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The distribution, expression of the Cu/Zn superoxide dismutase in *Apostichopus japonicus* and its function for sea cucumber immunity

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ABSTRACT

Cu/Zn superoxide dismutases (SODs) are antioxidative metalloenzymes that exist ubiquitously in different species and are distributed widely in various tissues and cell types. In this study, the distribution and biological function of the Cu/Zn superoxide dismutase in *Apostichopus japonicus* (AjSOD1) is first characterized. The AjSOD1 cDNA is 1219 bp in length and contains an open reading frame (ORF) of 459 bp that encodes a protein of 152 amino acids with a deduced molecular weight of 15.47 kDa and a predicted isoelectric point of 5.65. The Cu^{2+}/Zn^{2+} binding domain and conserved residues were found in the AjSOD1 amino acid sequence. A quantitative reverse transcriptase real-time PCR (qRT-PCR) assay was developed to assess the expression of AjSOD1 in different tissues. Spatial distribution analysis showed that AjSOD1 was constitutively expressed in all tested tissues, with strong expression in the intestine and weak expression in the respiratory tree. mRNA Expression of AjSOD1 was significantly upregulated when challenged with the pathogen *Vibrio splendidus*. Functional investigation revealed that recombinant AjSOD1 displayed good antioxidant activity. More importantly, the addition of AjSOD1 resulted in a significant decrease in coelomocyte apoptosis by LPS/H_2O_2 challenge *in vitro*. The results indicate that sea cucumber SOD1 may play critical roles not only in the defense against oxidative stress but also in the innate immune defense against bacterial infections.

1. Introduction

The sea cucumber, *Apostichopus japonicus (A. japonicus)*, is an important commercial marine culture species in coastal areas of southern Southeast Asia. In China, the aquaculture of *A. japonicus* is the largest industry with an annual production of approximately 180,000 tons valued at 30 billion Yuan [1,2]. In recent years, the development of the large-scale mariculture industry for this species has caused *A. japonicus* to suffer serious problems, such as mortality from environmental contamination, habitat loss, and disease epidemics [3–5]. *Vibrio* infection has resulted in incalculable loss in sea cucumber farming. It would be beneficial to maintain the healthy production of *A. japonicus* in aquaculture by understanding the defense mechanisms of the sea cucumber.

Aerobic organisms that derive their energy from the consumption of oxygen produce small amounts of hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{-}\bullet$), and hydrogen ions (OH-), which are referred to as reactive oxygen species (ROS). In times of environmental stress, such as pathogen infection or oxidative stress, the host needs more ATP to support the initiation of an immune response, which results in increased ROS production from the electron transport chain and dramatically increases ROS levels in the host [6–9]. The high level of ROS could damage the microbes during the course of pathogens phagocytosis. However, when immune responses are activated, the highly reactive nature of ROS can be cytotoxic at concentrations above a basic threshold. They can damage important cellular building blocks such as DNA, proteins and lipids and result in immune dysfunction or cell death, which is thought to be the pathogenesis of various diseases [10]. In marine organisms, uncontrolled levels of ROS are prevalent due to the frequent occurrence of abnormal environmental factors and may cause mass mortality [11,12]. Normally, aerobic organisms develop efficient defense systems to ameliorate and cope with injury from ROS damage and main redox homeostasis by the system of enzymatic and nonenzymatic antioxidants. It has been shown that antioxidant systems act as an important part of the innate immune response to limit the harmful effect of ROS production because the rapid elimination of these excessive ROS is essential for the proper functioning of cells and the

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survival of the host [13]. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) play a crucial role in fighting excess ROS [14].

One of the most important families of antioxidant enzymes, the superoxide dismutase family (SODs) specializes in eliminating ROS and constitutes the first line of defense against ROS. The SODs catalyze the disproportionation of superoxide anion radicals ($O_2{}^{-}\bullet$) in a two-step reaction that eventually generates molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) [15,16]. Eukaryotic SODs are classified into Cu/Zn-SOD and Mn-SOD according to their metal cofactors. Among these, Cu/Zn-SOD is widely distributed in eukaryotes and comprises approximately 90% of the total SODs [17]. Many studies have indicated the importance of SODs in the immune response as well as their role in protecting hosts against viruses [18,19], bacteria [20,21], and physical and chemical challenges [22,23]. In marine organisms, the Cu/Zn-SOD protein genes have been investigated in *Chlamys farreri* [16], *Meretrix* [6] and *Litopenaeus vannamei* [24], which all exhibited a corresponding immune response after bacteria or virus infection. These results revealed that Cu/Zn-SOD may play a pivotal role in immune response processes by regulating reactive oxygen species production in sea cucumbers.

The immune-defensive mechanisms and the regulatory processes of the sea cucumber almost completely rely on the innate immune system, which consists of a nonspecific immune system. Investigation of the components of the innate immune system of the sea cucumber may provide new insights into their defensive mechanisms and the regulatory processes that are of pressing practical relevance for disease control and drug exploitation [1,25]. Pathogens can induce respiratory outbreaks in sea cucumbers, destroying the homeostasis of ROS in the body, thereby affecting the body's immune status. Therefore, studying the functional genes of immune resistance is important. However, the function of Cu/Zn-SOD in *A. japonicus,* named AjSOD1, in the immune process is still not very clear. To gain new insights into the synergy of AjSOD1 in the immune system of *A. japonicus*, we analyzed the full-length AjSOD1 coding sequence and amino sequence using various bioinformatics tools and quantified the spatial expression in different tissues. The immune role of AjSOD1 was investigated by comparing the mRNA expression patterns of coelomocytes and intestines after *Vibrio splendidus (V. splendidus)* challenge. Furthermore, the antioxidant activity of AjSOD1 was investigated. The results will help us to understand the role of AjSOD1 in the immune response of *A. japonicus*.

2. Materials and methods

2.1. Culture conditions for A. japonicus

Healthy *A. japonicus* (weighting 5–10 g) used in this study were collected from the Dalian Pacific Aquaculture Company and acclimated for one week in the laboratory. The culture conditions for *A. japonicus* were 28.30‰ salinity with a pH 8.1 at 16 °C. The basal diet was provided by Dalian Boss-all Bio-Tech. Ltd., China.

2.2. Amplification of the AjSOD1 gene

Total RNA was extracted with TRNzol Universal (TRIzol) reagent (TIANGEN, China) from fresh tissues from *A. japonicus*. First-strand cDNA was synthesized from the total RNA with M-MLV reverse transcriptase and oligo (dT) primer suing the Reverse Transcription Kit (TIANGEN, China) according to the manufacturer's protocol.

Based on the sequence found in the NCBI (JX097096.1), two primers for AjSOD1 were synthesized: P_1 (5′-ATGCTCTACAAGCCGTTTG CGTTT-3′) and P_2 (5′-TTAGACCTGTTTG ATACCAATGA-3′). The coding sequences of AjSOD1 were cloned using the total cDNA template. The PCR program was 1 denaturation cycle of 94 °C for 4 min, followed by 35 cycles of 94 °C for 20 s, 50 °C for 20 s, 72 °C for 90 s, and 1 cycle at 72 °C for 10 min. The PCR product of expected size was extracted with the Gel Extraction Kit (TIANGEN, China), and the purified product was subcloned into pMD18-T (TaKaRa, China) to construct the recombinant vector pMD18-AjSOD1 and sequenced by the United Gene Company.

