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

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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 common ability to accomplish a dual role: as an endopeptidase and a dipeptidyl-carboxypeptidase (McGrath, 1999). Studies on humans have evidenced 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.

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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 *Peneaus monodon* (GenBank accession no. EF213113) and a long EST from *monodon* on the basis of a DNA sequence encoding a cathepsin B of *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 dyeoxy-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 (Blat 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 CINBOR 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 examined 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.

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 SybrGreen 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 TryLvr1; Sanchez-Paz et al., 2003), chymotrypsin (ChymolLvfw1 and ChymolLvr1; GenBank accession nos. X66415, Y10664 and Y10665), cathepsin L (CattLfw4 and CattLrv4; X99730.1), cathepsin B (CatBFwLvL and CatBRvLv1; GU571199.1), and the ribosomal protein L21 (L21Lfw2 and L21Lrv2; 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.

### Table 1

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Sequence (5′–3′)</th>
<th>cDNA position (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBvanFw3</td>
<td>CCGAGAGGGGCTACATAG</td>
<td>686–703</td>
</tr>
<tr>
<td>CBvanRv4</td>
<td>GTTAGCGTTGTTGATTGTTG</td>
<td>1443–1422</td>
</tr>
<tr>
<td>CBvanFw6</td>
<td>ACAGCTGGCGGAGCCTGT</td>
<td>18–35</td>
</tr>
<tr>
<td>CBvanRv6</td>
<td>GGAGGCTCGGTCCATCCC</td>
<td>517–499</td>
</tr>
<tr>
<td>CBLvMFw</td>
<td>ATGGCCGATTCGCTGCTTG</td>
<td>61–81</td>
</tr>
<tr>
<td>CBLvSRw</td>
<td>CTAATTCACTTAGGCGAACC</td>
<td>1056–1036</td>
</tr>
</tbody>
</table>
Data analysis was performed based on the comparison of PCR reaction efficiencies of each amplified gene, and the Ct value of each sample (data not shown). The \( -\Delta\Delta C_{t} \) method was used to evaluate changes in the relative mRNA amount of target genes. To determine the effects of starvation data were obtained from the following formula \( \Delta C_{t} = \text{mean Ct target} - \text{mean Ct control} \), to compare among tissues the same formula was used and all tissues mRNA amounts were calculated relative to ampule that equals to 1 (Livak and Schmittgen, 2001).

Statistical analyses were performed using Statistica 8 software (StatSoft, Inc). Chi-squared test was used to determine normal distribution, and Levene's test confirmed variances homogeneity. One-way ANOVA was performed to test the significance of starvation effects, and among tissues Duncan's test was used to determine differences between means. Statistical significance differences were considered at \( p<0.05 \) (Zar, 1984).

2.5. **LvCathB** enzyme activity assay

Cathepsin B (EC 3.4.22.1) specific activity was measured by using the fluorogenic substrate Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (Z-Arg-Arg-AMC) (Sigma) in protein extracts obtained from different tissues of *L. vannamei* including midgut gland, intestine, hemocytes, gills, eye stalk, muscle and pleopods; additionally gastric juice was obtained by leaking the liquid contained in the shrimp gastric chamber.

Assays were performed at 30 °C in black 96-well plates in a Synergy 4 (BioTek) fluorometer as follows: 5–20 μL of every tissue shrimp extract (ranging from 0.4 to 500 μg of protein depending on the assayed tissue) were mixed with a buffer solution containing 100 mM sodium acetate, 1.5 mM EDTA, 2 mM DTT, 0.05% and Triton X-100, pH 5.5. The reaction was set by adding the fluorogenic substrate to a final concentration of 100 μM. Rates of hydrolysis were recorded by measuring the increase of fluorescence in arbitrary units (relative fluorescence units, RFU). The excitation and emission wavelengths were 360 nm and 440 nm, respectively. A calibration curve was constructed by measuring the fluorescence of known concentrations of fluorochrome 7-amino-4-methoxy coumarin (AMC) and by plotting the RFU versus picomoles of AMC. Cathepsin B activity is expressed in picomol of MCA liberated per minute per μg of protein. One unit of activity was defined as the release of 1 μmol of MCA per min per μg protein.

