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D. Lemos · M. P. Hernández-Cortés · A. Navarrete
F. L. Garcia-Carreño · V. N. Phan

Ontogenetic variation in digestive proteinase activity of larvae and postlarvae of the pink shrimp *Farfantepenaeus paulensis* (Crustacea: Decapoda: Penaeidae)

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Abstract Proteinase (endopeptidase), trypsin-like and chymotrypsin-like activities were examined throughout the ontogenetic development of cultured *Farfantepenaeus paulensis*. Whole individuals from different larval and postlarval stages, and the hepatopancreas of adults were homogenized and assayed to quantify the enzyme activities of specific substrates. Proteinase activity was identified by substrate-SDS-polyacrylamide gel electrophoresis. Specific inhibitors for trypsin (TLCK), chymotrypsin (TPCK) and serine proteinases (PMSF) were used to identify activity zones of these enzymes in gels. Protein-specific activity of total proteinases, trypsin and chymotrypsin was negligible at the egg stage and at Nauplius III, increasing in the first protozoeal substage (PZ I), and reaching a peak at PZ III; it decreased again in the subsequent postlarval substages. Different patterns of proteinase activity were observed in SDS-PAGE zymograms during ontogenetic development. Active bands of 14.6, 16.4, 17.5, 19.5, 22.5, 23.9, 25.8, 28.9, 32.0, 34.4, 37.7, and 42.2 kdaltons were detected in the adult hepatopancreas. Proteolytic activity was detected on gels in PZ I, and intense activity zones of 16.4, 17.5 and 19.5 kdaltons were found up to Mysis I (M I). Intense bands of 39.1 and 53.5 kdaltons were observed only at PZ III and M I. Band-activity intensity decreased after metamorphosis to the postlarval stage (PL). The chymotrypsin inhibitor TPCK had no effect on the proteinase bands. Active zones in gel inhibited with both TLCK and PMSF were considered to represent trypsin. The inhibitory effect of PMSF alone on proteinase extracts indicated chymotrypsin activity. TLCK and PMSF inhibition also varied during ontogenetic development. The inhibition of bands recorded between 14.6 and 21.7 kdaltons suggested the presence of low molecular weight trypsin in *F. paulensis*. The 39.1 kdaltons band observed at PZ III and M I were trypsin-like. On the other hand, bands of 28.9, 32 and 37.7 kdaltons from the adult hepatopancreas seem to represent a chymotrypsin. We conclude that the recorded variation in enzyme activity may be associated with morphological and behavioral changes during penaeid ontogenetic development. The higher enzyme activity at PZ II, PZ III and M I may reflect the increased energy turnover associated with intense swimming behavior and food ingestion.

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D. Lemos (✉) · V.N. Phan
Instituto Oceanográfico,
University of São Paulo, C.P. 66149,
São Paulo 05315-9370
Brazil

Fax: 55 11 2103092
e-mail: dellemos@usp.br

M.P. Hernández-Cortés · A. Navarrete · F.L. Garcia-Carreño
Centro de Investigaciones Biológicas del Noroeste,
A.P. 128, La Paz, Baja California Sur,
23000 Mexico

Introduction

The ontogenetic development of penaeid shrimp is unique among decapod crustaceans in that all their larval stages are free-swimming, and the transformation to adult morphology and habits is not attained through metamorphosis to a postlarva (decapodid) or during the stages immediately preceding or succeeding metamorphosis (Pérez-Pérez and Ros 1975; Felder et al. 1985; Lovett and Felder 1989; Dall et al. 1990). After hatching, penaeid larvae develop through 5 to 6 non-feeding naupliar, 3 protozoeal, and 3 mysid substages prior to metamorphosis to the postlarval stage. In the wild, larval development is generally completed in offshore waters. Within the first days of postmetamorphic life, the postlarvae penetrate into inshore, brackish, nursery grounds. During the sequential transformation of larvae and postlarvae, significant changes occur in both external and internal morphology (Lovett and Felder 1989; Dall et al. 1990; Abubakr and Jones 1992; Icely and

Nott 1992), habits (Emmerson 1980, 1984; Jones et al. 1992), metabolic rates (Chu and Ovsianico-Koulikowski 1994), and digestive enzyme activity (Laubier-Bonichon et al. 1977; Lovett and Felder 1990a; Fang and Lee 1992; Le Moullac et al. 1992; González et al. 1994). An understanding of digestive physiology is important for studies on the nutritional requirements and feeding ecology of marine invertebrates (Biesiot and Capuzzo 1990). Because protein is the major component in the natural food of penaeids (Smith et al. 1992), proteolytic enzymes may play a key role in the assimilation processes of these species. Among the proteolytic enzymes of shrimp, trypsin may be responsible for 40 to 60% of total protein digestion (Galgani et al. 1984, 1985; Tsai et al. 1986), and it has been characterized in several penaeid species (Honjo et al. 1990 for *Fenneropenaeus indicus*; Lu et al. 1990 and Jiang et al. 1991 for *Penaeus monodon*; Klein et al. 1996 for *Litopenaeus vannamei*). The occurrence of chymotrypsin activity has been controversial because it was believed to be either absent (Lee at al. 1980; Galgani et al. 1984; Lovett and Felder 1990a) or present (Tsai et al. 1986; Fang and Lee 1992; Van Wormhoudt et al. 1992; Le Moullac et al. 1994) in the shrimp digestive tract. Although changes in digestive enzyme activity during shrimp development have been studied in some species, characterization of the enzymes has received little attention.