2.3. Sequence analysis of AjSOD1

The AjSOD1 cDNA sequence was analyzed using the BLAST search program (http://www.ncbi.nlm.nih.gov/blast/). Translation and protein analyses were performed using ExPASy tools (http://us.expasy.org/tools/). The calculated molecular mass and the theoretical isoelectric point were predicated using the Protein Mol. Wt & AA Composition Calculator (http://www.proteomics.com.cn/proteomics/pi_tool.asp). The amino acid sequence of AjSOD1 was compared with that of other SOD1 sequences using Clustal W (http://www.ebi.ac.uk/clustalw/). Phylogenetic analysis was performed by comparing the amino acid sequence of AjSOD1 with those of the other 20 SOD1s, including SOD1s of invertebrates and vertebrates, using MEGA 6.0 on the basis of p-distances by the neighbor-joining method. For the construction of the phylogenetic tree, all positions containing gaps and missing data were eliminated from the multiple alignments.

2.4. Spatial expression analysis of AjSOD1 mRNA

The expression levels of AjSOD1 mRNA were analyzed in five tissues, the body wall, tentacles, intestines, respiratory trees, and coelomocytes, by quantitative reverse transcriptase real-time PCR (qRT-PCR) with a Roche LightCycler480-II real-time PCR detection system. Sea cucumber β-actin served as the internal control to verify successful reverse transcription and calibrate the cDNA template. The primers used are listed below.

Two primers for sea cucumber β-actin (288 bp) were synthesized: P_3 (5′-TTCGTTGCCAATGGTGATGACCT-3′) and P_4 (5′-AAATCGTTCGTGA CATCAAGGA GAA-3′). Two primers for detected the distribution of AjSOD1 (140 bp) were also synthesized: P_5 (5′-TCTCTACAAGCCGTTT GCGT-3′) and P_6 (5′- AATTGGTGGATGTGAAAGCCGT-3′). The qRT-PCR amplification was performed with a total volume of 20 μL containing 10 μL of 2 × SYBR Green Mix, 3 μL of diluted cDNA (1:20), 0.1 μL of each primer (10 mM), and 6.8 μL of PCR-grade water. The following program was used: denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 20 s, 60 °C for 20 s, and final extension at 72 °C for 20 s. The amplified products were subjected to melting analysis at the end of the PCR to confirm the generation of a single PCR product. The expression levels of AjSOD1 were analyzed with $2^{-\triangle\triangle Ct}$, and the values represented the n-fold difference relative to the control tissue intestines.

2.5. Response of AjSOD1 in coelomocytes against V. splendidus challenge

The pathogenic microorganism *V. splendidus* was kept in our laboratory. After inoculation in liquid 2216E medium at 28 °C overnight, the bacteria were harvested and resuspended in filtered seawater to obtain a stock solution of 1×10^{11} CFU/mL. Coelomic fluids were collected at 0, 6, 24, 48, 72, and 96 h after *A. japonicus* were cultured with *V. splendidus* at a final concentration of 1×10^{7} CFU/mL in the sea water. The coelomocytes were centrifuged and harvested to analyze the regulation of the AjSOD1 mRNA response to the *V. splendidus* challenge. Three biological replicates were obtained for each experimental group. qRT-PCR was performed to analyze the AjSOD1 mRNA expression at different times after the *V. splendidus* challenge. The expression levels of AjSOD1were analyzed by $2^{-\triangle\triangle Ct}$, and the values represented the n-fold difference relative to the control group. Data are presented as the mean ± SD (n = 3). The results were subjected to one-way ANOVA, followed by Duncan's multiple range tests to determine the differences between the challenged and control groups at each sampling time. P values less than 0.05 were considered significantly different.

2.6. Response of AjSOD1 in intestine against V. splendidus challenge

When detecting the temporal expression of the AjSOD1 in intestine of sea cucumber, the experimental condition is to inject *V. splendidus* through oral cavity directly into the intestine of sea cucumber. Fifty microliters of *V. splendidus* (1×10^9 CFU/mL) was introduced into the intestines through the mouthparts of *A. japonicus* using a sterile flexible microsyringe. The intestines were collected at 0 min, 10 min, 30 min, 1 h, 3 h and 6 h after the oral infection for total RNA extraction. AjSOD1 mRNA expression in the intestines was analyzed after *V. splendidus* challenge. The method of data analysis is the same as *2.5*.

2.7. Expression and purification of AjSOD1 in E. coli

The restriction sites *XhoI* I and 6 × His tag were added at the C-terminal of the AjSOD1 coding sequence, and *Nde* I was added at the N-terminal using the specific primers P_7 (5′-<u>CTCGAG</u>TTAATGATGATGATGATGGTGGACCTGTTTGATACCAA-3′) and P_8 (5′-<u>CATATG</u>CTCTACAAGCCGTTTGCGTTTTAC-3′). The recombinant vector pMD18-AjSOD1 was used as the PCR model. The PCR program was as follows: 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s and a final extension step at 72 °C for 10 min. The PCR products were digested with *XhoI* I/*Nde* I and ligated into the pET-30a plasmid, which was also digested by the two enzymes. The resulting ligation of pET-30a-AjSOD1 was transformed in *E. coli,* and the correct clones were screened through ampicillin resistance. The recombinant expression vector pET-30a-AjSOD1 was confirmed by the digestion of *XhoI* I/*Nde* I and sequencing.

An overnight culture of the genic engineering strain named BL21(DE3)/pET-30a-AjSOD1 was diluted in LB medium (1:100) and cultured for ≥3 h until the log phase. IPTG (isopropyl b-D-1-thiogalactopyranoside) was added to the fermentation at a final concentration of 1 mM, and the bacteria were induced for 14 h at 37 °C to induce the expression of recombinant protein AjSOD1 (rAjSOD1). The supernatant samples of the BL21(DE3)/pET-30a-AjSOD1 bacteria were analyzed by 15% SDS-PAGE and stained with Coomassie Brilliant Blue R-250. rAjSOD1 was purified using high-affinity Ni^{2+}-NTA resin (GenScript) following the manufacturer's instructions. In order to remove the endotoxin, the purified rAjSOD1protein was separated and purified by the Q-sepharose anion exchange column (GE, China). The purified protein was also detected through 15% SDS-PAGE. Western blot analysis was performed to detect protein expression.

2.8. Analysis of the H_2O_2 tolerance for rAjSOD1

The oxidative stress bioassay of the genic engineering strain BL21(DE3)/pET-30a-AjSOD1 was performed after IPTG induction as described previously [22,23]. The induced *E. coli* cells were then serially diluted (1:2, 1:5, 1:10, 1:20), and a 10 μL droplet of each dilution was inoculated on LB agar-ampicillin medium containing 0, 0.5, 1.0, 1.5 and 2.0 mM H_2O_2. All plates were then incubated at 37 °C overnight. In this assay, *E. coli* cells transformed with blank pET-30a plasmids induced by IPTG under the same conditions served as controls.

