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Aspartic Proteinases in the Digestive Tract of Marine Decapod Crustaceans

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ABSTRACT Decapod crustaceans synthesize highly active proteolytic enzymes in the midgut gland and release at least a part of them into the stomach where they facilitate the first step in peptide hydrolysis. The most common proteinases in the gastric fluid characterized so far are serine proteinases, that is, trypsin and chymotrypsin. These enzymes show highest activities at neutral or slightly alkaline conditions. The presence of acid proteinases, as they prevail in vertebrates, has been discussed contradictorily yet in invertebrates. In this study, we show that acid aspartic proteinases appear in the gastric fluid of several decapods. Lobsters *Homarus gammarus* showed the highest activity with a maximum at pH 3. These activities were almost entirely inhibited by pepstatin A, which indicates a high share of aspartic proteinases. In other species (*Panulirus interruptus*, *Cancer pagurus*, *Callinectes arcuatus* and *Callinectes bellicosus*), proteolytic activities were present at acid conditions but were distinctly lower than in *H. gammarus*. Zymograms at pH 3 showed in each of the studied species at least one, but mostly two–four bands of activity. The apparent molecular weight of the enzymes ranged from 17.8 to 38.6 kDa. Two distinct bands were identified which were inhibited by pepstatin A. Acid aspartic proteinases may play an important role in the process of extracellular digestion in decapod crustaceans. Activities were significantly higher in clawed lobster than in spiny lobster and three species of brachyurans. Therefore, it may be suggested that the expression of acid proteinases is favored in certain groups and reduced in others. *J. Exp. Zool. 305A:645–654, 2006.*

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Digestion of alimentary proteins and processing of endogenous proteins is one of the most ancient metabolic processes evolved (Neurath, '84). Highly active proteinases for efficient utilization of alimentary proteins have been reported in many marine species. Using fish meal as protein source, the apparent in vitro protein digestibility was similar in vertebrates and in invertebrates. Fishes such as gilbel carp showed a value of 91% (Yang et al., 2004), Atlantic salmon 85%, seabass juveniles 90–96% and European seabass 95% (Kaushik et al., 2004). In crustaceans, such as the whiteleg shrimp *Penaeus vannamei*, the apparent in vitro digestibility of protein amounted to 84–87% (Ezquerra et al., '97) in mud crab *Scylla serratus* to 95–97% (Catacutan et al., 2003) and in *Penaeus monodon* to 90% (Sudaryono et al., '99).

Proteolytic enzymes in the digestive organs of crustaceans have been well documented and

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characterized (e.g., Dall and Moriarty, '83; Glass and Stark, '94: Jones et al., '97; Le Vay et al., 2001). Different from vertebrates, the entire process of digestion in decapods happens exclusively enzymatically. The digestive gland (or midgut gland) is the site of digestive enzymes production. F- and B-cells secrete enzymes which facilitate the intra-luminal digestion of chyme from the gastric chamber (Ceccaldi, '98; Guillaume and Ceccaldi, 2001). The digestive gland produces a wide range of proteolytic enzymes: endopeptidases such as trypsin, chymotrypsin and astacin, and exopeptidases such as carboxypeptidases and aminopeptidases. Due to their common origin, the same proteinases are present in gastric juice as well as in the midgut gland. Generally, the activities are higher in the gastric fluid than in the midgut gland tissue (Figueiredo et al., 2001; Celis-Guerrero et al., 2004).

Most of the digestive proteinases belong to the families of serine and metallo-proteinases. The contribution of cysteine proteinases in digestion has been raised by Degkwitz ('57) and, recently, their presence has been reported in some caridean shrimps (Teschke and Saborowski, 2005). Serine proteinases show highest activities at neutral or mild alkaline pH, while cysteine proteinases are most active at slightly acidic conditions (pH 4.7–6.0) which prevail in the cardiac chamber of the stomach in most crustaceans (Vonk and Western, '84). The reason of the low pH is uncertain, yet. However, already early workers have suggested that it is caused by the presence of acid salts, such as mono-disodium phosphate, rather than the release of free acids (Jordan, '13).

In vertebrates, aspartic proteinases such as pepsin play important roles in protein digestion at low pH. These enzymes, however, seem to be rare or missing in crustaceans (reviewed by Gildberg, '88). Since then, it has been believed that invertebrates do not possess aspartic peptidase activities because of the lack of codifying genes for pepsin. To our knowledge, no further research on acid invertebrate digestive proteinases has been reported and also the role of cathepsins D and E in the digestive process has not been finally clarified yet. Cathepsin D may have an extracellular function as a digestive enzyme in addition to the intracellular lysosomal function in many invertebrates. This is particularly true for crustaceans such as *Astacus* sp., *Cancer* sp. and *Homarus* sp. (reviewed by Vonk and Western, '84) and for insects such as cockroach, *Blattella germanica* (Arruda et al., '95) and mosquito, *Aedes aegypti* (Cho et al., '91; Cho and Raikhel, '92).