The present study describes the activity of proteinases, also called endopeptidases, during larval and early postlarval development of *Farfantepenaeus paulensis*, and compares it with activity in the adult shrimp. The pink shrimp *F. paulensis*, also known as the São Paulo shrimp (Pérez-Farfante 1967), constitutes one of the main fishery resources of the Southern Brazilian coast and shelf (Valentini et al. 1991), and is an important component of the benthic megafauna of this area (Pires-Vanin 1989; Wakabara et al. 1996). To characterize trypsin and chymotrypsin in various developmental stages, protein extracts were assayed using specific inhibitors and synthetic substrates. Proteinase activity was correlated with other penaeid developmental events such as morphological and behavioral changes. The generic designations in the text follow the recently proposed nomenclature for members of the family Penaeidae (Pérez-Farfante and Kensley 1997; Holthuis 1998).

Materials and methods

Experimental shrimp

Farfantepenaeus paulensis were caught along the Santa Catarina State coast, Brazil (Latitude 27°18′S; Longitude 48°23′W). After a period of acclimation, wild females of 33.9 ± 4.7 g wet weight were induced to mature sexually by unilateral eyestalk ablation, and were maintained individually isolated in 500-liter fiberglass tanks. Spawning took place during the night, and viable eggs (~200 000 per female) were transferred to cylindrical conical tanks, in which they hatched 12 to 14 h later at 26 °C ± 1 C°. *F. paulensis* has 6 naupliar (N I to VI), 3 protozoeal (PZ I to III), and 3 mysid (M I to III) substages (Iwai 1978). Then nauplii were moved to 50 000-liter tanks, and were reared following commercial practice at 26 °C ± 1 C° and 34 ± 1‰S (Vinatea et al. 1993; Beltrame et al. 1996). Exogenous food was supplied from PZ I onward, and consisted of the diatom *Chaetoceros calcitrans* at (80 000 cells ml^{-1}) and artificial plankton (Nippai Shrimp Feed Inc., Japan; 0.03 mg $larva^{-1}$ d^{-1}, 30 μm particle-size). Freshly hatched nauplii of *Artemia* sp. (15 nauplii $larva^{-1}$ d^{-1}) were added to the diet at Stage PZ III. After metamorphosis to postlarva (PL), the diet was composed of microalgae with increasing amounts of artificial plankton and brine shrimp nauplii (*Artemia* sp.).

Pools of individuals were selected throughout ontogenetic development from a tank containing 7 to 9 synchronized spawns. A homogeneous sample was defined when >80% of individuals belonged to the same substage; the remaining individuals differed by a single substage only. Eggs were chosen 10 to 12 h after spawning at the "early nauplius" stage according to Primavera and Posadas (1981). During the naupliar stage, individuals were selected of N III. From the PZ I substage, each subsequent larval substage was analyzed until metamorphosis. Postlarvae from specific instars (PL I to PL VII) were sorted according to Iwai (1978). Samples were immediately frozen at −20 °C and transferred to liquid nitrogen. The hepatopancreas of three adult, sexually immature, females were extracted, frozen at −20 °C, and freeze-dried. These females were previously fed with frozen mussel and squid, and fasted for 12 h before dissection of the hepatopancreas.

Water and protein determination

Eggs and larvae were washed in distilled water and, after removal of excess water, 3 to 6 samples of pooled individuals were weighed prior to being oven-dried at 60 to 70 °C for 48 h. Wet and dry weight were measured on a Cahn C-31 microbalance (0.1 μg precision). Water content was determined by comparing fresh and dry weights. Total soluble proteins were determined in freeze-dried samples by the Coomassie blue dye method (Bradford 1976), with bovine albumin as the standard.

Quantification of enzyme activity

Approximately 0.7 to 1.2 g wet weight of whole larvae or postlarvae, and 0.02 to 0.08 g dried adult hepatopancreas were homogenized in chilled distilled water and centrifuged at 10 000 ×*g* for 20 min at 5 °C. After lipid elimination, total soluble proteins were determined in the supernatants (Bradford 1976). Total proteinase activity was measured by the rate of hydrolysis of 1.5% azocasein in 50 m*M* Tris buffer, pH 7.5 (Garcia-Carreño 1992). Triplicates of 10 μl enzyme extracts were mixed with 0.5 ml substrate solution at 25 °C. The reaction was stopped 10 min later by the addition of 0.5 ml of 20% trichloroacetic acid (TCA), and the mixture was centrifuged for 5 min at 6 500 ×*g*. The supernatants were separated from the undigested substrate, and the absorbance for the released dye was recorded at 440 nm. Trypsin and chymotrypsin activities were determined by the rate of hydrolysis of synthetic substrates (Table 1). Trypsin activity was measured in the early stages for both TAME (*N*α-*p*-toluenesulphonyl-L-arginine methyl ester) and BAPNA (*N*α-benzoyl-DL-arginine-*p*-nitroanilide) substrates. Adult hepatopancreas were assayed for trypsin activity using only BAPNA. Samples (n = 6 to 20) of 300 μl enzyme extract were mixed with 1.2 ml 10 m*M* TAME in 46 m*M* Tris buffer, pH 8.1, containing 11.5 m*M* $CaCl_2$ at 25 °C, and the change in absorbance at 247 nm was recorded over 3 min (Rick 1984). One millimolar BAPNA was dissolved in 1 ml dimethylsulfoxide (DMSO) and then made up to 100 ml with Tris, pH 7.5, containing 20 m*M* $CaCl_2$. Triplicates of 10 μl were added to 1 ml of substrate solution at 37 °C, and the changes at 410 nm were recorded over 5 min (Erlanger et al. 1961). Chymotrypsin activity was determined using 0.1 m*M* SAPFNA (*N*-succinyl-L-ala-L-ala-L-pro-L-phe-*p*-nitroanilide) in 0.1 *M* Tris, pH 7.8, containing 0.01 *M* $CaCl_2$. Triplicate samples of 10 μl were mixed with 0.69 ml substrate solution, and the absorbance at 410 nm was recorded over 3 min at

