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Identification and expression of cathepsin B from the freshwater mussel *Cristaria plicata*

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ABSTRACT

Cathepsin B plays crucial roles in host immune defense against pathogen infection. In present study, a cathepsin B gene from the freshwater mussel, *Cristaria plicata* (CpCathB) was cloned and characterized. The full-length cDNA of CpCathB was 1825 bp, and contained a 5′ untranslated region (UTR) of 36 nucleotides, an open reading frame (ORF) of 1044 bp and a 3′ UTR of 745 bp with a poly (A) tail. The deduced CpCathB protein was encoded as a preproenzyme with 347 amino acid residues and predicted molecular weight of 38.55 kDa. Sequence alignment revealed that CpCathB protein shared 56% - 60.7% identity comparison with other species. The predicted tertiary structure of CpCathB protein was highly similar to that of human. The CpCathB mRNA was expressed in hemocytes, hepatopancreas, adductor muscle, gills and mantle tissues of healthy mussels, and the highest expression level was in hepatopancreas. The transcripts of CpCathB were increased in hemocytes and hepatopancreas from mussels after *Aeromonas hydrophila* challenge. Moreover, the recombinant CpCathB was expressed in the *Escherichia coli* Rosetta-gami (DE3) strain. The maximum titer of the anti-CpCathB polyclonal antibodies was 1:640,000.The CpCathB protein had a higher expression in hepatopancreas and mantle and a lower level in hemocytes.

1. Introduction

Cathepsins are a group of proteolytic enzymes origins of lysosomal and are classified into aspartic proteases, serine and cysteine according to the amino acid residue in active site(Turk and Stoka, 2007; Conus and Simon, 2010). They have important functions of protein degradation, antigen processing, protein precursor processing, cell apoptotic processing, and immune responses (Honey and Rudensky, 2003; Hsing and Rudensky, 2005; Conus and Simon, 2010). Cathepsin B is a member of cysteine proteases that thus far found in all eukaryotic organisms (Sajid and McKerrow, 2002). Most of them exhibit the distinctive characteristics of endopeptidase and carboxyl dipeptidyl peptidase (exopeptidase) activities (McGrath, 1999). Cathepsin B is synthesized as an inactive preproenzyme, and is activated by autoproteolysis in acidic environment (Cathers et al., 2002). Studies in mammalian have evidenced that cathepsin B plays important roles in pathological and physiological processes, such as apoptosis (Blomgran et al., 2007), cancer (Peri et al., 2011), TLR9 signaling pathway (Matsumoto et al., 2008), and TNF-α post translational processing in macrophages (Ha et al., 2008).

The role of cathepsin B in the host defense systems of aquatic species have been described. For example, cathepsin B mRNA can be induced by virus, bacteria, Lipopolysaccharides (LPS) and Poly I:C in fish and decapods crustaceans (Aoki et al., 2003; Zhang et al., 2008; Whang et al., 2011; Stephens et al., 2012; Li et al., 2013; Wei et al., 2014; Dai et al., 2016). The expression level of cathepsin B mRNA from sea cucumber *Apostichopus japonicus* was upregulated after *Vibrio splendidus* and LPS exposed coelomocytes (Chen et al., 2017). In molluscs, only few cathepsin B proteases have been identified. The studies from *Meretrix meretrix* and *Ostrea edulis* suggested that cathepsin B proteases were involved in digestion, yolk processing, embryogenesis and in resistance to parasites infection (Wang et al., 2008; Yao et al., 2011; Morga et al., 2012). The activity of cathepsin B has been shown to increased during early development of *Crassostrea gigas* (Donald et al., 2003). The transtripts of cathepsin B from *Sinonovacula constricta* and *Haliotis discus hannai* were upregulated after bacteria challenge (Niu et al., 2013; Qiu et al., 2013), indicating that cathepsin B was involved in the immune response against bacteria in molluscs.

Cristaria plicata is one of economically important freshwater mussel species that is cultured in the pearl industry in China. In recent years, *C. plicata* has experienced considerable economic losses due to an alarming increase in the prevalence and virulence of pathogenic infections. In previous work, we have cloned a cathepsin L gene of *C. plicata*, and the transtripts of cathepsin L were upregulated after *A.*

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hydrophila challenge (Hu et al., 2014). In this study, a full-length cDNA of cathepsin B from *C. plicata*, was cloned and characterized, and the tissues distribution and spatial expression profiles were evaluated after *A. hydrophila* challenge. The titer of the anti-CpCathB polyclonal antibodies was examined.

