Fish & Shellfish Immunology 29 (2010) 615–622

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Molecular cloning, identification and functional characterization of a novel intracellular Cu–Zn superoxide dismutase from the freshwater mussel *Cristaria plicata*

Huan-Huan Xu [a,b,1], Hui Ma [b,1], Bao-Qing Hu [a], Douglas B. Lowrie [b], Xiao-Yong Fan [b,*], Chun-Gen Wen [a,*]

[a] School of Life Sciences and Food Engineering, Nanchang University, Nanchang 330031, China
[b] Shanghai Public Health Clinical Center Affiliated to Fudan University, Shanghai 201508, China

ARTICLE INFO

Article history:
Received 3 April 2010
Received in revised form
24 May 2010
Accepted 8 June 2010
Available online 16 June 2010

Keywords:
Cristaria plicata
Superoxide dismutase (SOD)
Enzyme activity
Oxidative damage
Protective effect

ABSTRACT

Superoxide dismutases (SODs, EC 1.15.1.1) are one family of important antioxidant metalloenzymes involved in scavenging the high level of reactive oxygen species (ROS) into molecular oxygen and hydrogen peroxide. In the present study, the intracellular CuZnSOD gene of *Cristaria plicata* (Cp-icCuZnSOD) was identified from hemocytes by homology cloning and the rapid amplification of cDNA ends (RACE) technique. The full-length cDNA of Cp-icCuZnSOD consisted of 891 nucleotides with a canonical polyadenylation signal sequence ATTAAA, a poly (A) tail, and an open-reading frame of 468 bp encoding 155 amino acids. The deduced amino acids of CpSOD shared high similarity with the known icCuZnSODs from other species, and several highly conserved motifs including Cu/Zn ions binding sites (His-46, His-48, His-63, His-120 for Cu^{2+} binding, and His-63, His-71, His-80, Asp-83 for Zn^{2+} binding), intracellular disulfide bond and two CuZnSOD family signatures were also identified in CpSOD. Furthermore, the recombinant Cp-icCuZnSOD with high enzyme activity was induced to be expressed as a soluble form by IPTG supplemented with Cu/Zn ions at 20 °C for 8 h, and then was purified by using the native Ni^{2+} affinity chromatography. The specific activity of the purified rCp-icCuZnSOD enzyme was 5368 U/mg, which is 2.6-fold higher than that of zebrafish *Danio rerio* rZSOD and 5.3-fold higher than that of bay scallop *Argopecten irradians* rAi-icCuZnSOD. The enzyme stability assay showed that the purified rCp-icCuZnSOD enzyme maintained more than 80% activity at temperature up to 60 °C, at pH 2.0–9.0, and was resistant to 8 mol/L urea or 8% SDS. In addition, the addition of active rCp-icCuZnSOD enzmye could protect hepatocyte L02 cells from oxidative damage as assessed using an alcohol-injured human liver cell model.

1. Introduction

The freshwater mussel *Cristaria plicata*, which is of great economical importance and known as “pearl bivalves” in the aquaculture industry of China, has been suffering serious problems due to the outbreak of diseases, especially wound infection caused by the pearl nucleus inserting operation that leads to high mussel mortality in the process of pearl production [1]. Thus, understanding of the immunity of freshwater mussel is crucial for diseases management and development of sustainable mussel culture and pearl production.

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH^-) are generated from normal metabolic process in all aerobic organisms. The damages from ROS include lipid peroxidation, cross-linking and inactivation of proteins, breaks in DNA and RNA, and cell death [2,3]. Aquatic organisms are often subjected to enhanced “oxidative stress” by ROS due to chronic exposure to pollutants in their environments [4,5]. To limit the harmful effect of ROS production and prevent damage from oxidative stress, cells have evolved to use antioxidant systems as part of the innate immune defense to maintain reactive oxygen species at low basal levels and protect themselves from the constant oxidative challenge [5,6].

Superoxide dismutases (SODs; EC1.15.1.1), are one family of important antioxidant metalloenzymes that catalyze superoxide radicals from cellular oxidative metabolism into hydrogen peroxide and molecular oxygen [3,7]. According to the metal content, SODs are classified into four distinct groups including MnSOD, CuZnSOD,

* Corresponding authors.
E-mail addresses: xyfan008@fudan.edu.cn (X.-Y. Fan), cgwen@ncu.edu.cn (C.-G. Wen).
[1] These authors contributed equally to this work.

1050-4648/$ – see front matter
doi:10.1016/j.fsi.2010.06.013

FeSOD and NiSOD. MnSOD and CuZnSOD are found in both prokaryotes and eukaryotes, FeSOD is found in prokaryotes and plants [3], and NiSOD has recently been purified from several aerobic soil bacteria of the *Streptomyces* genus [8]. Among these SODs, CuZnSOD is important because of its physiological function and therapeutic potential [9,10]. The enzyme needs Cu and Zn ions for its biological activity, and the loss of Cu^{2+} results in complete inactivation [3,11], and induces a range of diseases in humans [12,13] and animals [14,15]. There are two isoforms of CuZnSOD enzyme existing in most organisms [16]. Extracellular CuZnSOD (ecCuZnSOD) with an N-terminal signal cleavage peptide for secretion is found in the extracellular matrix of tissues and the nucleus of human cells [17]. Intracellular CuZnSOD (icCuZnSOD) without a signal peptide is mainly found in the intracellular space, and it was also noted to be in the intermembrane space of mitochondria [18] and in nuclei [16].

