

## Digestive peptidases and proteinases in the midgut gland of the pink shrimp *Farfantepenaeus paulensis* (Crustacea, Decapoda, Penaeidae)

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### Abstract

Proteases from the midgut gland of the *Farfantepenaeus paulensis* juveniles were assessed. Enzyme activity was determined using protease substrates and inhibitors. The effect of pH, temperature and calcium on proteolytic activity was assayed. Caseinolytic activity was analysed in substrate-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Trypsin, chymotrypsin and leucine aminopeptidase activity was detected. Proteolytic activity was strongly inhibited by the specific trypsin inhibitors. Tosyl-phenylalanine chloromethyl ketone inhibited 59.3% of chymotrypsin activity. The greatest trypsin-like activity occurred at pH 8.0 and 45 °C. Chymotrypsin-like activity reached maximal values at alkaline pH (7.2–9.0) and 55 °C. CaCl<sub>2</sub> did not increase trypsin-like activity, but rather inhibited it at concentrations of 30 (20%), 50 (30%) and 100 mM (50%). The substrate-SDS-PAGE zymogram revealed eight proteinase bands. Two possibly thermal-resistant (85 °C, 30 min) chymotrypsin isoforms were found, which were inhibited by phenyl-methyl-sulphonyl-fluoride. Aminopeptidase activity of enzyme extracts (Arg, Leu, Lys, Phe and Val) and the recommended concentrations of these essential amino acids in penaeid shrimp diets were positively correlated ( $P < 0.05$ ). Because protein digestion involves the combined action of different enzymes, adequate knowledge of shrimp digestion and enzyme characteristics is required for the assessment of the digestive

potential of different feed sources and development of *in vitro* digestibility protocols.

**Keywords:** trypsin, chymotrypsin, aminopeptidase, protein digestion, substrate-SDS-PAGE, *Farfantepenaeus subtilis*

### Introduction

Shrimp use energy and monomers obtained from their diet to synthesize the molecules needed for growth, survival, reproduction, tissue repair and defence. Ingested food is subjected to enzymes that break it down into compounds, which are absorbed by cells in the gut (Shiau 1998; Córdova-Murueta, Garcia-Carreño & Navarrete del Toro 2003).

Digestion is a rather well-studied subject in the field of shrimp nutrition (Fernández-Gimenez, García-Carreño, Navarrete del Toro & Fenucci 2001) and is mainly focussed on enzyme properties that help determine digestive capabilities (Vega-Villasante, Nolasco & Civera 1995). An understanding of digestive enzymes is important for the rational use of feed resources in shrimp farming (Fernández, Oliva, Carrillo & Van Wormhoudt 1997; Muhlia-Almazán, García-Carreño, Sánchez-Paz, Yepiz Plascencia & Peregino-Uriarte 2003). Proteases are the most assessed digestive enzymes in crustaceans and play a key role in the overall assimilation of nutrients (Fernández-Gimenez, García-Carreño, Navarrete del

Toro & Fenucci 2002). These enzymes are also very important for the metabolism and growth of penaeids due to their fundamental role in providing essential amino acids. Thus, the release of essential amino acids may be dependent on the effective hydrolysis of proteins by digestive proteases (Sánchez-Paz, García-Carreño, Muhlia-Almazán, Hernández-Saavedra & Yepiz-Plascencia 2003).

The pink shrimp *Farfantepenaeus paulensis* is a highly valued fishery resource on the southern coast of Brazil (Peixoto, Soares, Wasielesky, Cavalli & Jensen 2004). Reproduction and hatchery techniques are rather well established for seed production (Vinatea & Andreatta 1997). The species is considered to be a potential alternative for the currently cultured species *Litopenaeus vannamei* in sub-tropical and temperate areas. However, *F. paulensis* farming has been restricted by a lack of information on nutrition and feeds for sustaining suitable growth at the commercial level (Lemos, Navarrete del Toro, Córdova-Murueña & García-Carreño 2004). The aim of the present study was to assess the basic functional characteristics of proteases in the midgut gland of *F. paulensis* juveniles, which is essential information for further application of enzymes in nutritional studies.

## Materials and methods

### Materials

All reagents were of analytical grade and purchased from Sigma (St Louis, MO, USA) and Merck (Darmstadt, Germany).

### Organisms and sampling

*Farfantepenaeus paulensis* postlarvae were obtained from a public hatchery and reared for 190 days until reaching 6–10 g of live weight. The shrimp were raised at a temperature of 24–28 °C and fed a commercial feed with 35% crude protein. Following sacrifice, midgut glands were excised from healthy individuals predominantly in intermolt (Dall, Hill, Rothlisberg & Staples 1990). The glands (0.12–0.2 g wet weight) were pooled and immediately stored in liquid nitrogen (–180 °C). Recovery of enzyme extracts began with homogenization of the midgut glands (40 mg mL<sup>-1</sup>) in chilled 0.15 M NaCl using a Potter–Elvehjen tissue homogenizer (Bodine Electric, Chicago, IL, USA) at 40–50 rpm for 3 min at 4 °C. Crude enzyme extracts were obtained from superna-

tants after centrifugation at 10 000 × *g* for 25 min at 4 °C and lipid removal, followed by storage at –20 °C for later use. The total soluble protein content of the enzyme extracts was determined using bovine serum albumin as the standard protein (Bradford 1976).

