



Molecular characterization of a cytosolic manganese superoxide dismutase from the Chinese mitten crab, *Eriocheir sinensis*

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ABSTRACT. A cytosolic manganese superoxide dismutase gene (*Es-cMnSOD*) was cloned from the Chinese mitten crab *Eriocheir sinensis*, using reverse transcription-polymerase chain reaction and the rapid amplification of cDNA ends. The open reading frame of *Es-cMnSOD* is 867 bp in length and encodes a 288-amino acid protein without a signal peptide. The calculated molecular mass of the translated protein of *Es-cMnSOD* is 31.43 kDa, with an estimated isoelectric point of 6.30. The deduced amino acid sequence of *Es-cMnSOD* has similarities of 90, 89, 84, 87, and 81% to those of white shrimp *Litopenaeus vannamei* MnSOD, black tiger shrimp *Penaeus monodon* MnSOD, giant freshwater prawn *Macrobrachium rosenbergii* MnSOD, blue crab *Callinectes sapidus* MnSOD, and red swamp crayfish *Procambarus clarkii* MnSOD, respectively. *Es-cMnSOD* contains a manganese superoxide dismutase domain (DVWEHAYY) and 4 conserved amino acids responsible for binding manganese. *Es-cMnSOD* was expressed

in the hemocytes, eyestalk, muscle, intestine, gill, and hepatopancreas. *Es-cMnSOD* transcripts in hemocytes of *E. sinensis* increased at 1.5 and 48 h after injection of *Aeromonas hydrophila*, indicating that the induction of the SOD system response occurred within a short period of time. This study suggests that MnSOD may play a critical role in crab immunity, allowing efficient activation of an early innate immune response in the crab.

Key words: Cytosolic manganese superoxide dismutase; Hemocyte; *Eriocheir sinensis*; *Aeromonas hydrophila*

INTRODUCTION

Phagocytosis is an important mechanism for immune defense in crustacean via engulfing bacteria in macrophage when hemocytes become infected with bacteria (Lee and Söderhäll, 2002; Smith et al., 2003). At this time, reactive oxygen species (ROS), including superoxide anion and hydrogen peroxide, are produced to kill the invading bacteria. However, when the ROS level exceeds the threshold level of the antioxidant system, cellular redox-homeostasis is disturbed, thus leading to oxidative stress (Trachootham et al., 2008). ROS are widely recognized as dual-role players since they act as key mediators in a variety of cellular processes, and an elevated ROS level can have deleterious effects to cellular macromolecules (Muñoz et al., 2000; Johnson, 2002). To defend against oxidative damage, cells are equipped with myriad antioxidant enzymes to scavenge and detoxify the accumulated oxyradicals (Vaughan, 1997; Park et al., 2004; Arenas-Ríos et al., 2007).

Among the antioxidant enzymes, the superoxide dismutases (SODs) comprise the first defense system that catalyzes the dismutation of superoxide radicals to hydrogen peroxide and oxygen (McCord and Fridovich, 1969). Since the SODs were discovered in the 1960s (McCord and Fridovich, 1969), many types of SODs have been reported as metalloproteins in the cells of a wide range of organisms, including cytosolic Cu/ZnSOD, MnSOD, FeSOD and NiSOD (Bannister et al., 1987; Fridovich, 1995).

MnSOD containing manganese ions was primarily found in the mitochondrial matrix (mMnSOD) (Weisiger and Fridovich, 1973), but some cytosolic MnSODs (cMnSOD) have also been reported in crabs, lobsters, prawns, and shrimp (Brouwer et al., 2003). The former was found in bacteria, plants, invertebrates, and vertebrates, whereas the latter was found only in crustaceans (Lin et al., 2010). Recently, MnSOD has received much attention because it is a major scavenger of damaging ROS metabolites in the mitochondrial matrix (Li et al., 2011). More importantly, the SOD protein has been also demonstrated to play an important role in cellular differentiation and tumorigenesis (St Clair et al., 1994) in the immune response induced by bacteria (Jung et al., 2005; Cheng et al., 2006), viruses (Zhang et al., 2007), or exposure toxic chemical (Kim et al., 2007).

