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Isolation, biochemical characterization, and molecular modeling of American lobster digestive cathepsin D1

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ABSTRACT

An aspartic proteinase was isolated from American lobster gastric fluid. The purified cathepsin D runs as a single band on native-PAGE displaying proteolytic activity on a zymogram at pH 3.0, with an isoelectric point of 4.7. Appearance of the protein in SDS-PAGE, depended on the conditions of the gel electrophoresis. SDS treatment by itself was not able to fully unfold the protein. Thus, in SDS-PAGE the protein appeared to be heterogeneous. A few minute of boiling the sample in the presence of SDS was necessary to fully denature the protein that then run in the gel as a single band of ~50 kDa. The protein sequence of lobster cathepsin D1, as deduced from its mRNA sequence, lacks a 'polyproline loop' and β-hairpin, which are characteristic of some of its structural homologues. A comparison of amino acid sequences of digestive and non-digestive cathepsin D-like enzymes from invertebrates showed that most cathepsin D enzymes involved in food digestion, lack the polyproline loop, whereas all non-digestive cathepsin Ds, including the American lobster cathepsin D2 paralog, contain the polyproline loop. We propose that the absence or presence of this loop may be characteristic of digestive and non-digestive aspartic proteinases, respectively.

1. Introduction

Aspartic proteinases comprise a protein superfamily present across different taxa including bacteria (Hill and Phylip, 1997), yeast (Tang and Wong, 1987), plants (Runeberg-Roos et al., 1991), viruses (Wlodawer and Gustchina, 2000) and vertebrates (Fruton, 2002). Based on their characteristics and tissue/cellular locality, aspartic proteinases of vertebrates have been classified as cathepsin D, cathepsin E, chymosin, pepsin and renin (Barrett et al., 1998).

Eukaryotic aspartic proteainases exhibit some key features, including the well conserved triad Asp-Thr-Gly around the two aspartic residues of the active site (D_{32} and D_{215}, from the human pepsin A numbering) (Fusek and Větvička, 2005). Although similarities surpass this characteristic, aspartic proteinases are also diverse in a number of ways, including the shape of their binding sites and cellular localization. These features point to their particular physiological functions (Davies, 1990) including extracellular protein hydrolysis at acid pH, digestion of intracellular proteins and activation of proenzymes (Benes et al., 2008) to name a few.

After pepsin, cathepsin D is the second most studied aspartic proteinase (Benes et al., 2008). The physiological functions of cathepsin D that have been explored include intracellular protein hydrolysis (Metcalf and Fusek, 1993), activation of some proenzymes (Khan et al., 1999), regulation of programmed cell death (Gui et al., 2006) and digestion of food proteins in invertebrates (Blanco-Labra et al., 1996; Williamson et al., 2002; Padilha et al., 2009; Rojo et al., 2010). Cathepsin D has been known to function at acid pH (Takahashi and Tang, 1981). Its structure may contain high mannose oligosaccharides linked at the so-called N-sites to the polypeptide chain (Takahashi et al., 1983). Cathepsin D pre-pro-enzyme is synthesized in the rough endoplasmic reticulum. Some posttranslational modifications must occur before its conversion to a fully active form, first the removal of a signal sequence of ~20 amino acid occurs within the rough endoplasmatic reticulum (Richter et al., 1998) to form a zymogen. Then, a 44 amino acid propetide is split-off the zymogen forming a single-chain intermediate form that is subsequently further processed in the so-called processing region (Yonezawa et al., 1988) and then converted to a fully activated two-chain linked proteinase (Richo and Conner, 1994).

In cathepsin D enzymes, amino acid sequences around the activation peptides and the position of cysteine residues are highly conserved (Fusek et al., 1992; Richo and Conner, 1994). Two loops surround the active site and they are probably involved in substrate binding. One of these, the polyproline loop contains three consecutive prolines, the other loop, the "Y_{75} flap" (Tyr_{75} from porcine pepsin numbering) is flexible and partly covers the active site (Metcalf and Fusek, 1993).

