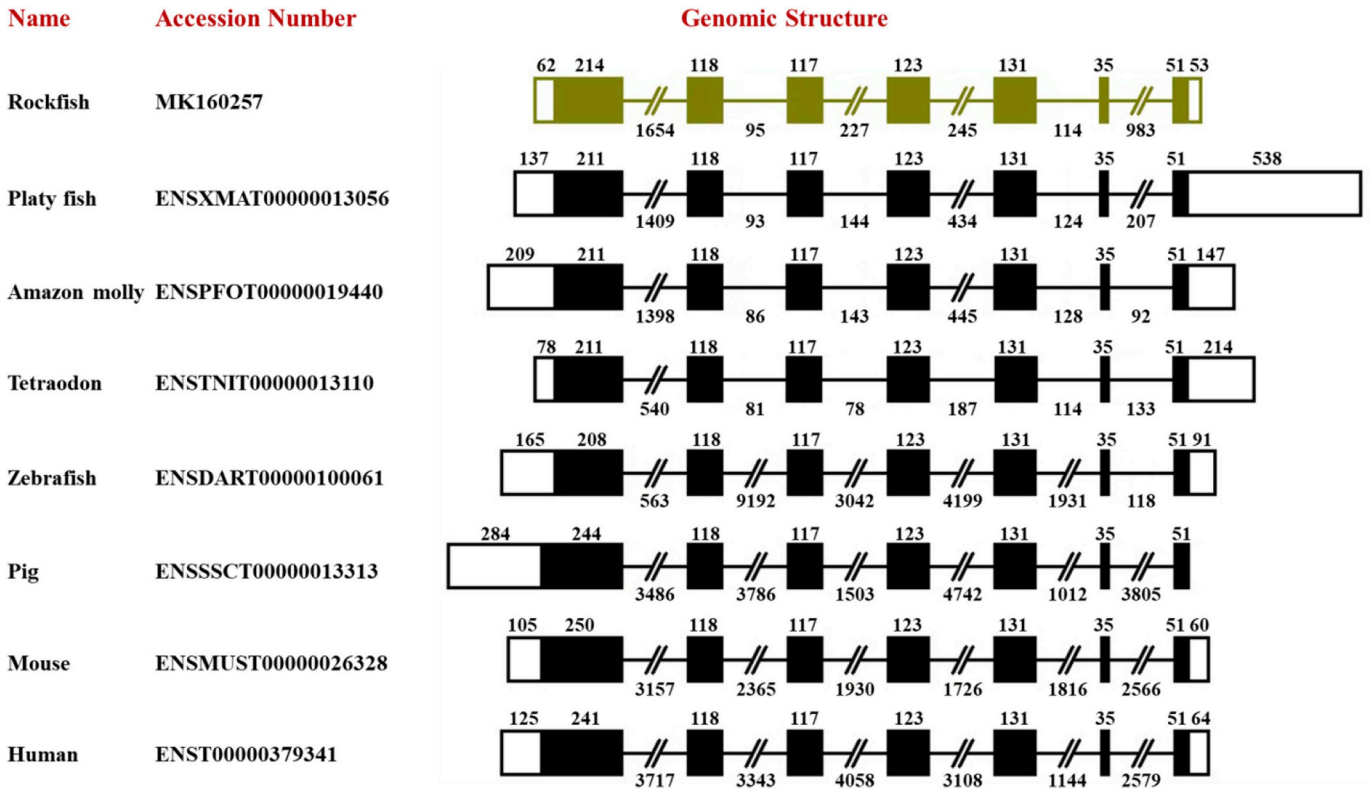

Fig. 5. Schematic genomic structure arrangement of Prx4 orthologs. Exons with coding sequences are shaded, exons with UTRs are in empty boxes, and introns are marked with straight lines. Exon sizes are mentioned on top of the boxes and intron lengths are mentioned below the lines. Ortholog sequence details were obtained from Ensemble and are shown next to the organism.