In this work, we analyzed a number of ecologically and economically important decapod crustaceans from Northwest Europe and Pacific America for the presence of acid proteinases in their digestive tract. The study was carried out using non-reduced electrophoresis (substrate-SDS-PAGE), which allows for the simultaneous comparison of crude extracts and determination of the apparent molecular masses of the enzymes. Furthermore, the effects of inhibitors and pH were evaluated. The role of acid proteinases in the digestive process is discussed.

MATERIAL AND METHODS

Origin of animals

The study was carried out at facilities of Centro de Investigaciones Biológicas del Noroeste (CIBNOR, La Paz, BCS, México), the University of Almería (Almería, Spain) and at the marine station of the Alfred Wegener Institute for Polar and Marine Research (AWI, Helgoland, Germany). Five species of decapod crustaceans were studied. Three of them (Spiny lobster, *Panulirus interruptus*, and the Blue crabs *Callinectes bellicosus* and *Callinectes arcuatus*) were sampled of the Mexican Pacific coast and in the Gulf of California. Edible crabs, *Cancer pagurus*, were sampled in the German North Sea at Helgoland. European lobster, *Homarus gammarus* came from Bretany (France) and were obtained from a trader (CuxFish, Cuxhaven, Germany) (Table 1). Live

TABLE 1. Details on the species studied, origin and dates of sampling

Species	Abbrev.	Infraorder/Family	Origin	Date of sampling
Panulirus interuptus	*P.i.*	Achaelata/Palinuridae	Pacific, BCS, Mexico	Nov. 2003
Homarus gammarus	*H.g.*	Astacidae/Nephropoidae	Bretany, France	Sep. 2004
Cancer pagurus	*C.p.*	Brachyura/Cancridae	North Sea, Germany	Sep. 2002
Calinectes arcuatus	*C.a.*	Brachyura/Portunidae	Gulf of California, BCS, Mexico	Jun. 2002
Calinectes belicosus	*C.b.*	Brachyura/Portunidae	Gulf of California, BCS, Mexico	Jun. 2002

organisms were taken to the lab and acclimatized for 1 week in 40-L aquarium tanks with running and aerated seawater at ambient temperatures of 22°C at CIBNOR (Mexico) and 18°C in the BAH-Ecolab at Helgoland (Germany). Animals were fed daily ad libitum with fish, shrimp or squid meat.

Enzyme preparations

Gastric juice was sampled with a disposable syringe which was equipped with a 5–10 cm long Teflon tube smaller in diameter than the oral cavity. The Teflon tube was carefully inserted into the stomach through the esophagus. Depending on the size of the animals, up to 2 ml of gastric juice was obtained by gently drawing the syringe. Samples were taken 12 hr after the last feeding. Each animal was treated only once. In total, 6–20 animals per species were sampled. Immediately after sampling, the pH of the gastric fluid was measured with a pH meter using a microelectrode. Then the gastric juices were transferred into 1.7 ml microtubes and centrifuged for 10 min at 10,000*g* and 4°C to separate solids. A small subsample of the supernatant was used for immediate enzyme activity measurements. The rest of the supernatants were lyophilized and subsequently stored at −20°C. Prior to enzyme evaluations, the powder was dissolved in cold distilled water (1:50; w/v). The solution was centrifuged at 10,000*g* and 4°C for 10 min. The supernatants, always kept on ice, were subsequently used for enzyme assays or electrophoresis. The recovery of enzyme activities was checked for each species by comparing activities of the original supernatant measured immediately after sampling and activities of re-dissolved enzymes. The recovery amounted on average to 90%.

Protein quantification and enzyme activity assays

Total soluble protein was evaluated with the Coomassie blue dye method according to Bradford ('76) using serum bovine albumin as the standard.

Acid proteinase activity at pH 3 was assayed after the method originally described by Anson ('38) and modified by Celis-Guerrero et al. (2004) in a solution of 0.5% (w/v) bovine hemoglobin (Sigma H-2625) in 0.1 M Glycine • HCl buffer. One milliliter of the substrate solution was mixed in a reaction tube with 10 µl of enzyme preparation and incubated for 10 min at 25°C. The reaction was stopped by adding 500 µl of 20% (w/v) trichloroacetic acid (TCA) and cooling on ice for 10 min. The undigested substrate precipitated was separated by centrifugation for 5 min at 10,000*g*. The absorbance of the supernatants was measured spectrophotometrically at 280 nm against distilled water. Control assays (blanks) received TCA solution before the substrate was added.