Table 1 Substrates and inhibitors used in proteolytic enzyme assays during ontogenetic development of *Farfantepenaeus paulensis*

Enzyme	Substrate	Inhibitor
Total proteinases	Azocasein or casein[a]	
Chymotrypsin	SAPFNA[b] (*N*-succinyl-L-ala-L-ala-L-pro-L-phe-*p*-nitroanilide)	TPCK[e] (tosyl-phenylalanine chloromethyl ketone)
Trypsin	TAME[c] (*N*α-*p*-toluenesulphonyl-L-argenine methyl ester) BAPNA[d] (*N*α-benzoyl-DL-argenine-*p*-nitroanilide)	TLCK[e] (tosyl-lysine chloromethyl ketone)
Serine proteinases		PMSF[e] (phenylmethylsulphonyl fluoride)

[a] Garcia-Carreño et al. (1993)
[b] Del Mar et al. (1979)
[c] Rick (1984)
[d] Erlanger et al. (1961)
[e] Beynon and Salvesen (1990)

25 °C (Del Mar et al. 1979). Each assay included blanks in which the changes in absorbance were recorded without the enzyme extract. Total proteinase, trypsin, and chymotrypsin activities were expressed as the change in absorbance per min per mg protein of the enzyme extract used in the assays (ΔA min^{-1} mg^{-1} protein).

Enzyme characterization

Proteinase activity was studied throughout ontogenetic development after separation of proteins by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). The Electrophoresis was carried out using 12% acrylamide (Laemmli 1970). The 0.1% concentration of SDS used does not affect proteinase activity in PAGE (Garcia-Carreño et al. 1993). Enzyme preparations (0.02 mg protein per lane) of each substage were loaded in a temperature-controlled (4 °C) vertical electrophoresis device (Hoefer, San Francisco, California). Molecular weight standards (0.0175 mg) were included on each plate. Proteinase activity of the extract was characterized according to Garcia-Carreño et al. (1993). After electrophoresis, gels were immersed in 3% casein in 50 m*M* Tris, pH 7.5, for 30 min at 5 °C to allow the substrate to diffuse into the gel at low enzyme activity. The temperature was then raised to 25 °C for 90 min for the digestion of the protein substrate by the active fractions. Gels were next washed in water, and immediately fixed and stained by immersion in a solution containing 40% ethanol, 10% acetic acid, and 0.1% Coomassie brilliant blue R-250. After staining period, of at least 24 h, gels were de-stained with the same solution without the Coomassie dye, and dried using a Bio-Rad 443 slab-gel dryer. Clear zones on a blue background indicated proteinase activities and could be compared to molecular weight standard bands. A regression between the distance of molecular weight standard bands from the top border of the gel (cm) and the log of their molecular mass (kdaltons) determined the molecular weight of active bands.

Enzyme extracts were incubated with proteinase inhibitors (Table 1) to identify possible trypsin and chymotrypsin activities in different life stages (Garcia-Carreño and Haard 1993). Solutions of 20 m*M* TLCK (tosyl-lysine chloromethyl ketone) in 1 m*M* HCl, 200 m*M* PMSF (phenylmethylsulphonyl fluoride) in 2-propanol, and 10 m*M* TPCK (tosylphenylalanine chloromethyl ketone) in MeOH were separately added to enzyme extracts at a ratio of 1:10 (inhibitor:extract) and incubated at 25 °C for 1 h. Distilled water replaced inhibitors in samples for total proteinase separation. Samples were loaded onto the gel as described above. After electrophoresis, gels were separated into molecular weight standard and inhibition lanes. The former lanes were immediately stained. Control and inhibition lanes were immersed in 3% casein following the described procedures. Bands with proteinase inhibitors were compared with control proteinase lanes (without inhibitor) to identify inhibitory effects on active bands. Since TLCK, TPCK, and PMSF are specific trypsin, chymotrypsin and serine proteinase inhibitors, respectively (Table 1), inhibited bands on PAGE could be attributed to the presence of one of these enzymes. The degree of inhibition was not measured in PAGE zymograms, and results were interpreted by the presence or absence of inhibition in the activity bands. Casein was used as substrate for substrate-gel electrophoresis because it is digested by penaeid proteinases such as trypsin and chymotrypsin (Jiang et al. 1991; Le Moullac et al. 1996; Ezquerra et al. 1997). This technique detects only proteinases (or "endopeptidases") by the production of short-chain polypeptides. Peptidases (or "exopeptidases") do not generate clear zones in the blue background and are not detected.

The substrates azocasein, casein, TAME, BAPNA, SAPFNA, the inhibitors TLCK, PMSF, TPCK, molecular weight markers, bovine albumin, and SDS–PAGE reagents were obtained from Sigma Chemical Co.

