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Short communication

Molecular cloning, characterization and expression analysis of cytoplasmic Cu/Zn-superoxid dismutase (SOD) from pearl oyster *Pinctada fucata*

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ABSTRACT

Because of its capacity to rapidly convert superoxide to hydrogen peroxide, superoxide dismutase (SOD) is crucial in both intracellular signalling and regulation of oxidative stress. In this paper we report the cloning of a Cu/Zn SOD (designated as pfSOD) from the pearl oyster (*Pinctada fucata*) using rapid amplification of cDNA ends (RACE) PCR. The full-length cDNA of this Cu/Zn SOD contains an open reading frame (ORF) of 471 bp coding for 156 amino acids. No signal peptide was identified at the N-terminal amino acid sequence of Cu/Zn SOD indicating that this pfSOD encodes a cytoplasmic Cu/Zn SOD. This is supported by the presence of conserved amino acids required for binding copper and zinc. Semi-quantitative analysis in adult tissues showed that the pfSOD mRNA was abundantly expressed in haemocytes and gill and scarcely expressed in other tissues tested. After challenge with lipopolysaccharide (LPS), expression of pfSOD mRNA in haemocytes was increased, reaching the highest level at 8 h, then dropping to basal levels at 36 h. These results suggest that Cu/Zn SOD might be used as a bioindicator of the aquatic environmental pollution and cellular stress in pearl oyster.

1. Introduction

The superoxide dismutases (SODs) are the first and most important of the antioxidant enzyme defense systems against reactive oxygen species, particularly the breakdown of superoxide anion into oxygen and hydrogen peroxide that prevents generation of highly toxic hydroxyl radicals. Superoxide dismutase (EC 1.15.1.1) is divided into four distinct groups according to their metal content: iron SOD (FeSOD), manganese SOD (MnSOD), copper/zinc SOD (Cu/Zn SOD), and nickel SOD (NiSOD). MnSOD and Cu/Zn SOD are found in both prokaryotes and eukaryotes, FeSOD is found in prokaryotes and plants [1], and NiSOD has recently been purified from several aerobic soil bacteria of Streptomyces [2]. SOD is one of the sensitive biomarker to indicate organisms being under stress [3,4].

Cu/Zn SOD is very important because of its physiological function and therapeutic potential. This enzyme requires Cu and Zn for its biological activity; the loss of Cu results in its complete inactivation, and is the cause of multiple diseases in human and animals [5–9]. There are two types of Cu/Zn SOD, extracellular Cu/Zn SOD with an N-terminal signal peptide for secretion, and cytoplasmic Cu/Zn SOD without signal peptide [10–13].

Both the transcription and enzyme activity of Cu/Zn SOD are sensitive to stresses such as exposure to heavy metals or biocides, like tributyltin, heat shock, and anoxia [3,4,14,15]. Cu/Zn SOD genes have been cloned from several aquatic species including frog, *Xenopus laevis* [16], grouper, *Epinephelus malbaricus* [17], Pacific oyster, *Crassostrea gigas* [18] and the abalones *Haliotis discus discus* [4]. So far, the Cu/Zn SOD from pearl oysters has not been elucidated. The present study is the first report of the characterization of Cu/Zn SOD in pearl oyster *Pinctada fucata*.

Pearl oyster, *P. fucata* is distributed along the South coast of India and is the most important bivalve mollusc for seawater pearl production in India. In 1972, the Central Marine Fisheries Research Institute started pearl culture research at natural pearl oyster beds in Tuticorin. The development of the pearl oyster hatchery technology in India in 1981 opened the way for commercial culture of this bivalve species. Recent decline in pearl production is mainly due to mortality of pearl oyster. The cause for high mortality is related to ocean pollution, disease outbreaks and stock degeneration [19,20]. In order to control disease and enhance the yields and quality of seawater pearls, it is necessary to study the innate immune defense mechanisms of pearl oysters, which lack the adaptive immune system. One major strategy to combat disease problem is to identify disease resistance genes and employ them for genetic improvement of cultured stock. Therefore, the aims of

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the present study were (1) to determine the nucleotide sequence of Cu/Zn SOD from the pearl oyster *P. fucata* and compare its deduced amino acid sequence to other known Cu/Zn SOD proteins; (2) to examine the expression of pfSOD in various tissues; and (3) to evaluate pfSOD expression after LPS challenge.

