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Analysis of the expression and antioxidative property of a peroxiredoxin 6 from *Scophthalmus maximus*

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ABSTRACT

Peroxiredoxins (Prxs) are a group of antioxidant proteins that protect cells from oxidative damage caused by various peroxides. To date, six different isoforms of peroxiredoxin (Prx1 to Prx6) have been identified, of which, Prx6 belongs to the 1-Cys Prx subfamily. Although Prx6 of several fish species have been reported at sequence level, there are very few documented studies on the potential function of fish Prx6. In this report, we describe the identification and analysis of a Prx6 homologue, SmPrx6, from turbot *Scophthalmus maximus*. The full length cDNA of SmPrx6 contains a 5′- untranslated region (UTR) of 60 bp, an open reading frame of 666 bp, and a 3′-UTR of 244 bp. The deduced amino acid sequence of SmPrx6 shares 81–87% overall identities with known fish Prx6. In silico analysis identified in SmPrx6 a conserved Prx6 catalytic motif, PVCTTE, and the catalytic triads putatively involved in peroxidase and phospholipase A2 activities. Expression of *SmPrx6* was detected in most fish organs, with the highest expression levels found in blood and heart and the lowest level in spleen. Experimental challenges with bacterial pathogens and poly(I:C) upregulated *SmPrx6* expression in liver and spleen in a manner that is dependent on the challenging agent and the tissue type. Treatment of cultured primary hepatocytes with H_2O_2 enhanced *SmPrx6* expression in a dose-dependent manner. Recombinant SmPrx6 expressed in and purified from *Escherichia coli* exhibited thiol-dependent antioxidant activity and could protect cultured hepatocytes from H_2O_2-induced oxidative damage. Taken together, these results indicate that SmPrx6 is a Prx6 homologue with antioxidative property and is likely to be involved in both cellular maintenance and protective response during host immune defense against bacterial infection.

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1. Introduction

Reactive oxygen species (ROS), which are produced by cells during normal physiological process and in response to stress conditions, participate in many important biological processes such as immune response and signal transduction [1–3]. However, owing to the destructive effect of ROS on cellular components, excessive accumulation of ROS is detrimental to the cell. As a result, the intracellular level of ROS is under tight control. One of the proteins that are involved in ROS homeostasis is peroxiredoxin (Prx), which represents a group of antioxidant enzymes that exist ubiquitously in almost all living organisms (4). Unlike most peroxidases, which depend on prosthetic groups or metal ions as cofactors for activity, peroxiredoxins rely on a conserved cysteine residue called the peroxidatic cysteine to cleave the peroxyl bonds of various peroxide substrates [4,5]. According to the number of cysteine residues involved in catalysis and the type of disulfide bond formed, peroxiredoxins are divided into three subfamilies: (i) 1-Cys Prxs, which contain only one conserved cysteine, i.e., the redox-active peroxidatic cysteine, (ii) typical 2-Cys Prxs, which contain two conserved cysteines, and (iii) atypical 2-Cys Prxs, which contain one conserved cysteine but require a non-conserved cysteine for catalytic activity [6–9]. All peroxiredoxins share a common catalytic cycle, in which the peroxidatic cysteine is oxidized by peroxide to a sulfenic acid, which is subsequently reduced back to thiol state by electron donors such as glutathione, dithiothreitol (DTT), and ascorbic acid [10,11]. In mammals, six different isoforms of peroxiredoxin (Prx1–Prx6) have been identified, of which, Prx1–Prx4 belong to the 2-Cys Prx subfamily, while Prx5 and Prx6 belong to the atypical 2-Cys and the 1-Cys Prx subfamilies, respectively.

Prx6 is called a bifunctional enzyme on the account that it possesses both glutathione peroxidase activity and phospholipase A2 (PLA2) activity [12]. Studies of human Prx6 have indicated that Prdx6 forms a thioredoxin "fold" containing four central β strands and two α helices which are essential to the higher-order structure

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of the protein [13,14]. The peroxidase activity of Prx6 depends on a conserved structural site called the catalytic center with the sequence feature of PVCTTE. The physiological importance of Prx6 has been demonstrated by mutational studies, which showed that, for example, mice lacking Prx6 are viable but sensitive to oxidative stress [15].

