



ELSEVIER

Comparative Biochemistry and Physiology Part B 135 (2003) 373–383

CBP

www.elsevier.com/locate/cbpb

Effects of dietary protein on the activity and mRNA level of trypsin in the midgut gland of the white shrimp *Penaeus vannamei*

Adriana Muhlia-Almazán^a, Fernando L. García-Carreño^{a,*}, J. Arturo Sánchez-Paz^a,
Gloria Yepiz-Plascencia^b, Alma B. Peregrino-Uriarte^b

^aLaboratorio de Bioquímica, Centro de Investigaciones Biológicas del Noroeste (CIBNOR), A.P. 128, La Paz,
Baja California Sur 23000, Mexico

^bCentro de Investigación en Alimentación y Desarrollo (CIAD), A.P. 1735, Hermosillo, Sonora 83000, Mexico

Received 4 July 2002; received in revised form 20 March 2003; accepted 21 March 2003

Abstract

Protein food modulates the activity of proteases of the midgut gland of *Penaeus vannamei*. Shrimp fed with food containing 15, 30 and 50% protein exhibited differences in trypsin and chymotrypsin activity and trypsin mRNA levels. Shrimp fed with 30% protein showed higher trypsin and chymotrypsin activities than those fed 15 or 50% protein. An additional paralogous trypsin was observed with electrophoretic analysis in shrimp fed 30% protein. Shrimp fed 30% protein showed the highest trypsin to mRNA concentration, suggesting that trypsin genes expression is regulated transcriptionally.

© 2003 Elsevier Science Inc. All rights reserved.

Keywords: Chymotrypsin; Midgut gland; mRNA; Protein; Proteolytic enzymes; Regulation; Shrimp; Trypsin

1. Introduction

Researchers have demonstrated that food quality influences adaptation of digestive enzyme activities (Noriega et al., 1994; Ezquerro et al., 1997). Understanding the mode of regulation of digestive enzymes is important for rational use of type and amounts of feed ingredients used for cultured marine organisms. In decapods, proteolytic enzymes synthesized in the midgut gland (hepatopancreas) play a key role in assimilation of food protein. Trypsin and chymotrypsin are the most abundant proteolytic enzymes in the midgut gland of decapods (Lemos et al., 2000) and are responsible for 60% of protein digestion in the midgut

gland of Penaeids (Galgani et al., 1984). From previous studies in penaeids, activity of the digestive enzymes changes according to size and stage of growth (Lee et al., 1984; Rodríguez et al., 1994; Lemos et al., 1999), temperature (Galgani, 1985), molting stage (Al-Mohanna and Nott, 1989) and circadian rhythm (Hernandez et al., 1999). Activity of digestive proteolytic enzymes is affected by alimentary stress, including low quality food or food containing anti-nutritional factors. This process has been called 'enzymatic adaptation' (García-Carreño and Hernández, 1996). Trypsin is transcriptionally modulated by food protein content in rats (Lhoste et al., 1994) and transcriptionally induced by blood meal in the mosquito *Aedes aegypti* (Noriega et al., 1994). Proteolytic changes occur in fish (Zambonino-Infante and Cahu, 1994), compensating protease inhibitors by secret-

*Corresponding author. Tel.: +52-612-123-8484; fax: +52-612-125-3625/4710.

E-mail address: fgarcia@cibnor.mx (F.L. García-Carreño).

ing more proteases (Olli et al., 1994) and responding to dietary protein and carbohydrates by modulating enzymes and mRNA levels (Peres et al., 1998). In shrimp, feeding habits as well as protein source affect the synthesis of proteolytic enzymes (Le Moullac and van Wormhoudt, 1994).

This work studied the effect of different protein concentration in the feed on the trypsin and chymotrypsin synthesized by the midgut gland of the shrimp *Penaeus vannamei*, and tested if changes in these activities correlate with trypsin mRNA levels, as part of our general interest in synthesis of digestive enzymes in marine organisms. We use the term ‘orthologues’ to name homologous molecules of other species, in contrast to ‘paralogues’ to name homologous molecules from the same species, according to the nomenclature proposed by Southan (2000).