Statistical analysis

Data were expressed as mean ± standard deviation. Differences among means were analyzed by ANOVA followed by a Tukey multicomparison test. Since trypsin activity data (TAME and BAPNA) deviated from the normal distribution, a Kruskal–Wallis rank test (non-parametric ANOVA) was applied followed by Nemenyi's test for means comparison. A Pearson product-moment analysis of correlation was applied between protein and water contents. Differences are reported as statistically significant when $p < 0.05$ (Zar 1984).

Results

The adult hepatopancreas of *Farfantepenaeus paulensis* weighed 1.43 ± 0.27 g as dry matter. Larval water content increased significantly throughout development, reaching a maximum at PZ II (94.3%, $p < 0.05$) (Table 2). A significant decrease was observed between PZ III and the postlarval substages ($p < 0.05$). Protein content as percent of dry weight also varied significantly during ontogenetic development (Table 2). There was a significant decrease between N III and PZ I ($p < 0.05$). Values were low during the following protozoeal substages and M I. An increase was observed from M II through the subsequent developmental stages ($p < 0.05$). In protozoeal substages and M I, lower

Table 2 *Farfantepenaeus paulensis*. Water and protein content (means ± SD; $n = 3$ to 6) during early life stages at 26 °C ± 1 C° (*N* nauplius; *PZ* protozoea; *M* mysis; *PL* postlarva; *roman numerals* substages; – not determined)

Substage	Age (days after spawn)	Water (%)	Protein (% dry wt)
Egg	0.5	83.1 ± 1.05	49.7 ± 1.12
N III	1.3	87.0 ± 1.03	54.3 ± 6.30
PZ I	2.5	92.3 ± 1.37	33.5 ± 3.30
PZ II	3.5	94.3 ± 2.0	30.7 ± 2.85
PZ III	4.5	92.5 ± 1.79	27.7 ± 7.66
M I	5.5	89.2 ± 2.08	24.9 ± 3.64
M II	6.5	84.5 ± 2.4	38.08 ± 3.92
M III	7.5	86.6 ± 3.64	41.03 ± 3.96
PL I	8.5	87.2 ± 1.27	44.07 ± 2.75
PL II	10	87.2 ± 0.85	37.6 ± 2.32
PL III	11.5	–	–
PL IV	13	–	–
PL V	15.5	86.1 ± 1.31	43.2 ± 1.57
PL VI	17.5	–	–
PL VII	20.5	–	–

protein values coincided with a higher water content. On a percentage basis, protein correlated negatively with water ($r = -0.84$, $p < 0.05$) at early ontogenetic stages (Fig. 1).

Proteinase activity varied significantly during the early life stages of *Farfantepenaeus paulensis* ($p < 0.05$). Total proteinase, chymotrypsin, and trypsin (using TAME) activities showed a similar pattern (Fig. 2a, b, c). Activity was low in the egg and N III, increased in PZ I, and peaked in PZ III. A subsequent decrease was observed between M I and PL I, which remained fairly constant until PL VII. Trypsin activity using the substrate BAPNA increased from the egg to N III, thereafter decreasing until PZ II. Activity almost doubled in PZ III following a decrease over the course of development (Fig. 2d). Total proteinase, trypsin (BAPNA as substrate), and chymotrypsin activities in the adult hepatopancreas were 0.81 ± 0.02, 2.6 ± 0.006, and 7.03 ± 0.01 $\Delta A\ min^{-1}\ mg^{-1}$ protein, respectively.

Proteolytic activity was detected from PZ I onward, and intense activity zones of 16.2, 17.5, and 19.5 kdaltons were observed through the protozoeal and mysis substages (Fig. 3). The intensity of these bands decreased after metamorphosis (PL I to PL VII). In the adult hepatopancreas, 12 active bands, distributed from 14.6 to 42.2 kdaltons, were detected (Fig. 3). Their molecular weight was 14.6, 16.4, 17.5, 19.5, 22.5, 23.9, 25.8, 28.9, 32.0, 34.4, 37.7, and 42.2 kdaltons. In PZ III and MI only, two intense bands of 39.1 and 53.5 kdaltons were found. Active bands heavier than 66 kdaltons appeared in the early stages (PZ I to M III), decreased in number and intensity during postlarval substages, and were absent from the adult hepatopancreas. Bands of 20.6 and 21.7 kdaltons appeared at M III, decreased in intensity in postlarval substages, and were absent from the adult hepatopancreas.

Specific proteinase activities were identified with synthetic inhibitors (Fig. 4). There was no inhibitory effect on activity when the extracts were incubated with TPCK. Active bands inhibited with both TLCK and PMSF were considered to represent trypsin, while the inhibition by only PMSF was evidence of chymotrypsin (Table 3). Several trypsin bands were found in larval and adult stages. Low molecular weight trypsin forms (14.6 to 21.7 kdaltons) varied in number and intensity (Fig. 3) through larval (3 forms), postlarval (4 in PL I and 2 from PL III to PL VII) and adult (4 forms) stages (Table 3). The 39.1 kdalton trypsin was found only in PZ III and M I, while the trypsin of 14.6 kdaltons was found exclusively in the adult hepatopancreas (Table 3, Figs. 3, 4). Mysis III and postlarval substages presented

