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A superoxide dismutase (MnSOD) with identification and functional characterization from the freshwater mussel *Cristaria plicata*

Wanying Yang, Wenxiu Liu, Chungen Wen*, Baoqing Hu**, Shaoqing Jian, Yang Gang

College of Life Science, Education Ministry Key Laboratory of Poyang Lake Environment and Resource Utilization, Nanchang University, Nanchang, 330031, China

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ABSTRACT

Manganese superoxide dismutase (MnSOD) is a sort of important metalloenzyme that can catalyze ROS in the organisms. In this study, MnSOD cDNA of *C. plicata*, designated as CpMnSOD (accession no. MK465057), was cloned from hemocytes. The full-length cDNA of MnSOD was 1096 bp with a 672 bp open reading frame encoding 223 amino acids. The deduced amino acid sequence contained a mitochondrial-targeting sequence (MTS) of 18 amino acids in the N-terminus, and four conserved amino acids for manganese binding (H^{49}, H^{97}, D^{182}, H^{186}). CpMnSOD showed a high level (65–73%) of sequence similarity to MnSODs from other species. The results of Real-time quantitative PCR revealed that CpMnSOD mRNA constitutively expressed in tissues. The highest expression level was in hepatopancreas, followed by muscle, mantle and gill, and the lowest expression level was in hemocytes. After microcystin challenge, the expression levels of CpMnSOD mRNA were up-regulated in hemocytes and hepatopancreas. The cDNA of CpMnSOD was cloned into the plasmid pColdI-ZZ, and the recombinant protein was expressed in *Escherichia coli* BL21 (DE3). The enzyme stability assay showed that the purified CpMnSOD protein maintained more than 80% enzyme activity at temperature up to 70 °C, at pH 2.0–10.0, and resistant to 8 mol/L urea or 8% SDS.

1. Introduction

Cellular antioxidant defense systems are categorized to primary, including antioxidant enzymes and small antioxidant molecules, and secondary defense systems, including proteolytic and lipolytic enzymes and the DNA repair systems [1]. When aquatic animals are under stressful environmental conditions, such as hyperthermia, hypoxia, low salinity, and toxin invasion and pathogen infection, as a part of their integrated stress response, which generate elevated levels of reactive oxygen species (ROS), reactive oxygen intermediate (ROI) and free radicals, and subsequently undergo oxidative stress [2,3]. To limit the harmful effect of ROS production and prevent damage from oxidative stress, the cells evolve to use antioxidant systems to maintain ROS at basal levels [4].

One of the most effective enzymatic antioxidants is superoxide dismutase (SOD) (EC 1.15.1.1) that catalyzes the dismutation of $O_2^{\bullet -}$ to O_2 and to the less-reactive species H_2O_2 [5]. SODs are originally found in prokaryotic organisms, which play importance role in protecting organisms against virus [6,7], bacteria [8,9], parasites [8], physical and chemical challenges [10,11]. They are classified into four groups of iron SOD (FeSOD), manganese SOD (MnSOD), copper-zinc SOD (CuZnSOD), and nickel SOD (NiSOD).

Two types of MnSOD, known as cytosolic and mitochondrial MnSOD, are reported, the former lacks a mitochondrial transit peptide and is retained in the cytosol, and the latter is transported to mitochondria with the help of mitochondrial transit peptide after translation [12]. Mitochondrial MnSOD is encoded by the nuclear gene, and is synthesized and translocated into the mitochondrial matrix with mature enzyme activity [13]. The mitochondrial matrix is the major site to produce cellular energy, which is also a major source for the single-electron reduction of O_2 to produce. O_2^-, is served as a common stress-responsive element of defense system, and is modulated by various factors including environmental change, chemical pollutants (heavy metal), biological stimuli (pathogens) and several drugs [14–16]. MnSOD of human can be induced by mediators of oxidant stress, including tumor necrosis factor, interleukin 1, and lipopolysaccharide, and the overexpression of MnSOD alters the phenotype of cultured cancer cells [17]. MnSODs are identified and cloned in several Seawater molluscs, such as *Argopecten irradians* (ACU00737), *Tegillarca granosa* (ADC34695), *Chlamys farreri* (AFN29183.1), *Patinopecten yesoensis* (AHX22598.1).

Microcystins (MCs) are cyclic hepatotoxic heptapeptides produced

* Corresponding author.Tel.: +86 791 83969534; fax: +86 791 83969530.
** Corresponding author.
E-mail addresses: cgwen@ncu.edu.cn (C. Wen), baoqinghu@sina.com (B. Hu).

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by certain strains of freshwater microcystis, which not only cause damage to aquatic organisms, but also can accumulate in the bodies of organisms [18,19]. MCs exerts toxicity by inhibition of serine/threonine protein phosphatases (PP) 1 and 2A, and induction of oxidative stress in the hepatocyte ultrastructure via cytoskeletal dysfunction [20,21]. Besides inhibition of PPs, oxidative stress may play an important biochemical mechanism of MCs toxicity in both animal and plant cells [22]. The concentration of MCs is the highest in the hepatopancreas followed by gills and muscle in *Unio douglasiae*, and microcystin RR is also detected in *Anadonta woodiana* [23].

The freshwater mussel *Cristaria plicata*, which is of great economic importance, is well known as one of "freshwater pearl bivalve" in the aquaculture industry of China. However, the farming of freshwater pearl has been suffering serious problems due to the outbreak of mussel diseases in the cultivation process [24]. Thus, it is crucial for diseases management and development of sustainable mussel culture and pearl production to research the immunity of freshwater mussel.