### Enzyme activity assays

The total protease activity was assayed through hydrolysis of 1% azocasein dissolved in 0.1 M Tris-HCl buffer, pH 8.0 (García-Carreño 1992). Triplicate samples (30 µL) of enzyme extract were incubated with substrate solution (50 µL) for 60 min at 25 °C in a microtube (Bezerra, Lins, Alencar, Paiva, Chaves, Coelho & Carvalho Jr 2005). The reaction was stopped with the addition of 10% trichloroacetic acid (240 µL) and the mixture was centrifuged at 8000 × *g* for 5 min. Unhydrolysed substrate was sedimented and the supernatant was recovered and then further mixed (70 µL) with 1 M NaOH (130 µL). The absorbance of supernatants was measured in a microtitre plate reader (Bio-Rad 680, Japan) at 450 nm. Changes in absorbance over time were calculated by the difference from reactions stopped at zero (blank controls) and after 10 min. One unit of total protease activity was expressed as the amount of enzyme required to hydrolyse azocasein and produce a 0.001 change in absorbance per millilitre per minutes. Specific protease activity was expressed in units per milligram of protein.

Trypsin activity was determined using benzoyl-DL-arginine *p*-nitroanilide (BAPNA). Chymotrypsin activity was measured using either succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (SAPNA) or *N*-succinyl-L-phenylalanine-*p*-nitroanilide (Suc-Phe-*p*-Nan) as specific substrates. Leucine aminopeptidase activity was detected using leucine-*p*-nitroanilide (Leu-*p*-Nan). All substrates were dissolved in dimethylsulphoxide (DMSO) to a final concentration of 0.6 mM (Bezerra *et al.* 2005). The change in absorbance at 405 nm was recorded for 15 min using a microtitre reader (Bio-Rad 680). One unit of activity was defined as the amount of enzyme required to produce 1 µmol of *p*-nitroaniline per minute ( $\epsilon = 9100 \text{ M}^{-1} \text{ cm}^{-1}$ ). Specific activity was expressed as units per milligram of protein.

Aminopeptidase activity was also evaluated using aminoacyl  $\beta$ -naphthylamide (AA-NA) with the following substrates: Arg, Leu, Phe, Val and Lys. The procedure adapted from Oliveira, Freitas Jr and Alves (1999) was carried out by incubating 4.2 mM

substrate (50  $\mu$ L), 50 mM sodium phosphate buffer, pH 7.0, (600  $\mu$ L) and dionized H<sub>2</sub>O (50  $\mu$ L) at 37 °C. After temperature equilibration, the enzyme extract (50  $\mu$ L) was added. After 120 min, the reaction was stopped by adding 1 mg mL<sup>-1</sup> fresh Garnet reagent (250  $\mu$ L) prepared in 0.2 M sodium acetate buffer, pH 4.2, containing 10% v/v Tween 20. Absorbance was measured at 525 nm and the amount of  $\beta$ -naphthylamine was determined using a standard  $\beta$ -naphthylamine curve. Activity was expressed as protease milliunits per milligram of protein. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyse 1  $\mu$ mol  $\beta$ -naphthylamine min<sup>-1</sup>.

### Inhibition assays

To determine the effect of specific inhibitors on protease activity, equal volumes (25  $\mu$ L) of crude extract and inhibitor were incubated for 30 min at 25 °C before the determination of residual activity. Phenyl-methyl-sulphonyl-fluoride (PMSF) was used as an inhibitor for serine proteases; tosyl-lysine chloromethyl ketone (TLCK) and benzamidine were used as inhibitors for trypsin; and tosyl-phenylalanine chloromethyl ketone (TPCK) was used as an inhibitor for chymotrypsin. Volumes were then adjusted to 170  $\mu$ L with 0.1 M Tris-HCl buffer, pH 8.0, and the respective substrate (30  $\mu$ L). Trypsin assays: BAPNA with PMSF, TLCK or benzamidine. Chymotrypsin assays: SAPNA with PMSF and TPCK. All inhibitors were prepared in DMSO to a final concentration of 1.0 mM (Bezerra, Santos, Paiva, Correia, Coelho, Vieira & Carvalho Jr 2001). The change in absorbance at 405 nm was recorded for 15 min using a microtitre reader (Bio-Rad 680). Inhibitory effects on enzyme activity were expressed in relation to controls (without specific inhibitors).

