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Characterization of 2-Cys peroxiredoxin 3 and 4 in common carp and the immune response against bacterial infection

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ABSTRACT

Accumulating evidence suggests that peroxiredoxins (Prxs) eliminate excessive cellular H_2O_2 and are important factors in redox signaling pathways. In this study, we cloned the full-length cDNAs and genomic sequences of *Prx3* and *Prx4* from common carp. The common carp *Prx3* and *Prx4* open reading frames were 753 base pairs (bp) and 783 bp in length, respectively, and contained seven exons and six introns. Multiple sequence alignment and phylogenetic analyses revealed that the common carp Prx1–4 proteins share high identities and similar characteristics with other known animal Prxs. *Prx3* and *Prx4* mRNA were constitutively expressed in all tissues, and the highest *Prx3* and *Prx4* transcript abundances occurred in head kidney. Although the highest Prx4 protein and mRNA expression were also observed in head kidney, many differences were detected between Prx4 mRNA and protein expression levels in other tissues. *Prx3* expression increased significantly in the head kidney 12 h after an *Aeromonas hydrophila* challenge. The *A. hydrophila* challenge upregulated *Prx3* mRNA expression in liver and spleen, increased *Prx4* mRNA expression levels in liver and spleen excluding at 36 h in spleen, but decreased *Prx4* mRNA expression level in the head kidney. The mature Prx4 peptide was recombinantly expressed and purified using Dextrin Beads 6FF and it exhibited thioredoxin (Trx)-dependent peroxidase activity. These data suggest that Prx3 and Prx4 are constitutive and inducible proteins that might play important roles in innate immune function. The Trx-dependent peroxidase activity analysis of recombinant Prx4 further verified the important role of Prxs in the redox system of fish.

1. Introduction

Reactive oxygen species (ROS) are produced in eukaryotic cells mainly by mitochondria as a result of aerobic respiration and associated redox reactions (Finkel, 1998; Valero et al., 2015). Low levels of ROS have beneficial effects, as they act as signaling molecules to regulate diverse biological processes (Holmstrom and Finkel, 2014). However, excessive ROS production causes serious oxidative damage to biological macromolecules and is associated with various diseases (Yu, 1994; Ye et al., 2015). Hydrogen peroxide (H_2O_2) is one of the most important ROS (Schwarz, 1996; Chen et al., 2013). Enzymes that eliminate H_2O_2 include catalases (CAT), glutathione peroxidases (GPx) and peroxiredoxins (Prxs) (Chae et al., 1994). In contrast with other peroxidases, Prxs have no special cofactor, but simply use cysteine residues for catalysis (Karplus, 2015). It has been observed that tumorous and virus-infected cells are able to secrete Prx proteins, which bind to Toll-like receptor 4 (*TLR4*), linking peroxiredoxin proteins with the immune response (Ishii et al., 2012; Ishii, 2015; Valero et al., 2015). Based on the number of conserved Cys per monomer, the Prx family is divided into either 2-Cys (Prx1–5) (Prx1 and Prx2 were also named as natural killer enhancing factor (NKEF)-A and NKEF-B, respectively) or 1-Cys peroxiredoxins (Prx6). Prx1–4 function as dimers in solution and use the peroxidatic Cys residue in NH_2-terminus of one monomer to reduce hydroperoxides; the resultant oxidized (sulfenic, -SOH) Cys interacts with the resolving Cys residue in C-terminus of the other monomer to generate a protein disulfide (Fisher, 2016). Prx5 is classified as an atypical 2-Cys Prx with both the peroxidatic and the resolving Cys on the same monomer forming an internal disulfide during the reaction cycle. Prx6 is the sole member of the 1-Cys group (Park et al., 2016). Similar to the mechanism for the 2-Cys Prx enzymes, reduction of hydroperoxides by 1-Cys Prx occurs through oxidation of its single conserved Cys (Fisher, 2016). Thioredoxin (Trx) provides the electron for reducing the oxidized Prx1–5, whereas glutathione is likely to be employed to reduce the oxidized Prx6 (Park et al., 2016).

