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RESEARCH REPORT

Comparative study of two novel extracellular copper/zinc superoxide dismutase (Cu-Zn SOD) genes from white shrimp *Litopenaeus vannamei*

J Sun[1], Y Wang[1,3], F Hu[1], J-J Hu[1,2,3], M-Q Wang[1,2,3*]

[1]MOE Key Laboratory of Marine Genetics and Breeding (Qingdao 266003), and Key Laboratory of Tropical Aquatic Germplasm of Hainan Province of Sanya Oceanographic Institute (Sanya 572024), Ocean University of China, China
[2]Laboratory for Marine Fisheries Science and Food Production Processes, and Center for Marine Molecular Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China
[3]Hainan Yazhou Bay Seed Laboratory, Sanya 572024, China

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Abstract

Reactive oxygen species (ROS) is a product of normal metabolism of aerobic cells, but excess ROS is harmful to the organism. Copper/zinc superoxide dismutase (Cu-Zn SOD) is a metalloenzyme for scavenging ROS. In this study, two extracellular Cu-Zn SOD genes (designated as LvEcSOD1 and LvEcSOD2) were cloned by rapid amplification of cDNA ends (RACE) technique. The cDNA length of LvEcSOD1 is 801 bp, with an open reading frame (ORF) of 555 bp, encoding a peptide of 184 amino acids. The cDNA length of LvEcSOD2 is 934 bp with an ORF of 678 bp, encoding a peptide of 225 amino acids. The predicted amino acid sequences of the two LvEcSOD both contained conserved four Cu^{2+} binding sites and four Zn^{2+} binding sites. The mRNA scripts of LvEcSOD1 and LvEcSOD2 were widely detectable in the eyestalk, gill, gonad, heart, hemocytes, hepatopancreas, intestine, muscle, nerve, and stomach of *Litopenaeus vannamei*. Both LvEcSOD1 and LvEcSOD2 exhibited the highest expression levels in hemocytes and hepatopancreas. After the white shrimp was stimulated by white spot syndrome virus (WSSV) or *Vibrio parahaemolyticus*, the mRNA expression levels of these two genes were up-regulated to varying degrees. The relative expression level of LvEcSOD2 was significantly increased after stimulation by two pathogenic microorganisms, which was generally later than that of LvEcSOD1. These results indicated that the two genes are both involved in the innate immunity of *L. vannamei* with different functions.

Key Words: *Litopenaeus vannamei*; extracellular Cu-Zn SOD; innate immunity

Introduction

Reactive oxygen species (ROS) are produced by aerobic organisms in the process of cellular oxygen metabolism, which includes superoxide anion (O^{-}_{2}), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) (Nosaka and Nosaka, 2017). Previous studies have shown that ROS were involved in various cell signaling pathways and played an important role in the elimination of harmful pathogens (Zelko *et al.*, 2002; Yang *et al.*, 2019). However, overproduction of ROS, also known as oxidative stress, may lead to damage to cell structure, such as DNAs, proteins and lipids (Lushchak, 2014). To keep ROS at a normal concentration, aerobic organisms have evolved an antioxidant enzyme defense system composed of various enzymes, such as superoxide dismutase (SOD, EC: 1.15.1.1), catalase, glutathione peroxidase and so on. Among them, SOD is an important enzyme for eliminating ROS by converting superoxide radicals into hydrogen peroxide and oxygen (Sheng *et al.*, 2014).

Based on the metal co-factor, SODs in eukaryotes could be classified into three isoforms: the cytosolic copper-zinc dimeric form, known as SOD1 or cytCuZnSOD; the mitochondrial tetrameric manganese superoxide dismutase, as SOD2 or MnSOD; and the extracellular tetrameric Cu-Zn superoxide dismutase, as SOD3 or EC-SOD (Mondola *et al.*, 2016). Among them, the two forms of Cu-Zn SOD have high sequence and structural similarity to each other. EC-SOD with an

Corresponding author:
Meng-Qiang Wang
MOE Key Laboratory of Marine Genetics and Breeding (Qingdao 266003), and Key Laboratory of Tropical Aquatic Germplasm of Hainan Province of Sanya Oceanographic Institute (Sanya 572024)
Ocean University of China, China
E-mail: wangmengqiang@ouc.edu.cn

N-terminal signal cleavage peptide for secretion which directs this enzyme exclusively to extracellular spaces is found in the extracellular matrix of tissues and the nucleus of human cells (Lin *et al.*, 2008). While cytCuZnSOD without a signal peptide is mainly found in the intracellular space, and it was also noted to be in the intermembrane space of mitochondria and in nuclei (Okado-Matsumoto and Fridovich, 2001; Zelko *et al.*, 2002).

EC-SOD was first detected in human lymph, plasma, ascites, and cerebrospinal fluids (Marklund *et al.*, 1982). The expression pattern of EC-SOD varies widely in different cell types and tissues, and its activity may exceed that of cytCuZnSOD and MnSOD in some cells (Zelko *et al.*, 2002). Subsequently, EC-SOD gene has been reported in many species such as *Hydra vulgaris* (Dash *et al.*, 2007)*, Procambarus clarkia* (Meng *et al.*, 2013), *Argopecten irradians* (Bao *et al.*, 2009), *Phaedon cochleariae* (Gretscher *et al.*, 2016), *Tribolium castaneum* (Ferro *et al.*, 2017) *and Scylla serrata* (Lin *et al.*, 2008). Previous studies have shown that the relative mRNA expression level of EC-SOD in the hemocytes, hepatopancreas, and gills of *Procambarus clarkii* significantly increased after being challenged by pathogenic microorganisms, suggesting that EC-SOD plays an important role in the innate immune response of *P. clarkia* (Meng *et al.*, 2013). However, information on EC-SOD from marine animals is still rare and more research is needed to demonstrate its potential roles.