To date, many MnSOD orthologs have been characterized in mammalian (Ho et al., 1991; Wan et al., 1994; Meyrick and Magnuson, 1994; Jones et al., 1995), fish (Lin et al., 2009), and invertebrate (Gómez-Anduro et al., 2006; Bao et al., 2008; Zhang et al., 2011; Yu et al., 2011) species. In decapod crustaceans, some MnSODs have also been cloned, including the pacific white shrimp *Litopenaeus vannamei* (DQ005531; DQ029053), tiger shrimp

Penaeus monodon (AY726542), Chinese shrimp *Fenneropenaeus chinensis* (GQ168792), kuruma shrimp *Marsupenaeus japonicus* (GQ181123), freshwater prawn *Macrobrachium rosenbergii* (EU077526), grass shrimp *Palaemonetes pugio* [AY211084, partial complementary DNA (cDNA)], giant mud crab *Scylla serrata* (GU213434), blue crab *Callinectes sapidus* (AF264030), and hydrothermal crab *Bythograea thermydron* (FM242567). Recently, the characterization of cMnSOD and its role of immunomodulation in *M. rosenbergii* (Cheng et al., 2006), *L. vannamei* (Gómez-Anduro et al., 2006), *M. japonicas* (Lin et al., 2010), and *B. thermydron* (Marchand et al., 2009) have also been reported.

In the last few decades, the cultured freshwater Chinese mitten crab *Eriocheir sinensis* has been an important economical species resource. Farming of this species has been a challenge due to opportunistic pathogens such as bacteria, viruses and rickettsia-like organisms (Wang and Gu, 2002; Wang et al., 2002; Xu et al., 2002) in deteriorated environments. Therefore, investigating the mechanisms of immune defense against pathogens in crabs may be beneficial to the management of crustacean-farming practices.

We previously identified an expressed sequence tag (EST) from *E. sinensis* cDNA library with sequence similarity to the freshwater prawn *M. rosenbergii* SOD (Zhao et al., 2009). Given the importance of SOD in immunity and the limited amount of molecular data available for *E. sinensis*, we further studied the SOD identified from this species in our previous EST study. The present study aimed at cloning and characterizing the *E. sinensis* SOD (designated as *Es-SOD*) by comparing its sequence to other known SODs, examining the expression levels of SOD in various tissues, and evaluating SOD expression in the hemocytes of crabs challenged with *Aeromonas hydrophila*. These results will provide a better understanding of the mechanisms underlying disease resistance and the process coping mechanisms with regard to oxidative stress in this species.

MATERIAL AND METHODS

Animals

Healthy crabs averaging 100 g (wet weight) were collected from a commercial crab farm near Shanghai, China, and acclimated at $22^{\circ} \pm 2^{\circ}\text{C}$ for 1 week prior to being used for experimentation.

RNA isolation from hemocytes and reverse transcription (RT)

Hemolymph sampling and collection of hemocytes were conducted according to previously reported methods (Zhao et al., 2009). Total RNA was isolated from the hemocyte pellet and further purified using the Unizol Reagent Kit (Biostar, Shanghai, China) according to the manufacturer protocol. The total concentration of RNA was estimated by measuring the absorbance at 260 nm. Total RNA (5 μg) was reversely transcribed using the MMLV reverse transcriptase (TaKaRa, Dalian, China) with the adapter primer (5'-GGCCACGCGTCGACT AGTACTTTTTTTTTTTTTTTTTT-3') for 3'-rapid amplification of cDNA ends (RACE) and the 5'-CDS primer A [(5'-(T) 25 VN-3', N = A, C, G, or T; V = A, G, or C)] for 5'-RACE to obtain the first-strand cDNA. Reaction conditions followed the recommendations of the kit manufacturer.

Cloning the full-length cDNA of SOD

The full-length SOD cDNA of *E. sinensis* was obtained by the RT-polymerase chain reaction (RT-PCR) and the RACE methods. Two gene-specific primers, P1 (GSP1: 5'-CCGCC TCTAGCCTCCACTTGC-3') and P2 (GSP2: 5'-TTCTTGGAGCAGTAGCCGAGCC-3') were designed based on the sequence of hem_0002_D01.ab1 (Zhao et al., 2009). The 5'-region of the *Es-SOD* transcript was obtained via the 5'-RACE reaction using the SMART RACE cDNA Amplification Kit (TaKaRa) according to manufacturer instructions. The primers consisted of GSP1 with the universal primer A mix (5'-CTAATACGACTCACTATAGGGCAAGCAG TGGTATCAACGCAGAGT-3') for RACE to derive the 5'-untranslated region (UTR) and the signal peptide sequences. For 3'-RACE, the primers of GSP2 with the abridged universal amplification primer (5'-GGCCACGCGTCTGACTAGTAC-3') were used for amplification of the target cDNA. The PCR fragments were subjected to electrophoresis on 1.0% agarose gel to determine differences in length. The amplified cDNA fragments were cloned into the pMD18-T vector (TaKaRa) following manufacturer instructions. Recombinant bacteria were identified by blue/white screening and confirmed by PCR. Plasmids containing the inserted GST fragment were used as a template for DNA sequencing.