2.9. Detection of the rAjSOD1 protection function for coelomocytes in vitro in response to H_2O_2/LPS challenge

Primary coelomocytes were prepared according to previously described methods [26]. The coelomic fluid from living *A. japonicus* was extracted and filtered by 70 μM membrane to remove tissue fragments and harvest the primary coelomocytes. Then, the cells with a final concentration of 1×10^6 cells/mL were cultured in L-15 medium (Invitrogen, USA) containing 0.39 M NaCl, 100 U/mL penicillin and 100 mg/mL streptomycin sulfate.

Coelomocytes were treated with H_2O_2 and LPS (10 mg/mL) for 24 h to detect cell injury and apoptosis. To detect the AjSOD1 protection function in coelomocytes in response to the H_2O_2/LPS challenge, the coelomocytes were treated with purified rAjSOD1 (1.0 mg/mL) for 2 h, and then the same concentrations of H_2O_2 and LPS were added. Cells without any treatment (LPS-NC) served as the control group. Coelomocyte apoptosis was analyzed using a Hochest33342/PI Apoptosis Detection Kit (Beyotime, China) under a fluorescence microscope according to the method described previously [18]. The 10 μL Hoechst 33342 stock solution was incubated for 15 min. Then, the cells were resuspended in 1 mL isotonic buffer, and 5 μL PI stock solution was added. Cells were observed under a fluorescence microscope (IX73P2F, TOKYO 163–0914, JAPAN). Apoptotic cells at the early and late stages were dyed for blue and red fluorescence, respectively. All experimental groups were run in triplicate.

3. Results

3.1. CDNA cloning and sequence characterization of AjSOD1

The full-length cDNA of AjSOD1 was 1501 bp long and contained 136 bp in the 5′ untranslated region, 906 bp in the 3′ untranslated region with a poly A signal (ATTAAA), and an open reading frame (ORF) of 459 bp that encoded 153 amino acids (Fig. 1). The deduced amino acid sequence had no putative signal peptide by SignalP software (ExPASy) (Fig. 1), which indicated that AjSOD1 is a cytoplasmic protein. The deduced molecular mass of mature AjSOD1 was 15.47 kDa, and its theoretical isoelectric point was 5.65. There were four amino acid residues (His-46, -48, −63, and −120) that are essential for AjSOD1 binding to copper and four amino acid residues (His-63, -71, −80 and Asp-83) that are essential for protein binding to zinc. AjSOD1 was also found to contain two cysteines (Cys-57 and Cys-146) that formed an intramolecular disulfide bond and had a free cysteine (Cys-7).

According to the comparison results in the NCBI database, the amino acid sequences of Cu/Zn SODs in different species have very high sequence similarity. The conserved amino acid sequence for AjSOD1 was aligned with those of selected Cu/Zn SODs from the NCBI database using Clustal X. Ten other Cu/Zn SODs were used for comparison. AjSOD1 had 79%, 73%, 70%, 68%, 66%, 65%, 65%, 65%, 64% and 62% identity with orthologs of *Sterechinus neumayeri* (AMH40758.1), *Harpegnathos saltator* (XP_025158148.1), *Perinereis nuntia* (AHY86344.1), *Crassostrea madrasensis* (AFV52312.1), *Mus musculus* (NM_011434.2), *Danio rerio* (NP_571369.1), *Ctenopharyngodon idella* (ADF31307.1), *Homo sapiens* (AY450286.1), *Gallus* (NP_990395.1) and *Xenopus laevis* (XP_018104357.1), respectively. The results showed that the N-terminal and C-terminal sequences of the protein are highly conserved AVCVL (M) and GVIGI, which are key binding sites for the protein dimer. Furthermore, AjSOD1 had two Cu/Zn SOD family signature sequences, GFHIHQFGDTT and GNAGGRLACGVI (Fig. 2). Studies have shown that SOD1 needs to be in the dimer state to have antioxidant effects. The potential tertiary structure of AjSOD1 was established using the SWISS-MODEL prediction algorithm. It demonstrated that each AjSOD1 monomer was a barrel-like structure consisting of eight sheets containing one copper ion and one zinc ion. The three-dimensional structure of the Cu/Zn SOD from *A. japonicus* was a dimer (data was not shown).

3.2. Phylogenetic characterization of the AjSOD1 gene

A phylogenetic tree was constructed based on the coding nucleotide sequences of selected Cu/Zn SODs using the Bayesian method (Fig. 3). It had demonstrated that the Cu/Zn SODs of vertebrata were clustered relatively closer, and the invertebrate Cu/Zn SODs were clustered as a subgroup. AjSOD1 is close to *Strongylocentrotus purpuratus* but is very distant from fishes and mammals, for it belongs to the subgroup of invertebrates. And it knows that sea cucumbers and sea urchins are both belong to echinoderms and the AjSOD1 among the two species were highly similar.