The main objectives of the present study were to: 1) to clone the full-length CpMnSOD cDNA from the freshwater mussel *C. plicata* and to compare its deduced amino acid sequence with known MnSODs from other organisms, 2) to screen for regulatory elements in 5′- flanking sequence of CpMnSOD. 3) to examine the tissues expression profiles of CpMnSOD and the dynamic change after challenged by Microcystins, 4) to express recombinant CpMnSOD in *Escherichia coli* and purify the recombinant, 5) to detect the enzyme activity of CpMnSOD in vitro.

2. Materials and methods

2.1. Collection and maintenance of mussels

The bivalve *C. plicata*, averaging 18–25 cm in shell length, collected from Poyang Lake in Jiangxi province, China, was maintained at 25 ± 2 °C in freshwater tanks with continuous oxygenation, changing water every day for one week before processing.

2.2. RNA extraction and cloning the full-length cDNA of CpMnSOD

Total RNA was extracted from hepatopancreas and haemocytes using Trizol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. The extracted RNA was treated with RQ1 RNase-free DNase I (Promega, Madison, WI) to remove any possible contaminating DNA. Smart cDNA was synthesized from total RNA by using SMAR™ cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA). Based on the highly conserved sequences from *Haliotis discus discus* (ABF67504) and *Tegillarca granosa* (ADC34695.1), two degenerated primers CpMnSOD-F1 and CpMnSOD-R1 (Table 1) were designed to obtain the mid-fragment of CpMnSOD from *C. plicata.* The 5′-end and 3′-end of CpMnSOD were cloned by using SMART-RACE technique. Gene-specific primers CpMnSOD-F2, CpMnSOD-F3, CpMnSOD-R2 and CpMnSOD-R3 were designed from the partial sequence of CpMnSOD cDNA (Table 1). All of PCR products were sequenced, and then the mid-fragment, 5′-end and 3′-end of CpMnSOD were spliced to get the full-length cDNA.

Table 1
The primers used in this study.

Primer	Usage	Sequence (5′- 3′)
CpMnSOD-F1	Initial PCR	CTAGTTGGCCATTTTAGCAGCATGA
CpMnSOD-R1	Initial PCR	ATGGCTGTACGAATGAAACACACC
CpMnSOD-F2	3′-RACE	CACTGAAGTTTAATGGAGGTGGT
CpMnSOD-R2	5′-RACE	TCTGTTGATCAATTCATTTTTCATG
CpMnSOD-F3	3′-RACE	GATCAACAGATCTGTGGCAATTC
CpMnSOD-R3	5′-RACE	GAGTGATTAATATGACCACCTCCAT
CpMnSOD-F4	Real-time PCR	AGTTGGAGCATTGAGGAGAG
CpMnSOD-R4	Real-time PCR	CCGCAGAGATGTAGGGTT
B-actinF	Real-time PCR	AAGGTTACGCCCTTCCTCAT
B-actinR	Real-time PCR	GCCATTTCCTGCTCAAAGTC
CpMnSOD-F5	Expression	CGGAATTCATGGCTGTACGAATGAAACAC
CpMnSOD-R5	Expression	CCGACGTCGACCTAGTTGGCCATTTTAGCAG

2.3. Genome walking of CpMnSOD

CpMnSOD promoter was cloned using Genome walking. Total DNA was extracted from *Cristaria plicata* by Animals DNA Kit (Sangon, Shanghai City, China). The qualified CpMnSOD DNA was digested with HindIII and purified. The digested products were then ligated to the Genome Walker™ Adaptors, and the ligated products were used as templates. Two reverse complementary primers were designed near the 5′-end of CpMnSOD genomic sequence and were paired with the primers AP1 and AP2, respectively. The two-step cycle program was executed and was detailed in the Genome Walking™ Universal Kit (CLONTECH) specification. The primers of CpMnSOD-AP1, CpMnSOD-AP2, CpMnSOD-GW-R6 and CpMnSOD-GW-R7 were designed (Table 1). The PCR products were separated by 1.2% agarose gel electrophoresis, and then the band of the desired size was excised and was purified using a DNA Gel Extraction Kit (Sangon). Finally, the purified DNA fragments were cloned into the pMD18-T vector (TaKaRa) and were sequenced.

2.4. Sequence analysis of CpMnSOD

CpMnSOD gene sequence was analyzed by using the BLAST algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov/blast), and the deduced amino acid sequence, the cellular localization prediction, signal peptide, structure domain analysis and open reading frame (ORF) were analyzed with the Expert Protein Analysis System (http://www.expasy.org/). AliBaba 2.1 software (http://www.generegulation.com/pub/programs/alibaba2/) was used to predict putative transcriptional factor binding sites. The protein sequences were aligned by the ClustalW multiple sequence alignment program (version 1.8). The molecular mass was calculated, and the theoretical isoelectric points were predicted by Protein MolWt & AA Composition Calculator (http://www.proteomics.com.cn/proteomics/pi_tool.asp). The phylogenetic tree was constructed from the deduced amino acid sequences using the Neighbor-Joining (NJ) algorithm within MEGA version 4.1.

