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A manganese superoxide dismutase with potent antioxidant activity identified from *Oplegnathus fasciatus*: Genomic structure and transcriptional characterization

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ABSTRACT

In this study, we describe the identification and characterization of manganese superoxide dismutase, an important antioxidant enzyme acting as the chief reactive oxygen species (ROS) scavenger, from rock bream *Oplegnathus fasciatus* (Of-mMnSOD) at genomic- and transcriptional-levels as well as the biological activity of recombinant protein. The Of-mMnSOD protein portrayed distinct MnSOD family features including signature motifs, metal association sites and the typical active site topology. It was also predicted to be localized in mitochondrial matrix. The *Of-mMnSOD* had a quinquepartite genome organization encompassing five exons interrupted by four introns. Comparison of its sequence and gene structure with that of other lineages emphasized its strong conservation among different vertebrates. The *Of-mMnSOD* was ubiquitously transcribed in different rock bream tissues with higher levels in blood cells and metabolically active tissues. Transcription of *Of-mMnSOD* was kinetically modulated in response to investigational challenges using mitogens (lipopolysaccharide and poly I:C) and live-pathogens (*Edwardsiella tarda* and rock bream irido virus) in blood cells and liver tissue. The purified recombinant Of-mMnSOD possessed potential antioxidant capacity and actively survived over a range of pH (7.5–11) and temperature (15–40 °C) conditions. Collectively, findings of this study suggest that Of-mMnSOD combats against oxidative stress and cellular damages induced by mitogen/pathogen-mediated inflammation, by detoxifying harmful ROS ($O_2^{\bullet -}$) in rock bream.

1. Introduction

Although aerobic metabolism is an indispensable mechanism in most of the biological systems for energy-generation, it could also yield reactive oxygen species (ROS) as byproducts. Moreover, ROS-production is inducible by a variety of endogenous and exogenous sources [1]. When ROS level exceeds the threshold level of anti-oxidant system, the cellular redox-homeostasis is disturbed leading to a phenomenon known as oxidative stress. ROS are widely recognized as dual-role players; they act as key mediators in a variety of cellular processes, and at elevated levels can cause deleterious effects to cellular macromolecules. To protect themselves from such oxidative damages, cells are equipped with myriad antioxidant enzymes to scavenge and detoxify increased oxyradicals.

The superoxide dismutases (SODs) are a family of metalloenzymes that constitute the first line of defense against ROS and found in virtually all kingdoms of aerobic life. The SODs play a central role in intercellular defense machinery against harmful consequences of ROS by catalytically converting the $O_2^{\bullet -}$ to O_2 and H_2O_2 [2]. They are classified into four groups based on the metal-ligand they harbor: copper/zinc SOD (Cu/ZnSOD), manganese SOD (MnSOD), iron SOD (FeSOD) and nickel SOD (NiSOD). Although, the fundamental role of different SODs is almost same, each SOD group has distinctive genomic- and proteomic-structural characteristics and subcellular distribution [3].

The MnSOD, a homo -dimeric or -tetrameric enzyme bound with Mn ion through an invariant active site, is principally localized to the

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mitochondrial matrix [4]. This enzyme is well-conserved in terms of its structure and function across different lineages. While MnSOD accomplishes its chief role in combating ROS produced locally in mitochondrion, the close association of MnSOD with innate immunity with possible physiological relevance is evident. Under normal physiological conditions, basal MnSOD expression maintains required ROS levels by establishing a balance between production and scavenging. However, the modulation of MnSOD transcription in response to endotoxic shock [5] and invading pathogens [6–8] suggest that it also protects host cells from pathogen-induced ROS during self-defense strategies like phagocytosis.

To date, many MnSOD orthologs have been characterized in mammals [9–12], fish [13,14] and invertebrate species [8,15–17]. Nevertheless, the mechanisms governing the transcriptional regulation of MnSOD have been rarely investigated in fish compared to that in mammals [18–20]. Despite the study reporting the *Hemibarbus mylodon MnSOD* by Cho and co-workers, no study has described any teleostean *MnSOD* at genomic level [6]. The scarce information available for teleostean *MnSOD* at genome and promoter levels become a barrier in understanding the regulated expression and physiological roles of this vital gene in fish.

Rock bream *Oplegnathus fasciatus* is a commercially important farm fish widely cultured across the eastern Asia. However, serious disease outbreaks in cultured rock bream caused by rock bream irido virus (RBIV) has been increasingly reported in recent years [21]. The *Edwardsiella tarda* bacterium is also an emerging pathogen in aquaculture [22]. Unfortunately, the detailed characteristics of complicated defense mechanisms in rock bream are poorly understood. Thus, unraveling such mechanisms against invading pathogens at molecular level may give new insights into diseases control schemes in rock bream cultivation.

