Two cysteine proteinases respond to bacterial and WSSV challenge in Chinese white shrimp *Fenneropenaeus chinensis*

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**ABSTRACT**

The cDNAs encoding CathL and legumain from Chinese white shrimp *Fenneropenaeus chinensis* (FcCathL, FcLegu) were obtained. Both FcCathL and FcLegu mRNA were expressed mainly in the hepatopancreas of unchallenged shrimp. Time-course analysis of FcCathL showed that FcCathL was upregulated in the hepatopancreas of shrimp challenged with white spot syndrome virus (WSSV) at 12 h. FcLegu mRNA in hepatopancreas was down-regulated by *Vibrio*. FcLegu transcript first declined from 2 h to 6 h and then recovered from 12 h to 24 h in hepatopancreas challenged with WSSV. FcCathL protein was detected in the hemocytes, hepatopancreas, gill, stomach, and intestine of unchallenged shrimp. Three bands of FcCathL protein detected in some tissues may represent preproenzyme, single chain and mature double chain form respectively. In hepatopancreas, FcLegu was detected in the proenzyme form. In other tissues, only active form could be detected. The protein of FcLegu was down-regulated by *Vibrio* or WSSV challenge in the stomach and gills. FcCathL and FcLegu were proposed to play a role in shrimp innate immunity for the first time.

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1. Introduction

Cysteine proteases (CPs) play a vital role in many biological processes including physiological and pathological reactions [1,2]. CPs can be classified into different clans and families depending on their molecular characterization and substrate specificity. The CPs of clan CA include papain, cathepsin L (CathL), CathB, CathS, and CathH [3,4]. The CPs of Clan CD include legumains (family C13), caspases (family C14), separases (family C50), gingipains (family C25 of gram negative eubacteria), and clostripains (family C11 of clan CA CPs) [5]. The different substrate specificities and protease inhibitors of clan CD CPs allow them to be experimentally distinguished from clan CA CPs [5].

CathL is involved in the clearance of unwanted proteins [6]. Further investigations on CathL have confirmed that it functions in many physiological and pathological reactions. In invertebrates, CathL is predicted to have a digestive role. The CathL from *Metapenaeus ensis* (McCathL) has a function in food digestion. However, it has also been found that McCathL exists in the nucleus of the oocyte and may have a specified physiological role in its nucleus [6]. Two CathLs found in *Penaeus vannamei* may take part in the intermolt cycles [7].

Legumain is a type of asparaginyl endopeptidases (AEP) that hydrolyzes proteins on the carboxyl side of asparagine residues. Legumain was first identified from plants where it functioned in the processing of seed precursor proteins to their final forms [8,9]. Legumain (HlLgm) in *Haemaphysalis longicornis* plays an important role in host blood-meal digestion [10]. The IrAEP/legumain in hard tick *Ixodes ricinus* may also have functions in the gut digestive processes [11].

CPs were reported to play an important role in host immune system. Among these CPs, the most important are the clan CA papain proteases and clan CD C13 legumain-like AEP, which are involved in major histocompatibility complex (MHC) class II antigen-processing pathway in mammals [12]. CathL and legumain are involved in vertebrates adaptive immunity. Shrimp rely on innate immunity only. Whether CathL and legumain have roles in shrimp innate immunity is not known. In our study, we attempt to identify the immunity function of CathL and legumain in shrimp by comparing its expression with that in normal and microbe-infected shrimp.

2. Materials and methods

2.1. Preparation of viral inoculums

The WSSV inoculum was prepared and quantified based on the previously described method [13]. WSSV was extracted from the...
gills of naturally heavily infected Fenneropenaeus chinensis that have been stored at −80 °C. The gill tissue (1 g) was homogenized in 10 mL PBS (140 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4, and 1.8 mmol/L KH2PO4) and then centrifuged at 5000g for 10 min at 4 °C. The supernatant was passed through a 450 nm membrane filter and used as inoculum.

2.2. Immunity challenge of shrimp and isolation of total RNA

Vibrio anguillarum (approximately 3 × 10^7 cells per individual) or WSSV (3.2 × 10^7 per shrimp) was injected into the abdominal segment of the shrimp [13]. Isolation of total RNA from different tissues with microbe challenge at specific time was according to the published paper [14].