### The effects of pH, temperature and concentration of calcium on enzyme activity

To evaluate the effects of pH and temperature on proteolytic activity, crude extracts were assayed with Tris-HCl buffer, with pH ranging from 7.2 to 9.0 and temperature ranging from 25 to 65 °C. Enzyme activity was determined as described above, using specific substrates. Thermal stability was evaluated by recording enzyme activity at 25 °C after pre-incubation for 30 min at temperatures ranging from 25 to 85 °C.

The effect of calcium on trypsin activity of the midgut gland extract was determined. CaCl<sub>2</sub> was added to the standard reaction assay to obtain final concentrations ranging from 1 to 100 mM. Residual activity was determined as described above, using BAPNA as the substrate. The effect on enzyme activity was expressed in relation to the control (without CaCl<sub>2</sub>).

### Enzyme characterization in substrate-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Zymograms were prepared according to García-carreño, Dimes and Haard (1993). Crude enzyme extracts were dialysed and loaded onto gels at 10  $\mu$ g protein per lane, corresponding to 60–80 U of enzyme activity. Following electrophoresis of crude extracts in SDS-PAGE (12% acrylamide, 0.1% SDS), gels were immersed in 0.1 M buffer Tris-HCl, pH 8.0, containing 2.5% Triton X-100 (100 mL) for 30 min at 4 °C to remove the SDS. The Triton X-100 buffer was removed by washing the gels three times with 100 mL of 0.1 M Tris-HCl buffer, pH 8.0. The SDS-free and Triton X-100-free gels were then incubated with 100 mL of 3% casein (w/v) in 0.1 M Tris-HCl buffer, pH 8.0, for 30 min at 4 °C. The temperature was raised to 25 °C and maintained for 90 min to allow the digestion of casein by active enzyme fractions. Gels were then stained with 0.18% (w/v) Coomassie brilliant blue and destained in 10% (v/v) acetic acid and 25% (v/v) methanol. For the determination of enzyme thermal stability in the zymogram, samples of enzyme extract were incubated for 30 min at temperatures ranging from 25 to 85 °C before loading onto the gel. For the enzyme inhibition zymogram, enzyme extracts were pre-incubated with specific inhibitors of serine proteases, trypsin and chymotrypsin, as described above (inhibition assays). These enzyme inhibition zymograms were compared with those with no inhibitor (control).

### Statistics

Data (mean  $\pm$  standard deviation) were processed using the MICROCAL ORIGIN 6.0 software program. Differences between means (effect of CaCl<sub>2</sub>) were analysed using one-way ANOVA, followed by Tukey's multi-comparison test and considered to be significant when  $P < 0.05$  (Zar 1984).

**Table 1** Proteolytic activity in midgut gland extracts from *Farfantepenaeus paulensis* juveniles using different substrates

Substrates	Enzyme activity (mU mg <sup>-1</sup> protein ± SD; n = 5)
Azocasein	6.49 ± 0.20
BAPNA	5.13 ± 0.55
SAPNA	12.20 ± 1.29
Suc-Phe- <i>p</i> -Nan	Not detected
Leu- <i>p</i> -Nan	0.20 ± 0.02

BAPNA, benzoyl arginine *p*-nitroanilide; SAPNA, succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide; Suc-Phe-*p*-Nan: *N*-succinyl-L-phenylalanine-*p*-nitroanilide; Leu-*p*-Nan: leucine *p*-nitroanilide.

**Table 2** Effect of specific inhibitors on protease activity in midgut gland extracts from *Farfantepenaeus paulensis* juveniles

Inhibitors	Activity inhibition (% ± SD; n = 5)
PMSF*	37.3 ± 0.43
PMSF†	27.1 ± 0.94
TLCK	86.1 ± 0.06
Benzamidine	89.9 ± 0.14
TPCK	59.3 ± 0.60

Maximal specific proteolytic activity (100%) was 5.46 mU mg<sup>-1</sup> protein and 11.05 mU mg<sup>-1</sup> using BAPNA and SAPNA as substrates, respectively.

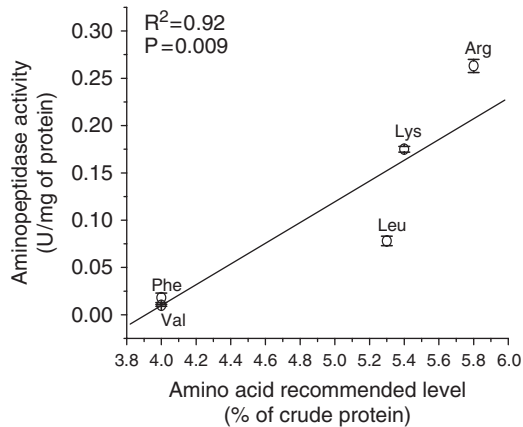
\*PMSF inhibition using BAPNA as substrate.

†PMSF inhibition using SAPNA as substrate.