2. Materials and methods

2.1. Animals and feeds

Juvenile *P. vannamei* shrimp ($N=230$), weighing 8.0 ± 1.0 g, were obtained from intertidal ponds at CIBNOR. Three groups of 25 shrimp (three replicates each) were randomly chosen. During the 15-day acclimatization period, shrimp were kept in nine 1000-l plastic tanks under controlled conditions (salinity 34 ppt, dissolved oxygen 7.3 mg/l, 28 °C). Shrimp were fed commercial feed (Silvercup™ 35% protein, El Pedregal, Mexico, under license of Sterling H. Nelson & Sons, Inc).

Three isocaloric feeds were prepared containing 15, 30 and 50% protein. Feeds ingredients were evaluated for their content of protein, carbohydrates, lipids, ash, wet, crude fiber and energy, feeds were designed and formulated using the Mixit-[win] computer software (Agricultural Software Consultants, Inc., USA).

After acclimatization, the shrimp were fed with one of the experimental feeds, twice a day ad libitum for 3 weeks. Daily water exchange was 70%. Once shrimp were acclimatized to a food, they were weighed and selected at inter-molt stage, by setogenesis (Chan et al., 1988). Eight shrimp from each experimental tank were collected, decapitated and the midgut gland extracted and frozen at -80 °C, a total of 72 shrimp. Five were used for proteolytic activity evaluation and electro-

phoretic analysis, and three to evaluate relative concentrations of trypsin mRNA. All determinations were performed in triplicate and with appropriate standards and controls. Differences among treatments were analyzed by one-way and two-way ANOVA, followed by a multiple comparison test. Differences are reported as statistically significant when $P < 0.05$ (Zar, 1984).

2.2. Enzyme activity evaluation

Dissected midgut glands were homogenized individually with distilled water (1:4 w/v). Homogenates were centrifuged for 30 min at $10\,000 \times g$ at 4 °C, and the supernatant, containing soluble proteins (enzyme extract), was stored at -20 °C. Total soluble protein content of each enzyme extract was determined as described by Bradford (1976), using bovine serum albumin as protein standard. Total proteinase activity was measured by hydrolysis of 1% azocasein in 50 mM Tris–HCl buffer, pH 7.5, 25 °C. The reaction was stopped by the addition of trichloroacetic acid (TCA) to a final concentration of 10%. Unhydrolyzed casein was precipitated. The amount of hydrolyzed casein was measured at 366 nm by spectrophotometry. Trypsin activity was measured with *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as substrate. BAPNA (1 mM) was dissolved in 1 ml dimethylsulfoxide and diluted to 100 ml with Tris–HCl buffer, pH 7.5, containing 20 mM CaCl₂. Triplicates of each midgut gland extract (5 μl) were added to 750-μl substrate solution at 37 °C, and the change of absorbance at 410 nm was recorded for 10 min. Chymotrypsin activity was measured using *N*-succinyl-ala-ala-pro-phe-*p*-nitroanilide (SAPNA) in 0.1 M Tris–HCl buffer, pH 7.5 and 20 mM CaCl₂. Midgut gland extracts (5 μl) were mixed with 750-μl substrate solution and the absorbance at 410 nm was recorded for 3 min, as described by García-Carreño et al. (1994). Each assay included blanks and commercial enzymes (1 mg/ml) as internal controls.

Total proteinase, trypsin and chymotrypsin activity units were expressed as the change in absorbance per minute per milligram of protein of the enzymes used in the assays ($\Delta\text{abs}/(\text{min mg protein})$) (Fernandez-Gimenez et al., 2001; Lemos et al., 2000). Each enzyme extract was analyzed by 12% SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis; Laemmli, 1970). Individual protein extracts (20 μg) were loaded

per lane and the gels run at 15 mA per gel at 4 °C. The gels were stained with 0.1% Coomassie Brilliant Blue R-250. After 24 h of staining, gels were destained with the same solution without Coomassie dye (Laemmli, 1970).

