Fish & Shellfish Immunology 33 (2012) 28–35

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

Molecular characterization, immune responses and DNA protection activity of rock bream (*Oplegnathus fasciatus*), peroxiredoxin 6 (Prx6)

Mahanama De Zoysa [a,g], Jae-Ho Ryu [b,c], Hee-Chung Chung [b,c], Cheol-Hee Kim [b,c], Chamilani Nikapitiya [d], Chulhong Oh [e], Hyowon Kim [f], K. Saranya Revathy [f], Ilson Whang [f], Jehee Lee [f,*]

[a] College of Veterinary Medicine, Chungnam National University, Yuseong-gu, Daejeon 305-764, Republic of Korea
[b] Department of Biology, Chungnam National University, Daejeon 305-764, Republic of Korea
[c] GRAST, Chungnam National University, Daejeon 305-764, Republic of Korea
[d] Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881, USA
[e] Korea Ocean Research and Development Institute, Ansan 426-744, Republic of Korea
[f] Department of Marine Life Sciences, School of Marine Biomedical Sciences, Jeju National University, Jeju Special Self-Governing Province 690-756, Republic of Korea
[g] Research Institute of Veterinary Medicine, Chungnam National University, Yuseong-gu, Daejeon 305-764, Republic of Korea

ARTICLE INFO

Article history:
Received 7 December 2011
Received in revised form
14 March 2012
Accepted 22 March 2012
Available online 29 March 2012

Keywords:
Antioxidant
Oxidative stress
Peroxiredoxin 6
Rock bream
Oplegnathus fasciatus

ABSTRACT

In this study, we describe the molecular characterization, immune responses of rock bream, *Oplegnathus fasciatus* peroxiredoxin 6 cDNA (RbPrx6) and DNA protection activity of its recombinant protein. The full-length cDNA sequence of RbPrx6 was identified after pyrosequencing of rock bream cDNA library. RbPrx6 consists of 663 bp open reading frame (ORF) that codes for a putative protein of 221 amino acids with predicted molecular mass of 27 kDa. It showed characteristic peroxiredoxin super-family domain similar to vertebrate Prx counterparts. In the pair-wise comparison, RbPrx6 showed the highest amino acid identity (92.8%) to *Scophthalmus maximus* Prx6.

Real-time RT-PCR analysis revealed that constitutive expression of RbPrx6 transcripts in eleven tissues selected from un-challenged fish showing the highest level in liver. Synthetic polyinosinic:polycytidylic acid (poly I:C) and iridovirus containing supernatant, up-regulated the RbPrx6 mRNA in liver. Purified recombinant RbPrx6 protein was able to protect supercoiled plasmid DNA from damages that is induced by metal-catalyzed generation of reactive oxygen species. Our results suggest that RbPrx6 may play an important role in regulating oxidative stress by scavenging of ROS, involving immune reactions and minimizing the DNA damage in rock bream.

1. Introduction

Living organisms are constantly exposed to many natural sources of oxidative stress that can be classified into biological (pathogenic microorganisms, toxic algae etc.), physical (heat, UV etc.) and chemical factors (heavy metals, pollutants etc.). Oxidative stress arises from a significant increase of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS are produced in all aerobic organisms mainly through cellular respiration by reduction of single electron from molecular oxygen [1]. ROS include a number of reactive molecules including superoxide radicals (O^{-}_{2}), singlet oxygen ($^{1}O_{2}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH.) [2]. High levels of ROS can react with bio-molecules such as DNA, protein, and lipid and exert a toxic effect leading to have oxidative damage in diverse cellular locations with serious cell damage. On the other hand, ROS like H_2O_2 are known as mediators of cell proliferation, differentiation, various intracellular signaling cascades [3,4] and immune response reactions [5]. Antioxidant enzymes play an important role to reduce the excessive levels of ROS by detoxification reactions that helps to minimize the oxidative stress [3]. These enzymatic antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidase and peroxiredoxins (Prxs) [4].