The Pacific white shrimp (*Litopenaeus vannamei*) is a worldwide farmed aquaculture species. The annual production of the Pacific white shrimp reaches 4 million tons and accounted for 70% of the world's total shrimp production (Shen *et al.*, 2022; Yin *et al.*, 2023). However, with the expansion of aquaculture scale and the continuous increase of aquaculture intensity, the ecological environment of aquaculture is also deteriorating. The diseases of shrimp are becoming more and more serious and have caused great economic losses. White spot syndrome virus (WSSV) and *Vibrio parahaemolyticus* have been reported to be the main pathogenic bacteria causing large mortalities of shrimp and the shrimp farming industry has lost billions of dollars due to these two pathogens (Zhang *et al.*, 2022; Zhou *et al.*, 2022). As for invertebrate, the innate immune system is almost the only important defense line for shrimp against pathogenic microorganisms (Han *et al.*, 2020). As an enzyme related to the innate immune system in many invertebrates (Lin *et al.*, 2008; Bao *et al.*, 2009; Ferro *et al.*, 2017), EC-SOD in the Pacific white shrimp is still insufficient in research. In this study, we cloned two EC-SOD genes from *L. vannamei*, examine the mRNA expression of the two EC-SODs in various tissues and investigated their expression profiles upon stimulation by the microorganisms WSSV and *V. parahaemolyticus*, which would provide new insight into the function on this important, widespread and functionally diverse enzyme.

Materials and Methods

Shrimp culture, tissues sample collection and RNA isolation

About 480 white shrimps with body weight 8-12 g, were obtained from Ruizi Seafood Development Co. Ltd., Qingdao, China, and acclimated at 20 ± 1 °C in 640 L cylindrical tanks with 500 L air-pumped circulating seawater (salinity 30 ‰) for two weeks before processing. Tissues, including eyestalk, gill, gonad, heart, hemocytes, hepatopancreas, intestine (mid gut), muscle, nerve and stomach, were collected from at least fifteen shrimps and each sample was a mixture from three individuals. Hemolymph was extracted from the abdominal sinuses of shrimps using a sterile syringe with equal volume of anticoagulation buffer (NaCl 510 mmol L^{-1}, glucose 100 mmol L^{-1}, citric acid 200 mmol L^{-1}, tri-sodium citrate 30 mmol L^{-1} and EDTA·2Na 10 mmol L^{-1}, pH 7.3), and then hemocytes were collected using centrifugation at 800 g for 10 min at 4 °C.

WSSV and V. parahaemolyticus *challenge and sample collection*

WSSV and *V. parahaemolyticus* were prepared according to the previous reports (Yi *et al.*, 2014). 480 shrimps were divided into three groups and each group of shrimps was cultivated in separate tanks. Different groups of shrimps were injected with 100 μL of phosphate buffered saline (PBS, pH 7.4, 10010023, Thermo Fisher Scientific, USA), *V. parahaemolyticus* suspension (1×10^4 CFUs μL^{-1}, in PBS) or WSSV stock (1×10^4 copies μL^{-1}, in PBS). At each time point of 0h, 3 h, 6 h, 12 h, 1 d, 2 d, 3 d, 4 d and 5 d post injection, hepatopancreas and hemocytes samples were extracted from fifteen shrimps and each sample was a mixture from three individuals. All samples were immediately stored in RNA*later* and stored at -80 °C until RNA isolation.

Cloning the full-length cDNA of LvEcSOD1 and LvEcSOD2

Total RNA was extracted from different samples using TRIzol reagent (15596026, Thermo Fisher Scientific, USA). The synthesis of first strand was conducted by the Promega M-MLV using the DNase I (RQ1, M6101, Promega, USA) treated total RNA as template and adaptor primer-oligo (dT) as primer (Table 1). Partial length sequences of LvEcSOD1 and LvEcSOD2 cDNAs was obtained from a transcriptome database of Pacific white shrimp (Qi *et al.*, 2017; Zhao *et al.*, 2017). All primers were designed using Primer Premier 5.00 and all PCR amplification was performed in a MiniAmp Thermal Cycler (Thermo Fisher Scientific, USA). The 3' end of LvEcSOD1 and LvEcSOD2 cDNAs were obtained using rapid-amplification of cDNA ends (RACE) technique. Gene-specific primers, LvEcSOD1-RACE-F1/2 and LvEcSOD2-RACE-F1/2 (Table 1), and adaptor primer-oligo (dT) were used in the semi-nested PCR for cloning the 3' end of LvEcSOD1 and LvEcSOD2. The coding sequence (CDS) of LvEcSOD1 and LvEcSOD2 were amplified by