2.5. Tissue distribution and temporal expression of CpMnSOD after microcystin (MC) challenge

The tissue-specific expression of CpMnSOD was detected in tissues of normal mussels, including hemocytes, mantle, hepatopancreas, gill and muscle from five individuals. Forty mussels were selected to measure the temporal expression of CpMnSOD, which were randomly divided into control and challenged groups in the same tank, and each group included 20 mussels in per tank. The adductor muscle of individual mussel in control or challenged groups was injected with 0.2 mL MC (Solarbio, 1.0 mg/mL), or 0.2 mL 0.8% normal saline (NS), respectively. The hemocytes and hepatopancreas were obtained separately from four mussels at 3, 6, 12, 24 and 48 h post-injection of each group, and were immediately stored in liquid nitrogen until used. Total RNA extraction, cDNA synthesis was performed as described above.

2.6. Real-time quantitative PCR

The transcripts of CpMnSOD in different tissues were determined by RT-qPCR. The primers of CpMnSOD -F4 and CpMnSOD -R4, and of β-actin-F and β-actin-R (Table 1) were designed to amplify specifically partial cDNA sequence of the CpMnSOD and the internal reference gene Cp-β-actin, respectively. RT-qPCR was performed in a total volume of 25 μL containing 10 μL of 2 × SYBR Green Real-time PCR Master Mix (TaKaRa, DRR041A), 1 μL of cDNA, 1 μL of each primer and 12 μL of PCR-grade water, were conducted on an Eppendorf Mastercycler ep

Real-plex 2 PCR system. Triplicate reactions were performed for each sample. The synthesis reaction was performed at 94 °C denaturations for 5 min, 40 cycles for 94 °C 30 s, 57 °C for 30 s, 72 °C for 15 s, and finally 72 °C extensions for 3 min. Fluorescence readings were performed at the end of each cycle. The result was calculated by using the $2^{-\triangle\triangle CT}$ method. All group data were given in terms of relative mRNA expressed as the mean (n = 4) ± SD, and then subject to Student's *t*-test.

2.7. PCR amplification of CpMnSOD coding sequence and construction of the prokaryotic expression vector

The ORF sequence of CpMnSOD was amplified by a pair specific primer CpMnSOD-F5 and CpMnSOD-R5 with the corresponding restriction enzyme sites of *EcoR* I and *Sal* I (Table 1). The PCR program was performed at 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 60 s, with an additional extension step at 72 °C for 10 min. The amplicon was first cloned into the pMD18-T vector, and the sequence was verified by DNA sequencing. The prokaryotic expression plasmid pColdI -ZZ (pColdI vector adds solubilized tag ZZ sequence) was digested with *EcoR* I and *Sal* I and then ligated with the same digested and recovered CpMnSOD gene from recombinant T vector that was designated to pcoldI-ZZ-CpMnSOD.

2.8. Prokaryotic expression of recombinant pColdI-ZZ-CpMnSOD

The recombinant expression plasmid pColdI-ZZ-CpMnSOD was utilized to transform *E. coli* BL21 (DE3) competent cells that were cultivated at 37 °C, 200 r/min in 10 mL LB medium containing 100 mg/mL ampicillin. When OD_{600} reached about 0.4, final concentrations of 0.5 mM isopropy β-D-1-thiogalactopyranoside (IPTG) was added to the culture that was induced at 18 °C, 200 r/min. 1 mL culture was removed at 0, 2, 4, 6, 8 h respectively. A blank group was cultured at 0 h, and induced groups were done at other time buckets. The cells were harvested by centrifugation at 12000*g* for 5 min at 4 °C. The recombinant expression of the target protein was determined by 15% SDS-PAGE assay.

2.9. Purification of soluble protein

In order to obtain more fusion protein, the culture volume was increased to 300 mL. The transformed *E. coli* was incubated at 18 °C,220 rpm for 8 h. After being induced, the cultures were collected by centrifugation at 4 °C, the harvested cells were pelleted by centrifugation at 4 °C, which were resuspended in 30 mL 1 × TBS buffer (20 mM Tris, pH 8.0, 150 mM NaCl), and then were sonicated on ice using a probe sonicator. The supernatants of sonicates were collected and loaded to 4 mL chealting Sepharose FastFlow column (Amersham Biosciences, GE Healthcare, Uppsala, Sweden). The column was washed with 50 mL TBS buffer containing 20 mM imidazole, and the target protein was eluted with TBS buffer containing 250 mM imidazole. After the elutions were mixed and dialyzed against the stepwise decrease of imidazole concentration in TBS buffer overnight at 4 °C, the purity of the recombinant protein was analyzed by 15% SDS-PAGE assay, and protein concentration was measured by Bradford protein assay (Bio-Rad, Hercules, CA).

2.10. Analysis of thermostability of recombinant CpMnSOD

The enzyme activity of the purified soluble CpMnSOD protein was measured by SOD assay kit (Nanjing Jiancheng Institute of Biological Engineering, China) according to manufacturer's instructions, based on SOD ability to inhibit the oxidation of hydroxylamine by the xanthine-xanthine oxidase system. The reaction was terminated and the absorbance at 450 nm was recorded for the calculation of SOD activity. Under the assay conditions used, 1 unit (U) of SOD activity was calculated as that inhibiting 50% of the oxidation of hydroxylamine without an enzyme source. SOD activity was expressed as U/mg with the following formula: SOD activity 1/4 (A_0 -A_1) D/(A_0 × 50% × C), in which A0 and A1 are the optical density at 450 nm for blank group or sample group, respectively, D is the dilution rate, and C is the protein concentration of purified SOD at mg/mL.