Alkaline proteinase at pH 8 was assayed as described above, however, with 1% (w/v) of casein as substrate (Sigma C-5890) in 50 mM Tris · HCl buffer. Ten microliters of the enzyme preparation was mixed in a microtube with 0.5 ml of 50 mM Tris · HCl. Substrate solution (500 µl) was added and the tube was incubated for 10 min at 25°C. The reaction was stopped by the addition of 0.5 ml of 20% TCA. Then the tubes were centrifuged for 5 min at 10,000*g*. The absorbance of the supernatants was recorded at 280 nm against distilled water. For the blanks, TCA solution was added before the substrate was supplied. Total protease units of activity were expressed as change in absorbance per minute per milligram of protein ($U = \mathrm{Abs}_{280}\,\mathrm{min}^{-1}\,\mathrm{mg}^{-1}$ protein).

The effect of pH on enzyme activity

The effect of pH on proteases was evaluated in the range between pH 2 and 10 on pooled samples of three individuals. For pH 2–4, hemoglobin (0.5% in 100 mM universal buffer) was used as substrate. Between pH 5 and 10, casein (1% in universal buffer) was used as substrate (Stauffer, '89). The assays with both substrates were performed as described above for hemoglobin and casein.

The effect of inhibitors on enzyme activity

In order to evaluate the major classes of proteolytic enzymes, preparations were incubated with specific inhibitors (García-Carreño, '92). Pepstatin A (1 mM in dimethylsulfoxide, DMSO) was used to inhibit aspartic proteinases including cathepsin D-like and cathepsin E-like enzymes, 10 mM TLCK (tosyl-lysine chloromethyl ketone) in 1 mM HCl to inhibit trypsin and cysteine activity, and 1 mM E-64 in DMSO to inhibit cysteine proteinases including cathepsin B-like and cathepsin L-like proteinases. Aliquots of 10 µl of each inhibitor stock solution were mixed separately with 10 µl enzyme extracts and subsequently incubated for 60 min at 25°C. Thereafter, the samples which were treated with the inhibitors were assayed for activity at pH 3 as described above for total proteinase. Assays were run in

triplicate. Control assays contained inhibitor solvent without the inhibitor. Residual activity was calculated in relation to uninhibited activity.

In order to identify the molecular mass, class and family of proteases, enzyme samples were incubated with specific inhibitors after the method of García-Carreño and Haard ('93). Zymograms with and without inhibitors were run and analyzed for the presence or absence of activity bands.

SDS-PAGE and substrate SDS-PAGE

Proteins and enzymes present in the preparations were separated by 14% SDS-PAGE according to Laemmli ('70). Enzyme preparations were mixed with sample treatment buffer (1:2) but were neither boiled nor treated with mercaptoethanol. Fifteen microliters of samples (on average 100 μg protein) and 4 μl of low-molecular-mass standards (Pharmacia, 17-0446-01) were loaded into individual gel slots in a vertical electrophoresis device (Hoeffer SE260, gel size 8×10 cm) and were run at 4°C at maximum 15 mA per gel. After electrophoresis, gels were stained with 0.05% Coomassie Brillant Blue R-250 in an aqueous solution of 40% methanol and 7% acetic acid for at least 4 hr and then distained with the same solution without dye.

The composition of endopeptidases was evaluated by substrate-SDS-PAGE (García-Carreño et al., '93). For acidic proteinase activity, gels were immersed after electrophoresis in 1 mM HCl solution for 5 min several times until the bromophenol blue dye front changed to yellow. Then the gels were immersed in 100 mM Glycine • HCl pH 3 for 10 min. Then the gels were transferred to a tray containing 100 ml of 0.25% (w/v) hemoglobin in 100 mM Glycine • HCl buffer pH 3. The tray was placed in an ice bath and remained there for 30 min under slow speed in an orbital shaker to allow the substrate to penetrate into the gels. Then the temperature was raised to 25°C and the gel was incubated for another 90 min. The gels were thoroughly washed with distilled water and then were stained and distained as described above. For alkaline protease activity 3% (w/v) casein in 50 mM Tris • HCl, pH 8.0 was used as the substrate while the procedure remained the same as described above. Activity band appeared pale on an otherwise blue dyed background.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Differences among means were analyzed by ANOVA followed by a Tukey's multi-comparison test. Differences are reported as statistically significant when $P < 0.05$ (Zar, '84). Statistical differences of data sets in tables and graphs are indicated by different letters. Statistical analysis was carried out with the computer program SigmaStat 2.03 (SPSS Inc.).

RESULTS

pH of gastric juice (Table 2)

The gastric juices of all species studied here were different in color ranging from dark olive over brownish to reddish or dark yellow. The appearance of colors was not related to species, nor gender, size or pH. In all species, the pH of the gastric juices was slightly acidic ranging from 4.7 in *H. gammarus* to 6.1 in *C. arcuatus*. The pH of the gastric juice of *H. gammarus* was significantly lower than the gastric pH values of all other species.