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   1 ACGCGGGGATAGCTGAACTGTTTGTGTAGCTAGCTACTGCGAAGACATTCACTTAACCGACCAAACTATCCTCTCAAAAT
  81 CCTTGACGAGTTTTGAAGCAATTGAAATATGTCTCTACAAGCCGTTTGCGTTTTACATGTCTCTACAAGCCGTTTGCGTT
   1                                                         M  S  L  Q  A  V  C  V
 161 TTACAAGGGCAGACTGTGTCGGGCACTATTACCTTCACTCAAGAGGGGAATTCTGTGACAGTCACTGGTGAAGTGAGGGG
   9  L  Q  G  Q  T  V  S  G  T  I  T  F  T  Q  E  G  N  S  V  T  V  T  G  E  V  R G
 241 ACTTACAAGAGGACTACACGGCTTTCACATCCACCAATTCGGTGACAATACAAATGGATGCACTTCAGCAGGGGGACATT
  36  L  T  R  G  L  H  G  F  H  I  H  Q  F  G  D  N  T  N  G  C  T  S  A  G  G  H
 321 TCAACCCTACTGGCAAGACTCACGGTGGACCAGCAGATGAAGAAAGACATGTAGGTGATCTAGGCAATGTGGATGCCGAT
  62 F  N  P  T  G  K  T  H  G  G  P  A  D  E  E  R  H  V  G  D  L  G  N  V  E  A  D
 401 GATAATGGTGTGGCTAGTGTTAACATAACTGATACAATCATATCGCTATCCGGCCCTAATTCCATCATTGGACGATCTCT
  89  D  N  G  V  A  S  V  N  I  T  D  T  I  I  S  L  S  G  P  N  S  I  I  G  R  S L
 481 TGTGGTCCACGAAGGAGTTGATGATTTAGGGAGAGGTGGCCATGAACAGAGCAAAACAACTGGCAACGCTGGAGGGCGTG
 116 V  V  H  E  G  V  D  D  L  G  R  G  G  H  E  Q  S  K  T  T  G  N  A  G  G  R  A
 521 CGGCGTGTGGTGTCATTGGTATCAAACAGGTCTAGATGGTGGTGATTCATGAAAACCATCACTGTTCTGTCCTTTCATCA
 143  A  C  G  V  I  G  I  K  Q  V  *
 601 GTAATCTTGTACGGCCCAGGCCATTTGAGGGCACTTCATTTGTGTTTTGGACTATTTCCTTATTATGTATCATTCAGAAT
 681 TGTTTCCTTTTAAGTTCACATGTATACCTTAATGATATTTTTCATCACAACTGAAATTTTTTCTACCGGCAGGCTTGTCG
 761 GCACAATAATAACAGCACTTTGCAGTGATCCAAAGTAACCCTGGCAATTCCTAGTGTGTCTGTCGAATCAATTTGCAATT
 821 GTACAATTTTTAAGAACTTTGTTTTATGTTAGGGTTTTTCCAAGACAGGAGTAACATGTTCAAGGGTTATTCTTAGGTCC
 901 AGATTTCTTCAGAGATAGCTTCTCTATCTTATGTAAGCATCTATCTTATGTAAGCATCTGGGTGAGTTTTCAAAATATTG
 981 AAGAATGTCTGGATGTCAGGTAGTGTCAACAAATACCTTTTACCTGACAGTGGGTTGGGACATGTGTAGCTACTCTAATT
1061 TGAGCACAGAAAGGCAACTTCTGAGTAGAAACAGTCCTAATAATAGTCATCATGCTACATATTTCTTTTTTTCGCTGGGA
1141 GCAGTGCAGGAATTTTCATTTTAATGGAGGTGATCAGTATTCTGTAAGGTAGAAACATTAATAAATGTCCAAATTTATCC
1221 TTGATTTTCACTCATTTTGCTGATTTATGATTAATTGGTCAAAAAGTTGAATACTCATTGGCAGGGGGGGGGAATGGGGG
1301 GGTATGTTCAGTACCTATTAATGGCAGAAAGTTGTTGACTTTGATATTTCCATCTATGTTATATGTTTTGTAATTAATCA
1381 AGAAAAGAGATTAATGGAAATAAAATCTTGAGAAAGGTGATCAAAAAAAAAAAAAAAAAAAAAAAAA
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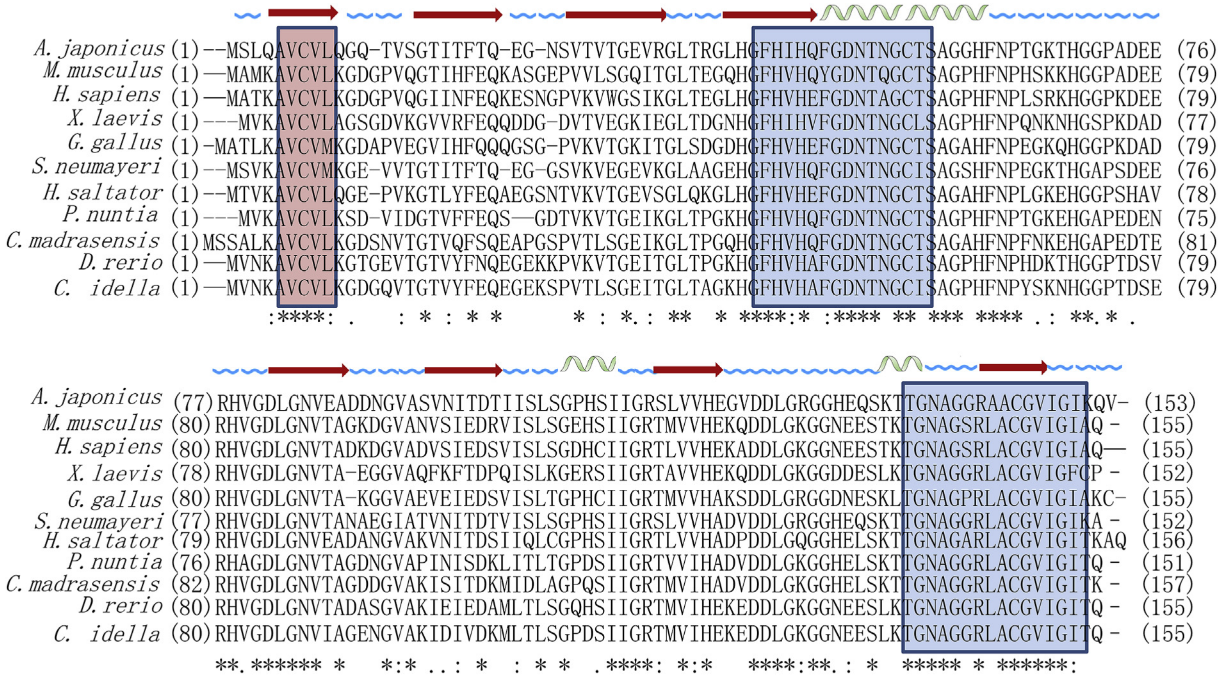
Fig. 1. The nucleotide sequence (above, black font) and deduced amino acid sequence (bottom, red font) of AjSOD1. The bold letters are the start codon (ATG), the stop codon (TGA) and the polyadenylation signal sequence (AATAAA). The amino acids required for copper (H-44, -46, −61, −118 and V-116) and zinc (H-61, -69, −78 and D-81) binding are also the bold letters in black. Two cysteines (Cys-55 and Cys-144) that might form an intramolecular disulfide bond were indicated in blue boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Tissue expression patterns of AjSOD1 mRNA

Quantitative real-time PCR was performed to quantify the expression of AjSOD1 mRNA in five different tissues, i.e., the intestine, respiratory tree, tentacle, body wall and coelomocytes (Fig. 4). The amplification specificity was determined by analyzing the dissociation curves; only one peak was observed in the dissociation curves for each gene. The highest mRNA level of AjSOD1 was detected in the intestine which was as the control level. The mRNA level of AjSOD1 was also higher in the respiratory tree and body wall, but there was no significant difference. The lowest mRNA expression of AjSOD1 was observed in coelomocytes (Fig. 4). The distribution of AjSOD1 in each analyzed tissue was relatively uniform which suggesting it might be a ubiquitous enzyme without tissue specificity.