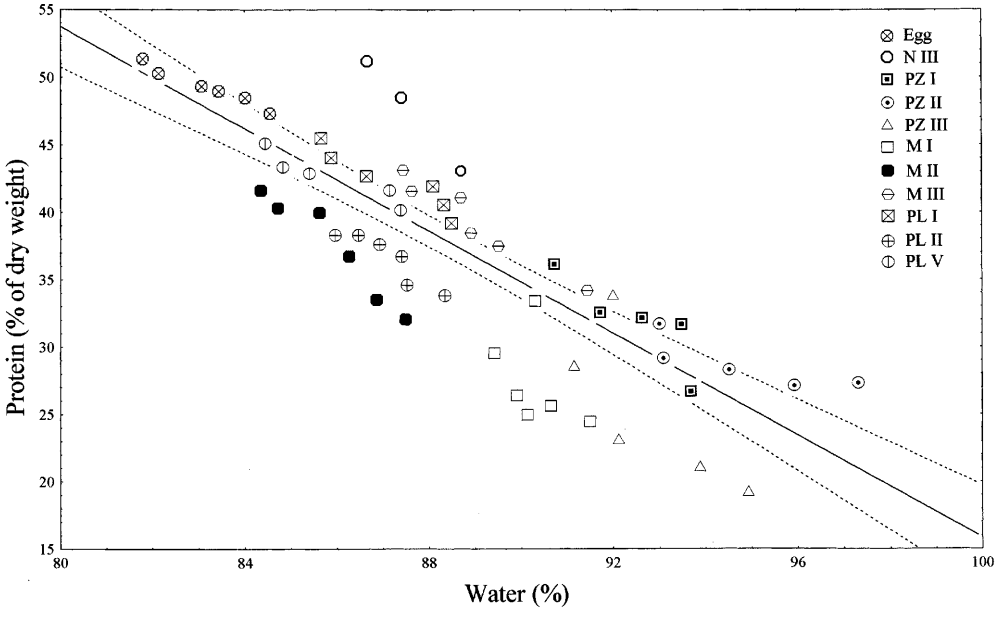

Fig. 1 *Farfantepenaeus paulensis*. Correlation between relative contents of protein and water during larval and postlarval development at 26 °C ± 1 C°. Protein = 205.44 – 1.896 water, $r = -0.84$ ($p < 0.05$) (*N* nauplius; *PZ* protozoea; *M* mysis; *PL* postlarva; *dotted lines* 95% confidence intervals)