Total proteolytic activity (Fig. 1a and b)

Enzyme preparations from all species hydrolyzed casein at pH 8 showing activities from $1.53 \pm 0.21\ \mathrm{Abs_{280}\,min^{-1}\,mg_{prt}^{-1}}$ in *C. pagurus* to $2.51 \pm 0.06\ \mathrm{Abs_{280}\,min^{-1}\,mg_{prt}^{-1}}$ in *P. interuptus* (Fig. 1b). Casein digestion in *H. gammarus* amounted to $0.16 \pm 0.04\ \mathrm{U\,mg_{prt}^{-1}}$ and was significantly lower than in all other species.

TABLE 2. pH values and the protein content of the gastric juice of decapods (means ± SEM)

Species	pH of gastric juice	(*n*)	Proteins ($\mathrm{mg\,ml^{-1}}$)[1]	(*n*)
Palinurus interruptus	6.0 ± 0.06	15	11.9 ± 0.44	3
Homarus gammarus	4.7 ± 0.02	5	6.71 ± 0.52	3
Cancer pagurus	5.8 ± 0.3	8	7.40 ± 2.92	3
Callinectes arcuatus	6.1 ± 0.24	6	7.96 ± 1.81	3
Callinectes bellicosus	n.d.		6.11 ± 1.82	3

[1]Values obtained from solubilized freeze-dried samples (50 mg of dry sample in 1 mL of water),

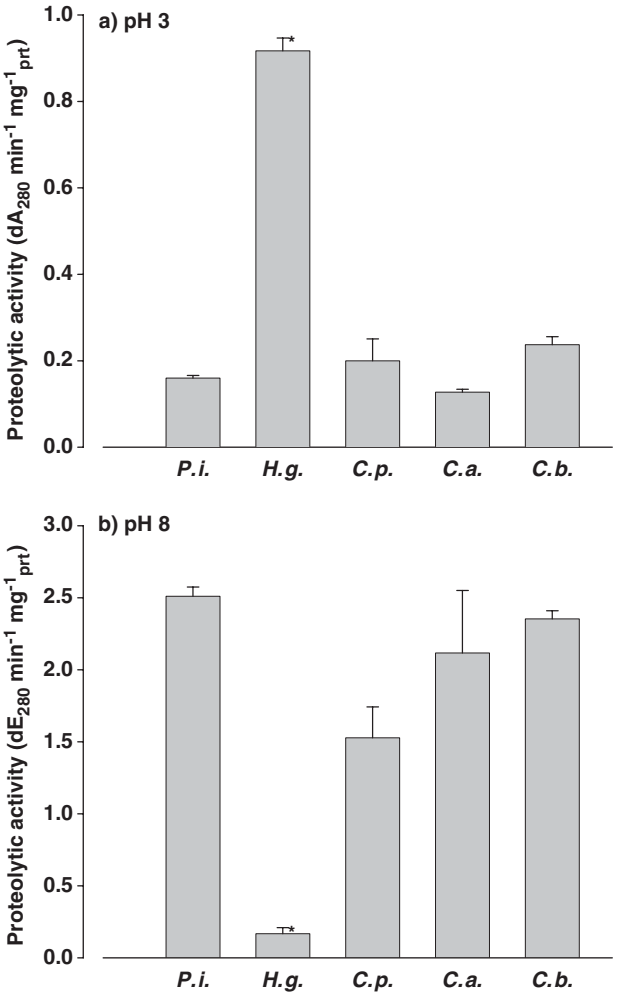


Fig. 1. Activities of (**a**) acid proteinase at pH 3 and (**b**) alkaline proteinases at pH 8 of decapod crustaceans. Means ± SEM, $n = 3$–5. Significantly different values ($P < 0.001$) are indicated by asterisks.

Total proteolytic activity of gastric juices at pH 3 using hemoglobin (Fig. 1a) as the substrate was highest in *H. gammarus* $0.92 \pm 0.05\,\mathrm{U\,mg_{prt}^{-1}}$, followed by *C. bellicosus* $0.24 \pm 0.03\,\mathrm{U\,mg_{prt}^{-1}}$, *C. pagurus* $0.20 \pm 0.08\,\mathrm{U\,mg_{prt}^{-1}}$, *P. interuptus* $0.16 \pm 0.006\,\mathrm{U\,mg_{prt}^{-1}}$ and *C. arcuatus* $0.13 \pm 0.01\,\mathrm{U\,mg_{prt}^{-1}}$ (Fig. 1a).