Table 1 Primer sequences used in this study

Name	Sequence (5'-3')	Tm (°C)	Brief information
adaptor primer	GGCCACGCGTCGACTAGTAC	60	Anchor primer for 3` RACE
adaptor primer-oligo (dT)	GGCCACGCGTCGACTAGTACT_{17}VN	-	Olido (dT) for cDNA synthetize
LvEcSOD1-RACE-F1	TATCATTTTAGACACGACTGCCATTGT	64	Gene specific primer for 3` RACE
LvEcSOD1-RACE-F2	TATCAGTGACAGGCAGCACCTTGCTGC	72	Gene specific primer for 3` RACE
LvEcSOD2-RACE-F1	GTCATAGGTCACGCTTGAGGGATGTCA	70	Gene specific primer for 3` RACE
LvEcSOD2-RACE-F2	TGACCTGTAATGAAGAACGAAACAAACGAC	69	Gene specific primer for 3` RACE
LvEcSOD1-CDS-F	ATGATGTTGGCTGGACTCCTGTGCCTCTCA	76	Gene specific primer for CDS
LvEcSOD1-CDS-R	TTAATAGTATTTTGTTTGTGTGTATCGCTGGGCTTG	73	Gene specific primer for CDS
LvEcSOD2-CDS-F	ATGGGACTGATCACACCGTTGCTA	65	Gene specific primer for CDS
LvEcSOD2-CDS-R	TCAAGCGTGACCTATGACCCCACA	68	Gene specific primer for CDS
LvEF-1α-qRT-F	GTATTGGAACAGTGCCCGT	60	Internal control for real-time PCR
LvEF-1α-qRT-R	CATCTCCACAGACTTTACCTCAG	60	Internal control for real-time PCR
LvEcSOD1-qRT-F	CGGACACTTCAACCCTCTC	55	Gene specific primer for real-time PCR
LvEcSOD1-qRT-R	GAATAAGGAGATGCGAGCCA	58	Gene specific primer for real-time PCR
LvEcSOD2-qRT-F	GGCAACGACGAGAGTTTGAAGAC	63	Gene specific primer for real-time PCR
LvEcSOD2-qRT-R	TCACATTTGACCTCTGACATCCCTC	64	Gene specific primer for real-time PCR
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	56	Vector primer for sequencing
RV-M	GAGCGGATAACAATTTCACACAGG	56	Vector primer for sequencing

gene-specific primers, LvEcSOD1-CDS-F/R and LvEcSOD2-CDS-F/R (Table 1). The PCR products were gel-purified, ligated into the pMD18-T simple vector (D103A, Takara, Japan), and transformed into the competent *Escherichia coli* DH5α (CB101-03, Tiangen, China). Positive clone was identified via anti-ampicillin selection and verified by PCR screening using M13-47 and RV-M primers (Table 1) and then sequenced using a PRISM 3730XL automated sequencer (Thermo Fisher Scientific, USA).

Bioinformatical analysis

Similarities to other EC-SOD nucleotide and protein sequences was identified by the BLAST web server with default settings (http://www.ncbi.nlm.nih.gov/Blast). The function domains of LvEcSOD1 and LvEcSOD2 were predicted by the SMART program (http://smart.embl-heidelberg.de/) using default pattern definitions. The search for signal peptide was carried out by SignalP 5.0 Server (https://services.healthtech.dtu.dk/service.php?SignalP-5.0/). The prediction of the potential N-glycosylation sites was performed by NetNGlyc 1.0 Server (https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0/). Euk-mPLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/) was employed to predict the subcellular localization of proteins. Based on the selected Cu-Zn SOD sequences (Table 2), MEGA 7.0.21 was used for multiple sequence alignment and construction of neighbor-joining (NJ) phylogenetic trees, in which bootstrap trials were replicated 1000 times.

Expression pattern analysis by quantitative real-time PCR

Quantitative real-time PCR (qPCR) technique was used to investigate the mRNA expression patterns of LvEcSOD1 and LvEcSOD2 in different tissues and after stimulated with various microbes. All the primers for qPCR were designed using PerlPrimer 1.1.21 and listed in Table 1. The mRNA expression levels of LvEcSOD1 and LvEcSOD2 were normalized to those of elongation factor 1α (EF-1α) and processed using comparative CT method ($2^{-\Delta\Delta Ct}$ method) (Schmittgen and Livak, 2008; Wang *et al.*, 2011). The data were subjected to one-way analysis of variance (ANOVA) followed by a multiple comparison using IBM SPSS Statistics 26.0.0.0, and the *p* values less than 0.05 were considered statistically significant.

Results

Sequence analysis of LvEcSOD1 and LvEcSOD2 cDNAs

The full-length cDNA sequences of two Cu-Zn SODs were obtained by 3' RACE technique and submitted to the GenBank database under the accession number MF318887 and MF318886. The nucleotide and predicted amino acid sequences of LvEcSOD1 and LvEcSOD2 cDNAs are shown in Figure 1. LvEcSOD1 comprised 801 bp, containing a 5' untranslated regions (UTR) of 42 bp, a 3' UTR of 204 bp with a poly A tail and an open reading frame (ORF) of 555 bp. Its ORF encodes a 184

Table 2 Sequences used in the alignment and phylogenetic tree

Accession no.	Species name	Type of CuZnSOD	Abbreviation
BAP28202	*Penaeus japonicus*	Extracellular	PjEcSOD
EFA10685	*Tribolium castaneum*	Extracellular	TcEcSOD
ABC25025	*Hydra vulgaris*	Extracellular	HvEcSOD
ACG80589	*Argopecten irradians*	Extracellular	AiEcSOD
AGH30392	*Procambarus clarkia*	Extracellular	PcEcSOD
NP_035565	*Mus musculus*	Extracellular	MmEcSOD
NP_001106630	*Xenopus tropicalis*	Extracellular	XtEcSOD
NP_037012	*Rattus norvegicus*	Extracellular	RnEcSOD
2JLP	*Homo sapiens*	Extracellular	HsEcSOD
QOE76459	*Rimicaris exoculate*	Extracellular	ReEcSOD
XP_001332758	*Danio rerio*	Extracellular	DrEcSOD
XP_015141186	*Gallus gallus*	Extracellular	GgEcSOD
NP_000445	*Homo sapiens*	Cytoplasmic	HsIcSOD
NP_777040	*Bos taurus*	Cytoplasmic	BtIcSOD
NP_571369	*Danio rerio*	Cytoplasmic	DrIcSOD
AAW59361	*Salmo salar*	Cytoplasmic	SsIcSOD
ACI28282	*Cristaria plicata*	Cytoplasmic	CpIcSOD
ABD58974	*Azumapecten farreri*	Cytoplasmic	AfIcSOD
ACM48346	*Argopecten irradians*	Cytoplasmic	AiIcSOD
NP_476735	*Drosophila melanogaster*	Cytoplasmic	DmIcSOD
AAP93581	*Apis mellifera ligustica*	Cytoplasmic	AmIcSOD

