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Molecular characterization and expression analysis of cathepsin L1 cysteine protease from pearl oyster *Pinctada fucata*

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ABSTRACT

Cathepsin L is one of the crucial enzyme superfamilies and involved in the immune responses. In this study, a cDNA encoding cathepsin L cysteine protease was identified and characterized from pearl oyster *Pinctada fucata* (designated as poCL1). The poCL1 cDNA was 1160 bp long and consisted of a 5′-untranslated region (UTR) of 15 bp, a 3′-UTR of 149 bp with a polyadenylation signal (AATAAA) at 11 nucleotides upstream of the poly(A) tail, and an open reading frame (ORF) of 996 bp encoding a polypeptide of 331 amino acids, which contained a typical signal peptide sequence (Met_1–Ala_{16}), a prodomain (Thr_{17}–Asp_{113}), and a mature domain (Leu_{114}–Val_{331}). The preproprotein contained the oxyanion hole (Gln), the active triad formed by Cys, His and Asn, and the conserved ERFNIN, GNFD motifs, which is characteristic for cathepsin L proteases. Homology analysis revealed that the poCL1 shared 62.5–72.5% similarity and 42.9–56.0% identity to other known cathepsin L sequences. The phylogenetic tree showed that the poCL1 clustered with the invertebrate cathepsin L cysteine proteases and was closely related to *Stichopus japonicus* CL, *Strongylocentrotus salar* CL1 and *Radix peregra* CL. The mRNA expression of the poCL1 in blank group and bacterial challenge group could be detected in all studied tissues with the higher level in digestive gland. The expression level of poCL1 mRNA was significantly up-regulated at 4 h and 8 h, and then significantly down-regulated at 12 h and 24 h in digestive gland after *Vibrio alginolyticus* stimulation. These results provided important information for further exploring the roles of pearl oyster cathepsin L in the immune responses.

1. Introduction

Pearl oyster *Pinctada fucata* is the most popular farming shellfish for seawater pearl production in Guangdong, Guangxi and Hainan province of China. In recent years, as other marine animals, higher frequencies of disease epidemics and the emergence of new diseases have been reported in artificial cultivation of pearl oyster [1,2]. Many researchers considered the reasons for high mortality were ocean pollution, disease outbreaks and stock degeneration [3,4]. In order to control disease and enhance the yields and quality of seawater pearl, it is necessary to further research the innate immune mechanisms of pearl oyster.

According to variety of the active sites, proteases are generally classified into four different families: cysteine proteases, aspartic proteases, serine proteases and metallo proteases [5]. Cathepsin L (EC: 3.4.22.15) is a member of the papain family C1 of cysteine proteases, which consist of four active residues: Gln, Cys, His and Asn. Like many other endoproteases, it is synthesized as an inactive proenzyme and activated by autocatalysis or other proteases to become active enzyme [6,7].

Cathepsin L is involved in many crucial biological functions in living organisms. In mammals, cathepsin L plays physiological role in the initiation of protein degradation as an important lysosomal cysteine protease, which is involved in antigen presentation [8], parasitic infection [9], MHC degradation and T-cell development [10], maturation of exopeptidase [11], and many immune system-related diseases. For example, Liu and Sukhova reported that there were higher serum levels of cathepsin L in patients with atherosclerosis, they thought cathepsin L take part in the pathogenesis of atherosclerosis and abdominal aortic aneurysm [12]. Arthropod cathepsin L seems to be responsible for food digestion. The cathepsin L exists in the large vacuole of B cell which is the digestive cell and enzyme secretion cell of the digestive gland of shrimp *Metapenaeus ensis*, which suggested that it may be necessary for digestion [13]. Recently, Hu and Leung's studies demonstrated that

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M. ensis cathepsin L has a food digestion role both intracellularly and extracellularly, and its digestive model in hepatopancreas is formulated [14]. Cathepsin L as a kind of parasite proteases also plays the vital functions in nutrient acquisition by catabolizing host proteins to absorbable peptides, excystment, encystment, immune evasion, and cell and tissue invasion [15–17]. Additionally, the presence of cathepsin L from oncomiracidia to adults indicates that cathepsin L may be involved in development of *Neobenedenia melleni* and the parasite–host interaction [18]. Recent data showed that cathepsin L is important to yolk processing during oocyte maturation and embryogenesis in many invertebrates and vertebrates. By analysing the temporal and spatial patterns of cathepsin L-a, cathepsin L-b and cathepsin L-c gene expression during development and in adult tissues, Rudi considered only cathepsin L-a was involved in yolk processing during oogenesis and embryogenesis in zebrafish [19]. Wang and Zhao examined the developmental expression of cephalochordate amphioxus cathepsin L in the adult and developing embryos and larvae of *Branchiostoma belcheri tsingtauense*, the results suggested that the cathepsin L participated in proteolytic events [20]. Moreover, Britton and Murray identified a cathepsin L (CPL) cysteine protease, which is essential for embryonic development of the free-living nematode *Caenorhabditis elegans* [21]. To investigate gene expression trends of mussel *Mytilus galloprovincialis* against water pollution, Venier and De Pitta constructed a EST library of multiple tissues and microarray of digestive gland, the results of microarray revealed cathepsin L may be a marker gene of mussels exposed to heavy metals [22].

In contrast with the abundant knowledge on cathepsin L in other organisms, the information of cathepsin L in bivalve mollusk is underdeveloped. Recently, several cathepsin L ESTs and EST homologs were identified from pacific oyster *Crassostrea gigas* and their expression after bacterial challenge has been investigated [23,24]. To study the gene expression regulation and its roles in immune responses, a full-length cDNA of cathepsin L was cloned and characterized (designated as poCL1) from pearl oyster *P. fucata*, and its expression patterns in different tissues and *Vibrio alginolyticus* stimulation condition were investigated in this study.