To date, a number of Prx6 have been identified in marine species such as Antarctic bivalve *Laternula elliptica* [16], Chinese shrimp *Fenneropenaeus chinensis* [17], disk abalone *Haliotis discus discus* [18], Pacific oyster *Crassostrea gigas* [19], and *Arenicola marina* [20]. However, only four fish Prx6 have been reported at sequence level, which are Prx6 from *Salmo salar*, *Oncorhynchus mykiss*, *Ictalurus punctatus*, and *Danio rerio* (GenBank accession nos: ACI67571, NP_001158604, ABG77029, and NP_957099, respectively). In addition, a partial Prx6 sequence from winter flounder (*Pseudopleuronectes americanus*) has also been reported [21]. The expression and function of these fish Prx6 proteins are largely uninvestigated. In turbot (*Scophthalmus maximus*), although no Prx6 has been reported, a Prx1 homologue called natural killer cell enhancing factor (NKEF) has been identified and its expression was found to be modulated by bacterial challenge and development [22].

In this report, we described the identification and analysis of a Prx6 homologue from turbot, which is one of the most important cultured fish species in northern China. We found that SmPrx6 was regulated at expression level by bacterial infection and that purified recombinant SmPrx6 possessed apparent antioxidant activity and could protect cultured cells against oxidative damage.

2. Materials and methods

2.1. Fish

Turbot (*S. maximus*) were purchased from a commercial fish farm in Shandong Province, China and maintained at 17 °C in aerated seawater. Fish were acclimatized in the laboratory for two weeks before experimental manipulation. Fish were anaesthetized with tricaine methanesulfonate (Sigma, St. Louis, MO, USA) prior to experiments involving injection, blood collection, or tissue removal.

2.2. Bacterial strains

Streptococcus iniae G26 and *Edwardsiella tarda* TX1 are fish pathogens that have been described previously [23,24]. *Listonella anguillarum* C312 is a pathogenic strain isolated from diseased flounder; it was virulent to both Japanese flounder and turbot (median lethal doses less than 5×10^6 CFU) in live animal infection studies. *Escherichia coli* BL21(DE3) was purchased from Tiangen, Beijing, China. All strains were cultured in LB medium [25] at 37 °C (for *E. coli*) or 28 °C (for all others).

2.3. Bacterial challenge and tissue collection for cDNA library construction

S. iniae G26 and *E. tarda* TX1 were cultured in LB medium to mid-logarithmic phase and resuspended in phosphate-buffered saline (PBS). Turbot (860–910 g) were randomly divided into two groups (3 fish/group) named A and B. Group A was injected intraperitoneally (i.p.) with 1×10^8 CFU of G26 and TX1, while group B was injected with PBS. At 24 h post-infection, the fish were sacrificed with a lethal dose of tricaine methanesulfonate, and the spleen, liver, and head kidney were collected under aseptic conditions. Tissues from the same fish were pooled together at equal amount and frozen in liquid nitrogen. The pooled tissues were grounded in a mortar, and equal amounts of grounded tissues from each fish were mixed and used for RNA preparation.

2.4. RNA extraction and cDNA library construction

Total RNA was isolated from the pooled fish tissues with the RNAprep Tissue Kit (Tiangen, Beijing, China). The RNA was used for the construction of cDNA library with the Super SMART PCR cDNA Synthesis Kit (Clontech, Mountain View, CA, USA) according to manufacturer's instructions.

2.5. Cloning of SmPrx6

Plasmid DNA was isolated from ~2000 clones of the cDNA library and subjected to DNA sequencing with the standard T7 primer. One of the clones was found to contain the full length cDNA of a Prx6 homologue (named *SmPrx6*). The 5′ and 3′ untranslated regions (UTRs) of *SmPrx6* were confirmed by rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to manufacturer's recommendations. The nucleotide sequence of *SmPrx6* has been deposited in GenBank database under the accession number GU561990.

2.6. Sequence analysis

The cDNA and amino acid sequences of SmPrx6 were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI. The calculated molecular mass and theoretical isoelectric point were predicated by EditSeq in DNASTAR software package (DNASTAR Inc. Madison, WI, USA). Signal peptide search and subcellular localization prediction were performed with SignalP 3.0 and CELLO v.2.5, respectively.