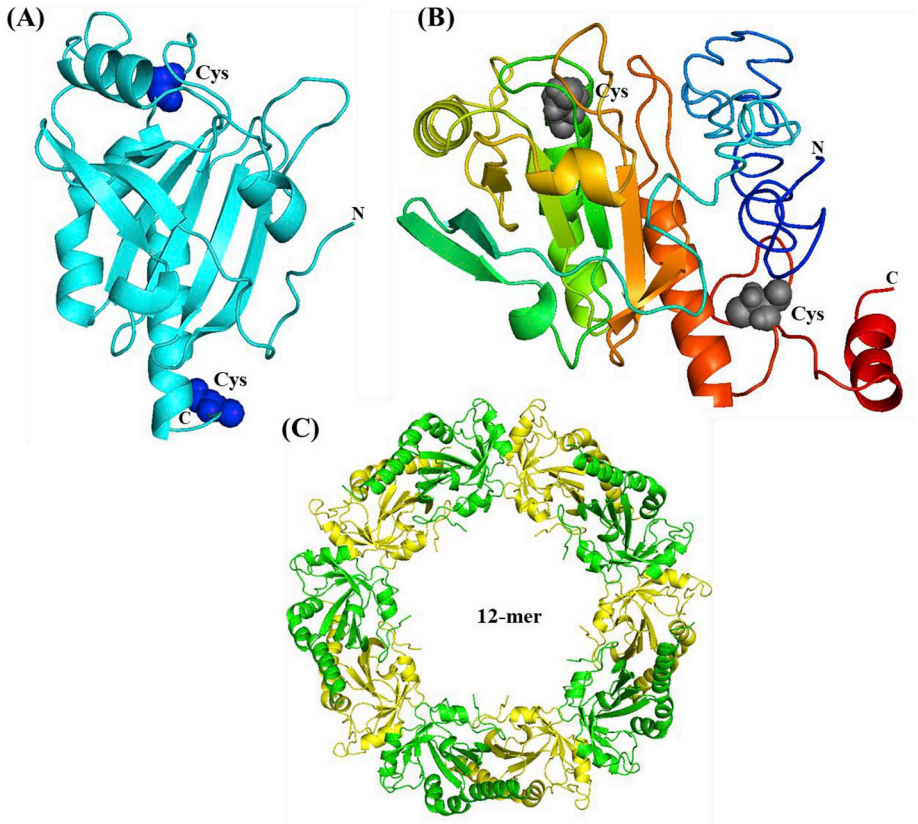

Fig. 6. The human Prx4 crystal structure (A), predicted 3D structure of SsPrx4 (B), and predicted 3D structure of SsPrx4 homo-12-mer (C). SsPrx4 monomer was generated in ITASSER server (TM-score = 0.53 ± 0.15, RMSD = 9.2 ± 4.6 Å, C-score = −1.46). SsPrx4 homo-12-mer was generated in SWISS-MODEL platform based on wild type human prx4 homo-12-mer (PDB, 3tjb). Terminals are marked and the positions of cysteine residues possibly forming an intra-molecular disulfide bond are indicated.

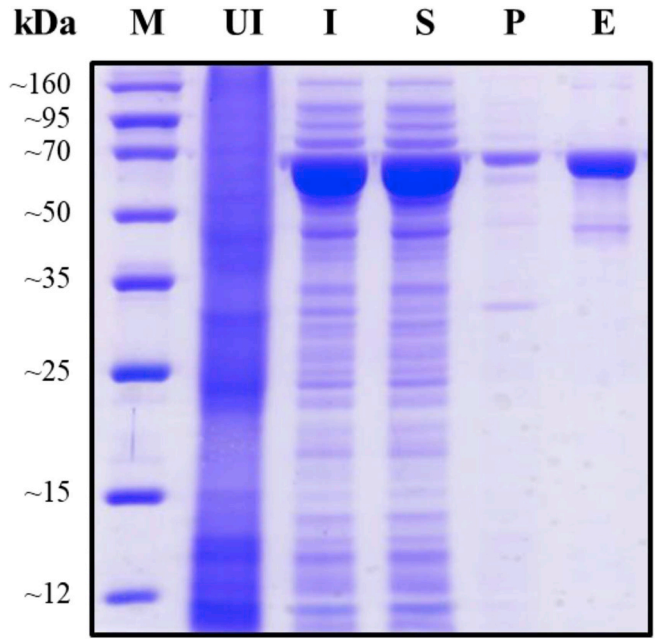

Fig. 7. The rSsPrx4 protein expression and purification analysis in SDS-PAGE. Lanes: M; unstained protein marker (Enzynomics, Korea), UI; total cellular extract prior to IPTG induction, I; total cellular extract after IPTG induction, S; supernatant (crude protein), P; pellet, and E; purified fraction.

is confirmed that SsPrx4 belongs to the fish Prx4. According to the molecular phylogenetic tree, SsPrx4 was closely cladded with the *Stegastes partitus* counterpart. Similar evolutionary studies related to the Prx orthologs were found in past studies consistent with the present results [17–20]. Moreover, this consistent taxonomical data revealed that Prx4 has extended evolutionary antiquity.

3.3. Genomic analysis

The SsPrx4 genomic sequence is 4222 bp in length. The data of the present genomic analysis clearly specified that the structure of the SsPrx4 gene is highly conserved, with seven exons interrupted by six introns (Fig. 5). However, the lengths of the introns vary markedly among different species, resulting in different genome lengths in Prx4 orthologs. Specifically, mammalian and zebrafish Prx4 genomic sequences are comparatively larger than other fish species, indicating extensive nucleotide insertion at their introns during the evolutionary process. The conserved thioredoxin 2 domain was coded by the nucleotides in the first five coding exons, which were mostly identical among the different taxa. Hence, antioxidant function of the Prx4 may be static in all of these Prx4 orthologs. In fact, the present genomic analysis result is consistent with a previous report [20].

3.4. Tertiary structure analysis and purity of recombinant protein

Human Prx4, predicted SsPrx4, and the predicted homo-12-mer of SsPrx4 were constructed using a homology modeling approach (Fig. 6). The globular structure of SsPrx4 highly resembled its human counterpart. Recombinant SsPrx4 was successfully overexpressed in ER2523 competent cells and purified while the different phases of purification were loaded in a reducing SDS-PAGE analysis (Fig. 7). A strong and clear protein band of ~71.5 kDa (fusion with rMBP; 42.5 kDa), which is its expected molecular weight, confirmed the purity of rSsPrx4.

3.5. Functional aspects of rSsPrx4

Prx4 is a well-known and essential antioxidant enzyme that combats ROS and protects the live cells from cellular and molecular level damage. Functional aspects with regards to the peroxidase activity, oxidoreductase activity, and the DNA protection activity of rSsPrx4 were evaluated via different planned experiments.