2. Materials and methods

2.1. Pearl oyster and immune challenge

Pearl oyster *P. fucata* (shell length 4.5–5.2 cm, body weight 18.2–22.5 g) was obtained from pearl oyster culture base of South China Sea Fisheries Research Institute in Xincun village, Hainan province, China and maintained at 25–27 °C in tanks with recirculating seawater for one week before experiment. The pearl oyster was fed twice daily on *Tetraselmis suecica* and *Isochrysis galbana* in the whole experiment process. Pearl oysters were injected into the adductor muscle 100 μl of PBS (Phosphate Buffered Saline) as control group. The bacterial challenge group was performed by injecting with 100 μl of *V. alginolyticus* resuspended in PBS to $OD_{600} = 0.4$ ($1\ OD = 5 \times 10^8$ bacteria ml^{-1}) into the adductor muscles of each pearl oyster. At each time point (0, 2, 4, 8, 12, 24, 48 and 72 h), digestive gland was collected and stored in liquid nitrogen from control group and bacterial challenge group until used. Unchallenged pearl oyster's digestive gland, gonad, haemocytes, gills, mantle, adduct muscle and intestine were collected as blank group and stored in liquid nitrogen until used. The same tissues were also collected and stored from bacterial challenge group at 8 h post-injection. The pearl oysters of each group (control group, bacterial challenge group and blank group) were divided into three replicates with equal amounts and fed in three tanks. Five pearl oysters were randomly sampled from each group at each time point, and mixed corresponding tissues with equal amounts as one sample.

2.2. cDNA library construction and EST analysis

A cDNA library was constructed from the whole body of a pearl oyster challenged by *V. alginolyticus*, using the ZAP-cDNA synthesis kit and ZAP-cDNA GigapackIII Gold cloning kit (Stratagene). Random sequencing of the library using T3: AATTAACCCTCACTAAAGGG yielded 6741 EST sequences, which were clustered into 808 contigs and 2456 singlets. BLAST analysis of all the EST sequences revealed that an EST of 492 bp (EST no. pmpca0_004006) was homologous to the cathepsin L of *Strongylocentrotus purpuratus* (XM_775620), *Ciona intestinalis* (XM_002130126) and *Monodelphis domestica* (XM_001367558). Based on the sequence of this EST, the corresponding colony was picked up and resequenced to obtain the complete sequence of the poCL1.

2.3. Sequence analysis of the poCL1

The poCL1 amino acid sequence was predicted using DNATool version 6.0 software. The position of the putative signal peptide cleavage site was predicted using the SignalP 3.0 software [25] (http://www.cbs.dtu.dk/services/SignalP/). The percentage of similarity and identity of the known cathepsin L sequences was calculated by the MatGAT program [26] with default parameters. The protein domain was predicted with the simple modular architecture research tool SMART version 4.0 program [27,28] (http://www.smart.emblheidelberg.de/) and ScanProsite (http://expasy.org/tools/). The protein sequence of the poCL1 was compared to its counterpart sequences currently available in GenBank using the BLAST program [29] (http://www.ncbi.nlm.nih.gov). Position of prodomain cleavage site and conserved cysteine residues are based on the N-terminal sequence information from HumanCL1 (*Homo sapiens*, P07711), RatCL1 (*Rattus norvegicus*, P07154), BovineCS (*Bos taurus*, P25326), and ChickenCL (*Gallus gallus*, P09648) [30]. Multiple alignment of the poCL1 was carried out with the Clustal W program (http://www.ebi.ac.uk/clustalw/). The phylogenetic tree was constructed with MEGA program version 3.1 [31] based on amino acid sequences alignment. The phylogenetic tree was tested for reliability using 1000 bootstrap replications.

2.4. Real-time quantitative RT-PCR analysis of the poCL1

The expression pattern of the poCL1 in digestive gland, gonad, haemocytes, gills, mantle, adduct muscle and intestine from blank group and bacterial challenged group at 8 h post-injection were detected by real-time quantitative RT-PCR. Temporal expression level in digestive gland after bacterial challenge was also detected by real-time quantitative RT-PCR. Total RNA samples were extracted using RNeasy Mini Kit (QIAGEN Cat: no. 74104) according to the manufacture's instructions, and treated with DNase I (QIAGEN Cat: no. 79254) to remove contaminated DNA. Subsequently, the first-strand cDNA was synthesized based on manufacture's instruction of PrimeScript™ RT reagent Kit (Perfect Real Time) (TaKaRa DRR037S) using total RNA as template. cDNA mix was diluted to 1:5 and stored at −80 °C for subsequent real-time quantitative RT-PCR. Two poCL1 gene-specific primers, poCL1-F: CAGAACAGAACCTTATTGACTGC and poCL1-R: CACCTTACCTTTGTCTGTAGCAC were designed to amplify a product of 197 bp. The β-actin gene was used as an internal control to verify the real-time quantitative RT-PCR reaction and adjust the cDNA templates. Two β-actin gene-specific primers β-actin-F: GCCGAAA GAGAAATCGTCAG and β-actin-R: TGGCTGGAATAGGGATTCTG were designed to amplify a fragment of 183 bp.

The real-time quantitative RT-PCR was performed in a total volume of 20 μl containing 10 μl of 2× SYBR Green Real-time PCR Master Mix (TaKaRa DRR041A), 1 μl of cDNA, 0.16 μM of each