2.3. Cloning the full-length cDNA of FcCathL and FcLegu

Based on the published sequences of other shrimp’s CathL, one primer (FcCathL-F: 5'-CTACCGTCAGGCTAGCAGGCGGTAA-3') was designed, and rapid amplification of the cDNA ends (RACE) was employed to obtain the 3' fragment, with the 3' adaptor primer (5'-GACCACCGTATCGATGTCGAC-3') provided by the 3'-Full RACE Core Set (TaKaRa). The Smart cDNA was produced according to the published paper (Ren et al., 2009) and was used as the template for the SMART primer (5'-TACGGCTGGCAAGAAGACAGAAGAG-3') and primer FcCathL-R (5'-GTTGCTGATGCGCTTATTGCT-3').

The 3’ fragment of FcLegu was obtained with primer F (5’-ATGCGTGCAAGGGAAGAG-3’) and 3’ anchor R. The 5’ end of FcLegu was also cloned with a specific primer R (5’-CAGGGTCCACATCGTCTGACGAC-3’) based on the known sequence and a universal primer T3 using the ZAP cDNA library of hepatopancreas from shrimp under 24 h bacterial challenge. The full-length of FcLegu was obtained by splicing these two fragments.

2.4. Phylogenetic and sequence analysis of FcCathL and FcLegu

The FcCathL and FcLegu cDNA sequences were analyzed and compared using the BLASTX and BLASTP search programs through the GenBank database search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Translation of cDNA and predictions of the deduced proteins were performed using ExtPASy (http://www.expasy.org/). Signal sequence and motif prediction were performed using SMART (http://smart.embl-heidelberg.de/). Phylogenetic trees were constructed using the Neighbor-joining (NJ) method using the MEGA 4 software [15].

2.5. Tissue distribution and responses to bacterial or WSSV challenge of FcCathL and FcLegu

Tissue distribution of FcCathL and FcLegu and the variations of these two genes post 24 h Vibrio challenge were investigated through a semi-quantitative RT-PCR using the primers (FcCathL-RT-F: 5’-ATGAGCTTCGTGACACTTTGGTTCG-3’; FcCathL-RT-R: 5’-CTGGTCATGCGCTTTATTTTCC-3’; FcLegu-RT-F: 5’-GATGAAAGTTCCTGACAGTGTTTGCTTGC-3’; FcLegu-RT-R: 5’-TACTCACATTGACACTTTGGTTCG-3’). The primers used in qRT-PCR were (CalQRT-F: 5’-AAGAGGGTCAGGACGTG TTCAG-3’; CalQRT-R: 5’-AGTTGTAGAC-3’).

2.6. Construction of the expression plasmid

The mature FcCathL and FcLegu were amplified by the primers (FcCathL-exF: 5’-TACTCACATTGACCTCTCCACCCCGCGG-3’; FcCathL-exR: 5’-AACCTGCGCGCCGCCTACGACCGGGGTAGAGCC-3’; FcLegu-exF: 5’-TACCAATGACGCTCCAGCACCCGAGAGGC-3’; and FcLegu-exR: 5’-TACCAATGACGCTCCAGACCGGGGTAGAGGC-3’). The sites of EcoR I, Xho I, and Not I were underlined. The constructed plasmid was transformed into competent cells of Escherichia coli BL21-DE3 for recombinant expression.

2.7. Recombinant expression and purification

Overnight culture transformants (2 mL) were added into 200 mL kanamycin-containing Luria–Bertani broth for the large scale culture. When the A600 value was up to 0.6, the final concentration of 1 mmol/L isopropyl b-D-thiogalactopyranoside (IPTG) was added for the induction of FcCathL or FcLegu expression. Cultured at 37 °C for 4 h, the bacteria were collected by centrifugation at 6000g for 5 min. The pellets were resuspended in 20 mL of PBS with 0.2% TritonX-100 and lysed by probe sonication. The lysates were centrifuged at 10,000 rpm for 20 min at 4 °C to discard the supernatants. Inclusion bodies were purified and denatured according to the method of Kuhelj et al. [17]. Correct refolding of the FcCathL and FcLegu was reached by gradient dialyzation according to the published paper [14]. The recombinant protein was then purified through affinity chromatography using His Bind resin chromatography (Novagen) following the manufacturer’s instructions. Routine protein estimation was assessed through the Bradford method [18] using bovine serum albumin (BSA) as the standard.

2.8. Anti-FcCathL and FcLegu rabbit serum preparation and Western blot analysis of FcCathL and FcLegu

The methods of rabbit serum preparation were as previously described (Ren et al., 2009). To investigate the expressions of FcCathL and FcLegu at the protein level, Western blot analysis was performed. The total protein concentrations of the different tissues were determined using a protein assay kit (QIAGEN). The methods of Western blot analysis were performed as described by Bio-Rad. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in TBST buffer for 1 h at room temperature and then incubated with primary antibodies (1:2000 dilution) overnight at 4 °C. After washing with TBST buffer, the membrane was incubated with secondary antibodies (1:10000 dilution) for 1 h at room temperature. The bands were visualized by using an enhanced chemiluminescence reagent (Perkin Elmer).
were determined by Bradford method [18]. The same quantity of protein was loaded onto the gel, and SDS-PAGE was run following the method of Laemmli [19]. After SDS-PAGE, the proteins were transferred onto the nitrocellulose membrane electrically. The protocol of Immunoblotting was as previously described [20].

