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# Is digestive cathepsin D the rule in decapod crustaceans?

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ABSTRACT

Cathepsin D is an aspartic endopetidase with typical characteristics of lysosomal enzymes. Cathepsin D activity has been reported in the gastric fluid of clawed lobsters where it acts as an extracellular digestive enzyme. Here we investigate whether cathepsin D is unique in clawed lobsters or, instead, common in decapod crustaceans. Eleven species of decapods belonging to six infraorders were tested for cathepsin D activity in the midgut gland, the muscle tissue, the gills, and when technically possible, in the gastric fluid. Cathepsin D activity was present in the midgut gland of all 11 species and in the gastric fluid from the seven species from which samples could be taken. All sampled species showed higher activities in the midgut glands than in non-digestive organs and the activity was highest in the clawed lobster. Cathepsin D mRNA was obtained from tissue samples of midgut gland, muscle, and gills. Analyses of deduced amino acid sequence confirmed molecular features of lysosomal cathepsin D and revealed high similarity between the enzymes from Astacidea and Caridea on one side, and the enzymes from Penaeoidea, Anomura, and Brachyura on the other side. Our results support the presence of cathepsin D activity in the midgut glands and in the gastric fluids of several decapod species suggesting an extracellular function of this lysosomal enzyme. We discuss whether cathepsin D may derive from the lysosomal-like vacuoles of the midgut gland B-cells and is released into the gastric lumen upon secretion by these cells.

## 1. Introduction

Protein is the main component in the food of heterotrophic organisms. Therefore, the importance of proteolytic enzymes in digestion. Neurath (1984) stated that proteases are presumed to have arisen in the earliest phases of biological evolution since even the most primitive organisms must have required them for digestion and for the metabolism of their own proteins. In crustaceans, digestive enzymes are synthesized in the midgut gland, likewise called hepatopancreas. This organ also plays a fundamental role in the absorption and storage of nutrients (Loizzi, 1971; McGaw and Curtis, 2013; Saborowski, 2015; Vogt, 1993). Mechanisms of production and storage of digestive enzymes in crustaceans differ significantly from those in vertebrates (Vogt et al., 1989). In crustaceans, digestive enzymes are synthesized in the F-cells of the midgut gland and secreted into the midgut gland lumen (Caceci et al., 1988; Ceccaldi, 1998; Vogt et al., 1989). Then, the enzymes accumulate in the gastric chamber and contribute to the extracellular digestion of food items (Saborowski, 2015; Vogt et al., 1989).

Animals possess many types of proteases, distinguished by the amino acid at the active site and the location in which it functions. Serine proteases, including trypsin and chymotrypsin, are the most abundant and, hence, the best described endopeptidases in the digestive system of decapods. Their functions are well established as well as the mechanisms of synthesis, storage, activation, and secretion (Celis-Guerrero et al., 2004; Díaz-Tenorio et al., 2006; Garcia-Carreño et al., 1994; Hernández-Cortés et al., 1997; Muhlia-Almazán et al., 2008). Klein et al. (1996) and Sainz et al. (2004) showed that trypsin isoforms are synthesized as inactive precursors but are rapidly activated.

Digestive endopeptidases belonging to the classes of metallo-, cysteine, and aspartate-proteases have been reported and characterized as well although for a limited number of decapod species (Laycock et al., 1989; Navarrete del Toro et al., 2006; Rojo et al., 2010b; Stöcker et al., 1988; Teschke and Saborowski, 2005). Additionally, the aspartic endopeptidase, cathepsin D, was found in the gastric fluid of the clawed lobsters *Homarus gammarus* and *H. americanus* and was identified as a fully operational extracellular digestive enzyme at the acid pH of gastric fluid (Navarrete del Toro et al., 2006; Rojo et al., 2010a). The presence of cathepsin D in the gastric fluid seems unusual as this enzyme is known and well characterized as typical lysosomal and, thus, intracellular endopeptidase.

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Cathepsin D (EC 3.4.23.5) plays a crucial role within the cells. By hydrolyzing intracellular proteins it participates in various physiological processes involved in maintaining tissue homeostasis, regulation of apoptosis, activation of hormones and growth factors (Benes et al., 2008), to mention a few. In insects, cathepsin D-like peptidases showed specialized functions like cellular remodelling during metamorphosis (Gui et al., 2006). In humans, cathepsin D is synthesized in the rough endoplasmic reticulum as a pre-pro-enzyme but rapidly loses the signal peptide. The enzymatically inactive pro-cathepsin D, now called precursor of cathepsin D or zymogen, is glycosylated in the endoplasmic reticulum and directed into the Golgi complex. Once in the Golgi complex the precursor is tagged with mannose-6-phosphate (M6P) and directed into the endosomes due to specific M6P receptors. The endosomes will eventually transport the precursor to lysosomes (Nicotra et al., 2010) where they will be activated by the action of cysteine lysosomal proteases. Finally the mature enzyme consists of two chains, one of 14 kDa in the amino-terminal domain and the other of 34 kDa in the carboxyl-terminal domain (Laurent-Matha et al., 2006). Once activated, the cathepsin D will participate in the hydrolysis of proteins within the lysosomes.

In contrast to serine or cysteine peptidases that form a covalent intermediate in the hydrolysis of the peptide bond, aspartyl peptidases catalyze the hydrolysis of the peptide bond by means of an acid-base mechanism that involves the coordination of a water molecule. One of the two aspartic acid residues forming the catalytic dyad activates the water molecule by taking a proton. This makes the water nucleophilic and allows it to attack on the carbonyl carbon of the substrate scissile bond, creating a tetrahedral oxyanion intermediate. Upon electron rearrangement the scissile amide is protonated which results in the splitting of the substrate peptide into two peptides (Brik and Wong, 2003; Conner, 2004).