Sequence analysis

The homology searches of nucleotide and protein sequences were conducted with the BLAST algorithm at the National Center for Biotechnology Information (NCBI). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (<http://www.expasy.org/>). The SignalP 3.0 program was used to predict the presence and location of the signal peptide and the cleavage sites in amino acid sequences.

Multiple-sequence alignment and phylogenetic analysis

The full-length multiple alignment of the *Es-SOD* sequence was compared to that of other SODs. Amino acid sequences from various species of arthropods were retrieved from the NCBI GenBank database and analyzed using the ClustalW Multiple Alignment program and the Multiple Align Show program. A neighbor-joining (N-J) phylogenetic tree was constructed using the MEGA software version 3.1 (Kumar et al., 2004). The reliability of the branching was tested using bootstrap re-sampling with 1000 pseudo-replicates.

Expression of the *Es-SOD* gene in tissues

The expression of *Es-SOD* messenger RNA (mRNA) in tissues was demonstrated by quantitative real-time RT-PCR (qRT-PCR) analysis. The hemocytes, hepatopancreas, muscles, gills, stomachs, and intestines were removed from the crabs. The tissues were blotted and weighed; they were then homogenized using the Unizol reagent (Biostar). Total RNA was extracted as described above. The first-strand cDNA was synthesized using the MMLV reverse transcriptase with 5 µg total RNA. The gene-specific primer pairs of *Es-SOD* QF (5'-CCCAGTTGATGACGTTGAAG-3') and *Es-*

SOD QR (5'-CTCGGCTACTGCTCCAAGAA-3') were used to amplify the SOD transcript. The primers, β -actin F (5'-GCATCCACGAGACCACTTACA-3') and β -actin R (5'-CTCCTGCTTGCTGATCCACATC-3') were used to amplify the β -actin fragment that was used as the internal control.

SOD expression in hemocytes after *A. hydrophila* challenge

A total of 72 crabs were challenged. The crabs were randomly divided into 2 groups, and each treatment was applied in triplicate. Each crab in the challenge group received an injection of 100 μ L live *A. hydrophila* suspended in saline (7.2×10^8 CFU/mL), while the crabs in the control group received the same volume of saline only. After treatment, the crabs were returned to the tanks, and 2 individuals from each group were randomly sampled at 0, 1.5, 3, 6, 12, 24, and 48 h post-injection. The qRT-PCR assay was performed using the SYBR Premix Ex Taq (TaKaRa) on an ABI StepOne real-time PCR system (Applied Biosystems, USA). Gene-specific primers of the Es-SOD QF and the Es-SOD QR with cDNA that had been reversely transcribed were used for the reaction according to manufacturer instructions. The primers β -actin F and β -actin R were used to amplify the β -actin fragment that was used as the internal control.

Data analysis for SYBR Green-based qRT-PCR

On a 96-well plate, each sample was run in triplicate along with the internal control gene. A dissociation curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. After the PCR run, data analysis was performed with the StepOne Software version 2.0 (Applied Biosystems). Briefly, the threshold PCR cycle (C_t) was defined as the cycle number at which a statistically significant increase in fluorescence of SYBR Green against the internal passive dye ROX (ΔR_n) was first detected. The copy number of the target gene and C_t values are inversely related; thus, a sample containing a higher number of copies of the target gene would have a lower C_t value than that of the sample with a lower number of copies of the same target gene. The differences in the C_t values of SOD and the corresponding internal control β -actin, (i.e., ΔC_t), were calculated to normalize any differences in the amount of total RNA added to the cDNA reaction mixture and the efficiency of RT reaction. The ΔC_t for the control sample was subtracted from the ΔC_t of the challenged sample. The difference was expressed as a $\Delta \Delta C_t$ value that allowed measurement of the change in SOD expression in the challenged crabs relative to the control crabs. The expression level of *Es-SOD*, calculated by the formula $2^{-\Delta \Delta C_t}$, was represented as an n-fold difference relative to the control. The relative expression values were exported into a Microsoft Excel sheet for subsequent data analyses.

Statistical analysis

A multiple-comparison Duncan test was used to compare significant differences in *SOD* gene expression between the control and challenged samples using the SPSS15.0 software. A significance level of $P = 0.05$ was chosen.

