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Polymorphism and partial characterization of digestive enzymes in the spiny lobster *Panulirus argus*

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ABSTRACT

We characterized major digestive enzymes in *Panulirus argus* using a combination of biochemical assays and substrate-(SDS or native)-PAGE. Protease and amylase activities were found in the gastric juice while esterase and lipase activities were higher in the digestive gland. Trypsin-like activity was higher than chymotrypsin-like activity in the gastric juice and digestive gland. Stability and optimal conditions for digestive enzyme activities were examined under different pHs, temperature and ionic strength. The use of protease inhibitors showed the prevalence of serine proteases and metalloproteases. Results for serine proteases were corroborated by zymograms where several isotrypsins-like (17–21 kDa) and isochymotrypsin-like enzymes (23–38 kDa) were identified. Amylases (38–47 kDa) were detected in zymograms and a complex array of non-specific esterases isoenzymes was found in the digestive gland. Isoenzyme polymorphism was found for trypsin, amylase, and esterase. This study is the first to evidence the biochemical bases of the plasticity in feeding habits of *P. argus*. Distribution and properties of enzymes provided some indication on how the digestion takes place and constitute baseline data for further studies on the digestion physiology of spiny lobsters.

1. Introduction

Spiny lobsters are predators in tropical and temperate seas feeding on a wide variety of benthic and infaunal species including molluscs, crustaceans, polychaetes and other invertebrates. Ecological studies on the natural diet of *Panulirus argus* have shown the carnivorous and opportunistic nature of their feeding habits (Herrera et al., 1991; Cox et al., 1997). *P. argus* has proven to rely on proteins and lipids for energy when feeding on main items in their natural diet, whereas no sparing of proteins by dietary lipids is possible when lobsters are fed with less important natural preys or unsuitable formulated diets (Díaz-Iglesias et al., 2002, Perera et al., 2005). Yet, digestion in spiny lobsters is poorly understood as evidenced in recent reviews (Williams, 2007a,b) and should be closely related to both feeding ecology and metabolic requirements.

Digestive enzymes of decapod crustaceans have been the subjects of many studies. As a result much information is now available on the digestive biochemistry of different species mainly those cultivated worldwide (e.g. shrimps). In contrast, little information is available on properties of digestive enzymes in spiny lobsters. To date, this knowledge is limited to proteases isolated from the digestive gland or gastric juice of *Panulirus japonicus* (Galgani and Nagayama, 1987) and to the characterization of proteases from crude extract in *Jasus edwardsii* (Johnston, 2003) and *Panulirus interruptus* (Celis-Gerrero et al., 2004). Trypsin and chymotrypsin have been shown to be the major proteinases in spiny lobsters (Johnston, 2003; Celis-Gerrero et al., 2004) although no evidence for chymotrypsin was noted by Galgani and Nagayama (1987). Exopeptidases (Galgani and Nagayama, 1987; Johnston, 2003) and collagenolytic serine proteinases (Iida et al., 1991) have been also found in spiny lobsters. Interestingly, the presence of an acid aspartic proteinase has been recently reported in the gastric juice of *P. interruptus* (Navarrete del Toro et al., 2006). There is little information on features of digestive enzymes other than proteases in palinurids. This paper deals with partial characterization of the main enzymes involved in food digestion in *P. argus*.

2. Materials and methods

2.1. Reagents, sample collection and preparation of extracts

All chemicals were reagent grade and obtained from Sigma-Aldrich, except for casein and DMSO (Merck). Lobsters were collected

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in the Golf of Batabanó, Cuba. Only intermolt lobsters were used and determination of molt state was done according to Lyle and MacDonald (1983). Animals were placed on ice for 10 min to obtain a chill coma before hepatopancreas extraction. Gastric juice was obtained through the oral cavity into a disposable plastic pipette. Samples were immediately frozen in liquid nitrogen and then lyophylised to be stored at −20 °C. Before analysis the powders were homogenised in cold distilled H_2O and centrifuged at 4 °C at 8000 *g* for 15 min. Supernatants were immediately used for enzyme assays or electrophoresis.

Crude extracts for each assay were diluted with reaction buffer to measure enzyme activities at initial rates. Assays were always run in triplicate and activities were expressed as change in absorbance per minute per milligram of protein (ΔAbs min^{-1} mg $protein^{-1}$) or in percent. The protein content of enzyme extracts was measured according to Bradford (1976) using BSA as standard.

2.2. Enzyme assays for total proteases

Total protease (alkaline) activity was measured by the casein hydrolysis assay (Kunitz, 1947) as modified by Walter (1984). The reaction mixture of 0.3 mL 1% casein in 200 mM Tris–HCl pH 7, 0.5 mL of the same buffer and 0.3 mL crude enzyme extract, was incubated at 25 °C for 1 h. Then 0.3 mL of 20% TCA was added to stop the reaction. Tubes were placed for 60 min at 4 °C, followed by centrifugation at 8000 g for 15 min. The absorbance of supernatant was recorded at 280 nm. The blank used for this assay was prepared by incubating the crude enzyme extract and buffer for 1 h at 25 °C, followed by the addition of TCA and casein. Additionally, hemoglobin denatured with urea was used as the substrate to assess acid proteolytic activity according to Sarath et al. (2001).

2.3. Trypsin and chymotrypsin-like activities

Trypsin-like activity was measured using 1.25 mM *N*-benzoyl-DL-arginine *p*-nitroanilide (BApNA) in 200 mM Tris–HCl, 20 mM $CaCl_2$, and pH 8.4. Chymotrypsin-like activity was measured with 0.1 mM Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (SApNA) in the same buffer. Substrate stock solutions of BApNA (125 mM) and SApNA (10 mM) were prepared in DMSO and brought to working concentration by diluting with buffer prior the assays. In a 96-well microplate, 10 µL of enzyme extract were mixed with 200 µL of respective substrate, and liberation of *p*-nitroaniline was kinetically followed at 405 nm in a microplate reader Multiscan EX (Thermolab Systems).