2.11. Effects of temperature, pH and denaturant on CpMnSOD activity

In the thermal stability assay, the enzyme samples were incubated for 20 min at 10, 20, 30, 40, 50, 60, 70, 80 and 90 °C in 1 × PBS buffer, and then immediately transferred and kept on ice for the determination of residual enzymatic activity. In the pH stability assay, the enzyme samples were incubated in 50 mM buffer at different pH values for 20 min at 40 °C, 0.2 M citrate buffer (pH 1.0, 2.0, 3.0, 4.0 and 5.0), 0.2 M Trise-HCl buffer (pH 6.0, 7.0, 8.0 and 9.0), 0.2 M glycine/NaOH buffer (pH 10.0 and 11.0). In the denaturant effect assay, the enzyme samples were added with SDS to the final concentration of 1–10%, or with urea to the final concentration of 2–8 M, and then incubated at 40 °C for 1 h. The maximum of enzyme activity was defined as 100% in each assay, and the standard curve was drawn based on the percentage that experiment groups account for the control group (residual activity).

2.12. Data processing and statistical analysis

All assays were executed in triplicate, and were repeated at least twice. The statistical analysis was examined with SPSS Statistics 13.0 software, and the data were calculated as the mean ± S.D. Differences were considered to be statistically significant when P values were lower than 0.05.

3. Result

3.1. The full-length cDNA of CpMnSOD

The full-length sequence of CpMnSOD cDNA was 1096 bp, and contained a 672 bp open reading frame (ORF) that encoded 223 amino acids. The ORF was flanked by 100 and 324 bp of 5′ and 3′-untranslated regions, respectively. A poly (A) tail was stretched at the 3′-end of the cDNA. The sequence of encoding CpMnSOD was deposited into GenBank (accession no. MK465057). The calculated molecular mass of the mature protein (286 amino acids) was 41.64 kDa with an estimated pI of 5.17. Signal IP 3.0 analysis showed that the sequence contained a putative signal peptide of 18 amino acids. Four putative amino acid residues (H^{49}, H^{9}, D^{182}, H^{186}) were located in highly conserved regions of CpMnSOD cDNA (Fig. 1).

3.2. Screening for regulatory elements in 5′- flank sequence of CpMnSOD

CpMnSOD region was located at 907 nucleotide upstream of the transcription start site. The online software of TRANSFAC database showed that CpMnSOD promoter sequence had one TATA-box (−280, −276), one CCAAT-box (−523, −519). CpMnSOD promoter lacked the canonical CpG islands, and contained a variety of transcription factor binding sites, GATA-1, AP-1, C/EBPα, C/EBPβ SP-1 and a ARE element (CTTGAGTAGCAAT, spanning −100 to −90). These sites were in promoter and were positive regulatory domain of CpMnSOD (Fig. 2).

3.3. Homology and phylogenetic analysis of CpMnSOD

ClustalW analysis revealed that the deduced amino acid sequence of CpMnSOD displayed high identity with that of selected MnSOD proteins from NCBI database. The sequence of CpMnSOD exhibited similarities with the putative MnSOD (73% identity) from *Haliotis discus discus* (ABF67504.1) and *Reishia clavigera* (AET43974.1), (72% identity) from