3.5.1. Extracellular H_2O_2 scavenging assay

Cell viability upon oxidative stresses can be used to discover their ability to scavenge extracellular H_2O_2 through peroxiredoxin protein. Here, we have treated the THP-1 cells with rSsPrx4, then exposed them to H_2O_2. Interestingly, the results showed that in the presence of rSsPrx4, the percentage cell viability was increased close to that of the control treatment (Fig. 8). Moreover, the percentage cell viability was increased with the dose of rSsPrx4, revealing its dose dependency. However, there was no significant result displayed by rMBP, as it was an inert fusion protein of rSsPrx4. When H_2O_2 was exposed to the cells, the rSsPrx4 in the medium reacted with H_2O_2 and converted it to H_2O and O_2. Thus, the amount of cytotoxic H_2O_2 was reduced and cell

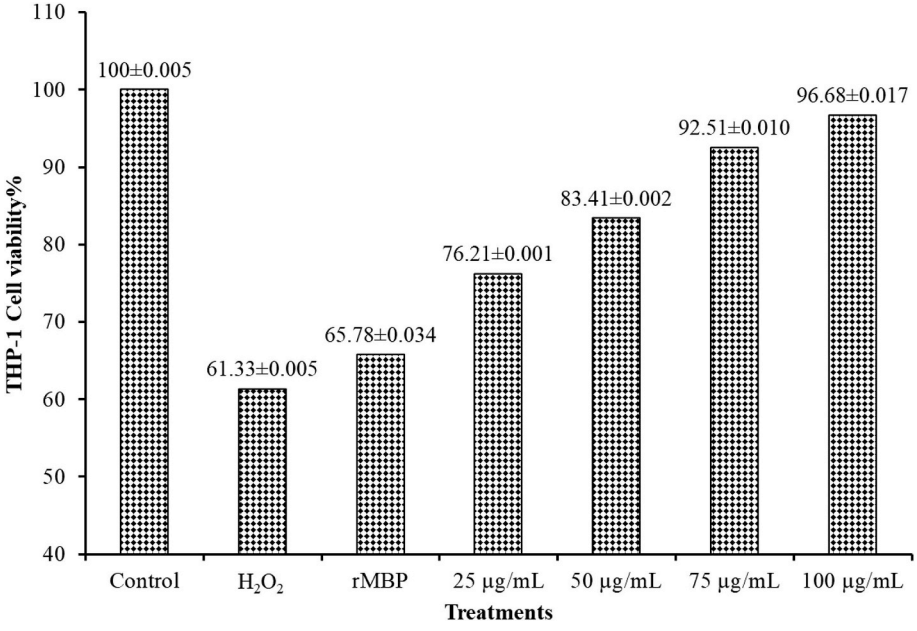

Fig. 8. rSsPrx4 treated THP-1 cell survival percentage upon exposure to 400 µmol of H_2O_2 and extracellular H_2O_2 scavenging activity of rSsPrx4. In the presence of DTT, cells were exposed to 100 µg/mL of rMBP, 25–100 µg/mL of rSsPrx4, followed by 400 µmol of H_2O_2. Cell survival rate was measured with an MTT assay. Vertical columns represent the mean ± SD (N = 3).

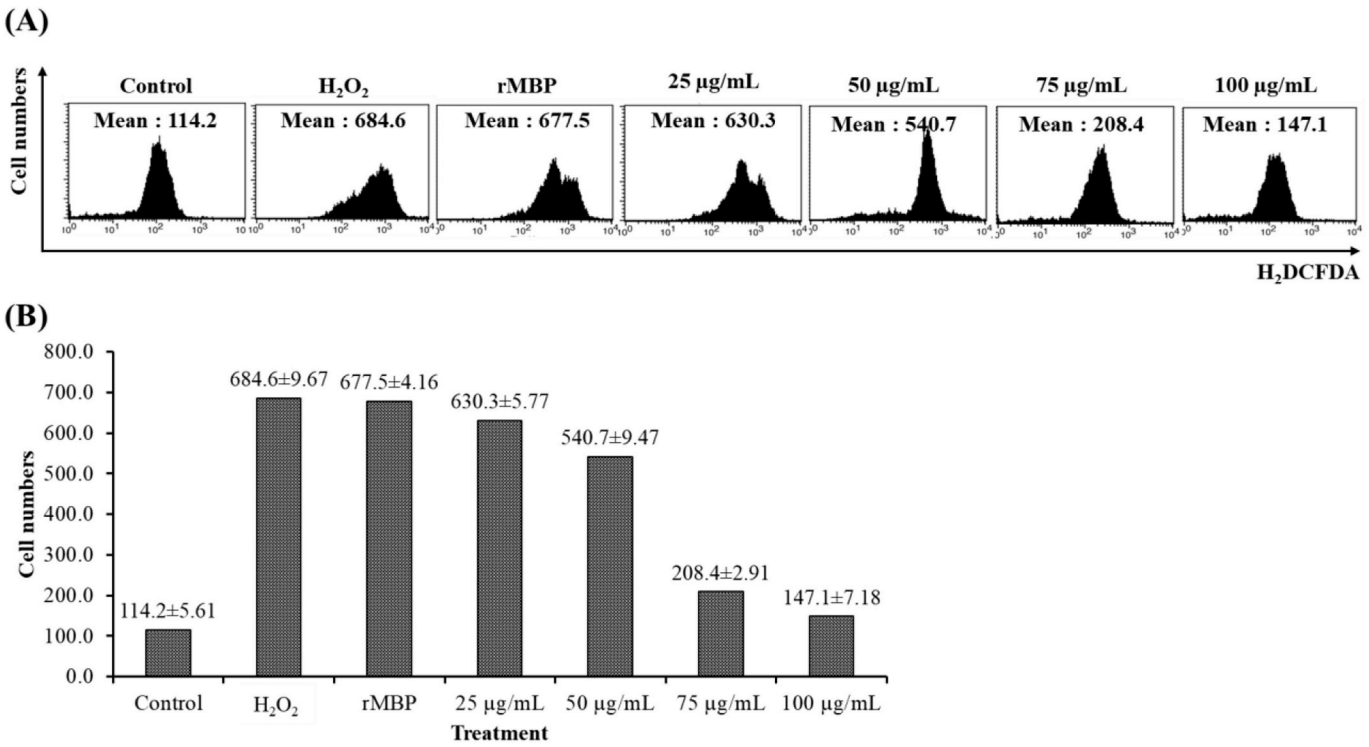

Fig. 9. (A) Flow cytometry assay of rSsPrx4 on THP-1 cells exposed to 400 μmol of H_2O_2 and (B) H_2DCFDA stained cell count. In the presence of DTT, cells were exposed to 100 μg/mL rMBP, 25–100 μg/mL of rSsPrx4, then 400 μmol of H_2O_2. The intracellular ROS levels are expressed as ROS generated cell count mean ± SD (N = 3).