3.4. AjSOD1 mRNA expression in response to V. splendidus exposure

The transcriptional profiles of AjSOD1 mRNA in the intestine were



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   A. japonicus (1)--MSLQAVCVLQGQ-TVSGTITFTQ-EG-NSVTVTGEVRGLTRGLHGFHIHQFGDNTNGCTSAGGHFNPTGKTHGGPADEE (76)
    M. musculus (1)—MAMKAVCVLKGDGPVQGTIHFEQKASGEPVVLSGQITGLTEGQHGFHVHQYGDNTQGCTSAGPHFNPHSKKHGGPADEE (79)
     H. sapiens (1)—MATKAVCVLKGDGPVQGIINFEQKESNGPVKVWGSIKGLTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHGGPKDEE (79)
      X. laevis (1)---MVKAVCVLAGSGDVKGVVRFEQQDDG-DVTVEGKIEGLTDGNHGFHIHVFGDNTNGCLSAGPHFNPQNKNHGSPKDAD (77)
      G. gallus (1)-MATLKAVCVMKGDAPVEGVIHFQQQGSG-PVKVTGKITGLSDGDHGFHVHEFGDNTNGCTSAGAHFNPEGKQHGGPKDAD (79)
   S. neumayeri (1)--MSVKAVCVMKGE-VVTGTITFTQ-EG-GSVKVEGEVKGLAAGEHGFHVHQFGDNTNGCISAGSHFNPEGKTHGAPSDEE (76)
    H. saltator (1)--MTVKAVCVLQGE-PVKGTLYFEQAEGSNTVKVTGEVSGLQKGLHGFHVHEFGDNTNGCTSAGAHFNPLGKEHGGPSHAV (78)
      P. nuntia (1)---MVKAVCVLKSD-VIDGTVFFEQS—GDTVKVTGEIKGLTPGKHGFHVHQFGDNTNGCTSAGPHFNPTGKEHGAPEDEN (75)
 C. madrasensis (1)MSSALKAVCVLKGDSNVTGTVQFSQEAPGSPVTLSGEIKGLTPGQHGFHVHQFGDNTNGCTSAGAHFNPFNKEHGAPEDTE (81)
       D. rerio (1)—MVNKAVCVLKGTGEVTGTVYFNQEGEKKPVKVTGEITGLTPGKHGFHVHAFGDNTNGCISAGPHFNPHDKTHGGPTDSV (79)
      C. idella (1)—MVNKAVCVLKGDGQVTGTVYFEQEGEKSPVTLSGEITGLTAGKHGFHVHAFGDNTNGCISAGPHFNPYSKNHGGPTDSE (79)
                        :****: .    : * : * *      * : *. : **  * ****:* :**** ** *** **** .: **.* .

   A. japonicus (77)RHVGDLGNVEADDNGVASVNITDTIISLSGPHSIIGRSLVVHEGVDDLGRGGHEQSKTTGNAGGRAACGVIGIKQV- (153)
    M. musculus (80)RHVGDLGNVTAGKDGVANVSIEDRVISLSGEHSIIGRTMVVHEKQDDLGKGGNEESTKTGNAGSRLACGVIGIAQ - (155)
     H. sapiens (80)RHVGDLGNVTADKDGVADVSIEDSVISLSGDHCIIGRTLVVHEKADDLGKGGNEESTKTGNAGSRLACGVIGIAQ— (155)
      X. laevis (78)RHVGDLGNVTA-EGGVAQFKFTDPQISLKGERSIIGRTAVVHEKQDDLGKGGDDESLKTGNAGGRLACGVIGFCP - (152)
      G. gallus (80)RHVGDLGNVTA-KGGVAEVEIEDSVISLTGPHCIIGRTMVVHAKSDDLGRGGDNESKLTGNAGPRLACGVIGIAKC- (155)
   S. neumayeri (77)RHVGDLGNVTANAEGIATVNITDTVISLSGPHSIIGRSLVVHADVDDLGRGGHEQSKTTGNAGGRLACGVIGIKA - (152)
    H. saltator (79)RHVGDLGNVEADANGVAKVNITDSIIQLCGPHSIIGRTLVVHADPDDLGQGGHELSKTTGNAGARLACGVIGITKAQ (156)
      P. nuntia (76)RHAGDLGNVTAGDNGVAPINISDKLITLTGPDSIIGRTVVIHADVDDLGKGGHELSKTTGNAGGRLACGVIGITQ - (151)
 C. madrasensis (82)RHVGDLGNVTAGDDGVAKISITDKMIDLAGPQSIIGRTMVIHADVDDLGKGGHELSKTTGNAGGRLACGVIGITK - (157)
       D. rerio (80)RHVGDLGNVTADASGVAKIEIEDAMLTLSGQHSIIGRTMVIHEKEDDLGKGGNEESLKTGNAGGRLACGVIGITQ - (155)
      C. idella (80)RHVGDLGNVIAGENGVAKIDIVDKMLTLSGPDSIIGRTMVIHEKEDDLGKGGNEESLKTGNAGGRLACGVIGITQ - (155)
                    **.****** *   *:* ..: *  : * *  .****: *:*   ****:**.: *  ***** * ******:
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Fig. 2. Alignment of deduced amino acid sequences of the selected Cu/Zn SODs. (A) Identities are signed as “*”, “: ”, “.”. Two Cu/Zn SOD signature motifs (GFHIHQFGDTT and GNAGGRLACGVI) are boxed. Six helices are shown above the alignment. Beta turns were indicated with β.

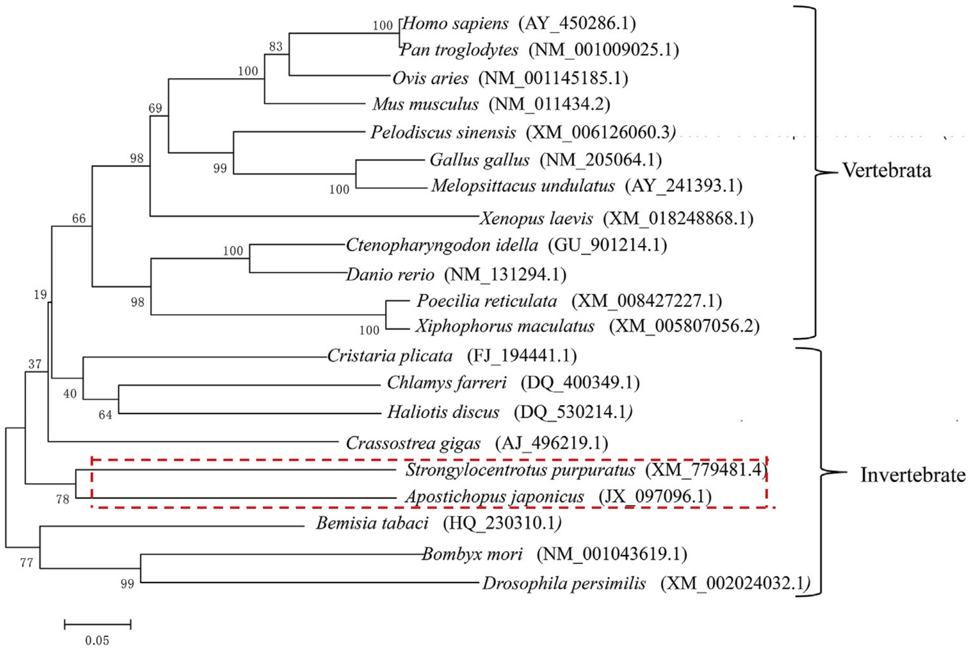

Fig. 3. Phylogenetic tree of Cu/Zn SOD amino acid sequences from different species. The abbreviation of Cu/Zn SOD and the GenBank accession numbers used to construct phylogenetic tree are given in figure. Numbers at branch nodes represent the confidence level of posterior probability.