Enzyme activity was individually evaluated by 12% substrate-PAGE, using aliquots containing 7 mU of total proteinase activity for each sample. After electrophoresis, the gels were immersed in 3% casein in 50 mM Tris–HCl buffer, pH 7.5, for 30 min at 5 °C to allow casein to diffuse into gel, at reduced enzyme activity. Afterwards, temperature was raised to 25 °C for 90 min and the gels were rinsed with distilled water and Coomassie blue stain. All protein and activity bands corresponding to trypsin and chymotrypsin enzymes, were individually compared between shrimp subjected to different treatments.

To determine the class and specificity of the enzyme, extracts were incubated with specific protease inhibitors (García-Carreño et al., 1993). Phenylmethylsulfonyl fluoride (PMSF) was used as inhibitor of serine proteinases and *N*α-*p*-tosyl-lysine chloro-methylketone (TLCK) as specific trypsin inhibitor. Solutions containing 20-mM TLCK in 1-mM HCl, pH 3 and 200-mM PMSF in 2-propanol were separately mixed with enzyme extracts in a ratio of 1:10 v/v (inhibitor/extract) and incubated at 25 °C for 1 h. Distilled water was used instead of inhibitors in controls. The inhibitor–enzyme mixture was diluted with loading buffer and loaded into 12% acrylamide gels.

2.3. Trypsin mRNA concentration (RT-PCR)

Total RNA was extracted from the midgut gland of each shrimp using a TRIzol[®] reagent (GIBCO-BRL, New York) and analyzed on a 1.2% agarose–formaldehyde gel (Sambrook et al., 1989). Intact total RNA was used for reverse transcription; cDNA was synthesized from each individual sample using the Superscript[™] first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). Reverse transcription was performed using 5-μg total RNA, 1-μl (10 mM) dNTP mix, 1-μl (0.50 μg/μl) oligo (dT)_{12–18} and DEPC water adjusted to 10 μl. The reaction mixture was incubated for 10 min at 70 °C and then placed on ice for 2 min. Then, 2-μl (10X) RT buffer, 4-μl (25 mM) MgCl₂, 2-μl (0.1 M) DTT, 1-μl RNase inhibitor and 1-μl (50 units) Superscript II[™] RT were

added to a final reaction volume of 20 μl and incubated for 50 min at 42 °C.

Trypsin primers for PCR amplification were based on three trypsin genes reported for *P. vannamei* (Klein et al., 1996). Primer sequences were TryEx3Fw:5'-TCCTCTCCAAGATCATCCAA-3' and TryRev:5'-ATTGGCCTTAATCCA-ATCGAC-3' that match positions 854–873 and 1306–1326, respectively. Two primers were designed for the ribosomal protein L21 mRNA for *P. vannamei* (GenBank accession number BE188654). Primer sequences were L21Fw:5'-GGGCTTCTTTCCCGTTTCAGCG-3' and L21 Rev:5'-CCGGTCTGAACTCAGATCACGT-3', corresponding to positions 2–23.

PCR amplifications were performed in a 40-μl final reaction mixture containing 20.4-μl H₂O, 4-μl (10X) PCR buffer, 6-μl (25 mM) MgCl₂, 1.6-μl (2.5 mM) dNTP mix, 0.8 μl (20 mM) of each primer, 4 μl of the obtained cDNA of each sample and 2.4 U of Taq DNA polymerase. A thermocycler (PTC 200 DNA Engine, MJ Research) was used with the following program: 3 min at 94 °C, 1 min at 55 °C, 3 min at 72 °C (1 cycle); 1 min at 94 °C, 1 min at 60 °C and 3 min at 72 °C (34 cycles); and an over-extension step for 10 min at 72 °C. The resulting PCR products for trypsin and L21 were analyzed in a single 2.0% agarose gel and stained with ethidium bromide (Sambrook et al., 1989).

Gel image was obtained and analyzed using DIGITAL SCIENCE 1D software (Kodak, Rochester, NY) and the intensity of the bands evaluated by densitometry and compared to a known concentrations standard DNA marker.