PMSF: phenylmethylsulphonyl fluoride, serine protease inhibitor; TLCK: tosyl lysine chloromethyl ketone, trypsin inhibitor; TPCK: tosyl phenylalanine chloromethylketone, chymotrypsin inhibitor; BAPNA, benzoyl-DL-arginine *p*-nitroanilide; SAPNA, succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide.

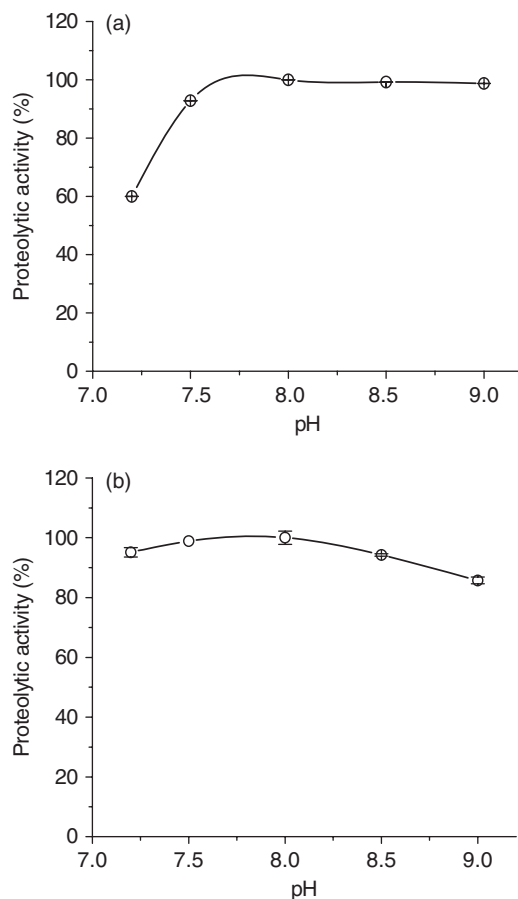
## Results

Table 1 displays the activity of digestive proteases in *E. paulensis*. Trypsin-like activity (BAPNA) was observed in midgut gland extracts, whereas no activity was detected using Suc-Phe-*p*-Nan as the specific chymotrypsin substrate. However, enzyme activity was verified with the SAPNA chymotrypsin substrate, which contains more than one amino acid. Table 2 displays the effects of four different inhibitors on digestive proteases in the midgut gland of *E. paulensis*. Proteolytic activity using BAPNA was 37.3% and was inhibited by PMSF, which is a serine protease inhibitor. Tosyl-lysine chloromethyl ketone and benzamidine (both synthetic trypsin inhibitors) demonstrated a strong inhibitory effect (87.1% and 89.9% respectively) using the same substrate. Phe-

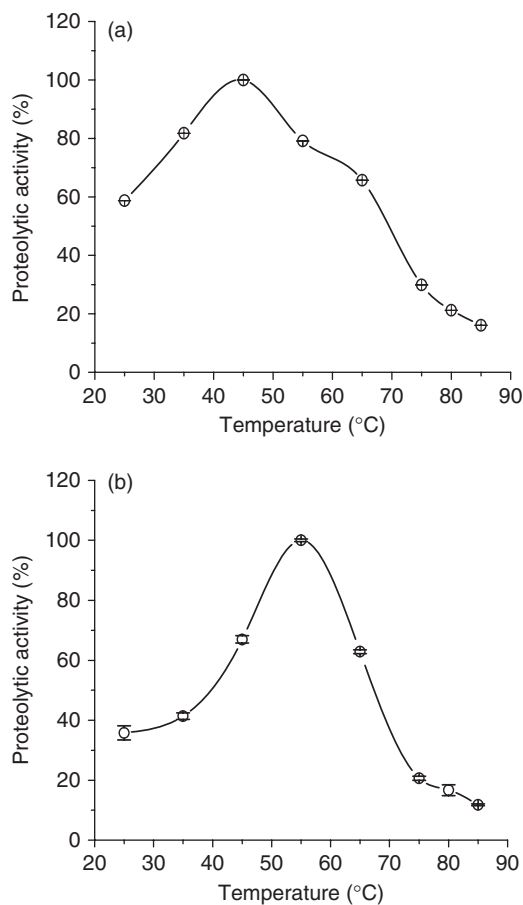
**Figure 1** Regression between aminopeptidase activity in midgut gland extracts from *Farfantepenaeus paulensis* juveniles using aminoacyl β-naphthylamide as substrates (Arg, Leu, Lys, Phe and Val) and recommended concentrations for these essential amino acids in penaeid shrimp diets (Guillaume 1997). Values expressed as mean ± SD (n = 5).

nyl-methyl-sulphonyl-fluoride was also capable of inhibiting chymotrypsin activity by 27.1%, as determined by SAPNA hydrolysis. Proteolytic activity was also inhibited by TPCK (59.3%), which is a specific bovine chymotrypsin inhibitor, using SAPNA as the substrate. Activity with the Leu-*p*-Nan substrate revealed the presence of leucine aminopeptidase-like enzymes (Table 1). Furthermore, plotting aminopeptidase activity using specific AA-NA (Arg, Leu, Lys, Phe and Val β-naphthylamide) versus recommended concentrations of respective essential amino acids in penaeid shrimp diets resulted in a significant positive correlation ( $P < 0.05$ ) (Fig. 1).