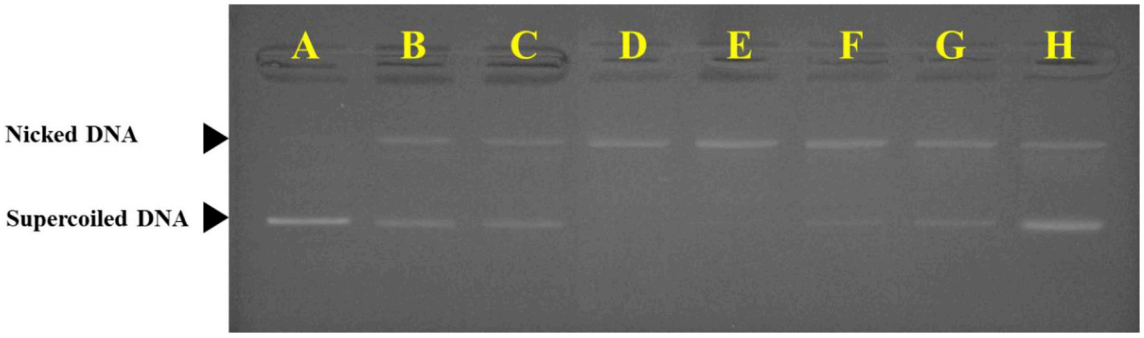

Fig. 10. Protection of supercoiled DNA cleavage by rSsPrx4 in an MCO system. A) pUC19 without incubation; B) pUC19 with MCO system; C) pUC19 + MCO system + 100 μg/mL rMBP; D) pUC19 + MCO system + 6.25 μg/mL rSsPrx4; E) pUC19 + MCO system + 12.5 μg/mL rSsPrx4; F) pUC19 + MCO system + 25 μg/mL rSsPrx4; G) pUC19 + MCO system + 50 μg/mL rSsPrx4; and H) pUC19 + MCO system + 100 μg/mL rSsPrx4.

survivability was increased. Interestingly, it has been recorded that mammalian Prx4 provided a cellular protective effect against H_2O_2 [3].

3.5.2. Flow cytometry analysis

To further evaluate the peroxidase activity of rSsPrx4, we conducted flow cytometry assays in the presence of rSsPrx4. According to the results, developed internal ROS of THP-1 cells were reduced in the presence of rSsPrx4 (Fig. 9). As we observed in the MTT assay, dose-dependent activity of rSsPrx4 was observed where no significant activity was observed for the rMBP. External H_2O_2 may have led to the generation of intracellular ROS in the THP-1 cells. Those cells were stained with H_2DCFDA and recorded as cells with ROS. However, rSsPrx4 may reduce the externally supplied H_2O_2 by converting them to H_2O and O_2. This could lead to a reduction of the generation of ROS in the THP-1 cells. It is a well-known fact that peroxiredoxins react with H_2O_2 and convert it into H_2O and O_2 [21,22]. Moreover, Prx4 is an ER-resident protein and it is important to reduce the H_2O_2 produced by ER membrane-associated oxidoreductin 1 [22]. Therefore, the results of this assay further confirmed the ability of peroxidase of Prx4, revealing that SsPrx4 is an important antioxidant in rockfish which mediates the redox homeostasis.

3.5.3. MCO assay

In order to investigate whether rSsPrx4 has the ability to protect DNA from nicking, we conducted a DNA protection assay. Here, MCO systems form a platform for the auto-oxidation of DTT by varying ROS, including; $O_2^{\cdot-}$, $O^{\cdot-}_2$, H_2O_2, and $^1O_2$. The pUC19 super-coiled plasmid DNA was used to observe the presence and extent of nicking and undamaged DNA. According to the results, the pUC19 DNA was nicked by the MCO system, whereas it reduced the DNA cleavage via rSsPrx4 and

