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Two metalloenzymes from rockfish (*Sebastes schligellii*): Deciphering their potential involvement in redox homeostasis against oxidative stress

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ABSTRACT

Disturbance in the balance between pro-oxidants and anti-oxidants result oxidative stress in aerobic organisms. However, oxidative stress can be inhibited by enzymatic and non-enzymatic defense mechanisms. Superoxide dismutases (SODs) are well-known scavengers of superoxide radicals, and they protect cells by detoxifying hazardous reactive oxygen species. Here, we have identified and characterized two different SODs, CuZnSOD and MnSOD, from black rockfish (RfCuZnSOD and RfMnSOD, respectively). *In silico* analysis revealed the well-conserved molecular structures comprising all essential properties of CuZnSOD and MnSOD. Phylogenetic analysis revealed that both RfCuZnSOD and RfMnSOD cladded with their fish counterparts. The recombinant RfSOD proteins demonstrated their potential superoxide scavenging abilities through a xanthine oxidase assay. The optimum temperature and pH conditions for both rRfSODs were 25 °C and pH 8, respectively. Moreover, the potential peroxidation function of rRfCuZnSOD was observed in the presence of HCO_3^-. The highest peroxidation activity was observed at 100 μg/mL of rRfCuZnSOD using the MTT cell viability assay and flow cytometry. The analogous tissue-specific expression profile indicated ubiquitous expression of both *RfCuZnSOD* and *RfMnSOD* in selected tissues of healthy juvenile rockfish. An immune challenge experiment illustrated the altered expression profiles of both RfCuZnSOD and RfMnSOD against lipopolysaccharide, *Streptococcus iniae*, and polyinosinic-polycytidylic acid (poly I:C). Collectively, these results strengthen the general understanding of the structural and functional characteristics of SODs within the host defense system.

1. Introduction

Pro-oxidants and anti-oxidants maintain a physiological equilibrium in a healthy life where alterations in this balance may cause oxidative stress that damages cells and tissues. As a result of metabolic reactions, pro-oxidants/free-radicals are formed and damaged macromolecules including proteins, carbohydrates, lipids, and nucleic acids [1]. Free radicals are reactive chemical species with an unpaired electron in their outer orbit [2]. The energy released by this unstable configuration leads to cellular damage. However, aerobic organisms are equipped with a variety of antioxidants that are important in mediating redox balance and defending against reactive oxygen species to counterbalance the effect of oxidants. Endogenous antioxidants can be classified as enzymatic and non-enzymatic antioxidants. Superoxide dismutases (SODs), peroxidases, and thioredoxins are key enzymatic antioxidants that play crucial roles in the host antioxidant defense system.

SODs are metalloenzymes that are thought to serve as the first line of antioxidant defense by catalyzing the dismutation of superoxide anions into H_2O_2 and O_2 [3]. Depending on the metal content, SODs can be broadly classified into four distinct groups: copper-zinc SOD (CuZnSOD), manganese SOD (MnSOD), iron SOD (FeSOD), and nickel SOD (NiSOD) [4–6]. Cytosolic CuZnSOD is the major intracellular SOD found in most aerobes [7]. MnSOD is a mitochondria-resident enzyme that is synthesized in the cytoplasm and then directed to the mitochondria *via* a signal peptide [3]. It is thought that FeSOD originated in the plastid and then moved to the nuclear genome in plants and prokaryotes [8,9]. NiSOD is the most recent SOD to be discovered in *Streptomyces* [5,10] and cyanobacteria [11].

CuZnSOD and MnSOD have been shown to exert great antioxidant activities, tumor suppressor activities, and modulations against different pathogenic stimuli. It has been observed that overexpression of CuZnSOD leads to extended lifespan in *Drosophila* [12], and overexpression of MnSOD weakened cancer cells in human [13]. Moreover, evidences for the immune responses against bacterial [14–16], viral [17,18], parasitic stimuli [15], and thermal stresses [19] were reported in both CuZnSOD and MnSOD. Therefore, the therapeutic functions of CuZnSOD and MnSOD are important for the survival of the host.

Black rockfish (*Sebastes schligelii*) is a highly valued aquatic crop in

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the Asian-Pacific region due to its specific features including tolerance to low water temperature, high survival rate, and fast growth [20,21]. Rockfish is the second most highly produced fish in the Republic of Korea [22]. However, due to the massive culturing systems, the prevalence of infectious diseases was found to be drastically increased over time [23]. Moreover, oxidative stress caused by intense aqua-farming may also lead to weakened immune responses [24] in these species, leaving them more vulnerable to pathogenic infections [23,25–27]. Studying the molecular defense mechanisms of aqua-farming species could be of great importance for establishing a worldwide sustainable mariculture system. Therefore, in the current study, we identified two SODs from black rockfish that are known to be powerful antioxidants, catalyzing the dismutation of superoxide radicals. First, the characteristic features were analyzed through web-based bioinformatics tools, followed by use of recombinant proteins in order to decipher their functional roles as antioxidants. Physiological behavior and the responses towards the pathogenic infections were determined through immune challenge experiments.

2. Methodology

2.1. Construction of rockfish cDNA and gDNA databases

The cDNA sequence database of black rockfish was created by 454 GS-FLX™ sequencing [28]. Three black rockfish were dissected and total RNA was extracted from blood, ovary, brain, skin, kidney, testis, liver, head kidney, intestine, spleen, muscle, heart, stomach, and gill. Then, the extracted total RNA was purified using the RNeasy Mini Kit (Qiagen, USA) following the manufacturer's instructions. Finally, the rockfish transcriptomic library was constructed using fragmented RNA samples (Macrogen, Korea).