2.7. Quantitative real time reverse transcriptase PCR (qRT-PCR) analysis of SmPrx6 expression in fish tissues

Brain, heart, gill, kidney, spleen, liver, muscle, and blood were taken aseptically from five fish and used for total RNA extraction as described above. One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR was carried out in an ABI 7300 Real-time Detection System (Applied Biosystems, Foster City, CA, USA) by using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described previously [26]. Each assay was performed in triplicate with β-actin mRNA as the control. The primers used to amplify the β-actin gene were ATF1 (5′-TGAACCCCAAAGCCAACAGG-3′) and ATR1 (5′- AGAGGCATACAGGGACAGCAC-3′); the primers used to amplify *SmPrx6* were Prx6RTF (5′- TGCTGGACCCCGATGAGATAGA -3′) and Prx6RTR (5′- TCGTCAAAGTTCCTCCCTGTGG -3′). All data are given in terms of relative mRNA, expressed as means plus or minus standard errors of the means (SE).

2.8. Expression of SmPrx6 in response to experimental challenge

L. anguillarum C312 and *S. iniae* G26 were cultured in LB medium and resuspended in PBS as described above to 5×10^7 CFU/ml. Polyinosinic-polycytidylic acid {poly(I:C)} (Sigma, USA) was suspended in PBS to 0.5 mg/ml. Turbot (~8.7 g) were divided randomly into four groups and injected i.p. with 100 μl of C312, G26, poly(I:C), and PBS, respectively. Fish were sacrificed at various times (5 fish/time) post-challenge, and spleen and liver were excised under aseptic conditions. Total RNA extraction from tissues, cDNA synthesis, and qRT-PCR were performed as described above.

2.9. Cell culture

Primary cultures of turbot hepatocytes were established as described by Schmid et al. [27]. In brief, turbot liver was removed under aseptic conditions and washed three times with PBS containing 100 U of penicillin and streptomycin (Thermo Scientific HyClone, Beijing, China). The liver was cut into small pieces and digested with trypsin (Sigma, USA). The digested solution was centrifuged at 500×*g* for 10 min, and cell pellet was resuspended in RPMI 1640 (Thermo Scientific HyClone, Beijing, China) containing 15% fetal bovine serum (FBS) (Thermo Scientific HyClone, Beijing, China) and 100 U of penicillin and streptomycin (1640P). The cells were seeded in monolayers in 96-well culture plates with 1640P and cultivated at 25 °C.

2.10. Expression of SmPrx6 in response to H_2O_2 treatment

Turbot primary hepatocytes were maintained in 1640P as described above; H_2O_2 was added into the cell culture at the final concentrations of 10 μM, 20 μM, and 30 μM, respectively. After incubation at 25 °C for 8 h, the cells ($\sim 4 \times 10^5$) were collected and used for RNA extraction with the Total RNA Kit I of Omega Bio-tek (Beijing, China). *SmPrx6* expression was determined by qRT-PCR as described above.

2.11. Plasmid construction

pET259 was constructed by inserting linker L811 (5′- TATGGCAT TTAAATCTC -3′) into pET258 [23] between NdeI/XhoI sites. To construct pETPrx6, the coding region of SmPrx6 was amplified by PCR with primers Prx6F1 (5′- GATATCGCCACCATGCCTGGACTTCTG CT -3′; underlined sequence, EcoRV site) and Prx6R1 (5′- CGCGATATCGGGCTGAGTGTAGCG -3′; underlined sequence, EcoRV site); the PCR products were ligated with the T-A cloning vector pBS-T (Tiangen, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the 0.6 kb fragment, which was inserted into pET259 at the SwaI site.

2.12. Expression and purification of recombinant SmPrx6 (rSmPrx6)

E. coli BL21(DE3) was transformed with pETPrx6. The transformant BL21(DE3)/pETPrx6 was cultured in LB medium at 37 °C to mid-log phase, and expression of *SmPrx6* was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.6 mM. After growth at 37 °C for an additional 4 h, the cells were harvested by centrifugation and lysed with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). His-tagged rSmPrx6 was purified under native conditions using nickel–nitrilotriacetic acid columns (GE Healthcare, Piscataway, NJ, USA) as recommended by the manufacturer. The purified protein was dialyzed against PBS for 32 h at 4 °C and concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250.