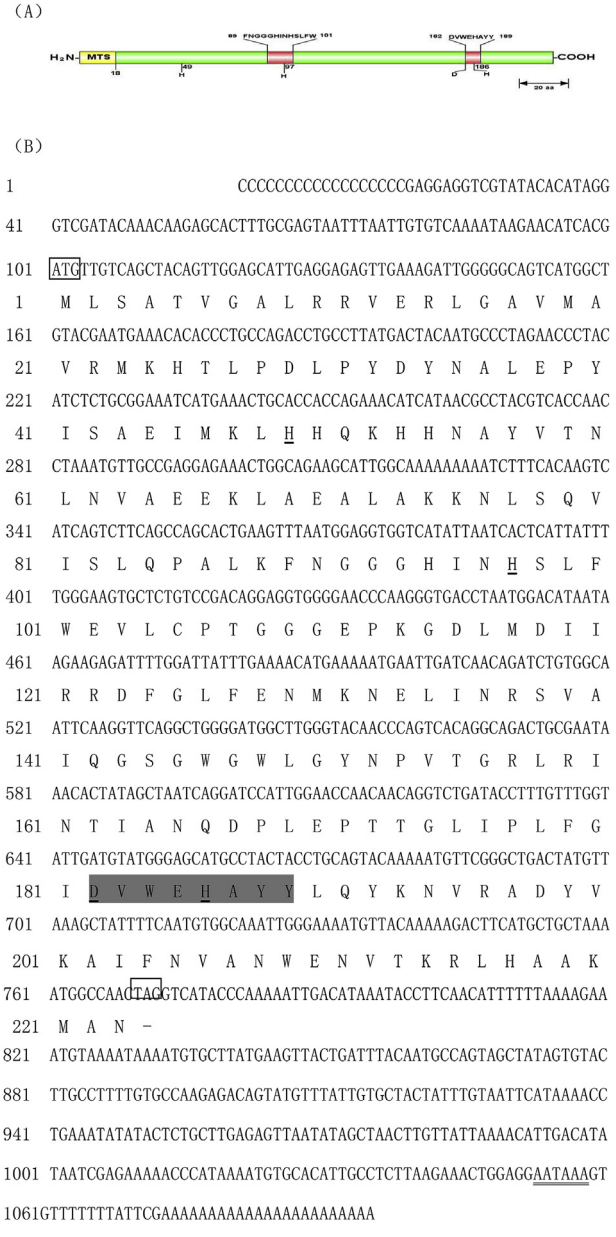

Fig. 1. The domain architecture (A) and nucleotide and deduced amino acid sequence of MnSOD cDNA gene from *Cristaria plicata* (B). Note: (A) The CpMnSOD is composed of a MTS of 18 residues (yellow). (B) The start codon (ATG) and the end codon (TAG) are boxed. The potential metal-binding sites for Mn^{2+} (His49, His97, Asp182 and His 186) are shown with single linear underlining. The CpMnSOD domain (DVWEHAYY) was shaded by gray and the polyadenylation signal sequence (AATAAA) is doubly underlined. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Mimachlamys nobilis (AHX22598.1) and *Argopecten irradians* (ABW98672.1.). The sequence of CpMnSOD exhibited similarities with the multiple alignments of MnSOD proteins were shown in Fig. 3. Two signature sequences (FNGGGHINHSLFW and DVWEHAYY) of MnSOD family were in highly conserved region. The constructed molecular phylogenetic tree based on amino acid sequences of MnSOD proteins was constructed by the N-J method as shown in Fig. 4. MnSODs from invertebrates and vertebrates were separated to two sub-clusters. CpMnSOD was located in the branch of invertebrate.

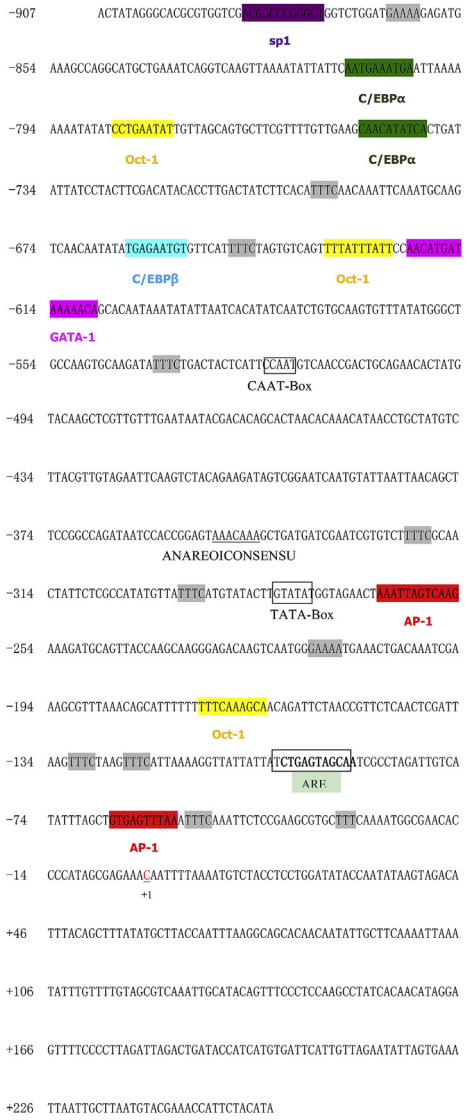

Fig. 2. Potential functional elements of CpMnSOD promoters. Note: Promoter sequences of CpMnSOD are shown in capital letters, respectively. ARE are marked by the bold letters in box. The transcriptional start site, TATA box and CAT box initiation elements are marked by box. Highly conserved sequences (GAAA/TTTC) are marked by the gray shading. All predicted functional sites are underlined. Putative SP1, AP-1,Oct-1,GATA-1,C/EBPα,C/EBPβand binding sites are differently marked in different color. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. The tissue expression pattern of CpMnSOD mRNA

CpMnSOD mRNA was expressed in detected tissues (Fig. 5). The highest expression level was in hepatopancreas, followed by muscle, mantle and gill, and the lowest expression level was in hemocytes.

3.5. Temporal expression of the CpMnSOD after MC challenge

The expression of the CpMnSOD mRNA was up-regulated in hemocytes and hepatopancreas after MC challenge. The expression of CpMnSOD mRNA in hemocytes was increased at 3, 6, 12 and 24 h, and dropped to normal levels at 48 h (Fig. 6A). The expression of CpMnSOD mRNA in hepatopancreas enhanced at 3 h, and the highest expression was observed at 6 h (Fig. 6B).