2.6. **Phylogenetic analysis**

A multiple alignment analysis including the cathepsin B deduced amino acid sequences from selected taxa and LvCathB was performed. Digestive cathepsin B sequences from 11 invertebrate species: *Triatoma infestans* (Kollien et al., 2004), *Tenebrio molitor* (Prabhakar et al., 2007), *Pandanus borealis* (Aoki et al., 2003), *Haemonchus contortus* (Yatsuda et al., 2006), *Diatrobotica virgifera* (Bown et al., 2004), *Iodes ricus* (Sojka et al., 2008), *Ascaris suum* (Rehm and Jasmer, 1999), *Ancylostoma caninum* (Harrop et al., 1995), *Schistosoma mansoni* (Sajid et al., 2003), *Necator americanus* (Ranjit et al., 2008) and *Fasciola gigantica* (Wilson et al., 1998) were included. The selected sequences were aligned for comparison using Clustal X v. 1.81 software (Thompson et al., 1994), and the alignment was used to construct a cladogram. For phylogenetic analysis, 259 positions from the mature cathepsin B amino acid sequences from 26 species were used; pre-pro regions and C-terminal extensions were excluded because of intrinsic high sequence variability. Based on the Jones et al. (1992) model of substitution, a cladogram using the neighbor-joining algorithm (Saitou and Nei, 1987) was constructed, the Bootstrap method was applied for assessing confidence analysis of the clades (Hillis and Bull, 1993) based on 1000 replicates in MEGA 4.1 software (Tamura et al., 2007).

3. **Results**

3.1. **LvCathB** cDNA and protein sequences

The full-length LvCathB cDNA sequence was shown to be 1560 nucleotides long, including a 60 nucleotides 5′-UT region, a 996 nucleotides coding region, and a 504 nucleotides 3′-UTR. Two putative polyadenylation signals AATAAA occurred at positions 1522 and 1544 and a poly A tail at position 1561 (Fig. 1). Although multiple transcripts have been found to result from alternative splicing in the untranslated regions of rat cath B (Ellis, 2004), no clear evidence of other LvCathB mRNA isoforms in shrimp was found since several cDNA sequences from different tissues (data not shown) were analyzed and no major differences among them were found.

The LvCathB deduced protein is 331 residues long, it is encoded as a preproenzyme that includes a 16-residue putative signal peptide (Dyrlov et al., 2004). Also it contains a propeptide_C1 at positions 21–60, which is found in the N-terminal of those proteins that belong to the peptidase_C1 family as cathepsins, and the active peptidase cathepsin B which is coded by residues 79 to 326 (248 residues). The putative LvCathB protein showed a high degree of identity to other known cathepsin B from crustacean species, especially with those from the Peneidae family, sharing 97 and 94% identity with *P. monodon* and *Marsupenaeus japonicus*, respectively. However, the protein sequence shared only 55% identity with that from the northern shrimp *P. borealis* that belongs to the Pandalidae family (Fig. 2). When compared to crustacean cathepsins as *P. monodon*, *M. japonicus*, and *P. borealis*, LvCathB showed a similar protein size, but when compared with cathepsins from various insect and vertebrate species it showed to be slightly smaller.

The mature protein LvCathB of 248 amino acid residues long has a calculated molecular weight of 26.9 kDa and a theoretical pl of 6.1. As observed in Fig. 2, all compared sequences share some conserved characteristics of cathepsin B proteinases, as the three highly conserved residues forming the active site at C107, H276, and N296 and the glutamine oxyanion hole at residue Q101, which is known to stabilize the transition state of the reaction catalyzed by cystein proteinases (Menard et al., 1991). However, some other elements like the amino acid residues at the “occluding loop”, the GCNGG motif at positions 147–151, and the six S2 sites located at F152-P153, A250, G274, A277 and E322, seem to be conserved only in the non-digestive and *L. vannamei* cathepsin Bs (Fig. 2).