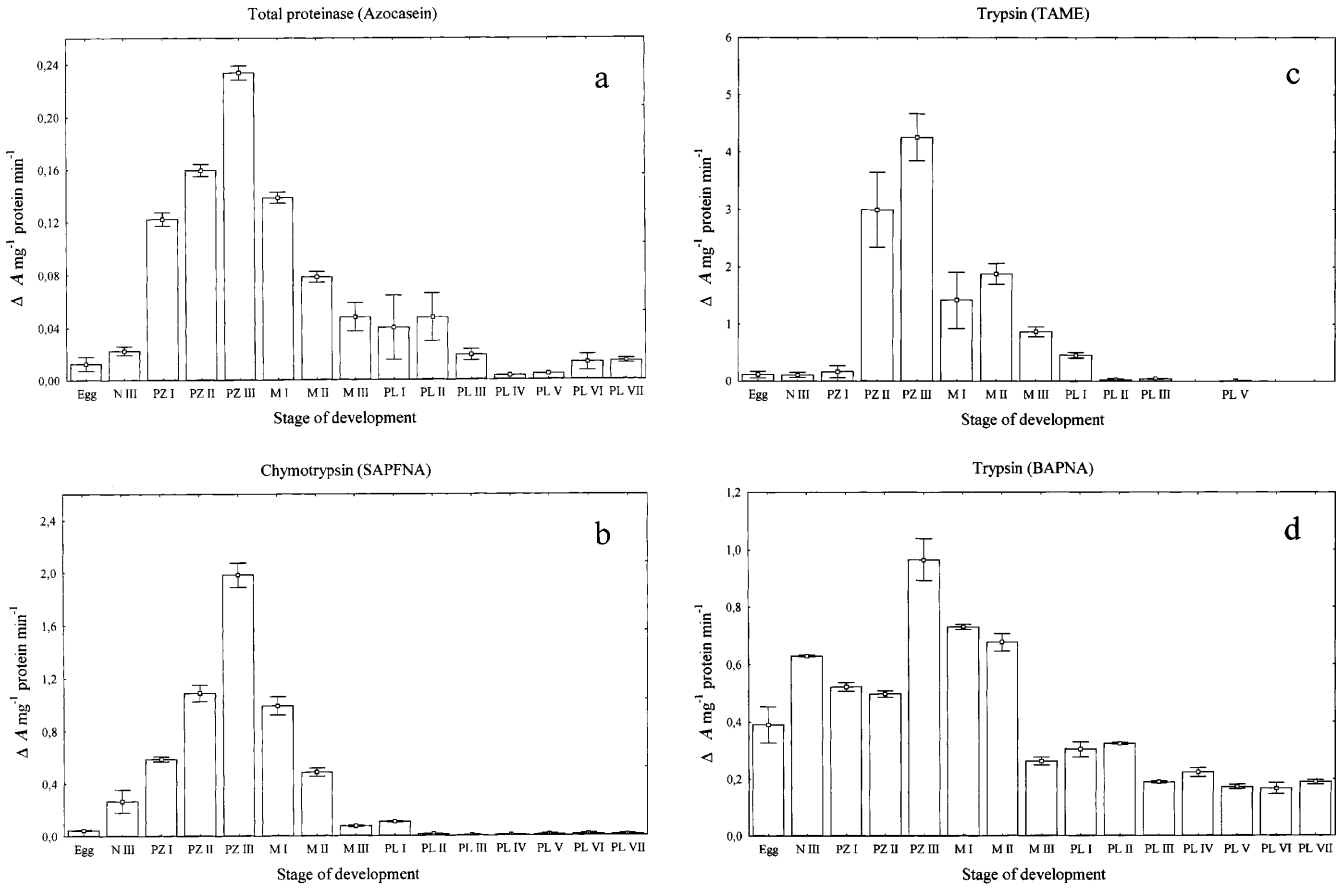

Fig. 2 *Farfantepenaeus paulensis*. Total proteinase (**a**), chymotrypsin (**b**) and trypsin (**c, d**) activity using azocasein, *N*-succinyl-L-ala-L-ala-L-pro-L-phe-*p*-nitroanilide (SAPFNA), *N*α-*p*-toluenesulphonyl-L-arginine methyl ester (TAME) and *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as substrates, during larval and postlarval development at 26 °C ± 1 C°. Sample size (*n*) of means = 3, except in *c*, where *n* = 6 to 20 (*Error bars* standard deviation; *abbreviations* as in key to Fig. 1)

trypsins of 20.6 and 21.7 kdaltons (data not shown). In the adult stage, bands of 28.9, 32.0, and 37.7 kdaltons were inhibited by PMSF, but not by TLCK (Fig. 4). Bands heavier than 45 kdaltons were seen in postlarval substages, but identification was not possible. High molecular-mass active bands (68 to 74 kdaltons) could not be characterized with inhibitors because of their low activity.

Discussion

The protein values of *Farfantepenaeus paulensis* decreased to a minimum in Protozoea Stages I–III and M I, and thereafter gradually increased until postlarva stage; this is similar to observations of the larval development of *Marsupenaeus japonicus* (Laubier-Bonichon et al. 1977) and *Litopenaeus setiferus* (Lovett and Felder 1990a). The reduced protein content in protozoea coincides with a low RNA:DNA ratio, indicating a decreased cellular multiplication rate and consequently less protein synthesis (Laubier-Bonichon et al. 1977). As observed in *Metapenaeus ensis*, the water content of *F. paulensis* was maximum during the larval stages.

Specific proteinase activity was higher in the adult hepatopancreas than in early stages. Nevertheless, if the hepatopancreas occupies 5% of the total body volume, as observed in postlarvae (Lovett and Felder 1990a), then adult whole-body proteinase activity would be much lower than at certain larval stages. The highest activity in early stages occurred at the PZ III–M I interval in *Farfantepenaeus paulensis*, as found for other penaeid species such as *Marsupenaeus japonicus*, *Penaeus monodon*, *Litopenaeus schmitti*, *L. setiferus* and *L. van-*

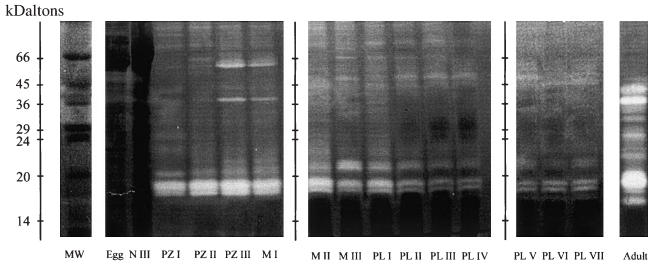

Fig. 3 *Farfantepenaeus paulensis*. Proteinase activity bands in substrate-SDS–PAGE zymograms during ontogenetic development at 26 °C ± 1 C° (*MW* molecular weight; *A* adult; *other abbreviations* as in legend to Fig. 1)

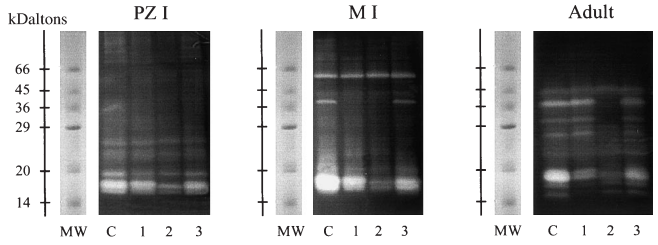

Fig. 4 *Farfantepenaeus paulensis*. Inhibition of proteinase activity in substrate-SDS–PAGE zymograms in Protozoea I (*PZ I*), Mysis I (*M I*) and adult extracts. C, 1, 2, 3 lanes denote activity pattern incubated with different inhibitors [*C* control (without inhibitor); *1* TLCK; *2* PMSF; *3* TPCK; *MW* molecular weight]

namei (Laubier-Bonichon et al. 1977; Lovett and Felder 1990a; Fang and Lee 1992; Le Moullac et al. 1992; González et al. 1994; Rodríguez et al. 1994,). The egg, N III, and postlarval stages are characterized by reduced proteinase activity.

Variations in digestive-enzyme activity may be related to the characteristic life history during the early ontogenetic development of penaeids, which are unique among decapods in displaying so many morphological, metabolic, and behavioral changes during early development (Dall et al. 1990; Icely and Nott 1992; Chu and Ovsianico-Koulikowsky 1994). Anterior and lateral midgut caeca are believed to be the main enzyme- secretion sites in larvae, reaching their maximum volume at PZ III–M I (Lovett and Felder 1989; Abubakr and Jones 1992). A reduction in the size of the midgut caeca begins at the mysid stage and extends through the early postlarval substages (Lovett and Felder 1989; Icely and Nott 1992). Unlike most crustaceans, in penaeids the adult form of the gut is not achieved just until after the metamorphosis (Felder et al. 1985; Lovett and Felder 1990b). A structure similar to adult hepatopancreas derives from the lateral midgut caeca and appears only after 5 wk of postlarval life (Lovett and Felder 1989). Thus, the low enzyme activity of early postlarvae registered in the present study may have resulted from an incomplete digestive system. Reduced metabolic activity in postlarvae (Laubier-Bonichon et al. 1977; Chu and Ovsianico-Koulikowsky 1994) coincides with this low digestive-enzyme activity and may reflect an adaptation to limited food conditions during this transitional period (Lovett and Felder 1990a).