2.4. Calculation of relative mRNA concentrations of trypsin and L21

Aliquots of 2- and 4-μl commercial standard DNA markers, with known DNA concentrations per band, were loaded onto the 2% agarose gel. Net intensity data (pixels) from standard bands were obtained by image analysis, and a linear equation was obtained to calculate DNA concentrations. Net intensity (pixels) of each experimental sample was also obtained by image analysis, and relative concentration of trypsin and L21 PCR products (ng) were calculated from net intensity data. The ribosomal protein L21 mRNA abundance determined as the RT-PCR product concentration, was used to normalize against trypsin mRNA

Table 1
Shrimp feed composition

Protein content (%)	Wet (%)	Protein ^a (%)	Lipids (%)	Ash (%)	Crude fiber (%)	N.F.E. ^b	Energy (cal/g)
15	14.36±0.21	15.24±0.25	3.06±0.08	5.82±0.09	2.77±0.07	73.11	4562±13.0
30	12.75±0.05	31.82±0.02	4.49±0.07	6.41±0.15	5.79±0.09	51.49	4461±8.79
50	17.19±0.08	49.85±0.26	5.65±0.21	10.65±0.21	6.26±0.24	27.59	4602±24.3

^a Fish meal was used as the main protein source in feeds formulation which contained 70.3% of protein.

^b Nitrogen-free extracts were assumed as carbohydrates.

levels. The number of PCR cycles was kept constant and chosen to obtain comparable PCR product amounts of the different amplified gene fragments (Dimopoulos et al., 1998). Thirty cycles were used to reach an end point reaction (Yu and Kanost, 2000).

Statistical analysis was made by two-way ANOVA and differences between treatments were evaluated by Duncan's comparison test ($P < 0.05$) (Zar, 1984).

3. Results

3.1. Feed, shrimp and midgut gland weight

Composition of feed is shown in Table 1. At the end of the experiment, shrimp body weight varied between 8.32 and 9.36 g. Weight was higher in shrimp fed 50% protein feed, and the differences were significant only between groups fed 15 and 50% protein (Table 2). In contrast, midgut gland weight was not significantly different among groups fed with the three experimental treatments (Table 2). Shrimp fed 15 and 30% protein had midgut gland weight 10.1 and 12.6% lower than those fed 50% protein.

3.2. Enzyme activity

Soluble protein content of the midgut gland showed a significant increase as feed protein con-

centration increased (Table 2). Protein content of the midgut gland from shrimp fed 50% protein showed 54% more soluble protein than those fed 15% protein. Total proteolytic activity in the three groups was not significantly different, but a higher activity value (U/(mg protein)) was observed in shrimp fed 30% protein food (Table 2).

Trypsin-specific activity (U/(mg protein)) was significantly different between the 30 and 50% treatments. Shrimp fed 30% protein had 30% higher trypsin activity in the midgut gland than those animals fed 50% protein (Fig. 1). Also, chymotrypsin activity was higher in shrimp fed 30% protein (Fig. 2). A difference of 35% was observed in chymotrypsin activity (U/(mg protein)) between shrimp fed 30 and 50% protein.

Electrophoretic analyses of midgut gland extracts showed differences in the number of paralogous trypsins with feed protein concentration. Activity bands inhibited by TLCK (specific inhibitor of trypsin) and PMSF were considered trypsins, and bands inhibited only by PMSF are evidence of chymotrypsin activity (Fig. 4; Lemos et al., 2000).

Protein and activity bands of each shrimp were compared and common patterns were observed. Shrimp fed 30% protein consistently showed three paralogous trypsins (19.4, 20.7 and 22.0 kDa). Shrimp fed 15 or 50% protein showed two paralogous trypsins (20.7 and 22.0 kDa) (Fig. 3). Two

Table 2
Total body weight, midgut gland weight, protein content and proteolytic activity of the midgut gland of white shrimp *P. vannamei*

Protein content (%)	Body weight (g)	Midgut weight (g)	Midgut gland protein (mg/ml)	Proteolytic activity (U/(mg protein))
15	8.32±1.2 <i>a</i>	0.305±0.07 <i>a</i>	4.99±1.4 <i>a</i>	0.80±0.16 <i>a</i>
30	8.51±1.1 <i>ab</i>	0.297±0.07 <i>a</i>	7.14±1.3 <i>b</i>	0.83±0.15 <i>a</i>
50	9.36±1.1 <i>b</i>	0.340±0.04 <i>a</i>	11.01±1.5 <i>c</i>	0.61±0.10 <i>a</i>

Different italic letters show statistical differences between treatments.