2.13. Assay for the antioxidant activity of rSmPrx6

The assay was performed in 50 mM Hepes–NaOH buffer (pH 7.0) containing 100 μM H_2O_2 and 10 μg/ml native or heat-denatured (by boiling for 15 min) rSmPrx6 in the presence or absence of 10 mM DTT. Recombinant FliC, a bacterial flagellin protein purified from *E. coli* BL21(DE3) [28], was used as a negative control. Reduction of H_2O_2 was measured by monitoring decrease in absorbance at A_{240} as described previously [29,30].

2.14. Effect of rSmPrx6 on cultured cells against H_2O_2 challenge

Turbot primary hepatocytes ($\sim 1 \times 10^6$) cultured as described above were treated with 5 μg/ml native or heat-denatured rSmPrx6 and H_2O_2 at the concentrations of 20 μM, 40 μM, 80 μM, and 100 μM, respectively. After incubation at 25 °C for 8 h, the cells were determined for viability using the MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Beijing, China) according to manufacturer's instructions.

2.15. Statistical analysis

All statistical analyses were performed with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as means ± SE, and statistical significances were determined with Student's *t*-test. In all cases, significance was defined as $P < 0.05$.

3. Results

3.1. Sequence characterization of SmPrx6

The full length cDNA of SmPrx6 contains a 5′- untranslated region (UTR) of 60 bp, an open reading frame (ORF) of 666 bp, and a 3′-UTR of 244 bp (Fig. 1). The cDNA ends with a poly-A tail which is preceded at 14 bp upstream by a polyadenylation signal, AATAAA. The ORF encodes a putative protein of 221 amino acid residues which shares 87%, 86%, 83%, and 81% overall identities with the Prx6 of *O. mykiss*, *S. salar*, *I. punctatus*, and *D. rerio*, respectively (GenBank accession nos: NP_001158604, ACI67571, ABG77029, and NP_957099,respectively). SmPrx6 also shares high (74–75%) sequence identities with the mammalian Prx6 from *Bos Taurus*, *Sus scrofa*, and *Homo sapiens* (GenBank accession nos: AAX08984, NP_999573, and AAH35857, respectively). In contrast, SmPrx6 shares only 27.8% overall identity with the turbot Prx1 homologue NKEF. The predicted molecular mass and theoretical isoelectric point of SmPrx6 were 24.3 kDa and 5.44, respectively. Conserved domain search identified SmPrx6 as a member of the 1-Cys Prx

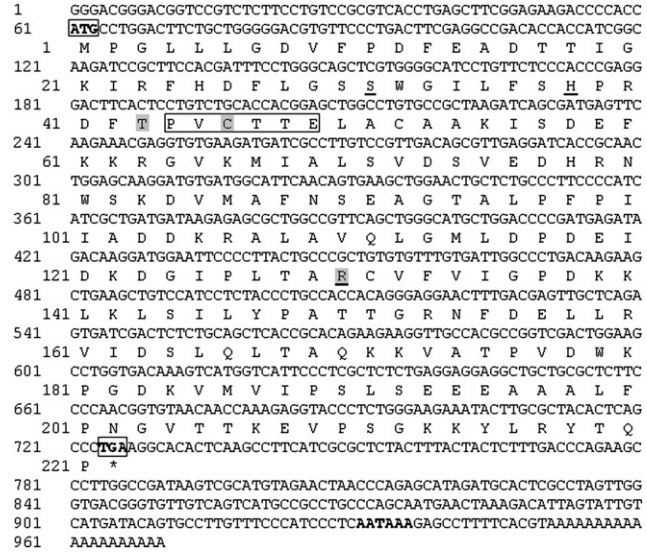

Fig. 1. The nucleotide and deduced amino acid sequences of SmPrx6. The nucleotides and amino acids are numbered along the left margin. The translation start (ATG) and stop (TGA) codons are boxed and in bold, the conserved catalytic center (PVCTTE) is boxed, the catalytic triad (T43, C46, and R130) involved in peroxidase activity is shaded, the catalytic triad (S31, H38, and R130) for phospholipase A2 activity is underlined, and the polyadenylation signal (AATAAA) is in bold.

subfamily with the conserved catalytic triad formed by T43, C46, and R130. The Prx6-specifc catalytic center, PVCTTE, is completely preserved in SmPrx6. In addition, a putative catalytic triad for PLA2 activity, composed by S31, H38, and R130, was also found in SmPrx6. Signal peptide search discovered no potential signal sequence in SmPrx6. Consistently, subcellular localization prediction indicated that SmPrx6 is a cytoplasmic protein.

