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ORIGINAL ARTICLE

Cold-Adapted Digestive Aspartic Protease of the Clawed Lobsters *Homarus americanus* and *Homarus gammarus*: Biochemical Characterization

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Abstract Aspartic proteinases in the gastric fluid of clawed lobsters *Homarus americanus* and *Homarus gammarus* were isolated to homogeneity by single-step pepstatin-A affinity chromatography; such enzymes have been previously identified as cathepsin D-like enzymes based on their deduced amino acid sequence. Here, we describe their biochemical characteristics; the properties of the lobster enzymes were compared with those of its homolog, bovine cathepsin D, and found to be unique in a number of ways. The lobster enzymes demonstrated hydrolytic activity against synthetic and natural substrates at a wider range of pH; they were more temperature-sensitive, showed no changes in the K_M value at 4°C, 10°C, and 25°C, and had 20-fold higher k_{cat}/K_M values than bovine enzyme. The bovine enzyme was temperature-dependent. We propose that both properties arose from an increase in molecular flexibility required to compensate for the reduction of reaction rates at low habitat temperatures. This is supported by the fast denaturation rates induced by temperature.

Keywords Lobster · Aspartic proteinase · Cathepsin D · Enzyme kinetics

Introduction

The role of crustacean digestive proteinases has been studied by many authors; most of them designate trypsin and chymotrypsin as the main enzymes in the food-protein digestion process (Galgani et al. 1985; Hernández-Cortés et al. 1999; Córdova-Murueta et al. 2003; Perera et al. 2008), where the focus is on characterization of enzymatic, molecular, and catalytic properties (Lee et al. 1984; Galgani et al. 1985; Hernández-Cortés et al. 1997), genetic organization (Klein et al. 1998; Sainz et al. 2004a), and regulation of the codifying genes (Muhlia-Almazan and García-Carreño 2002; Sainz et al. 2004b). Beside serine proteinases, cysteine proteinases have also been described in the digestive system of crustaceans (Le Boulay et al. 1995; Le Boulay et al. 1998; Aoki et al. 2003; Hu and Leung 2007). Aspartic proteinases in the digestive fluid of the European clawed lobster have been reported (Navarrete del Toro et al. 2006); however, there are no reports on the biochemical characterization of enzymes belonging to the aspartic proteinases in crustaceans. The study of biochemical properties of digestive enzymes can lead us to a deeper understanding of their physiological roles.

As far as we know, clawed lobsters *Homarus americanus* and *Homarus gammarus* are the only crustaceans where aspartic proteinases are confirmed to play a role in digestion (Navarrete del Toro et al. 2006; Rojo et al. 2010a). Lobsters live in marine environments where temperature vary from 0°C to 25°C, depending on season, winds, and tides (Cobb and Phillips 1980), and it is expected that their digestive enzymes exhibit adaptations similar to those described for other cold-blooded species that have higher catalytic efficiencies at low temperatures and can be inactivated at relatively low temperatures and mild pHs (Smalås et al. 1994; Gerday et al. 2000; Leiros et al. 2000; Gudmundsdóttir and Pálsdóttir 2005).

Aspartic proteinases have been classified as cathepsins, renins, pepsins, and chymosins and are commonly active at acid pH. Enzymes belonging to this class show a high degree of similarity, resulting in typical bi-lobular, three-

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dimensional structures (Davies 1990; Andreeva 1992), but they can vary in the topography of the binding site and the tissue of synthesis. These characteristics determine their physiological function (Szecsi 1992). Aspartic proteinases have been extensively studied and characterized in mammals (Pardesi et al. 2004), plants (Simões and Faro 2004), fungi, and viruses (Khan et al. 1999). The physiological role of aspartic proteinases has been characterized in hematophagous parasites (Harrop et al. 1996; Brinkworth et al. 2001; Boldbaatar et al. 2006; Pinho et al. 2009; Suttiprapa et al. 2009), sharing topology with cathepsin D proteases, and their participation in hemoglobin digestion is well understood (Williamson et al. 2002, 2003a).

In mammals, cathepsin D (EC 3.4.23.5) is a widely studied aspartic proteinase; this enzyme is synthesized in most cells, is one of the major contributors to the proteinolytic activity of lysosomes (Barrett 1979), and contains highly structured mannose oligosaccharides linked to asparagine (N) residues (Takahashi and Tang 1981). Research related to cathepsin D protease has lead to the discovery and description of multiple physiological functions of this proteinase (Benes et al. 2008), including intracellular protein hydrolysis (Metcalf and Fusek 1993), zymogen activation (Khan and James 1998), regulation of programmed cell death (Gui et al. 2006), and digestion of protein from food in parasitic invertebrates (Harrop et al. 1996; Williamson et al. 2002; Delcroix et al. 2006).