amino acid sequence with a calculated molecular mass of approximately 19.39 kDa and theoretical isoelectric point (pI) of 7.72. Four Zn^{2+} binding sites (H-82, H-90, H-99 and D-102) and four Cu^{2+} binding sites (H-65, H-67, H-82 and H-141) were found in the amino acid sequence. The SMART program revealed that the LvEcSOD1 possessed a conserved Cu-Zn SOD domain at position 45-164. The full-length cDNA of the LvEcSOD2 was 934bp, containing a 5' UTR of 48bp, a 3' UTR of 208 bp with a poly A tail and a 678bp ORF encoding a 225 amino acid sequence with a predicted molecular mass of approximately 23.24 kDa and theoretical pI of 6.22. The LvEcSOD2 protein possessed two potential N-linked glycosylation sites (N110LSP and N171ITD). Four Zn^{2+} binding sites (H-136, H-144, H-153 and D-156) and four Cu^{2+} binding sites (H-119, H-121, H-136 and H-193) were found in the amino acid sequence. SignalP and TargetP predicted that LvECSOD1 contained a putative 17 amino acid signal peptide and LvEcSOD2 contained a putative 21 amino acid signal peptide. These results combined with the prediction of Euk-mPLoc 2.0 indicated that these SODs are two extracellular enzymes.

Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment revealed that LvEcSOD1 and LvEcSOD2 share high similarity with previously identified EC-SODs and are conserved in Zn^{2+} and Cu^{2+} binding sites (Fig. 2). In order to clarify the evolutionary positions of LvEcSOD1 and LvEcSOD2, a phylogenetic tree was constructed based on the amino acid sequences of EC-SOD and cytCuZnSOD from different species using the neighbor-joining method (Fig. 3). These results showed that EC-SOD of different species, including LvEcSOD1 and LvEcSOD2, were clustered together, and cytCuZnSOD was clustered in other groups. In the subgroup of EC-SOD, LvEcSOD1 was clustered with EC-SOD from the kuruma shrimp *Penaeus japonicus* and LvEcSOD2 was clustered with EC-SOD from the hydrothermal vent shrimp *Rimicaris exoculata*.

Tissue distribution of SOD expression

As shown in Figure 4, LvEcSOD1 and LvEcSOD2 was widely detectable in the eyestalk, gill, gonad, heart, hemocytes, hepatopancreas, intestine, muscle, nerve and stomach of *L. vannamei*. The highest mRNA transcripts level of LvEcSOD1 was detected in hemocytes, which was 410.9-fold of that in muscle ($p < 0.05$), followed by hepatopancreas, which was 46.72-fold of that in muscle ($p < 0.05$). The highest expression of the LvEcSOD2 was detected in hepatopancreas, which was 1026.39-fold of that in muscle ($p < 0.05$), then in hemocytes, which was 72.39-fold of that in muscle ($p < 0.05$).

A

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1          AATCACTGGCTGGTCGCCGCACGAGGAGGAGAAAACACGACGATGATGTTGGCTGGACTC
1                                                    M  M  L  A  G  L
61         CTGTGCCTCTCAGCGGCTTGCCTTGTCGCAACCACAGGCCCTGACGCGGTGGTGGACATC
7           L  C  L  S  A  A  C  L  V  A  T  T  G  P  D  A  V  V  D  I
121        GTGCCAGGCTCCAACCCCAACATCAGAGGCGCGCTTTACCTGTACAGACGTCGCAGTGGA
27          V  P  G  S  N  P  N  I  R  G  A  L  Y  L  Y  R  R  R  S  G
181        GGTGTCGACATCCATGGTACGGTTGGCGGTCTGAAGCCGGGGCTGCACGGGTTCCACGTG
47          G  V  D  I  H  G  T  V  G  G  L  K  P  G  L  H  G  F  H  V
241        CACGCGGAGGGGAACCTGGGCGACTCGTGCAAGGCGGCAGGCGGACACTTCAACCCTCTC
67          H  A  E  G  N  L  G  D  S  C  K  A  A  G  G  H  F  N  P  L
301        ATGAAAAACCACGGCAGCCCCTTGGACTTCCACCGCCACGCCGGCGACTTGGGCAACGTC
87          M  K  N  H  G  S  P  L  D  F  H  R  H  A  G  D  L  G  N  V
361        ATCGCCGACTACAACGGCGTGGCTCGCATCTCCTTATTCGACAGGCACATTTCCCTGGAC
107         I  A  D  Y  N  G  V  A  R  I  S  L  F  D  R  H  I  S  L  D
421        TGGAACTCTCCGGTATACATCGGCGGGCTCGCCTTCGTCATCCACGCCGGCGAGGACGAC
127         W  N  S  P  V  Y  I  G  G  L  A  F  V  I  H  A  G  E  D  D
481        CTGGGGCGCGGCGGGGACGCCGAGAGCCTCAAGACGGGCAACGCTGGCGGCCGCGAAGGG
147         L  G  R  G  G  D  A  E  S  L  K  T  G  N  A  G  G  R  E  G
541        TACGGCATCGTCCGCGTGGCGCAAGCCCAGCGATACACACAAACAAAATACTATTAAATG
167         Y  G  I  V  R  V  A  Q  A  Q  R  Y  T  Q  T  K  Y  Y
601        GATCTCTTTTTATTTACGTAACTCCATGTATCATTTTAGACACGACTGCCATTGTTATAG
661        TAATTATTAAGGGCTATATCAGTGACAGGCAGCACCTTGCTGCCGATTATTCAAAGGCAT
721        TATCATACGCATATCAGTGACAGGCAGCACCTTGCTGCCGATTATTCAAAGGCATTATCA
781        TACGAAAAAAAAAAAAAAAA
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B