LvCathB possesses 15 cystein residues, with Cys107 (or Cys29 according to human cathepsin B structure; Musil et al., 1991) representing the active site cystein, 12 additional cystein residues which are presumed to be involved in six disulphide bridges formation at positions C92–C121, C104–C148, C140–C205, C141–C144, C177–C209, and C185–C196 are highly conserved among species, and an additional cystein residue which is not observed in species besides penaeids and marine fish was found at C294, and finally C317 which is also a conserved cystein (Fig. 2).
The disulphide bridge between residues C185 and C196 in L. vannamei cathepsin B sequence (Cys108 and Cys119 in human sequence) is suggested as the region were the occluding loop is formed. Also, H110 and H111 from human sequence are found in L. vannamei sequence at H187 and H188, suggesting that the side chains of both residues are implicated in exopeptidase activity (Musil et al., 1991). Finally LvCathB contains at the carboxylic terminus, a five residues extension (L327–N331) with no resolved function.

3.2. Shrimp LvCathB tissue-specific expression and the effect of starvation

The LvCathB steady state mRNA levels of different shrimp tissues were evaluated in triplicate by quantitative PCR. Each of the melting curves generated at the end of the real-time-PCR reactions contained a single PCR product for each gene in all samples. Fig. 3 shows the statistical differences detected among tissues relative to the L21 ribosomal protein transcripts (p < 0.05). Higher mRNA amounts were found in gills > hemocytes > midgut gland > gut > terminal ampullae > muscle. The higher values were 2 to 3-fold higher than ampullae considered the basis, since it was one of the tissues with lower LvCathB mRNA amounts and equals to 1. No detectable LvCathB mRNA amounts were observed in pleopods and eye stalk. Fig. 4 shows the changes produced by starvation in the amount of mRNA for four proteinases in the midgut gland of the white shrimp L. vannamei. Start and stop codons are marked in black squares. Both putative polyadenylation signals are double underlined. The shaded box indicates the predicted signal peptide; the open box indicates the predicted propeptide. The active peptide is underlined.
an increase was detected in their mRNA amounts after 120 h of starvation (p<0.05); also a statistically significant increase of LvCathB was detected at 24 h (p<0.05). Trypsin and cathepsin L were less affected by starvation, however, significant changes were detected between 0 and 120 h (p<0.05), both changed in a same manner. Among all evaluated enzymes chymotrypsin and LvCathB were detected in higher amounts at the end of the starvation period than trypsin and cathepsin L.

3.3. LvCathB enzyme activity

The resulting activities of cathepsin B from different shrimp tissues are shown in Table 3. Results show high specific activities in all those tissues tightly related to the digestive function as the midgut gland, the intestine and also in the collected gastric juice. Also, LvCathB activity was recorded in gills, but not in the hemocytes where high amounts of mRNA were previously found. Not enough tissue was available for determining LvCathB activity in the terminal ampule.

