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Cathepsin B from the white shrimp *Litopenaeus vannamei*: cDNA sequence analysis, tissues-specific expression and biological activity

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ABSTRACT

Cathepsin B is a cystein proteinase scarcely studied in crustaceans. Its function has not been clearly described in shrimp species belonging to the sub-order Dendrobranchiata, which includes the white shrimp *Litopenaeus vannamei* and other species from the Penaeidae family. Studies on vertebrates suggest that these lysosomal enzymes intracellularly hydrolize protein, as other cystein proteinases. However, the expression of the gene encoding the shrimp cathepsin B in the midgut gland was affected by starvation in a similar way as other digestive proteinases which extracellularly hydrolyze food protein. In this study the white shrimp *L. vannamei* cathepsin B (LvCathB) cDNA was sequenced, and characterized. Its gene expression was evaluated in various shrimp tissues, and changes in the mRNA amounts were compared with those observed on other digestive proteinases from the midgut gland during starvation. By using qRT-PCR it was found that LvCathB is expressed in most shrimp tissues except in pleopods and eye stalk. Changes on LvCathB mRNA during starvation suggest that the enzyme participates during intracellular protein hydrolysis but also, after food ingestion, it participates in hydrolyzing food proteins extracellularly as confirmed by the high activity levels we found in the gastric juice and midgut gland of the white shrimp.

1. Introduction

Cathepsin B (CathB; EC 3.4.22.1), is the most well studied intracellular, acidic cystein proteinase in vertebrates and it shows the uncommon ability to accomplish a dual role: as an endopeptidase and a dipeptidyl-carboxypeptidase (McGrath, 1999). Studies on humans and rats have evidenced that CathB plays an essential role in the intracellular activation of trypsin zymogens during the premature activation of proteinases on human pancreatitis (Halangk et al., 2000; Lerch and Halangk, 2006), and it participates in the enzymatic cascade associated to some human pathologies (Sloane, 1990; Ellis, 2004).

In invertebrate hematophagous species, as the nematode *Haemonchus contortus* and the tick *Ixodes ricinus*, CathB has been confirmed to contribute on the digestion of blood protein (Williamson et al., 2003; Yatsuda et al., 2006; Franta et al., 2010). However, the information concerning cystein-proteinases and their function from marine and freshwater invertebrates is still scarce (Le Boulay et al., 1996; Cardenas-Lopez and Haard, 2005). In the northern shrimp *Pandalus borealis* the cDNA encoding a CathB, was found to be exclusively expressed in the midgut gland (Aoki et al., 2003), and since extracellular activity of this enzyme was detected at acidic pH, it was suggested that it may play a role on digestion. Recently it was reported that the genes expression of two cystein proteinases was affected during bacterial and viral challenges in the midgut gland of the Chinese shrimp, *Fenneropenaeus chinensis*, suggesting that both enzymes may play a role in shrimp innate immunity (Ren et al., 2010).

The midgut gland of decapod crustaceans is recognized as the organ where a battery of proteinolytic enzymes is synthesized to digest food protein. A series of studies have contributed to identify different classes of enzymes, their biological function, and the conditions governing its activity during protein hydrolysis (Muhlia-Almazan et al., 2008; Rojo et al., 2010). Among cystein-proteinases synthesized in the midgut gland of shrimp, cathepsin L (CathL) is commonly recognized as the enzyme responsible for protein hydrolysis (Hu and Leung, 2004). It has been reported that in caridean shrimp, *Crangon* spp., serine proteinases, as trypsin and chymotrypsin, are substituted by cystein proteinases such as Cath L, this replacement seems not to be related to the mode of feeding or to the nutritive status of shrimp (Teschke and Saborowski, 2004).