Recently, much more attention has been paid to icCuZnSOD for its multiple functions in addition to superoxide dismutation [19]. The icCuZnSOD was shown to promote peroxynitrite-mediated nitrotyrosine formation in vitro. Peroxynitrite ($ONOO^-$) is formed by a rapid diffusion-limited, radical–radical coupling reaction between NO and O_2^- [19]. The ability of mollusc to produce NO in response to various stimuli has been shown previously [20]. The direct toxicity of NO is modest but is greatly enhanced by reacting with superoxide to form $ONOO^-$ [21]. Thus, icCuZnSOD protects molluscs from superoxide and NO under pathological conditions by preventing $ONOO^-$ formation. At present, icCuZnSOD cDNAs have been cloned from some aquatic animals [17,22–26], most of which studies primarily focused on cDNA cloning and mRNA expression. There are reports on the expression and purification of recombinant SOD enzyme from the zebrafish *Danio rerio* [24], bay scallop *Argopecten irradians* [25] and blue mussel *Mytilus edulis* [26]. However, little is known about the molecular features and characterization of SODs of freshwater mussels, especially little information is available about antioxidant effects of their recombinant SODs.

The main aims of the present study were: 1) to clone the full-length icCuZnSOD cDNA from the freshwater mussel *C. plicata* and compare its deduced amino acid sequence with known icCuZnSODs from other organisms, 2) to express recombinant Cp-icCuZnSOD (rCp-icCuZnSOD) enzyme in *Escherichia coli* and purify it, 3) to characterize the enzyme activity of rCp-icCuZnSOD protein *in vitro*, and 4) to evaluate protective effects of rCp-icCuZnSOD on alcohol-injured human hepatocyte L02 cell line.

2. Materials and methods

2.1. Animal and tissue collection

The bivalves *C. plicata*, averaging 18–25 mm in shell length, were collected from Poyang Lake in Jiangxi province, China, and acclimatized in water tanks for one week till further processing. The hemolymph was collected using a syringe and then centrifuged at 2000×*g*, 4 °C for 10 min to harvest the hemocytes.

2.2. RNA extraction and cloning the full-length cDNA of Cp-icCuZnSOD

The total RNA was extracted from hemocytes by using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The extracted RNA was then treated with RQ1 RNase-free DNase I (Promega, Madison, WI) to remove any possible contaminating DNA. SMART cDNA was synthesized from total RNA by using SMART™ cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA). Two degenerate primers CpSOD-F1 and CpSOD-R1 (Table 1) were designed based on the highly conserved sequences from *Chlamys farreri* [23] and *Crassostrea gigas* [27] to clone the mid-fragment of SOD gene from *C. plicata*. The PCR product was then cloned into the pGEM-T vector (Promega) and was sequenced in both directions with primers T7 and SP6.

The 5′-end and 3′-end of the CpSOD fragment were cloned by using SMART-RACE technique. Four gene-specific primers CpSOD-F2, CpSOD-F3, CpSOD-R2 and CpSOD-R3 (Table 1) were designed based on the sequenced partial sequence of CpSOD fragment. The 3′-end of CpSOD was cloned by using primer CpSOD-F2 and long primer (Table 1) in the first round PCR and CpSOD-F3 and long primer in the second round PCR. The 5′-end of CpSOD was cloned by using primers CpSOD-R2 and short primer (Table 1) in the first round PCR and CpSOD-R3 and short primer in the second round PCR. After all of the PCR products were sequenced, the mid-fragment, 5′-end and 3′-end of CpSOD were then spliced to get the full-length cDNA of Cp-icCuZnSOD (GenBank accession no. FJ194441).

2.3. Sequence analysis of Cp-icCuZnSOD

The Cp-icCuZnSOD gene sequence was analyzed by using the BLAST algorithm at the NCBI web site (http://www.ncbi.nlm.nih.gov/blast), and the deduced amino acid sequence and open reading frame (ORF) were analyzed with the Expert Protein Analysis System (http://www.expasy.org/). The similarity and identity of Cp-SOD with known SOD enzymes from other organisms were calculated by the online program (http://www.biosoft.net/sms/index.html). The multiple sequence alignment was created with ClustalW program (http://www.ebi.ac.uk/clustalw/), and the calculated molecular mass and the theoretical isoelectric point were predicted by Protein MolWt & AA Composition Calculator (http://www.proteomics.com.cn/proteomics/pi_tool.asp).

Table 1
Primers used in this present study.

Primers	Sequence (5′ → 3′)[a,b]	Sequence information
CpSOD-F1	MGACAAYACHAATGGYTGTA	Degenerate primer
CpSOD-R1	CCRATSACHCCACAMGCCA	Degenerate primer
CpSOD-F2	GACCAGAAGATGCATCCAGAC	3′-RACE
CpSOD-R2	CACCAGCATTGCCAGTTGTCTTG	5′-RACE
CpSOD-F3	GCAGGAGAGGATGGAGTGGCTCA	3′-RACE
CpSOD-R3	CCTGCCAAGGTCATCTTCATCTGC	5′-RACE
Long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	3′-RACE
Short	CTAATACGACTCACTATAGGGC	5′-RACE
CpSOD-F4	AAG GGT ACC ATG TCC ATT AAG GCT GTT TGC G	ORF cloning
CpSOD-R4	GGA GAA TTC TCA ATC CAA TTT TGA AAT TCC A	ORF cloning

[a] M = A or C; Y = C or T; H = A or C or T; R = A or G; S = C or G.
[b] The restriction enzyme sites are underlined.