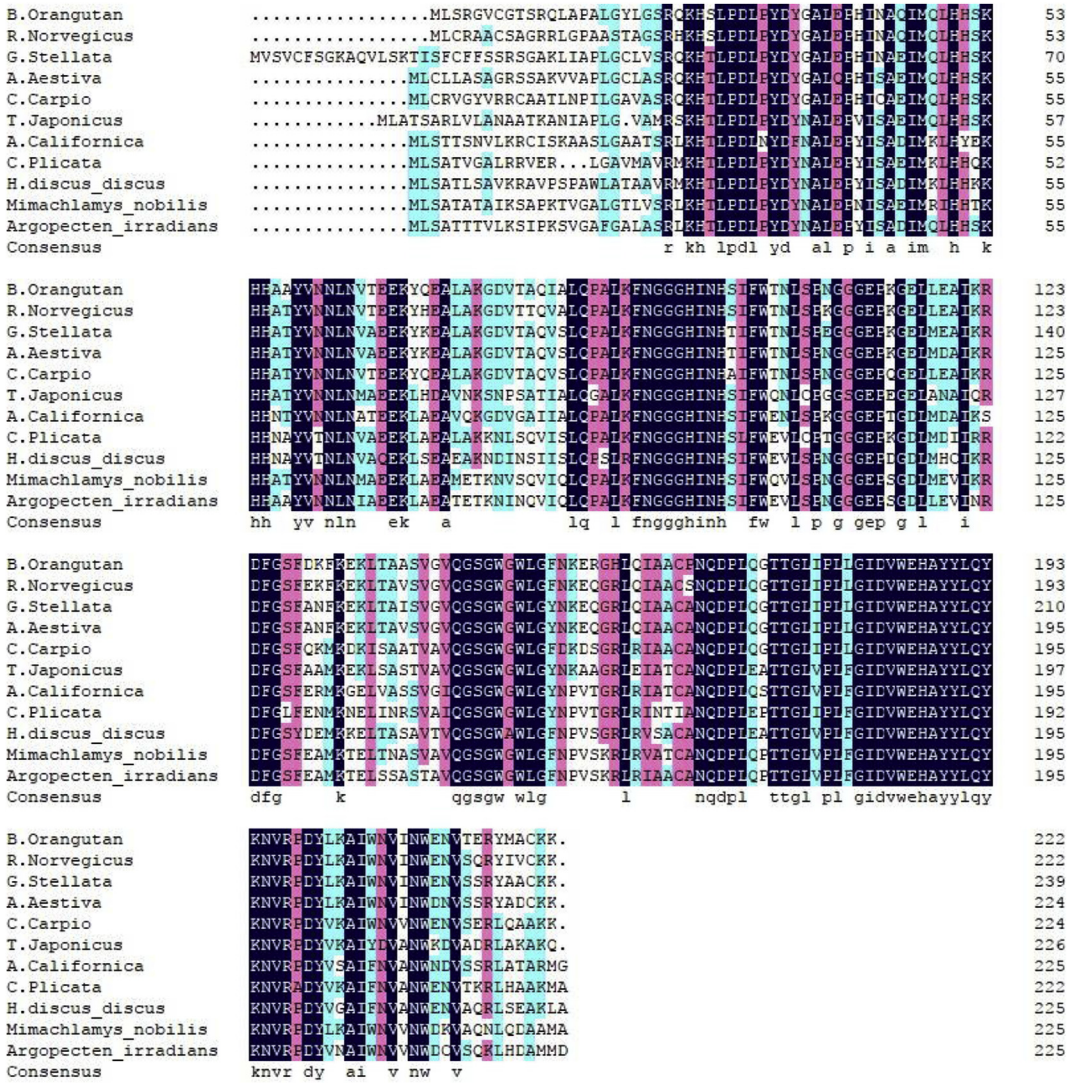

Fig. 3. Multiple alignment analysis of CpMnSOD. Note: GenBank accession numbers for the protein sequences are as follows. *Mimachlamys nobilis*: AHX22598.1, *Argopecten irradians*: ABW98672.1, *Haliotis discus discus*: ABF67504.1, *Aplysia californica*: XP_005104703.1, *Bornean orangutan*: Q8HXP6.3, *Rattus norvegicus*: NP_058747.1, *Gavia stellate*: XP_009818437.1, *Amazona aestiva*: KQK81588.1, *Cyprinus carpio*: KTF74341.1, *Tigriopus japonicus*: AEM66982.1.

3.6. Expression and purification of the CpMnSOD fusion protein

Comparison with non-induced CpMnSOD, DE3 with pcoldI-ZZ-CpMnSOD was expressed, and a fusion protein was approximately 41.64 kDa (including 17 kDa His-tag), the protein was determined by SDS-PAGE matched the theoretic size calculated from recombinants (Fig. 7A). A large amount of the protein was expressed in the inclusion body, a small amount of the protein is also existed in the supernatant. The recombinant was collected in the supernatant, and was purified by Ni-affinity chromatography (Fig. 7B).

3.7. Characterization of temperature, pH and denaturant effects on CpMnSOD activity

The enzyme activity of CpMnSOD at different temperatures was examined. The recombinant enzyme maintained more than 80% activity between 10 and 70 °C, at 80 °C, the SOD activity was 63% and was rapidly inactivated down 50% at 90 °C (Fig. 8A). At pH ranging from 2.0 to 10.0, the recombinase had more than 87% activity, and the optimum pH was at 5.0, SOD activity was 87%, while pH was 11.0, the enzyme activity retained only 38% (Fig. 8B). The concentration of SDS increased from 1% to 10%, the activity of recombinase gradually decreased. The enzyme activity maintained more than 50% under 1–8% SDS treatment, and had only 31% activity after 10% SDS treatment (Fig. 8C). The enzyme activity kept more than 90% under treatment with 2–7 M urea, while the concentration of SDS was to up to 8 M urea, and the enzyme activity was more than 80% (Fig. 8D).

4. Discussion

In general, MnSOD is synthesized in the cytosol and then translocated into mitochondrial matrix, and the translocation is conducted by the conserved mitochondrial targeting sequence (MTS) [25,26]. mitochondrial targeting sequence were also observed in MnSODs of some organisms, such as *Carassius auratus* [27], *Argopecten irradians* [28] and *Scapharca broughtonii* [29]. Most mitochondrial MnSODs contain short signal peptides at the N-terminal region, which might be essential for translocation into the mitochondria [30]. Mitochondria are the major producer of ROS in the electron transport chain, and are directly attacked by these ROSs [31]. Therefore, mitochondrial MnSODs may play a crucial role in eliminating mitochondrial ROS.