Cathepsin D evolved to an enzyme with digestive function in invertebrates like insects (Srp et al., 2016), mites (Wajahat Mahmood et al., 2013) and bloodsucking parasites as part of a multienzyme proteolytic complex (Sojka et al., 2016) and in vertebrates like bloodsucking fish (Xiao et al., 2015) and now we found it in crustaceans (Navarrete del Toro et al., 2006; Rojo et al., 2010a). The presence of cathepsin D in the gastric fluid of clawed lobsters that contributes to the extracellular digestion (Rojo et al., 2010b) raises the question whether this situation is unique within the genus of clawed lobsters (*Homarus* sp.) or spread among other decapod taxa. Therefore, in this study we assessed the presence of cathepsin D in the gastric fluid and other tissues of 11 decapod species from six infraorders by means of catalytic activity as well as the expression of cathepsin D mRNA. The data presented here may contribute to a better understanding of the function and evolution of the digestive processes in the midgut gland of decapod crustaceans.

## 2. Materials and methods

### 2.1. Studied species

Adults of 11 species of decapod crustaceans belonging to six different infraorders (Table 1) were analysed for the presence of cathepsin D activity and mRNA. Species from the North Sea were caught during two sampling campaigns, one in September 2013 and the other in April 2016, near the island of Helgoland (54°11′N, 7°55′E), by bottom trawling. American lobster, *H. americanus*, were purchased from a local seafood merchant (Edelfisch Kontor, Bremerhaven, Germany) and the European crayfish *Astacus astacus* from a crayfish rearing facility (Edelkrebszucht Göckemeyer, Neustadt-Poggenhagen, Germany). Specimens were processed in the laboratories of the Alfred Wegener Institute in Bremerhaven, Germany. Red or California spiny lobster *Panulirus interruptus* were purchased from a local seafood merchant in La Paz, Baja California Sur, in February 2014. The Blue crab *Callinectes bellicosus* and the Whiteleg shrimp *Penaeus vannamei* were caught in the Gulf of California and at the Mexican Pacific coast in August and October 2013, and September 2016. Specimens were processed in the laboratories of the Centro de Investigaciones Biológicas del Noroeste in La Paz. BCS, Mexico.

After collection, the specimens were maintained without food for 24 h in running seawater to level their dietary status and to empty their stomachs of food remains. The gastric fluids from *H. americanus*, *A. astacus*, *P. interruptus*, *Pagurus bernhardus*, *Liocarcinus depurator*, *C. bellicosus*, and *Cancer pagurus* were sampled from the gastric chambers by inserting a flexible tube through the oesophagus which was attached to a 10 ml syringe. Depending on the specimens' size, 0.5 to 2 ml of gastric fluid was obtained. Each specimen was sampled only once. Immediately after sampling the gastric fluid was transferred into 1.5-ml reaction tube and stored at −80 °C until used. It was not possible to obtain gastric fluid from *Pandalus montagui*, *Crangon crangon*, *P. vannamei*, and *Nephrops norvegicus*.

Subsequently, tissue samples of midgut gland (MG), muscle (M), and gills (G) were taken. All samples were divided into two subsamples, one was transferred into a 1.5-ml tube and frozen at − 80 °C for further protein and enzyme analysis. The other was transferred into 1.5-ml tubes containing 0.5 ml of RNAlater (AM7020, Life Technologies, ONT, Canada) for future RNA extraction.

### 2.2. Enzyme preparation

Samples of gastric fluid were thawed and centrifuged for 15 min at 10,000 × *g* and 4 °C to discard solids. Samples of midgut gland, muscle, and gills (75 to 100 mg) were thawed and homogenates of individual tissues were prepared on ice by ultrasonication (Branson Sonifier Cell Disruptor) with three bursts of 5 s and 10 s breaks in between. Homogenates were centrifuged for 30 min at 10,000 × *g* and 4 °C, and the supernatants, which contained the soluble protein, were aliquoted and stored at −80 °C. The sample was aliquoted to avoid repeated thawing and freezing. Each aliquot was used only once. During the assays the samples were cooled on ice. No loss of activity was detected between fresh and frozen samples.

### 2.3. Quantification of soluble protein and cathepsin D activity

Protein concentration was quantified after Bradford (1976) with serum bovine albumin as the standard (B-4287, Sigma-Aldrich). Cathepsin D activity was measured in a microplate reader using the fluorogenic substrate 7-methoxycoumarin-4-acetyl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys-(DNP)-DArg-amide (M0938, Sigma-Aldrich) in 50 mM sodium acetate buffer at pH 4.0 (Rojo et al., 2010b). Excitation and emission wavelengths were 320 nm and 380 nm, respectively. Rates of hydrolysis of the substrate were recorded by measuring the increase of fluorescence every 30 s for 10 min and expressed as change of relative fluorescence units per minute (RFU $min^{-1}$). The linear part of the kinetic curve was used to calculate activity. A calibration curve was established by measuring the fluorescence of increasing concentrations of the fluorochrome 7-methoxycoumarin-4-acetic acid (MCA) (235,199, Sigma-Aldrich). One unit of activity was expressed as one nmol of MCA liberated per minute per mg of protein in 50 mM of sodium acetate buffer at pH 4.0 and room temperature.

### 2.4. Inhibition of cathepsin D with pepstatin A

Specific inhibition of cathepsin D was done using pepstatin A (Sigma, P 5318), an inhibitor specific for aspartic peptidases (Navarrete del Toro et al., 2006). Ten µl of enzyme preparation were incubated with 10 µl of 10 mM pepstatin A at room temperature and the remaining activity was measured after 30 min as above.