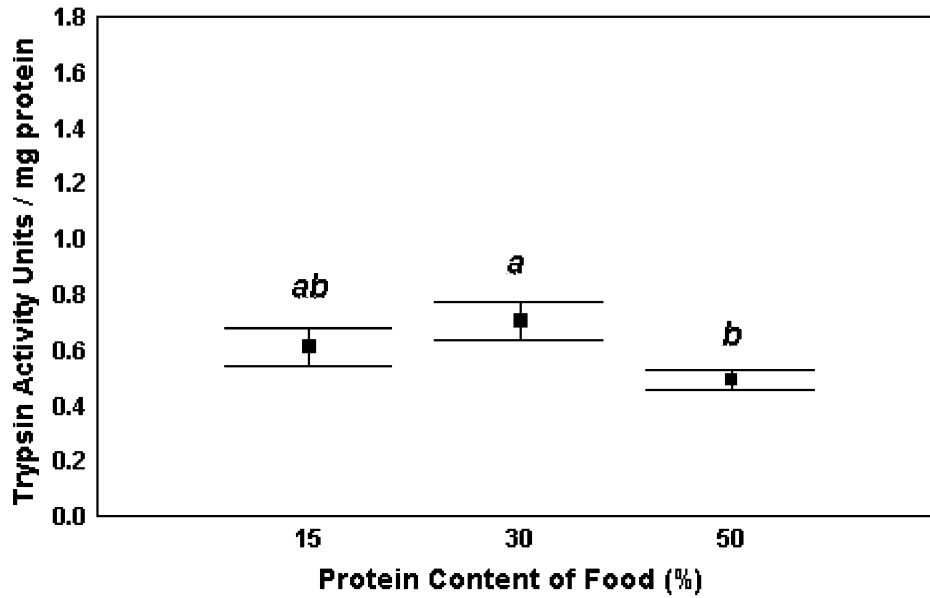


Fig. 1. Trypsin activity (U/(mg protein)) in the midgut gland of white shrimp *P. vannamei* fed different protein concentrations. Different italic letters indicate statistical differences between groups ($P < 0.05$). Each value represents mean \pm S.E. ($n = 15$).

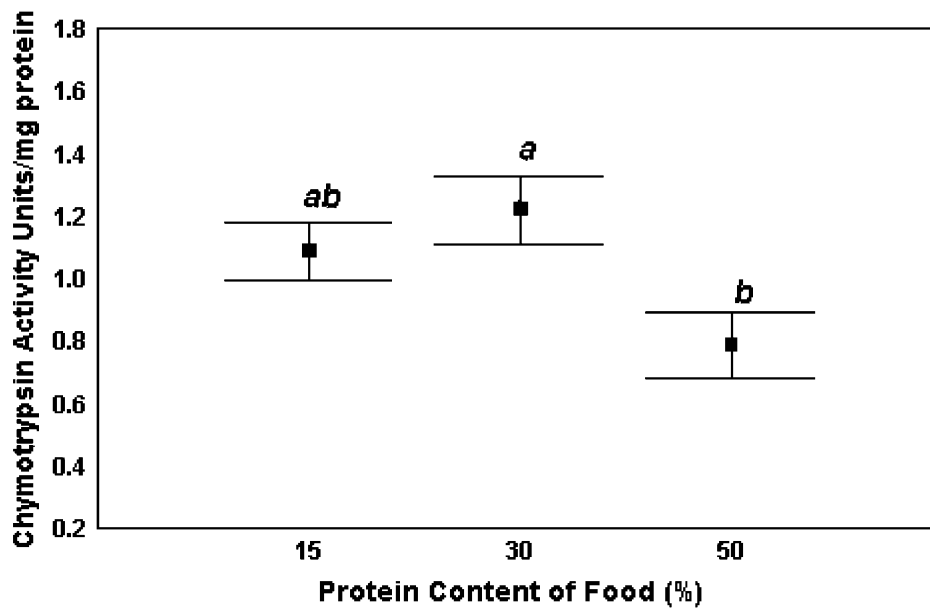


Fig. 2. Chymotrypsin activity (U/mg protein) of the midgut gland of white shrimp *P. vannamei* fed different protein concentrations. Different italic letters indicate statistical differences between groups ($P < 0.05$). Each value represents mean \pm S.E. ($n = 15$).