3.4. Phylogenetic analysis

Since major differences were observed between the cathepsin B sequences of Penaeidae and Pandalidae, an unrooted neighbor-joining tree was constructed to assess on the phylogenetic relationship among the Crustacean cathepsin B and that of several other taxa, including digestive enzymes from various invertebrates (Fig. 5). While non-digestive and L. vannamei cathepsin B amino acid sequences show to be highly conserved; the enzymes with digestive function showed a wide range of variability including non conservative amino acid substitutions.
substitutions at sites related to substrate recognition, binding and hydrolysis, e.g. the presence and sequence of the occluding loop including the HH residues involved in the exopeptidase activity, and the GCNGG motif. The inconsistencies in the amino acid composition of digestive cathepsin Bs are reflected on their distribution on the constructed cladogram; e.g. all of the non-digestive cathepsin Bs from insects form a well resolved clade in which the digestive cathepsin B of the species *T. infestans*, *Diabrotica vigatedera* and *T. molitor* are not included in such clade, but distributed on non-resolved branches of the cladogram. The same is observed for Decapods, *P. borealis* cathepsin B (digestive enzyme) is not included in the Decapod branch (Fig. 5). Cathepsin B enzymes responsible for hydrolysis of hemoglobin by blood-feeding helminthes share a unique motif, YWLIANSWxxDWGE (Baig et al., 2002), and helminths included in this analysis are not the exception.

### 4. Discussion

The deduced amino acid sequence for cathepsin B from *L. vannamei* shares most of the common features from cystein proteinases (McGrath, 1999). The LvCathB pro-region, which is strictly required in all cystein proteinases for the expression of the native enzyme (Vernet et al., 1995), is less conserved than the mature protein as commonly observed among these proteinases, except when compared with closer species as those of the Penaeidae family. The cathepsin B deduced proteins of the three shrimp species included in the multiple alignment (Fig. 2) do not include the conserved GNDF motif in the pro-region sequence, which is in agreement with previous observations since this motif appears in all papain group members, except cathepsins B and C (Vernet et al., 1995).
Our analysis showed that LvCathB protein contains twelve conserved cysteine residues, which suggests the potential formation of six disulphide bridges, however, two additional cysteines at the very end of the carboxyl-terminal are also found. One of these cysteines, C294, is not conserved among species but present in the three penaeid shrimp sequences, and C317 is unique to cathepsin B but conserved among species (Musil et al., 1991). Further studies constructing a predictive model or the solved crystal structure of *L. vannamei* cathepsin B, and the evaluation of kinetic parameters of the enzyme may help to understand the biological function of these cysteine residues.

As part of our results, and in agreement with other lysosomal enzymes from shrimp species (Hu and Leung, 2004), the LvCathB mRNA was detected in most of the evaluated shrimp tissues. Large amounts of the LvCathB mRNA were found on both the midgut gland and gut, organs primarily involved in the process of food digestion, just as reported for cathepsin L in the midgut gland of *Metapenaeus ensis* (Hu and Leung, 2004). However, transcripts and the active enzyme were also detected in no-digestive tissues including hemocytes, gills, muscle, and terminal ampoule. Studies dealing with cathepsin B were also detected in no-digestive tissues including hemocytes, gills, muscle, and terminal ampoule. Studies dealing with cathepsin B from invertebrate species have reported that it is involved in control over the processes of food digestion, just as in regulating larval metamorphosis and development in species as *Culex pipiens pallens* (Uchida et al., 2006). However, in the white shrimp that inhabits tropical waters, LvCathB was found to be highly active in those tissues related to digestive processes, confirming its participation during food protein hydrolysis.

According to the phylogenetic analysis performed in this study, LvCathB groups with other decapods’ cathepsin B lysosomal enzymes. In this regard we suggest that LvCathB functions primarily as a lysosomal enzyme, and given the holocrine secretion found in the midgut gland of crustaceans, this enzyme is released once the cells disrupt their plasma membrane. However, the high amount of LvCathB mRNA detected in gills and hemocytes, remain to be explained.

A high degree of variability in the amino acid sequences at sites related to substrate recognition, binding and catalysis was found among digestive cathepsin B’s which may be related to structural and functional implications, influencing in their substrate specificity and susceptibility to the effect of inhibitors (Cathers et al., 2002), such effects may lead to lack of classic cathepsin B role in lysosomal physiology and signalling (Krupa et al., 2002).

