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Short communication

Characterization and function of a cathepsin B in red crayfish (*Procambarus clarkii*) following lipopolysaccharide challenge

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ABSTRACT

Cathepsin B is a lysosomal cysteine protease of the papain-like enzyme family with multiple biological functions. In the present study, a cathepsin B gene (named *PcCTSB*) was cloned and characterized from the red crayfish, *Procambarus clarkii*. The cDNA fragments of *PcCTSB* was 990 bp in length. It encoded a putative protein of 329 amino acid residues with predicted molecular weight of 36.4 kDa and isoelectric point of 7.020. Sequence alignment revealed that *PcCTSB* protein is 53.6%–80.4% identical with those from other 10 species. The predicted tertiary structure of *PcCTSB* protein was highly similar to that of animals. The results of the phylogenetic analysis indicated that the *PcCTSB* protein could be clustered with the *Eriocheir sinensis* cathepsin B protein. The recombinant protein of *PcCTSB* was expressed successfully in *Escherichia coli* cells. The mRNA expressions of *PcCTSB* were detected in all tested tissues, particularly high in the hepatopancreas. After lipopolysaccharide (LPS) challenge, the expression levels of *PcCTSB* were up-regulated significantly at different time points compared with control. Our results suggested that the *PcCTSB* might play an important role in defending against the pathogenes infection.
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1. Introduction

Lysosomes contain a large number of cathepsins. Based on the amino acid residues in their active sites, these cathepins can be divided into three groups: cysteine proteases (including cathepsins B, C, F, H, K, L, O, S, T, U, V, W, and X), serine proteases (including cathepsins A and G) and aspartate cathepsins (including cathepsins D and E) [1]. The 11 types of members (including cathepsins B, C, H, F, K, L, O, S, V, W and X) have been identified in human cathepsins [2]. Previous studies have demonstrated that cysteine proteinases perform crucial functions in different levels of biological processes, such as intracellular protein degradation [3,4], antigen processing [5], and inflammatory responses [6–8]. Most cathepsins are endopeptidases, whereas cathepsin B function as both exopeptidase and endopeptidase [9]. The first cathepsin B was identified from rat and published in the 1983s [10].

In recent years, with the employment of molecular biology techniques and with the help of largely increased databases, the structures and characters of cathepsins had been well characterized in many species [11]. In animals, cathepsin B had been identified in *Gallus gallus*, *Sus scrofa*, *Pandalus borealis*, *Myzus persicae*, *Bombyx mori*, and so on [12–16]. In plants, cathepsin B had been reported in *Triticum aestivum* and *Picrorhiza kurrooa* [17,18]. In protists, cathepsin B had been reported in *Uronema marinum* [19]. In addition, the high expression of cathepsin B and cathepsin L had been detected in non-Hodgkin lymphoma (NHL) samples [20]. In the channel catfish, the wide distribution and regulation across catfish tissues suggested catfish cathepsin B was not only involved in the acute inflammatory responses, but also played key roles in the pathogen triggered immune response after pathogen entry and recognition [21]. In the tongue sole, the cathepsin B showed a significant trend of up-regulated after bacterial challenge, which indicated an involvement of cathepsin B in host immune response against bacterial infection [22]. However, further investigation is required to clarify the role of cathepsin B in the immune responses and in the other physiological processes.

To date, no cathepsin B had been reported in the red cratfish (*Procambarus clarkii*). In the present study, we cloned and characterized a cathepsin B (*PcCTSB*) from *P. clarkii*. Moreover, a recombinant expression of the protein was carried out in *Escherichia coli*. Finally, the expression profiles of *PcCTSB* in different tissues of the cratfish were analyzed. Our present results could provide insight

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for better understand the biological function of *PcCTSB* in crayfish as well as other species.

2. Materials and methods

2.1. Immune challenge of crayfish

P. clarkii crayfishes of both sexes, each about 10 g in mass, were brought from an aquatic products market of Hefei (Anhui, China) in May. Before tissue collection, crawfish were cultured in a tank of continuously flowing water (circulating water system, Goldbill Ltd., Ningde City, China) which had been filtered through a Brimak/carbon filtration unit (Silverline Ltd., Winkleigh, UK) [23,24]. These crayfishes were randomly divided into two groups, one group was challenged by injecting 10 μl LPS (1 μg/μl) into the coelomic cavity, the control group was injected with 10 μl PBS. Eight crayfishes from each group were dissected to collect gill, hepatopancreas, hemocytes, gut, muscle and stomach at different time points after injection, respectively. These tissues were frozen immediately in liquid nitrogen and stored at −80 °C.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from each collected tissue using RNAiso Plus (TaKaRa, Dalian, China) according to the protocol. RNase-Free DNase I was used to remove contaminating genomic DNA (Promega, USA). The purity and quantity of the extracted RNA were quantified by the ratio of OD_{260}/OD_{280} by a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Wilmington, DE). Then, 1 μg of total RNA was used to synthesize first strand of cDNA using a TUREscript cDNA Synthesize Kit (Aidlab Biotech Co. Ltd., Beijing). The cDNA samples were diluted to 150 ng/μl with RNase-free water and employed as templates in quantitative real-time PCR (qRT-PCR) analysis.