End point assays were performed for estimating optimal temperature for trypsin-like and chymotrypsin-like activities. Crude extract (70 µl) was incubated with 500 µL BApNA or SApNA in buffer, for 10 min at a different temperature. To stop the reaction 570 µL of 30% acetic acid was added and absorbance was recorded at 405 nm in a spectrophotometer.

2.4. Inhibition assay for proteases

Classes of proteases in the digestive gland were characterized by the effect of protease inhibitors on caseinolytic activity. Inhibitors employed were soybean trypsin inhibitor (SBTI), benzamidine, and aprotinin for serine proteases. Leupeptin was used for serine-cysteine protease inhibition, NEM for cysteine protease inhibition and EDTA for metalloprotease inhibition. Concentrations of inhibitors were as in Table 3.

Additionally, specific inhibitors were used to inhibit trypsin-like and chymotrypsin-like activities on *N*-benzoyl-DL-arginine *p*-nitroanilide (BApNA) and Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (SApNA), respectively. Final concentration of inhibitor for trypsin was 0.5 mM Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) while for chymotrypsin were: 0.3 mM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 0.3 mM carbobenzoxy-Phe chloromethyl ketone (ZPCK) and 0.5 mM chymostatin.

The mixture enzyme: inhibitor was incubated for 60 min at room temperature and then assayed using appropriate substrate as above. Enzyme extracts incubated with buffer instead of inhibitors were used as controls and referred as 100% of enzyme activity.

2.5. Non-specific esterase activity

Esterase activity in extracts was assessed by the hydrolysis of 0.3 mM of *p*-nitrophenyl acetate (*p*NPA) and 0.3 mM *p*-nitrophenyl butyrate (*p*-NPB) according to Gilham and Lehner (2005) with slight modifications. Stock solutions (100 mM) were prepared for the *p*-nitrophenyl esters in CH_2Cl_2 and diluted immediately prior the assays with 20 mM Tris–HCl, 150 mM NaCl, pH 8.0. Twenty µL of enzyme extract were mixed with 200 µL substrate solution in 96-well microplates and the liberation of *p*-nitrophenol was measured kinetically at 405 nm in a microplate reader.

For optimal temperature and pH, an end-point assay was used. Ten µL extract were incubated with 490 µL substrate solution for 10 min at 37 °C. Then, 700 µL of 5:2 (v:v) acetone/ hexane solution were used to stop reaction followed by 300 µL 200 mM Tris–HCl, pH 8. After centrifugation (2 min at 10,000 *g*) the absorbance of the lower phase was recorded at 405 nm (López-López et al., 2003).

2.6. Lipase activity

Lipase activity was measured using ß-naphthyl caprylate in DMSO. The assay mixture contained: 100 µL of 100 mM sodium taurocholate, 900 µL of 50 mM Tris–HCl pH 7.5, 10 µL enzyme extract, and 10 µL of substrate stock solution (100 mM). The reaction mixture was incubated for 30 min at 37 °C for the reaction to proceed and then 10 µL of 100 mM Fast Blue BB in DMSO were added. The reaction was stopped with 100 µL TCA 12%. Finally, 1.35 mL of 1:1 (v:v) ethyl acetate/ethanol solution were added and absorbance was recorded at 510 nm.

2.7. Amylase activity

Amylase activity was determined according to the Somogy–Nelson method using soluble starch (2% w:v) as substrate, as described in Robyt and Whelan (1968). Briefly, 20 µL of enzyme extract and 125 µL of 100 mM phosphate-citrate pH 7.5 were incubated with 125 µL of starch for 30 min. Activity was measured by calculating the reducing sugars released at 600 nm.

2.8. Effects of pH and temperature on the activity and stability of digestive enzymes

The effect of pH on enzyme activity was evaluated using Universal Buffer (Stauffer, 1989). Excluding the reaction pH, enzyme activities were determined as above. Optimal temperature for each enzyme was determined as described earlier by assaying at the optimum pH over the range 20 °C to 80 °C. The effects of different pH and temperature on the stability digestive enzymes were studied by preincubating the enzyme extract at different pH and temperature for 15, 30 and 60 min prior to the enzyme assays. Results were expressed as residual activity in %.

2.9. Effect ionic strength on digestive enzyme activities

The effect of different NaCl concentrations on the activity of the digestive enzymes was examined including the following NaCl concentration in reaction buffers: 0, 20, 100, 200, 500, 1000, 1500 and 2000 mM. Blanks were prepared using buffer at the appropriate salt concentration.

2.10. Substrate-PAGE of digestive enzymes

Substrate-SDS-PAGE (5% stacking gel, 13% separating gel) was used to determine the composition of proteases in digestive tract (García-Carreño et al., 1993). The enzyme extract was incubated with the various inhibitors for 60 min at 25 °C prior to electrophoresis. Distilled water was used instead of inhibitors in the control. Samples were neither boiled nor treated with mercaptoethanol before loading into the gel; they were run at 15 mA and 4 °C in a vertical electrophoresis device (Hoeffer SE260, 8×10×0.75 cm). The gel was then immersed in 3% casein solution for 45 min at 4 °C and thereafter, the temperature was raised to 25 °C for an additional 90 min. The gel was thoroughly washed with distilled water, fixed in TCA 12% for 30 min, stained with 0.05% Coomassie Brillant Blue in 40% methanol, 7% acetic acid, and finally distained with the same solution without dye. The absence of spots in presence of specific inhibitors indicates a specific type of protease. Molecular mass markers (14–97 kDa, Pharmacia) without reducing agent were used for apparent MW.