Aspartic proteinases along with cysteine proteinases are the main contributors to the proteolytic activity found in the gastric fluid in American and European lobster (Laycock et al., 1989; Navarrete del

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Toro et al., 2006); the so-called cathepsin D1 seems to be in charge of the aspartic proteinase activity (Rojo et al., 2010). Occurrence of digestive aspartic enzymes in ectothermic animals living at low temperatures has been proposed as a possible evolutionary strategy to achieve a normal level of proteolysis at low temperatures (Carginale et al., 2004; De Luca et al., 2009). The present study describes the isolation and some unusual structural characteristics as deduced by electrophoresis analysis and *in silico* modeling of a digestive aspartic proteinase found in the gastric fluid of American lobster. The structural properties of this proteinase are also compared to those of lysosomal cathepsins D.

2. Materials and methods

2.1. Isolation of cathepsin D1 from American lobster gastric fluid

Lyophilized gastric fluid was reconstituted with 50 mM sodium citrate buffer pH 4.0 to yield 25 mg/mL and centrifuged for 10 min at 12 000 *g*. The resulting supernatant, containing approximately 4 mg protein in a volume of 500 μL, was applied to a pepstatin A–agarose (P2032, Sigma-Aldrich Chemical) affinity column. Briefly, 1 mL of matrix was equilibrated in 50 mM sodium citrate buffer, pH 4.0; then 1 mL of gastric fluid was loaded into the column and washed with 5 mL of the same buffer and 5 more times with 50 mM sodium citrate buffer (pH 4.0, 1 M NaCl). The proteins bound to the column were gradually eluted with 100 mM Tris-HCl, pH 7.0, 1 M NaCl and then with 100 mM Tris-HCl, pH 7.5, 1 M NaCl. Ten 0.5 mL fractions of each step were collected and their protein content was assessed with spectrophotometry at A_{280}. Their protein composition was checked by a 12% SDS-PAGE under reducing conditions. The enzyme-containing fractions were pooled and concentrated by ultra-filtration. A summary of the purification procedure is presented in Table 1.

Throughout the purification process of cathepsin D1, proteolytic activity of the fractions was measured by specific substrate 7-methoxycoumarin-4-acetyl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-D-Arg-amide (Sigma) (↓ shows the site where the peptide is cleaved), (Yasuda et al., 1999), the cathepsin D activity was monitored by using a microplate reader (Synergy 4, BioTek). Rates of hydrolysis were recorded by measuring the increase of fluorescence in arbitrary units (relative fluorescence units, RFU). The excitation and emission wavelengths were 328 nm and 393 nm, respectively. A calibration curve was constructed by measuring the fluorescence of known concentrations of flourochrome 7-methoxycoumarin-4-acetic acid (MCA) and by plotting the RFU versus picomoles of MCA. Cathepsin D activity is expressed in pmol 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.

2.2. Analysis of cathepsin D1 by gel electrophoresis

The homogeneity of our cathepsin D preparations was checked by PAGE under different conditions. Unless it is noted, the samples loaded onto the gel were not boiled. The conditions were as follows: 12% PAGE in the absence of denaturants and reducing agents (NATIVE-PAGE), 12% SDS-PAGE in the absence of reducing agent (SDS-PAGE), and SDS-PAGE in the presence of DTT (SDS-DTT-PAGE). SDS-PAGE with proteins that were boiled for two min in the sample buffer before they were loaded onto the gel (SDS-B-PAGE), SDS-B-PAGE in the presence of dithiothreitol and boiled (SDS-DTT-B-PAGE). Additionally, the proteolytic activity of cathepsin D1 was monitored by substrate PAGE as described by García-Carreño et al. (1993). Hemoglobin (0.25%) dissolved in 100 mM glycine buffer of pH 3.0 was used as substrate. A few gels were also run in the presence of another commonly used denaturing agent (urea) at different concentrations (2, 4, and 8 M) co-polymerized into the gel.

Analytical isolelectric focusing (IEF) was performed with the Phast System (Pharmacia Biotech, Uppsala, Sweden) using IEF gels at pH ranging from 3 to 9. A protein mixture (Broad *pI* 3.5–9.3) was used as the *pI* marker. The gel was silver-stained, using the modified method described by Switzer et al. (1979).