2. Materials & methods

2.1. Animal culture

Live individuals of adult *P. fucata* (about 4.5–5.5 cm in shell length and body weight 20–30 g) were collected from the Pearl Farm in Tuticurin, and maintained at 25 °C in tanks containing static aerated seawater (0.5 L/oyster) in the laboratory. The seawater was changed every day and the pearl oysters were fed with *Isochrysis galbana* twice daily. Animals were kept 2 weeks for acclimatization before they were used.

2.2. RNA isolation and cDNA cloning

Total RNA was extracted from the haemocytes of the adductor muscles using NucleoSpin RNA II reagent (MACHEREY-NAGEL GmbH & Co, Germany) as per the manufacturer's instructions and stored at −80 °C until further use. cDNA was synthesized with iScript cDNA synthesis (Bio-rad) in accordance with the manufacturer's protocols. Finally, synthesized cDNA was diluted 10 fold (total 200 μl) and stored at −20 °C. Primers were designed using Beacon designer (Bio-rad) from the sequence information of Pacific oyster *C. gigas* available in the data base (GenBank accession AJ496219). Polymerase chain reactions (PCR) were carried out using sense and antisense primers (Table 1) to obtain the open reading frame (ORF) of pfSOD. The reaction volume of 25 μl consisted of 2.5 μl of 10× PCR buffer, 0.5 μl of dNTP (10 mM), 1 μl of each primer (10 mM), 18.7 μl of PCR-grade water, 0.3 μl (1 U) of Taq polymerase (Sigma Aldrich) and 1 μl of cDNA. The PCR program consisted of an initial denaturation of 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s and the final extension step of 72 °C for 7 min. PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide (EB). The PCR product was then eluted and cloned into the pJET vector (Fermentas, EU) and transformed into competent *Escherichia coli* TOP10 cells. Positive clones were identified as white colonies on LB (Luria broth) agar and were used for sequencing in both directions.

The full-length Cu/Zn SOD cDNA of *P. fucata* was obtained by the reverse-transcription polymerase chain reaction (RT-PCR) and RACE methods. The 5′ region of the transcript was obtained in 5′-RACE reactions using the SMARTScribe™ Reverse Transcriptase (Clontech) according to the manufacturer's instructions. The primers were the pfSOD-specific antisense primer GSP1in combination with the universal primer mix (UPM) (Table 1) for RACE to derive the 5′-terminal untranslated region (UTR). For 3′-RACE, the pfSOD-specific sense primerGSP2and the universal primer mix (UPM) (Table 1) were used for amplification of the target cDNA. The PCR fragments were subjected to electrophoresis on 1.5% agarose gels to determine length differences. The amplified cDNA fragments were cloned into the pJET vector (Fermentas, EU) following the manufacturer's instructions. Recombinant clones were identified as white colonies on LB (Luria broth) agar and confirmed by colony PCR. Plasmids containing the inserted fragment were used as a template for DNA sequencing.

Table 1
Primers used in this study.

Primer	Sequence (5′–3′)
For conventional PCR	
Sense primer	ATGTCATCTGCTCTGAAGGCCGT
Antisense primer	CTACTTGGTGATACCGATCACTCCACA
For RACE PCR	
GSP1	GGTGATCCTGGAGCCTCTTGG
GSP2	AATCAGCATCACCGACAA
UPM mix	AAGCAGTGGTATCAACGCAGAGT–CTAATACGACTCACTATAGGGC
For RT-PCR	
pfSOD-F	AATCAGCATCACCGACAA
pfSOD-R	TTGGTGATACCGATCACTCCACA

2.3. Homology analysis

The sequence was analysed for identity and similarity to known sequences by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and multiple sequence alignment was generated using the CLUSTAL W program (http://www.ebi.ac.uk/clustalw/index.html). Signal peptide prediction was performed by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) [21] Protein family signatures were identified using InterPro program (http://www.ebi.ac.uk/InterProScan/).