We recently demonstrated that the acid proteinase in clawed lobsters (Navarrete del Toro et al. 2006) is a cathepsin D, as indicated by mass mapping, N-terminal, and full-length cDNA sequencing; we also showed that *H. gammarus* and *H. americanus* transcribe two cathepsin D mRNAs, namely cathepsin D1 and cathepsin D2. Cathepsin D1 mRNA was detected only in the midgut gland, suggesting its function as a digestive enzyme (Navarrete del Toro et al. 2006; Rojo et al. 2010a). Later, we isolated and characterized the enzyme from American lobster gastric fluid and characterized it; it is a cathepsin D that runs as a single band on native-polyacrylamide gel electrophoresis (PAGE), displaying proteolytic activity on a zymogram at pH 3.0, with an isoelectric point of 4.7. The protein sequence of lobster cathepsin D1, as deduced from its mRNA sequence, lacks a 'polyproline loop' and β-hairpin, which is characteristic of invertebrate cathepsin Ds involved in food digestion, whereas all non-digestive cathepsin Ds, including the American lobster cathepsin D2 paralogs, contain the polyproline loop (Rojo et al. 2010b). We present a detailed characterization of the biochemical properties of this enzyme from the American lobster (*H. americanus*) and the European lobster (*H. gammarus*) and compare them to the homeothermic homolog, bovine cathepsin D.

Materials and Methods

Isolation of an Aspartic Proteinase from Gastric Fluid

Live adult clawed American and European lobsters, *H. americanus* and *H. gammarus*, were purchased in Cuxhaven, Germany. In the laboratory, the animals were maintained in aquariums with continuously flowing filtered seawater. The temperature of the water was maintained at 13±0.5°C, and specimens were fed ad libitum with fish or small crustaceans.

Gastric fluid of lobsters was obtained by introducing a probe through the esophagus to reach the gastric chamber and aspirate digestive fluid (physiological pH ranging from 4.7 to 5.5). The sample was transferred to a 1.5-mL tube and centrifuged for 10 min at 10,000×*g* at 4°C to remove solids. The supernatant (enzyme extract) was freeze-dried and stored at −20°C until used. Further assays were carried out with solutions of the freeze-dried gastric fluid (25 mg enzyme powder in 1 mL 50 mM sodium citrate buffer, pH 4.0).

The presence of aspartic proteinase activity in the gastric fluid was previously identified by the diminishment of activity by the class-specific inhibitor pepstatin A (Navarrete del Toro et al. 2006). We further identified this activity as cathepsin D by measuring the hydrolysis of the fluorogenic substrate 7-methoxycoumarin-4-acetyl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-D-Arg-amide (#M0938, Sigma-Aldrich), specific for cathepsin D.

The aspartic proteinase from the gastric fluid of the lobsters were purified to homogeneity, based on the affinity of these enzymes for pepstatin A (Rojo et al. 2010b). Briefly, 1 mL of reconstituted gastric fluid (25 mg enzyme powder in 1 mL 50 mM sodium citrate buffer, pH 4.0) was loaded into a chromatographic column packed with 1 mL pepstatin A-agarose (#P2032, Sigma-Aldrich) and equilibrated with 50 mM sodium citrate buffer at pH 4.0. After loading the sample, the column was washed with 5 mL of the same buffer, then with 5 mL 50 mM sodium citrate buffer at pH 4.0 and 1 M NaCl. Undesired proteins bound to the column were eluted with 5 mL 100 mM Tris–HCl at pH 7.0 and 1 M NaCl. Finally, cathepsin D was eluted with 100 mM Tris–HCl at pH 7.5 and 1 M NaCl; 0.5 mL fractions were collected. All fractions were monitored at 280 nm and assayed for cathepsin D activity, as described below. All fractions revealing cathepsin D activity were pooled. Ultrafiltration on a 10-kDa cut-off membrane was used to concentrate and desalt the fractions. This concentrate was used for further analysis.