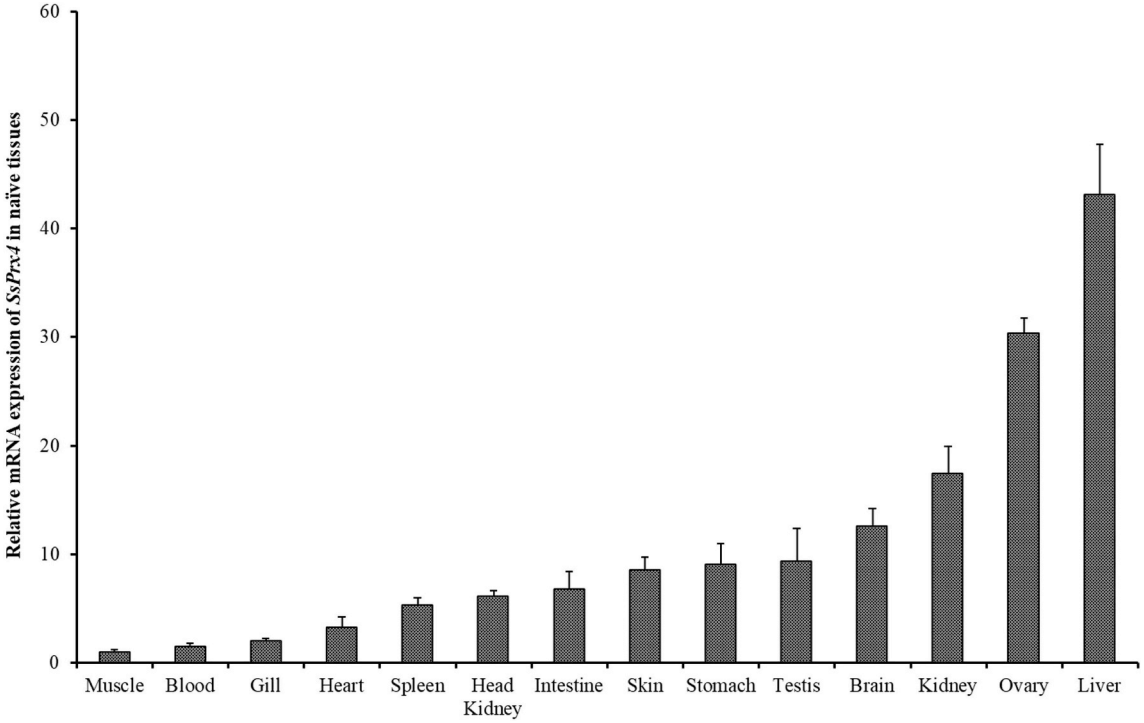

Fig. 11. Tissue distribution of SsPrx4. Data were obtained from triplicate qPCR reactions (N = 3) and presented as average values with error bars representing ± SD ($P < 0.05$). The mRNA level in muscles was set as a basal expression.

less nicking was detected in the higher concentrations of rSsPrx4 (Fig. 10). The DNA protection activity of rSsPrx4 was dose-dependent and its DNA protection significantly increased with the higher rSsPrx4 protein. On the contrary, there was no significant DNA protection observed with rMBP in the MCO system. So far, similar DNA protection was also observed with the other Prx orthologs in previous studies [23–26]. Taken together, these results verified that rSsPrx4 is capable enough to protect the DNA from nicking by the deleterious ROS.

3.6. Tissue expression kinetics of SsPrx4 mRNA

A tissues specific transcriptional level of SsPrx4 was calculated through quantitative real-time PCR to reveal the possible physiological characteristic features of SsPrx4. A ubiquitous expression of SsPrx4 mRNA was observed with different magnitudes in all the selected tissues (Fig. 11). The highest expression was detected in liver tissue followed by the ovary, kidney, brain, and testis. Many studies have shown that fish liver primarily expresses many antioxidant genes to combat ROS generated by multiple oxidative reactions [27]. Interestingly, *Mylopharyngodon piceus* Prx4 [28] and *Sparus aurata* Prx4 [29] displayed greater mRNA levels in the liver. *Procambarus clarkii* Prx4 also showed the highest mRNA level in the hepatopancreas [26]. The tissue distribution of Prx4 was intensively expressed in digestive and reproductive organs in both humans and mice [30]. Moreover, in *Penaeus monodon* and *Fenneropenaeus chinensis,* Prx4 expression was at a relatively high level in the gonads and hepatopancreas [18,31]. *Cyprinus carpio* Prx4 transcripts are also highly expressed in gonads and head kidney tissues [20]. Therefore, this indicates that Prx4 may have an important role in the reproductive process and peroxidase-dependent innate immune response. To date the Prxs modulate immune response in many species has been proven [20,26,32–38]. Therefore, we have conducted an immune challenge experiment for the rockfish with several immune stimulants.

3.7. Temporal expression kinetics of SsPrx4 mRNA

According to the results of the immune challenge, it is clear that SsPrx4 has been modulated against the different stimuli in both liver and spleen tissues (Fig. 12). The highest up-regulation against *S. iniae* and LPS was observed at 48 h p.i. in both liver and spleen tissues. However, the highest up-regulation against poly I:C was observed at 72 h p.i. in both liver and spleen tissues. Interestingly, several studies have reported regarding the upregulation of Prx4 against different immune stimuli [18,26,28,29,31,39]. Moreover, large yellow croaker Prx4 regulated pro-inflammatory responses through the inhibition of the activation of NF-κB to protect the large yellow croaker from bacterial infection [40]. The overexpression of Drosophila Prx4 triggered an NF-κB-mediated pro-inflammatory response in the fly [41]. SsPrx4 may be important in the innate immune system of rockfish, as well as its role as an antioxidant.

4. Conclusion

To conclude, we have obtained the sequence of SsPrx4 from black rockfish and found that it is constitutively and widely distributed in the rockfish tissues. Characteristic features were analyzed through bioinformatics tools and the recombinant protein was purified prior to evaluating its functional characteristics. Functional characterization of the rSsPrx4 confirmed its peroxidase function, oxidoreductase function, and the DNA protection ability as an important antioxidant. Moreover, the modulation of the SsPrx4 transcripts suggests that it may be involved in the innate immunity-related function and be important in the immune defense mechanism of the rockfish.