2.3. In silico analysis of structural features

The recently reported amino acid sequence of cathepsin D1, deduced from the mRNA sequence (Rojo et al., 2010) was aligned for comparison with other digestive aspartic proteinases from invertebrates, using the Clustal X v. 1.81 software (Thompson et al., 1994). The tertiary structures of bovine cathepsin D was predicted by the homology modeling method with the 3D-JIGSAW program (Bates et al., 2001) which is available at http://www.bmm.icnet.uk/servers/3djigsaw. The theoretical model for lobster cathepsin D1 was obtained with the MOE software (ChemComp) 2009.10 using pig pepsin as a template (PDB 2PRP), and the model was obtained using 25 intermediate models. Three-dimensional structures were visualized by using the program PyMOL. Molecular characteristics as disulphide and hydrogen bonds were predicted using the software PredictProtein (Rost et al., 2004).

2.4. Phylogenetic analysis

Phylogenetic analysis using digestive and non-digestive cathepsin D-like proteinases was performed with the program MEGA 4.1 (Tamura et al., 2007). Distance analysis was carried out using the substitution model described by Jones et al. (1992), from which we constructed a cladogram using the neighbor-joining algorithm (Saitou and Nei, 1987). The Bootstrap method was applied for assessing confidence analysis of the clades (Hillis and Bull, 1993), based on 1000 replicates.

3. Results

3.1. Isolation of a digestive aspartic proteinase from clawed lobster

By affinity chromatography of the digestive fluid from *H. americanus*, two A_{280} peaks were obtained (Fig. 1). The first peak eluted with Tris-HCl buffer pH 7.0; the second peak with Tris-HCl buffer pH 7.5. These fractions were assayed for cathepsin D activity and only the second one showed activity on the synthetic substrate (results not shown). The eluted proteins were run in an SDS-B-DTT-PAGE (for abbreviation, see Section 2.2). In the two fractions, we detected two different proteins: of ~20 kDa for the first peak and ~50 kDa for the second peak (Fig. 1). The protein in the second

Table 1
Purification of American lobster cathepsin D1.

Steps	Volume (μL)	Protein concentration (mg/mL)	Total protein (mg)	Activity (U/μg)*	Total activity (U)	Specific activity (U/mg)	Purification Fold	Yield (%)
10,000 *g* supernatant	450	3.51	1 575	31.62	14 229	9.03	1	100
Pepstatin A-agarose and centrifugal filtration	500	0.24	120	267.05	13 3525	1 112.70	123.16	9.3

* One unit of activity is 1 μMol of AMC per min per μg protein.

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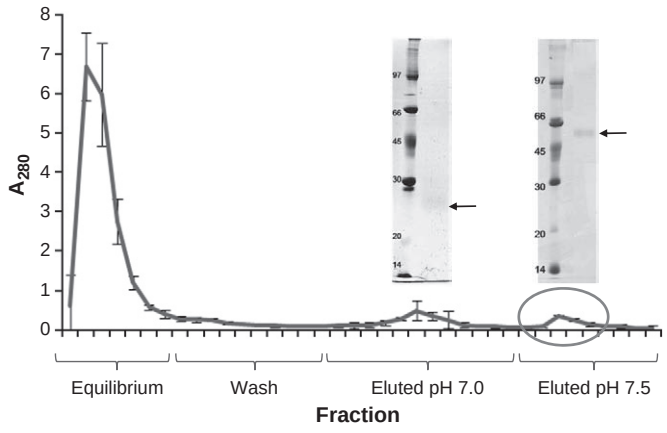

Fig. 1. Chromatography fractions of Pepstatin-A agarose column equilibrated with 50 mM citrate buffer, pH 4.0, and eluted with 0.5 M Tris-HCl, pH 7.0, 1 M NaCl followed by 0.5 M Tris-HCl, pH 7.5, 1 M NaCl. Gel electrophoresis patterns of eluted fractions are shown. Cathepsin D active fractions are circled.

fraction was identified as cathepsin D1 (Rojo et al., 2010). The overall purification of the enzyme by pepstatin A–agarose chromatography was 123-fold with 9.3% enzyme activity (Table 1).

3.2. Analysis of cathepsin D structural features by gel electrophoresis

By the comparative use of SDS-PAGE in the presence or absence of reducing agents, such as DTT, one is able to estimate the number and loop size of the disulfide bridges of a particular protein, since SDS is believed to work by disrupting the intramolecular non-covalent interactions within the protein, thus exposing the amino acid side chains to the solvent. As a consequence, the proteins lose their native shape and structure. While SDS, by itself, may partially affect the shape of the protein molecule, DTT reduces the disulfide bonds whereas SDS fully uncoils the molecules. The extent of the DTT effect depends on the number and loop-size of the disulfide bridges.