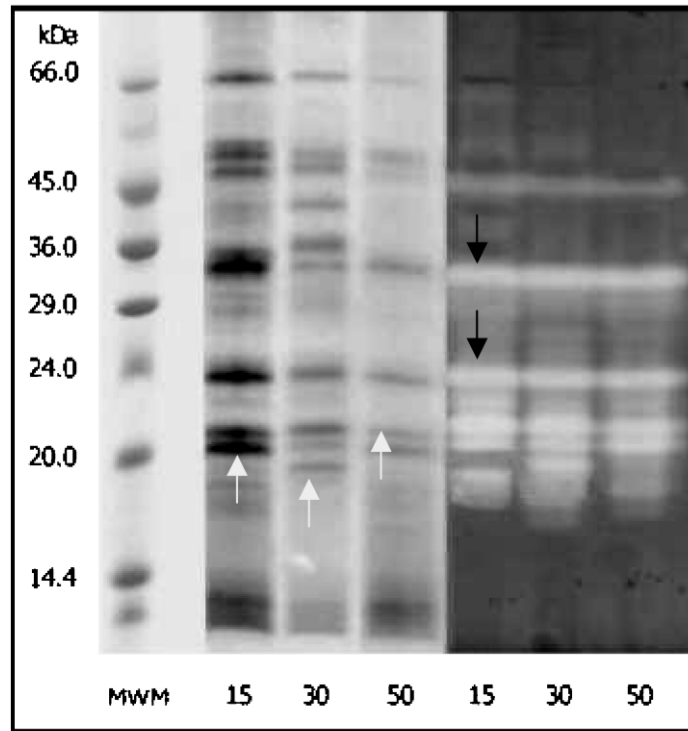


Fig. 3. SDS-PAGE. Protein and substrate gels of midgut gland pooled enzyme extracts of *P. vannamei*. MWM: molecular weight markers. Lane 1: protein bands of shrimp fed 15% protein. Lane 2: protein bands of shrimp fed 30% protein. Lane 3: protein bands of shrimp fed 50% protein. Lane 4: activity bands of shrimp fed 15% protein. Lane 5: activity bands of shrimp fed 30% protein. Lane 6: activity bands of shrimp fed 50% protein. White arrows show trypsin bands; black arrows show chymotrypsin bands.

chymotrypsin bands were observed with molecular weight approximately 24 and 30 kDa (Fig. 3). Chymotrypsin paralogues in the experimental groups remained unchanged, regardless of treatment.

3.3. Relative mRNA concentrations of trypsin and L21

Amplification of internal fragments of trypsin and L21 genes by RT-PCR produced the expected sizes (410 and 517 bp, respectively; Fig. 5). Restriction digest and nucleotide sequencing confirmed their identity (data not shown).

To obtain an estimate of the effect of variable feed protein content on the relative mRNA levels of trypsin in the midgut gland, concentrations of the fragments produced by RT-PCR for trypsin and the ribosomal protein L21 (used as a constitutive gene) were evaluated. Concentrations of trypsin/L21 RT-PCR products were calculated to normalize for the effect of general transcription, which resulted in different values: 0.57, 0.95 and

0.91 for the 15, 30 and 50% protein feeds, respectively (Fig. 6b, relative scale). These values indicate a higher relative mRNA concentration of trypsin in the shrimp fed 30 and 50% protein feed and were 40 and 37% higher, respectively, than values observed for shrimp fed 15% protein (Fig. 6b).

An index based on trypsin activity/(trypsin/L21 relative mRNA concentration) was calculated to obtain understanding of the relationship between the observed changes in both variables. The index calculated for the three protein levels of feed were 0.78, 0.89 and 0.51 for the 15, 30 and 50% protein treatments, respectively (Fig. 7). Higher values were observed in the shrimp fed 30% protein, while a decrease in the index occurred in the 50% group, as previously seen for trypsin activity.

