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Transcriptional regulation of extracellular copper zinc superoxide dismutase from white shrimp *Litopenaeus vannamei* following *Vibrio alginolyticus* and WSSV infection

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ABSTRACT

The cDNA encoding an extracellular copper zinc superoxide dismutase (LvECSOD) was cloned from the hepatopancreas of white shrimp *Litopenaeus vannamei*. It consisted of 915 bp nucleotides with an open reading frame corresponding to a deduced protein of 178 amino acids. The LvECSOD contains a putative signal peptide of 16 amino acids, two potential N-linked glycosylation sites (N^{115}GTA and N^{135}ITG) and a copper zinc superoxide dismutase family signature sequence (G^{162}NAGaRvACctI^{173}). It was found that four copper binding sites, four zinc binding sites and two cysteines involving in the formation of the disulfide bridge were conserved in the protein. LvECSOD shared 33–58% identity to ECSODs from other organisms. Expression analysis revealed that LvECSOD mRNA was widely distributed in all the tissues examined. When the shrimp challenged with *Vibrio alginolyticus* or white spot syndrome virus (WSSV), expression of LvECSOD mRNA in the hepatopancreas and hemocytes was mediated responsively. Our results suggested that LvECSOD was implicated in the immune response induced by *V. alginolyticus* and WSSV.

1. Introduction

Reactive oxygen species (ROS) including superoxide anion (O_2^-), hydroxyl radical ($OH^\bullet$) and hydrogen peroxide (H_2O_2) are continuously formed as a consequence of cellular oxygen metabolism in aerobic organism [1]. ROS are essential for the elimination of harmful pathogens like bacteria and virus [2,3], and are necessary in immune signal transduction [4,5], synaptic plasticity and memory formation [6]. However, extremely high levels of ROS may lead to irreversible cell damage and eventually to cell death [7]. Concentrations of ROS are exquisitely balanced in part by antioxidant enzyme defense system including superoxide dismutase (SOD), catalase and glutathione perixidase, etc. The first step of ROS eliminated from the cells is performed by SODs, which convert the O_2^- into O_2 and H_2O_2 that passes freely through membranes.

As a sort of metalloenzymes in animals, SODs were classified into two groups based on the metal co-factor they harbor: manganese SODs (MnSOD) and copper-zinc SODs (CuZnSOD). Cytoplasmic MnSOD and mitochondrial MnSOD are two types of MnSODs. There are also two forms of CuZnSODs: one is cytoplasmic CuZnSOD (cytCuZnSOD) without N-terminal signal peptide found in the intracellular cytoplastic compartments, the other is extracellular CuZnSOD (ECSOD) with N-terminal signal peptide circulated in the extracellular fluids [8] or localized to the plasma membrane [9]. These two forms share high sequence and structural homology but distinct from MnSODs [10].

ECSOD, which was first identified in human plasma, lymph, ascites and cerebrospinal fluids in 1982, is a slightly hydrophobic glycoprotein [8,11]. It is the only antioxidant enzyme that scavenges superoxide anion specifically in the extracellular compartment and thereby prevents the formation of many other reactive oxygen metabolites [12]. Although ECSOD has been reported to play an important role in maintaining vascular tone, lung function, the metabolism of NO, and in the pathology of some diseases (see review [13]), it is the least characterized member of the SODs so far [14].

ECSOD has been identified in variety of species such as mouse [15], frog (Genbank accession number, NP_001106630), zebrafish (Genbank accession number, CAX13027), wasp (Genbank accession number, xp_001602916), Hydra [10], bay scallop [16], blue crab

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[17], mud crab [18], crayfish [9] and giant freshwater prawn [19]. ECSOD exhibited tissue-specific expression pattern in most organisms except in mud crab. It is ubiquitously expressed in mud crab tissues with highest transcript levels in hemocytes and muscle [18]. As far as we know, there is little information on penaeid shrimp ECSOD and its responses to the foreign infections. Here, we cloned ECSOD cDNA (LvECSOD) from *Litopenaeus vannamei*, the most widely cultured shrimp species in the world and analyzed its mRNA expression in hepatopancreas and hemocytes after challenged with *Vibrio alginolyticus* and white spot syndrome virus (WSSV), respectively.

2. Materials and methods

2.1. Animals

White shrimps *L. vannamei* weighing 5–6 g were obtained from a shrimp farm in Panyu (Guangdong, China). Shrimps were acclimated in 250 L recirculating water tanks and fed the commercial diet three times daily for one week. During the acclimation and experiment, the water salinity and temperature in tanks was consistent with that of the culture ponds (salinity 4‰ and temperature 27 ± 1 °C). Only shrimps in the intermolt stage were used for the study.

2.2. LvECSOD cDNA cloning

Total RNAs were extracted with Trizol (Invitrogen, Gaithersburg, USA) from hepatopancreas of *L. vannamei* and quantified spectrophotometrically. First-strand cDNA synthesis was performed with reverse transcription system (Promega, Madison, USA) using oligo d (T) primer with the DNaseI(Promega, Madison, USA)-treated total RNA as template. Reaction conditions recommended by the manufacturer were followed. Degenerate primers LvECSODF1 and LvECSODR1 (Table 1) used for cloning the initial fragment were designed based on the highly conserved nucleotides of ECSODs from some arthropods. The PCR program was 94 °C for 1 min, 37 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 45 s, followed by 72 °C for 5 min. PCR products were cloned into pMD-20T (TAKARA, Dalian, China) and sequenced by Sangon Corp (Shanghai, China).