3.2. Constitutive expression of SmPrx6 in turbot tissues

To examine the expression profile of *SmPrx6* in turbot tissues, total RNA was extracted from muscle, liver, spleen, kidney, heart, brain, blood, and gill and used for qRT-PCR analysis. The results showed that *SmPrx6* expression was highest in blood and heart and lowest in spleen (Fig. 2). Compared to *SmPrx6* expression in spleen, *SmPrx6* expression in gill, brain, liver, kidney, muscle, heart, and blood were, respectively, 2-, 3.2-, 5.3-, 6.6-, 10.8, 18.3-, and 18.9-fold higher.

3.3. SmPrx6 expression in response to experimental challenge

To examine whether *SmPrx6* expression was affected by microbial infection, turbot were challenged with the Gram-negative fish pathogen *L. anguillarum*, the Gram-positive fish pathogen *S. iniae*, and the synthetic double-stranded RNA poly(I:C). *SmPrx6* expression in spleen and liver was determined by qRT-PCR at 4 h, 8 h, 12 h, 24 h, 48 h, and 72 h post-challenge. The results showed that *SmPrx6* expression in spleen was drastically induced by *L. anguillarum* and poly(I:C), with maximum induction folds reaching 42.6 and 29, respectively, at 24 h and 4 h post-challenge (Fig. 3A). *S. iniae* also significantly enhanced *SmPrx6* expression, though to a much lesser degree than *L. anguillarum* and poly(I:C). However, *S. iniae* infection induced drastic *SmPrx6* expression in liver, with maximum induction (~50-fold) occurring at 4 h and 48 h post-challenge (Fig. 3B). In liver, poly(I:C) treatment caused significant, though low levels of, *SmPrx6* induction at all the examined time points, while *L. anguillarum* challenge induced weak *SmPrx6* expression which reached significant levels at 12 h and 72 h post-challenge.

3.4. SmPrx6 expression in response to oxidative stress

To examine *SmPrx6* expression under stress conditions induced by oxidant, turbot primary hepatocytes were exposed to challenge with different concentrations of H_2O_2. Subsequent qRT-PCR analysis showed that *SmPrx6* expression was upregulated by H_2O_2 in a manner that depended on the dose of H_2O_2 (Fig. 4).

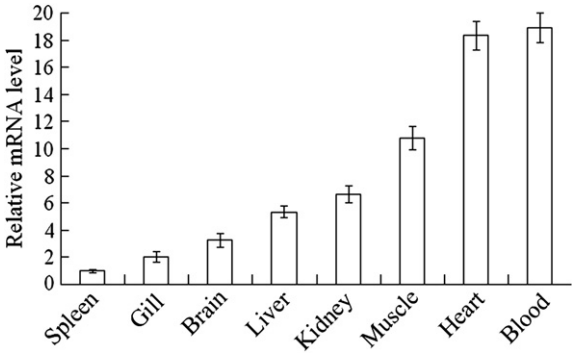

Fig. 2. *SmPrx6* expression in turbot tissues detected by quantitative real time reverse transcriptase PCR. *SmPrx6* expression levels in gill, brain, liver, kidney, muscle, heart, and blood are normalized to that in spleen. Vertical bars represent means ± SE ($N = 5$).