Substrate-SDS-PAGE for amylase was performed on 5% stacking gel and 12% resolving gel in the same conditions as for proteases. Gels were immersed in a starch solution (1%) at pH 6 for 60 min and then stained with iodine/KI solution (10%). Molecular weight markers (14–97 kDa, Pharmacia) without reducing agent were used for apparent MW.

Activity and band profile of lobster esterases were strongly affected by SDS; thus zymograms for esterases were performed under native conditions (5% stacking gel and 8% resolving gel). After electrophoresis, the gel was introduced into a mixture of 50 mM Tris–HCl (pH 8), 100 mM α-naphthyl acetate or ß-naphthyl acetate and 100 mM fast blue, and incubated until activity bands were revealed (López-López et al., 2003).

For assessing isoenzyme polymorphism, 40 intermolt individuals were analyzed by zymograms as describe above.

3. Results

3.1. Distribution of enzyme activities

Specific activities for the different enzymes are presented in Table 1. Trypsin-like activity was 2.4 times higher than chymotrypsin-like activity in gastric juice whereas tryptic activity was 4.7 times higher than chymotryptic activity in the digestive gland. Activity on *p*-NPB was 2.6 times greater than on *p*NPA in the digestive gland, thus *p*-NPB was selected for further characterization of esterase activity. Proteases and amylase were several times more active in the gastric juice while esterase and lipase activities were higher in the digestive gland.

3.2. Optimal condition for enzyme activities

Optimal pH for total proteolysis, trypsin and chymotrypsin activities was found to be 7 and activity of these proteases remained high over a broad alkaline pH range (Table 2). Acid protease activity on Hb increased 7–8 fold from pH 2.5 to pH 3. Activity continued to increase slowly until pH 3.5–4.5 (0.04 ΔAbs/min/mg prot.). Similar to protease activities, esterase and lipase activities were optimally active over the wide range of pH (Table 2). In contrast, amylase activity had pH optima over the narrow acidic pH range of 4–5 (Table 2). Optimal temperature for the different enzymes varied from 40 to 60 °C. (Table 2).

Enzyme activities were observed to increase with ionic strength to maximal activity at 20–100 mM NaCl for amylase, at 100–200 mM NaCl for trypsin, at 200–500 mM NaCl for esterase, and 20 mM NaCl for lipase. Decrease in activity was observed at high salt concentration for all enzymes studied but chymotrypsin. Severe effect of salt was observed on lipolytic activity above 1 M NaCl (Fig. 1).

Table 1
Specific activities (ΔAbs min^{-1} mg $protein^{-1}$) of major digestive enzymes in *P. argus*

Enzyme	Gastric juice	Digestive gland
Trypsin	20.9±4.462	1.32±0.422
Chymotrypsin	8.56±0.564	0.28±0.078
Amylase	33.38±8.537	10.64±4.330
Esterase (*p*-NPB)	1.76±0.320	20.60±6.305
Esterase (*p*-NPA)	1.81±0.278	7.91±3.265
Lipase	0.07±0.011	4.25±2.732

Activities were assayed at optimal pH for each enzyme.

Table 2
Optimal temperature and pH for digestive enzymes activities in the spiny lobster *P. argus*

Enzyme activity	Optimal *T* (°C)	Optimal pH	Observations
Proteases	60	7	A minor peak was observed at pH 10–10.5
Trypsin	60	7 and 10–12	Neutral and alkaline peaks are similar in activity. At pH 6 the activity is still high but strongly affected at pH 5
Chymotrypsin	50	7 and 11	Alkaline peak is slightly higher than at neutral pH. Activity is affected at pH 6
Esterase	40–50	7, 9 and 11	Three peaks but high activity all over the range pH 7–11
Lipase	40	7–9	High activity all over the range pH 6–9
Amylase	50	4–5	Strong reduction of activity below pH 4. At pH 6 activity is around 50% of maximal activity

3.3. pH and temperature stability of enzymes

Enzymes responsible for tryptic and chymotryptic activities were stable up to 55 °C for at least 1 h but activities were significantly affected at 60 °C. Chymotrypsin-like activity was more stable than trypsin-like activity conserving around 40% of activity after 30 min at 60 °C whereas trypsin-like activity was almost abrogated after 15 min at this temperature (Fig. 2). Chymotrypsin-like activity was also more stable than trypsin-like activity under acid conditions retaining 50–60% of activity after 1 h incubation (Fig. 2). Amylase activity was stable up to 50 °C and had 30–40% activity after 1 h at 60 °C. Amylase activity was unstable below pH 4 and lost 20% activity at pH 8 (Fig. 3).

Esterase and lipase activities were found to be less tolerant to high temperature than protease and amylase activities. A significant reduction in esterase activity was observed after 15 min at 50 °C while almost 20% lipolytic activity was lost after 1 h at 30 °C (Fig. 4).

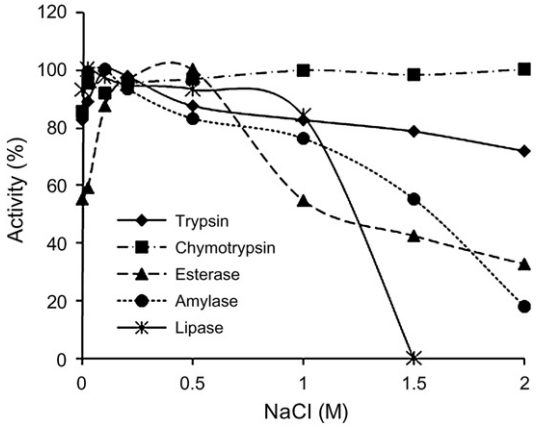

Fig. 1. Effect of NaCl on digestive enzyme activities in *P. argus*.