In lanes 2 and 4 of Fig. 2A, the SDS-DTT treatment, by itself, only slightly affects the mobility of the upper protein band. This is clearly related to the sizes of the three disulfide bonds forming structural loops of cathepsin D1, which are relatively small compared to the full size of the molecule (see Fig. 3), and the disruption of these bonds may only cause a relatively small change in the shape of the SDS-treated protein. This effect has been noticed in other crustacean digestive protease, Graf (2008) observed that crayfish trypsin and its complex with BPTI also exhibit an extremely high stability towards SDS treatment. In both cases, two minute boiling of the proteins dissolved in the sample buffer was necessary to denature and fully uncoil them. This appears in lanes 3 and 5 of Fig. 2A. Since this upper band is most likely composed of cathepsin D1 fully saturated by SDS, the mobility of this protein should reciprocally relate to its molecular mass. The estimated value of ~50 kDa is somewhat higher than what can be predicted from the amino acid composition of cathepsin D1 (42.6 kDa). This difference may have something to do with the unique structural features of the enzyme.

Substrate-PAGE gels (zymograms) of the same samples (see the black panel of Fig. 2A) clearly show that only the lower bands of the gels contain active proteinase. NATIVE-PAGE (lane 1 in Fig. 2A) also confirmed that this lower band represents a fraction of the SDS-treated sample that resists SDS treatment, suggesting that, under such conditions, this enzyme can bind smaller amounts of SDS than the average protein binds. Experimental results shown in Fig. 2B further confirm our hypothesis about the unique structural properties and stability of lobster cathepsin D1. Though 2 M urea causes a dramatic structural transition of the protein (lanes 1 and 2 in Fig. 2B), further conformational changes occur at higher concentrations of urea (lanes 3 and 4 in Fig. 2B). An interesting feature of this pattern is that the conformational changes of cathepsin D1 occur in a stepwise manner: in the four different concentrations of the denaturing agent, the enzyme appears to display four different structural conformers.

3.3. Analysis of cathepsin D structural features by amino acid sequence comparison

Based on amino acid sequence comparisons and the known tertiary structure of bovine cathepsin D (Krieger and Hook, 1992; Metcalf and Fusek, 1993), Fig. 3A shows the positions of signal peptide and activation peptide and the locations of the disulfide bonds in the primary structures of bovine cathepsin D and lobster cathepsin D1. Homology pairings of the cysteine residues revealed three homologous disulfide bonds in the structures. In Fig. 3B, the most notable differences of the modeled tertiary structures are shown. Besides the common aspartic proteinase topology for the main body of mature cathepsin D1, structural comparisons of the enzymes indicated some particularities of cathepsin D1. In addition to the two identical catalytic sites, cathepsin D1 contains two loops that are presumed to close over the substrate binding cleft, displays the conserved tyrosine residue of the so-called Y_{75} flap (corresponding to Tyr_{75} in the porcine pepsin numbering and residue 174 in lobster), and a loop located in