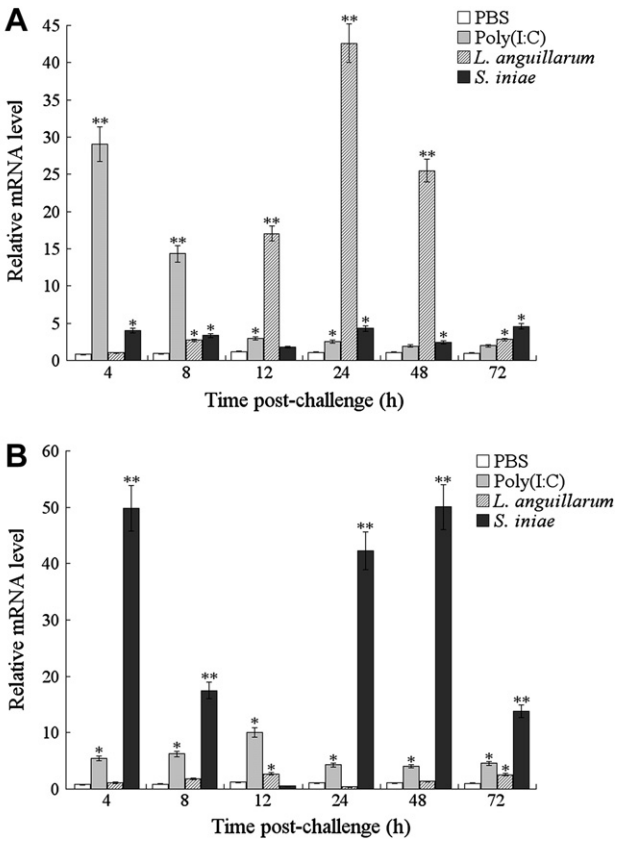

Fig. 3. *SmPrx6* expression in turbot tissues in response to experimental challenge. Turbot were challenged separately with poly(I:C), *Listonella anguillarum*, *Streptococcus iniae*, and PBS. *SmPrx6* expression in spleen (A) and liver (B) was determined by quantitative real time reverse transcriptase PCR at various times post-challenge. The mRNA level of *SmPrx6* was normalized to that of β-actin. Values are shown as means ± SE ($N = 5$). Significances between PBS-challenged fish and bacterium/poly(I:C)-challenged fish are indicated with asterisks. $^*P < 0.05$; $^{**}P < 0.01$.

3.5. Purification of recombinant SmPrx6 (rSmPrx6) from E. coli

In order to examine the biological property of SmPrx6, the coding sequence of SmPrx6 was subcloned into *E. coli*, and rSmPrx6

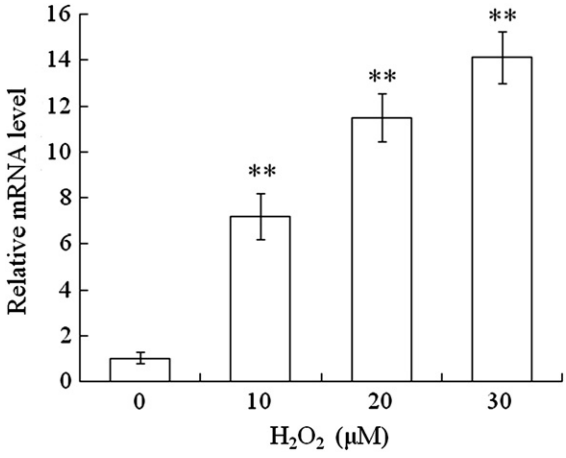

Fig. 4. *SmPrx6* expression in turbot cultured hepatocytes in response to H_2O_2 challenge. Turbot cultured primary hepatocytes were treated with different concentrations of H_2O_2, and *SmPrx6* expression was determined by quantitative real time reverse transcriptase PCR. The mRNA level of *SmPrx6* was normalized to that of β-actin. Values are shown as means ± SE ($N = 4$). Significances between control (untreated cells) and H_2O_2-treated cells are indicated with asterisks. $^*P < 0.05$; $^{**}P < 0.01$.

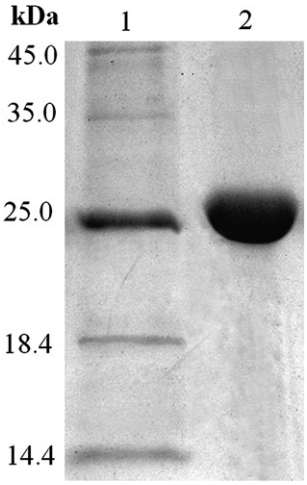

Fig. 5. SDS-PAGE analysis of recombinant SmPrx6. Recombinant SmPrx6 (lane 2) was analyzed by SDS-PAGE and viewed after staining with Coomassie brilliant blue R-250. Lane 1, protein markers.

was purified from the *E. coli* transformants under native conditions. SDS-PAGE analysis showed that the purified protein exhibited a single band with a molecular mass comparable to that predicted for rSmPrx6 (25.2 kDa) (Fig. 5).