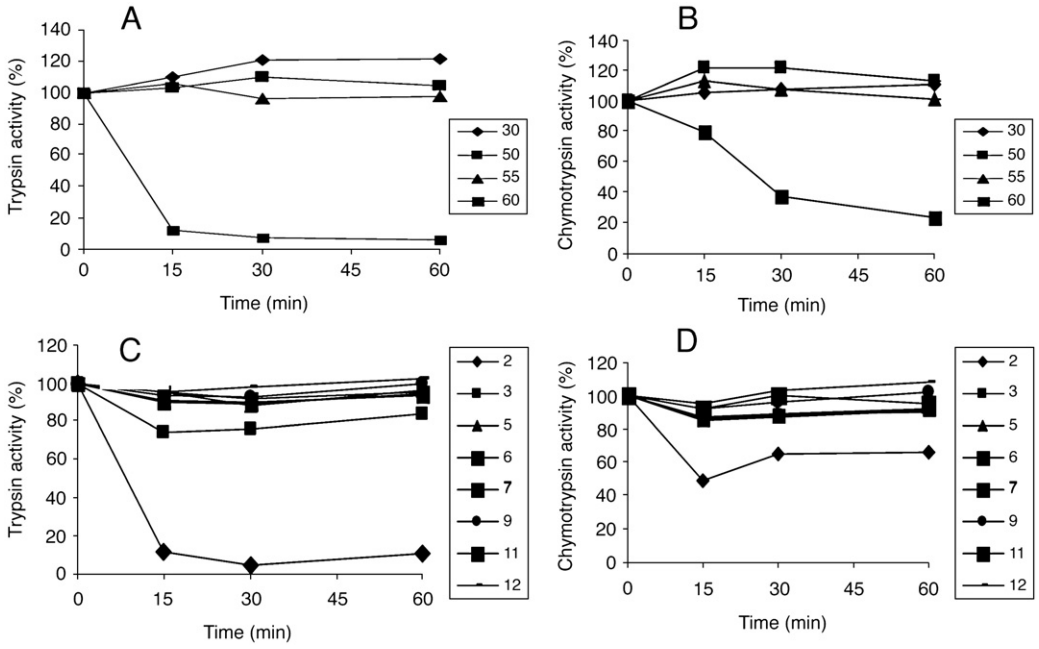

Fig. 2. Stability of trypsin and chymotrypsin activities from digestive gland of *P. argus* incubated at different temperatures (°C) (A, B) and pH (C, D).

Non-specific esterase and lipase activities were stable over a wide range of pH from neutral to alkaline (Fig. 4). Both activities were more affected by acid media than proteases and amylase (Fig. 4).

3.4. Effect of specific protease inhibitors

Leupeptin and aprotinin produced 37% and 47% inhibition respectively while SBTI suppressed 54%–56% of the activity of controls (Table 3). Surprisingly, benzamidine did not inhibit more than 17% of activity (Table 3). An inhibitor for metalloproteases, EDTA, abolished 40%–42% the activity. Results obtained with SBTI and EDTA suggests that significantly higher percentage of inhibition of serine and metalloproteases cannot be obtained by increasing the amount of inhibitor (Table 3). The SH-enzyme inhibitor NEM produced low percentage of inhibition.

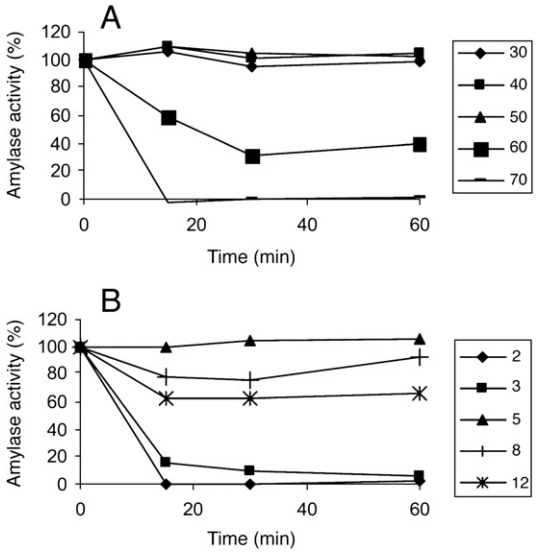

Fig. 3. Stability of amylase activity from digestive gland of *P. argus* incubated at different temperatures (°C) (A) and pH (B).

Preincubation with TLCK was always accomplished at pH 6 to ensure stability of inhibitor but activity was evaluated at different pH values. The hydrolysis of BApNA (trypsin-like) at pH 7 was strongly inhibited (80%) by TLCK whereas it only abolished 67% of amidase activity on BApNA above pH 9. Few or no reduction of the hydrolysis of SApNA (chymotrypsin-like) was observed with TPCK or ZPCK at any pH whereas chymostatin suppresses 100 % of activity at pH 7–8.