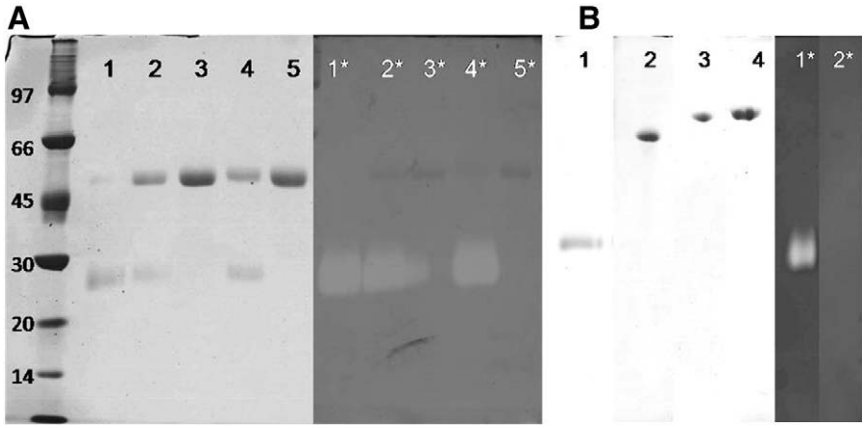

Fig. 2. Electrophoresis profiles of American lobster cathepsin D1 obtained by pepstatin A-agarose column. SDS-PAGE (black bands) and substrate PAGE (white bands). (A) SDS-PAGE and zymogram. 1: NATIVE-PAGE, 2: SDS-PAGE, 3: SDS-B-PAGE, 4: SDS-DTT-PAGE, 5: SDS-DTT-B-PAGE. Asterisks represent zymograms samples. (B) Electrophoretic migration of American lobster cathepsin D1 on Urea gels. 1: Native gel, 2: 2 M urea gel, 3: 4 M urea gel, 4: 8 M urea gel. Asterisks represent zymograms samples.

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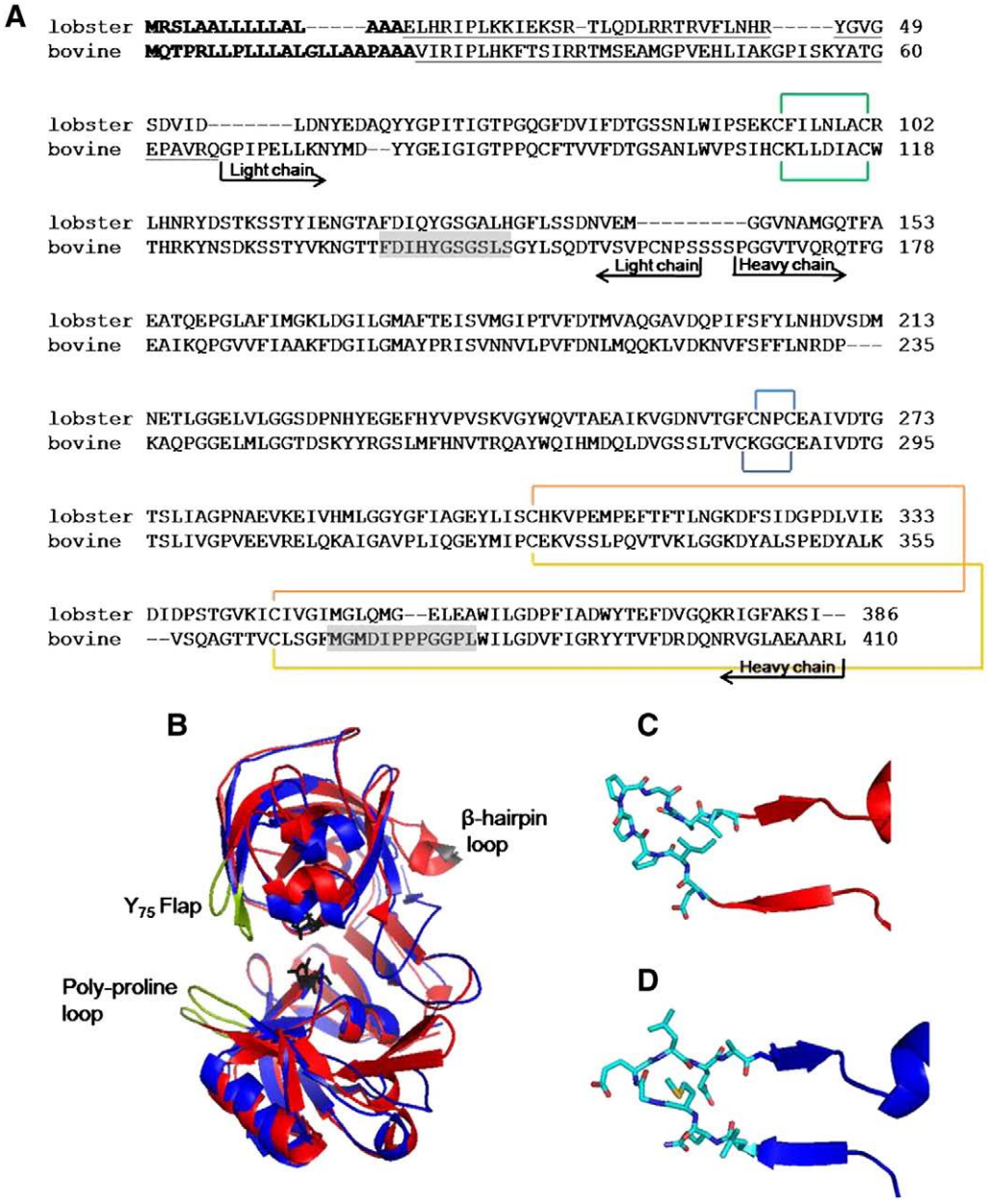