The effect of pH on enzyme activity revealed the most trypsin-like activity at pH values ranging from 8.0 to 9.0 (Fig. 2a), whereas chymotrypsin-like activity was greatest at a slightly lower pH range (7.5–8.0) (Fig. 2b). Temperature exerted pronounced effects on the proteolytic activity of *E. paulensis* enzyme extracts (Fig. 3). Enzyme activity reached maximal values at 45 °C (A) and 55 °C (B) in assays with BAPNA (trypsin-like activity) and SAPNA (chymotrypsin-like activity) respectively. Trypsin-like activity was ≥60% of maximal activity between 25 and 65 °C, whereas chymotrypsin-like activity was ≥60% of maximal activity between 45 and 65 °C. Figure 4a shows the influence of heat treatment for 30 min on trypsin-like stability. The activity exhibited no marked change up to 35 °C, but was drastically re-



**Figure 2** Effects of pH on proteolytic activity in midgut gland extracts from *Farfantepenaeus paulensis* juveniles using BAPNA (a) and SAPNA (b) as substrates. Values expressed as mean  $\pm$  SD ( $n = 5$ ). BAPNA, benzoyl arginine *p*-nitroanilide; SAPNA, succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide.

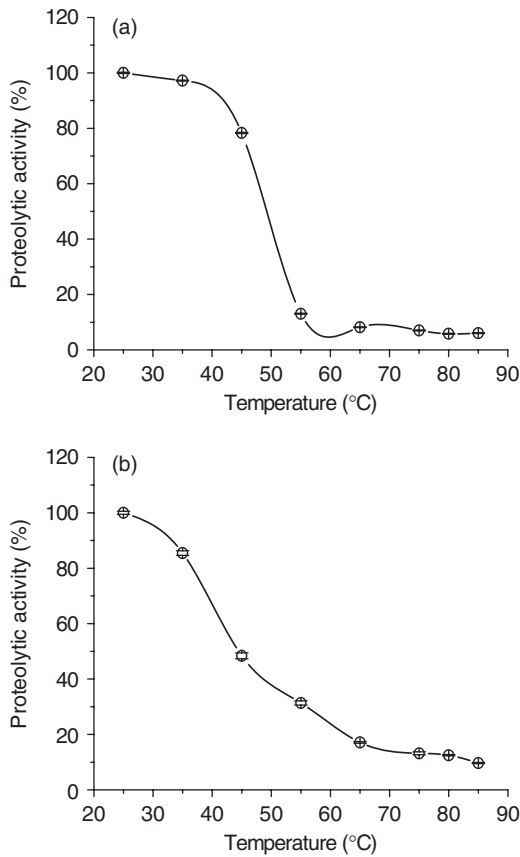


**Figure 3** Effects of temperature on proteolytic activity in midgut gland extracts from *Farfantepenaeus paulensis* juveniles using BAPNA (a) and SAPNA (b) as substrates. Values expressed as mean  $\pm$  SD ( $n = 5$ ). BAPNA, benzoyl arginine *p*-nitroanilide; SAPNA, succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide.

duced after 45 °C, with nearly no activity detected at temperatures above 55 °C. Chymotrypsin-like activity demonstrated lesser stability in comparison with trypsin (Fig. 4b). However, overall higher thermal stability was noticed at temperatures between 5 and 65 °C. No significant difference ( $P > 0.05$ ) was observed in trypsin activity between 1 and 10 mM  $\text{CaCl}_2$ . At these concentrations, activity remained between 80% and 110% of controls. Decreasing activity was recorded at concentrations of 30, 50 and 100 mM ( $P < 0.05$ ) (Fig. 5).

The caseinolytic activity of *E. paulensis* midgut gland extracts revealed eight active bands in substrate-SDS-PAGE at 25 °C (Fig. 6). The activity of heat-treated extracts (35–55 °C) exhibited a band pat-

tern similar to the control (25 °C). However, there was a less intense band at 55 °C (Fig. 6, lane 4, white dashed arrow). Surprisingly, two bands remained active at temperatures up to 85 °C (Fig. 6; white arrow). Enzyme extracts demonstrated specific inhibition in substrate-SDS-PAGE (Fig. 7). Proteolytic bands were strongly or slightly inhibited by PMSF, with the exception of bands with a lower molecular weight (Fig. 7, lane 2, double white arrow). Tosyl-lysine chloromethyl ketone (Fig. 7, lane 3) and benzamidine (Fig. 7, lane 4) were responsible for inhibiting five and three proteolytic bands, respectively. The band with the lowest molecular weight was only inhibited by TLCK (Fig. 7, lane 3). In contrast, TLCK was unable to inhibit a band previously affected by PMSF (Fig. 7,