3.5. Substrate-(SDS or native)-PAGE

Digestive gland and gastric juice exhibited the same composition of proteases in preliminary experiments and therefore gastric juice was selected as source of proteases for electrophoresis. Zymograms illustrated 13 active zones with caseinolytic activity (Fig. 5, control lane). EDTA and 1, 10-phenanthroline did not affect any activity band (not shown). Several bands were inhibited by SBTI and PMSF corroborating the prevalence of serine proteases in digestive system of lobster. Bands of approximately 43, 32 and 29 kDa were not inhibited by serine protease inhibitors (Fig. 5) and remain unclassified. Bands with approximate molecular masses of 17, 18, 19, 20 and 21 kDa were inhibited by serine protease inhibitors and TLCK and thus classified as trypsin-like proteases (Fig. 5). A band of 38 kDa was inhibited by serine proteases inhibitors and TPCK, but not by chymostatin. A 35 kDa enzyme was inhibited by serine proteases inhibitors, TPCK and chymostatin. Other band with high caseinolytic activity of around 23 kDa was inhibited to some extent by TPCK, but strongly inhibited by chymostatin and serine protease inhibitors. The last three bands were classified as chymotrypsin-like proteases (Fig. 5). ZPCK did not affect any activity band. Two bands of around 25 and 27 kDa were inhibited by serine protease inhibitors, but do not by TLCK, TPCK, ZPCK or chymostatin (Fig. 5) and therefore they could not be assigned to either trypsin-like or chymotrypsin-like protease groups. Proteinase polymorphism could be detected only for trypsin-like enzymes (Fig. 5, Panel B).

Zymograms showed the existence of up to four starch degrading enzymes in *P. argus* (Fig. 6) with apparent molecular masses of 38, 43, 44 and 47 kDa. Several esterases were evidenced in gels with a complex isozyme pattern using α-naphthyl acetate (Fig. 7). The same

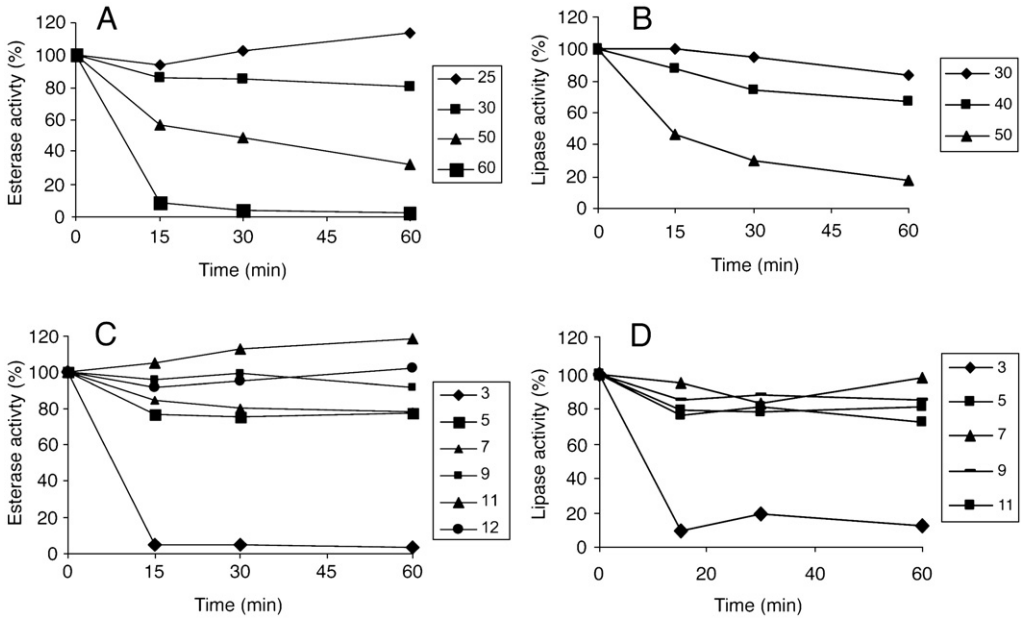

Fig. 4. Stability of esterase (C4) and lipase activities from digestive gland of *P. argus* incubated at different temperatures (°C) (A, B) and pH (B, C).

band pattern as in Fig. 7 was obtained with ß-naphthyl acetate (not shown).

4. Discussion

Properties of digestive enzymes from Palinuridae have been reported for just a few species (Galgani and Nagayama, 1987; Iida et al., 1991; Johnston, 2003; Celis-Gerrero et al., 2004; Navarrete del Toro et al., 2006). With few exceptions, such studies have covered mainly biochemical aspects of proteinases. Herein, activities of major digestive enzymes in *P. argus* are presented for the first time and partially characterized.

The lipid content of the digestive gland has been positively correlated with growth in spiny lobster (Johnston et al., 2003). We have previously demonstrated that lipids are an important energy source for *P. argus* (Díaz-Iglesias et al., 2002; Perera et al., 2005). Non-specific esterases in digestive gland were more reactive toward *p*-NPB than on *p*-NPA, both showing normal Michaelis–Menten kinetics in the absence of emulsified agents. Lipase activity was obtained with ß-naphthyl caprylate in the presence of sodium taurocholate, largely in the digestive gland. Lipase activity has been reported in spiny lobsters before (Johnston, 2003) and in other crustaceans like clawed lobsters (Brockeroff et al., 1970), shrimp (González et al., 1994) and crayfish (Figueiredo et al., 2001). Specific activities for esterase and lipase were higher in the digestive gland despite the high content of non-enzyme proteins in this tissue.

In correspondence with predacious behaviour of spiny lobsters, high protease activity was found in the digestive system of *P. argus* especially in the gastric juice. Trypsin-like proteases were more active than chymotrypsin-like enzymes. Results compare well with those presented for *P. interruptus* (Celis-Gerrero et al., 2004) with the same substrates (BApNA and SApNA) but higher activity for chymotrypsin in *J. edwardsii* was found by Johnston (2003) when using benzoyl-L-tyrosine ethyl ester (BTEE) as the substrate.

As observed for proteases, higher amylolytic activity was evidenced in the gastric juice. Our results suggest that efficient lipid digestion starts later in the digestion process whereas a high rate of hydrolysis of dietary proteins and carbohydrates start just after ingestion in the gastric chamber of *P. argus*.