The 5′ and 3′ RACE were obtained by nested PCR using the BD SMART RACE cDNA amplification kit (Clontech, Palo Alto, USA) according to the manufacture's instructions. Specific primers LvECSOD5R1 and LvECSOD5R2 (Table 1) for 5′ end, and LvECSOD3R1 and LvECSOD3R2 (Table 1) for 3′ end, were designed based on the initial fragment obtained by the degenerate primers. The full-length cDNA was verified by primers LvECSODF2 and LvECSODR2. All the PCR products were sequenced as described above.

Table 1
Primers or probes used in the experiment.

Name	Sequence (5′-3′)
LvECSODF1	TGCCG(C/G)CCA(C/T)TTCAAC
LvECSODR1	CG(A/G)GC(A/T)CCCGCATTGCCAG
LvECSODF2	GGAGACACAGCTGATTTC
LvECSODR2	GAGAGGGAGAGCTTAGAACC
LvECSODF3	GGACACGACCATTAGCC
LvECSODR3	GCCAGTAGCGAGTGA ACC
LvECSOD5R1	CCCAGGTCGTCTTCCAGGGCGTG
LvECSOD5R2	GCTCCAGGTCGTACAGGCTAATGATCGTG
LvECSOD3R1	GGGATCTCGGGAACATCGACGCC
LvECSOD3R2	TTAGCCTGTACGACCTGGAGCGGAAC
actinF	CAACAAGATGTGTGACGACGAAGTAGC
actinR	GTTTCTCCTTGATGTCACGAACGATT
For real-time	
LvECSODrtF	ATGAAGACGTTGGCAACTCTG
LvECSODrtR	CTCGCAGGTGGAGTGGAG
LvECSODprobe	(FAM)-CGCCGTCGCCGCCACAGCC(Eclipse)
actinrtF	TGCTACGTGGCCCTGGAC
actinrtR	GTTGTAGGTGGTCTCGTGGATG
actinprobe	(FAM)-ACCACCGCTGCTTCCTCCTCCTCG(Eclipse)

2.3. Sequence analysis

The cDNA sequence was analyzed for coding probability with the DNATools program [20]. Comparison against the GenBank protein database was performed using the BLAST network server at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple protein sequences were aligned using the MegAlign program by the CLUSTALW method in DNASTAR software package [21]. A phylogenetic tree was constructed by the neighbor-joining method within the PHYLIP 3.5c software package [22] using 1000 bootstrap replicates. The search for signal peptide in the protein was carried out by the program of SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The prediction of the potential N-glycosylation sites was performed by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). The identification of motif sequences was done using the software of InterPro Scan (http://www.ebi.ac.uk/InterProScan/).

2.4. Tissue expression of LvECSOD

Haemolymph was withdrawn from the ventral sinus of healthy shrimps using a 1.0-ml sterile syringe (25-gauge), diluted 1:1 in precooled anticoagulant (27 mM trisodium citrate, 385 mM sodium chloride, 115 mM glucose, pH 7.5). Haemolymph was centrifuged at 800 × *g*, 4 °C for 10 min to separate the hemocytes. Total RNAs were extracted with Trizol (Invitrogen, Gaithersburg, USA) from tissues including hemocytes, hepatopancreas, heart, stomach, intestine, eyestalk, gill and muscle of eight healthy shrimps and 1 μg total RNA from each tissue was reversely transcribed as mentioned above. Semi-quantitative RT-PCR was conducted to investigate the tissue expression of LvECSOD. LvECSOD specific primers LvECSODF3 and LvECSODR3 (Table 1) were used to amplify a product of 119 bp, while the primers actinF and actinR (Table 1) of β-actin (Genbank accession number, AF300705) were used to amplify a product of 656 bp fragment as internal control. The PCR reactions were performed at 94 °C for 1 min, 33 cycles for LvECSOD or 25 cycles for β-actin of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 45 s, followed by 72 °C for 5 min. 5 μl of the reaction products were separated on a 1.5% agarose gel and stained with ethidium bromide.

2.5. LvECSOD mRNA expression assays after V. alginolyticus and WSSV challenge

2.5.1. V. alginolyticus and WSSV challenge and sample collection

For *V. alginolyticus* challenging experiment group, shrimps were injected individually with 10 μl of bacterial suspension (9×10^7 cfu ml^{-1}) in saline solution (0.85%) into the abdominal segment, resulting in 9×10^5 cfu shrimp^{-1}. Shrimps injected with 10 μl saline solution were used as the control. For WSSV challenging, the treatment and control shrimps were injected with 10 μl of tissue homogenate prepared from WSSV-infected and WSSV-free shrimp, respectively. Following injection, shrimps were raised in plastic tanks containing about 100 L air-pumped water with salinity 4‰ and temperature 27 ± 1 °C. Haemolymph of 4 shrimps in each group was sampled and centrifuged as mentioned above at the beginning (initial control) and then 3, 6, 12, 24 and 48 h post-injection, respectively. Hemocytes were collected for total RNA extraction. Following haemolymph preparation, the