RESULTS

Nucleotide sequence of SOD cDNA and the deduced amino acid sequence

A singleton sequence with significant similarity to the grant freshwater shrimp *M. rosenbergii* SOD gene was found from the crab hemocyte EST library. This partial sequence provided crucial DNA sequence information required for the 3'-RACE cloning and 5'-amplification of the complete SOD of the crab. A 426-bp product of the 3'-end of SOD was obtained by 3'-RACE. Approximately, 295 bp of the 5'-end of SOD were obtained by the RACE method. Finally, the full-length SOD cDNA fragment of *E. sinensis* was obtained by overlapping 3 cDNA fragment sequences. The nucleotide sequence and the deduced amino acid sequence are shown in Figure 1. The full-length of *Es-SOD* cDNA comprised of 1339 bp, containing 20 bp in the 5'-UTR, 867 bp in the open reading frame (ORF), 452 bp in 3'-UTR with a poly(A) tail of 25 bp, and a putative polyadenylation consensus signal (aataaa) of 19 nucleotides upstream of the poly(A) tail. The ORF encode a polypeptide of 288 amino acids without a signal peptide. The calculated molecular mass of the mature protein was 31.43 kDa, with an estimated isoelectric point of 6.30. The *Es-SOD* cDNA sequence and its deduced amino acid sequence were submitted to the NCBI GenBank under accession No. FJ617306.

Comparison of SOD sequences and motifs of *E. sinensis* with other species

Sequence comparisons of the *Es-SOD* deduced amino acids showed 90, 89, 84, 87, and 81% similarities to white shrimp *L. vannamei* MnSOD, *P. monodon* MnSOD, *M. rosenbergii* MnSOD, *C. sapidus* MnSOD, and *Procambarus clarkii* MnSOD, respectively. Multiple alignment of *Es-SOD* with the SOD of different crustaceans indicated that they share some conserved motifs, including a general MnSOD domain from the 172nd to the 279th amino acid, a putative cMnSOD signature sequence from the 244-251th amino acids (DVWEHAYY), and 4 putative manganese binding sites (i.e., H111, H159, D244, and H248) (Figure 2). The high homology of the *Es-SOD* sequence to the MnSOD of other decapod species indicates that the *Es-SOD* cloned in *E. sinensis* belonged to the MnSOD family (designated as *Es-MnSOD*) and might play a similar role as that of other MnSOD in response to similar stresses in other species.

Phylogenetic analysis

To examine the molecular phylogeny of *Es-MnSOD*, 53 *MnSOD* orthologs from different taxa of arthropods were aligned and a phylogenetic tree was constructed using the N-J method (Table 1). The mitochondrial and cytosolic branches split and formed 2 major groups. All of the mMnSODs were clustered together into a subgroup, and the cMnSODs were clustered into another group, which was consistent with the belief of a common origin for these 2 SODs with different subcellular compartmentalizations. The *Es-MnSOD* was placed within the cMnSOD monophyletic clade, close to its ortholog from *Cardisoma armatum* (Figure 3), indicating that the SOD obtained from *E. sinensis* should be classified as a cMnSOD (designated as *Es-cMnSOD*).

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1          acgcgggacaactgccagc
21 atggcggatgcgaaagaagcgtatattagcattctggaaaaaaa
   M A D A K E A Y I S I L E K K   15
66 ctggcggaaactaccggcattgaagtggatcagattaaaaaaac
   L A E L T G I E V D Q I K K N   30
111 cagctggcgaacgcgcggtgaagcgcgtgattcgtgaaatg
   Q L A N A A D E A R V I R E M   45
156 gcgagctatgtgaagcatttggtgcagcaggcggcgctggcg
   A S Y V E G I V V Q Q A G V A   60
201 caggcggcctggtgagcccgagattgcggcattgttagccat
   Q A G L V S P Q I A A M F S H   75
246 attaacgggaactggcgaaagcgtggcgcgcattgcctgccc
   I N A E L G E A R G A H A L P   90
291 ccgctgaaatgatgatgatgcctggaaccgatattagcggc
   P L K Y D Y D A L E P H I S G   105
336 atgattatggaattcatcacaacaaatcatcaggcctatatt
   M I M E I H H T K H H Q G Y I   120
381 aacaacctgattgcgccgacaaaaaactggtggaagcgggaagc
   N N L I A A T K K L V E A E A   135
426 gcgaacgatgtggcgcgatgaacgcctgctccggcgattaaa
   A N D V G A M N A L L P A I K   150
471 aaatttaacggcgccatctgaaccataccatttttgaccaa
   K F N G G H L N H T I F W T N   165
516 atggcggcgatgcgggcggaaccgagcggcgcggtggcgat
   M A P D A G G E P S G A V A D   180
561 gcgattaacgaaagccttggcagctttcagagccttaagataaa
   A I N E S F G S F Q S F K D K   195
606 tttagcggcgagcgtggcgctgaaagcagcggctggcgctgg
   F S A A S V G V K G S G W G W   210
651 attggctattgccccaaaaacgataaactggcattgacacctgc
   I G Y C P K N D K L A I A T C   225
696 cagaaccaggatccgctgcagctgaccatggcctggtgcccgtg
   Q N Q D P L Q L T H G L V P L   240
741 ctggcctggatgtgtgggaacatcgcttattatctgcagcgtaaa
   L G L D V W E H A Y Y L Q R K   255
786 aacctgcgtgaggattatgtgaaagcgtttttaacgtgattaac
   N L R A D Y V K A F F N V I N   270
831 tggagcaacgtgaacgaacttatgaaacgcgctgtaaacgc
   W S N V N E R Y E N A R R K A   285
876 gcgggccattgactcgcacgggtcccccaaccaccggcccgcctc
   A G H *   288
921 tagcctccacttgctaactgcttgccagctgctgctacatagtc
966 ttgtactttttgtgtgtgtgtgtaggttctgtancctcct
1011 cctttgtcattgactgaagtagtttgtgtttgtttctgttctc
1056 acttagttaaagatcagaaggttaataatacttgatttagtcagt
1101 gtggttctaaggattggacttggttaacagaccttcaataattt
1146 gtgagtaagaaagaactgcttgggatgtagctccttgcattggtt
1191 tggcagcctgcctcaagagccttcacatattctgctattggtt
1236 gactcatatccatattccggtgtccatgttatacagaagaatgat
1281 cagaagtcaaataataactatttgaaacctcaaaaaaaaaa
1326 aaaaaaaaaaaaaa