Optimal temperature obtained here for the different enzymes are similar to those reported elsewhere. Enzymes responsible for tryptic, chymotryptic and amylase activities in *P. argus* showed to be thermally robust. Chymotrypsins-like enzymes were more stable than trypsin-like proteases facing high temperature and extreme pH. Similar results were reported for crabs (Díaz-Tenorio et al., 2006).

Enzymes with trypsin and chymotrypsin activities were stable and highly active under neutral and alkaline conditions and interestingly, they were resistant to pH 5 where they did not exert significant activities. Weak bounds that stabilize these proteases could be fairly resistant to extreme pH values or able to renature the disrupted enzymes as environmental pH moves toward the optimum. Whether this behavior only reflects molecular features of enzymes or has any physiological meaning remains to be clarified. As stated before high amount of enzymes with trypsin-like and chymotrypsin-like activities occur in the gastric juice of *P. argus*, where acidic pH occurs (5.9±0.2, mean±standard deviation, unpublished results) as in other spiny lobsters (Johnston, 2003; Navarrete del Toro et al., 2006). As these proteases are fairly resistant to the pH conditions in the gastric juice, they can aid the proteases in hepatopancreas in later digestion if they migrate to the gland along with food particles which is likely to occur.

Table 3
Effect of inhibitors on caseinolytic activity of digestive gland extracts of *P. argus*

Protease inhibitor	Target	Concentration	% of inhibition
NEM	Binds to SH groups	1 mM	10.1
		10 mM	13.3
EDTA–Na_2	Metallo-proteases	10 mM	40.4
		25 mM	41.8
Leupeptin	Trypsin-like serine proteases and some cysteine proteases	0.1 mM	34.0
		1 mM	37.0
Aprotinin	Serine-proteases	15 mM	40.7
		30 mM	46.7
Benzamidine	Serine-proteases	1 mM	7.10
		10 mM	17.0
SBTI	Serine-proteases	10 µM	53.6
		20 µM	55.8
		50 µM	54.2

Concentration values in the table mean final concentration of inhibitor. The hydrolysis of casein without previous incubation of the crude extract with inhibitor was referred as 100%.

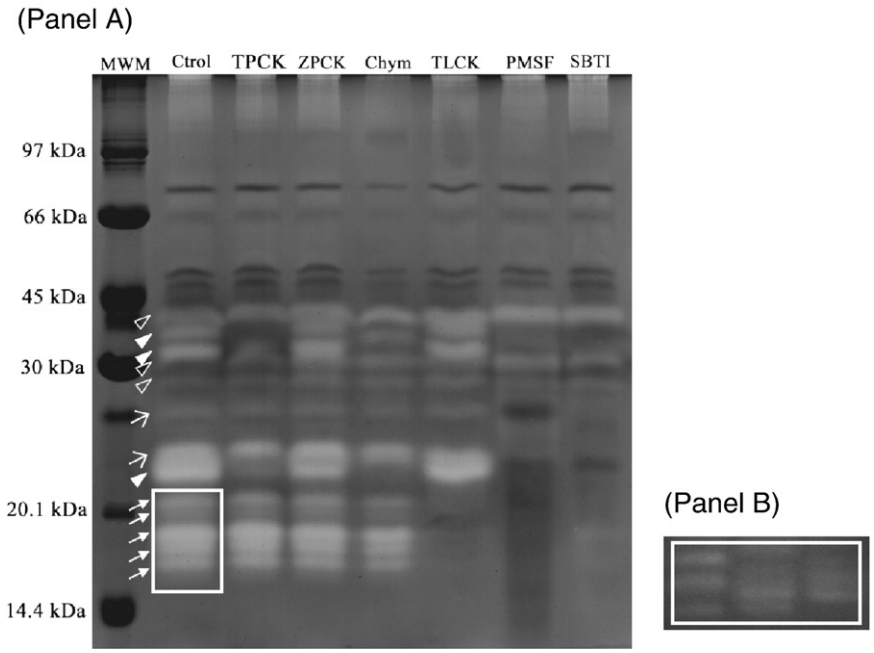

Fig. 5. Panel A, 13% substrate SDS-PAGE showing caseinolytic activity bands in gastric juice of *P. argus* (Ctrol) and inhibition by specific inhibitors for chymotrypsin (TPCK, ZPCK and Chymostatin), trypsin (TLCK) and serine proteinases (PMSF and SBTI). Type of proteases is indicated in the control lane as follows: serine proteinases (→), chymotrypsin like proteinases (▶), trypsin like proteinases (➞) and unclassified proteinases (▷). Panel B, 14% substrate SDS-PAGE showing polymorphism for trypsin like proteinases within the white rectangle in panel A.

Optimal pHs for esterase and lipase activities in *P. argus* were similar to those for proteases. However, esterase and lipase activities were more sensitive to heat and acid pH than proteases and amylase, suggesting a more complex three-dimensional architecture of the active enzymes. Pattern of loss of activity for the different enzymes due to extreme conditions could be affected by the presence of isoforms (Figs. 5–7).

The occurrence of acid proteases for early digestion as in the stomach of terrestrial vertebrates and some fishes has been discussed contradictorily in crustaceans. Sometimes the activity at acid pH could not be inhibited by pepstatin A nor separated from the alkaline peak by gel filtration or anion-exchange (Glass and Stark, 1994) suggesting absence of a distinct acidic protease, whereas activity was completed abolished by pepstatin A in other studies (Navarrete del Toro et al., 2006) suggesting the presence of an aspartic proteinase. Also, cysteine proteinases have been found in crustaceans (Laycock et al., 1991; Le Boulay et al., 1995; Le Boulay et al., 1996; Aoki et al., 2003; Hu and Leung, 2007). Weak acid protease activity in gastric juice of *P. argus* increased 7–8 fold from pH 2.5 to pH 3 and continued to increase slowly until pH 3.5–4.5. The general pattern of activity at acid pH in *P. argus* matched the one for the spiny lobster *P. interruptus*, and the crabs *C. pagurus*, *C. arcuatus* and *C. belicosus* (Navarrete del Toro et al., 2006), but differed from *H. gammarus* (Glass and Stark, 1994; Navarrete del Toro et al., 2006) and *Homarus americanus* (Brockehoff et al., 1970; Biesiot and Capuzzo, 1990) where high activities and clear peaks are evident. Since palinurids are primitive decapods and brachyurans have long been considered morphologically as a much evolved taxon, similarities between spiny lobsters and crabs have been supposed to be