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1          CCCGGTTTCAGGCAGTCTGTCGATGTCCTTGGTAGGAACACCATCACCATGGGACTGATC
1                                                          M  G  L  I
61         ACACCGTTGCTAGCGTGGGCGTTGTTGGGCTTAGCCGTGGGCGCCGCAGCAGAACCCGCC
5           T  P  L  L  A  W  A  L  L  G  L  A  V  G  A  A  A  E  P  A
121        GGGCGCCATGTCGTGTACCTGAGCCAGAACAACTACCCGTCGCTGCTGTACATCAACTCC
25          G  R  H  V  V  Y  L  S  Q  N  N  Y  P  S  L  L  Y  I  N  S
181        GCCCATGGAACCGCCTCTATGGACCACCGGGCTGACACCTCTGACATCATACAGCTGATT
45          A  H  G  T  A  S  M  D  H  R  A  D  T  S  D  I  I  Q  L  I
241        CTTCATCCGTCGAATCCGGAAGCCAAACAGACGCGCCGCGCTGCCGTCGTGCTCACAGGG
65          L  H  P  S  N  P  E  A  K  Q  T  R  R  A  A  V  V  L  T  G
301        GAGGCCCAAGGAACACTAACCCTAACGCAGAGCAACCCTCCTGTAGGACCCACTGTCATC
85          E  A  Q  G  T  L  T  L  T  Q  S  N  P  P  V  G  P  T  V  I
361        GAGGGCGTGATCTCTAACCTCTCCCCGGGACTGCATGGCTTTCACATCCACCAGCTGGGC
105         E  G  V  I  S  N  L  S  P  G  L  H  G  F  H  I  H  Q  L  G
421        GACCTGACCGGAGGATGCGTGTCCGCCGGGGGCCATTACAATCCGTATATGCGCCCCCAC
125         D  L  T  G  G  C  V  S  A  G  G  H  Y  N  P  Y  M  R  P  H
481        GGCTCCCCCGAGGACCGCGAGAGACACGTCGGTGACCTCGGCAACATCCTGGCTGACGCG
145         G  S  P  E  D  R  E  R  H  V  G  D  L  G  N  I  L  A  D  A
541        ACCGGCCGGGCAGAGGTCAACATCACTGACCCCCTGGTGACCTTAGTGGGACCTCGTACG
165         T  G  R  A  E  V  N  I  T  D  P  L  V  T  L  V  G  P  R  T
601        GTGCTGGGTCGTGCGGTCGTCGTCCACGCGGGTGAGGACGACCTCGGCGACGGAGGCAAC
185         V  L  G  R  A  V  V  V  H  A  G  E  D  D  L  G  D  G  G  N
661        GACGAGAGTTTGAAGACTGGCAACGCTGGCGGTCGGGTGGCGTGTGGGGTCATAGGTCAC
205         D  E  S  L  K  T  G  N  A  G  G  R  V  A  C  G  V  I  G  H
721        GCTTGAGGGATGTCAGAGGTCAAATGTGACGGTGTATGTTTTTTTAAAATTAATTTTCAT
225         A
781        CTTTACATGACCTGTAATGAAGAACGAAACAAACGACTTGCAGAAACAATACAATTGAGA
841        TTTTTTTTTAAAAACTAAAACTCGAATTTTCTCATAATATGTTCTGGAAATGAATACAAA
901        AAAGTCTCTGGAGATCCAAAAAAAAAAAAAAAAA
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Fig. 1 Nucleotide and deduced amino acid sequences of LvEcSOD1 (A) and LvEcSOD2 (B), the translational start codon and stop codon are bolded. The signal peptide is underlined and the italic and bold letters represent the amino acids required for Cu2+ and Zn2+ binding. The Cu-Zn SOD family signature sequence is boxed and the predicted N-link glycosylation sites are double underlined