To end this, we designed an investigation to characterize the *MnSOD* identified from rock bream (*Of-mMnSOD*) at genomic-, transcriptional- and functional-levels. The exon-intron structure and promoter proximal region of *Of-mMnSOD* were determined using genomic sequence obtained from bacterial artificial chromosome (BAC) library. We demonstrated the basal expression and spatial distribution of *Of-mMnSOD* in different rock bream tissues and temporal expression modulated by mitogens and pathogens. Finally, the full-length *Of-mMnSOD* cDNA determined by transcriptome analysis was cloned and recombinant Of-mMnSOD was expressed to purify and demonstrate its antioxidant activity.

2. Materials and methods

2.1. The cDNA library construction and identification of putative MnSOD cDNA

We have previously established a rock bream cDNA database using the Roche 454 Genome Sequencer FLX System (GS-FLX™) [23]. Briefly, the total RNA was extracted from pooled-multiple tissues of three fish using Tri Reagent™ (Sigma, USA) and mRNA was then purified using FastTrack® mRNA isolation kit (Invitrogen, USA). The first strand cDNA synthesis and normalization were carried out using the Creator™ SMART™ cDNA library construction kit (Clontech) and Trimmer-Direct cDNA normalization kit (Evrogen), respectively, as recommended by manufacturers. Finally, the sequencing of cDNA library was performed by the Roche 454 platform and a GS-FLX™ shotgun database was established (DNA Link, Inc.). We identified a putative cDNA demonstrating significant homology to known mitochondrial MnSOD members using the BLAST program available at the NCBI, GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and designated as *Of-mMnSOD*.

2.2. BAC library construction, screening and genome sequencing

We have recently custom built a bacterial artificial chromosome (BAC) library for rock bream (Lucigen®, USA). The genomic DNA extracted from fish blood was used in BAC construction using random shearing approach and 92,160 independent clones were recovered. With two consecutive rounds of PCRs using gene specific primers (F1: 5′- AGTTCAACGGAGGAGGCCACATTA -3′ and R1: 5′-TGTGGCAGCAGACATCTTCTCCTT-3′), the library was screened as per manufacturer's recommendation and a clone bearing the *Of-mMnSOD* was localized. The BAC DNA was isolated from this positive clone and purified using QIAGEN Plasmid Midi Kit. Subsequently, it was sequenced by GS-FLX™ system (Macrogen) and the complete genomic sequence of *Of-mMnSOD* was obtained.

2.3. Molecular and genomic characterization of Of-mMnSOD

Based on full-length *Of-mMnSOD* cDNA sequence, a putative open reading frame (ORF) and corresponding amino acid sequence were determined using DNAssist (2.2). The Of-mMnSOD sequence was subjected to a homology search through the BLAST program and homologous sequences were retrieved. The deduced Of-mMnSOD amino acid sequence and its architecture were analyzed using the ExPASy Resource Portal (http://www.expasy.org/). The genome structure and exon–intron junctions were deduced based on an alignment of cDNA and genomic sequences of *Of-mMnSOD* using the Spidey program (http://www.ncbi.nlm.nih.gov/spidey/). By subjecting the 5′-flanking promoter proximal region to AliBaba 2.1 (http://www.gene-regulation.com/pub/programs/alibaba2/index.html) program, putative cis acting elements were determined. Subcellular localization of Of-mMnSOD was predicted using the MitoProt II (http://ihg.gsf.de/ihg/mitoprot.html). Pairwise comparison and sequence alignments were performed using the Needle tool and ClustalW2 at the EBI (http://www.ebi.ac.uk/Tools/), respectively. To establish the tertiary structure of mature Of-mMnSOD, a 2.4 Å crystal structure of human MnSOD dimer from PDB database (ID, 2adqB) was selected as template by Swiss-Model (http://swissmodel.expasy.org/). The monomer structure of Of-mMnSOD was constructed by I-TASSER server through multiple-threading alignments with potential templates. The structural models were visualized with DeepView and RasMol programs. A phylogenetic tree rooted with two MnSODs from plant species was reconstructed, based on an alignment of full-length amino acid sequences of MnSODs from different species by the Poisson model and neighbor joining (NJ) algorithm embedded in MEGA 5.0 (http://www.megasoftware.net/). The topological stability of the tree was evaluated by 5000 bootstrapping replications.

2.4. Animal rearing

Healthy fish with an average body weight of ~50 g were obtained from the Jeju Special Self-Governing Province Ocean and Fisheries Research Institute (Jeju, Republic of Korea) and maintained in 400 L aquaria filled with aerated sand-filtered seawater (salinity 34 ± 1%, pH 7.6 ± 0.5) at 24 ± 1 °C. All fish were acclimatized to laboratory conditions for 1 week before processing.

2.5. In vivo investigational challenges and sampling of tissues

Challenge experiments were conducted as previously described [23]. Briefly, *E. tarda* strain was obtained from the Department of Aqualife Medicine, Chonnam National University, Korea and grown in a brain-heart infusion broth at 30 °C for 12 h. Cells were harvested by centrifugation, re-suspended in 1 × phosphate-buffered