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Figure 1. Complete complementary DNA (cDNA) and deduced amino acid sequences of *Eriocheir sinensis* cytosolic manganese superoxide dismutase (Es-cMnSOD). Nucleotides are numbered from the first base at the 5'-end. Amino acids are numbered from the initiating methionine start codon. The general MnSOD domain is underlined. The putative MnSOD signature sequences (DVWEHAYY) are boxed. Putative manganese binding sites (i.e., H111, H159, D244, and H248) are double underlined. The asterisk (*) indicates the stop codon. The polyadenylation signal (aataaa) is in bold font and underlined. The full sequence was submitted to GenBank under accession No. FJ617306.

in the mRNA expression profile following stimulation. Temporal expression of the *Es-cMnSOD* gene after bacterial challenge was up-regulated at 1.5 h after bacterial injection. Then, a step-by-step recovery at 24 h was conducted; at 48 h after bacterial injection, the expression level of the *Es-cMnSOD* gene was similar to that observed at 1.5 h after bacterial injection.

Table 1. Accession number, scientific name, and source of MnSOD amino acid sequences used to construct the phylogenetic tree.

Accession No.	Scientific name	Type of MnSOD
DQ298208.1	<i>Litopenaeus vannamei</i>	cMnSOD
AF264030.1	<i>Callinectes sapidus</i>	cMnSOD
AF264029.1	<i>Callinectes sapidus</i>	mMnSOD
KC461130.1	<i>Penaes monodon</i>	mMnSOD
JQ040506.1	<i>Cherax quadricarinatus</i>	cMnSOD
HQ852225.1	<i>Macrobrachium nipponense</i>	mMnSOD
HQ852226.1	<i>Macrobrachium nipponense</i>	cMnSOD
FM242573.1	<i>Carcinus maenas</i>	cMnSOD
EF427949.1	<i>Fenneropenaeus chinensis</i>	cMnSOD
FJ031018.1	<i>Portunus trituberculatus</i>	cMnSOD
GU213434.1	<i>Scylla paramamosain</i>	cMnSOD
FJ605170.2	<i>Scylla paramamosain</i>	mMnSOD
EU077526.1	<i>Macrobrachium rosenbergii</i>	cMnSOD
EU077525.1	<i>Macrobrachium rosenbergii</i>	mMnSOD
EU254488.3	<i>Procambarus clarkii</i>	cMnSOD
DQ205424.1	<i>Fenneropenaeus chinensis</i>	mMnSOD
GQ403706.1	<i>Eriocheir sinensis</i>	cMnSOD
JQ763321.1	<i>Cherax quadricarinatus</i>	mMnSOD
FJ892724.1	<i>Procambarus clarkii</i>	mMnSOD
HQ848280.1	<i>Tigriopus japonicus</i>	mMnSOD
FM213481.1	<i>Perisesarma bidens</i>	mMnSOD
FM213479.1	<i>Carcinus maenas</i>	mMnSOD
FM213477.1	<i>Cardisoma armatum</i>	mMnSOD
FM213475.1	<i>Segonzacia mesatlantica</i>	mMnSOD
FM213473.1	<i>Bythograea therymydron</i>	mMnSOD
FM213471.1	<i>Atelecyclus undecimdentatus</i>	mMnSOD
FM213469.1	<i>Necora puber</i>	mMnSOD
FM213480.1	<i>Cardisoma armatum</i>	mMnSOD
FM213478.1	<i>Perisesarma bidens</i>	mMnSOD
FM213476.1	<i>Xantho poressa</i>	mMnSOD
FM213474.1	<i>Cyanagraea praedator</i>	mMnSOD
FM213472.1	<i>Dromia personata</i>	mMnSOD
FM213470.1	<i>Cancer pagurus</i>	mMnSOD
AY211085.1	<i>Farfantepenaeus aztecus</i>	cMnSOD
AY211086.1	<i>Farfantepenaeus aztecus</i>	mMnSOD
AY211084.1	<i>Palaemonetes pugio</i>	cMnSOD
AY726542.1	<i>Penaes monodon</i>	cMnSOD
FJ892725.1	<i>Uca pugnax</i>	mMnSOD
FM242571.1	<i>Cardisoma armatum</i>	cMnSOD
FM242569.1	<i>Segonzacia mesatlantica</i>	cMnSOD
FM242567.1	<i>Bythograea therymydron</i>	cMnSOD
FM242565.1	<i>Atelecyclus undecimdentatus</i>	cMnSOD
FM242563.1	<i>Necora puber</i>	cMnSOD
FM242572.1	<i>Perisesarma bidens</i>	cMnSOD
FM242570.1	<i>Xantho poressa</i>	cMnSOD
FM242568.1	<i>Cyanagraea praedator</i>	cMnSOD
FM242566.1	<i>Dromia personata</i>	cMnSOD
FM242564.1	<i>Cancer pagurus</i>	cMnSOD
DQ029053.1	<i>Litopenaeus vannamei</i>	cMnSOD
GQ181123.1	<i>Marsupenaeus japonicus</i>	cMnSOD
GQ478988.1	<i>Marsupenaeus japonicus</i>	mMnSOD
HM371157.1	<i>Litopenaeus vannamei</i>	Cu-Zn MnSOD
NM_001043619.1	<i>Bombyx mori</i>	Cu-Zn MnSOD