Enzymatic Activity Assay

Hydrolysis of 7-methoxycoumarin-4-acetyl-GKPILF↓FRLK (DNP)-D-Arg-amide, where ↓ represents the site where the

peptide is cleaved (Yasuda et al. 1999) by lobsters' cathepsin Ds (hereafter called cathepsin D1) was monitored at pH 4.0 and 25°C, using 50 mM sodium acetate buffer in a microplate reader (Synergy 4, BioTek). Rates of hydrolysis were determined by monitoring the increase in fluorescence measured in arbitrary units (relative fluorescence units (RFU)) at excitation and emission wavelengths of 328 and 393 nm, respectively. A calibration curve was constructed by measuring the fluorescence of known concentrations of flouro-chrome 7-methoxycoumarin-4-acetic acid (MCA) and by plotting the RFU versus the nanomoles of MCA. Once linearity of the curve was validated, the slope of the curve was used to calculate the activity units of cathepsin D present in the sample. Cathepsin D activity was defined and expressed in nanomoles MCA liberated per minute per microgram of protein.

Evaluation of the Effect of pH on Cathepsin D Activity

The pH optimum for clawed lobster cathepsin D1 (0.137 pmoles) and bovine cathepsin D (1.33 pmoles) (spleen cathepsin D, C3138, Sigma-Aldrich) activity was determined by assaying the hydrolysis of 7-methoxycoumarin-4-acetyl-GKPILFFRLK(DNP)-D-Arg-amide in 50 mM sodium acetate at 0.5-unit pH increments from pH 2 to 6 at 25°C. The measurement at pH 4.5 was omitted since it is close to the lobsters' cathepsin D1 isoelectric point (4.7). The final substrate concentration was 2 μM, and the final volume of each reaction was 100 μL.

pH and Thermal Stability

The effect of pH on enzyme stability was assessed by measuring the residual activity after incubation of the enzymes in 50 mM sodium acetate buffer at pH 2.5, 4.0, 5.0, and 6.0 at 25°C for 30 min. For thermal stability, the enzyme solution was heated at 25°C, 50°C, or 60°C for up to 90 min in a temperature-controlled water bath. Thereafter, treated samples were suddenly cooled in ice water. Immediately after incubation, the remaining activities were assessed by measuring hydrolysis of the fluorogenic substrate 7-methoxycoumarin-4-acetyl-GKPILFFRLK(DNP)-D-Arg-amide in 50 mM sodium acetate buffer at pH 4.0 and 25°C. The final substrate concentration was 2 μM, and the final volume of each reaction was 100 μL. Results are expressed in percent of residual activity. Differences among treatments were analyzed by two-way ANOVA, followed by a Tukey's test. Statistical significance was set at $P<0.05$ (Zar 1984).

Evaluation of Kinetic Parameters at Different Temperatures

The clawed lobster cathepsin D1 catalytic efficiency was assessed at pH 4.0 (50 mM sodium acetate buffer; temperature coefficient, –0.0002 per °C) (see Stoll and Blanchard 1990). We measured the hydrolysis rate of the fluorogenic substrate 7-methoxycoumarin-4-acetyl-GKPILFFRLK (DNP)-D-Arg-amide at nine concentrations, ranging from 0.2 to 30 μM; the activity is expressed in nanomoles MCA released per minute as the initial velocity. The catalytic constants k_{cat}, K_M, and k_{cat}/K_M were established from the resulting Michaelis–Menten equation after plotting the double reciprocal of the initial velocity versus the concentrations of the synthetic substrate according to the Lineweaver–Burk plot.

The temperature dependence of kinetic parameters was assessed by calculating the catalytic constants at 4°C, 10°C, and 25°C; reaction temperature was attained by water-bathing the reaction components at these temperatures; for comparison, we monitored the reaction to confirm that the temperature (±1°C); measurements were made in parallel with bovine cathepsin D. Values of the turnover number (k_{cat}) were obtained from the equation, $V_{max}/[E]$, where [E] is the enzyme concentration (determined by its deduced amino acid composition and protein concentration data under the assumption that 100% of the enzyme is active). The resulting kinetic values are the average of three independent determinations. Differences among kinetic values at the assay temperatures were analyzed by parametric and non-parametric statistics, including ANOVA and Kolmogorov–Smirnov followed by a Tukey's test. Statistical significance was set at $P<0.05$ (Zar 1984).

Digestion of Bovine Serum Albumin by Cathepsin Ds

The ability and pH dependence of lobster and bovine cathepsin D to digest bovine serum albumin (BSA) was assayed with 12% sodium dodecyl sulfate (SDS)-PAGE, where 50 μg BSA was diluted in 6.25 μL McIlvaine's buffer solution (0.2 M disodium phosphate, 0.1 M citric acid) at pHs 2.2, 2.7, 3.0, 3.6, 4.0, 4.6, 5.0, and 6.0 and incubated with 0.2 μg cathepsin D at 25°C. After 15 min, the reaction was stopped with electrophoresis loading buffer. Hydrolysis of BSA was monitored by image analysis of the gels, where the hydrolytic products were determined with image analysis software (Digital Science 1D, Eastman Kodak, Rochester, NY). As reference, we used BSA at pH 4.0, in which water instead of an enzyme was added.