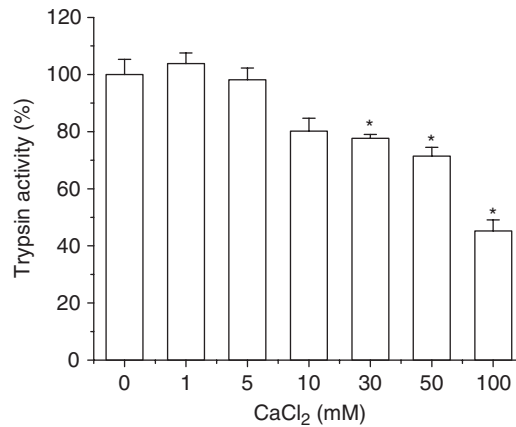


**Figure 4** Thermal stability of proteolytic activity in midgut gland extracts from *Farfantepenaeus paulensis* juveniles using BAPNA (a) and SAPNA (b) as substrates. Enzyme activity was evaluated by recording activity at 25 °C after pre-incubation for 30 min at test temperatures. Values expressed as mean ± SD (*n* = 5). BAPNA, benzoyl arginine *p*-nitroanilide; SAPNA, succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide.

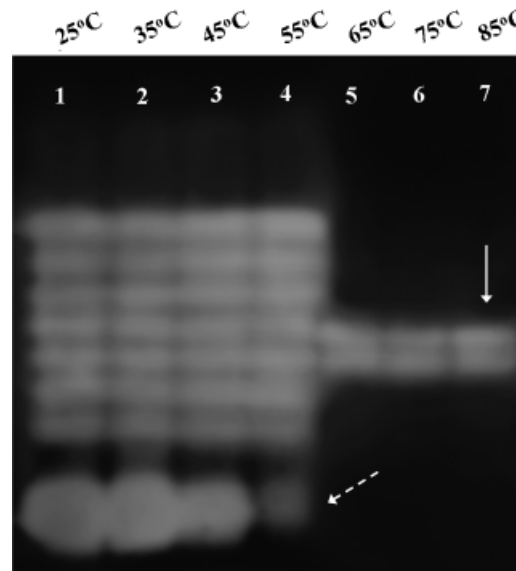
lane 3, white arrow). The thermoresistant bands shown in Fig. 6 (lanes 5–7) were strongly inhibited by PMSF and slightly inhibited by TPCK (Fig. 7, lane 5, white dashed arrows). The inhibitory effects of TPCK on proteases from *F. paulensis* were not determined using the caseinolytic zymogram (Fig. 7, lane 5). However, the caseinolytic band with the highest molecular weight appeared to be inhibited by all the inhibitors used.

**Discussion**

Studies on digestive physiology and enzyme activity provide important basic knowledge for the assessment of nutritional status in farmed shrimp (Jones,

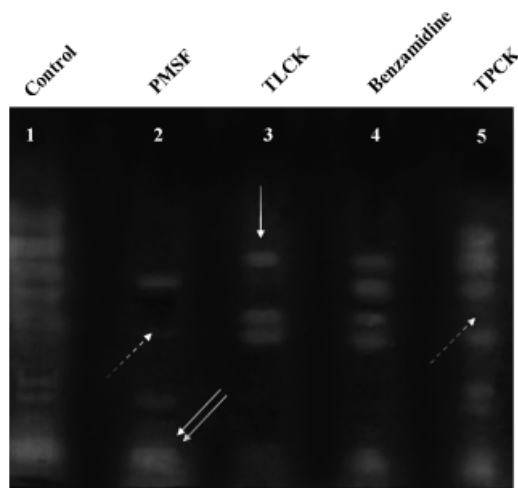


**Figure 5** Effect of CaCl<sub>2</sub> concentration on tryptic activity in midgut gland extracts from *Farfantepenaeus paulensis* juveniles. CaCl<sub>2</sub> and NaCl were added for final concentrations of 1–100 mM. Tryptic activity was determined using BAPNA as substrate. Values expressed as mean ± SD (*n* = 5). Bars with asterisks are significantly different (*P* > 0.05). BAPNA, benzoyl arginine *p*-nitroanilide.