The digestive-enzyme activity of shrimp can be modulated by the quality of their food. High activity levels may maximize the assimilation of scarce compounds such as protein or carbohydrates (Ceccaldi 1986; Rodríguez et al. 1994; Lemos and Rodríguez 1998), or increase the absorption of feeds (Jones et al. 1992; Le Vay et al. 1993; Ezquerra et al. 1997). Under laboratory conditions, penaeid larvae feed on microalgae in the first protozoeal substages, with the onset of omnivory at PZ III (Emmerson 1984; Yúfera et al. 1984; Rodríguez et al. 1994). Higher proteinase activity of the PZ III stage may indicate a physiological response to a different feeding condition (Ceccaldi 1986; Harms et al. 1991). In the present experiment, the diet of larval and postlarval *Farfantepenaeus paulensis* included artificial plankton and microalgae. Thus, the food supplied during development was of the same quality throughout, and therefore the proteinase activity varied independent of diet.

This study examined digestive-enzyme activities at several transitional periods of penaeid development. Nauplii shift from endogenous feeding to herbivorous protozoeae, which become omnivorous at PZ III. A change from a planktonic to a benthic existence takes place during the first postlarval substages. Proteinase activity was correlated with some of these transitional periods in *Farfantepenaeus paulensis*, since increasing activity marked the N III–PZ III interval whereas postlarvae displayed a constant low activity. Behavioral events also accompany the variety of body forms noted through the ontogenetic development of penaeids. As an exception, PZ III and M I are almost morphologically identical (Dall et al. 1990); this was also reflected by their proteinase pattern in SDS–PAGE. The profiles of enzyme activity obtained for *F. paulensis* may reflect different patterns of energy storage and use during early development (Biesiot and Capuzzo 1990). In protozoea, swimming and feeding are virtually continuous (Dall et al. 1990), and the relative ingestion rate increases through each substage, reaching its maximum during larval development in PZ III (Emmerson 1980; Loya-Javellana 1989). The retention time of food in the gut is lower than in mysis and postlarva, whereas feces production is high (Jones et al. 1992). Such strategies indicate an intense food-energy turnover achieved by higher ingestion and digestive-enzyme activity. The lower protein content of protozoeal *F. paulensis* confirms a reduced accumulation of body reserves. On the other hand, the subsequent mysid and postlarval stages exhibit less-active swimming behavior, and filter-feeding efficiency declines at M II, when a shift to raptorial feeding is typical (Emmerson 1980, 1984). In late larval stages, thoracic appendices are now more specialized, enabling a better manipulation and selection of food particles (Jones et al. 1992; Marín-Magán and Cañavate 1995), leading to the ingestion of more digestible parts of food (Cushing and Vucetic 1963; Corner et al. 1972). The retention time of food in the gut is longer (Jones et al. 1992), and the development of the gastric mill may contribute to the processing of food during early postlarval stages (Lovett and Felder 1989). The morphological and behavioral changes in mysis and early postlarvae may compensate for the lower digestive-enzyme activity when the transition to the benthic life begins. The reduced metabolic activity, food uptake, and assimilation capacity of early postlarval stages accompany the search for a suitable new habitat after migration into inshore, brackish, nursery grounds.

Literature detailing the characterization of digestive enzymes during the ontogeny of marine animals is relatively scarce. Vacquier et al. (1971) found differences in

molecular weight of amylase between the larval and adult sand dollar *Dendraster excentricus*, suggesting age-related changes in isozyme production. A study on the larval development of the gilthead sea bream *Sparus aurata* also detected variation in digestive proteinase patterns in SDS–PAGE zymograms (Diaz et al. 1997). Among penaeids, changes in isozyme patterns during larval and postlarval development have been detected (Lester and Cook 1987; Fang and Lee 1992). In the present study, the zymogram of adult *Farfantepenaeus paulensis* showed a higher number of active bands than early stages. This may reflect an increase in the cellular and physiological complexity of the decapod hepatopancreas during development (Morgan et al. 1978; Biesiot and Capuzzo 1990). The absence of active zones in the egg and N III agree with the low activity of total proteinase, trypsin, and chymotrypsin (Fig. 2a, b, c), and may be related to the lack of a functional gut. Higher trypsin activity recorded at these stages using BAPNA (Fig. 2d) may be due to hydrolysis of the substrate by other enzymes such as non-specific esterases (Hess and Pearse 1958; Lovett and Felder 1990a). In PZ III and M I, the presence of 39.1 kdalton (trypsin) and 53.5 kdalton (not identified) bands coincided with the peak of enzyme activity during early development. The occurrence of these bands may be associated with the drastic increase in activity observed between PZ II and PZ III. A reduction in the number and intensity of active bands in the postlarval period also coincided with low enzyme activity. However, as previously stated, the morphometric relation between the volume of digestive structures and the muscle fraction seems to decrease in early postlarvae (Lovett and Felder 1989; Icely and Nott 1992). Since the same amount of protein from developmental stages was used in the analysis, the reduction in bands intensity in postlarvae (Fig. 3) may also be due to a reduced amount of digestive enzymes than in earlier stages.