3. Results

3.1. Gene cloning of FcCathL and FcLegu in Chinese white shrimp

The complete sequence of FcCathL was composed of 1078 bp with an open reading frame (ORF) of 987 bp encoding a protein of 328 amino acids. A putative 16-residue signal peptide, an Inhibitor_I29 domain of 61-aa, and the 217-residue Pept_C1 domain were predicted in FcCathL through the SMART analysis (http://smart.embl-heidelberg.de/) (Supplemental Fig. 1A). Three conserved residues necessary for enzyme activity existed in the FcCathL (C135, H274 and N295). The amino acid sequence of FcCathL was similar with those of Litopenaeus vannamei (cathepsin L, 87.805%,CAA59441) and other species. The full-length of legumain from F. chinensis (FcLegu) was 1444 bp including an 1320 bp ORF encoding a 439-aa peptide including a 20-aa signal peptide and a Peptidase_C13 domain from position 28 to 285. Within the Peptidase_C13 domain, two active residues (H147 and C188) forming a catalytic dyad are necessary for enzyme activity (Supplemental Fig. 1B). BLASTP analysis showed that it was similar to legumains from other species.

3.2. Phylogenetic analysis of FcCathL and FcLegu

Phylogenetic analysis of the cysteine protease family was performed at the amino acid level. The results showed that FcCathL and FcLegu belonged to different clusters. FcLegu together with legumains from other species were grouped together and belonged to the clan CD family. FcCathL together with Cathepsin L from other species were clustered into another group and belonged to the clan CA family (Supplemental Fig. 2).

3.3. Expression and tissue distribution of FcCathL and FcLegu mRNA

The FcCathL was expressed only in the hepatopancreas of the unchallenged shrimp. The expression of the FcCathL was detected in the hepatopancreas, stomach, and intestine of V. anguillarum challenged shrimp (Fig. 1A). The transcript of FcLegu was detected only in the hepatopancreas of normal shrimp. After injection with V. anguillarum for 24 h, the expression level of FcCathL decreased in the hepatopancreas (Fig. 1B). Time-course analysis was performed using qRT-PCR and the results showed that FcCathL was upregulated in the hepatopancreas of shrimp challenged with WSSV at 12 h and then recovered to normal level at 24 h (Fig. 2B). There was no significant difference in the statistical analysis post Vibrio challenge (Fig. 2A). The transcript of FcLegu went down after injection with V. anguillarum (Fig. 3A). FcLegu went down from 2 h to 6 h and recovered from 12 h to 24 h after WSSV challenge in the time-course analysis (Fig. 3B).

3.4. Recombinant expression, purification and Western blot analysis of FcCathL and FcLegu in tissues post bacterial or WSSV challenge

The FcCathL and FcLegu were recombinantly expressed in E. coli. (Supplemental Fig. 3A, B). Western blot analysis was used to analyze the change under WSSV or Vibrio challenge at protein level. The results show that three bands of FcCathL protein detected in some tissues may represent preproenzyme, single chain and mature double chain form respectively (Fig. 4A). An additional band could be detected only in gills. In hemocytes, only a band representing its preproenzyme form could be detected. Although WSSV or Vibrio challenge could not activate FcCathL, the preproenzyme of FcCathL in hemocytes was upregulated post 24 h WSSV or...
Vibrio challenge. In hepatopancreas, two bands representing the preproenzyme and single chain form could be detected and WSSV or Vibrio challenge had no impact on FcCathL. In intestine and gills, single chain of FcCathL was upregulated by Vibrio or WSSV. In stomach, FcCathL single chain was upregulated only by WSSV. In stomach, partial of FcCathL single chain was activated into double chain post 24 h WSSV challenge. In intestine, single chain converted into double chain was also observed post 24 h WSSV or Vibrio challenge (Fig. 4B). FcLegu protein was detected in all detected tissues. In hepatopancreas, FcLegu was detected mainly in the proenzyme form and weak signal of its active form could also be detected. In other tissues, only active form could be detected (Fig. 5A). The protein of FcLegu was down-regulated by Vibrio or WSSV challenge in the stomach and gills (Fig. 5B, C).