The effect of the concentration of pepstatin A on activity was also assayed by incubating 0.2 μg cathepsin D for 15 min in the presence of pepstatin A at 14.5 and 145 μM final concentration. After incubation, the enzyme–inhibitor mixture was combined with 50 μg BSA in McIlvaine's buffer solution for 15 min at pH 4.0 and immediately mixed with loading buffer and loaded into 12% SDS-PAGE for image analysis.

Results

The Digestive Aspartic Proteinase from Clawed Lobsters

An aspartic proteinase was purified to homogeneity from the gastric juice of American and European lobsters by affinity chromatography on pepstatin A–agarose column. Analysis by SDS-PAGE of the purified enzymes showed a single band of about 40 kDa (see Rojo et al. 2010b for details). Enzymes from both species were previously identified, and some of their peculiarities have been described. In brief, the molecule is ~40 kDa, a cathepsin D proteinase, according to its modeled structural features and their deduced amino acid sequence (Rojo et al. 2010a). The identity of the enzymes as aspartic proteinases was further corroborated by the observed effect of the specific inhibitor pepstatin A, which reduced the proteinolytic activity of the isolated enzymes in a concentration-dependent manner (Fig. 1a), just like typical cathepsin D proteases (Knight and Barrett 1976). The deduced lobster aspartic proteinase amino acid sequence shares 47% identity with bovine cathepsin D, 41% with human renin, and 36% with human pepsin A.

Effect of pH on the Digestive Aspartic Proteinase

The effect of pH on clawed lobster and bovine cathepsin D activity are shown in Fig. 1b–e. Optimal activity of bovine cathepsin D on the fluorogenic substrate was observed at pH ~4.0. It is notable that, for the same substrate, clawed lobsters' cathepsin D1 possess optimal activity over a wider pH range (3.5 to 5.0) than bovine cathepsin D. Lobster proteinases also had higher activity than their mesophilic counterparts at the flanks of the measured values (Fig. 1b).

Based on the data generated by the image analysis software, we assayed the number of BSA hydrolytic products visualized in 12% SDS-PAGE. The three enzymes yielded more digestion bands (indicated by arrows) from BSA at pH 3.0 than at other pH values. At all pH values, clawed lobster enzymes yielded more hydrolytic products than bovine cathepsin D. Additionally, activity over a wider range of pH occurred in clawed lobsters digesting BSA, since these enzymes generated hydrolytic products at all tested pH values; even at pH 6.0, some hydrolytic products were generated, whereas bovine cathepsin D did not show activity at pH 2.2 or at pH 6.0 (Fig. 1c–e). No hydrolytic products were observed in the control.

pH Stability

The effect of pH at 25°C is shown in Fig. 2. All cathepsin Ds are stable at pHs 4.0 and 5.0 given that 100% of its original activity was retained after incubation for 30 min. Lobster enzymes were poorly stable at pH 2.5, losing more than 40% of activity after 30 min. American lobster enzyme was less stable (higher loss of activity) at pH 2.5 and 6.0.

Thermostability

Comparison of temperature stability of the three cathepsin Ds is shown in Fig. 3. They were stable and active at 25°C for at least 90 min. Cathepsin D1 of the two lobsters did not remain active as long as bovine cathepsin D. At 50°C, European lobster enzyme is less stable, remaining active for 30 min, compared with bovine and American lobster enzymes that remain active for >90 min. At 60°C, cathepsin D1 of lobsters lost up to 80% activity after 15 min; bovine cathepsin D maintained 100% of the original activity after 15 min.

Kinetic Constants

The effect of temperature of cathepsin D1 of clawed lobsters on kinetic parameters K_M, and k_{cat} and the catalytic constant k_{cat}/K_M, using the synthetic substrate 7-methoxycoumarin-4-acetyl-GKPILFFRLK(DNP)-D-Arg-amide, was assayed. All measurements were made parallel with bovine cathepsin D (Table 1).

At 25°C, K_M was similar among the lobster and bovine cathepsin Ds. At lower temperatures (4°C and 10°C), higher K_M values were measured; however, K_M for lobsters was markedly lower than the bovine enzyme, indicating a higher substrate affinity at low temperatures (Fig. 4a). The low K_M affected the catalytic efficiency values of k_{cat}/K_M, which was >20-fold for the lobsters enzymes catalyzing at temperatures close to the mammalian physiological temperature of 25°C (Fig. 4b; Table 1).