In addition, it is worth noting that the nematode and trematode digestive cathepsin B protein sequences included in the analysis share the motif YWLIANSWxxDWGE previously described by Baig et al. (2002). This motif surrounds the N219 residue of LvCathB from the active site region where hydrogen binding plays a key role in catalysis (Wang et al., 1994). It has been suggested that single- or double-point mutations occurring at the motif region may provoke critical modifications in the proteinolytic character of cysteine proteinases affecting its substrate specificity. Furthermore, the evidence suggests that this motif evolved independently in different lineages, and that it may have arisen through convergent evolution, since the motif is present in phylogenetically diverse helminthes, being the mammalian hemoglobin the selective force leading to the presence of the motif (Baig et al., 2002).

Arthropod cathepsin B activity with extracellular digestive function has been reported in a broad diversity of taxa exhibiting wide feeding habits (Aoki et al., 2003; Prabhakar et al., 2007; Sojka et al., 2008), therefore the presence of digestive cathepsin B enzymes in arthropods cannot be explained in terms of a single alimentary selective force. Since *P. borealis* digestive cathepsin B shows characteristics of a cold-adapted enzyme, its presence has been explained as an adaptation to the cold environments where this species is distributed (Aoki et al., 2003). However, in the white shrimp that inhabits tropical waters, LvCathB was found to be highly active in those tissues related to digestive processes, confirming its participation during food protein hydrolysis.

Table 3

LvCathB activity on various tissues of the white shrimp *Litopenaeus vannamei*.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific activity (U/μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric juice</td>
<td>270.42 ± 62.01</td>
</tr>
<tr>
<td>Midgut gland</td>
<td>666.95 ± 96.53</td>
</tr>
<tr>
<td>Intestine</td>
<td>63.29 ± 13.7</td>
</tr>
<tr>
<td>Hemocytes</td>
<td>0.01 ± 0.006</td>
</tr>
<tr>
<td>Gills</td>
<td>0.170 ± 0.008</td>
</tr>
<tr>
<td>Eye stalk</td>
<td>ND</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.001 ± 0.002</td>
</tr>
<tr>
<td>Pleopods</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND = not detected.

### Fig. 3.
LvCathB mRNA expression relative to ribosomal protein L21 in six different shrimp tissues. (Hm) haemocytes, (M) muscle, (Amp) terminal ampoule, (Gut) intestine, (MG) midgut gland, and (G) gills. Data represent mean values ± std. error. Different letters denote statistical differences (p<0.05).

### Fig. 4.
Relative expression of four proteinases in the midgut gland of shrimp at different starvation times. Data represent mean values ± std. error. Asterisks denote statistical differences (p<0.05).
on the effect of starvation in the midgut gland proteinases from shrimp have shown that both the gene expression of trypsin (Sanchez-Paz et al., 2003) and the enzyme activity of trypsin and chymotrypsin are significantly affected (Muhlia-Almazan and Garcia-Carreño, 2002). Additionally, the participation of cathepsin L, another lysosomal cystein proteinase found in the midgut gland of shrimp species, during protein hydrolysis in the midgut gland during extracellular food protein digestion has been observed in the penaeid shrimp *M. ensis* (Hu and Leung, 2007).

Finally, we conclude that since the LvCathB mRNA is significantly affected by starvation, in the same way as other digestive enzymes are, their activities could be complemented, operating in a cooperative manner as a network. We believe that the pH changes found along the shrimp digestive tract, including those in and out the midgut gland, may explain the spatio-temporal secretion and participation of each enzyme, however, whether cathepsins L or B are the major cystein proteases, acting in food protein digestion remains to be analyzed.

Further studies are required to reveal the complete protein digestion machinery working after shrimp food intake, including the specific sites of synthesis and activation of each enzyme. To date, there are no reports of aspartic proteinases as cathepsin D, found in the midgut gland of shrimp; however, other marine crustacean species as lobsters are known to synthesize these enzymes as part of their extracellular digestion mechanism (Rojo et al., 2010).

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**References**