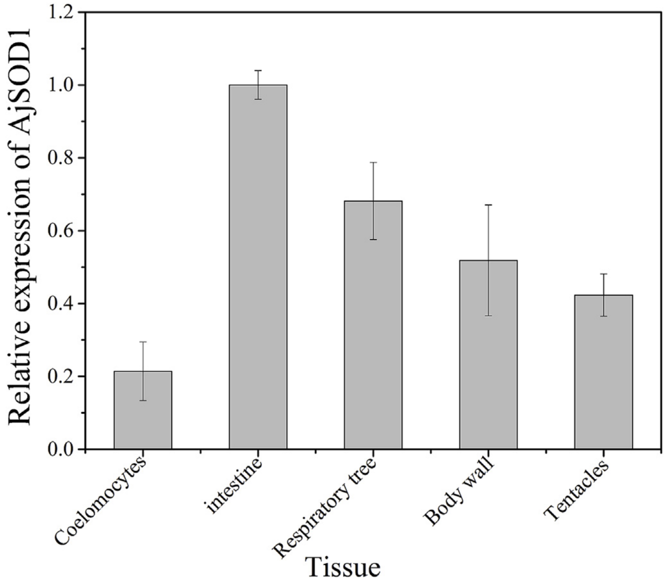

Fig. 4. The qRT-PCR analysis of AjSOD1 mRNA expression levels in the different tissues. The expression levels of AjSOD1 mRNA were normalized to intestine. All obtained data are expressed as mean values ± SD of three independent assays.

detected after *V. splendidus* oral infection. The results of qRT-PCR analysis showed that the expression of AjSOD1 mRNA was significantly upregulated and reached a maximum value at 3 h ($P < 0.01$), approximately 30.0-fold greater than that observed in the control group. Then, the AjSOD1 mRNA significantly decreased at 6 h ($P < 0.05$), but it was still approximately 5.0-fold greater compared with that of the control group. Expression recovered to normal levels at 12 h (Fig. 5).

After stimulation by *V. splendidus in vitro*, the temporal mRNA changes of AjSOD1 in coelomocytes *in vivo* were detected by quantitative real-time PCR. The expression trend of AjSOD1 mRNA in the coelomic cells was consistent with that of the intestine. Compared with the control groups, the expression of AjSOD1 mRNA was significantly up-regulated after induction by the pathogenic bacteria (Fig. 6). It demonstrated that the expression was gradually upregulated from 1 h and reached a maximum value at about 12 h ($P < 0.05$), approximately 14.5-fold greater than that observed in the control group. Then, the AjSOD1 mRNA level decreased gradually from 24 h to 72 h ($P < 0.05$). The AjSOD1 mRNA level remained at a higher level from 12 to 24 h. Levels then declined at 48 h ($P < 0.05$) and it remained at a lower level at 72 h. The infection of pathogenic bacteria *V. splendidus* can abnormally regulate the expression of AjSOD1 at the transcriptional level which suggesting that AjSOD1 plays a role in the sea cucumber immune.

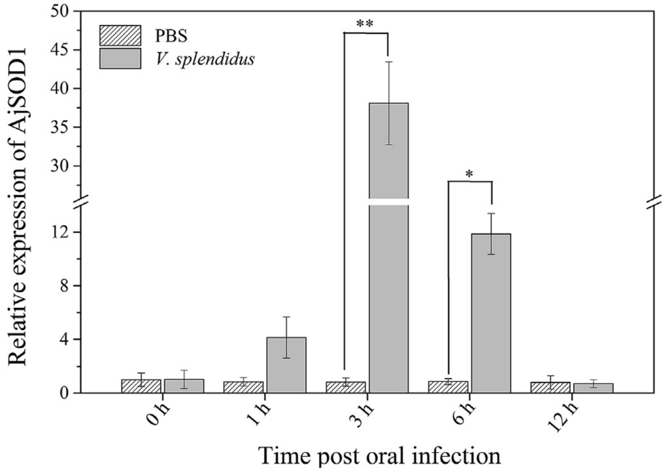

Fig. 5. Temporal expression of the AjSOD1 transcripts in intestine challenged by *V. splendidus* for the *A. japonicus.* All obtained data are expressed as mean values ± SD of three independent assays. Asterisks indicate significant differences (*: $p < 0.05$, **: $p < 0.01$) analyzed by *t*-test.

3.5. Expression and purification of AjSOD1 by engineered bacteria and its H_2O_2 tolerance

To investigate the biological property of AjSOD1, rAjSOD1 with a

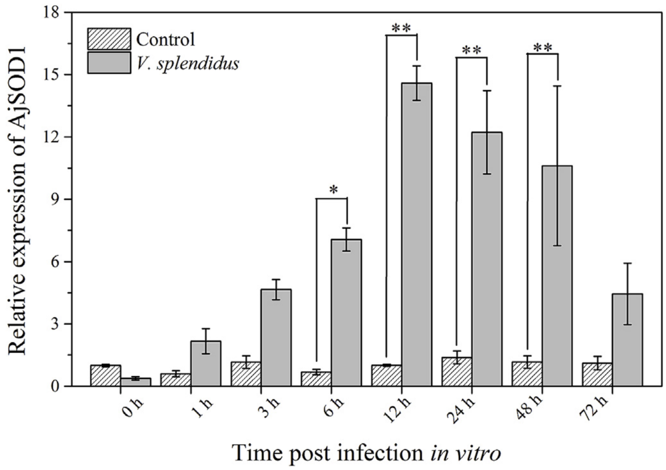

Fig. 6. Temporal expression of the AjSOD1 transcripts in coelomocytes challenged by *V. splendidus in vitro* for the *A. japonicus*. All obtained data are expressed as mean values ± SD of three independent assays. Asterisks indicate significant differences (*: $p < 0.05$, **: $p < 0.01$) analyzed by *t*-test.

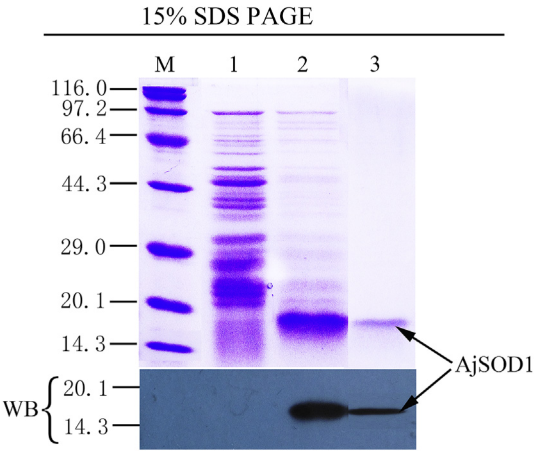

Fig. 7. SDS-PAGE (above) and Western blot (below) analysis of expression and purification of rAjSOD1. 1: cell lysate transformed with pET30a after IPTG induction; 2: cell lysate transformed with pET30a-AjSOD1 after IPTG induction; 3: purified rAjSOD1; M: protein marker. Specificity of 6 × His monoclonal antibody was determined by Western-blot.

6 × His tag at the C-terminal region was overexpressed in the recombinant, engineered bacteria BL21(DE3)/pET30a-AjSOD1. Protein expression was induced by 1 mM IPTG for 6 h at 37 °C. A specific band (molecular weight approximately 15–20 kDa) appeared in the whole cell lysate of *E. coil* with pET30a-AjSOD1 compared with *E. coil* with an empty pET-30a plasmid induced by the same conditions. The results showed that rAjSOD1 accumulated in very high levels in the fusion state (Fig. 7). The rAjSOD1 proteins were purified by Ni^{2+}-NTA and were resolved by SDS-PAGE and Coomassie staining. One major protein band with a molecular weight of approximately 15–20 kDa was detected (Fig. 7). Furthermore, the same experiments were conducted using a monoclonal antibody specific for histidine labeling (6 × His) as the primary antibody. It reacted with the histidine-labeled protein to produce one clear band. These were specific bands that indicated that the rAjSOD1 protein was expressed successfully *in vitro*.