**Table 1**
Systematic classification, origin and sampling date of the studied species.

| Suborder | Infraorder | Species (abbrev.) | Origin | Date of sampling |
|---|---|---|---|---|
| Pleocyemata | Astacidea | *Homarus americanus* (*H.a.*) | Atlantic coast of Canada | May 2014/Apr. 2016 |
| Pleocyemata | Astacidea | *Nephrops norvegicus* (*N.n.*) | North Sea, Germany | Sep. 2013/Apr. 2016 |
| Pleocyemata | Astacidea | *Astacus astacus* (*A.a.*) | Crayfish Farm, Germany | May 2013/Apr. 2016 |
| Pleocyemata | Caridea | *Pandalus montagui* (*P.m.*) | North Sea, Germany | Sep. 2013/Apr. 2016 |
| Pleocyemata | Caridea | *Crangon crangon* (*C.c.*) | North Sea, Germany | Sep. 2013/Apr. 2016 |
| Pleocyemata | Palinura | *Panulirus interruptus* (*P.i.*) | Pacific, BCS, Mexico | Feb. 2014/Sep. 2016 |
| Pleocyemata | Anomura | *Pagurus bernhardus* (*P.b.*) | North Sea, Germany | Sep. 2013/Apr. 2016 |
| Pleocyemata | Brachyura | *Liocarcinus depurator* (*L.d.*) | North Sea, Germany | Sep. 2013/Apr. 2016 |
| Pleocyemata | Brachyura | *Callinectes bellicosus* (*C.b.*) | Gulf of California, BCS, Mexico | Sep. 2013/Sep. 2016 |
| Pleocyemata | Brachyura | *Cancer pagurus* (*C.p.*) | North Sea, Germany | Sep. 2013/Apr. 2016 |
| Dendrobranchiata | Penaeoidea | *Penaeus vannamei* (*P.v.*) | Pacific, BCS, Mexico | Oct. 2013/Sep. 2016 |

### 2.5. Assessing of cathepsin D mRNA in tissues

Tissue samples, stored in RNAlater, were thawed and 30 mg of each one were taken for RNA extraction. The Precellys keramik-kit 1.4 (91-PCS-CKM, PEQLAB, Erlangen, Germany) was used for the cell lysis by 3 cycles of 15 s shaking and 30 s pause in between shakes. Subsequently the samples were centrifuged for 3 min at 13,000 × *g* and at room temperature. Total RNA was isolated using the RNeasy Mini Kit Spin Columns (74,104, QIAGEN, Texas, USA), following the manufacturer's instructions. Total RNA concentration was assessed spectrophotometrically with a NanoDrop device at 260 nm and purity was assessed by the ratio of absorption at 260/280 nm. DNA was removed by digestion with one unit of DNase I (AMPD-1, Sigma-Aldrich) for 15 min at 25 °C. Reverse transcription was performed with the Reverse Transcription System (A3500, Promega, Madison, WI, USA) using 1 μg of total RNA in 20 μl reactions using oligo-dT as anchor primer following the manufacturer's recommendations.

The presence of cathepsin D transcripts was assessed by RT-PCR. One μl of cDNA from each species and tissue were used as a template for the RT-PCR reactions using the specific primers based on *H. americanus* (5′ → 3′) cathepsin D Fwd. CTCAGTACTACGGCCCCATC and cathepsin D Rev. CCSAGGATGCCGTCYAACTT. S and Y indicate degenerate sites (S═C or G. Y═C or T; Rojo et al., 2010b). PCR amplifications (final volume of 12 μl) were carried out with GoTaq® Green Master Mix (M7122, Promega, WI, USA). The PCR amplification program was: 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 59 °C and 30 s at 68 °C. In the last cycle, the extension step at 68 °C lasted for 5 min. PCR products were separated on 1.5% agarose gels (16,500 Invitrogen, C.A, U.S.A) and stained with SYBR® Safe DNA gel stain. Target bands were cut out of the gel and the DNA was extracted with the peqGold extraction kit (VWR. Pensilvania. USA). Sequencing PCR was carried out with the BigDye terminator V 3.1 Cycle sequencing Kit (4,336,923, Applied Biosystems, USA). The primers designed for cathepsin D were used as initiators. The sequencing PCR program consisted of 1 min at 96 °C, followed by 30 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C and after the last cycle 15 °C indefinitely. The products were treated with the kit Dye-Ex (63,204, QIAGEN, Germany) to remove the terminator dye, which was not incorporated. Finally the DNA sample was sequenced in the department of Chemical Ecology in the Alfred Wegener Institute, Bremerhaven Germany. A BLAST analysis (Altschul et al., 1990) confirmed the obtained sequences as cathepsin D. They were translated into amino acid sequences for further analysis. An alignment was carried out using ClustalW at EMBL-EBI (Larkin et al., 2007).

### 2.6. Statistical analysis

Levene test was used to analyse the activity data for normal distribution and homogeneity of variances. To test if there are differences in the activity of the cathepsin D between midgut gland and the gastric fluid among the different species and tissues the data were analysed by one-way ANOVA. If homogeneity of variances failed, the ANOVA was combined with a Welch test. Differences among groups were subsequently identified by pairwise comparison with the Tukey's post-hoc test. The level for statistical significance was set at $P < 0.05$. All statistical tests were carried out with the software PAST (Version 3.0).

### 2.7. Cladogram of partial sequences of cathepsin D

A cladogram based on nucleotide sequences was constructed using MEGA 6 (Tamura et al., 2013). Evolutionary history was interfered by the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969). The initial tree for the heuristic search was obtained by applying Neighbour-Join algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. A Bootstrap method based on 10,000 permutations was applied for assessing confidence analysis of the clades. The gap sites were ignored in the analysis.