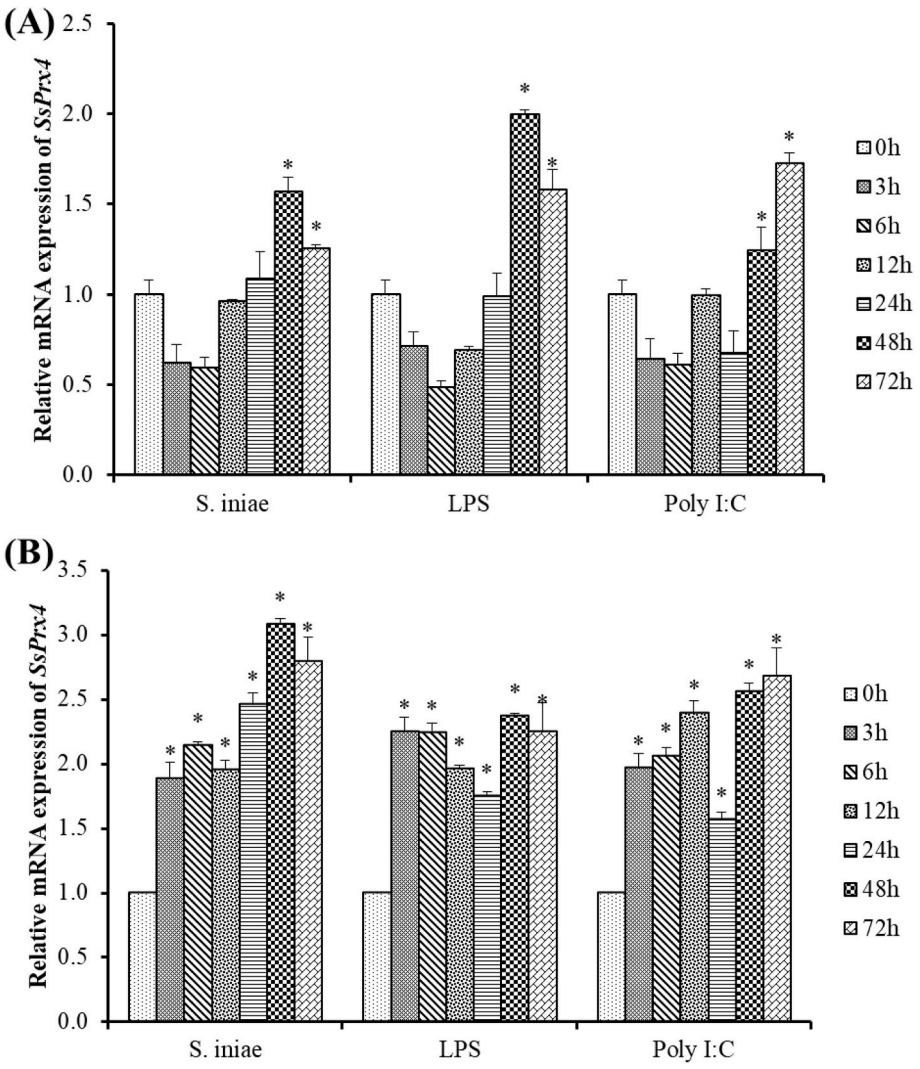

Fig. 12. SsPrx4 mRNA expression in liver (A) and spleen (B) after the challenge experiment. Data were obtained from triplicate qPCR reactions (N = 3) and presented as average values with error bars representing ± SD. Data marked with * indicates significantly different expression levels with respect to the 0 h control ($P < 0.05$).

Acknowledgements

This research is part of the project titled 'Fish Vaccine Research Center' funded by the Ministry of Oceans and Fisheries, Korea.

References

[1] G.Y. Liou, P. Storz, Reactive oxygen species in cancer, Free. Radic. Res. 44 (2010) 479–496 https://doi.org/10.3109/10715761003667554.

[2] S.G. Rhee, H.A. Woo, Multiple functions of peroxiredoxins: peroxidases, sensors and regulators of the intracellular messenger H_2O_2, and protein chaperones, Antioxidants Redox Signal. 15 (2011) 781–794 https://doi.org/10.1089/ars.2010.3393.

[3] T.J. Tavender, N.J. Bulleid, Peroxiredoxin IV protects cells from oxidative stress by removing H_2O_2 produced during disulphide formation, J. Cell Sci. 123 (2010) 2672–2679 https://doi.org/10.1242/jcs.067843.

[4] S.T. Wen, R.A. Van Etten, The PAG gene product, a stress-induced protein with antioxidant properties, is an Abl SH3-binding protein and a physiological inhibitor of c-Abl tyrosine kinase activity, Genes Dev. 11 (1997) 2456–2467 https://doi.org/10.1101/gad.11.19.2456.

[5] S.G. Rhee, H.Z. Chae, K. Kim, Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling, Free Radic. Biol. Med. 38 (2005) 1543–1552 https://doi.org/10.1016/j.freeradbiomed.2005.02.026.

[6] S.G. Rhee, S.W. Kang, W. Jeong, T.S. Chang, K.S. Yang, H.A. Woo, Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins, Curr. Opin. Cell Biol. 17 (2005) 183–189 https://doi.org/10.1016/j.ceb.2005.02.004.

[7] S. Yamada, Y. Ding, Y. Sasaguri, Peroxiredoxin 4: critical roles in inflammatory diseases, J. UOEH 34 (2012) 27–39.

[8] N.C. Perera, G.I. Godahewa, B.H. Nam, J. Lee, Molecular structure and immune-stimulated transcriptional modulation of the first teleostean IFP35 counterpart from rockfish (Sebastes schlegelii), Fish Shellfish Immunol. 56 (2016) 496–505 https://doi.org/10.1016/j.fsi.2016.08.010.

[9] G.I. Godahewa, N.C.N. Perera, B.H. Nam, J. Lee, Antioxidative properties and structural features of atypical 2-Cys peroxiredoxin from Sebastes schlegelii, Dev. Comp. Immunol. 82 (2018) 152–164 https://doi.org/10.1016/j.dci.2018.01.015.

[10] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254 https://doi.org/10.1016/0003-2697(76)90527-3.