2. Materials and methods

2.1. Experimental animal and bacteria challenge

The freshwater mussels *Cristaria plicata* with shell length of 20.2 ± 4.7 cm, were collected from Poyang Lake in Jiangxi province, China, and were cultured in plastic water tanks for one week before experiment. Water temperature was 25 °C, pH 6.7, dissolved oxygen in water was continuously supplied by electric air compressors and was > 5.0 mg/L, and partial water was changed every day. The mussels were fed twice daily on *Scendesmus obliquus* and *Chlorella vulgaris* in the whole experiment process. *A. hydrophila* strain (No. XS 91-4-1) was purchased from Institute of Hydrobiology, Chinese Academy of Science, which was grown in LB broth at 37 °C with shaking at 210 rpm until reaching OD600 to 0.8. The culture was collected by centrifuge at 7000 rpm for 10 min. 135 mussels were randomly divided into three parallel challenge groups and one control groups in the aerated tanks. Each challenge group included 30 mussels and control groups included 45 individuals. 0.1 mL living *A. hydrophila* with 10^7 cell/mL was re-suspended in phosphate buffer soltion (PBS, pH 7.2), and was injected into the anterior adductor muscles of mussels in the challenge group. The control group received an injection with 0.1 mL PBS. All injected mussels were returned to water tanks. No mussel death was found during the whole experiment. After 6, 12, 24 and 48 h, three mussels from each challenge groups and nine individuals from control group were randomly removed at each time point. 0.1 g hepatopancreas and gill tissues from individual mussel were collected. Ten mL hemolymph were collected using a syringe from posterior adductor muscle and were centrifuged at 3000 *g*, 4 °C for 10 min to harvest the hemocytes. For the challenge group, hepatopancreas, gill and hemocytes of three individuals from parallel groups were mixed to homogenize as a sample. Likewise, tissues of three mussels from the control group were mixed as a sample. Tissue collected from mussels at 0 h served as the black.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from each collected tissue using Trizol reagent (TaKaRa, DaLian, China) according to the protocol. RNase-Free DNase I was utilized to remove contaminating genomic DNA (Promega, USA). The purity and quantity of the extracted RNA was quantified by the ratio of OD 260/OD 280 from the Ultraviolet spectrophotometer UV-5100 (Metash). Then, five μg of total RNA was used to synthesize smart cDNA by the PrimeScript II 1st strand cDNA Synthesis kit (TaKaRa, DaLian, China). The cDNA mix was stored at −20 °C. Rapid amplification of cDNA ends PCR (Race-PCR) and quantitative real-time PCR (qRT-PCR) were performed to clone the full-length cDNA, and tissue-specific expression changes were determined in response to bacteria challenge.

2.3. Cloning the full-length cDNA of CpCathB

The EST sequence of cathepsin B was searched in the hemocytes cDNA library of *C. plicata* from our laboratory. Based on the known fragment, four pair gene specific primers (Table 1) were designed using Primer 5.0 software to amplify the 3′ and 5′ end of CpCathB by nest PCR and rapid amplification of cDNA ends, The 3′ -RACE PCR was performed with smart cDNA template using the primer pair of CB-3F1/UPM in the first round PCR and CB-3F2/UPM in the second round. For the 5′ -RACE, CB-5R1/UPM and CB-5R2/UPM was used in the first round and the second round PCRs, respectively. All of the PCR programs were performed in a 50 μL reaction volume containing 33.7 μL PCR-gradewater, 5.0 μL 10 × Ex Taq Buffer, 4.0 μL dNTP Mix (2.5 mM), 5.0 μL UPM primer, 1.0 μL gene-specific primer, 1.0 μL cDNA template (smart cDNA for the first round PCR, the product of the first round PCR diluted by 1: 50 for the second round), 0.3 μL ExTaq Polymerase (1 U/μL) (TaKaRa, DaLian, China). PCR amplification conditions were an initial denaturation for 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 35 s at 68 °C, 1 min at 72 °C, and a final extension step of 72 °C for 10 min. All PCR reactions were performed in the T100™ Thermal Cycler (BIO-RAD, USA). The PCR products were resolved by 1% agarose gel electrophoresis and were purified using a DNA gel extraction kit (TaKaRa). Purified PCR fragments were ligated into the PMD-18-T-vector which was transformed into competent cells of *E. coli* DH5α, and recombinants were identified by PCR using primers in Table 1. Positive clones were sequenced by Sangon Biotech (Shanghai) Co. Ltd. Full length cDNA sequences were obtained by combining the 3′-end and corresponding sequences.

2.4. Sequence analysis

Nucleotide sequence similarities were identified by the BLASTn algorithm at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The open reading frame and deduced amino acid sequence, molecular mass, and theoretical isoelectric point were analyzed with the Expert Protein Analysis System (http://www.expasy.org/). Multiple sequence alignment was performed by ClustalW (http://www.ebi.ac.uk/clustalw/). The putative signal peptide prediction was made by SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/). The protein domain feature of CpCathB was predicted using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). The phylogenetic tree was constructed from the deduced amino acid sequence using the Neighbor-Joining (NJ) method of MEGA version 5.01. Reliability of the tree was assessed by 1000 bootstrap repetitions. GenBank accession number of the amino acid sequences in the phylogenetic tree was shown in supplemental file 1. The presumed tertiary structures were established for the CpCathB protein using the SWISS-MODEL prediction algorithm (http://swiss.model.expasy.org/) and displayed by DeepView/Swiss-Pdb Viewer version 4.1. The overall stereo chemical quality of the final model was assessed by the program PROCHECK (http://services.mbi.ucla.edu/SAVES/PROCHECK). Ramachandran plot statistics of the model was also calculated. The structural quality of the model was also verified using the program Verify-3D (http://services.mbi.ucla.edu/ SAVES/Verify_3D/).

2.5. Tissues distribution and temporal expression of CpCathB mRNA post A. hydrophila challenge

The mRNA expression of CpCathB in various tissues from healthy mussels, including hepatopancreas, gill, muscle, hemocytes and mantle tissue, and the temporal expression of CpCathB in hemocytes, gills, hepatopancreas of *C. plicata* challenged with *A. hydrophila* were determined by quantitative real-time-PCR (qRT-PCR). Total RNA extraction and cDNA synthesis were as described above. SYBR Green qRT-PCR was carried out in the CFX96™ Real-Time System (BIO-RAD). The β actin gene was amplified as the internal control with the primers β-actin F and β-actin R, a 150 bp CpCathB fragment was amplified using CBTF and CBTR (Table 1). The amplification efficiency of all primers was determined by standard curves, and the primers with amplification efficiency between 0.9 and 1.0 were chosen for use in the present study. The qRT-PCR was performed in a total volume of 20 μL containing 10 μL 2 × SYBR Premix Ex Taq™ buffer (TaKaRa, Dalian, China), 1 μL each primer, 1 μL cDNA, and 7 μL RNase-free H_2O. The PCR procedure was initial denaturation at 95 °C for 10 min, followed by 40 cycles of at