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These Prxs isoforms also differ in their cellular distributions (Ambruso, 2013). Prx1, 2 and 6 are localized in the cytosol whereas Prx3 exists in the mitochondria and Prx4 has an N-terminal signal peptide targeting it for secretion from the cell. Prx5 is localized intracellularly to the cytosol, mitochondria, nuclei and peroxisomes (Shi et al., 2014). The Prx cDNAs have been cloned from many fish species, such as *Prx1* from common carp et al. (Shin et al., 2001; Zhang et al., 2001; Chen et al., 2006; Dong et al., 2007; Huang et al., 2009; Zheng et al., 2010; Ren et al., 2013; Ren et al., 2014; Wu et al., 2016); *Prx2* from common carp et al. (Shin et al., 2001; Dong et al., 2007; Huang et al., 2009; Sutton et al., 2010; Ren et al., 2013; Ren et al., 2014; Wu et al., 2016); *Prx3* from black carp (Wu et al., 2016); *Prx4* from yellowtail kingfish et al. (Loo and Schuller, 2010; Ren et al., 2013; Ren et al., 2014; Wu et al., 2016); *Prx5* from black carp (Wu et al., 2016); *Prx6* from rock bream et al. (Chen et al., 2006; Zheng et al., 2010; De Zoysa et al., 2012; Godahewa et al., 2015; Saranya Revathy et al., 2015; Priyathilaka et al., 2016; Tolomeo et al., 2016; Wu et al., 2016). Prx transcripts have been detected in all of these species but different Prx isoforms are expressed at different levels in different fish species and tissues. Previous studies have shown the tissue mRNA expression pattern of *Prx3* from black carp (Wu et al., 2016) and miiuy croaker (Ren et al., 2014), *Prx4* from miiuy croaker (Ren et al., 2013; Ren et al., 2014), black carp (Wu et al., 2016), gilthead sea bream and yellowtail kingfish (Loo and Schuller, 2010; Pérez-Sánchez et al., 2011). However, characterization at the protein level has received much less attention. Proteomic studies have identified Prx proteins from several fish species, and found that their roles have been related to oxidative stress and immunity (Valero et al., 2015). The Prx functions in fish have been mainly studied after exposure to immune stimuli (Valero et al., 2015). Moreover, most of the Prx information available for teleost fish has mainly concerned Prx1 and Prx2. Cloning of *Prx3* and *Prx4* and functional characterization of the Prx proteins have not been reported in common carp.

Common carp is an important commercial cyprinid species, accounting for 10% of worldwide freshwater aquaculture production (Xu et al., 2014). Intensive culture of common carp has led to their high susceptibility to various disease agents, such as *Aeromonas hydrophila* (Feng et al., 2016), spring viremia of carp virus (Ashraf et al., 2016) and others. It would be meaningful to investigate the roles of Prxs in the innate immune and metabolic systems of common carp, considering the association between Prxs expression level and disease susceptibility. Thus, the objectives of the present study were: 1) to clone the *Prx3* and *Prx4* genomic sequences from common carp (*Cyprinus carpio*), 2) to investigate *Prx3* and *Prx4* tissue expression distributions, 3) to determine the relative changes of *Prx3* and *Prx4* mRNA expression after *A. hydrophila* challenge, 4) to express the Prx4 protein in *E. coli* and analyze peroxidase activity of recombinantly expressed Prx4.