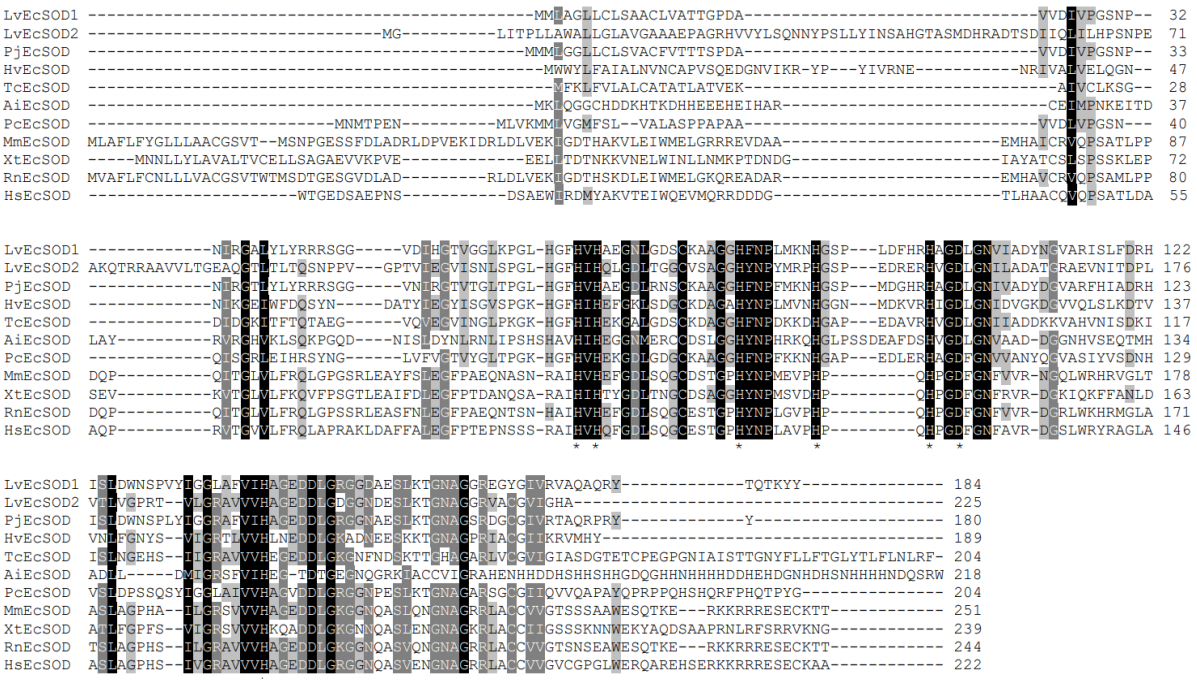

Fig. 2 Multiple sequence alignment of EC-SOD proteins with those from other animals. Taxa information are shown in Table 2. The same amino acid residues are shaded in black and similar amino acids are shaded in grey. Zn^{2+} and Cu^{2+} binding site are marked by (*)

LvEcSOD1 and LvEcSOD2 mRNA expression analysis post WSSV and V. parahaemolyticus *stimulation*

The mRNA expression levels of LvEcSOD1 and LvEcSOD2 mRNA were investigated in hemocytes and hepatopancreas after stimulation with two pathogenic microorganisms. After being stimulated by *V. parahaemolyticus*, the mRNA expression levels of LvEcSOD1 and LvEcSOD2 in hemocytes were significantly up-regulated after 3h and 6h (8.28-fold and 20.70-fold compared with time 0 of the control group, $p < 0.05$), and reached the highest level at 6h and 12h (38.09-fold and 168.53-fold, $p < 0.05$). In hepatopancreas, the mRNA transcripts of LvEcSOD1 were significantly up-regulated and reached the highest level after 3h (7.82-fold, $p < 0.05$), while the mRNA transcripts of LvEcSOD2 was significantly up-regulated after 12h (14.83-fold, $p < 0.05$) and at 1d reached to the peak (63.87-fold, $p < 0.05$). After being stimulated by WSSV, the mRNA expression levels of LvEcSOD1 in both hemocytes and hepatopancreas were significantly up-regulated after 6h (5.14-fold and 18.38-fold, $p < 0.05$), and reached the highest at 12h (17.95-fold and 59.99-fold, $p < 0.05$). As for LvEcSOD2, the mRNA expression levels in hemocytes and hepatopancreas were significantly up-regulated and reached to the peak at 1d and 2d (11.91-fold and 5.11-fold, $p < 0.05$). The transcripts of these two EC-SOD mRNAs dropped back to the original level in all tissues after 3d. No significant change in the control group was observed throughout the experiment (Fig. 5).

Discussion

SOD enzymes play an important role in both controlling ROS damage and regulating ROS signaling in aerobic organisms (Wang *et al*., 2018). In this study, we characterized and compared two Cu-Zn SOD genes in an important aquaculture species, *L. vannamei*, and then analyzed their mRNA expression profiles of them. Based on the signal peptide prediction by signalP and TargetP software and subcellular localization prediction by Euk-mPLoc 2.0, the two SOD enzymes were determined to be two extracellular enzymes. These two genes all have the conserved Cu^{2+} binding sites and Zn^{2+} binding sites, indicating that they have Cu-Zn SOD family characteristics. High similarity with other identified EC-SOD and the phylogenetic relationship collectively suggested that LvEcSOD1 and LvEcSOD2 were two novel members of invertebrate Cu-Zn SOD family, and it could have similar functions to those from other invertebrates. The LvEcSOD1 and LvEcSOD2 exhibited only 37.94 % and 33.36 % similar to a previously studied LvECSOD, which indicates the diversity of EC-SOD genes in *L. vannamei* (Tian *et al.*, 2011). Two EC-SOD genes were also identified in *Marsupenaeus japonicus* and they have different functions against pathogenic microorganisms WSSV and *V. parahaemolyticus* challenge (Hung *et al.*, 2014). We deduced that the redundancy of gene products for EC-SODs in *L. vannamei* might have contributed to the functional specialization in the innate immunity system, which could increase their survival during microbial infections.

