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Molecular characterization and expression analysis of extracellular copper/zinc superoxide dismutase (ecCuZnSOD) from oriental river prawn, *Macrobrachium nipponense*

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ABSTRACT

Superoxide dismutase (SOD, EC 1.15.1.1) is an enzyme catalyzing the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide. In the present study, the full length cDNA of extracellular copper–zinc superoxide dismutase (MnECSOD) from *Macrobrachium nipponense* was cloned (GenBank accession no. JX045662). Analysis of the nucleotide sequence revealed that the MnECSOD cDNA consisted of 817 bp with an open reading frame (ORF) of 597 bp encoding a polypeptide of 198 amino acids with a putative signal peptide of 19 amino acids. The mature protein had a predicted molecular weight of 18.71 kDa with an estimated *p*I of 4.87. Four copper binding sites, 4 zinc binding sites and 2 cysteines involved in the formation of the disulfide bridge were conserved in the protein. Sequence comparison showed that the MnECSOD protein had 86% and 55% similarity to that of freshwater prawn *Macrobrachium rosenbergii* and white shrimp *Litopenaeus vannamei*, respectively. After challenge with *Aeromonas hydrophila*, the expression of MnECSOD mRNA in the hepatopancreas was increased responsively. After a drastic decrease at 12 h, the expression level increased again by 6.75-fold compared to that in the control group at 48-h post-infection. These results may indicate that MnECSOD was involved in the innate immune responses of *M. nipponense*.

1. Introduction

Reactive oxygen species (ROS) including hydroxyl radical, superoxide anion, hydrogen peroxide, and singlet oxygen, are constantly generated in all aerobic biological systems. On one hand, ROS are essential for the elimination of harmful pathogens such as bacteria and viruses (Chanock et al., 1994; Muñoz et al., 2000) and are also involved in immune signal transduction (Schreck et al., 1991; Xiang, 2001), synaptic plasticity and memory formation (Kishida and Klann, 2007). On the other hand, high level of ROS tends to cause oxidative damage to cellular components such as proteins, nucleic acids and lipids (Johnson, 2002). In order to limit the harmful effect of ROS, organisms have evolved antioxidant systems as part of the innate immune defense to maintain ROS at low levels (Manduzio et al., 2004).

Superoxide dismutase is one of the important antioxidant enzymes directly participating in the balance of ROS by converting O_2^- into O_2 and H_2O_2. Depending on the metal content, SODs are classified into three distinct groups: copper–zinc SOD (CuZnSOD), iron SOD (FeSOD) and manganese SOD (MnSOD) (Gutteridge and Halliwell, 1999; Plantivaux et al., 2004). CuZnSOD is an important type of SOD because of its physiological function and therapeutic potential (Ni et al., 2007). CuZnSOD requires Cu and Zn for its biological activity. Loss of Cu can result in its complete inactivation, and can induce many diseases in human and animals (Concetti et al., 1976; Mavelli et al., 1984; Mizuno, 1984). Two CuZnSODs encoded by two different genes are found in eukaryotes: intracellular CuZnSODs (icCuZnSODs) and extracellular CuZnSODs (ecCuZnSODs or ECSODs). The icCuZnSOD is present in the cytoplasm and nucleus, whereas ecCuZnSOD is found in the extracellular matrix of tissues such as lymph and plasma (Fattman et al., 2003). In decapod crustaceans, ecCuZnSOD might mediate or regulate cell adhesion and phagocytosis, and have been cloned from crayfish *Pacifastacus leniusculus* (Johansson et al., 1999), giant freshwater prawn *Macrobrachium rosenbergii* (Cheng et al., 2006), blue crab *Callinectes sapidus* (Brouwer et al., 2003), mud crab *Scylla serrata* (Lin et al., 2008), swimming crab *Portunus trituberculatus* (Li et al., 2011), shore crab *Carcinus maenas* (Orbea et al., 2000) and Chinese mitten crab *Eriocheir sinensis* (Meng et al., 2011). Up to now, only ecCuZnSOD has been cloned in decapod crustaceans, whereas in bacteria, fungi, plants, platyhelminths, mollusks and fish, just icCuZnSOD has

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been cloned. Interestingly, both ecCuZnSOD and icCuZnSOD have been cloned from the cnidarians, nematodes, insects and mammals (Lin et al., 2008; Meng et al., 2011).

M. nipponense is a kind of freshwater or brackish prawn and it is often a commercially important species in China, Japan and Vietnam (Uno, 1971; Wang et al., 2002). Two types of SOD: MnSOD and CuZnSOD were purified from the hemolymph of *M. nipponense* and partial characterizations were studied (Yao et al., 2004, 2007). The aim of the present study was to gain new insights into the characterization of CuZnSOD and its role in prawns. Here, the full length cDNA of ecCuZnSOD was cloned from the oriental river prawn *M. nipponense*. The deduced amino acid sequence was compared with other known ecCuZnSODs from other decapod crustaceans. The expression of MnECSOD in various tissues was investigated, and its expression profiles in hepatopancreas challenged with *Aeromonas hydrophila* were studied.

2. Materials and methods

2.1. Animal and RNA extraction

The oriental river prawns, *M. nipponense* (2–3 g in weight, 4–5 cm in length) purchased from a market in Nanjing, China, were cultivated in 100 L aquaculture tanks at 20 °C with freshwater and an aeration system. The prawns were acclimated for 10 days before processing. Randomly selected specimens were checked using *A. hydrophila* diagnostic PCR (Pollard et al., 1990) and were all found to be PCR negative.