## 3. Results

### 3.1. Activity of cathepsin D

Cathepsin D activity was found in all species analysed (Fig. 1). Among those species from which gastric fluid was studied, highest activity of cathepsin D was detected in *H. americanus* ($251.5 \pm 24.6\ U\ mg^{-1}$). It was significantly higher than in all other species ($P < 0.005$, Fig. 1a). The gastric fluid of *L. depurator* showed quite high activity as well ($147.5 \pm 36.4\ U\ mg^{-1}$). Lowest activity was present in the gastric fluid of *C. bellicosus* ($28.2 \pm 6.6\ U\ mg^{-1}$). No gastric fluid was obtained from *N. norvegicus* or the small species *P. montagui*, *C. crangon*, and *P. vannamei*.

Activity of cathepsin D was quantified in the midgut glands of all species investigated. Again, the highest activity was present in *H. americanus* ($73.9 \pm 10.0\ U\ mg^{-1}$, Fig. 1b). *P. interruptus*, *C. bellicosus* and *P. vannamei*, showed intermediate activity of $24.9 \pm 5.5\ U\ mg^{-1}$ to $30.1 \pm 14.3\ U\ mg^{-1}$. Lowest activity was obtained from the midgut gland of *A. astacus* ($8.7 \pm 1.7\ U\ mg^{-1}$) (Fig. 1b). The cathepsin D activity in each tissue is listed in Table 2. The activities in muscle and gills were orders of magnitude and significantly lower than in the midgut glands. Muscles of *A. astacus* and *P. bernhardus* showed highest activities among species ($0.984 \pm 0.166\ U\ mg^{-1}$ and $0.924 \pm 0.348\ U\ mg^{-1}$, respectively). Negligible activities were detected in the muscles of *P. interruptus* and *P. vannamei* (Table 2). In all species, gills showed higher cathepsin D activities than muscles. They ranged from a minimum value of $0.030 \pm 0.008\ U\ mg^{-1}$ in *P. vannamei* to a maximum value of $5.648 \pm 1.349\ U\ mg^{-1}$ in *C. crangon* (Table 2).

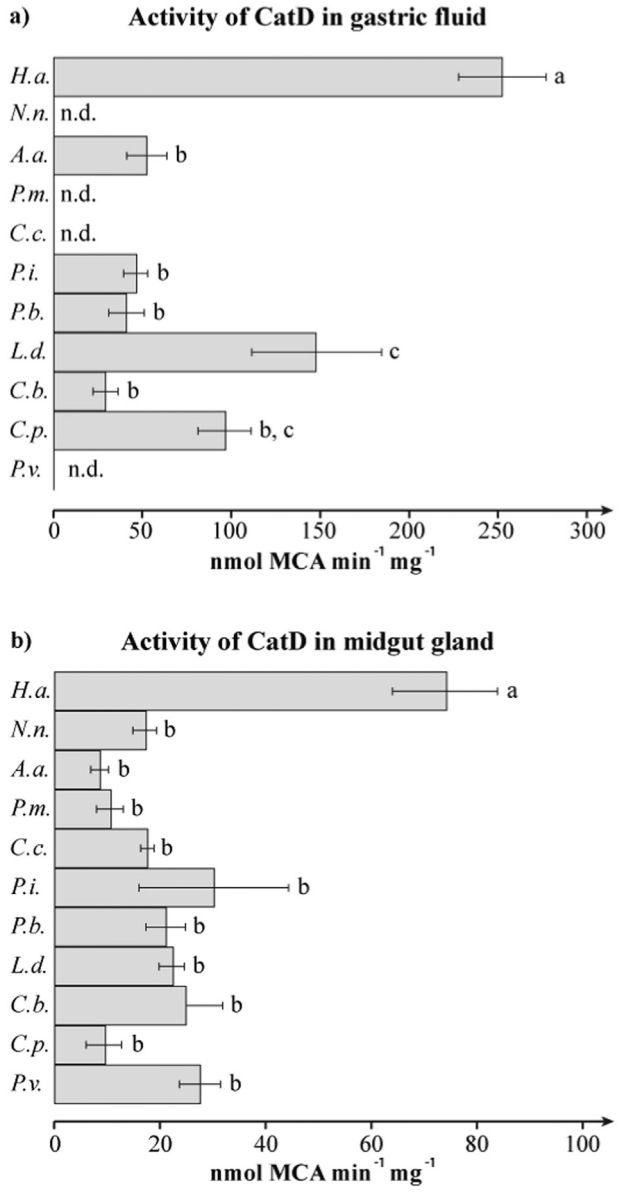


**Fig. 1.** Activity of cathepsin D in the gastric fluid (a) and in the midgut gland tissue (b) from studied species (means ± SEM, n = 6, for each species). Different letters indicates significantly different values.

### 3.2. Inhibition of cathespin D with Pepstatin A

Because the substrate used to quantify the activity of cathepsin D is not 100% specific and some other peptidases may hydrolyze it at low rate, we run a confirmative test for cathepsin D. Pepstatin A is a peptidase competitive inhibitor with one of the lowest dissociation constant ($4.5 \times 10^{-11}$) in nature for aspartic peptidases and is used due to its ability to inhibit pepsin at picomolar concentrations. Cathepsin D activity in samples was inhibited by pepstatin A. In midgut glands the highest percentage of inhibition was present in *H. americanus*, *P. bernhardus* and *P. vannamei* accounting for 94.5%, 95.5% and 96.5% respectively. In *C. crangon* and *P. montagui* the inhibition was 76.9% and 77.3%. In the gastric fluids the maximum inhibition was detected in *P. bernhardus* and *L. depurator* with 97.7% and 96.9% (Table 3).