Discussion

Aspartic proteinases that participate in food protein digestion have been found in many eukaryotes: protozoans (Goldberg et al. 1991), nematodes (Williamson et al. 2003b), platyhelminthes (Brindley et al. 2001), and arthropods (Sojka et al. 2008), which share hematophagy. For arthropods, digestive aspartic proteinases have been reported in a limited number of species (Matsumoto et al. 2009; Padilha et al. 2009), including crustaceans (Navarrete del Toro et al. 2006; Rojo et al. 2010a), but until now, the catalytic properties and biological function of aspartic proteinases in crustaceans have never been investigated.

We investigated some features of aspartic digestive enzymes of lobsters. Our findings show that lobster enzymes are adapted to efficiently digest proteins from food at low temperature. In the environments where lobsters are found, they cope with conditions faced by other cold-blooded

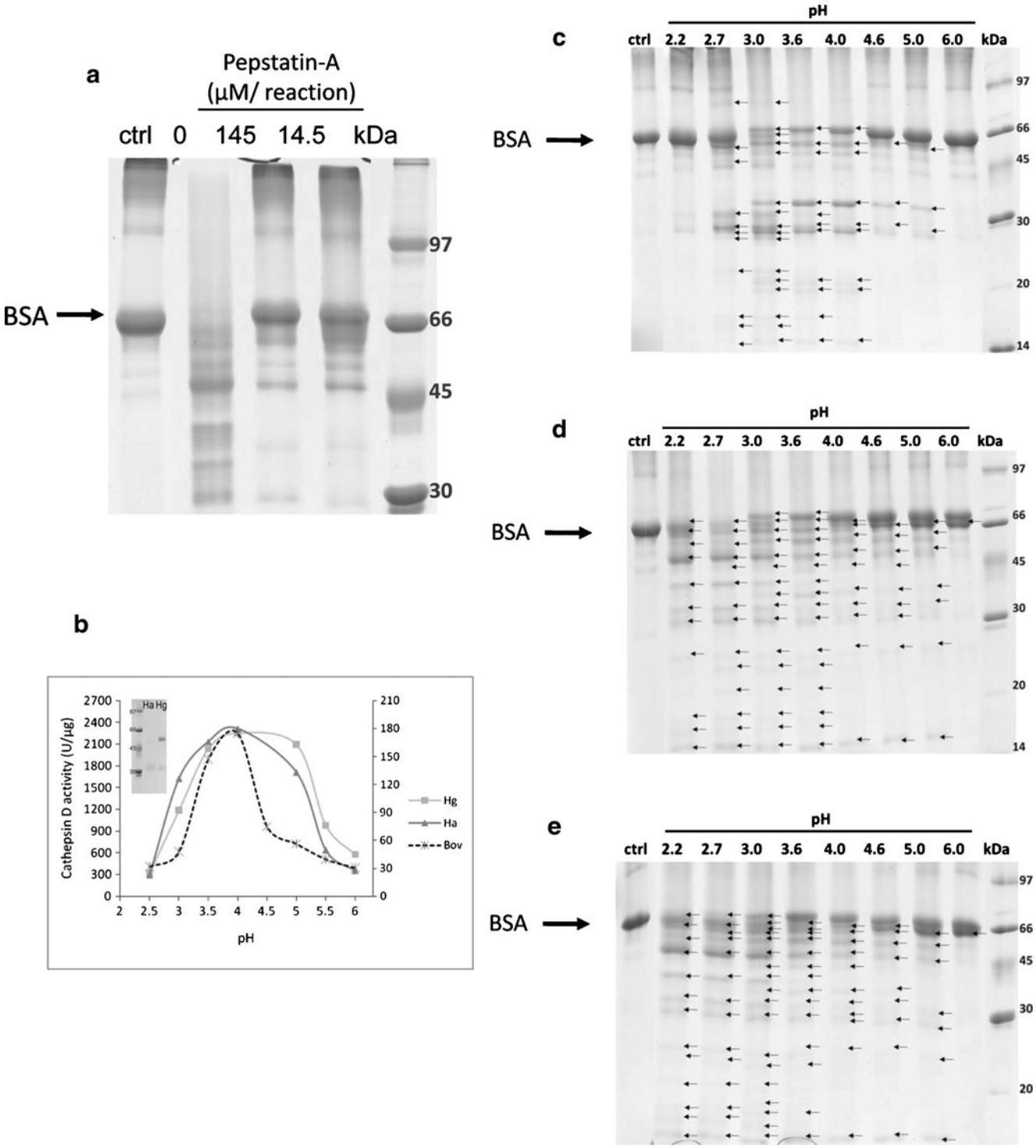

Fig. 1 Cathepsin D activity. **a** American lobster cathepsin D1 activity towards bovine serum albumin (BSA) and the effect of pepstatin-A at different concentrations; 50 μg BSA diluted in McIlvaine's buffer, pH 4.0, 25°C was incubated for 15 min with ~2 μg American lobster cathepsin D1 or in its absence (*Ctrl*). **b** The effect of pH on the lobster (*left axis*) and bovine (*right axis*) cathepsin D activity using the fluorogenic substrate 7-methoxycoumarin-4-acetyl-GKPILFFRLK (DNP)-D-Arg-amide; the final substrate concentration was 2 μM, and the final volume of each reaction was 100 μL; SDS-PAGE of the purified American (*Ha*) and European (*Hg*) lobsters' enzyme is shown. Visual examination of the pH dependence of **c** bovine cathepsin D, **d** European lobster cathepsin D1, and **e** American lobster cathepsin D1, or in its absence (*ctrl*), after 15 min reaction; the resultant proteinolysis products, indicated by *arrows*, were analyzed by 12% SDS-PAGE

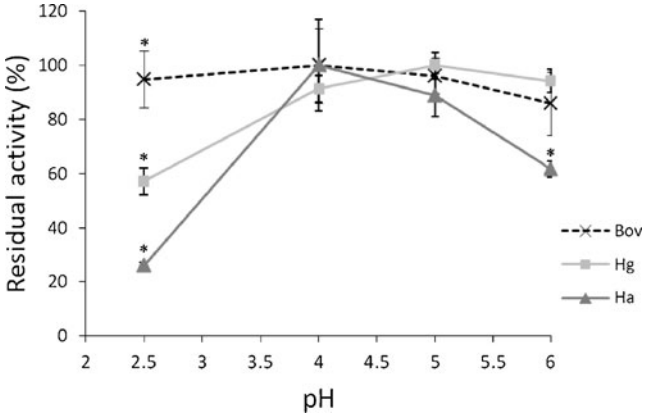

Fig. 2 Stability of Bovine (*Bov*), European lobster (*Hg*), and American lobster (*Ha*) cathepsin D1 incubated at various pHs (2.5, 4.0, 5.0, and 6.0) for 30 min. The values are given as means from three different measurements carried out in triplicate; datasets significantly different ($p<0.05$) from each other at the given pH point are marked with an *asterisk*

animals living at low temperatures, where adaptation of enzymes is involved (Smalås et al. 1994; Carginale et al. 2004; Mukhin et al. 2007). This is supported by our findings.