The administration of toxin in *C. plicata* possibly cause a systemic imbalance in redox state through the excessive accumulation of ROS, which may in turn stimulate CpMnSOD transcription via controlling

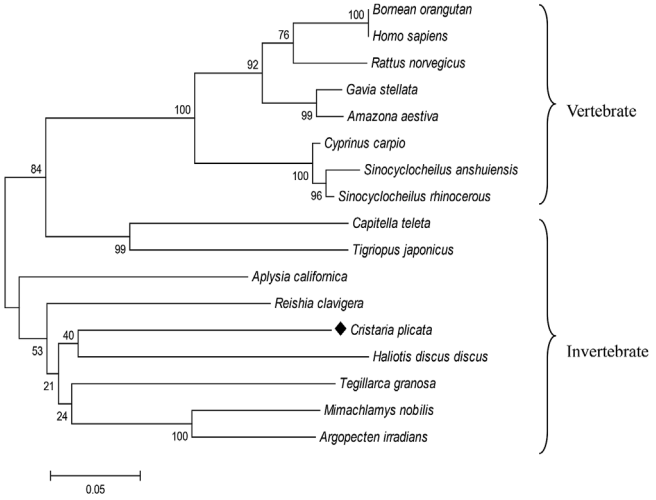

Fig. 4. Neighbor-joining phylogenetic tree of MnSOD amino acid sequences from sixteen species of animals. Note: GenBank accession numbers for the protein sequences are as follows. Homo sapiens: NP_001309749.1, *Sinocyclocheilus anshuiensis*: XP_016351771.1, *Sinocyclocheilus rhinocerous*: XP_016391578.1, *Reishia clavigera*: AET43974.1, *Capitella teleta*: ELU11436.1, *Tegillarca granosa*: ADC34695.1. Other abbreviations and accession numbers are the same as in Fig. 3.

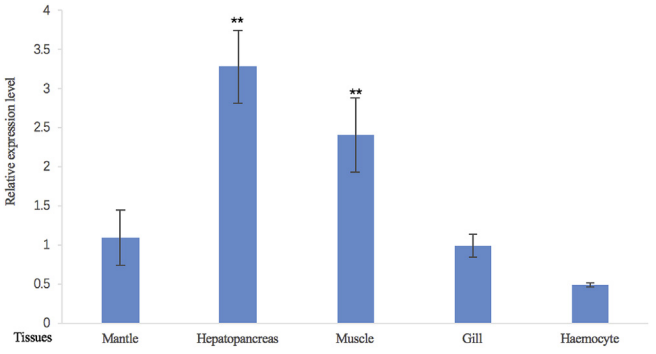

Fig. 5. The expression of CpMnSOD mRNA in different tissues of *Cristaria plicata*.

elements. Subsequently, screened promoter region of CpMnSOD for the presence of *cis*-regulatory elements. CpMnSOD promoter was 907 bp of the 5′-flanking region upstream from the transcription start site. Previous studies demonstrated that MnSOD promoter of vertebrate lacks TATA box and CAAT box [8,32–37]. In invertebrates, PoMnSOD promoter which from pearl oyster *Pinctada fucata* also lacks CAAT box, but it contains TATA box [38]. However, CpMnSOD promoter has TATA box and CAAT box, and also contains a variety of transcription factor binding sites associated with immune modulation and stress response, such as AP-1, Sp1, GATA-1, C/EBPα, and C/EBPβ. The transcription factors already play the important roles in modulation MnSOD

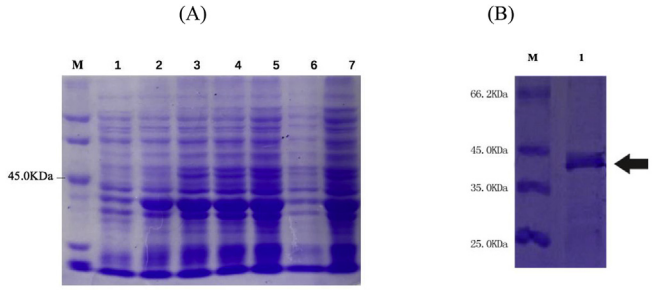

Fig. 7. Expression (A) and Purification (B) of recombinant plasmid in *Escherichia. coli* by SDS-PAGE. Note: M. molecular weight marker, (A1) Uninduced plasmid, (A2) Plasmid with IPTG induced 2 h, (A3) Plasmid with IPTG induced 4 h, (A4) Plasmid with IPTG induced 6 h, (A5) Plasmid with IPTG induced 8 h, (A6) Supernatant after ultrasonic broken, (A7) Inclusion body after ultrasonic broken. (B) Supernatant of CpMnSOD fusion protein after purification.

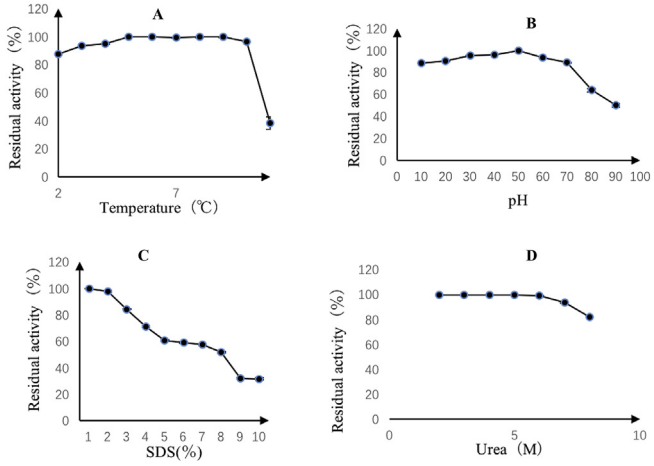

Fig. 8. Enzyme activity assay of CpMn-SOD with different treatments in vitro. Note: Enzyme samples are incubated for 10 min at different temperature from 10 °C to 90 °C (A), or are incubated at 40 °C for 1 h at the different pH values (B), or treated with different concentration of urea (C) or SDS (D). All data are averages from the multiple measurements, and the error bars represent standard deviations.

genes of mammalian under stimulatory conditions [14,34,39–42]. These elements might regulate the temporal expression of CpMnSOD in different tissues post-administration of stimulants and infection of toxin. Promoter element distribution became valuable information for understanding the regulated expression and physiological role of this important gene in molluscs.