The expression vector BL21(DE3)/pET30a-AjSOD1 was used to confirm the potential antioxidant role of rAjSOD1. *E. coli* cells BL21 (DE3) with the rAjSOD1 expression vector pET30a-AjSOD1 or the blank pET30a plasmid (as a control) were cultured in LB medium. After induction by IPTG for 6 h, the *E. coli* cells were dropped in LB medium containing 0, 0.5, 1.0, 1.5 and 2.0 mM H_2O_2. Compared to *E. coli* cells with rAjSOD1, the control *E. coli* cells were more sensitive to the medium containing H_2O_2 (Fig. 8). Control *E. coli* cells barely grew at lower concentrations (0.5 mM H_2O_2) at a dilution of 1:2, but the growth of control cells were completely inhibited at dilutions of 1:5, 1:10 and 1:20. Control cells could not grow on the 1.0 mM or higher H_2O_2. The BL21(DE3)/pET30a-AjSOD1 strain grew on 0.5 mM H_2O_2 LB medium. In LB medium containing 1.0, 1.5 and 2.0 mM H_2O_2, the growth of *E. coli* was inhibited to varying degrees. Compared with the growth of the control group, it suggested that rAjSOD1 protected cells from H_2O_2 stress.

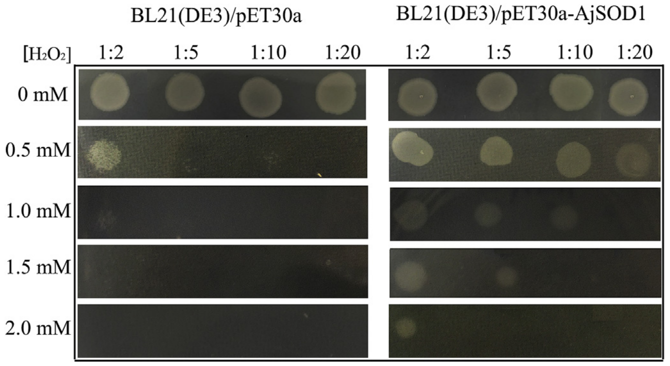

Fig. 8. H_2O_2 tolerance of rAjSOD1. *E. coli* cells incubated in the LB-agar medium containing 0, 0.5, 1.0, 1.5 and 2.0 mM H_2O_2. BL21(DE3)/pET30a: E. coli cells transformed with empty pET30a plasmid; BL21(DE3)/pET30a-AjSOD1: *E. coli* cells transformed with recombinant pET30a-AjSOD1 plasmid.

3.6. rAjSOD1 modulation of the apoptosis of coelomocytes challenged by H_2O_2/LPS in vitro

The modulation and protection function of AjSOD1 for the cultured coelomocyte cells *in vitro* challenged by H_2O_2/LPS was detected by an apoptosis and necrosis assay kit using the double staining method with Hoechst 33342 and pI. When the cells were treated with H_2O_2, the stain intensity increased compared with the intensity of the control cells, which indicated that H_2O_2 mediated many cells at the stage of early apoptosis (Fig. 9B). Additionally, the intensity of the red stain

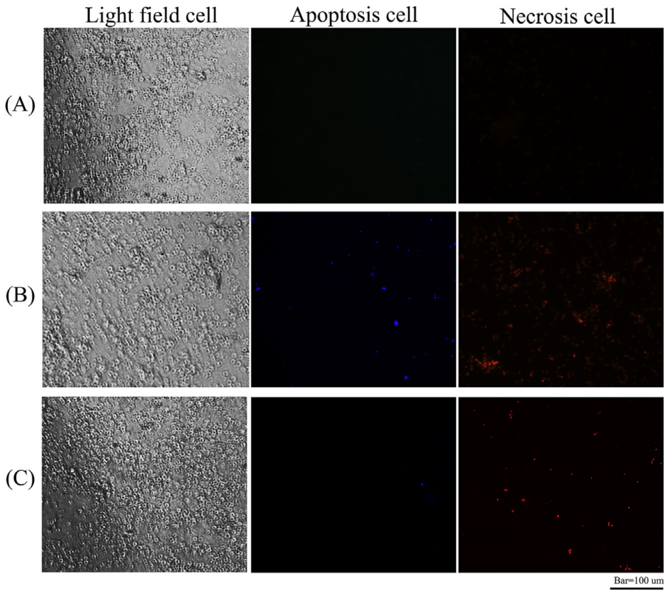

Fig. 9. Apoptosis assay of H_2O_2-exposed coelomocytes by the double staining method. A: untreated cell. B: H_2O_2 exposure cells. C: H_2O_2/rAjSOD1 exposure cells.

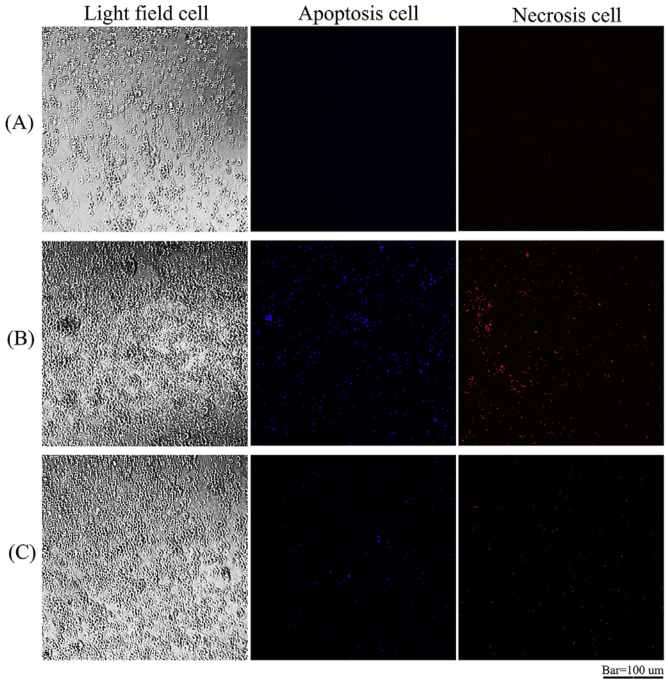

Fig. 10. Apoptosis assay of LPS-exposed coelomocytes by the double staining method. A: untreated cell. B: LPS exposure cells. C:LPS/rAjSOD1 exposure cells.

significantly increased compared with that of the control cells (Fig. 9A). These results showed that H_2O_2 caused coelomocytes to clearly induce late necrosis. Additionally, we analyzed the protective function of rAjSOD1 against oxidative damage by H_2O_2 in cultured coelomocyte cells *in vitro*. The early apoptosis and late necrosis of coelomocyte cells significantly decreased when rAjSOD1 was added with the H_2O_2 simultaneously (Fig. 9C).

LPS also significantly induced cell apoptosis compared with that of normal coelomocyte cells (Fig. 10A). For the LPS challenge, we also detected apoptosis in cells in which rAjSOD1 was added simultaneously. The apoptosis of cells significantly decreased in the groups containing AjSOD1 (Fig. 10C) compared with that in the LPS-treated group (Fig. 10B). The results demonstrated that rAjSOD1 could protect cells against oxidation or LPS damage.