The effect of pH on proteolytic activity (Fig. 2a–e)

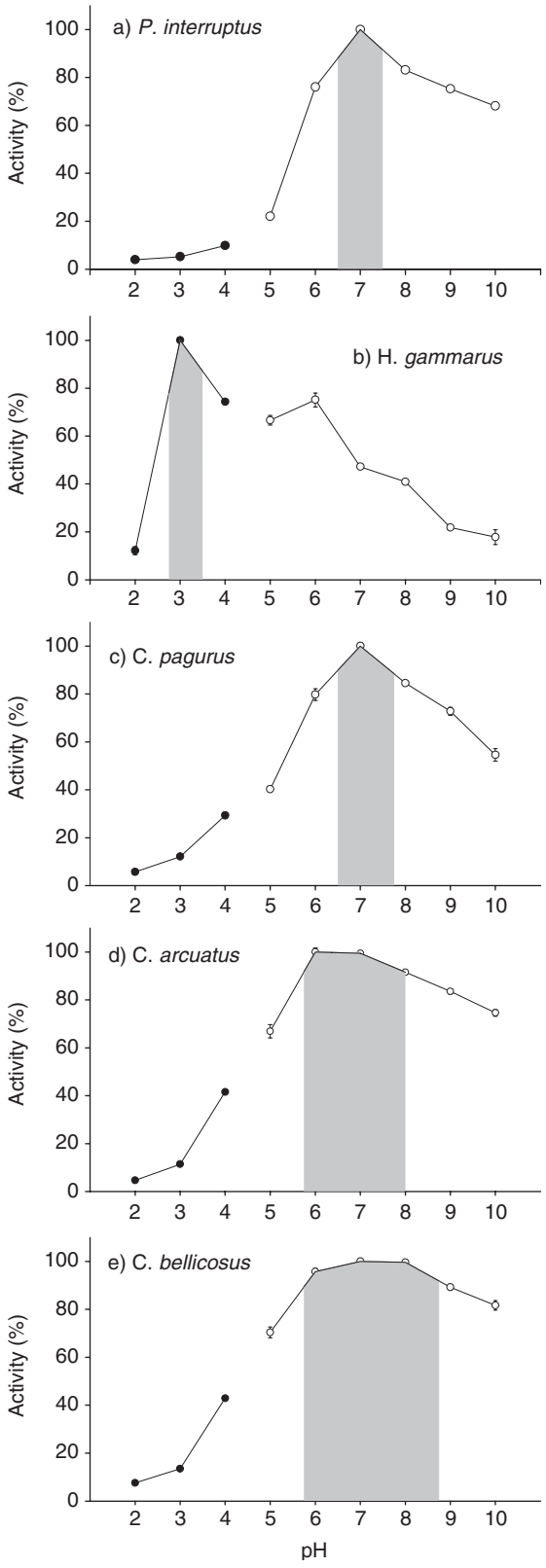
The effect of pH on the total proteolytic activity was assayed by hemoglobin and casein digestion (Fig. 2). *P. interruptus* had maximum activity at pH 7, showing lowest activities at acid pH but still elevated values at alkaline condition above pH 7 (Fig. 2a). In *H. gammarus* the proteolytic activity was highest at pH 3 (Fig. 2b). A second but lower maximum of activity appeared at pH 6. Different to *P. interruptus*, activities rapidly decreased when the pH further increased. All of the brachyuran species showed maximum activities between pH 6 and 8 (Fig. 2c–e). In *C. pagurus*, maximum activity appeared at pH 7. At pH 5 the activity amounted to less than 50% of the maximum (Fig. 2c). In both species of *Callinectes*, proteolytic activities were lower at acid pH than at alkaline pH (Fig. 2d). Broad maxima of activity were present between pH 6 and 8 in *C. arcuatus* (Fig. 2d) and between pH 6 and 9 in *C. bellicosus* (Fig. 2e). The activity remained high above pH 9 showing more than 75% of the maximum activity.

Effects of inhibitors on proteolytic activity (Fig. 3)

The effect of inhibitors on proteolytic activities in gastric juices of decapods was evaluated at pH 3. In *H. gammarus*, the proteolytic activity was entirely abolished by pepstatin A. In *P. interruptus*, pepstatin A reduced proteolytic activity to one third of the control. In the brachyuran species the inhibitory effect of pepstatin A was less distinct. The resulting activities amounted to 45% in *C. arcuatus* and to 66% in *C. pagurus*. Different to papstatin A, the effect of trypsin and cysteine inhibitor TLCK was uniform in all species, resulting in residual activities of 68–77%. E-64 inhibited significantly the proteolytic activity in *H. gammarus* (21%) and in *C. arcuatus* (38%). No significant inhibition was observed in *P. interruptus*, *C. pagurus* and *C. bellicosus*.