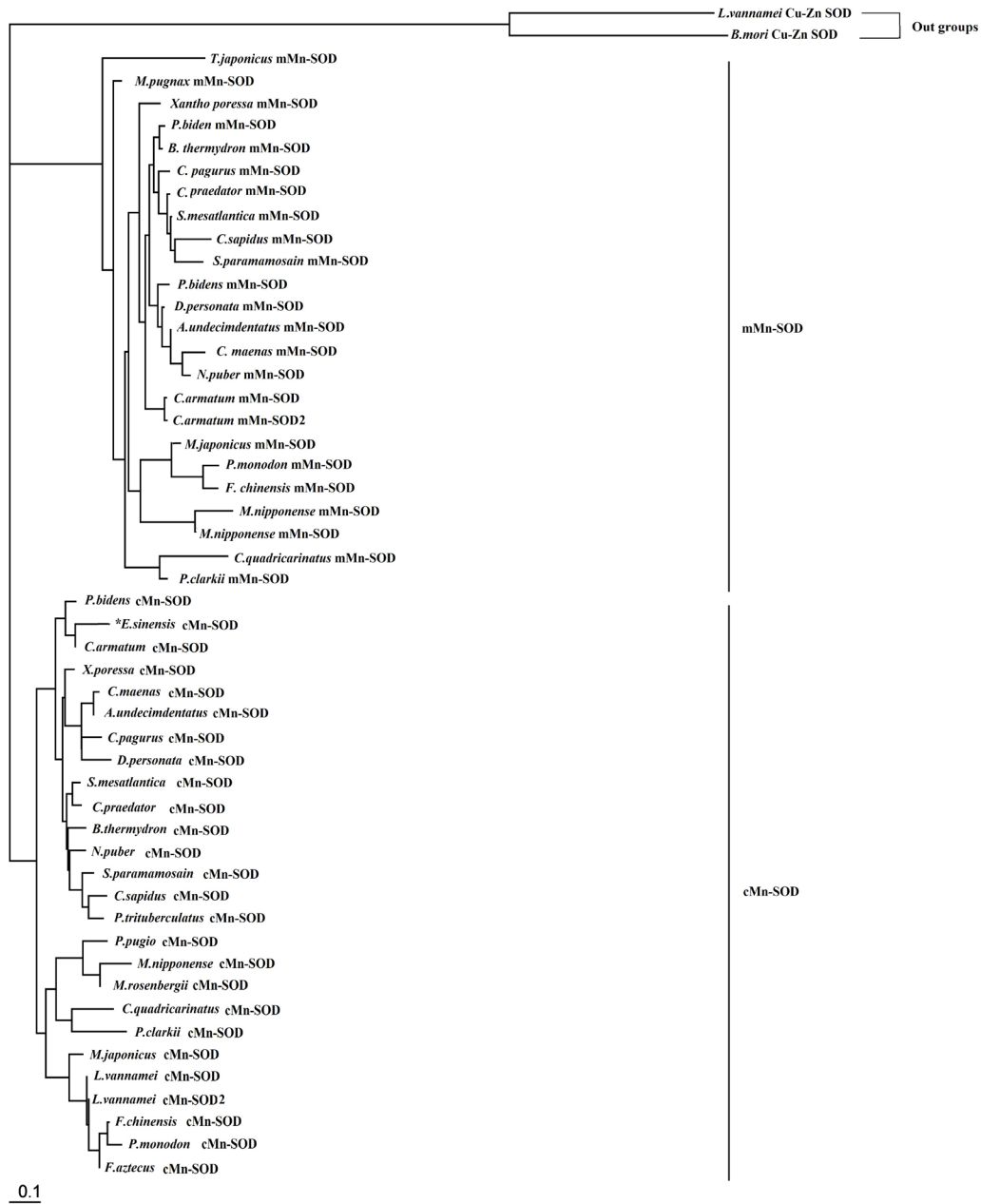


Figure 3. Neighbor-joining phylogenetic tree of Es-MnSOD from different species. The abbreviations of these proteins and their GenBank accession numbers used to construct the phylogenetic tree are given in Table 1. The Es-MnSOD is indicated with an asterisk (*).