Most of the digestive enzymes from decapods are serine proteinases (Garcia-Carreño et al. 1994). Trypsin and chymotrypsin belong to this class of enzymes. The inhibitor PMSF was found to be specific for decapod serine proteinases (Kim et al. 1992; Garcia-Carreño et al. 1994), and TLCK inhibited trypsin activity in shrimp extracts (Jiang et al. 1991; Ezquerra et al. 1997). As in other decapod species (Garcia-Carreño et al. 1994; Ezquerra et al. 1997), the proteinases of *Farfantepenaeus paulensis* were insensitive to the mammalian chymotrypsin inhibitor TPCK. This may be the reason for the controversy in the literature regarding its presence in the penaeid digestive system. Although some investigators believed that chymotrypsin was absent from the shrimp digestive tract (Lee et al. 1980; Galgani et al. 1984; Lovett and Felder 1990a), activity was detected in some penaeid species by using SAPFNA as substrate (Tsai et al. 1986; Fang and Lee 1992; Van Wormhoudt et al. 1992; Garcia-Carreño et al. 1994; Le Moullac et al. 1994; Ezquerra et al. 1997; Hernández-Cortés et al. 1997). Recently, the purification and sequence of chymotrypsin from *Litopenaeus vannamei* (Sellos and Van Wormhoudt 1992; Hernández-Cortés et al. 1997) corroborate its existence in the shrimp digestive tract. In the present study, the activity with SAPFNA and the bands inhibited only by PMSF in SDS–PAGE provide additional evidence of the presence of chymotrypsin in adult *F. paulensis*.

Proteinase fractions observed in SDS–PAGE zymograms may appear in different forms during the ontogenetic development of *Farfantepenaeus paulensis* (Table 3). Low molecular mass trypsin (14.6 to 21.7 kdaltons) characterized the majority of ages. Other decapod species such as the crayfish *Astacus fluviatilis* (Titani et al. 1983) and *Procambarus clarkii* (Kim et al. 1992) also exhibited low molecular weight trypsin bands (Table 4). The band of 39.1 kdaltons in PZ III and MI may be a trypsin, although its weight is higher than that of other bands identified in these substages. On the other hand, it was similar to the trypsin found in *F. indicus* (Honjo et al. 1990; present Table 4). Bands of 28.9, 32.0 and 37.7 kdaltons in adults may be chymotrypsin-like (Fig. 4). If so, the number of chymotrypsin forms in adult *F. paulensis* (3) is higher than in *Penaeus monodon*

Table 3 *Farfantepenaeus paulensis*. Schematic representation of proteinase activity bands inhibited by tosyl-lysine chloromethyl ketone (TLCK) and phenylmethylsulphonyl fluoride (PMSF) in SDS–PAGE zymograms during ontogenetic development (*MW* molecular weight; *A* adult. *T*, *P*, *T–P* inhibition by TLCK, PMSF and by both, respectively; * no inhibition detected; *other abbreviations* as in legend to Table 2)

MW (kdaltons)	PZ			M			PL							A
	I	II	III	I	II	III	I	II	III	IV	V	VI	VII	
39.1			T–P	T–P										
37.7														P
32														P
28.9														P
21.7						T–P	T–P	T–P	T–P	T–P	T–P	T–P	T–P	
20.6						T–P	T–P	T–P	T–P	T–P	T–P	T–P	T–P	
19.5	T–P	T–P	T–P	T–P										T–P
17.5	T–P	T–P	T–P	T–P	T–P	T–P	T–P	T–P	*	*	*	*	*	T–P
16.2	T–P	T–P	T–P	T–P	T–P	T–P	T–P	T–P	*	*	*	*	*	T–P
14.6														T–P

Table 4 Characteristics of trypsin and chymotrypsin from adult penaeids and other decapods (*MW* molecular weight in kdaltons; – not determined)

Species	Trypsin form	(MW)	Chymotrypsin form	(MW)	Source
Penaeus monodon	4	(–)	2	(–)	Fang and Lee (1992)
	2	(27, 29)			Lu et al. (1990)
	3	(18.5, 23.3, 50.1)	1	(20.9)	Jiang et al. (1991)
			2	(26, 27)	Tsai et al. (1991)
Litopenaeus vannamei			2	(27)	Van Wormhoudt et al. (1992)
	3	(31–32)			Klein et al. (1996)
			1	(33.2)	Hernández-Cortés et al. (1997)
Fenneropenaeus indicus	7	(36)			Honjo et al. (1990)
Astacus fluviatilis	1	(25.03)			Titani et al. (1983)
Procambarus clarkii	4	(23.8, 24.8, 27.0, 31.4)			Kim et al. (1992)

(2) or *Litopenaeus vannamei* (2), while the more intense band (37.7 kdaltons) is heavier than reported for other crustaceans (Table 4).

Several forms of serine proteinases have been found in fish such as salmon (Male et al. 1995) and cod (Gudmundsdottir et al. 1996). Trypsins from salmon may present either cationic or anionic forms. The methionine content of cod elastase is similar to that recorded earlier for enzymes of psychrophilic fish enzymes (Genicot et al. 1996). In penaeids, trypsin and chymotrypsin forms have not been characterized in terms of differences in their catalytic or molecular characteristics. Moreover, the ontogenetic pattern of *Farfantepenaeus paulensis* confirms that some forms can appear in certain stages but not in others, since activity may differ between stages (Fang and Lee 1992).

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