4. Discussion

Crustaceans fed high concentrations of protein tend to grow and survive better. Presumably, they take advantage of the protein content in food and

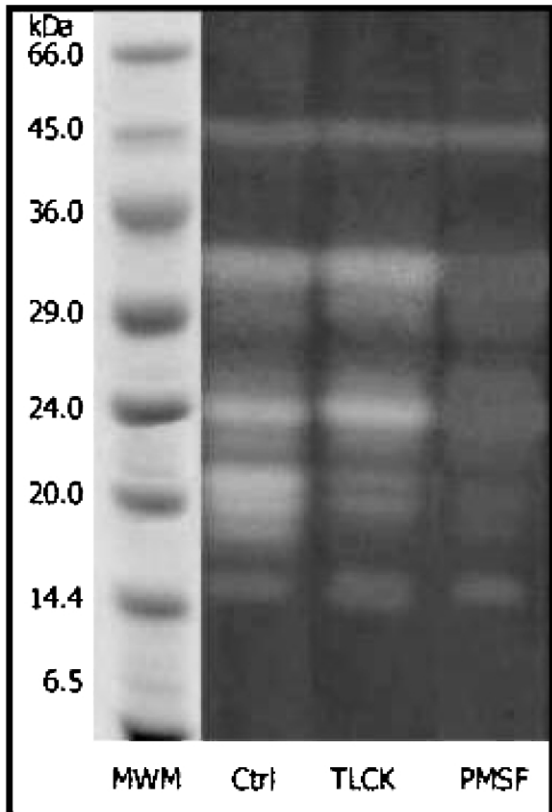


Fig. 4. SDS-PAGE of enzyme extracts of the midgut gland of white shrimp *P. vannamei*. Lane 1: control sample of pooled midgut gland extract from shrimp fed the 30% protein diet. Lane 2: TLCK-incubated enzyme extract. Lane 3: PMSF-incubated extract.

acquire more building blocks for tissue construction and energy reserves for metabolic functions (Koshio et al., 1993; Le Moullac and van Wormhoudt, 1994). A significantly higher body weight was observed after 3 weeks in shrimp fed 30 and 50% protein than those fed 15% protein, which agrees with the assumption that crustaceans fed high protein grow faster.

Midgut gland weight showed no statistical differences among treatments and no correlation with changes in body weight. Some reports indicate that midgut gland weight is influenced by physiological processes, such as molting cycle and starvation (Al-Mohanna and Nott, 1989; Fernandez-Gimenez et al., 2001). Differences in concentration of feed ingredients besides protein (Table 1), like nitrogen-free extracts (mostly carbohydrates), affect biochemical processes that

were not evaluated in this study, hence, midgut gland weight response remains to be addressed.

Total soluble protein concentration (mg/ml) in the midgut gland increased significantly as feed protein increased, which correlate positively with the changes we observed in total body weight. Le Moullac and van Wormhoudt (1994) reported similar results for *P. vannamei* larvae, suggesting that, when more protein is available above a threshold, the organ is triggered to build reserves to face molting, growth and reproduction processes.

We observed that enzymatic activity results showed highest values when shrimp were fed 30% protein, and the lowest values when shrimp were fed 50% protein. Similar results occurred in shrimps fed casein and this agrees with Le Moullac et al. (1996). However, there were no statistical differences in total proteolytic activity between the three experimental groups. Total proteolytic activity results from all active proteases in the midgut gland of shrimp, including trypsin, chymotrypsin, carboxy- and aminopeptidases, cathepsin and collagenases.

We assume that, although trypsin and chymotrypsin are the main proteases in the midgut gland, activity of other active proteases precludes detection of significant changes since the feed protein concentration as detected for specific trypsin and chymotrypsin activities.

