Fish & Shellfish Immunology 26 (2009) 821–827

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Molecular cloning and characterization of peroxiredoxin 6 from Chinese mitten crab *Eriocheir sinensis*

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ARTICLE INFO

Article history:
Received 28 July 2008
Received in revised form
1 October 2008
Accepted 13 October 2008
Available online 19 October 2008

Keywords:
Eriocheir sinensis
Peroxiredoxin 6 (Prx6)
Innate immunity
Gene cloning
mRNA expression
Bacteria challenge

ABSTRACT

Peroxiredoxin is a superfamily of antioxidative proteins that play important roles in protecting organisms against the toxicity of reactive oxygen species (ROS). In this study, the full-length cDNA encoding peroxiredoxin 6 (designated EsPrx6) was cloned from Chinese mitten crab *Eriocheir sinensis* by using rapid amplification of cDNA ends (RACE) approaches. The full-length cDNA of EsPrx6 was of 1076 bp, containing a 5′ untranslated region (UTR) of 69 bp, a 3′ UTR of 347 bp with a poly (A) tail, and an open reading frame (ORF) of 660 bp encoding a polypeptide of 219 amino acids with the predicted molecular weight of 24 kDa. The conserved Prx domain, AhpC domain and the signature of peroxidase catalytic center identified in EsPrx6 strongly suggested that EsPrx6 belonged to the 1-Cys Prx subgroup. Quantitative real-time RT-PCR was employed to assess the mRNA expression of EsPrx6 in various tissues and its temporal expression in haemocytes of crabs challenged with *Listonella anguillarum*. The mRNA transcript of EsPrx6 could be detected in all the examined tissues with highest expression level in hepatopancreas. The expression level of EsPrx6 in haemocytes was down-regulated after bacterial challenge and significantly decreased compared to the control group at 12 h. As time progressed, the expression level began to increase but did not recover to the original level during the experiment. The results suggested the involvement of EsPrx6 in responses against bacterial infection and further highlighted its functional importance in the immune system of *E. sinensis*.

1. Introduction

Aerobic organisms have developed a set of defence systems to mitigate the damaging effects of reactive oxygen species (ROS) [1]. In general, major ROS existing in cell include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), and they are important in many biochemical processes including immunity, cell proliferation, cell differentiation, signal transduction and ion transport [2]. However, high levels of ROS may cause metabolic malfunctions and damage to biological macromolecules, resulting in various diseases [3,4]. In normal physiological state, the balance between ROS and antioxidants is tightly controlled [5,6]. To maintain this homeostasis and counteract potential ROS-induced damage, cellular defence mechanisms have evolved to protect against oxidative damage [7,8], such as to minimize the formation of ROS or to inactivate them if they are produced [9].

Peroxiredoxin (Prx) is a superfamily of nonselenium peroxidases, which has been demonstrated to play important roles in reducing and detoxifying hydrogen peroxide [10,11], peroxinitrite and a wide range of organic hydroperoxides (ROOH) [12]. Several lines of evidence suggested that peroxiredoxins played a physiologically important role in the enzymatic removal of ROS [13] and to protect the organism cells against highly reactive oxidative stress. Recently, multiple *Prx* genes have been cloned and identified from a variety of organisms [12,14,15]. In mammalian, there are six isoforms of Prx (Prx1–Prx6), which are classified into three subgroups (2-Cys, atypical 2-Cys and 1-Cys) based on the number and position of cysteine residues participated in catalysis [12,16]. Prx1–Prx4 belongs to the 2-Cys subgroup, and Prx5 containing two additional cysteine residues constitutes the second subgroup, atypical 2-Cys. Prx6 is classified into 1-Cys Prx family for its one conserved cysteine residue. Several 1-Cys Prx (or Prx6) have also been isolated from vertebrate and invertebrate species [13,17–19]. However, no information about the Prx6 is currently available in crustacean.

Chinese mitten crab *Eriocheir sinensis* is an economical aquatic species widely farmed in the south of China. With the development of intensive culture and environmental deterioration, various diseases caused by bacteria, viruses and rickettsia-like organisms had frequently occurred in cultured *E. sinensis* populations [20–22],

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doi:10.1016/j.fsi.2008.10.006

which resulted in enormous losses to the crab aquaculture. Better understanding of the knowledge on the immune defence system of crab will be beneficial to the development of health management and disease control in crab aquaculture. The main objectives of this study were (1) to clone the full-length cDNA of Prx6 from *E. sinensis*, (2) to investigate the tissue distribution of EsPrx6 transcript, and (3) to characterize the temporal profile of EsPrx6 mRNA to *Listonella anguillarum* challenge.

2. Materials and methods

2.1. Animals, bacterial challenge and haemocyte collection

Healthy *E. sinensis*, averaging 36 g in weight, were collected from local market in Qingdao, China, and acclimated at 23 ± 2 °C for one week before processing. One hundred crabs were employed for the bacterial challenge experiment. The crabs were randomly divided into 3 groups and each group contained 30 individuals. The groups receiving an injection of 50 μL alive *L. anguillarum* suspended in 0.1 mol L^{-1} PBS (pH7.0) (O.D.600 = 0.4) or the same volume of PBS were used as challenge group and control group, respectively. The untreated group was employed as blank group. After treatment, the crabs were returned to water tanks and 5 individuals were randomly sampled at 3, 6, 12 and 24 h post-injection. The haemolymph was collected from cheliped using a syringe with an equal volume of anticoagulant (27 mmol L^{-1} sodium citrate, 336 mmol L^{-1} NaCl, 115 mmol L^{-1} glucose, 9 mmol L^{-1} EDTA, pH 7.0) [23], and centrifuged at 1000×*g*, at 4 °C for 10 min to harvest the haemocytes for RNA preparation. Gill, muscle, heart, gonad, haemocytes and hepatopancreas from untreated crabs were collected to determine the tissue distribution of EsPrx6 transcript. Total RNA was immediately extracted using Trizol reagent according to the manufacture's protocol (Invitrogen).