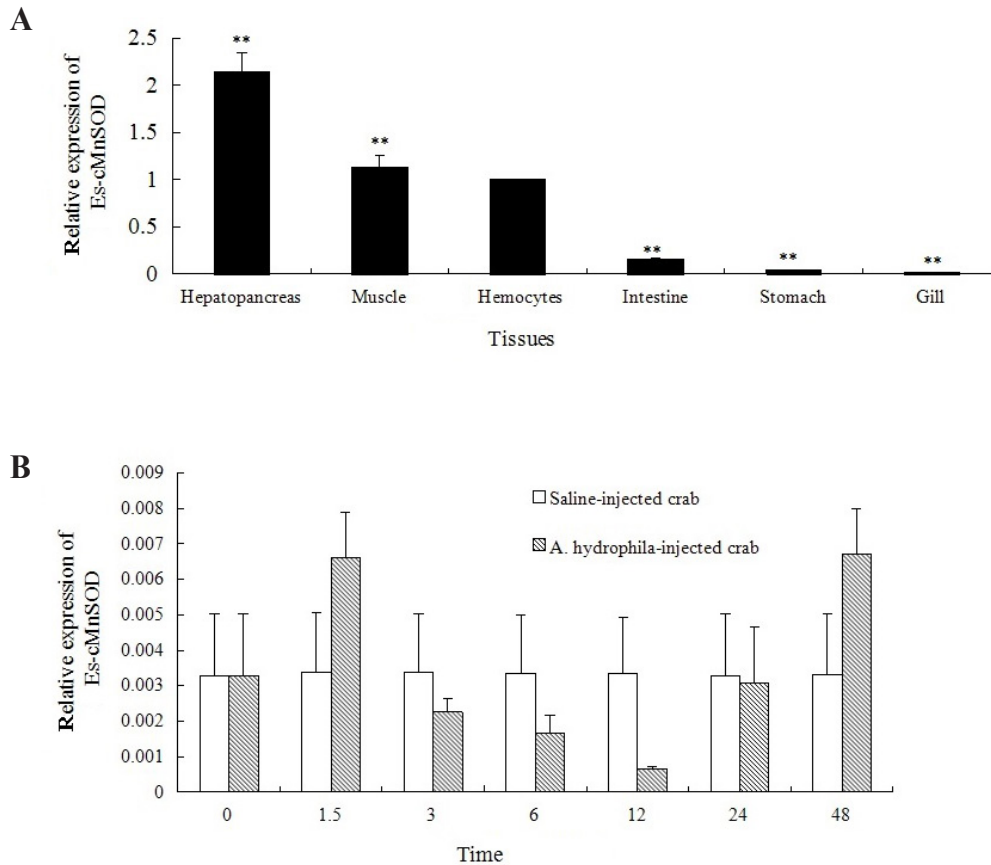


Figure 4. Expression and induction of Es-cMnSOD messenger RNA (mRNA). **A.** Tissue distribution of the Es-cMnSOD transcript measured by the SYBR-Green-based quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The tissues, including hepatopancreas, hemocytes, gill, stomach, muscle, and intestine, were collected from the crab. All values are relative to Es-cMnSOD expression in hemocytes, which was arbitrarily set to 1 after normalization by β -actin expression. ** $P < 0.01$ for statistical significance differences of Es-cMnSOD expression when compared to that in hemocytes. **B.** Temporal expression profile of the Es-cMnSOD transcript in hemocytes after *Aeromonas hydrophila* challenge as measured by SYBR-Green-based qRT-PCR. Comparisons of the Es-cMnSOD mRNA levels (relative to β -actin mRNA) among different times were performed by the *t*-test. Values are reported as means \pm SE (N = 3).

DISCUSSION

SODs play an important role in antioxidant defense pathways, and the MnSODs comprise a major type of SOD for crustaceans. There are 2 types of MnSODs (i.e., cMnSOD and mMnSOD). In this study, BLAST and multiple-alignment analyses demonstrated that the MnSOD isolated from *E. sinensis* shared high similarity with the cMnSOD in other crustacean species and was clustered into a subgroup of the cMnSODs according to the N-J-phylogenetic analysis, which indicates that the MnSOD cloned from the Chinese mitten crab is an MnSOD. We designated this gene as *Es-cMnSOD*.

The full-length of *Es-cMnSOD* was cloned based on the EST obtained in the study. In this study, both *Es-cMnSOD* and other arthropod *cMnSODs* showed several common features, including an MnSOD domain (DVWEHAYY) and 4 putative manganese binding sites (H, H, D, and H). An arginine (R258) located at the seventh amino acids after the MnSOD domain was present. This R residue near the MnSOD domain was considered necessary for the structural stability of MnSOD. Among the known MnSODs, most species have an R residue in this position and only a few possess the K residue. The position replaced by the K residue reduces MnSOD activity (Gabbianelli et al., 1997). Furthermore, a polyadenylation signal (aataaa), which is known to be involved in the regulation of mRNA stability, was observed in the 3'-UTR (Malter and Hong, 1991). The conservation sequence and motif indicate the same function with that of other organisms.