Fig. 3. Representation of primary and three-dimensional structures of American lobster cathepsin D1 predicted by molecular modeling and comparison with bovine cathepsin D. (A) Predicted arrangements of the disulfide bonds in cathepsin D1 (upper row) and bovine cathepsin D (lower row). Highlighted residues represent the signal peptide; underlined residues represent the activation peptide; residues outlining the Y75 flap and the polyproline loop are shaded. (B) Overlay comparison of bovine cathepsin D (blue) and American lobster cathepsin D1 (red), the Y_{75} flap, and the polyproline loop are represented in green. The β-hairpin loop is colored grey; amino acid residues of the active site are in black. Structural comparison of bovine is shown in (C) and the polyproline loop and homologous loop in lobster is shown in (D). Atoms are represented in colors, carbon in light blue, nitrogen in deep blue, oxygen in red.

the same position of the analogous polyproline loop of mammalian cathepsin Ds (Fig. 3C), although in cathepsin D1, this loop does not contain any proline (Fig. 3D). Cathepsin D1, lacks the β-hairpin loop (or processing loop) observed in mammalian cathepsin Ds (Fig. 3B). We can only speculate at this stage if these differences have any functional significance.

3.4. Phylogenetic analyses

The amino acid residues of the conventional polyproline loop are substituted in the American lobster cathepsin D1. The absence of a proline and processing loops in other invertebrate cathepsin D-like proteinases was previously noted by Padilha et al. (2009). In our analysis, we confirmed this by comparing a large number of amino acid sequences from invertebrates, including twelve cathepsin D-like proteinases reported to have a digestive function. Some cathepsin D-like amino acid sequences that are considered of lysosomal origin are also included (Table 2). These sequences were recovered from the nonredundant protein sequence data-bank of the NCBI (http//:www.ncbi.nlm.nih.gov). This analysis confirms that most of digestive enzymes from invertebrates, including American lobster cathepsin D1, lack the polyproline loop that is present in non-digestive cathepsin Ds of invertebrates, including American lobster cathepsin D2. The mRNA of the latter one was previously found in non-digestive tissues (Rojo et al., 2010). Together with all cathepsin Ds included in the analysis, cathepsin D1 and cathepsin D2 lack the sequence that

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Table 2
List of invertebrate species used for phylogenetic analyses.

Scientific name	Common name	Tissue	Gene	GenBank Accession no.	Reference
♦*Callosobruchus maculatus*	Cowpea weevil	Midgut	CmCatD*	**FJ360435**	(Ahn and Zhu-Salzman, 2009)
♦*Sitophilus zeamais*	Maize weevil	Alimentary tract	SAP1*	**AB457169**	(Matsumoto et al., 2009)
♦*Haemaphysalis longicornis*	Hard tick	Midgut gland	Longepsin*	**AB218595**	Boldbaatar et al. (2006)
♦*Ancylostoma caninum*	Dog hookworm	–	Acasp	**AAB06575**	Harrop et al. (1996)
♦*Necator americanus*	Human hookworm	–	Necepsin II	**AJ245459**	Ranjit et al. (2009) Williamson et al. (2002)
♦*Musca domestica*	House fly	Midgut	AspMD01	**EF151165**	Padilha et al. (2009)
♦*Musca domestica*	House fly	Midgut	AspMD02	**EF193384**	Padilha et al. (2009)
♦*Caenorhabditis elegans*	Soil nematode	–	ASP-1	**AF208526**	Tcherepanova et al. (2000)
♦*Schistosoma mansoni*	Blood fluke	–	SmCD1	**U60995**	Wong et al. (1997) Brindley et al. (2001)
♦*Schistosoma mansoni*	Blood fluke	–	SmCD2	**ABP48739**	Brindley et al. (2001)
♦*Homarus americanus*	American lobster	Midgut gland	CD1	**EU687261**	Rojo et al. (2010)
Homarus americanus	American lobster	Midgut gland	CD2*	**FJ943775**	Rojo et al. (2010)
Bombyx mori	Silkworm	–	BmCatD*	**AAP50847**	Gui et al. (2006)
Aedes aegypti	Mosquito	–	mLAP*	**M95187**	Cho and Raikhel (1992)
Penaeus monodon	Black tiger shrimp	Lymphoid organ	–*	**EF213114**	Unpublished data
Musca domestica	House fly	Midgut	AspMD03*	**EF193385**	Padilha et al. (2009)
Tribolium castaneum	Red flour beetle	Whole larvae	–*	**XM961424**	Morris et al. (2009)