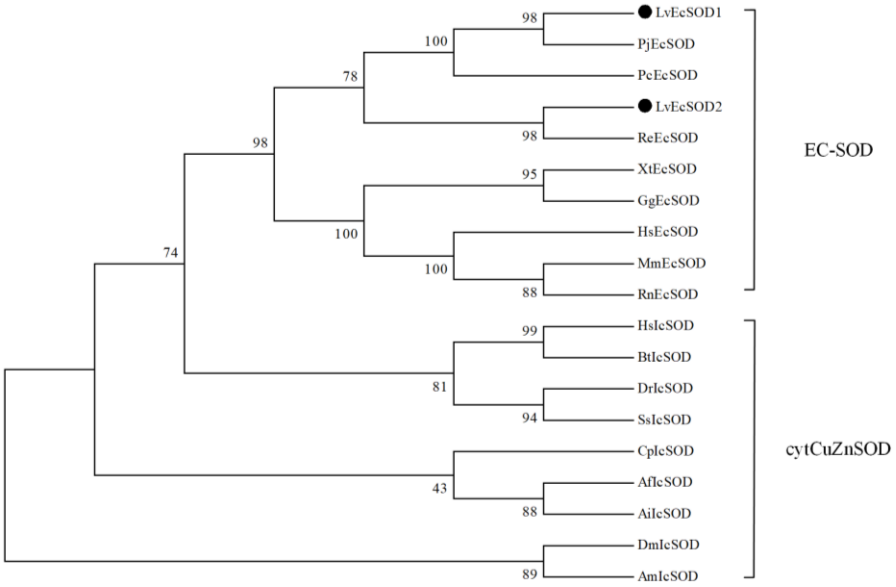

Fig. 3 Consensus neighbor-joining phylogenetic based on the protein sequences of EC-SOD and cytCuZnSOD from different animals. Taxa information are shown in Table 2. To derive the confidence value for the phylogeny analysis, bootstrap trials were conducted 1000 replicates. The numbers at the forks indicate the bootstrap value

The results of qPCR showed that LvEcSOD1 and LvEcSOD2 were expressed in various tissues of shrimps, and the highest relative expression levels were observed in hemocytes and hepatopancreas, which was significantly different from other tissues. Similarly, in *P. clarkii*, EC-SOD was expressed highest in hepatopancreas and hemocytes (Meng *et al.*, 2013). Previous studies have shown that hepatopancreas and hemocytes are considered to be the main immune organ and immune cells in crustaceans (Wang *et al.*, 2013; Wang *et al.*, 2017). Moreover, the hepatopancreas was also regarded as the main organ in which multiple oxidative reactions and antioxidant defenses occur with high metabolic activity (Wang *et al.*, 2015). The higher mRNA expression level of LvEcSOD1 and LvEcSOD2 in hemocytes and hepatopancreas implied that they might play important roles in the innate immunity and detoxification system in Pacific white shrimps. Additionally, the basal mRNA expression level of LvEcSOD1 in hemocytes was higher than that of LvEcSOD2 by approximately five times, which indicated that LvEcSOD1 might play a more routine role in the physiological activity of Pacific white shrimps.

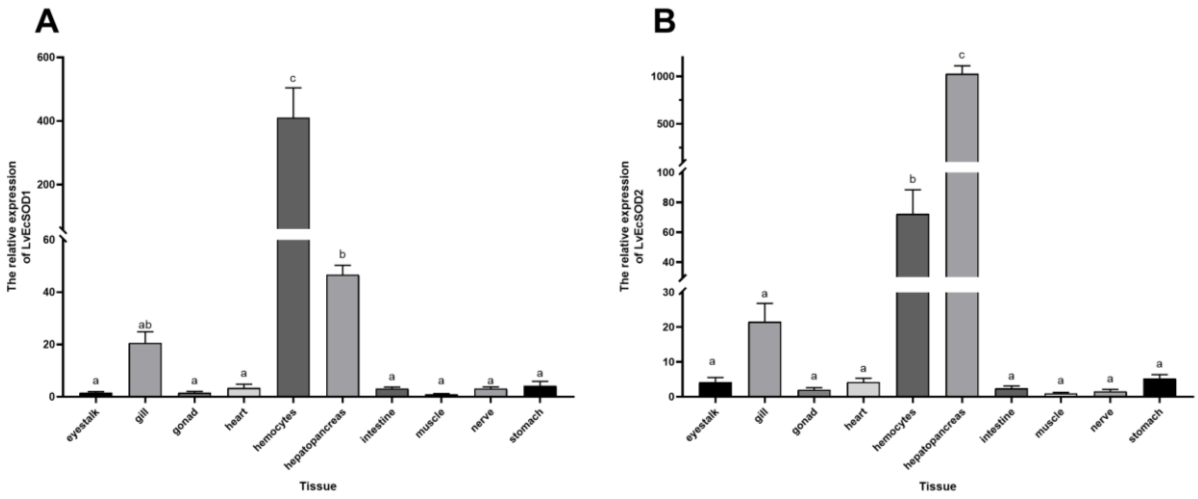

Fig. 4 Tissue distribution of LvEcSOD1 (A) and LvEcSOD2 (B) mRNA transcripts detected by qPCR technique. The mRNA transcripts levels in eyestalk, gill, gonad, heart, hemocytes, hepatopancreas, intestine, muscle, nerve and stomach of three untreated shrimps were normalized to that of muscle. The EF-1α gene was used as an internal control to calibrate the cDNA template for each sample. Vertical bars represent mean ± SD (n = 3), and bars with different characters were significantly different ($p < 0.05$), while bars with same characters were not significantly different