2.4. Phylogenetic analysis

A phylogenetic tree was constructed based on the amino acid sequences of the selected Cu/Zn SODs (Fig. 2) using the WAG + G method with MEGA, version 5 [22]. To derive the confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times.

2.5. Immune challenge

For stimulation with LPS, animals were injected with 50 μl of LPS (*E. coli* 055:B5, #62326, Sigma–Aldrich, Munich, Germany) dissolved in PBS (LPS 10 μg ml^{-1}) into the adductor muscles of each pearl oyster. The control groups were injected with 50 μl of PBS. At each time point (0, 4, 8, 12, 24 and 36 h), haemolymph was collected from the control group and the LPS stimulation group. Haemolymph samples were withdrawn from the adductor muscles using a syringe and immediately centrifuged at 5000× *g* at 4 °C for 10 min to harvest the haemocytes. At each time point, five control and five LPS injected individuals were sampled. The haemocyte pellets were immediately used for RNA extraction. The tissues including adductor muscle, gill filaments, mantle, digestive gland, gonad, heart and haemocytes were collected from five healthy individuals to investigate the tissue-specific expression of pfSOD.

2.6. Semi-quantitative PCR

Semi-quantitative PCR was conducted to determine the relative expression of pfSOD in *P. fucata*. At defined time points pfSOD in the challenged oysters and vehicle controls were processed and quantified based on the gel band intensity using ImageJ analysis software [23]. Primers for semi-quantitative PCR were designed from the pfSOD cDNA sequence and are shown in Table 1. The PCR condition for pfSOD and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: initial denaturation at 94 °C for 3 min, then different cycles of amplification of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The GAPDH was amplified in PCR reaction as a loading control. The products were analysed on 2.0% agarose gel containing ethidium bromide. The cycle numbers at half-maximal amplification were used for subsequent quantitative analysis of gene expression. The PCR cycles, 28 cycles for pfSOD and 25 cycles for GAPDH were optimized as such that the target gene and house-keeping gene amplification were in logarithmic phase.

