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Characterization, molecular modelling and developmental expression of zebrafish manganese superoxide dismutase

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ABSTRACT

A 977 bp cDNA containing an open reading frame encoding 224 amino acid residues of manganese superoxide dismutase was cloned from zebrafish (zMn–SOD). The deduced amino acid sequence showed high identity with the sequences of Mn–SODs from human (85.1%) to nematode (61.6%). The 3-D structure model was superimposed on the relative domains of human Mn–SOD with the root mean square (rms) deviation of 0.0919 Å. The recombinant mature zMn–SOD with enzyme activity was purified using His-tag technique. The half-life of the enzyme is approximately 48 min and its thermal inactivation rate constant k_d is 0.0154 min^{-1}at 70 °C. The enzyme was active under a broad pH (2.2–11.2) and in the presence of up to 4% SDS. Real-time RT-PCR assay was used to detect the zMn–SOD mRNA expression during the developmental stages following a challenge with paraquat. A high level expression of Mn–SOD mRNA was detected at the cleavage stage, but decreased significantly under paraquat treatment. The results indicated that Mn–SOD plays an important role during embryonic development.

1. Introduction

The immunological capacity is severely limited at the fish larval stage, and the main immune substances are maternal transfer to eggs during fish vitellogenesis (for review, [1]). Although fish larvae are protected by several innate and adaptive immune substances, it seems that the adaptive immunity may develop more slowly [2]. One important immune defense reaction of zebrafish embryos is phagocytosis that can engulf three to four bacteria in each macrophage when the embryos are infected by bacteria [3]. At such time, reactive oxygen species (ROS) including superoxide anion and hydrogen peroxide are produced and used to kill the infecting bacteria. Therefore, the rapid clearance of excessive ROS is important for normal tissue to avoid damage from ROS.

Among the antioxidant enzymes, the superoxide dismutases (SODs) play the first defense system that convert O_2^-• to O_2 and H_2O_2 [4]. Three classes of SODs including Mn, Cu/Zn and extracellular (EC)-SOD have been identified in mammals [5]. Mn–SOD containing manganese ion was found mostly in the mitochondrial matrix [6], but some cytosolic Mn–SOD was also reported [7]. Cu/Zn–SOD containing copper and zinc ions was not only found in the cytoplasm, but also in the intermembrane space of mitochondria [8]. EC–SOD also binds to Cu and Zn ions secreted to the extracellular region during its synthesis [9].

The yeast mutant without Mn–SOD was hypersensitive to oxygen toxicity [10]. Several cellular pathologies including cancer [11], ischemia/reperfusion (I/R) injury [12] and cell apoptosis [13] have been shown to correlate with a decrease in Mn–SOD activity. Mn–SOD was also reported to play an important immune response when organisms were infected by bacteria [14,15], virus [16] or treated with a toxic chemical [17].

Mitochondrial Mn–SOD is encoded by the nuclear gene; it is synthesized and translocated into the mitochondrial matrix with mature enzyme activity [18]. The mitochondrial matrix is the major site to produce cellular energy, and it is also a major source for the single-electron reduction of O_2 to produce O_2^-•. Therefore, Mn–SOD is thought to be a major scavenger of ROS in the mitochondrial matrix among the aerobic organisms [19].

It has been reported that ROS is produced during fish embryonic development [20], but the developmental gene expression of Mn–SOD has never been determined. Overexpressed Mn–SOD can induce cell differentiation, and Mn–SOD gene (−/−) homozygous knock-out mice died within 5–21 days of birth with serious oxidative damage [21,22]. These results indicate that the Mn–SOD is

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very important for embryonic development. The oxidative stress regulation of fish Mn–SOD gene expression during embryonic development is also of interest.

Many Mn–SOD genes have been cloned from various organisms, but only a few reports concern teleost fish. In this study, we cloned Mn–SOD cDNA from zebrafish, the gene expression was analyzed by real time RT-PCR during the developmental stages under paraquat (PQ) induced oxidative stress. In addition, the antioxidant activity and biochemical properties of zMn–SOD were analyzed.

2. Materials and methods

2.1. Experimental fish and embryos

The Zebrafish (*Danio rerio*) AB strain [23] was reared at a constant temperature of 26 °C under 14 h light:10 h dark cycle in fresh water for 2 weeks before being used in the experiments. Twenty males and 12 females were used to produce fertilized embryos as reported in a previous study [23]. The collected embryos were washed 2 times with ddH_2O and kept in oxygen-saturated ddH_2O.

2.2. In silico cloning and subcloning of zMn–SOD cDNA

Three dbESTs including gene accession nos. BI879088, BU710351 and BM530205 were used as templates to clone zMn–SOD cDNA. A 977 bp full length cDNA was found with a 672 bp open reading frame that encoded a 224 amino acid sequence. To confirm this sequence, total RNA from zebrafish was extracted and the cDNA was synthesized as described in our previous work [24]. Two primers (zMn-a and zMn-b (Table 1)) were synthesized for zMn–SOD cDNA subcloning and the PCR program was 30 cycles of 95 °C for 60 s, 55 °C for 60 s, and 72 °C for 60 s. The PCR products were subcloned into pCR2.1 cloning vector (Invitrogen) and were determined in both directions according to autosequencing (ABI PRISM 3730 DNA sequencer, Perkin–Elmer).