**Table 3**
Percentage of inhibition of cathepsin D activity by 30 min incubation with pepstatin A. All assays were run in triplicate in each of four specimens.

| Specie | Percentage of cathepsin D inhibition | |
|---|---|---|
| | Gastric fluid | Midgut gland |
| *H. americanus* | 83.2 ± 5.0 | 94.5 ± 1.4 |
| *N. norvergicus* | n.d. | 80.9 ± 4.3 |
| *A. astacus* | 95.9 ± 0.5 | 89.3 ± 4.0 |
| *P. montagui* | n.d. | 77.3 ± 3.2 |
| *C. crangon* | n.d. | 76.9 ± 1.7 |
| *P. interruptus* | 86.4 ± 1.5 | 87.6 ± 2.4 |
| *P. bernhardus* | 97.7 ± 0.8 | 95.5 ± 1.3 |
| *L. depurator* | 96.9 ± 1.0 | 89.7 ± 2.6 |
| *C. bellicosus* | 79.2 ± 1.7 | 93.1 ± 1.8 |
| *C. pagurus* | 95.3 ± 1.7 | 90.8 ± 3.1 |
| *P. vannamei* | n.d. | 96.5 ± 0.3 |

### 3.3. Cathepsin D mRNA

Transcripts of cathepsin D were found in the midgut glands of all species, except for *P. interruptus* and *C. bellicosus*. In *P. bernhardus* and *C. pagurus* cathepsin D transcripts in midgut glands were negligible. Transcripts of cathepsin D were found in the muscle tissues of *A. astacus*, *P. montagui*, *P. bernhardus*, *C. bellicosus* and *C. pagurus* but not in *N. norvegicus*, *C. crangon*, *P. interruptus*, *L. depurator* or *P. vannamei*. In gills, transcripts of cathepsin D were present in *N. norvegicus*, *A. astacus*, *P. montagui*, *C. crangon, P. bernhardus, L. depurator*, and *C. pagurus* (data not shown in figure).

### 3.4. Decapod cathepsin D deduced amino acid sequence

Partial sequences of cathepsin D mRNA were obtained from the midgut glands of *N. norvegicus*, *A. astacus*, *C. crangon*, *P. bernhardus*, *L. depurator*, *C. pagurus* and *P. vannamei*. Based on the deduced amino acid sequence of the cathepsin D fragments from midgut gland of several decapod species, we identified conserved motifs, which are archetypal for cathepsin D. One is the conserved motif that codifies for one of the aspartic acid residue D (GAT and GAC; Fig. 2) in the catalytic motif DTGS (GATACGGGGTCC). This domain is exclusive to cathepsin D.

**Table 2**
Activities of cathepsin D at pH 4.0 in different tissues of decapods crustaceans (means ± SEM, n = 6). All assays were run in triplicate in each of four specimens.

| Species | Gastric fluid | Midgut gland | Muscle | Gill |
|---|---|---|---|---|
| *Homarus americanus* | 251. 5 ± 24.6 | 73.9 ± 10.0 | 0.519 ± 0.276 | 0.947 ± 0.356 |
| *Nephrops norvegicus* | n.d. | 17. 3 ± 2.2 | 0.348 ± 0.057 | 3.799 ± 0.626 |
| *Astacus astacus* | 51.0 ± 10.9 | 8.7 ± 1.7 | 0.984 ± 0.166 | 1.647 ± 0.329 |
| *Pandalus montagui* | n.d. | 10.6 ± 2.6 | 0.205 ± 0.024 | 3.205 ± 0.780 |
| *Crangon crangon* | n.d. | 17.5 ± 1.2 | 0.236 ± 0.107 | 5.648 ± 1.349 |
| *Panulirus interruptus* | 46. 1 ± 7.1 | 30.1 ± 14.3 | 0.008 ± 0.002 | 0.142 ± 0.055 |
| *Pagurus bernhardus* | 40. 1 ± 10.3 | 21.0 ± 3.6 | 0.924 ± 0.348 | 2.401 ± 0.417 |
| *Liocarcinus depurator* | 147. 5 ± 36.4 | 22.3 ± 2.5 | 0.627 ± 0.092 | 3.465 ± 0.526 |
| *Callinectes bellicosus* | 28.2 ± 6.6 | 24.8 ± 5.5 | 0.038 ± 0.010 | 0.089 ± 0.015 |
| *Cancer pagurus* | 96.2 ± 15.4 | 9.6 ± 3.4 | 0.705 ± 0.339 | 1.326 ± 0.912 |
| *Penaeus vannamei* | n.d. | 27. 5 ± 3.8 | 0.006 ± 0.001 | 0.030 ± 0.008 |

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        65        75        85        95       105       115       125
H.a.  GPITIGTPGQGFDVIFDTGSSNLWIPSEKCFILNLACRLHNRYDSTKSSTYIENGTAFDI
N.n.  GPITIGTPGQGFDVIFDTGSSNLWIPSEKCFILNLACRLHNRYDSTKSSTYIENGTAFDI
C.c.  GPITIGTPGQGFDVIFDTGSSNLWIPSEKCFILNLACRLHNRYDSTKSSTYIENGTAFDV
P.m.  GPITIGTPGQGFDVIFDTGSSNLWIPSEKCFILNLACRLHNRYDSTKSSTYIENGTAFDI
A.a.  GPITIGTPGQGFDVIFDTGSSNLWIPSEKCFILNLACRLHNRYDSTKSSTYIENGTTFDI
P.v.  GPISIGTPRQSFRVVFDTGSSNLWVPSKQCLYTNIACLLHNKYDSRKSSTFKKNGTEFAI
L.d.  GPISIGTPPQSFRVVFDTGSSNLWVPSKQCHYTNIACLLHNKYDSRKSSTFKKNGTEFAI
P.b.  GPISIGTPPQSFRVVFDTGSSNLWVPSKQCHFTNIACLIHNKYDARKSSTFKKNGTEFAI
C.p.  GPISIGTPPQSFRVVFDTGSSNLWVPSKQCHYTNIACLLHNKYDSRKSSTYKKNGTDFAI
      ***:**** *:* *:********:**::*   *:** :**:**: ****: :*** * :