The sequence of CpMnSOD shared relatively high similarity with MnSOD proteins from other species. The four metal binding sites for manganese (His49, His97, Asp182 and His 18) and two MnSOD signatures from 89 to 101(FNGGGHINHSLFW)and from 182 to 189

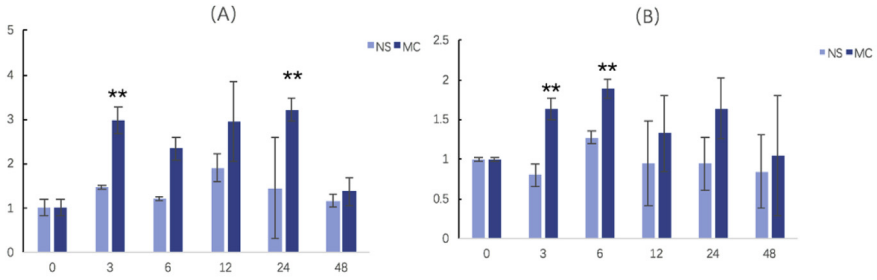

Fig. 6. The expression of CpMnSOD mRNA in hemocytes (A) and hepatopancreas (B) from *Cristaria plicata* after challenge by Real-time quantitative PCR. Note: All values represent the mean ± S.D. (n = 4). CpMnSOD transcript levels are normalized by injecting 0.1 mL NS (normal saline) at 0 h. Asterisk (*) are significantly different (*represent $P < 0.05$ and **represent $P < 0.01$; respectively, *t*-test).

(DVWEHAYY) were completely conserved among the selected animals. This was suggested that MnSOD in *C. plicata* could have the same function as that in other organisms due to the conserved sequence and motif. The deduced amino acid of CpMnSOD owned high conserved sequences with those from other species (Fig. 3). *C. plicata* had the closest relationship with *Haliotis discus discus*. CpMnSOD clustered together firstly with that of *Tegillarca granosa*, and then clustered with those from bivalve and gastropods. It was indicated that the cluster of MnSOD was mainly in accordance with the traditional classification.

MnSOD transcripts are abundant in metabolically active tissues, liver, brain and heart [32,33,43], and are related to the mitochondrial content and the oxidative burden of the tissues [44]. MnSOD mRNA of *Mytilus galloprovincialis* has a similar expression pattern, and the expression levels are higher in hepatopancreas and gonad [16], and that of *Ruditapes philippinarum* are highly expressed in haemocyte and gill, and significantly low in muscle, foot and siphon [15]. However, the highest expression of CpMnSOD mRNA was in hepatopancreas. The hepatopancreas seems to be the primary site for the production of immune recognition molecules, and act as an accessory to the gut in digestion and absorption of nutrients [45]. These results demonstrated that MnSODs expressed in species-specific patterns.

The expression level of CpMnSOD mRNA increased after intramuscular injection of Microcystins. The expressions of McSOD and MnSOD mRNA from *Mytilus coruscus* were up-regulated under pathogenic bacteria and heavy metals stress, what benefited the immune system to fight against oxidative stress [46]. After challenge with the immunostimulants, the activities of SOD in the haemocytes and muscles from *Litopenaeus vannamei* increas, and the survival rates also enhance after the infection with the pathogenic bacteria [47]. mMnSOD in hepatopancreas of *Fenneropenaeus chinensis* after WSSV challenge show the highest expression of mMnSOD after the infected group at 3 h, and then decreased to normal in 48 h [48]. The transcripts of CpMnSOD in haemocytes significantly increased at 3 h post challenge with MC. CpMnSOD raised in hepatopancreas at 3 and 6 h, but dropped around normal levels at 48 h, which is consistent with the results in hepatopancreas of WSSV virus-infected Chinese shrimps [48]. Therefore, we suggested that CpMnSOD might be important to resist oxidative stress and other insults by mediating stress reactions.

The optimum temperature and pH is slight differences among species [49–51]. In this study, CpMnSOD from *C. plicata* also maintained more than 80% activity at the temperature up to 70 °C (Fig. 8A). aMnSOD enzyme of *Haliotis discus discus* retained 100% activity during this range of buffer from pH 3.5–6.5, and then drastically decreased to 20% at pH 7.5. The enzyme is totally inactivated above pH 9.5 [52]. Unlike aMn-SOD, the enzyme activity of CpMnSOD had more than 87% activity after incubation in the buffer between pH 2.0 and 10.0, and the optimum pH was at 5.0, the SOD activity was 87%, while pH was 11.0, the enzyme activity retained 38%. This was suggested that CpMnSOD was a more acid-resistant enzyme. zMn–SOD from *Danio rerio* kept its activity after treatment with up to 4% SDS [53], but CpMnSOD retained the activities after treatment with even 8% SDS. The activity of CpMnSOD is maintained over 80% in the urea of 2–8 M. Therefore, we suggested that the recombinant enzyme had a certain adaptive capacity to a certain concentration of urea.

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