**Figure 6** Thermal stability of digestive proteinases in midgut gland extracts from *Farfantepenaeus paulensis* juveniles in substrate-SDS-PAGE. Lanes – proteinase activity after incubating crude extract for 30 min at each respective temperature. White dashed arrow: less intense bands at 55 °C. White arrow: thermoresistant bands at 85 °C. Before electrophoresis the samples were previously dialyzed. SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Kumlu, Le Vay & Fletcher 1997). The digestion of feed tested *in vitro* with specific enzyme extracts may indicate differences in the digestive potential of feed



**Figure 7** Inhibition of proteinase activity in midgut gland extracts from *Farfantepenaeus paulensis* juveniles in substrate-SDS-PAGE using specific inhibitors. Lanes: 1 – control (without inhibitors); 2 – PMSF; 3 – TLCK; 4 – Benzamidine; 5 – TPCK. Thermoresistant bands inhibited by PMSF and TPCK (white dashed arrows). Band inhibited by PMSF but not by TLCK (white arrow). Band with the lowest molecular weight not inhibited by PMSF (double white arrow). Before electrophoresis the samples were previously dialysed. TPCK, tosyl-phenylalanine chloromethyl ketone; PMSF, phenyl-methyl-sulphonyl-fluoride; TLCK, tosyl-lysine chloromethyl ketone; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

between species (Lemos, Ezquerro & Garcia-Carreño 2000; Lemos *et al.* 2004). Most digestive proteases from decapods are reported to be serine proteases (more recently called serine endopeptidases), including trypsin and chymotrypsin, which seem to be the most important crustacean digestive enzymes (García-Carreño, Hernández-Cortés & Haard 1994; Fernández *et al.* 1997). Trypsin- and chymotrypsin-like enzyme activity has been reported previously throughout the ontogenetic development of *F. paulensis* (Lemos, Hernández-Cortés, Navarrete del Toro, Garcia-Carreño & Phan 1999). The present study indicates that the midgut gland of *F. paulensis* juveniles contains trypsin, chymotrypsin and aminopeptidases. The presence of proteinases and peptidases in the same compartment is an important adaptive advantage. In fact, it makes the protein digestion more efficient. Following hydrolysis of proteins by proteinases into long-chained peptides, peptidases such as aminopeptidases further degrade it into smaller peptides and free amino acids, thus enhancing the absorption of these nutrients. It is also important to

note that a better understanding of the digestive physiology of this species is essential for an adequate shrimp feed formulation, a central topic in aquaculture. Also, a misplanned dietary management may lead to excessive feed loss and metabolic excretion, therefore generating environmental pollution.

Considering the importance of trypsin and chymotrypsin for protein digestion in penaeid shrimp, these findings may have nutritional relevance regarding the use of less complex molecules such as protein hydrolysates (Zambonino-Infante & Cahu 2007). Proteases from the midgut gland of *F. paulensis* exhibited properties from the serine class. These proteases were inhibited by PMSF, which is consistent with the presence of serine and histidine residuals at the catalytic site (Sainz, García-Carreño & Hernández-Cortés 2004) and agrees with previous findings on the inhibition of decapod crustacean serine proteases by PMSF (Lemos, Garcia-Carreño, Hernández & Navarrete del Toro 2002). The inhibition of three trypsin isoforms from the Pacific white shrimp *L. vannamei* has been reported with TLCK and benzamidine when BAPNA was used as the substrate (Sainz *et al.* 2004). Despite inhibiting 60% of the chymotrypsin activity in *F. paulensis* in the present study, TPCK has not proven effective on chymotrypsin activity in other penaeid species (Tsai, Liu & Chuang 1986; García-Carreño *et al.* 1994; Lemos *et al.* 1999).

The greatest trypsin-like activity in *F. paulensis* was found in the pH range 8.0–9.0 for the hydrolysis of BAPNA, thereby indicating a slightly higher optimal pH for the species. Jiang, Moody and Chen (1991) found optimal pH values of 7.0–8.0 for three trypsins from the midgut gland of *Penaeus monodon* using *p*-toluenesulphonyl-L-arginine methyl ester as the substrate. The highest hydrolysis of trypsins in other decapods, such as crayfish, has also been found to be at pH values between 7.0 and 8.0 (Dionysius, Hoek, Milne & Slattery 1993). The highest chymotrypsin-like activity in *F. paulensis* at pH values between 7.5 and 8.0 are also in contrast to values of around 7.0 and between 8.0 and 10.0 reported for this enzyme in the gastric fluid of the marine crab *Cancer pagurus* (Saborowski, Sahling, Navarrete del Toro, Walter & García-Carreño 2004) and the gut of *Daphnia* (Elert, Agrawal, Gebauer, Jaensch, Bauer & Zitt 2004) respectively. In view of such specificities in enzyme functioning, the determination of the optima pH is fundamental to the assessment of the digestive capacity of different feeds (e.g. degree of protein hydrolysis) in assays considering the relationship between