4. Discussion

The sea cucumber is an important economic marine species in Asia. In recent years, the breeding environment has gradually deteriorated, and diseases have become more frequent with the intensification of aquaculture. Therefore, understanding the immunity and the regulatory mechanisms of the sea cucumber is very important. Studies have shown that adverse environmental factors can cause abnormal ROS levels and disease in organisms. Excessive ROS can help the body eliminate harmful pathogens, but excessive ROS can also cause serious oxidative damage to the body [27]. Cu/Zn SOD is a key enzyme for scavenging superoxide anions and plays an important role in maintaining ROS homeostasis [28]. Therefore, the antioxidant enzyme Cu/Zn SOD can play an important role in immune regulation. The present study was the first to report the synergy of AjSOD1 in the immune system of the sea cucumber.

First, the coding genes of the AjSOD1 protein in sea cucumber were analyzed by bioinformatics. The full-length cDNA of AjSOD1 from *A. japonicus* was 459 nucleotides encoding a putative peptide of 152 amino acids. A phylogenetic tree was constructed and analyzed based on the coded nucleotide sequences of Cu/Zn SODs. The phylogenetic tree showed that the Cu/Zn SOD protein was mainly divided into two branches. The Cu/Zn SOD protein in vertebrates clustered into one branch and that in invertebrates clustered into another branch. Within that, the sea cucumber clustered with other invertebrates in a subgroup and had the closest Cu/Zn SOD relationship with the sea urchin. A BLAST search was conducted with the NCBI database for the protein sequence of AjSOD1. The results showed that the homology of the AjSOD1 protein with Cu/Zn SOD proteins from other species was high. Further amino acid sequence alignment showed that AjSOD1 had three highly conserved regions, the N-terminal region, the C-terminal region and the intermediate region. In humans, the N-terminal sequence of Cu/Zn SOD protein is used to mediate the formation of Cu/Zn SOD dimers and help the protein function; the highly conserved sequence at the other end is mainly involved in the binding of Cu/Zn SOD to copper and zinc ions, which is necessary for the catalytic function of Cu/Zn SOD [29]. These results suggest that AjSOD1 should have a similar biological function to that of the Cu/Zn SOD protein in humans and other organisms.

In this study, the mRNA expression levels of AjSOD1 were examined in different tissues. The results showed that AjSOD1 was expressed in all examined tissues, suggesting that it might be a ubiquitous enzyme without tissue specificity. However, its transcription level was higher in the intestine, respiratory tree and body wall and lower in the tentacles and coelomic cells. As two important organs in the cavity of sea cucumber, the intestine and respiratory tree are the main sites of microbial invasion in the sea cucumber. It was known that the intestine might be involved in the immune defense response, as it is the main tissue for the synthesis of immune defense factors and the main tissue for prevention of oxidative stress [30]. The respiratory organ is important in defending against invasion by microbes and toxins because it flushes water that contains a large number of pathogens and toxins [6]. The higher level of expression in the intestine and respiratory system suggested that AjSOD1 is a constitutive protein that may play important roles in sea cucumber defense.

On the basis of the tissue distribution, we studied the expression pattern of the AjSOD1 gene in both the intestine and coelomocytes after infection with *V. splendidus*. The innate immune system in sea cucumbers mainly includes two mechanisms: humoral immunity and cellular immunity [31–33]. The coelomocytes of the sea cucumber, which may originate from the axial organs, the blood system, the dermal connective tissue, and the coelomic epithelium, are immune effector cells with various cell types and immune histochemical features. Sea cucumber coelomic cells can penetrate into other tissues involved in the body's immune defense mechanism [31,33]. The regulation trend of SOD1 mRNA in the intestine and coelomocytes was similar. In the early stage of *V. splendidus* infection, the transcription levels of the AjSOD1 gene in the intestine at 1 h post-challenge and coelomic cells at 6 h post-challenge were significantly upregulated. The higher transcriptional level of the target gene remained for a long time. These results indicate that AjSOD1 is a very important defense mechanism in the innate immune system.

Further function of AjSOD1 was based on successful expression and purification of the recombinant AjSOD1 protein. Cu/Zn-SOD has no signal peptide, and intracellular experiments for Cu/Zn-SODs from both mammalian and marine species showed that Cu/Zn-SODs were distributed in the cytoplasm, serving as a classical intracellular protein [34,35]. In this study, the rAjSOD1 protein was purified from the soluble fraction of Cu/Zn-SOD overexpressed in *E. coli* cell lysate, indicating that the soluble rAjSOD1 with biological function could be generated in the bacterial host cells. In known species, Cu/Zn-SOD is mainly used to remove superoxide anions and has an antioxidant role. Therefore, the antioxidant activities of rAjSOD1 were first explored using rAjSOD1 over-expressed in *E. coli* cells. Our results showed that *E. coli* cells expressing the rAjSOD1 plasmid exhibited a higher tolerance to H_2O_2, which is a commonly used oxidative stress condition [36], than those with the blank plasmid, suggesting that rAjSOD1 protected cells against the oxidative environment.

As a unique enzyme capable of dismutating O_2^-, Cu/Zn-SOD is expected to be indispensable in the treatment of ROS-mediated normal tissue injuries originating from exposure to various factors. Studies of Cu/Zn-SOD in humans and other species showed that the expression of Cu/Zn-SOD was significantly upregulated after exogenous stimulation treatment, protecting cells against abnormal stress; therefore, Cu/Zn-SOD was considered to be a potential immune regulatory factor [37,38]. It was also observed that intravenous injection of bovine Cu/Zn-SOD into mice could significantly promote the recovery of erythrocytes, reticulocytes and white blood cells from X-irradiation-induced loss [38,39]. Additionally, the function of AjSOD1 was elevated by coelomocyte apoptosis when challenged with LPS or H_2O_2 compared with that of the groups treated with rAjSOD1 simultaneously. When cultured coelomic cells were treated with LPS or H_2O_2, extensive apoptosis and necrosis were observed. However, early necrosis (blue) and late apoptosis (red) of coelomic cells were significantly reduced when cells were treated with rAjSOD1. The results showed that the addition of rAjSOD1 could significantly reduce the damage to cells and improve the survival rate of cells. This demonstrated that the addition of rAjSOD1 protein into the culture medium of coelomic cells significantly reduced the lethal effects of LPS or H_2O_2 in the coelomocytes. Our results suggest that AjSOD1 could serve as an important immune protein in the sea cucumber innate immune system.

In conclusion, we found that AjSOD1 was distributed mainly in the intestine, which is the main defense tissue against pathogens and abnormal environments that can cause oxidative stress in sea cucumbers. The mRNA expression levels of AjSOD1 were upregulated in the intestine and coelomocytes, which play an important role in the innate immune system of the sea cucumber. The antioxidation activity of AjSOD1 was also demonstrated in this study. In addition, the addition of rAjSOD1 was found to significantly decrease the apoptosis of coelomocyte cells when challenged by LPS or H_2O_2 in primary culture experiments *in vitro*, which suggested that the AjSOD1 from the sea cucumber may play critical roles not only in the defense against oxidative stresses but also in the innate immune defense against bacterial infections.

Acknowledgments

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