2. Materials and methods

2.1. Fish culture and sampling

The common carp, *Cyprinus carpio*, (mass, 60.0 ± 10.0 g) used in this study originated from a full-sib family developed at Shandong Freshwater Fisheries Research Institute. The study was approved by Shandong Agricultural University Animal Care and Use Committee with approval number SDAUA-2013-002. The fish were maintained temporarily for 2 weeks in 0.5-m^3 tanks filled with aerated freshwater. After euthanizing with MS-222 (tricaine methanesulfonate, 0.38 mM), the tissue samples were prepared from various tissues including brain, liver, heart, swim bladder, gill, muscle, fin, eye, intestine, spleen, gonad, and head kidney obtained from three different common carp and stored in liquid nitrogen until use. *Aeromonas hydrophila* were cultured in Luria-Bertani (LB) medium at 28 °C for 12 h, and the bacterial cells were harvested when the optical density value at 600 nm (OD_{600}) reached 1.0. Following centrifugation, the pellet was resuspended with sterile phosphate buffer saline (PBS) and diluted to 1.0×10^6–5.0×10^8 colony-forming units (CFU)·mL^{-1} in PBS for use. In order to determine the 50% lethal dose (LD50) of *A. hydrophila* at 48 h, 1×10^6, 5×10^6, 1×10^7, 5×10^7, 1×10^8, 2×10^8, 3×10^8, 4×10^8, 5×10^8 cfu·mL^{-1} *A. hydrophila* was injected intraperitoneally into common carp. The control group injected equal amount (1 mL) of PBS. For each group, 40 common carp with mean body weight of 60.0 g were used. The 50% lethal dose (LD50) of *A. hydrophila* at 48 h was determined to be 2.0×10^8 CFU/fish in this study. A group of 60 common carp (mean body weight: 60.0 g) was injected intraperitoneally (i.p.) with 2.0×10^8 CFU/fish of *A. hydrophila* and the fish were maintained in 3 separate tanks before terminating the experiment. Another group of 60 common carp were injected equal amounts of PBS to serve as controls and the fish were also maintained in 3 separate tanks. The liver, spleen and head kidney were collected from three different injected individuals, one from each tank, at 6, 12, 24, 36, 48, and 72 h post injection (hpi), and frozen immediately in liquid nitrogen until examined. While, liver, spleen and head kidney of control group were collected at 6 h post injection from three different individuals, one from each tank.

2.2. DNA/RNA extraction and cDNA synthesis

DNA was extracted from gill tissue using the standard method with proteinase K digestion and phenol-chloroform purification (Ma et al., 2009). RNA was extracted from various tissues using Trizol reagent (Takara Bio, Dalian, China) following the manufacturer's instructions. RNA quantity, purity and integrity were verified using a spectrophotometer (Thermo Scientific) and 1.0% agarose gel electrophoresis. First-strand cDNA was reverse transcribed according to the instructions in the PrimeScript® First strand cDNA synthesis kit (Takara Bio).

2.3. Cloning and sequence analyses

The primers for cloning the *Prx3* and *Prx4* open reading frame (ORF) fragments were designed according to the published common carp whole genome sequence (Table 1) (Xu et al., 2014). The cloned *Prx3* and *Prx4* ORF sequences were blasted with known genomic sequences of fish species in GenBank database and the primers for cloning the *Prx3* and *Prx4* introns were designed according to their ORF sequences and the blast results (Table 1). The PCR conditions were: 1 cycle of 94 °C for 5 min; 35 cycles at 94 °C for 30 s, 55–60 °C for 45 s, 72 °C for 45 s, and one cycle at 72 °C for 10 min.

The online BLAST program (http://blast.ncbi.nlm.nih.gov/) was used to analyze sequence homology of the nucleotide and amino acid sequences. Signal and domain predictions were carried out using SMART software (http://www.smart.embl-heidelberg). A phylogenetic tree was constructed based on the Prx amino acid sequences of common carp and other vertebrates using Mega 5.0 software and the UPGMA method (Tamura et al., 2011).

2.4. Quantitative real time-polymerase chain reaction (qRT-PCR)

The tissue distribution of *Prx1–4* mRNA expression in 12 tissues and the changes in *Prx3* and *Prx4* expression after the bacterial challenge were determined by qRT-PCR. The qRT-PCR primers are shown in Table 1. qRT-PCR was conducted utilizing a Mx3000p™ real-time PCR system. The qRT-PCR reaction mixtures (20 μL) consisted of 10 μL SYBR Premix Ex Taq (2 ×), 0.4 μL of each gene specific primer (10 nmol), 2 μL cDNA, and 0.4 μL ROX reference dye II. The PCR amplification procedure was initial denaturation at 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s, followed by a dissociation curve analysis to determine target specificity. Each assay was performed with β-actin cDNA as the positive control and no template was used as negative control. The assessment of the exact amplification efficiencies of target and reference genes were carried out. The