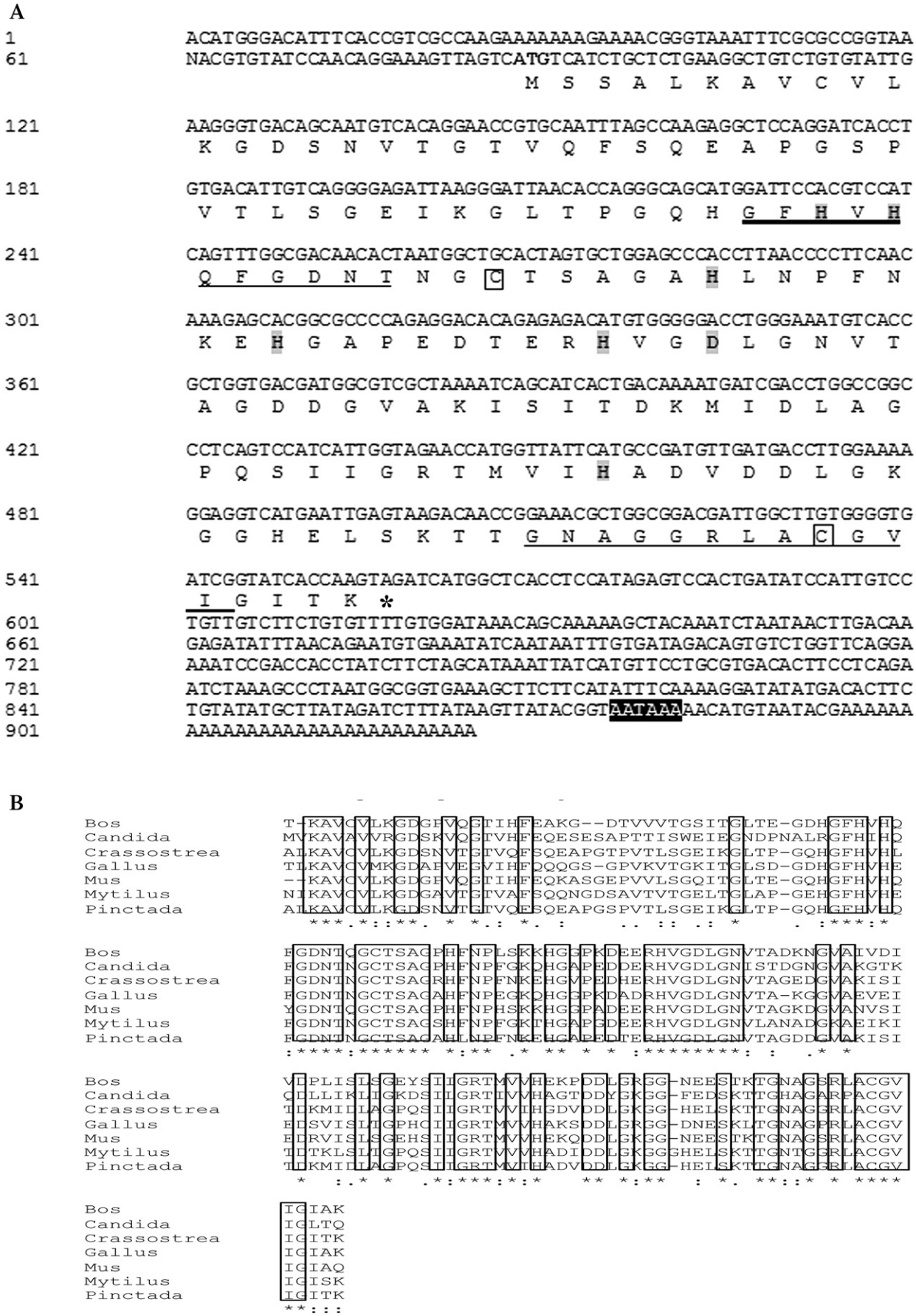

Fig. 1. (A) Nucleotide sequence of pfSOD cDNA from *P. fucata* and its deduced amino acid sequence. Two Cu/Zn SOD family signatures are underlined (_). The start codon is in bold and termination codon is indicated with asterisk (□). The amino acids required for binding of copper (His-49, -51, -66, and -123) and zinc (His-66, -74, and -83 and Asp-86) are shaded. Two cysteines (Cys 60 and Cys 149) predicted to be engaged in the disulfide bond formation were boxed. (B) The alignment of deduced amino acid sequence of pfSOD. Conserved regions are represented in box.

2.7. Statistical analysis

Multiple comparisons using Duncan's test were made to check the differences between the gene expression in the control and challenged oysters using SPSS13.0 software.

3. Results and discussion

RT-PCR was used to clone the open reading frame of SOD using total RNA extracted from haemocytes of *P. fucata*. A single PCR product of 471 bp was obtained. The size of this segment correlated well with SOD genes from other species. This partial cDNA sequence provided the necessary information to obtain an additional 368 bp sequence by 3′RACE, and an additional 87 bp sequence by 5′RACE. Finally, the full-length sequence information of the Cu/Zn SOD cDNA was obtained by overlapping the three cDNA sequences. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 1A. The full-length SOD cDNA is comprised of 924 bp, containing 87 bp in the 5′-terminal untranslated region (UTR), 471 bp in the ORF, 366 bp in 3′-terminal UTR with a poly(A) tail of 30 bp and a putative polyadenylation consensus signal (AATAAA). The ORF encodes a polypeptide of 156 amino acids. The SOD cDNA sequence and its deduced amino acid sequence were submitted to the NCBI GenBank under accession no (JX013537). No signal peptide was identified in the deduced amino acid sequence of Cu/Zn SOD by the signal P program, indicating that this pfSOD is a cytoplasmic Cu/Zn SOD.