The pH dependency on activity and stability of lobster and bovine cathepsin D helps us understand functional and structural integrity of enzymes (Barrett et al. 1998). As expected, cathepsins show a maximum of activity at acid pH on natural and synthetic substrates (Fig. 1). Optimal pH in the acid range is typical for aspartic proteinases (Davies 1990; Simões and Faro 2004); however, maximum activity varies with the substrate, illustrating a pH dependence on the substrate. Cathepsin D is probably one of the enzymes responsible for high proteolytic activity at acid pH described in the gastric fluid of European lobster (Navarrete del Toro et al. 2006). The effect of pH on the activity of the three enzymes on the synthetic substrate shows that, compared with bovine cathepsin D, clawed lobster enzymes have maximum activity at a considerably wider range of pH, 3.5 to 5.0 for European lobster and 3.5 to 4.0 for American lobster (Fig. 1b). Likewise, hydrolysis of BSA by lobster cathepsin D1 occurs at practically all tested pH values. This enzyme also generates more peptides compared with bovine cathepsin D, as indicated by arrows in Fig. 1c–e. Therefore, lobster cathepsin D seems to have a general ability to hydrolyze protein, pointing to broader specificity that aids digestion of food protein. This last statement is intuitively appealing, but remains to be proved; however, this finding seems to be common for other lysosomal proteinases described as digestive in other invertebrates (Brindley et al. 2001; Williamson et al. 2002; Aoki et al. 2004; Ranjit et al. 2009).

Our observations of thermostability of the enzymes reinforce the findings described above. Thermal stability of the cathepsins is depicted in Fig. 3. Thermostability indicates that thermally induced alterations of the catalytic mechanism occur at moderate temperatures in the case of lobster cathepsin D1. At 60°C, they most likely underwent denaturation and consequent rapid loss of activity. In a review of the characteristics of cold-adapted enzymes, Gerday et al. (2000) identified three major criteria to typify such proteins. One criterion involves thermal stability, claiming that, since all the cold-adapted enzymes so far characterized display thermostability that is considerably lower than their mesophilic counterparts, the authors ascribe this to a limited structural stability.