Several properties of CathL, as its gene characterization and expression, and protein and enzyme activity, have been extensively studied in some crustacean species including *Litopenaeus vannamei* (Le Boulay et al., 1996) and *Metapenaeus ensis* (Hu and Leung, 2004). Furthermore, its role during intracellular and extracellular protein hydrolysis has been previously described (Hu and Leung, 2007). However, the existence and the assumed role of other lysosomal cystein proteinases, especially cathepsin B, during digestion

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has not been assessed in the Penaeidae subfamily, which includes the most commercially important species of shrimp worldwide.

In this study biochemical and molecular biology approaches were used to identify and characterize the mRNA sequence encoding a cathepsin B in the midgut gland of shrimp, and to assess whether its gene expression is affected during starvation following the same pattern as the main digestive enzymes trypsin, chymotrypsin, and cathepsin L. In addition, the analysis of its gene expression and its enzymatic activity were evaluated in several tissues, to provide insights to better understand its basic biological function during protein degradation. Finally, a phylogenetic analysis was included to infer about shrimp species cathB from their sequence and structure homologies.

2. Materials and methods

2.1. LvCathB cDNA sequencing

The complete cDNA sequence of cathepsin B from *L. vannamei* (LvCathB) was obtained by using specific oligonucleotides designed on the basis of a DNA sequence encoding a cathepsin B of *Penaeus monodon* (GenBank accession no. EF213113) and a long EST from *L. vannamei* (www.marinegenomics.org; MGID996966). The set of oligonucleotides used to amplify LvCathB cDNA fragments is listed in Table 1.

The midgut gland cDNA used as template in the PCR reactions was synthesized as described below. The complete LvCathB coding sequence was amplified using oligonucleotides CBLvMFw and CBLvSRw, and the 3′-UT region was amplified using CBvanFw3 and an oligo-dT. PCR conditions were optimized to amplify different size DNA fragments at 95 °C for 3 min, 40 cycles of 94 °C for 1 min, 55 to 60 °C for 1 min, 72 °C for 1 min and a final extension of 72 °C for 10 min. During PCR amplification, reactions of 25 μL total volume included 1 μL of each oligonucleotide (100 pmol), 1 μL of template (250 ng of polyadenylated RNA equivalents), and 22 μL of supermix (Invitrogen, USA).

PCR products were analyzed by electrophoresis on a 1% agarose gel stained with SYBR safe (Molecular Probes, USA), and subsequently purified and sequenced by the dideoxy chain-termination method at the Laboratory of Molecular and Systematic Evolution at the University of Arizona. The complete sequence was compared using protein and nucleotide data bases (Blast algorithm N and P; Altschul et al., 1997).

2.2. Starvation assay and shrimp tissues collection

Thirty adult shrimp (*L. vannamei*) were selected by size (18.0 ± 1.0 g) and molt stage (intermolt) from CIBNOR facilities. Organisms were placed in 3 plastic tanks of 1200 L each (10 shrimp per tank) and maintained under controlled laboratory conditions during an acclimatization period of 15 days (28 °C, 34 ppt, 6.0 mg/L). Shrimp were fed once a day ad libitum with commercial food (Silver cup with 35% protein), uneaten food and feces were daily removed, and the total volume of marine water was daily exchanged.

Once acclimatized, shrimp were fed and their digestive tract was examinated to be full, and immediately after, two specimens were sampled from each tank, at 0 (control group), 24, 72 and 120 h (starved groups). Six individual shrimp were decapitated at each different starvation time and their midgut gland (MG), were dissected and frozen in liquid nitrogen. All samples were stored at −20 °C.

In order to understand LvCathB gene expression and enzyme activity in different shrimp tissues, one remaining control shrimp was decapitated and its various tissues were dissected. Samples of midgut gland (MG), gastric juice (GJ), intestine (gut), gills (G), muscle (M), hemocytes (Hm), pleopods (Pl), and terminal ampoule (Amp) were stored at −20 °C and then individually analyzed.

Table 1
Specific oligonucleotides used for LvCathB sequencing from the midgut gland of the white shrimp *L. vannamei*.