              135       145       155       165       175
H.a.  QYGSGALHGFLSSDNVEMGGVNAMGQTFAEATQE-PGLAFIMGKLDGILG
N.n.  QYGSGALHGFLSSDNVEMGGVNAMGQTFAEATQE-PGLAFIMGKLDGILG
C.c.  QNGSGALHGFLSSDNVEMGGVNAMGQTFAEATQE PGLAFIMGKLDGILG
P.m.  QYGSGALHGFLSSDNVEMGGVNAMGQTFAEATQE-PGLAFIMGKLDGILG
A.a.  QYGSGALHGFLSSDNVEMGGVNAMGQTFAEATQE-PGLAFIMGKLDG---
P.v.  QYGSGSLS----------------------------------------
L.d.  QYGSGSLSGYLSTDTVAVASIAVKEQTFAEARETRPGFCCCVRRHPG---
P.b.  QYGSGSLSGYLSTDTVTVASLSVKNQTFAEALSE-PGLAFVAAKLDGILG
C.p.  KYGSGSLSGYLSTDTVAIGSVAVKSQTFAEALSE-PGLAFVAAKLDGILG
      : ***:*
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**Fig. 2.** Multiple sequence alignment of the deduced amino acid sequences of cathepsin D of decapods using the software ClustalW. Sequence numbering according to *Homarus americanus* cathepsin D (Rojo et al., 2010b). The underlined and bold D represents the conserved aspartic amino acid residue of the catalytic site in the motif DTGS which is strictly conserved in cathepsin D. The cysteine residues that might form a disulphide bond are linked with a horizontal square bracket. The sequence motif in bold constitute the residues that form the Y75 flap. The tyrosine residue is shaded. The upright triangle highlights the presence of a potential asparagine glycosylation residue. Asterisks represent identical or conserved residues in all the sequences in the alignment, colons are conservative substitutions, periods are semi conservative substitutions.

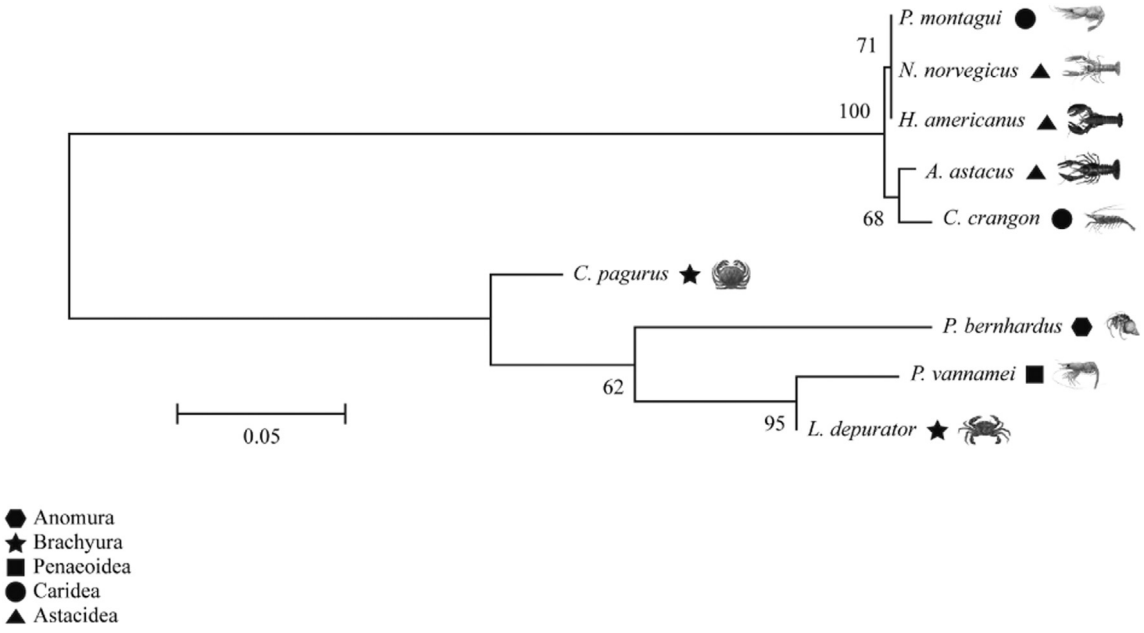


**Fig. 3.** Maximum Likelihood cladogram of partial nucleotide sequences of cathepsin D of the studied species and the sequence of the lobster *Homarus americanus* (Rojo et al., 2010b). Bootstrap values are noted at the nodes.