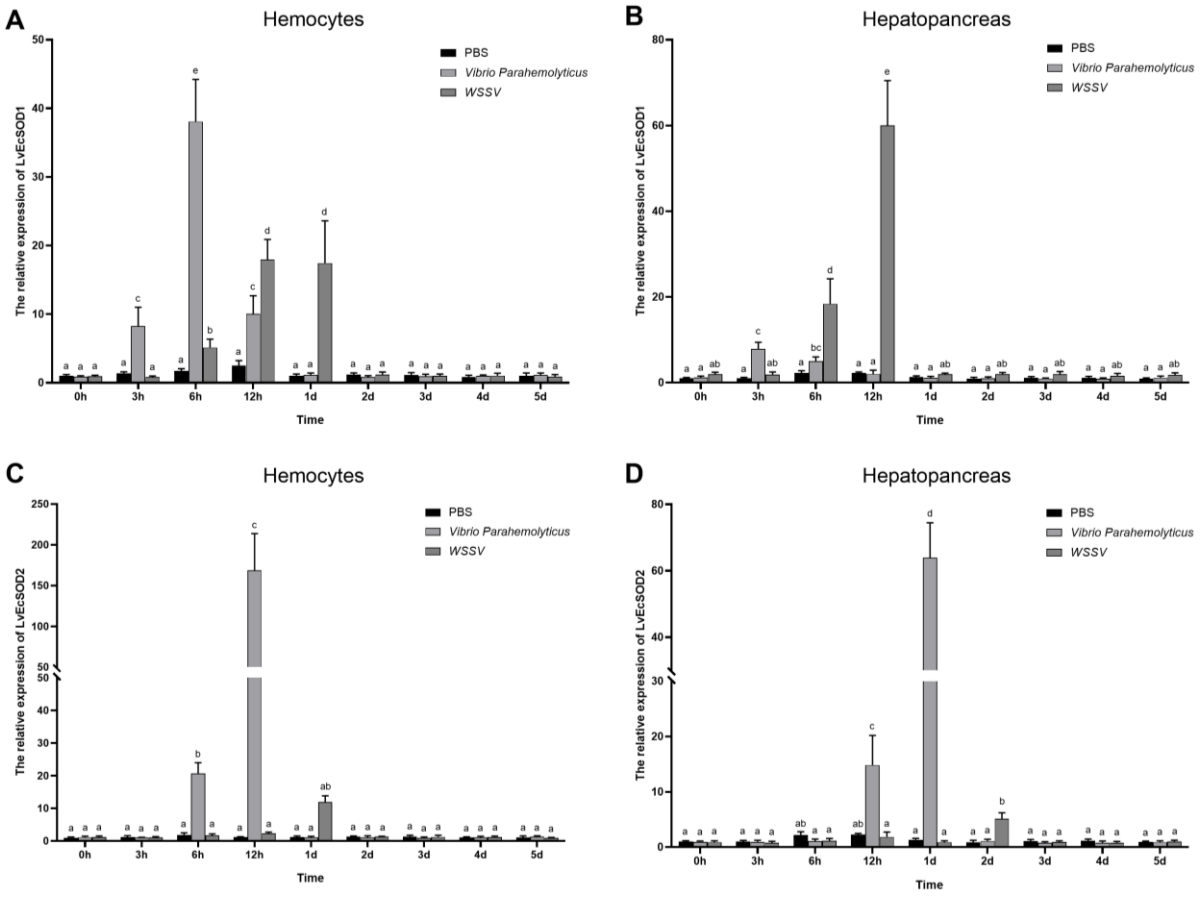

Fig. 5 Temporal mRNA expression profiles of LvEcSOD1 in hemocytes (A) and hepatopancreas (B), and LvEcSOD2 in hemocytes (C) and hepatopancreas (D), which were detected via qPCR technique at 0h, 3h, 6h, 12h, 1d, 2d, 3d, 4d and 5d post white spot syndrome virus (WSSV) and *Vibrio parahaemolyticus* stimulation. The EF-1α gene was used as an internal control to calibrate the cDNA template for each sample. Vertical bars represent mean ± SD (n = 3), and bars with different characters were significantly different ($p < 0.05$), while bars with same characters were not significantly different

It has been reported that *V. parahaemolyticus* and WSSV could activate the shrimp's antioxidant defense mechanisms, which include SOD scavenging of reactive oxygen species (Ji *et al.*, 2011). The depletion of SOD makes the host express more SOD to compensate, so the relative expression of mRNA will increase significantly (Jiravanichpaisal *et al.*, 2006). In the present study, both two EC-SOD gene expressions were significantly up-regulated after shrimp stimulation with *V. parahaemolyticus* and WSSV pathogens. After stimulated by WSSV, the expressions of both two genes started to be up-regulated were later than stimulated by *V. parahaemolyticus*. Different invading microorganisms induce different toxicity and produce different amounts of oxygen-derived products in the host. In *Chlamys farreri*, the temporal expression patterns of cytCuZnSOD challenged by *Listonella anguillarum*, *Micrococcus luteus* and *Candida lipolytica* were different (Ni *et al.*, 2007). The relative expression of LvEcSOD2 was significantly increased after stimulation by two pathogenic microorganisms, which was generally later than that of LvEcSOD1, which shows that the two different EC-SODs might have specific functions in shrimp innate immunity. ROS have the effect of killing pathogenic microorganisms in the phagosome and play an important role of immune signaling pathway in innate immune system (O'Neill *et al.*, 2015; Martinvalet and Walch, 2021). Therefore, a small amount of ROS may be beneficial for the innate immunity of *L. vannamei*, appropriately increase the concentration of ROS to allow them to survive the attack of pathogens, and then secrete SOD to remove ROS when the concentration of ROS is too high, which might be the reason for the slower response of LvEcSOD2. The role of SOD's can be viewed as regulators of ROS, the difference in expression of two EC-SOD genes in different tissues or in response to different pathogenic stimuli reflects the precise control of ROS by SOD. Our research further verified that two LvEcSODs participated in the immune response as a part of response against the pathogen.

In conclusion, we cloned and characterized the full-length cDNAs of LvEcSOD1 and LvEcSOD2 and investigated their expression profiles under the stimulation by pathogenic microorganisms WSSV or *V. parahaemolyticus*. Two LvEcSODs were found to be widespread in tissues, can be induced after WSSV or *V. parahaemolyticus* stimulation, and involved in innate immunity in shrimp.

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