In this study, the *Es-cMnSOD* was ubiquitous in all tissues examined, including the hepatopancreas, gill, muscle, hemocytes, stomach, and intestine. This finding is not surprising since the expression of *MnSOD* in a wide range of cell types has been reported previously in the white shrimp *L. vannamei* (Gómez-Anduro et al., 2006), giant freshwater prawn *M. rosenbergii* (Cheng et al., 2006), red swamp crayfish *P. clarkia* (Zhu and Doumen, 2009) and the swimming crab *Portunus trituberculatus* (Li et al., 2010).

MnSOD has been considered a stress-responsive factor and its expression at the transcriptional and translational levels might be influenced by a variety of intracellular and environmental cues (Fukuhara et al., 2002). This study investigated whether acute bacterial challenge could affect the expression of *cMnSOD* in the Chinese mitten crab. In a bacterial challenged crab, up-regulation of *Es-cMnSOD* gene expression from the infected crab at 1.5 h post-challenge showed that the gene might be involved in temporal expression because of the invasion of heterogeneous bacteria. The significant increase in the transcript level of SOD after bacterial invasion suggests that the gene transcriptions are inducible. At 48 h post-injection, the transcript level in the challenged crab was higher than that in the control, suggesting that during the phagocytosis on bacteria via crab immune cells, the level of ROS increases and more SODs are needed to offset the effects of ROS. In previous study, the Chinese shrimp *F. chinensis* MnSOD was activated in the hemocytes and hepatopancreas when cells were infected with the white spot syndrome virus (Zhang et al., 2007), while the *Crassostrea hongkongensis* SOD could be induced after infection by *Vibrio alginolyticus* (Yu et al., 2011). Therefore, the change in transcript levels of *Es-cMnSOD* implies that this gene may play an important role in the immune responses against infection.

In conclusion, *cMnSOD* cDNA was cloned for the first time from the hemocytes of *E. sinensis*. The sequence analysis makes it possible to visualize the specific responses of the SODs to various stress factors such as bacterial infection. The transcriptional quantification of *Es-cMnSOD* provides valuable and reliable information for measuring oxidative-stress-induced regulation in the crab during bacterial infection. Further study is suggested to focus on the immune regulation of *Es-cMnSOD* under different protein levels.

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