2.3. Sequence analysis and 3-D homology modelling of zMn–SOD

The identity and similarity of zMn–SOD were compared with human (M36693), mouse (AK002534), rat (BC070913), chicken (AF299388), fly (L18947) and nematode (X77021) by using the online software BLAST 2 SEQUENCES program [25] from NCBI (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). The multiple amino acid sequence alignment was performed using online software Clustal W [26]. The amino acid sequence of zMn–SOD was sent to SWIS S-MODEL program [27] to create the 3-D homology model. The data was then superimposed to human Mn–SOD with PDB ID 1MSD [28] by SYBYL 7.3 program (TRIPOS Associates, Inc.) to obtain better structure similarity.

Table 1
Primer sequences were used in this study.

Primer name	Sequence (5′–3′)
zMn-a(5′)[a]	GAATTCGATGCTGTGCAGAGTCGGATAT
zMn-b(3′)[a]	CGTTTCCAAGCTGCCAAGAAACTCGAG
zMnSOD-rbs-PB(5′)[b]	GAAGGAGATATACATATGAAGCACGCTCTCCC
zMnSOD-His-PC(3′)[b]	TCAGTGGTGGTGGTGGTGGTGTTTCTTGGCAGCTTGG
zMnSOD-RT-P1(5′)[c]	CTTGGGATAGATGTCTGGG
zMnSOD-RT-P2(3′)[c]	GTGGTCTGATTAATTGTGCG
z-Ef1a-Q-P3(5′)[c]	GGCGTCATCAAGAGCG
z-Ef1a-Q-P4(3′)[c]	CAACCTTTGGAACGGTGT
M13 forward primer[d]	GTAAAACGACGGCCAG
M13 reverse primer[d]	CAGGAAACAGCTATGAC
T7 promoter primer[d]	TTAATACGACTCACTATAGGG
T7 terminator primer[d]	CAATAACGAGTCGCCACCG

[a] Primers were used for clone zMn-SOD cDNA.
[b] Primers were used for overexpress zMn–SOD in *E.coli*.
[c] Primers were used for real-time RT-PCR.
[d] Primers were used for sequencing only.

2.4. Expression and purification of zMn–SOD

To express and to purify zMn–SOD in *E. coli*, we designed a 5′ upstream primer zMnSOD-rbs-PB, which contained a ribosomal binding site (GAAGGAG) just upstream of initiation codon (ATG) of zMnSOD cDNA, and a 3′ downstream primer zMnSOD-His-PC, which contained six His tail (Table 1). Using 0.02 μg of cDNA as a template, 10 pmol of each 5′ upstream and 3′ downstream primer were added, a 0.6 kb fragment was amplified by PCR (35 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s), and ligated with pGEM-T EASY vector (Promega), and then transformed into the *sodA* & *sodB* mutant *E. coli* QC779 host [29]. The transformed *E. coli* was grown at 37 °C in 200 mL of Luria Bertani medium containing 50 μg/mL ampicillin until A_{600} reached 0.6. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM followed by the addition of a 50 μM $MnCl_2$ to provide essential metal ions. The culture was incubated at 20 °C for 12 h at 150 rpm, and then the bacterial cells were harvested by centrifugation at 12,000 × *g* for 10 min. The induced zMn–SOD was purified by Ni-Sepharose (Amersham Biosciences) according to our previous study [30].

2.5. Characterization of zMn–SOD by activity staining assay

Each 3.6 μg recombinant zMn–SOD underwent the following tests. After the following treatments, each sample was divided into two parts, and then each part of the sample was electrophoresed into a 12% native polyacrylamide gel to determine the changes in the activity and protein levels according to our previous study [30].

(1) Thermal Stability. Enzyme samples were heated at 70 °C for 8, 16, 32 and 64 min.
(2) pH Stability. Enzyme samples were mixed with half a volume of buffer at different pH values: 0.2 M citrate buffer (pH 2.2, 3.0, or 5.8), 0.2 M Tris–HCl buffer (pH 7.4 or 9.0), or 0.2 M glycine–NaOH buffer (pH 10.4, or 11.22). Each sample was incubated at 37 °C for 3 or 8 h.
(3) SDS Effect. SDS was added to the enzyme sample to the level of 1, 2, 3, or 4% and incubated at 37 °C for 1 h.

2.6. Oxidative stress regulation of zMn–SOD gene expression assayed by real-time RT-PCR during embryonic development

Paraquat was purchased from Sigma Chemical Company (St. Louis, Mo.). Three 5 mL wells, each containing 50 one-cell stage fish embryos, were immersed with 0, 100, and 300 ppm PQ for 48 h at 25 °C. The survival rate was recorded. Seven 5 mL wells, each containing 50 embryos at one-cell stage were immersed with 100 ppm PQ for 4, 8, 12, 24, 30, 36 and 48 h at 25 °C. Embryos without any treatment were used as control. After treatment, total RNA was extracted and cDNA was synthesized according to our previous study [24]. Real time RT-PCR was performed on Light-Cycler 1.5 system, and FastStart DNA Master SYBR Green I was used as detection reagent (Roche Diagnostics, Mannheim, Germany). 2 μL cDNA was added to 8 μL of PCR master mix containing 4 μL of ddH_2O, 1 μL SYBR Green mix, 1 μL $MgCl_2$ stock (final 2.5 mM), and 1 μL (3 pmol) each of zMn–SOD-RT-P1 and zMn–SOD-RT-P2 primers (Table 1). The PCR program was set as follows: pre-incubation at 95 °C for 10 min; 50 cycles of amplification at 95 °C for 10 s, 62 °C for 7 s and 72 °C for 6 s; melting at 60 °C for 15 s, and