Multiple alignment of the deduced amino acid sequences (Fig. 1B) with other closely related cytoplasmic Cu/Zn SOD sequences showed that three cysteines (Cys 9, Cys 60 and Cys 149) are present in the mature pfSOD. Cys 60 and Cys 149 are conserved in all Cu/Zn SODs and it is believed that those form an intramolecular disulfide bond. The amino acids required for binding of copper (His-49, -51, -66, and -123) and zinc (His-66, -74 and -83 and Asp-86) are also conserved. Two Cu/Zn SOD family signature sequences were found in the deduced amino acid sequence of pfSOD; signature 1 (consensus sequences: [GA]-[IMFAT]-H-[LIVF]-H-{S}-x-[GP]-[SDG]-x-[STAGDE].) and signature 2 (consensus sequences: G-[GNHD]-[SGA]-[GR]-x-R-x-[SGAWRV]-C-x(2)-[IV]). These family signature sequences are conserved in all Cu/Zn SODs. Several reports have shown that copper and zinc ions have critical functions in stabilizing the quaternary structure and therefore in the kinetic properties of Cu/Zn SOD [24–26]. BLAST analysis shows that the deduced amino acid sequence of pfSOD has extremely high identity with the Cu/Zn SOD of *Crassostrea hongkongensis*, *C. gigas* and *Mytilus chilensis* (99%). Similarly, it has high identity with Cu/Zn SOD of *Candida ariakensis* (98%) and *H. discus discus* (97%).

Phylogenetic relationships of Cu/Zn SOD from pearl oyster and other invertebrates and vertebrates were estimated. Cu/Zn SOD of *Candida albicans* was used as the out-group. As shown in Fig. 2, Cu/Zn SODs of pfSOD formed a separate cluster with cytoplasmic Cu/Zn SODs from oyster *C. gigas* and mussel *Mytilus edulis* indicative of the closer evolutionary relationship of *P. fucata* with other aquatic invertebrates. Vertebrates are evolutionarily distinctly separated.

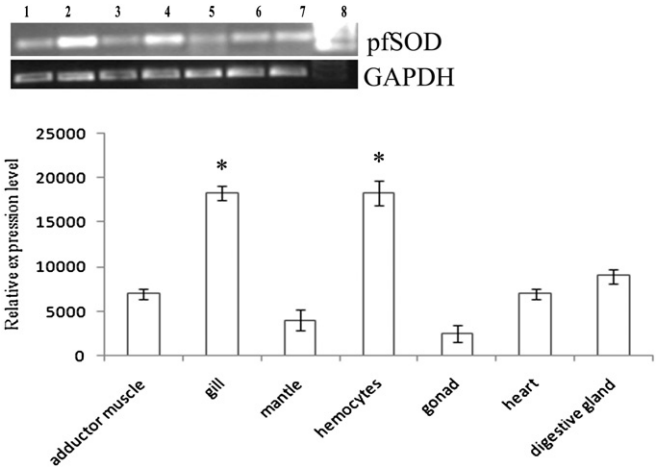

Fig. 3. Distribution of Cu/Zn SOD mRNA in different adult tissues of pearl oyster. Expression analysis of Cu/Zn SOD mRNA in different adult tissues of pearl oyster by RT-PCR.GAPDH was used as an internal control. Lane 1: adductor muscle. Lane 2: gill. Lane 3: mantle. Lane 4: haemocytes. Lane 5: gonad. Lane 6: heart. Lane 7: digestive gland. Lane 8: 100 bp ladder. The values are showed as mean ± S.E ($N = 5$).

RT-PCR was carried out to analyse the distribution of pfSOD mRNA in the adult tissues of the pearl oyster. RT-PCR analyses revealed that pfSOD mRNA is abundantly expressed in the gill and haemocytes. Levels are up to 3 fold higher than the moderately expressed pfSOD in the adductor muscle, mantle, gonad, heart and digestive gland (Fig. 3). Hence, haemocytes are considered as the most suitable tissue to analyse the pearl oysters immune function. This is in agreement with the report by Kuchel et al. [27] who found haemocytes defense enzyme expression in *Pinctada impricata*. As histological studies have revealed the presence of a large amount of haemocytes in bivalve gill tissues [28–30], a high expression level in gill is more likely associated with haemocyte abundance.