Prxs represent a large family of thiol-specific antioxidant enzymes that exist in a variety of prokaryotes and eukaryotes. Prxs are also named as thioredoxin peroxidases and alkyl-hydroperoxide reductase C22 proteins. Members of the Prx family enzymes exert antioxidant role through their peroxidase activity which catalyze the reduction of H_2O_2, peroxynitrite and wide range of organic hydroperoxides (ROOH) into alcohol (ROH), and water (H_2O), respectively. All Prx enzymes are reported to consist of

* Corresponding author. Tel.: +82 647543472; fax: +82 647563493.
E-mail addresses: jehee@jejunu.ac.kr, jeheedaum@hanmail.net (J. Lee).

1050-4648/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2012.03.029

conserved cysteine residue in the NH_2 terminal region which is the primary site of oxidation by peroxide substrate [5]. Prx sequences are classified into six isoforms (Prx I to VI), that are mainly included in three sub-groups namely 1-Cys, typical 2-Cys and atypical 2-Cys Prxs based on the number and position of Cys residues. The typical 2-Cys subgroup is the largest class of Prxs that includes Prx I ~ IV isoforms. The typical 2-Cys Prxs (PrxV) are consisted of highly conserved two redox active cysteines named as peroxidatic and resolving cysteines in the carboxyl-terminal region. The atypica 2-Cys Prxs subgroup members have the identical mechanism as typical 2-Cys but they have monomeric function. Members of the 1-Cys Prx subgroup (Prx VI) contain only one conserved Cys, which serves as the peroxidatic cysteine. Prx VI is the main mammalian 1-Cys bi-functional Prx that is generally located in cytosol [6–8].

Several reports on cloning, transcriptional responses and functional characterization of Prx6 from various invertebrates and vertebrates have been described. However, studies and sequence information on fish Prx 6 have been limited to few species such as *Danio rerio* [NP_957099], *Scophthalmus maximus* [ADJ57694], *Sparus aurata* [ADI78069], *Salmo salar* [ACI67571] and catfish, *Ictalurus punctatus* Rafinesque, 1818 [9]. Therefore, further studies could support to understand the specific immune responses and other associated functions of Prx 6 in lower order vertebrates such as fish. Rock bream is a commercially important fish in marine aquaculture, which is frequently affected by various infectious diseases and stress conditions. To date, no Prx 6 has been cloned or identified from the rock bream.

The next generation sequencing technology can be effectively used for gene discovery [10]. The method, Roche 454 sequencing has not yet been widely applied marine fish compared to mammalian. In this study, first we identified the full-length cDNA of Prx6 by transcriptome analysis of rock bream. Then, we analyzed the sequence and mRNA expression of Prx6 in different tissues to understand the expression profile and its immune responses after experimentally challenge of rock bream using poly I:C and iridovirus containing supernatant of kidney tissue. To determine the functional role of RbPrx6, we purified the recombinant protein and DNA protection activity was determined by metal-catalyzed oxidation (MCO) assay.

2. Materials and methods

2.1. Identification and molecular characterization of RbPrx6

The RbPrx6 was originally identified by pyrosequencing of normalized cDNA using Roche GS-FLX system (DNA Link, Republic of Korea). The cDNA sequence that showed higher sequence similarity to known Prx6 was selected by homology searches using the Basic Local Alignment Tool (BLAST) algorithm [11]. Nucleotide and predicted peptide sequence of RbPrx6 was analyzed using DNAsisst and BLAST programs. Characteristic domains or motifs were identified using the PROSITE profile database [12]. Signal sequence and putative cleavage site of RbPrx6 were identified using the Signal P 3.0 server. Identity, similarity and gap percentages were calculated using the FASTA program [13]. Pair-wise and multiple sequence alignment were analyzed using the ClustalW program, version 1.8 [14]. The phylogenetic tree was constructed using the Neighbor-Joining method and plotted with MEGA version 3.1 program [15].