♦Aspartic proteinases reported as digestive.
* Sequences exhibiting a polyproline loop.
–Not specified.

is characteristic of the β-hairpin loop of bovine cathepsin D. This suggests that Cathepsin D1 may have a function different from lysosomal cathepsin Ds with the polyproline loop.

As shown in the cladogram of amino acid sequences of digestive cathepsin Ds of invertebrates (Fig. 4), the large grouping divides into two branches. One contains all digestive cathepsin Ds, and the other all non-digestive cathepsin Ds. The latter group, however, also includes some that were reported to be digestive enzymes. All enzymes in the upper group contain the polyproline loop, the lower branch lack this loops.

4. Discussion

To our knowledge, Rojo et al., 2010 and this study are the first reports on isolation and structural characterization of a digestive crustacean cathepsin D, in this case, isolated from gastric fluid of

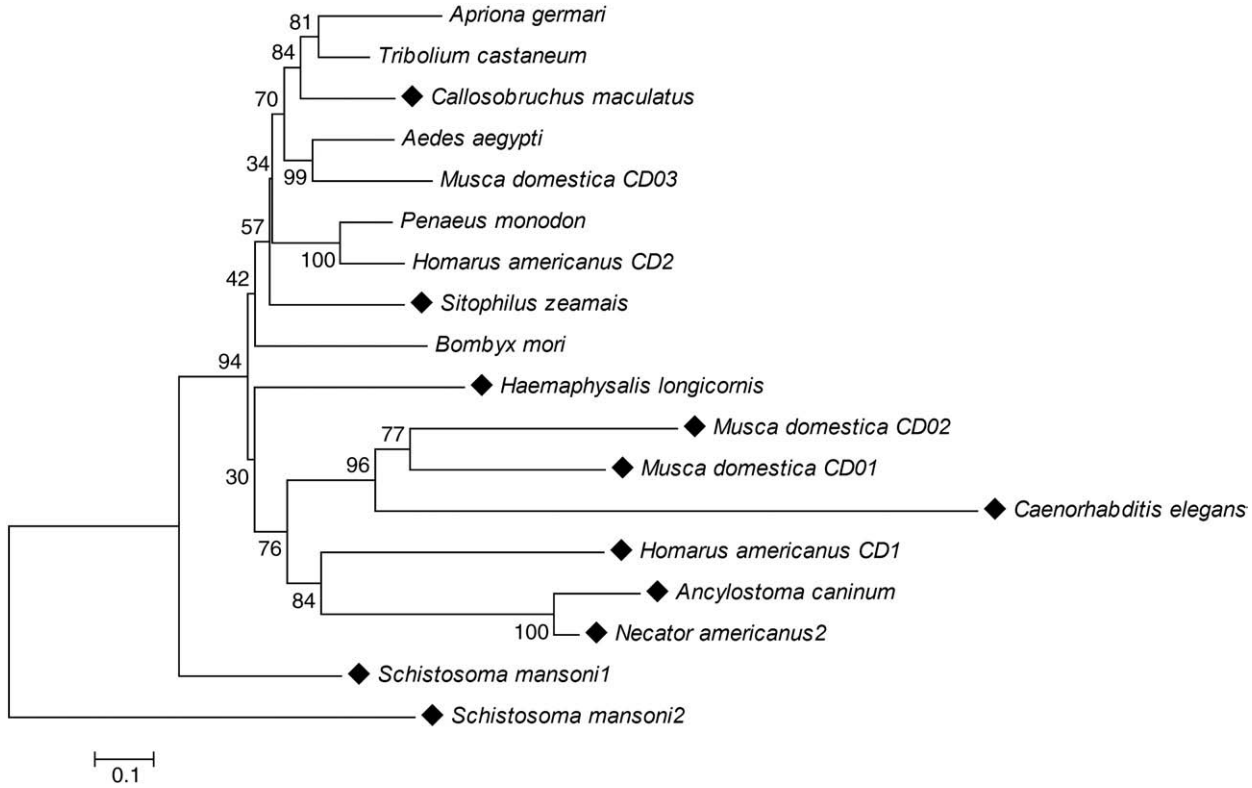

Fig. 4. Cladogram of digestive and non-digestive cathepsins D sequences deposited in GenBank. The dendrogram of cathepsin Ds was generated with the neighbor-joining algorithm. The branches were statistically supported by Bootstrap analysis (cut off 50) based on 1000 replicates. The sequences used for the analysis are summarized in Table 2. Aspartic proteinases reported as digestive are marked with a black diamond.

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American lobster. Unlike most known crustaceans that use serine proteinases to digest food (Hernández-Cortés et al., 1999; Celis-Guerrero et al., 2004; Sainz et al., 2004; Perera et al., 2008), in clawed lobsters, the main digestive proteinases are cysteine (Laycock et al., 1991) and aspartic proteinases (Navarrete del Toro et al., 2006).

In mammals, active cathepsin D is a dimeric protein (Yonezawa et al., 1988) with a mature form composed of a light (14 kDa) and a heavy (31 kDa) polypeptide chain that can be separated by SDS-PAGE in the presence of a reducing agent (Fusek et al., 1992; Partanen et al., 2003). Our SDS-PAGE experiments (Fig. 2) clearly show that cathepsin D1 from American lobster is a single polypeptide chain protein with a size similar to mammalian and invertebrate aspartic proteinases (Takahashi and Tang, 1981; Szecsi, 1992; Fusek and Větvička, 2005). Apparent size differences between species homologues of different cathepsin D enzymes, as estimated by SDS-PAGE, might result from some differences in the abilities of these proteins to bind SDS. A clear example of this phenomenon is cathepsin D1 from American lobster. Our studies provided evidence that under standard conditions, which most workers use for SDS-PAGE, cathepsin