In order to investigate the immunological function of pfSOD in pearl oyster, *P. fucata* we determined the levels of pfSOD cDNA in haemocytes after challenge with lipopolysaccharides (LPS). LPS stimulation significantly increased pfSOD mRNA expression in the haemocytes in a time-dependent manner (Fig. 4). Over time pfSOD mRNA expression reached a significant increase 4 h after exposure to LPS. pfSOD mRNA levels further increased to reach a maximum at 8 h post treatment and then dropped to basal levels at 36 h. At the maximum the relative mRNA expression of pfSOD increased to

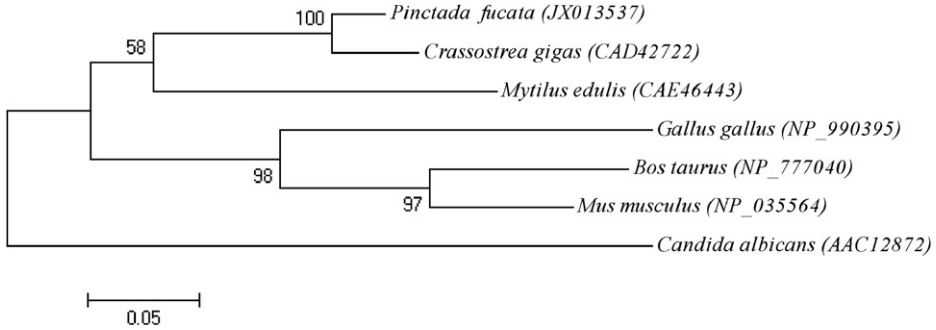

Fig. 2. Neighbour-joining phylogentic tree of pfSOD amino acid sequences from 7 species. Note: Numbers represent the bootstrap values. The amino acid sequences for the phylogenetic tree are shown in Fig. 1B.

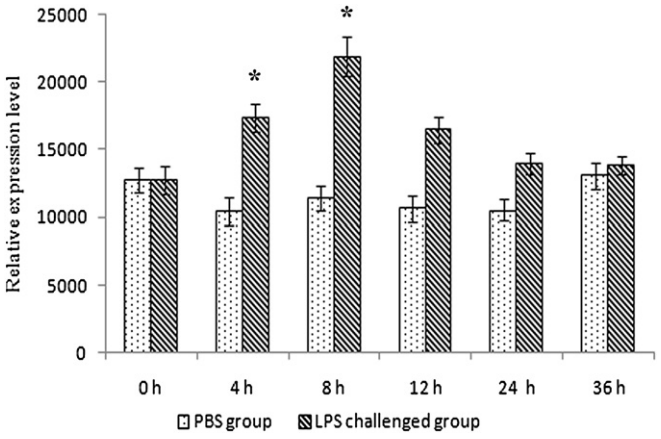

Fig. 4. Temporal expression pattern analysis of Cu/Zn SOD mRNA in haemolymph of the pearl oyster challenged with LPS. Vertical bars represent the mean ± S.E ($N = 5$). Significant differences ($P < 0.05$) are indicated with the asterisk (*).

2-fold over control. The high level of expression in haemocytes and gill suggest that pfSOD could be involved in the innate immune response.

In conclusion, the full-length cDNA of Cu/Zn pfSOD contains an open reading frame (ORF) of 471 bp coding for 156 amino acids. Semi-quantitative analysis in adult tissues showed that the pfSOD mRNA was abundantly expressed in haemocytes and gill. After challenge with lipopolysaccharide (LPS), expression of pfSOD mRNA in haemocytes was increased, reaching the highest level at 8 h, then dropping to basal levels at 36 h. These results suggest that Cu/Zn SOD could be used as a bioindicator of the aquatic environmental pollution and cellular stress in pearl oyster.

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