A direct relation between stability and molecular flexibility has not yet been shown, but it is likely that these features are related, as suggested by our kinetic analysis. So far, all reported cold-adapted enzymes display modifications in their kinetic parameters that allow catalytic reactions to take place at low temperatures (Feller and Gerday 1997; Fields 2001; Feller and Gerday 2003); therefore, we determined such parameters for lobster cathepsin D at low and room temperatures and ran the analysis in parallel with bovine cathepsin D, which is a mesophilic counterpart. At 25°C, we found

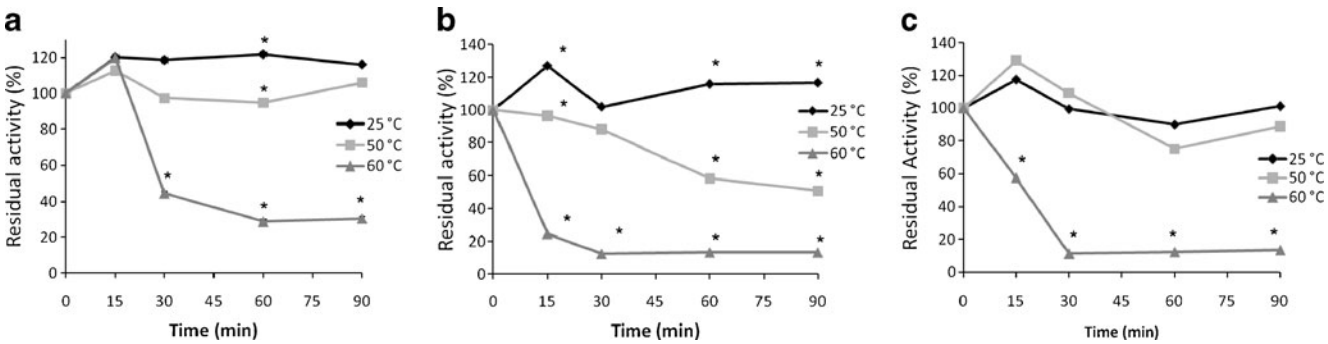

Fig. 3 Stability of **a** bovine, **b** European lobster, and **c** American lobster cathepsin D1 incubated at different temperatures (25°C, 50°C, and 60°C). The values are given as means from three different measurements carried out in triplicate; *asterisk* indicates datasets significantly different from each other at the correspondent time point ($p<0.05$)

Table 1 Kinetic parameters for the hydrolysis of the fluorogenic substrate 7-methoxycoumarin-4-acetyl-Gly-Lys- Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-D-Arg-amide by Bovine, European lobster (*Hg*), and American lobster (*Ha*) cathepsin D1

T (°C)	K_M, μM			k_{cat}, s^{-1}			k_{cat}/K_M, μM^{-1} s^{-1}		
	Bovine	Hg	Ha	Bovine	Hg	Ha	Bovine	Hg	Ha
4	132.91	25.41	31.07	64.4	259.2	385.1	0.46	12.4	24.2
10	34.89	12.35	11.4	28.1	190.5	222.7	0.82	16.1	19.7
25	16.05	10.95	9.36	25.5	262.4	412.5	1.51	27.7	45.3

Values were determined at pH 4.0 at the indicated temperatures

that the fluorogenic substrate value (K_M) of lobster proteinases is similar to what we found for bovine cathepsin D. Despite this similarity, at lower temperatures, there are notable differences between lobsters and bovine enzymes, even though, for all enzymes, substrate affinity tends to increase as temperature rises. K_M values of lobster cathepsin D1 at low temperatures are significantly lower, indicating a higher substrate affinity than bovine cathepsin D (Fig. 4a). K_M values are temperature-dependent; five relationships between these parameters has been described (Simpson and Haard 1987); according to this classification, clawed lobsters show a type 2 K_M-temperature response, where the value remains relatively constant at different temperatures; this relationship has previously been reported for digestive enzymes of aquatic organisms living at low temperatures (Hofer et al. 1975).

Though K_M is a good catalytic indicator of substrate affinity and catalytic efficiency, k_{cat}/K_M is generally a better indicator of catalytic evolution of digestive enzymes because the underlying reaction rates have to be taken into consideration when dealing with temperature adaptation of the catalytic function (Feller and Gerday 1997). Hence, increasing the k_{cat}/K_M ratio through a decrease in K_M can constitute a useful strategy to determine if some enzymes are suitable for adaptation in cold environments (Zecchinon et al. 2001).