2.3. Cloning and sequencing of PcCTSB cDNA

The cDNA fragments of *PcCTSB* were amplified by polymerase chain reaction (PCR) with primers (Table 1), which were designed with Primer premier 5.0 software package on the basis of a SSH pupal cDNA library constructed in our laboratory. PCR reactions were carried out in a 50 μl reaction volume, including 5 μl 10 × Taq buffer (Mg2+ plus), 4 μl dNTP (2.5 mM), 1.5 μl cDNA template from a single specimen, 2 μl each primer (10 μM), 35 μl sterilized distilled water and 0.5 μl (1 unit) taq (Aidlab Co., Beijing, China). Conditions for PCR amplification were as follows: an initial denaturation for 4 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 35 s at 51 °C, 1 min at 72 °C, and a final extension step of 72 °C for 10 min. All PCR reactions were performed in an BIO-RAD thermal cycler. The above PCR products were resolved by agarose gel electrophoresis (1% w/v) and purified using a DNA gel extraction kit (TaKaRa Co., Dalian, China). The purified PCR fragments were ligated into the T-vector (TaKaRa Co., Dalian, China) and then transformed into competent *Escherichia coli* DH5α. The positive recombinant clone with an insert was sequenced at least three times (Invitrogen Co., Ltd., Shanghai, China).

Table 1
The primers used in this study.

Primer no.	Primer sequences (5′–3′)	Purpose
F1	ATGAAGTCACTGGTGGTACTGGT	RT-PCR
R1	CTAAGGTAGACCAGCAGTAATCT	RT-PCR
F2	CCGGAATTCATGGCAGTAGTTGAGGATCCCA	Protein expression
R2	CCGCTCGAGCTAAGGTAGACCAGCAGTAATCT	Protein expression
F3	AGGCTGGTCGCAACTTCAACAAAC	qRT-PCR
R3	AATGCCCAACAAGATCCACACGAC	qRT-PCR
18S F	CTGTGATGCCCTTAGATGTT	qRT-PCR
18S R	GCGAGGGGTAGAACATCCAA	qRT-PCR

Note: “ ” present Restriction Enzyme cutting site: *EcoR* I.
“” present Restriction Enzyme cutting site: *Xho*I.

2.4. Sequence analysis

The data of DNA sequences were edited and analyzed using DNAStar software (DNASTAR Inc., Madison, Wisconsin, USA) and all sequences generated were used to search for similarity using BLASTN and BLASTP at web servers of the National Center of Biotechnology Information. The amino acid sequences of *PcCTSB* were predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The molecular weight (MW) and the isoelectric point (pI) of the deduced amino acid sequences were calculated by ExPASy (http://web.expasy.org/compute_pi/). In addition, ExPASy server was also used to analyze the *N*-glycosylation sites. The signal peptide and functional domain were predicted by using software SMART (http://smart.embl-heidelberg.de/). The deduced amino acid sequences of *PcCTSB* from *P. clarkii* were submitted for automated protein structure homology modeling using the swissmodel protein fold server (http://swissmodel.expasy.org).

2.5. Homologous alignment and phylogenetic analysis

In order to construct the phylogenetic tree of cathepsin B, the amino acids sequences of cathepsin B from *P. clarkii* as well as the other species including *Penaeus monodon*, *Fenneropenaeus chinensis*, *Litopenaeus vannamei*, *Marsupenaeus japonicus*, *Macrobrachium nipponense*, *Exopalaemon carinicauda*, *Eriocheir sinensis*, *Aedes aegypti*, *Lutjanus argentimaculatus*, *Ictalurus punctatus*, *Homo sapiens* and *Scophthalmus maximus* were selected and downloaded from NCBI databases. Multiple sequence alignments were carried out using Clustal X software with its default parameters [25]. Phylogenetic tree was constructed by MEGA version 5.0 using the neighbor-joining (NJ) method with bootstrap test of 1000 replications [26].

2.6. Construction of recombinant plasmids and protein expression

The forward primer F3 and reverse primer R3 (restriction enzyme sites for *EcoR* I and *Xho* I were underlined) were designed to amplify the complete open reading frame of *PcCTSB* by PCR under the same conditions (Section 2.3). The PCR product and pET-28a vector were ligated after they were both digested with restriction enzymes *EcoR* I and *Xho* I. The resulting recombinant plasmids were confirmed by DNA sequencing and then transformed to *Escherichia coli* BL21 (DE3) cells (TransGen, Beijing, China). After being induced by different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 4 h, the cells were harvested by centrifugation, resuspended, sonicated and then centrifuged again. The recombinant proteins were analyzed with 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After being separated by SDS-PAGE, the recombinant proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by an electrophoretic transfer system. Membranes were blocked with 5% non-fat milk in PBST (PBS contained 0.1% Tween-20) for 2 h at room temperature, washed with PBST and subsequently incubated with anti-His tag antibodies (diluted 1:2000 with 5% non-fat milk in PBST) for 2 h at room temperature. After washing with PBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (TransGen, Beijing, China) (diluted 1:8000 with 5% non-fat milk in PBST) for 50 min at room temperature. The final detection was performed with a HRP-DAB Detection Kit (Sangon, Shanghai, China).

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