D1 of American lobster remains more or less a native protein (Fig. 2A, lane 2). Consequently, gel electrophoresis under such conditions does not give us useful information on the molecular mass of the protein. However, boiling the sample in the presence of SDS results in a shift of mobility of the protein. This band represents the denatured SDS-bound form, with an apparent molecular mass of 50 kDa (Fig. 2A, lane 3). Monomeric cathepsin Ds seems to be a common feature in invertebrates (Cho and Raikhel, 1992; Harrop et al., 1996; Boldbaatar et al., 2006).

Resistance against the effect of surfactants is a property of proteins that many scientists try to enhance in proteins used in the industry, like proteinases of the laundry industry. The classic example comes from the pioneering protein engineering work on subtilisin (Wells and Estell, 1988). This property may have a structural basis that is clearly different from those that determine stability of proteins against urea or heat. Above certain concentration, urea causes a loss of native structure with an initial intrusion into the tertiary structure of proteins by competition with intramolecular hydrogen bonds (Rossky, 2008). As observed in Fig. 2B, it seems that unfolding of the protein occurs gradually, since in the presence of 2 M urea, cathepsin D1 migrates faster than at higher concentrations of urea (4 and 8 M). This phenomenon is not typical. Further studies could reveal the structural cause of this curious behavior.

Another interesting structural property of cathepsin D1 is the absence of the polyproline loop. This may be related to the extracellular role of digestive cathepsins (Padilha et al., 2009). This possibility was confirmed by the sequence analysis of digestive and non-digestive invertebrate cathepsin D enzymes (Table 2). In the cladogram based on these sequences, most of the digestive aspartic proteinases from a group, and the non-digestive cathepsin Ds form a separate group (separation supported by a bootstrap value of 93), although the non-digestive branch includes a few digestive cathepsin Ds (Fig. 4). The grouping pattern suggests that digestive aspartic proteinases share sequences that may lead to some specialized characteristics to fulfill a digestive function with less structural restrictions, such as the lack of a polyproline loop. The properties exhibited by lobster cathepsin D1, expressed exclusively in the midgut gland (Rojo et al, 2010) and lacking a polyproline loop like other digestive cathepsin Ds, support our proposal that cathepsin D1 is a proteinase with a valid role in digesting food.

Acknowledgments

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