2.2. cDNA library construction and EST analysis

A cDNA library was constructed from the haemocytes of *E. sinensis* challenged with *L. anguillarum* and *Micrococcus lysodeikticus*, using the ZAP-cDNA synthesis kit and ZAP-cDNA GigapackI II Gold cloning kit (Stratagene). Random sequencing of the library using T3 primer yielded 7500 successful sequencing reactions. BLASTx analysis of all the EST sequences revealed that one EST (EST No. ZCBA0358; Contig253; length: 441 bp) was homologous to Prx6 in sponge *Suberites domuncula* (CAC38779), and it was selected for further cloning of Prx6 from *E. sinensis* (designated EsPrx6).

2.3. Cloning the full-length cDNA of EsPrx6

Two specific primers P1 and P2 were designed based on the sequence of EST (ZCBA0358) to clone the full-length cDNA of Prx6 from *E. sinensis*. PCR reactions to get the 5′ and 3′ end of EsPrx6 were performed in a PTC-100 Programmable Thermal Controller Cycler (MJ Research) using sense primer P1 and reverse primer P3 or P2 and P4 (Table 1). The PCR products were gel-purified and cloned into pMD18-T simple vector (Takara, Japan). After being transformed into the competent cells of *Escherichia coli* Top10F', the positive recombinants were identified through anti-Amp selection and PCR screening with M13-47 and RV-M primers (Table 1). Three of the positive clones were sequenced on an ABI3730 Automated Sequencer (Applied Biosystem).

2.4. Sequence analysis

The homology searches of nucleotide and protein sequences were conducted with BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.gov/blast). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org). SignalP 3.0 program was utilized to predict the presence and location of signal peptide, and the cleavage sites in amino acid sequences (http://www.cbs.dtu.dk/services/SignalP).

Table 1
Oligonucleotide primers used in the experiment.

Primer	Sequence (5′–3′)	Sequence information
P1 (reverse)	TGCGGCAGTCTTTTCATCAG	EsPrx6 specific primer
P2 (forward)	CCCTTGGTATGATTGACCCTGATG	EsPrx6 specific primer
P3 (forward)	GGCCACGCGTCGACTAGTACG_{10}	Adaptor primer
P4 (reverse)	GGCCACGCGTCGACTAGTACT_{17}	Oligo (dT)
P5 (forward)	ACCCATCGGACTACACCCCAG	Real-time EsPrx6 primer
P6 (reverse)	GGACCAATGACAAAGACAGCA	Real-time EsPrx6 primer
P7 (forward)	GCATCCACGAGACCACTTACA	Real-time actin primer
P8 (reverse)	CTCCTGCTTGCTGATCCACATC	Real-time actin primer
T3 (forward)	AATTAACCCTCACTAAAGGG	Vector primer
T7 (reverse)	GTAATACGACTCACTATAGGGC	Vector primer
M13-47	CGCCAGGGTT TTCCCAGTCACGAC	Vector primer
RV-M	GAGCGGATAACAATTTCACACAGG	Vector primer

2.5. Multiple sequences alignment and phylogenetic analysis

Multiple alignment of the EsPrx6 was performed with the ClustalW multiple alignment program (http://www.ebi.ac.uk/clustalw) and multiple alignment show program (http://www.biosoft.net/sms/index.html). A phylogenic NJ tree was constructed with Mega 3.1 software package (http://www.megasoftware.net). To derive the confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times.

2.6. Tissues distribution and temporal expression of EsPrx6 mRNA in haemocytes post L. anguillarum challenge

The mRNA expression of EsPrx6 in various tissues, including hepatopancreas, gill, gonad, muscle, heart and haemocytes of unchallenged crabs, and the temporal expression of EsPrx6 in haemocytes of crabs challenged with *L. anguillarum* were determined by quantitative real-time RT-PCR. Total RNA from different tissues and haemocytes was extracted according to the protocol of Trizol (Invitrogen). M-MLV reverse transcriptase (Promega) was used to synthesize single-strand cDNA with the DNase I (Promega)-treated total RNA and oligo (dT) primer (Table 1). The mixture was incubated at 42 °C for 1 h, terminated by heating at 95 °C for 5 min, and subsequently stored at −80 °C.

The cDNA was diluted 100 times by the DEPC-treated water for the next step. The quantitative real-time RT-PCR was carried out in a total volume of 25 μL, containing 12.5 μL of 2 × SYBR Green Master Mix (Applied Biosystems), 2 μL of the diluted cDNA, 0.5 μL of each of primers (10 μmol L^{-1}), 9.5 μL of DEPC-water. A 295 bp product was amplified with P5 and P6 (Table 1, designed on the basis of the full cDNA sequence of EsPrx6) from cDNA template, and then sequenced to verify the PCR specificity. Two β-actin primers, P7 and P8 (Table 1) were used to amplify a 266 bp fragment as an internal control to verify the successful reverse transcription and to calibrate the cDNA template.

The SYBR Green RT-PCR assay was carried out in an ABI PRISM 7300 Sequence Detection System (Applied Biosystems). In a 96-well plate, each sample was run in triplicate along with the internal control gene. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. After the PCR program, data were analyzed with the ABI 7300 SDS software (Applied Biosystems). To maintain consistency, the baseline was set