3.6. Antioxidant activity of rSmPrx6

To examine the antioxidative potential of rSmPrx6, the protein was assayed for the ability to reduce H_2O_2 in the presence or absence of DTT. As a negative control, recombinant FliC, a bacterial flagellin purified from *E. coli* BL21(DE3), was also included in the assay. The results showed that rSmPrx6, but not FliC, caused apparent H_2O_2 degradation in the presence of DTT (Fig. 6). In contrast to native rSmPrx6, heat-denatured rSmPrx6 exhibited only weak peroxidase activity.

3.7. Protective effect of rSmPrx6 on cultured cells against oxidative stress

To examine the protective effect of rSmPrx6 against oxidative stress, cultured primary hepatocytes were treated with different concentrations of H_2O_2 in the presence or absence of rSmPrx6. The cells were then subjected to viability assay. The results showed

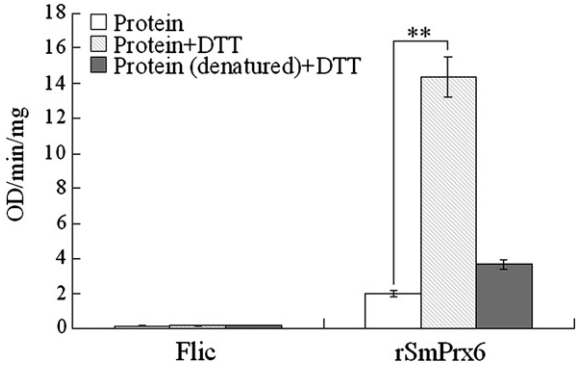

Fig. 6. Antioxidant activity of rSmPrx6. The ability of native and heat-denatured rSmPrx6 to reduce H_2O_2 in the presence or absence of dithiothreitol (DTT) was determined by measuring decrease in absorbance at A_{240}. FliC was used as a negative control. Data are shown as means ± SE ($N = 5$).

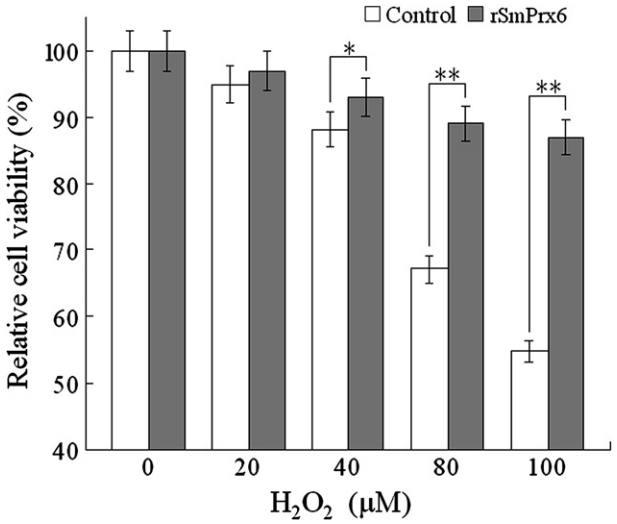

Fig. 7. Effect of rSmPrx6 on cultured cells against H_2O_2 challenge. Turbot cultured primary hepatocytes were treated with different concentrations of H_2O_2 in the presence or absence of rSmPrx6, and the cells were determined for viability. Data are shown as means ± SE ($N = 4$). Significances between untreated (control) and rSmPrx6-treated cells are indicated with asterisks. $^{*}P < 0.05$; $^{**}P < 0.01$.

that the presence of rSmPrx6 significantly enhanced the survival rate of hepatocytes, especially in treatments with high-dose of H_2O_2 (Fig. 7).