4. Discussion

Two cDNAs of CPs (FcCathL, FcLegu) belonging to different clans have been cloned from F. chinensis. FcCathL has three conserved active residues forming a catalytic triad by alignment with other CathL, while FcLegu has only two active residues necessary for the formation of catalytic dyads. The presence of a highly conserved motif (EX3RX2VFX2NX3IX3N), also referred to as the ERFNIN motif, exists in FcCathL, which distinguishes it from other cathepsins of the papain family [21]. AEPs, also referred to as legumains, were first translated as prepropeptides and then processed into active forms by corresponding enzymes at its N-end and C-end. It was reported that human prolegumain was activated by cleavage at a C-terminal asparagine residue [22].
CathL was generally thought to be a type of digestive enzyme. Little research focused on CathL function in shrimp innate immunity could be obtained up to date. In our study, FcCathL transcript was upregulated in hepatopancreas with WSSV challenge. Its protein has three different forms designated as preproenzyme, single chain and active double chain respectively which referred to the published paper [23]. In some of our detected tissues, WSSV or Vibrio could convert single chain of FcCathL into mature double chain. In some tissues, WSSV or Vibrio could upregulate the level of single chain of FcCathL in compared to the control. Although, WSSV or Vibrio could not activate the FcCathL, the proenzyme of FcCathL was upregulated by WSSV or Vibrio in hemocytes. In humans, clan CA papain proteases are reported to be involved in the MHC class II antigen-processing pathway [12]. It was also reported that CathL took part in the proteolytic processing of the Hendra virus fusion protein [24]. In 2007, there were reports about CathL in shrimp that were responsive to the virus challenge [25,26]. Our research also suggested FcCathL role in shrimp anti-WSSV innate immunity. CathL gene was upregulated by 5.7 fold in monocyte-derived macrophages (MDM) under 24 h avian pathogenic E. coli strain (APEC) [27]. CathL was upregulated in Chinese soft-shelled turtle (Trionyx sinensis) infected with Aeromonas hydrophila, a ubiquitous waterborne bacterium [28]. CathL is also a lysosomal CP that belongs to the papain superfamily, just like FcCathL. [29]. The temporal expression of PmCathC mRNA in the hepatopancreas was upregulated by lipopolysaccharide (LPS) stimulation, and it reached the maximum level at 4 h before gradually restored to its basal level. The results show that PmCathC was a constitutive and inducible acute-phase protein that could function in the innate immunity of Penaeus monodon [30]. In all, our research may indicate its role in shrimp innate immunity against bacteria or WSSV.

FcLegu mRNA was detected mainly in hepatopancreas in our study. Although FcLegu was specifically expressed at the transcript level, it was widely distributed in nearly all detected tissues at protein level. Legumain from amphibious Branchiostoma belcheri (BbLegu) was not widely distributed and two bands of BbLegu were detected only in hepatic caecum and hind-gut [31]. One band of BbLegu was its prionzyme and another was its active form. The proenzyme was converted into its active form by removing about a 10 kDa peptide through its C-terminal autocleavage at acid pH at Asn 147 [32]. The signal of FcLegu protein active form was weakened in the hepatopancreas, and the proenzyme was only detected in hepatopancreas. In other tissues, only active form could be detected. It could be speculated that FcLegu was first synthesized in hepatopancreas at its proenzyme form and then it was transported into hepatopancreas and was auto-activated at there. It also reported that vertebrate legumain could control antigen processing and LPS priming could down-regulate the expression of legumain in monocytes. However, the down-regulation of legumain in B-cells was observed with LPS priming [32]. In our study, FcLegu transcript went down in hepatopancreas with Vibrio or WSSV challenge. Active form of FcLegu protein in gills and stomach post Vibrio or WSSV challenge was also down-regulated. This is the first study which correlates the invertebrates legumain with immunity.

Pathogens are known to have evolved from a myriad of immune evasion mechanisms, many of which are aimed at blocking the MHC-linked antigen-processing pathways [33]. Legumain was down-regulated in bovine alveolar macrophages infected with ATCC35723, a virulent strain of Mycobacterium bovis compared with WAg520, and in attenuated isogenic strains of M. bovis [34]. So, it could be speculated that pathogens such as WSSV and Vibrio may escape shrimp immune system through the down-regulation of legumain. In all, FcLegu might play an important role in shrimp innate immunity.

From the molecular characterization and expression pattern, it can be speculated that FcCathL and FcLegu may have functions in shrimp innate immunity. However, the specific role and immune defense mechanism of these two CPs genes need further investigation.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsi.2010.03.002.

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