**Table 4**
Percentage of similarity between partial cathepsin D sequences from decapods and the sequence of the Lobster *Homarus americanus*.

| Species | Sequence similarity (in %) |
|---|---|
| *Nephrops norvegicus* | 100 |
| *Astacus astacus* | 98.7 |
| *Pandalus montagui* | 100 |
| *Crangon crangon* | 98.7 |
| *Pagurus bernhardus* | 69.2 |
| *Liocarcinus depurator* | 68.9 |
| *Cancer pagurus* | 67.6 |
| *Penaeus vannamei* | 66.5 |

Additional characteristics were found, such as two cysteine residues that might form one of the disulphide bonds (Cys45 and Cys50, porcine pepsin numbering), and the residues that form an extended loop, the so-called Y75 flap (from Tyr75, porcine pepsin numbering). Most sequences contain the conserved tyrosine residue (Fig. 2). The exception is *C. crangon* that bears a nonhomologous asparagine residue. Also, the potential asparagine glycosylation residue N (AAC; Asn67, porcine pepsin numbering, marked with a triangle in Fig. 2) was found, indicating that similar to mammalian enzymes, decapod cathepsin D might be tagged for transfer to lysosomes (Fig. 2). Based on the comparison of the cathepsin D partial sequences obtained from the midgut gland of several decapod species it was possible to create a cladogram (Fig. 3). There are two main branches in the cladogram: one of them includes the highly conserved cathepsin D from Astacidea and Caridea (98.7% to 100% similarity). The second branch is less conserved and

includes cathepsin D from Anomura, Brachyura and Penaeoidea. *C. pagurus* (Brachyura) shows more similarity with *P. bernhardus* (Anomura) than with *L. depurator* (Brachyura) or *P. vannamei* (Dendrobranchiata, Penaeoidea). Partial cathepsin D was compared with *H. americanus* cathepsin D (Accesion number: EU687261.1). The percentages of similarity are summarized in Table 4. Overall, for the region analysed, cathepsin D of *P. montagui* (Caridea) and *N. norvegicus* (Astacidea) show the highest similarity with *H. amercianus* cathepsin D. The sequence of cathepsin D of *P. vannamei* (Penaeoidea) shows the lowest similarity with the cathepsin D of *H. americanus*.

## 4. Discussion

In this study we showed that the lysosomal peptidase cathepsin D appears in the digestive organs and the gastric fluid of eleven decapod species from different infraorders suggesting the presence of cathepsin D as extracellular digestive enzyme in crustacea.

PCR-amplification of the partial sequence of cathepsin D cDNA obtained regions which clearly identified them as cathepsin D transcripts by the specific and conserved motif DTGS which is part of the catalytic site. The highly conserved cysteine residues that might form one of the disulphide bonds in the tertiary structure of the protein were identified in all the studied species. Additional characteristic is a region forming an extended loop, the so-called Y75 flap. In mammalian cathepsin D, the Y75 flap is flexible and partially covers the active site (Guha and Padh, 2008; Metcalf and Fusek, 1993). The phenolic-OH group of $Tyr_{75}$ donates proton to the amide nitrogen of the scissile bond of the substrate (Hsu et al., 1977). The highly conserved asparagine residue (Asn67 porcine pepsin numbering) found in crustacean cathepsin D (Rojo et al., 2010a), points toward the presence of a N-glycosylation tag for lysosome import as described for other cathepsin D enzymes.

We assayed cathepsin D activity by means of the substrate 7-methoxycoumarin-4-acetyl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys-(DNP)-DArg-amide and the inhibitor Pepstatin A. Although the substrate is widely used to determine the cathepsin D activity, the molecule was originally described as suitable to detect both cathepsin D and E activities (Yasuda et al., 1999). Cross reactivity of the assay with cathepsin E can be neglected in our study as to date no cathepsin E was described in crustaceans. Even recent transcriptome studies of the midgut gland of the shrimp *Crangon crangon* could not discover cathepsin E transcripts (Martínez-Alarcón, unpublished data).

The substrate specificity of human cathepsin D was studied by Pimenta et al. (2001) who described that S2 is a critical subsite for specificity and very sensitive to the length and geometry of the amino acid side chain at substrate P2, human cathepsin D has been reported to accommodate large hydrophobic amino acids at P2. This is partially concordant for invertebrate enzymes as well, lobster cathepsin D, for example, accommodates both hydrophobic and charged amino acids (Asp, Phe, Met, Ala) at P2 (Bibo-Verdugo et al., 2015). Moreover, mammalian cathepsin D shows preference for basic resides at P2' often accepting Arg and Lys. Lobster cathepsin D showed similar specificities when testing the suitability of 7-methoxycoumarin-4-acetyl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys-(DNP)-DArg-amide to detect crustacean cathepsin D activity (Bibo-Verdugo et al., 2015). Accordingly, 7-methoxycoumarin-4-acetyl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys-(DNP)-DArg-amide is an appropriate substrate for detecting cathepsin D activity from lobster. Since the deduced cathepsin D amino acid sequences of all studied crustaceans show highly conserved residues at the catalytic site we presume a high specificity of these enzymes for the substrate as well.

Pepstatin A is a competitive inhibitor of aspartic peptidases. It has been used as an efficient ligand in the purification of cathepsin D from the gastric fluid of lobster by affinity chromatography (Rojo et al., 2010b). Pepstatin A-agarose retained protein which, after elution, was detected as a single band by denaturing electrophoresis, into SDS-PAGE the protein migrated into two conformational states. One of these bands showed proteolytic activity at acid pH and was clearly identified as cathepsin D. Accordingly, the combined application of substrate and inhibitor provides high evidence for the authenticity of the enzyme and the suitability of the assay.