[11] G.I. Godahewa, W.D. Wickramaarachchi, I. Whang, S.D. Bathige, B.S. Lim, C.Y. Choi, M. De Zoysa, J.K. Noh, J. Lee, Two carboxypeptidase counterparts from rock bream (Oplegnathus fasciatus): molecular characterization, genomic arrangement and immune responses upon pathogenic stresses, Vet. Immunol. Immunopathol. 162 (2014) 180–191 https://doi.org/10.1016/j.vetimm.2014.10.005.

[12] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408 https://doi.org/10.1006/meth.2001.1262.

[13] G. Blobel, Protein targeting (Nobel lecture), Chembiochem 1 (2000) 86–102 https://doi.org/10.1002/1439-7633(20000818)1:2<86::AID-CBIC86>3.0.CO;2-A.

[14] C. Stapf, E. Cartwright, M. Bycroft, K. Hofmann, A. Buchberger, The general definition of the p97/valosin-containing protein (VCP)-interacting motif (VIM) delineates a new family of p97 cofactors, J. Biol. Chem. 286 (2011) 38670–38678 https://doi.org/10.1074/jbc.M111.274472.

[15] C.A. Tairum, M.C. Santos, C.A. Breyer, R.R. Geyer, C.J. Nieves, S. Portillo-Ledesma, G. Ferrer-Sueta, J.C. Toledo Jr., M.H. Toyama, O. Augusto, L.E. Netto, M.A. de