RNA was extracted from hepatopancreas using TRIzol Reagent (Invitrogen, USA) following the manufacturer's protocol. RNA quality was assessed by electrophoresis on 1.2% agarose gel and the total RNA concentration was determined by measuring the absorbance at 260 nm on a spectrophotometer.

2.2. cDNA synthesis and gene cloning

The cDNA was synthesized using about 5 μg total RNA using the Takara PrimerScript™ First Strand cDNA Synthesis Kit (TaKaRa, China) according to the manufacturer's instruction. Degenerate primers, CZF1 and CZR1, designed based on the highly conserved nucleotide region of CuZnSOD using the CLUSTAL program (Cheng et al., 2006) were used in the reverse-transcriptase polymerase chain reaction (RT-PCR). Amplification primers for MnECSOD are shown in Table 1.

2.3. Rapid amplification of cDNA ends (RACE) of MnECSOD

About 5 μg of total RNA was reverse-transcribed with CZR2 primer to generate 5′-RACE template. CZR2 primer was designed based on the sequence amplified by the degenerate primers. For the 5′-RACE, part of the MnECSOD gene was obtained using a 5′-RACE System (Invitrogen, USA) according to the manufacturer's instruction. The primer set consisted of CZR3 with Abridged Anchor Primer (AAP) for the first-run PCR, and CZR4 with the Abridged Universal Amplification Primer (AUAP) for the second-run PCR. For the 3′-RACE, the RT-PCR was performed using the Oligo-d$(T)_{18}$ ACP primer with CZF2, and the nested PCR was performed using CZF3 with 3′-RACE primer. The amplified products were cloned into pGEM-T Easy vector and sequenced.

Table 1
Primers used in the present study.

Name	Sequence
CZF1	5′-CCAGGAAAACATGGCTTCCAYGTNCA-3′
CZR1	5′-GGGTTGCCCCCGCGGCCCAARTCRTC-3′
CZR2	5′-GCAGTTCCATCCTCAC-3′
CZR3	5′-CAAATCACCAACGTGCCTCTG-3′
CZR4	5′-CCGTCTGGGTTGTAATGAGCC-3′
CZF2	5′-GAGGCTCATTACAACCCAGACGGATTCG-3′
CZF3	5′-GCAGTTGTAGGCCGAAGCATAGTTGTCC-3′
AAP	5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′
AUAP	5′-GGCCACGCGTCGACTAGTAC-3′
Oligo-d$(T)_{18}$ ACP	5′-CTGTGAATGCTGCGACTACGA$(T)_{18}$-3′
3′-RACE primer	5′-CTGTGAATGCTGCGACTACGA-3′
CZQF	5′-GGGCAGGCTTTACGGTGCTCTT-3′
CZQR	5′-CCCTCGATATGAATGGTGGTCG-3′
β-actinF	5′-AATGTGTGACGACGAAGTAG-3′
β-actinR	5′- GCCTCATCACCGACATAA-3′

2.4. Sequence analysis

The cDNA sequence of MnECSOD was analyzed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast), and the deduced amino acid sequence was analyzed with the ExPASy tools (http://us.expasy.org/). The ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) was used for the multiple sequence alignment. The presumed tertiary structures were established using SWISS-MODEL prediction algorithm (http://swissmodel.expasy.org/). A cladogram was constructed based on the amino sequences alignment by the neighbor-joining (NJ) algorithm embedded in MEGA 4 program. The reliability of the branching was tested by bootstrap resampling (1000 pseudoreplicates).

2.5. Tissue expression of MnECSOD

Tissue distribution of MnECSOD mRNA in hemocytes, heart, hepatopancreas, gill, intestine, nerve, and muscle was demonstrated by quantitative real-time PCR analysis. Total RNA was extracted as described above, and 5 μg of total RNA was used to synthesize the first strand cDNA. For quantification of the MnECSOD expression, a pair of gene specific primers (CZQF, CZQR) was used, and the primers *β-actin* F and *β-actin* R (Zhao et al., 2011) were used to amplify *β-actin* as an internal control. The different gene expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Statistical analysis was performed using SPSS software (Ver11.0). Data are presented as the mean ± standard error ($n = 3$). Statistical significance was determined by one-way ANOVA and post hoc Duncan multiple range tests. Significance was set at $P<0.05$.

2.6. Expression pattern of MnECSOD in hepatopancreas under A. hydrophila stimulation

For the experiment group, 30 prawns were injected individually with 50 μL of bacterial suspension (10^4 cells/mL). The bacterium *A. hydrophila* was kindly presented by Professor Hui Chen (Jiangsu Center for Control and Prevention of Aquatic Animal Infectious Disease) and was grown at 28 °C in TSB medium. At the same time, 30 prawns injected with 50 μL saline (0.85% NaCl) (pH = 7.0) were used as the control group. *M. nipponense* were sampled at 0 h, 1 h, 12 h, 24 h, 36 h and 48 h post-injection. For each treatment and each exposure time, the hepatopancreas of five prawns was sampled and total RNA was extracted as described above. The extracted RNA was determined quantitatively, and 5 μg of total RNA from hepatopancreas was used for reverse transcription. Gene expression of MnECSOD was determined by quantitative real-time RT-PCR. The quantitative real-time PCR method was the same as that described above.

3. Results

3.1. Sequence analysis of MnECSOD cDNA

A 301 bp partial cDNA fragment was obtained using CZF1 and CZR1. The full-length cDNA (GenBank accession number JX045662) was 817 bp in length including 88 bp in the 5′-untranslated region, an open reading frame (ORF) of 597 bp encoding a protein of 198 amino acids, and 132 bp in the 3′-untranslated region including a