Oligonucleotide name	Sequence (5′–3′)	cDNA position (nt)
CBvanFw3	GCGAGAAGGGCTACATAG	686–703
CBvanRv4	GTTAGCGTTGTTGTATTTTGTG	1443–1422
CBvanFw6	ACGACGGTAGGGAGTGTC	18–35
CBvanRv6	GGAAGCCTCCGTTACATCC	517–499
CBLvMFw	ATGAGGGTTATCCTGGTCTTG	61–81
CBLvSRv	CTAATTCAACTTAGGCAACCC	1056–1036

2.3. Total RNA isolation and complementary DNA synthesis

Total RNA was extracted from 100 mg of midgut gland from control and starved shrimp, and also from all the above mentioned shrimp tissues using the method described by Chomczynsky and Sacchi (1987). In brief, each tissue was homogenized in 500 μL of Trizol® reagent (Invitrogen) and samples were incubated at room temperature for 5 min. Then 200 μL of chloroform were added and tubes were shaken and incubated for 15 min, then centrifuged at 10,000 *g* for 15 min at 4 °C. The supernatant phase was transferred to clean tubes and 500 μL of additional TRizol were added. The following steps were repeated and once the new aqueous phase was transferred to clean microtubes, 500 μL of cold isopropyl alcohol were added to precipitate total RNA. Samples were incubated for 10 min at room temperature and centrifuged at 16,000 *g* for 10 min at 4 °C. Total RNA samples were washed once with 1 mL of 75% ethanol (prepared with DEPC water), and centrifuged at 5000 *g* for 10 min at 4 °C. Samples were air-dried and dissolved in nuclease-free water. Total RNA concentrations were measured at 260 nm using a spectrophotometer (NanoDrop 1000, Wilmington, DE, USA) and their integrity was confirmed by 1% agarose-formaldehyde gel electrophoresis (Sambrook and Russell, 2001).

Genomic DNA contamination of total RNA samples was eliminated using DNAse I (Sigma Aldrich, St. Louis, MO, USA) at 1 U/μg RNA. Samples were incubated for 10 min at 37 °C. Once gDNA was digested and its absence confirmed, RNA samples were used as template in the complementary DNA (cDNA) synthesis. Five micrograms of total RNA were reverse transcribed using the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and oligo-dT primers, following manufacturer instructions. cDNA samples were used as template to evaluate mRNA relative amounts.

2.4. LvCathB qRT-PCR by real time

The relative mRNA amounts of cathepsin B, cathepsin L, chymotrypsin, trypsin and L21 (as an internal control) were evaluated by real time PCR using an iQ5 real time PCR detection system (BioRad, CA, USA). All samples were PCR-amplified in triplicates using total volume reactions of 25 μL which included 12.5 μL of 2x iQ Sybr-Green Supermix (BioRad), 0.7 μL of each 5 mM forward and reverse oligonucleotides, water and cDNA (200 ng of polyadenylated RNA equivalents) from each sample.

Specific oligonucleotides were designed using mRNA sequences already reported at the GenBank (Table 2). Trypsin (TryLvFw1 and TryLvRv1; Sanchez-Paz et al., 2003), chymotrypsin (ChymoLvFw1 and ChymoLvRv1; GenBank accession nos. X66415, Y10664 and Y10665), cathepsin L (CATLFw4 and CATLRv4; X99730.1), cathepsin B (CatB Fw2Lv and CatBRv1Lv; GU571199.1), and the ribosomal protein L21 (L21LvFw2 and L21LvRv2; BE188654.1) was evaluated as an internal control to normalize target gene expression.

Amplification conditions were 95 °C for 5 min and 40 cycles of 95 °C for 30 s, 55–60 °C for 35 s, and 72 °C for 55 s. PCR products specificity was confirmed by constructing a melt curve after amplification raising temperature from 60 to 95 °C with an increase of 0.3 °C every 20 s and a fluorescence reading at each temperature increase. No-template controls were included during each gene amplification.