Zymograms at pH 8 and 3 (Fig. 4)

Each species expressed an individual pattern of proteolytic enzyme activities at pH 3 (Fig. 4a) and at pH 8 (Fig. 4b). *P. interruptus* showed at pH 8 ten activity bands of apparently 66–16.2 kDa. The highest activity band was of 21.4 kDa. However, at pH 3 no activity of these proteins was detected. Two bands around 25.7 and 20 kDa showed activities at both pHs. *H. gammarus* showed one band of activity at 20 kDa at pH 8. At pH 3, the same sample showed four activity bands, the highest at 30.2 and 17.8 kDa. *C. pagurus* had several bands around 75.0–20.4 kDa of proteolytic activity at pH 8. One band of 52.5 showed the highest activity. However, at pH 3 only very low proteolytic activities were observed at 36.7 and 26.1 kDa. Both *Callinectes* species had several bands of activity at pH 8, those of *C. arcuatus* around 53.7–23.4 kDa and those of *C. bellicosus* at



51.0–23.6 kDa. At pH 3, crabs showed four activity bands between 35.6 and 24.3 kDa.

Effects of inhibitors on activity displayed in zymograms (Fig. 5)

Gastric juice from *H. gammarus* was incubated prior to electrophoresis with specific inhibitors. The zymogram at pH 3 showed two bands of activity which were inhibited by pepstatin A. TLCK and E-64 did not affect the activities of both enzymes. The apparent sizes of the proteins were 30.2 and 17.8 kDa.

DISCUSSION

The presence of proteolytic enzymes active at acid pHs in digestive systems of invertebrates was discussed by Gildberg ('88) and eventually neglected in more recent studies. Our data, however, strongly support the presence of aspartic proteolytic enzymes in the gastric juice of decapods and, thus, indicate a potential role in the digestion of alimentary proteins.

Some of the most intensively investigated and, probably, most important digestive enzymes present in crustaceans may be those of the serine proteases family showing highest activities at neutral or slightly alkaline conditions (Dall and Moriarty, '83, Ceccaldi, '98). They are characterized by an active site consisting of histidine, aspartate and serine as the nucleophilic residue to attack the scissile peptide bond in proteins. In several previous studies, we found that not the entire proteolytic activities in the gastric juice of crustaceans could be assigned to serine proteinases, either because total activity was not entirely inhibited by specific inhibitors or because significant activity was detected at acid pH (García-Carreño et al., 2003; Celis-Guerrero et al., 2004).

Besides serine proteinases, also proteolytic enzymes belonging to the cysteine proteinase family have been identified in crustaceans (Le Boulay et al., '95). The nuclophile in the catalytic site of cysteine proteinases is formed by the sulfhydryl group of a Cys residue, active at slightly acidic and

Fig. 2. The effect of pH on the activities of proteolytic enzymes from the gastric juices of crustaceans. The substrates used were hemoglobin (pH 2–4) and casein (pH 5–10). Shaded areas emphasize the pH ranges of highest activities. Activity calculations are based on the absorption of digested substrate at 280 nm (means of pooled samples from three animals. $n = 3$, SEM was less than 3% of the mean).

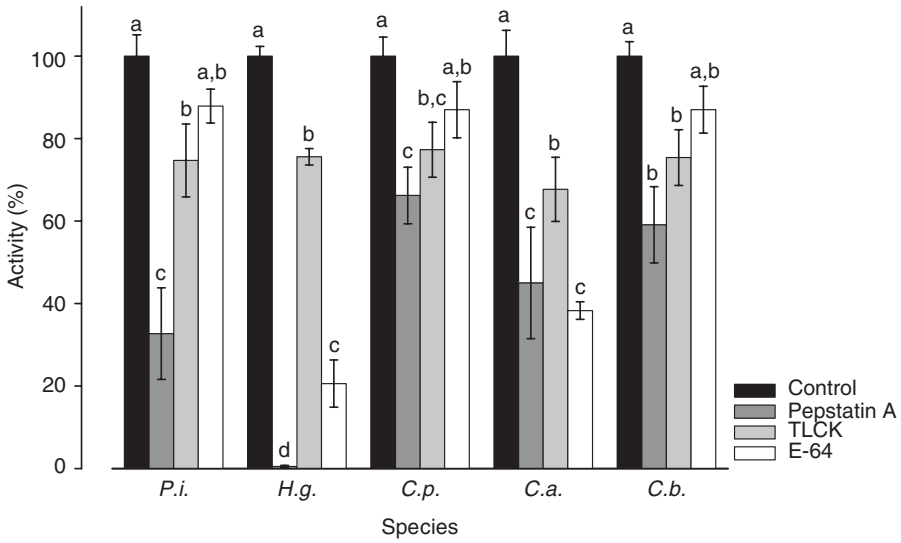


Fig. 3. The effect of inhibitors on the activities of proteolytic enzymes from the gastric juice of crustaceans. The activities were calculated in relation to a control assay which was not treated with any inhibitor (means ± SEM, $n = 6$ animals).