Cathepsin D evolved to an enzyme with digestive function in invertebrates like insects (Srp et al., 2016), mites (Wajahat Mahmood et al., 2013) and bloodsucking parasites as part of a multienzyme proteolytic complex (Sojka et al., 2016) and in vertebrates like bloodsucking fish (Xiao et al., 2015). How a typical intra-cellular lysosomal enzyme can appear in the gastric fluid of crustacea and act there as an extra-cellular enzyme (Rojo et al., 2010a) may be answered by the function and cytology of the digestive organs. In crustaceans, the midgut gland is the main organ involved in nutrition. This organ consists of hundreds of distally blind ending tubules, which are lined by four different cell types: the embryonic *E*-cell, the resorptive R-cells, the secretory F-cells, and blister-like B-cells. At the proximal end these tubules merge to larger ducts that are connected with the gastric chamber. The embryonic E-cells are located at the distal end of the midgut gland tubules and differentiate into R- and F-cells while the origin of B-cells is under debate. Some authors' support a two-cell-line genesis as first proposed by Hirsch and Jacobs (1928): R- and F-cells originate from E-cells while B-cells evolve from F-cells. This theory, however, has been questioned by cytological studies supporting the origin of B-cells directly from E-cells instead of F-cells (Vogt, 1993). The function of the R- and F-cells is well established, the R-cells resorb nutrients from the tubule lumen while the F-cells synthesize and release digestive enzymes into the tubule lumen, these enzymes are directed into the gastric chambers where they accumulate (Vogt et al., 1989). The function of the B-cells remains ambiguous, Vogt (1993) suggested an excretory function of the B-cells within the midgut gland. The early B-cells take up luminal remnants by pinocytosis, then, Golgi vesicles fuse with pinocytotic vesicles and form subapical vacuoles which can be considered as secondary lysosomes. Accordingly, the central vacuole which originates by coalescence of subapical vacuoles would represent a huge lysosome (Vogt, 1993). Cytological features indicate that biological material may be re-utilized within the B-cells while waste products are deposited in the huge vacuole of the B-cells. Finally, the B-cells are extruded from the midgut gland epithelium by holocrine secretion and leave the body as egesta through the gut (Vogt, 1993). Hu and Leung (2007) demonstrated, using immunohistology techniques, that decapod digestive enzymes are secreted both by exocytosis (apocrine secretion) and by the late disintegration of B-cells in the midgut gland (holocrine secretion) releasing the whole cellular content.

Cathepsin D activity is present in both the gastric fluid and the midgut gland of all species studied. Furthermore, this activity is up to two orders of magnitude higher than in the non-digestive tissues, muscle and gills. This suggests that the enzyme originates from enriched sources, which, presumably, are located in the hepatopancreatic B-cells. We hypothesize that the extruded B-cells or their large lysosome-like vacuoles may pass into the gastric chambers where they may essentially influence the chemistry of the gastric fluid. The huge vacuoles of the B-cells have an acidic content and are rich in lysosomal enzymes.

The pH of the gastric chamber varies between species. On one hand, it may be as high as pH 6.0 in the spiny lobster *P. interruptus* and pH 5.8 in the crab *C. pagurus*. On the other hand, it may be as low as pH 4.7 in the gastric fluid of the lobsters *H. gammarus* and *H. americanus* (Navarrete del Toro et al., 2006). Those species which show the highest cathepsin D activities in the gastric fluid also seem to have the lowest gastric pH. The intensity of lysosomal release (B-cell vacuoles) into the gastric chambers seems to vary between species as the average cathepsin D activities differ by the factor of approximately eight. The reason for the differences in activity and whether it is due to enzyme synthesis or different processing of B-cells among species remains to be investigated in future studies.

The partial primary structures of cathepsin D contain the highly conservative regions determining the active sites but also more variable regions. To evaluate whether sequence properties are related to the phylogeny of decapods, we compared the sequences and relate the results to established phylogenetic traits. The cathepsin D sequences show higher similarity between Astacidea and Caridea on one side and Brachyura, Anomura, and Penaeoidea on the other side. This does only partly match with the general phylogeny of decapods. According to Porter et al. (2005), Astacidea contain the nephropoid and astacid lineages. Furthermore, the lobster *H. americanus* is phylogenetically closer to *N. norvegicus* and both share a common ancestor with *A. astacus* that dates back 275 Myr. In this case our results are in accordance with Porter's hypothesis. The partial sequence obtained from the lobster *H. americanus* has 100% of similarity with the partial sequence from *N. norvegicus* and 98% with *A. astacus*. Our results, however, are not in line with the phylogenetic relationships between Astacidea, Caridea, and Brachyura as most of the phylogenetic analyses suggest that Astacidea are closer to Brachyura than to Caridea (Porter et al., 2005; Toon et al., 2009; Tsang et al., 2008). Today, there is little doubt that the divergence between Dendrobranchiata and Pleocyemata has occurred in the early phase of the evolutionary history of Decapoda. According to Porter et al. (2005) these two groups shared a common ancestor 437 Myr ago. Again, this phylogenetic relationship is not reflected in the cathepsin D sequences that show a quite close relationship between the representatives of Dendrobranchiata (*P. vannamei*) and Pleocyemata (*L. depurator*). Apparently, molecular evolution of digestive enzymes is somehow uncoupled from the phylogenetic traits based on morphological or molecular data. It remains to be investigated whether environmental and, particularly, nutritional factors are drivers of evolutionary traits in digestive enzymes.

In conclusion, the study shows that cathepsin D is a common extracellular digestive enzyme in decapod crustaceans although being a lysosomal enzyme according to its molecular properties. The origin of the digestive cathepsin D is to be disclosed, never the less, we hypothesize that it is present in the large lysosome-like vacuoles of hepatopancreatic B-cells. Upon extrusion of the cells it may accumulate in the gastric fluid. In this way lysosomal enzymes can remain in the stomach and contribute to the extracellular digestion of freshly ingested food.

## Conflict of interest

The authors declare that they have no conflict of interest.

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