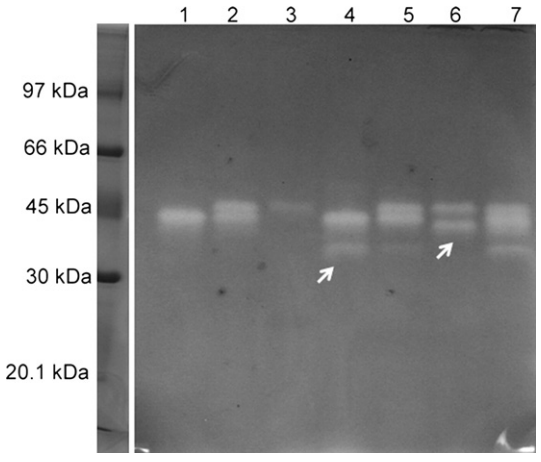

Fig. 6. SDS-PAGE in 12% acrylamide of amylolytic enzymes in digestive gland of *P. argus*. Lanes represent the different phenotypes found. One gene appears responsible for the starch degrading enzymes observed in lanes 1, 2 and 3, with two alleles of around 44 and 47 kDa. Band in lane 1 is the more frequent phenotype followed by phenotype in lane 2, whereas lane 3 shown the less common phenotype. Other faster bands of around 38 and 43 kDa (indicated by arrows) were clearly evidenced in just few individuals. From 40 individuals analyzed only one displayed the four enzymes as in lane 7.

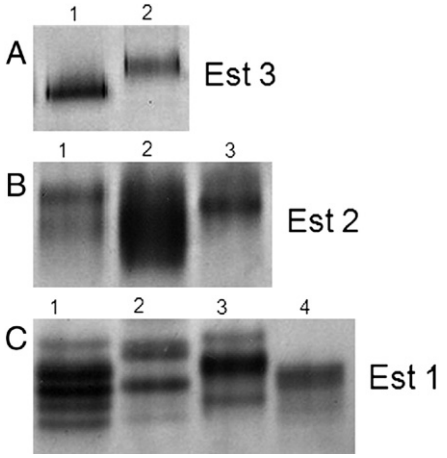

Fig. 7. Native-PAGE in 8% acrylamide of α-esterase isozymes in digestive gland of *P. argus*. Phenotypes in lane 1 of panels A, B and C are of the highest frequency ($n=40$).

contradictory (Navarrete del Toro et al., 2006), but recent analysis based on molecular data suggest a more basal position of the brachyurans in decapods phylogeny (Porter et al., 2005). Yet, phylogenetic relationships cannot totally explain the different traits since substantial share of proteolytic activity in Caridea is caused by cysteine proteinases (Teschke and Saborowski, 2005).

Amylase has been reported to be the most important carbohydrase in the spiny lobster *J. edwardsii* (Johnston, 2003). *P. argus* amylase activity showed an optimal pH of 4–5 in correspondence to the acidic pH in the gastric juice. Early studies divided crustacean amylases into two groups, one with optimal pH below 6.3 including isopods, amphipods and Astacura, and other group with higher pH optimum comprising shrimps and brachyurans (Robson, 1979). Results obtained here and those from Johnston (2003) in *J. edwardsii* indicate that amylases from spiny lobsters belong to the first group. Other spiny lobster carbohydrases like α and ß-glucosidase, ß-galactosidase and N-acetyl ß-D glucosaminidase are also more active in acidic media (Johnston, 2003). More remarkable is that *P. argus* amylase activity becomes compromised at slight alkaline pH values which might be a limiting factor in carbohydrate digestion in the digestive gland. Low specific activity for amylase in hepatopancreas extracts of spiny lobster in comparison to those exhibited by shrimps and crabs has been reported, although few differences were found between the purified enzymes (Van Wormhoudt et al., 1995). Medium pH appears relevant in regulating carbohydrate digestion in spiny lobsters.

Spiny lobsters are unable to regulate the osmolarity of internal media independently of the environment and thus confined to marine ecosystems with salinities varying in the narrow range of 34–36‰. High salt concentration affected most of the digestive enzymes studied. Electrostatic interactions are relevant for activity of many enzymes and usually decline as salt concentration rise. However, since both crustacean chymotrypsin-like enzymes and SApNA are anionic, increased salinity should reduce the electrostatic repulsion between the enzyme and the substrate, enhancing the activity. This kinetic-salt effect was observed in shrimp chymotrypsins toward SApNA, but not when a neutral substrate was used (Tsai et al., 1991). This salt effect was not observed here, suggesting that lobster chymotrypsin-like proteases could be less anionic than the shrimp enzymes. Another possibility is that the interaction between lobster chymotrypsin-like proteases and the substrate is mainly hydrophobic rather than electrostatic, resembling more the bovine than the shrimp chymotrypsin. Chymostatin could inhibit both shrimp (Tsai et al., 1991) and *P. argus* chymotrypsins (Fig. 5) but results with other inhibitors are inconclusive. Lipase activity in *P. argus* was more affected than any other enzyme by ionic strength. High salt concentration increase hydrophobic interactions, which can lead to the aggregation of lipolytic enzymes and lose of activity.

