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Copper-zinc-superoxide dismutase (CuZnSOD), an antioxidant gene from seahorse (*Hippocampus abdominalis*); molecular cloning, sequence characterization, antioxidant activity and potential peroxidation function of its recombinant protein

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ABSTRACT

Copper-zinc-superoxide dismutase (CuZnSOD) from *Hippocampus abdominalis* (HaCuZnSOD) is a metalloenzyme which belongs to the ubiquitous family of SODs. Here, we determined the characteristic structural features of HaCuZnSOD, analyzed its evolutionary relationships, and identified its potential immune responses and biological functions in relation to antioxidant defense mechanisms in the seahorse. The gene had a 5′ untranslated region (UTR) of 67 bp, a coding sequence of 465 bp and a 3′ UTR of 313 bp. The putative peptide consists of 154 amino acids. HaCuZnSOD had a predicted molecular mass of 15.94 kDa and a theoretical *p*I value of 5.73, which is favorable for copper binding activity. *In silico* analysis revealed that HaCuZnSOD had a prominent Cu-Zn_superoxide_dismutase domain, two Cu/Zn signature sequences, a putative *N*-glycosylation site, and several active sites including Cu^{2+} and Zn^{2+} binding sites. The three dimensional structure indicated a β-sheet barrel with 8 β-sheets and two short α-helical regions. Multiple alignment analyses revealed many conserved regions and active sites among its orthologs. The highest amino acid identity to HaCuZnSOD was found in *Siniperca chuatsi* (87.4%), while *Maylandia zebra* shared a close relationship in the phylogenetic analysis. Functional assays were performed to assess the antioxidant, biophysical and biochemical properties of overexpressed recombinant (r) HaCuZnSOD. A xanthine/XOD assay gave optimum results at pH 9 and 25 °C indicating these may be the best conditions for its antioxidant action in the seahorse. An MTT assay and flow cytometry confirmed that rHaCuZnSOD showed peroxidase activity in the presence of HCO_3^-. In all the functional assays, the level of antioxidant activity of rHaCuZnSOD was concentration dependent; metal ion supplementation also increased its activity. The highest mRNA expressional level of *HaCuZnSOD* was found in blood. Temporal assessment under pathological stress showed a delay response by *HaCuZnSOD*. Our findings demonstrated that HaCuZnSOD is an important antioxidant, which might be involved in the host antioxidant defense mechanism against oxidative stress.

1. Introduction

Biological and environmental factors including, the alterations in feed and husbandry practices, climatic variables, prophylactic activities, and various stresses can affect the quality or quantity of production by farm animals. Above stressful factors can mediate oxidative stress in the presence of oxygen *via* the generation of reactive oxygen species (ROS). For example, ingestion of a pathogen can activate phagocytic cells and stimulate the production of ROS [1]. This phenomenon was originally called a respiratory burst, which is characterized by the increased consumption of oxygen. ROS are reactive molecules and free radicals derived from the molecular oxygen cause a steady state of oxidative damage in cells, tissues or organs [2]. ROS are widely generated in body with the forms of hydroxyl radicals (•OH), superoxide anions ($O_2^{\bullet -}$), and singlet oxygen ($^1O_2$). Excessive levels of ROS can cause cellular

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damage through the oxidation of amino acids, generation of cross-links and inactivation of proteins, nucleic acid damage, and lipid peroxidation [3]. The production of antioxidants are one of the means by which cells attempt to detoxify hazardous ROS [4] to limit the damage caused.

Living organisms possess a variety of enzymatic and non-enzymatic antioxidant defense mechanisms that protect against the constant oxidative challenge by ensuring a proper balance between pro-oxidants and antioxidants. Superoxide dismutases (SODs) comprise a large family of such enzymatic antioxidants which are well-known metalloenzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide [5]. Depending on their metal content, they are classified into four groups: copper-zinc SOD (CuZnSOD), manganese SOD (MnSOD), iron SOD (FeSOD), and nickel SOD (NiSOD). FeSODs are mainly found in prokaryotes and plants, whereas NiSODs are predominant in bacteria. MnSODs are generally found in prokaryotes and the mitochondria of eukaryotes. CuZnSODs are found in the cytosol and extracellular compartments of eukaryotic cells and in the periplasm of Gram-negative bacteria [6]. This group is generally considered as the most important group of SOD because of their physiological and therapeutic importance [7].