peptide bond breakage and changes in pH values (Ezquerro, Garcia-Carreño, Civera & Haard 1997). Trypsin-like activity exhibited the greatest BAPNA hydrolysis at 45 °C, which is a lower optimal temperature than that found for other crustacean species, such as the digestive tract of *P. monodon* (55–65 °C) (Jiang *et al.* 1991); *L. vannamei* (50 °C) (Sainz *et al.* 2004); and *Triops* sp. (50–60 °C) (Maeda-Martínez, Obregón-Barboza, Navarrete del Toro, Obregón-Barboza & Garcia-Carreño 2000). The thermostability of trypsin in *F. paulensis* at temperatures higher than 45 °C contrasts trypsin from *C. pagurus*, which is reported to retain 70% of its initial activity after 60 min at 50 °C (Saborowski *et al.* 2004). On the other hand, chymotrypsin in *F. paulensis* had the highest activity at 55 °C, but lost 75% of this activity after incubation at this temperature for 30 min. Although the literature reports chymotrypsin-like activity in the digestive system of shrimp (Vega-Villasante *et al.* 1995; Fernández *et al.* 1997; Fernández-Giménez *et al.* 2001; Córdova-Murueta, García-Carreño & Navarrete del Toro 2004), information on its physicochemical characterization remains scarce. A similar study has reported chymotrypsin activity in the gastric fluid of *C. pagurus* extinguished after incubation at 60 °C for 20 min (Saborowski *et al.* 2004).

Thermal effects on proteolytic enzymes were also determined in substrate gel electrophoresis. All bands remained active up to a pre-treatment of 55 °C, although the enzymatic tube assay of proteolytic activity was reduced at this temperature. Indeed, the zymogram method seems to be more sensitive than the quantitative assay when using soluble substrates (Lemos *et al.* 2000). The fact that only one caseinolytic band (with the lowest molecular weight) was not inhibited by PMSF indicates that most of the proteases belong to the serine class. The protease with the lowest molecular weight in *F. paulensis* was inhibited by TLCK, which is a specific trypsin inhibitor, but was not inhibited by benzamidine, which is also a trypsin inhibitor. This suggests that its activity centre may not be homologous to the mammal trypsin (benzamidine-sensitive). On the other hand, a caseinolytic band (Fig. 7, lane 3, white arrow) that was inhibited by PMSF and benzamidine, but not by TLCK, possibly represents a trypsin-like enzyme. It is noteworthy that two bands (Fig. 7, lane 2, white dashed arrow) were only inhibited by PMSF. By exclusion, this finding suggests the presence of a thermal-stable chymotrypsin. Through inhibition by PMSF, chymotrypsin isoforms have been reported

previously in early and juvenile stages of *F. paulensis* (Lemos *et al.* 1999).

Calcium chloride is used in different concentrations as an enzyme stabilization factor for the determination of trypsin and chymotrypsin activity in protocols developed for mammal enzymes (Erlanger, Kokowsky & Cohen 1961). Trypsin activity in *F. paulensis* seemed to be sensitive to concentrations usually used in conventional assays (> 20 mM CaCl<sub>2</sub>), exhibiting significantly reduced enzyme activity. On the other hand, although the effect of certain calcium concentrations on enzyme activity may be non-significant, the hydrolytic potential of the activity of standardized enzyme extracts may be improved at some Ca<sup>2+</sup> concentrations, as observed in assays for protein hydrolysis (Pedersen & Eggum 1983). This may be particularly relevant to the formulation of shrimp feed, considering the variety of ingredients with different mineral contents and their potential effects on the digestive capacity in shrimp (Lemos 2006).

The significant correlation found between aminopeptidase activity (Arg, Leu, Lys, Phe and Val) and the recommended concentrations of these essential amino acids in shrimp diets (Guillaume 1997) possibly indicates a physiological response to protein hydrolysis based on the required concentrations of essential amino acids. To some extent, the data from the present study corroborate reported recommended concentrations for shrimp, with arginine and lysine requiring relatively high concentrations (Fox, Lawrence & Li-Chan 1995). Thus, increased hydrolytic efficiency is dependent on the composition of essential amino acids in commercial shrimp diets (Lemos & Nunes 2008). Further determination of aminopeptidase activity with additional essential AA-NA substrates (e.g. methionine, cystine and threonine) may contribute towards an understanding of the relationships between digestive enzyme activity, hydrolytic potential and diet composition in terms of nutrient requirements.

Enzyme technology has been useful in the development of specific *in vitro* methods for the quality control of diets and ingredients in the livestock feed industry (Fuller 1991). However, the different nature in the functioning, affinity and catalytic performance of enzymes from terrestrial versus aquatic animals entails the development of methods based on specific enzymatic digestion features (Dimes, Haard, Dong, Rasco, Forster, Fairgrieve, Arndt, Hardy, Barrows & Higgs 1994). The set-up of *in vitro* assay conditions for proper hydrolysis is mostly dependent on basic



conditions such as pH and temperature. Thus, the characterization of the functional properties of digestive enzymes in aquatic animals is a necessary starting point for further use of enzyme extracts as tools in the search for the proper nutrient sources in aquaculture.

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