Enzymes in cold-adapted species generally show a higher catalytic efficiency than their mesophilic counterparts over a range from 0°C to 30°C (Feller et al. 1996; Fields 2001). Lobster cathepsin D1 can be considered cold-adapted enzymes because they have k_{cat}/K_M values greater than values for bovine enzyme over a range of 4°C to >25°C (Fig. 4b). Although we observed an increase of catalytic efficiency values (k_{cat}/K_M) directly related to increasing temperature, it is notable that k_{cat}/K_M values of lobster cathepsin D1 at 4°C are higher than the corresponding values of the mammalian enzyme at the highest tested temperature (25°C), closer to the bovine physiological temperature (38°C). According to Feller and Gerday (1997), "…high activity at low temperatures showed by cold adapted enzymes seems to be achieved by destabilization of the active site or the whole protein, allowing the catalytic centre to be more mobile or flexible at temperatures that tend to freeze molecular motions…" From this view, together with the low thermal and pH stability in lobster enzymes and reduction in the number of proline residues in the protein structure typical of cold-adapted enzymes (Rojo et al. 2010b), we conclude that clawed lobster cathepsin D1 meets the criteria of cold-adapted enzymes.

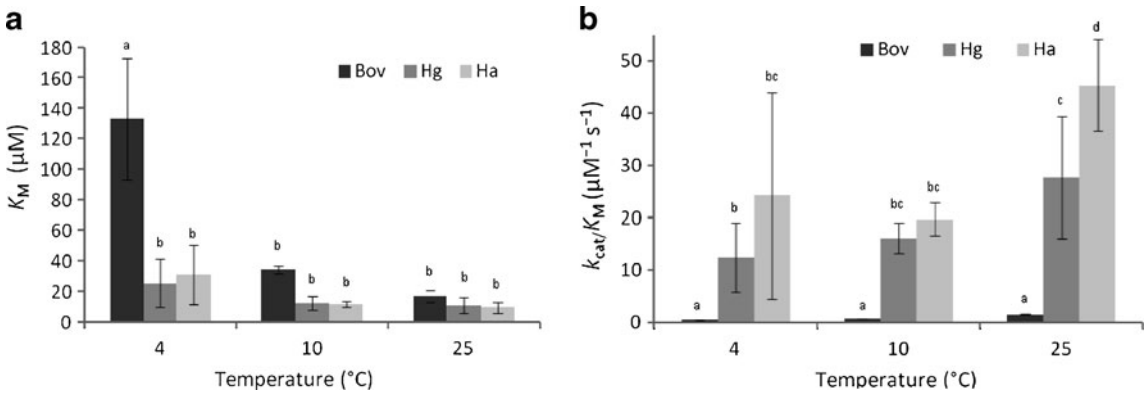

Fig. 4 Bovine (*Bov*), European lobster (*Hg*), and American lobster (*Ha*) cathepsin D1 temperature dependence of the kinetic parameters K_M **a** and k_{cat}/K_M **b**, distinct *letters* indicate datasets significantly different ($p<0.05$)

On the other hand, some authors claim that animals thriving at low temperatures are subject to strong selection for highly active enzymes to accomplish efficient food digestion at similar overall rates of digestion as their mesophilic counterparts (Leiros et al. 2000; D'Amico et al. 2002). Previous research on enzymes isolated from the gastric fluid of the American lobster found digestive cysteine proteinases that work at low temperatures, since cysteine digestive proteinases display an apparent lack of effect of temperature on k_{cat}/K_M from 10°C to 60°C (Laycock et al. 1989).

Physiological and biochemical adaptations in prokaryotic and eukaryotic organisms living at low temperatures have been widely studied, establishing that, to function at low temperatures, enzymes of cold-tolerant organisms have higher activity of k_{cat}/K_M than their counterparts from more temperate climates (Gerday et al. 2000; Ahsan and Watabe 2001; Fields 2001; Carginale et al. 2004; Siddiqui and Cavicchioli 2006). Our data extend this relationship to digestive aspartic proteases of clawed lobsters. Of special interest was its high catalytic efficiency and activity at low temperatures. Therefore, clawed lobster cathepsin D1 is a potential target for novel industrial applications because enzymes from cold-adapted species are generally better suited for enzymatic processes than their mesophilic counterparts (Gudmundsdóttir and Pálsdóttir 2005).

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