Oliveira, Catalytic Thr or ser residue modulates structural switches in 2-cys peroxiredoxin by distinct mechanisms, Sci. Rep. 6 (2016) 33133.
[16] P. Chen, J. Li, B. Gao, P. Liu, Q. Wang, J. Li, cDNA cloning and characterization of peroxiredoxin gene from the swimming crab *Portunus trituberculatus*, Aquaculture 322–323 (2011) 10–15 https://doi.org/10.1016/j.aquaculture.2011.09.009.
[17] L. Qiu, Z. Ma, S. Jiang, W. Wang, F. Zhou, J. Huang, J. Li, Q. Yang, Molecular cloning and mRNA expression of peroxiredoxin gene in black tiger shrimp (*Penaeus monodon*), Mol. Biol. Rep. 37 (2010) 2821–2827 https://doi.org/10.1007/s11033-009-9832-8.
[18] Q. Zhang, J. Huang, F. Li, S. Liu, Q. Liu, J. Wei, G. Liang, J. Xiang, Molecular characterization, immune response against white spot syndrome virus infection of peroxiredoxin 4 in *Fenneropenaeus chinensis* and its antioxidant activity, Fish Shellfish Immunol. 37 (2014) 38–45.
[19] X. Wang, B. Hu, C. Wen, M. Zhang, S. Jian, G. Yang, Molecular cloning, expression and antioxidative activity of 2-cys-peroxiredoxin from freshwater mussel Cristaria plicata, Fish Shellfish Immunol. 66 (2017) 254–263 https://doi.org/10.1016/j.fsi.2013.12.026.
[20] Y.Z. Yang, Y. Zhao, L. Yang, L.P. Yu, H. Wang, X.S. Ji, Characterization of 2-Cys peroxiredoxin 3 and 4 in common carp and the immune response against bacterial infection, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 217 (2018) 60–69 https://doi.org/10.1016/j.cbpb.2017.12.012.
[21] A. Nicolussi, S. D'Inzeo, C. Capalbo, G. Giannini, A. Coppa, The role of peroxiredoxins in cancer, Mol Clin Oncol 6 (2017) 139–153 https://doi.org/10.3892/mco.2017.1129.
[22] S.G. Rhee, H.A. Woo, I.S. Kil, S.H. Bae, Peroxiredoxin functions as a peroxidase and a regulator and sensor of local peroxides, J. Biol. Chem. 287 (2012) 4403–4410 https://doi.org/10.1074/jbc.R111.283432.
[23] K. Saranya Revathy, N. Umasuthan, I. Whang, H.B. Jung, B.S. Lim, B.H. Nam, J. Lee, A potential antioxidant enzyme belonging to the atypical 2-Cys peroxiredoxin subfamily characterized from rock bream, Oplegnathus fasciatus, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 187 (2015) 1–13 https://doi.org/10.1016/j.cbpb.2015.04.008.
[24] G.I. Godahewa, N.C.N. Perera, J. Lee, Insights into the multifunctional role of natural killer enhancing factor-A (NKEF-A/Prx1) in big-belly seahorse (Hippocampus abdominalis): DNA protection, insulin reduction, H2O2 scavenging, and immune modulation activity, Gene 642 (2018) 324–334 https://doi.org/10.1016/j.gene.2017.11.042.
[25] M. De Zoysa, J.H. Ryu, H.C. Chung, C.H. Kim, C. Nikapitiya, C. Oh, H. Kim, K. Saranya Revathy, I. Whang, J. Lee, Molecular characterization, immune responses and DNA protection activity of rock bream (Oplegnathus fasciatus), peroxiredoxin 6 (Prx6), Fish Shellfish Immunol. 33 (2012) 28–35 https://doi.org/10.1016/j.fsi.2012.03.029.
[26] L.S. Dai, X.M. Yu, M.N. Abbas, C.S. Li, S.H. Chu, S. Kausar, T.T. Wang, Essential role of the peroxiredoxin 4 in Procambarus clarkii antioxidant defense and immune responses, Fish Shellfish Immunol. 75 (2018) 216–222 https://doi.org/10.1016/j.fsi.2018.01.042.
[27] J.H. Kim, J.S. Rhee, J.S. Lee, H.U. Dahms, J. Lee, K.N. Han, J.S. Lee, Effect of cadmium exposure on expression of antioxidant gene transcripts in the river pufferfish, Takifugu obscurus (Tetraodontiformes), Comp. Biochem. Physiol. C Toxicol. Pharmacol. 152 (2010) 473–479 https://doi.org/10.1016/j.cbpc.2010.08.002.
[28] C. Wu, J. Gao, F. Cao, Z. Lu, L. Chen, J. Ye, Molecular cloning, characterization and mRNA expression of six peroxiredoxins from Black carp Mylopharyngodon piceus in response to lipopolysaccharide challenge or dietary carbohydrate, Fish Shellfish Immunol. 50 (2016) 210–222 https://doi.org/10.1016/j.fsi.2016.01.033.
[29] J. Perez-Sanchez, A. Bermejo-Nogales, J.A. Calduch-Giner, S. Kaushik, A. Sitja-Bobadilla, Molecular characterization and expression analysis of six peroxiredoxin paralogous genes in gilthead sea bream (Sparus aurata): insights from fish exposed to dietary, pathogen and confinement stressors, Fish Shellfish Immunol. 31 (2011) 294–302 https://doi.org/10.1016/j.fsi.2011.05.015.
[30] Y. Iuchi, F. Okada, S. Tsunoda, N. Kibe, N. Shirasawa, M. Ikawa, M. Okabe, Y. Ikeda, J. Fujii, Peroxiredoxin 4 knockout results in elevated spermatogenic cell death via oxidative stress, Biochem. J. 419 (2009) 149–158 https://doi.org/10.1042/BJ20081526.
[31] M.B. Bacano Maningas, T. Koyama, H. Kondo, I. Hirono, T. Aoki, A peroxiredoxin from kuruma shrimp, Marsupenaeus japonicus, inhibited by peptidoglycan, Dev. Comp. Immunol. 32 (2008) 198–203 https://doi.org/10.1016/j.dci.2007.07.004.
[32] D.D. Tu, Y.L. Zhou, W.B. Gu, Q.H. Zhu, B.P. Xu, Z.K. Zhou, Z.P. Liu, C. Wang, Y.Y. Chen, M.A. Shu, Identification and characterization of six peroxiredoxin transcripts from mud crab Scylla paramamosain: the first evidence of peroxiredoxin gene family in crustacean and their expression profiles under biotic and abiotic stresses, Mol. Immunol. 93 (2018) 223–235 https://doi.org/10.1016/j.molimm.2017.11.029.
[33] G.H. Loo, K.A. Schuller, Cloning and functional characterization of a peroxiredoxin 4 from yellowtail kingfish (Seriola lalandi), Comp. Biochem. Physiol. B Biochem. Mol. Biol. 156 (2010) 244–253 https://doi.org/10.1016/j.cbpb.2010.03.013.
[34] C. Kiruthiga, S. Rajesh, V. Rashika, R. Priya, R.B. Narayanan, Molecular cloning, expression analysis and characterization of peroxiredoxin during WSSV infection in shrimp Fenneropenaeus indicus, J. Invertebr. Pathol. 109 (2012) 52–58 https://doi.org/10.1016/j.jip.2011.09.006.
[35] J. Arockiaraj, S. Easwvaran, P. Vanaraja, A. Singh, R.Y. Othman, S. Bhassu, Immunological role of thiol-dependent peroxiredoxin gene in Macrobrachium rosenbergii, Fish Shellfish Immunol. 33 (2012) 121–129 https://doi.org/10.1016/j.fsi.2012.04.010.
[36] T.T. Priyathilaka, Y. Kim, H.M.V. Udayantha, S. Lee, H. Herath, H.H.C. Lakmal, D.A.S. Elvitigala, N. Umasuthan, G.I. Godahewa, S.I. Kang, H.B. Jeong, S.K. Kim, D.J. Kim, B.S. Lim, Identification and molecular characterization of peroxiredoxin 6 from Japanese eel (Anguilla japonica) revealing its potent antioxidant properties and putative immune relevancy, Fish Shellfish Immunol. 51 (2016) 291–302 https://doi.org/10.1016/j.fsi.2015.12.012.
[37] G.I. Godahewa, N.C.N. Perera, D.A.S. Elvitigala, R. Jayasooriya, G.Y. Kim, J. Lee, 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, Fish Shellfish Immunol. 57 (2016) 186–197 https://doi.org/10.1016/j.fsi.2016.08.028.
[38] G.I. Godahewa, Y. Kim, S.H. Dananjaya, R.G. Jayasooriya, J.K. Noh, J. Lee, M. De Zoysa, Mitochondrial peroxiredoxin 3 (Prx3) from rock bream (Oplegnathus fasciatus): immune responses and role of recombinant Prx3 in protecting cells from hydrogen peroxide induced oxidative stress, Fish Shellfish Immunol. 43 (2015) 131–141 https://doi.org/10.1016/j.fsi.2014.12.011.
[39] L. Ren, Y. Sun, R. Wang, T. Xu, Gene structure, immune response and evolution: comparative analysis of three 2-Cys peroxiredoxin members of miiuy croaker, Miichthys miiuy, Fish Shellfish Immunol. 36 (2014) 409–416 https://doi.org/10.1016/j.fsi.2013.12.014.
[40] S. Yu, Y. Mu, J. Ao, X. Chen, Peroxiredoxin IV regulates pro-inflammatory responses in large yellow croaker (Pseudosciaena crocea) and protects against bacterial challenge, J. Proteome Res. 9 (2010) 1424–1436 https://doi.org/10.1021/pr900961x.
[41] S.N. Radyuk, V.I. Klichko, K. Michalak, W.C. Orr, The effect of peroxiredoxin 4 on fly physiology is a complex interplay of antioxidant and signaling functions, FASEB J. 27 (2013) 1426–1438 https://doi.org/10.1096/fj.12-214106.