Additionally, a *de novo* genome assembly method was used to construct the black rockfish genome database. The DNA libraries were constructed through Illumina paired-end (PE) and mate-pair (MP) libraries according to the manufacturer's protocols (Illumina, San Diego, CA, USA).

2.2. Identification and sequence analysis of two SODs

Complete cDNA sequences of CuZnSOD and MnSOD were identified from the black rockfish cDNA database using the basic logical alignment search tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Putative coding sequences (CDS) of RfCuZnSOD and RfMnSOD were identified *via* DNAssist (v 2.2). Corresponding protein sequences were derived from the CDS of the two SODs. The identified sequences were characterized by using different bioinformatics tools to discover gene-specific structural and the functional features.

Insights of the domain architecture of RfCuZnSOD and RfMnSOD were determined using the ExPASy PROSITE (http://prosite.expasy.org/), SMART (http://smart.embl-heidelberg.de/), and Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) bioinformatics tools. The potential signal peptide and the predicted cellular locations of these two SODs were retrieved using SignalP (http://www.cbs.dtu.dk/services/SignalP/), and the MultiLoc tool (http://abi.inf.uni-tuebingen.de/Services/MultiLoc/), respectively. The Prot-Param tool on ExPASy was used to compute the predicted molecular mass, isoelectric point, and the instability index of the putative proteins including RfCuZnSOD and RfMnSOD. Homology analysis was conducted with the EMBOSS Needle program (http://www.ebi.ac.uk/Tools/psa/emboss_needle/), and the multiple sequence alignment was carried out *via* the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic trees were reconstructed with bootstrap values calculated for 5000 replications by using the minimum evolution method available in the Molecular Evolutionary Genetics Analysis (MEGA 5.0) tool [29]. Predicted three-dimensional structures were designed by the SWISS-MODEL protein modeling server (https://swissmodel.expasy.org/) and were visualized by the PyMOL v1.5 molecular graphics system. Genomic analysis was performed by aligning the complete cDNA and gDNA sequences of RfCuZnSOD and RfMnSOD with the Splign genomic alignment tool (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi) separately. Orthology of CuZnSOD and MnSOD genomic structures were collected from the Ensemble genome browser database (http://asia.ensembl.org/index.html) and all the genomic arrangements were visualized *via* GeneMapper (v2.5).

2.3. Preparation of the recombinant plasmids of CuZnSOD and MnSOD

Complete CDS of RfCuZnSOD and the RfMnSOD were amplified using gene-specific primers (Supplementary Table 1) and were appended with *Eco*RI and *Hin*dIII restriction enzymes [30]. Then, the constructed recombinant vectors were transformed into *Escherichia coli* DH5α competent cells and purified plasmids were sent for sequence verification (Macrogen, Korea).

2.4. Overexpression and purification of recombinant proteins

Sequence-verified recombinant vectors were then transformed into ER2523 (NEB Express) competent cells and overexpressed as fusion proteins with maltose binding protein (MBP) following the steps mentioned in our previous study [31]. Briefly, the ER2523 cells were cultured in 500 mL LB broth that was freshly supplemented with 100 mg/mL ampicillin and 100 mM glucose (at 37 °C until the OD_{600} reached ~0.5). Protein expression was induced by incubating with 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG) at 20 °C at 200 rpm for 10 h. Then the cells were harvested at 4000 × g for 20 min at 4 °C and stored at −20 °C for an overnight after the cells were re-suspended in column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl). The following day, recombinant proteins (rRfCuZnSOD and rRfMnSOD) were purified as mentioned elsewhere [30]. Cellular fractions collected during the purification steps were subjected to 12% SDS-PAGE analysis. Finally, the SDS-PAGE gel was stained with Coomassie blue R-250 (0.05%) and destained following the standard procedure.

2.5. Assessment of rRfCuZnSOD and rRfMnSOD scavenging activity and kinetics

A xanthine/xanthine oxidase (xanthine/XOD) assay [32] was used to measure the superoxide radical scavenging activity of both rRfCuZnSOD and rRfMnSOD, as previously mentioned in Perera et al. (2016) [30]. A conventional spectrophotometric technique was used to quantify the antioxidant functions of two SODs separately. A total volume of 160 μL was used per sample that consisted of 0.1 M glycine-NaOH buffer (pH 8), 6.75 μL of xanthine (3 mM), 6.75 μL 3 mM EDTA (ethylenediaminetetraacetic acid), 6.75 μL 0.15% BSA (bovine serum albumin), 6.75 μL 0.75 mM NBT (nitro blue tetrazolium chloride), and 20 μL of recombinant protein. Homogenized sample mixtures were incubated for 10 min at 25 °C. The reaction was initiated by adding 6 mU of XOD and incubated again at 25 °C for 20 min. Finally, OD was recorded at 560 nm using a microplate reader (Multiskan GO, Thermo Scientific). Recombinant MBP (rMBP) was simultaneously assessed as the control.

In order to determine the optimum pH for rRfCuZnSOD and rRfMnSOD activity, the XOD assay was conducted with a pH gradient using citrate buffer (pH 3, 4, 5), phosphate buffer (pH 6, 7, 8), and glycine-NaOH buffer (pH 9, 10, 11). The optimum pH buffer was used for the XOD assay to assess the optimum temperature for rRfCuZnSOD and rRfMnSOD activity. Here, temperatures of 10, 20, 25, 30, 40, 50, 60 and 70 °C were used. Next, the XOD assay was again conducted to determine the effect of protein concentration on the superoxide scavenging ability at both optimum pH and optimum temperature for rRfCuZnSOD and rRfMnSOD activity. Different amounts of rRfCuZnSOD and rRfMnSOD, and rMBP were used including 2.5, 5, 10,