Two isoforms of CuZnSOD exist in most organisms; extracellular CuZnSOD is present in the extracellular matrix, and intracellular CuZnSOD is found in the intracellular space, inter-membrane space of mitochondria, and in the nuclei [8]. CuZnSOD can form a homodimeric molecule in which two cysteines of each monomeric unit, that harbor Cu^{2+} and Zn^{2+} ions, form an intra-subunit disulfide bridge [9]. CuZnSOD combats ROS by catalyzing the disproportionation of $O_2^{\bullet -}$ anions to hydrogen peroxide which is then converted to water by other antioxidant enzymes such as peroxiredoxins [9]. The crucial role of this first line of defense is facilitated by Zn^{2+} and Cu^{2+} ions which respectively play a structural role and catalyze the disproportionation of $O_2^{\bullet -}$ anions in the redox cycle [10]. Previous investigations showed that overexpression of CuZnSOD extend the life span in Drosophila and reduces the main proximate of aging in yeast [11,12]. Catalyzing of protein nitration is another role of CuZnSOD other than its conventional activities [13]. Since the level of CuZnSOD gene expression varies in aquatic ecosystems following metal contamination, it can be used as an excellent biomarker in ecotoxicology and bioaccumulation studies [14,15].

The big-belly seahorse (*Hippocampus abdominalis*) is one of the largest seahorse species; it is popular in the aquarium industry and is used for traditional medicine in Asia. Unlike other aquaculture species, seahorses generally do not adapt to harsh conditions causing them to be more vulnerable to infections. This species is also listed in Appendix II by CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora). Although some studies have reported on different aspects of the biology of this species including its feeding and growth, little information is available on its immunological characteristics. The goal of the present study was to identify and characterize the *CuZnSOD* gene of *H. abdominalis* (*HaCuZnSOD*) and to carry out functional assays to clarify its role as an antioxidant enzyme.

2. Methodology

2.1. Construction of seahorse cDNA database

A seahorse cDNA sequence database was constructed using the 454 GS-FLX™ sequencing technique (Roche, USA). In brief, total RNA was extracted from blood, liver, kidney, gill, and spleen tissues of 18 seahorses. The extracted RNA was treated with the RNeasy Mini kit (Qiagen, USA) and the quality and quantity were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Canada); an RNA integration score (RIN) of 7.1 was obtained. For construction of a seahorse transcriptomic library, the RNA was fragmented into an average size of 1147 bp using the Titanium 454 sequencing system (Roche, USA). Finally, the sequencing was performed on one-half of the picotiter plate on a Roche 454 GS-FLX™ DNA platform at Macrogen, Korea.

2.2. Sequence analysis of HaCuZnSODs

We identified a cDNA sequence of *HaCuZnSOD* (Accession number KU665493) from the seahorse cDNA database using the basic local alignment search tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). DNAssist (v 2.2) was used to identify the putative coding sequence (CDS) of *HaCuZnSOD* and derive its corresponding protein sequence.

2.3. In silico sequence profiling

Domain and the signature analyses of HaCuZnSOD were carried using the ExPASy PROSITE Database (http://prosite.expasy.org/) and Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan). Putative cleavage sites of the signal peptide were retrieved using SignalP (http://www.cbs.dtu.dk/services/SignalP/). The MultiLoc tool (http://abi.inf.uni-tuebingen.de/Services/MultiLoc/) was used to predict the cellular location of the HaCuZnSOD peptide. Potential *N*-linked glycosylation sites were predicted using the NetNGlyc web server (http://www.cbs.dtu.dk/services/NetNGlyc/). Molecular mass, isoelectric point and instability index of the putative HaCuZnSOD protein were calculated by the ProtParam tool on ExPASy (http://web.expasy.org/protparam/). For the prediction of cysteine sites in the peptide, the DiANNA 1.1 web server (http://clavius.bc.edu/~clotelab/DiANNA/) was used. To analyze the homology and evolutionary relationships of the HaCuZnSOD with orthologs of other species, we performed a multiple sequence alignment and phylogenetic analysis using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the Neighbor-Joining (NJ) method at MEGA (ver. 5.0), respectively. The tertiary structure of HaCuZnSOD was predicted using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and SWISS-MODEL (http://swissmodel.expasy.org/) protein modeling servers and visualized using PyMOL v1.5 software.

2.4. Construction of the expression plasmid pMAL-c5X/HaCuZnSOD

To investigate the antioxidant role of HaCuZnSOD, a cDNA fragment including the CDS was amplified using gene-specific primers (Supplementary Table 1). The amplicon (465 bp) was then isolated from a 1% agarose gel using a Gel Purification Kit (Accuprep, Bioneer, Korea). The pMAL-c5X vector and the amplicon were digested with EcoRI and HindIII restriction enzymes (TaKaRa, Japan). The digested cDNA fragment and pMAL-c5X vector were gel purified and ligated using Mighty Mix DNA Ligation Kit (TaKaRa, Japan). Finally, the constructed vector was transformed into *Escherichia coli* DH5α competent cells. The plasmid construct was purified from bacterial cells and subjected to sequence verification (Macrogen, Korea). The HaCuZnSOD/pMAL-c5X recombinant vector was transformed into ER2523 (NEB Express) competent cells for protein expression.

2.5. Overexpression and purification of recombinant HaCuZnSOD (rHaCuZnSOD)

Protein expression and purification were carried out as described in our previous study [16] with slight modifications.