The inhibition assay showed the prevalence of serine and metalloproteases in the digestive tract of *P. argus*. Results for serine proteases were corroborated by zymograms where several bands with caseinolytic activity were inhibited by SBTI and PMSF. Digestive trypsin in shrimps is polymorphic both by cDNA (Klein et al., 1996) and biochemical (Sainz et al., 2004) studies. Here, five enzymes (17, 18, 19, 20 and 21 kDa) were observed to behave like trypsins since they were inhibited by serine proteases inhibitors and TLCK, with the three slighter bands as the most active enzymes. This pattern is similar to the previously presented for *P. interruptus* (Celis-Gerrero et al., 2004). Interestingly, the most reactive forms showed the highest degree of polymorphism. Crustacean trypsins have been estimated from 16 to 25 kDa (Brockeroff et al., 1970; Brun and Wojtowicz, 1976; Galgani and Nagayama, 1987; Celis-Gerrero et al., 2004; Díaz-Tenorio et al., 2006). Apparent molecular masses for trypsin-like enzymes in *P. argus* are consistent with data for other crustaceans, but probably underestimated due to the higher relative mobility of unreduced proteins (incomplete unfolding). The same is true for all enzymes evaluated here under non-reduced or native conditions.

Similar to the spiny lobster *P. interruptus* (Celis-Gerrero et al., 2004), chymotrypsin-like enzymes in *P. argus* have a wide range of molecular mass (23, 35 and 38 kDa) and can be divided into two groups according to electrophoretic mobilities. Crustacean chymotrypsin-like enzymes have been reported from 21 to 60 kDa (Tsai et al., 1986, Celis-Gerrero et al., 2004, Díaz-Tenorio et al., 2006). Activity of shrimp (Tsai et al., 1991) and *P. argus* chymotrypsins toward SApNA are poorly affected by TPCK. However, using casein as the substrate in gels, it was further demonstrated that TPCK inhibits the 38 and 35 kDa chymotrypsin-like enzymes; the smallest (23 kDa) chymotrypsin-like enzyme was poorly inhibited (Fig. 5).In contrast, hydrolysis of SApNA could be almost completely abrogated by chymostatin although this inhibitor totally suppresses only the smallest and most reactive form. All three enzymes were inhibited by serine protease inhibitors like SBTI and PMSF. Results indicate that (i) the two larger chymotrypsin-like enzymes of lobster are not very reactive toward SApNA and (ii) no inhibitor is equally effective on all lobster chymotrypsin-like enzymes. TPCK is known to produce inhibition by alkylation of the active-site histidine by the chloromethyl moiety, whereas the larger chymostatin, as PMSF, reacts with a serine residue in the active site. Altogether, results indicate that these aminoacids are involved in catalysis in all form of the enzyme. Thus, differences in reactivity toward a specific inhibitor are thought to result from differences in the geometry of the active sites. The pocket geometry is important for both the initial docking of the substrate and the formation of the transition state (Wouters et al., 2003). Tsai et al. (1991) suggested that the interaction between the active site of shrimp chymotrypsin and the substrate involve extended subsites, perhaps beyond the enzyme S_4. As in most crustaceans, the small inhibitor ZPCK was a poor affinity-label for *P. argus* chymotrypsin-like enzymes.

Although a collagenolytic metalloprotease has been described in a marine crab (Sivakumar et al., 1999) most crustacean collagenolytic proteases reported so far belong to the serine proteases family (Grant et al., 1983; Iida et al., 1991; Sellos and Van Wormhoudt, 1992, 1999) some times exhibiting tryptic or chymotryptic activity. The hydrolytic activity of identified serine proteases in *P. argus* toward native substrates including collagen should be examined, especially for enzymes for which tryptic or chymotryptic activity were assigned.

Some active bands were not inhibited by serine protease inhibitors. Results from test-tube inhibition assay indicate significant activity of metalloproteases, but this was not evidenced in gels. It has been stated before that these enzymes do not generate clear zones with this technique (Lemos et al., 2000).

Zymograms showed the existence of four starch degrading enzymes in *P. argus* giving rise to seven different phenotypes. Most individuals had one or two active bands. A high degree of polymorphism has been reported for crustacean amylase with up to 5–6 active bands in some species, but a single molecular form in the spiny lobster *P. interruptus* and other decapods (Van Wormhoudt et al., 1995). One gene appears responsible for the starch degrading enzymes observed in lanes 1, 2 and 3 of Fig. 6, with two alleles of around 44 and 47 kDa. Two faster bands (38 and 43 kDa) evidenced in just a few individuals were not specific for either sex or any molt stage (not shown). Doubt remains on whether the two smaller bands come from a second gene expressed in very few individuals or as a result of modification of 44 and 47 kDa enzymes. It has been suggested that glycosylation cannot explain the high degree of polymorphism in crustaceans (Van Wormhoudt et al., 1995), but other post-translational processes like C-terminal processing of amylase by carboxypeptidases (Søgaard et al., 1993) may produce different active forms of a single gene product.

Zymograms resulting from esterase stain showed several isoenzymes with a complex band pattern and different staining intensity as previously reported for shrimps (Lester and Cook, 1987) and spiny lobsters (Menzies and Kerrigan, 1978), but different from the crayfish *Cherax quadricarinatus* where only three bands were reported to

hydrolyze ß-naphthyl acetate and butyrate (López-López et al., 2003). Lipase activity could not be evidenced in gels.

The present work highlights for the first time the diversity of enzymes in the digestive system of *P. argus* supporting the plasticity in their feeding habits. Additionally, the distribution and properties of digestive enzymes have provided some indication on how the digestion process takes place. Yet, several issues remain to be studied to fully understand digestion in spiny lobsters.

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