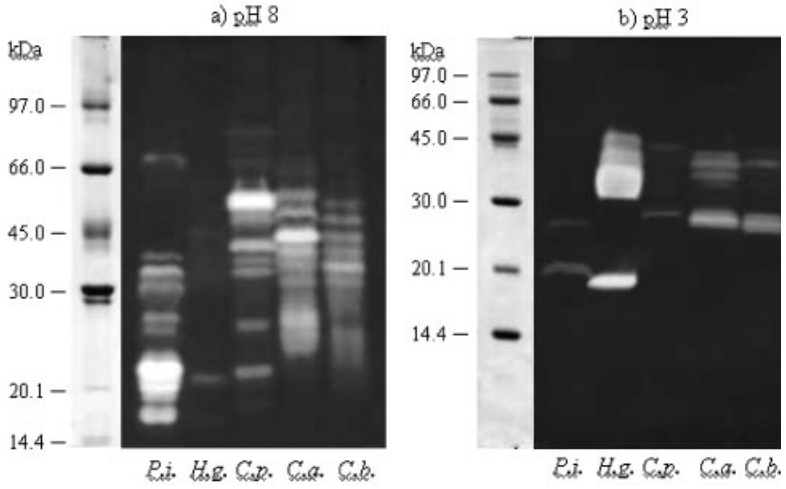


Fig. 4. Zymograms at pH 8. : (**a**) performed with casein as substrate. At pH 3 (**b**) hemoglobin was used as substrate. The amounts of protein applied to the gels were: 135 μg (*P.i.*), 68 μg (*H.g.*), 56 μg (*C.p.*), 80 μg (*C.a.*) and 80 μg (*C.b.*).

neutral pH. They have been isolated from a wide range of sources with several physiological roles, generally involved in intracellular lysosomal processes. Cysteine proteinases seem also to be involved in food digestion in invertebrates. For example, in the digestive fluid of the American lobster, *Homarus americanus*, a cysteine proteinase accounts for 80% of the proteolytic activity (Laycock et al., '89, '91) and in North Sea shrimps, *Crangon* sp. for 70% (Teschke and Saborowski, 2005). Cysteine proteinases were also present in the species studied here showing highest shares in *H. gammarus* and *C. arcuatus*. Particularly, our results on lobsters are in good agreement with the data presented previously by Laycock et al. ('89). Some cysteine proteinases have been identified as cathepsins. In the shrimp, *Pandalus borealis*, cathepsin B is transcribed exclusively in the digestive gland, suggesting a significant role as digestive enzyme (Aoki et al., 2003). A cathepsin L was isolated from the stomach of Norway lobster, *Nephrops norvegicus* (Le Boulay et al., '95) and was also identified in the digestive gland of shrimp, *Metapenaeus ensis* (Hu and Leung, 2004).

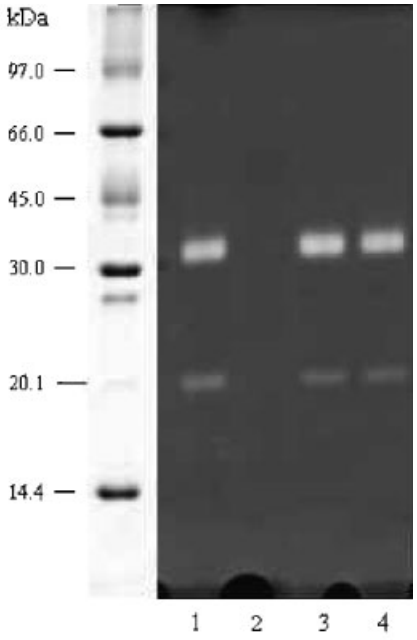


Fig. 5. Zymogram of endopeptidases from the gastric juice of *H. gammarus*.: Lanes: (1) Marker, (2) Control, (3) Pepstatin A, (4) TLCK, (5) E-64. The amount of protein applied was 18 μg.

Aspartic proteases, as they appear in the gastric juices of vertebrates, are active even at pH as low as 3. These enzymes are a class of endopeptidases in which the nucleophile that attacks the scissile peptide bond is an activated water molecule rather than the nucleophilic side chain of an amino acid. Aspartic residues of the active site are involved in catalysis, working as ligands of the activated water molecule. They are active in acidic pH and are inhibited reversibly by pepstatin A and irreversibly by a variety of diazoacetyl compounds in the presence of Cu^{2+}.

The most common technique to identify enzymes in crude extracts is to challenge the enzymes with specific substrates and inhibitors and to evaluate their activities. However, a problem arises in the case of aspartic peptidases. Although some inhibitors are quite sensitive, they often lack enough specificity for the class or type of enzyme due to their often similar tertiary structures. All enzymes belonging to the aspartic peptidase family A, such as pepsin, cathepsin D and cathepsin E, are bilobed molecules with the active site between the lobes. They can accommodate a relatively broad range of substrate residues in the P1-P1′ positions. Therefore, further analytical procedures are required to distinguish between them (Barrett et al., '98).