4. Discussion

In this report, we described the identification and analysis of a Prx6 homologue, SmPrx6, from turbot. Prx6 is known to be ubiquitous among all living organisms and highly conserved at DNA and amino acid sequence levels. Sequence analyses have indicated that the deduced amino acid sequences of Prx6 from human, cow, mouse, rat, and pig are more then 95% identical [8], which suggests from the evolutionary aspect the biological importance of Prx6. In our study, we found that SmPrx6 shares more than 80% overall sequence identities with other fish Prx6 and is also highly homologous to higher vertebrate Prx6. Like typical Prx6, SmPrx6 possesses the conserved catalytic domain and the catalytic triads putatively involved in peroxidase and PLA2 activities. These sequence and structural features classify SmPrx6 as a member of the Prx6 subfamily of peroxiredoxin. Consistently, purified rSmPrx6 exhibited apparent antioxidant activity in the presence of DTT, suggesting that rSmPrx6 is a thiol-dependent peroxidase. Together these results indicate that SmPrx6 is a Prx6 homologue and is likely to be a biologically active peroxidase in turbot.

Studies of bovine, rat, and mouse peroxiredoxins have shown that Prx6 expressed in all major mammalian organs such as lung, heart, liver, and spleen, with the highest level appearing in lung and the lowest level in spleen [9,31,32]. In fish, there is only one documented study on tissue-specific expression of Prx6, which showed that in channel catfish, the expression of Prx6 was most abundant in head kidney, liver, intestine, and skin but meager in gill and sometimes undetectable in spleen [33]. In turbot, it was reported that NKEF, a typical 2-Cys Prx, exhibits high levels of expression in kidney, liver, and spleen and low levels of expression in gill, muscle, and heart [22]. In our study, we found that, unlike NKEF, *SmPrx6* expression was high in blood, heart, muscle, and kidney and low in spleen, which is similar to what have been observed with mammalian and channel catfish Prx6. These results suggest that in turbot, SmPrx6 is distinct from NKEF in

tissue-specific distribution and that SmPrx6 is probably the peroxiredoxin isoform that is involved in cellular maintenance under normal physiological conditions in organs such as blood, heart, muscle, and kidney.

Induction of Prx6 expression by various stress signals have been observed by many research groups. For example, it is reported that transcription of a Pacific oyster Prx6 increased with pollution level [19] and expressions of Prx6 in Antarctic bivalve, Chinese shrimp, and zebrafish were induced by, respectively, thermal exposure, bacterial challenge, and brominated flame retardants [16,17,34,35]. On the other hand, negative regulation of Prx6 expression has also been reported. It is known that the transcription of a 1-Cys peroxiredoxin in the liver of winter flounder was down-regulated by chromium oxide exposure [21], the expression of a 2-Cys Prx from kuruma shrimp (*Marsupenaeus japonicus*) is decreased in heart and lymphoid tissues by peptidoglycan treatment [36], and the expression of a Prx6 in the gill of disk abalone is negatively regulated by viral infection [18]. In turbot, expression of NKEF in liver, spleen, and head kidney was reported to be induced by bacterial challenge [22]. In our study, we found that, similar to NKEF, *SmPrx6* expression in liver and spleen was upregulated by challenges with Gram-negative and Gram-positive fish pathogens and poly(I:C). However, the expression patterns and kinetics varied with the challenging agent and the tissue type. *L. anguillarum* induced strong *SmPrx6* expression in spleen but only weak *SmPrx6* expression in liver; in contrast, *S. iniae* caused drastic *SmPrx6* expression in liver but only moderate *SmPrx6* expression in spleen. The tissue-specific induction pattern is probably due to the difference in infection mechanism between *L. anguillarum* and *S. iniae* as well as to the difference in defending mechanism between spleen and liver against the infection of these pathogens. Bacterial infection is a classical stress condition that elicits in the host a series of immune response involving the production of ROS, which, if uncontrolled, can have detrimental effect on the host. A recent study by Yang et al. [37] has demonstrated that in mice, 2-Cys peroxiredoxin II is an essential negative regulator of LPS-induced inflammatory signaling and is crucial for the prevention of excessive host responses to microbial products. A similar protective role may also be played by SmPrx6, since its expression is enhanced by bacterial infection. This hypothesis is in accordance with the fact that rSmPrx6 can protect cultured hepatocytes against oxidant challenge.

In conclusion, the results of this study demonstrate that SmPrx6 is a Prx6-like protein which possesses conserved 1-Cys peroxiredoxin features and thiol-dependent antioxidant activity. Expression of SmPrx6 occurs in most turbot tissues and is regulated by bacterial and poly(I:C) challenge. These observations suggest the possibility that SmPrx6 plays a role in both cellular maintenance and protective response during host immune defense against microbial infections.

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