2.2. Isolation of rock bream tissues

Healthy rock breams (mean weight 50 g) were obtained from the Ocean and Fisheries Research Institute (Jeju, Republic of Korea) and acclimatized to laboratory conditions for one week in a 400 L stock tank at 22–24 °C. For tissue specific expression, various tissues (brain, gills, liver, kidney, head kidney, spleen, skin, intestine and muscle) were dissected from three healthy fish. Blood samples (approximately 1–2 mL/fish) were taken from the caudal fin using a 22 G syringe in to a tube with heparin as anticoagulant; samples were immediately centrifuged 3000 × g at 4 °C for 10 min to separate blood cells. All tissues and blood cells were directly frozen in liquid nitrogen before storage at −80 °C.

2.3. Poly I:C and iridovirus challenge of rock bream

To study the immune response of RbPrx6, we performed two immune challenge experiments using poly I:C and iridovirus containing supernatant. Three groups of 20 fish were established for challenging i) poly I:C (Sigma–Aldrich®), ii) iridovirus containing supernatant and iii) phosphate buffered saline (PBS) as a control. The first group was injected intraperitoneally (i.p.) with 150 μg of poly I:C (Sigma) dissolved in phosphate buffered saline (PBS). For virus challenge experiment, 100 μL (50 g fish) of iridovirus supernatant was injected by intramuscularly. Briefly, kidney tissue was isolated from iridovirus infected rock bream. After homogenizing and centrifugation it was filtered by a 0.45 μm syringe filter to collect supernatant of iridovirus. The control group was injected with the same volume (as used in treatments) of PBS. Rock bream liver tissue was removed at 3, 6, 12, 24, and 48 h post-challenge. Respective control samples were isolated from PBS-injected fish.

2.4. RNA isolation and cDNA synthesis

An equal amount (50 mg) of tissue was mixed from three fish to make a pooled sample for RNA isolation. The total RNA was extracted and processed from the pooled tissue (150 mg) and blood cells. Purified RNA was diluted up to 1 μg/uL before synthesis of cDNA. A sample of 2.5 μg RNA was used to synthesize cDNA from each tissue using a Superscript III first-strand synthesis system for RT-PCR (Invitrogen). Briefly, RNA was incubated with 1 μL of 50 μM oligo$(dT)_{20}$ and 1 μL of 10 mM dNTPs for 5 min at 65 °C. After incubation, 2 μL of 10 × cDNA synthesis buffer, 2 μL of25 mM $MgCl_2$, 2 μL of dithiothreitol (DTT, 0.1 M), 1 μL of RNaseOUT™ (40 U/μL) and 1 μL of SuperScript III reverse transcriptase (200 U/μL) were added and incubated for 1 h at 50 °C. The reaction was terminated by adjusting the temperature to 85 °C for 5 min. Then, 1 μL of RNase H was added to each cDNA reaction and incubated at 37 °C for an additional 20 min. Finally, synthesized cDNA was diluted 10-fold (total 200 μL) before storing at −20 °C.

2.5. Transcriptional analysis of RbPrx6 by quantitative real-time RT-PCR (qRT-PCR)

The expression of RbPrx6 was analyzed in various tissues in unchallenged and immune challenged fish by qRT-PCR. Gene-specific primers of forward (5′-TAACAGTGAGGCTGAGATTGCCCT-3′) and reverse (5′-TTGTCTGGGCCAATCACAAAGACG-3′) were designed to amplify corresponding regions of RbPrx6. The rock bream beta actin (Accession No. FJ975145) was selected as a reference gene, and it was amplified using gene-specific primers forward (5′-TCATCACCATCGGCAATGAGAGGT-3′) and reverse (5′-TGATGCTGTTGTAGGTGGTCTCGT-3′). Tissue-specific mRNA expression was analyzed in blood cells, gills, liver, spleen, head kidney, kidney, muscle and intestine. The RbPrx6 mRNA response was determined in liver and blood cells after poly I:C and iridovirus challenge. The qRT-PCR was carried out in a 20 μL reaction volume containing 4 μL of 1:10 diluted original cDNA, 10 μL of 2 × SYBR Green Master Mix, 1.0 μL of each primer (10 pmol/μL), and 4.0 μL of PCR grade water using Thermal Cycler Dice™ Real Time