We propose four explanations for the presence of aspartic proteases in the digestive system of crustaceans. (1) These enzymes are vestigial ones. They remained in the organisms with limited function during evolution. (2) These enzymes contribute to the hydrolysis of food protein as fully operational enzymes by keeping some activity at acid pH. (3) That all or at least most such enzymes may contribute to the activity by keeping some activity at acid pH. (4) Aspartic proteases isolated from the gastric juice were introduced with food or are gastric flora-born.

Premise (3) was discarded because the pattern of activity bands differed in all species distinctly between pH 3 and pH 8. Accordingly, not the same proteins showed activities at both pHs and, thus, the contribution of aspartic proteinases to proteolytic activity at acid pH seems to be limited to specific enzymes rater than to a broad majority of them.

Premise (4) was also discarded when we evaluated food. No evidence of the presence of such enzymes in food was found by test tube or substrate-SDS-PAGE (data not shown), and no evidence of a significant contribution of enzymes from bacteria was found, which agrees with previous studies by Donachie et al. ('95) on gastric bacteria in krill.

Finally, it is the presence of aspartic proteolytic enzymes of some use for digestion of food protein or is it just a vestigial character? Both possibilities are likely. Vestigial enzymes are not uncommon. Pepsin B has a weak general proteolytic activity, about 4% of that of pepsin A (Barrett et al., '98), and does not contribute much in digestion. However, in all of the organisms studied in the present work, enzyme activity at pH 3–5 was significant and Pepstatin A significantly inhibited the total activity at pH 3.

Synthesis of mRNAs for trypsin, amylase, chitinase and cathepsin-L happens in the F-cells of the epithelium lining of the digestive gland tubules in *P. monodon* (Lehnert and Johnson, 2002). Since the secretion of digestive enzymes is holocrine (Ceccaldi, '97), it is possible that, beside digestive enzymes, any compartments and, thus, enzymes from the cell may be secreted to the lumen of the midgut gland tubules. The fluid of the digestive gland enters the proventriculus where it is mixed with ingested food to initiate the first steps of digestion. Accordingly, it seems not exceptional finding enzymes in the gastric juice including those having higher activity at acid pH.

Decapod species exhibit a great variety in the composition of proteolytic activities, both quantitatively and qualitatively (García-Carreño and Haard, '93; García-Carreño et al., '93). On the first sight, no homogeneous pattern in the expression of digestive enzymes seems to exist. However, comparative studies showed similarities in the expression of proteinases classes in species from different phylogenetic branches (Teschke and Saborowski, '05; Saborowski, pers. com.) and, thus, may reflect evolutionary traits in proteinase expression within the decapods.

A discussion on this topic, however, raises immediately a number of unsolved questions. If ancient enzymes are considered to perform most ''simple'' or most ''basic'' reactions, then serine proteinases, such as trypsins-like enzymes may be called ancient (Pfleiderer and Zwilling, '72; Neurath, '84). They show a highly conservative molecular structure, act in the extra-cellular space and catalyze the first steps in proteolysis at an optimum pH similar to that of seawater. They already appeared with high activities in ''ancient'' species such as brine shrimp, *Artemia* sp. (Ezquieta and Vallejo, '85; Pan et al., '91) or the branchiopod *Triops* sp. (Maeda-Martínez et al., 2000). On the contrary, more modern proteinases should show properties derived from the ancient ones. These may comprise complex reaction mechanisms, altered conditions for optimum catalysis (i.e., pH) and/or compartmentalization. These properties seem better to apply for cysteine proteinases and aspartic proteinases, rather than for serine proteinases. These suggestions are in accordance with the hypothesized evolutionary pattern of acid proteinases by Gildberg ('88). The author suggested that aspartic proteinases may have evolved from a common ancestor similar to cathepsin D which progressed to pepsins in the acid stomachs of fish and finally, in the even more acid stomachs of vertebrates. However, the presumably most progressed proteinases do not inevitably appear in the morphologically most evolved crustacean taxa. The ''ancient'' serine proteinases are present in primitive groups such as palinurids. But they are also frequent and often even dominate in the gastric tracts of brachyurans, which represent the most modern crustaceans. On the contrary, presumably ''modern'' aspartic and cysteine proteinases appear in primitive groups such as lobsters, but are lacking in brachyurans. This apparent conflict cannot be resolved without additional information on the enzyme composition of crustacean species from the major phylogenetic groups.

In conclusion, our work has shown that acid aspartic proteinases seem to play an important role in the process of extracellular digestion in decapod crustaceans. Since activities were significantly higher in clawed lobster than in spiny lobster and three species of brachyurans, it may be suggested that the expression of acid proteinases is favored in certain groups and reduced in others. Again, additional systematic studies are required.

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