The electrophoretic analyses of enzyme extracts showed an additional third protein band with trypsin activity (Fig. 3) in shrimp fed 30% protein. We suggest that, even when shrimp grew faster on 50%-protein feed, synthesis of the three active trypsin enzymes in the midgut gland is induced

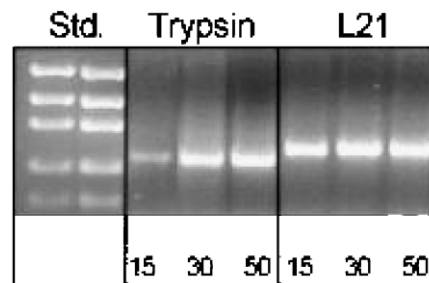


Fig. 5. Trypsin and L21 RT-PCR products from the midgut gland of white shrimp *P. vannamei*. Lanes 1–2: commercial DNA molecular markers. Lanes 3–5: trypsin PCR products of different protein concentration treatments. Lanes 6–8: L21 PCR product of different protein concentration treatments.

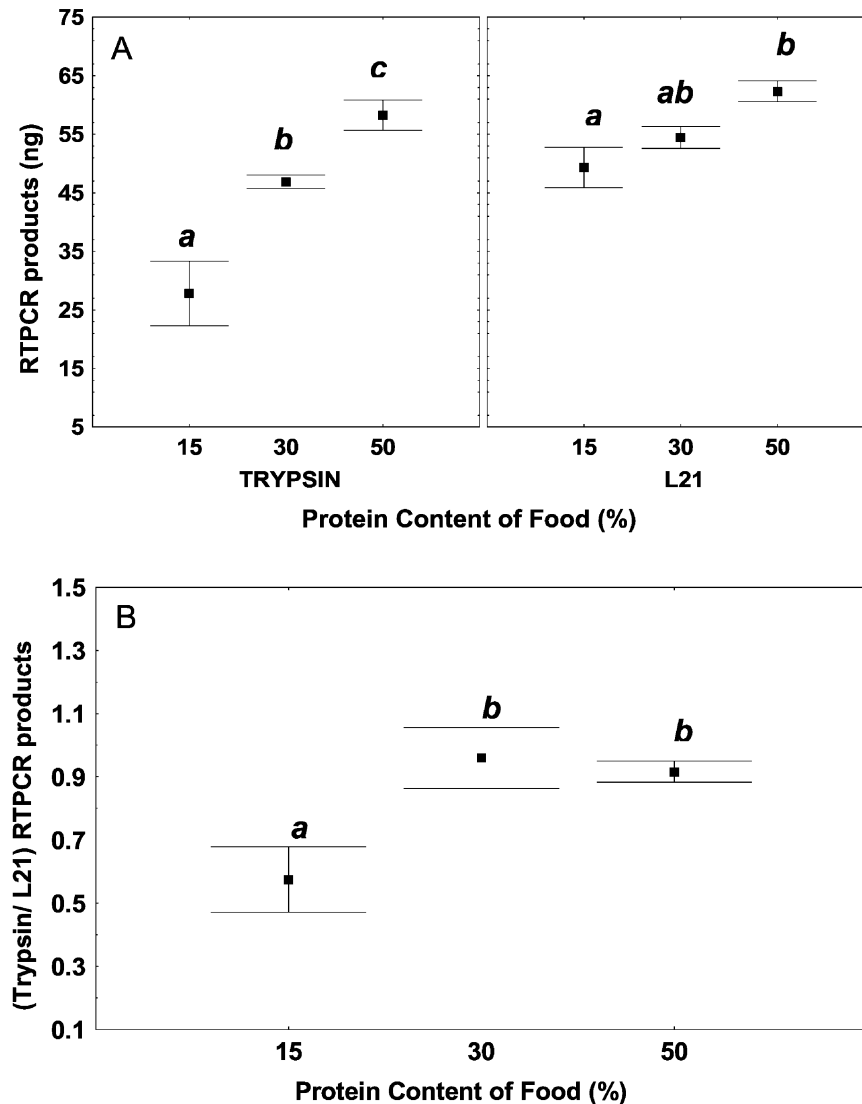


Fig. 6. (a) Trypsin and L21 relative mRNA concentrations of trypsin and L21 in the midgut gland of white shrimp *P. vannamei*, using RT-PCR. (b) Normalized data of relative mRNA concentrations of trypsin/L21. Different italic letters indicate statistical differences between groups ($P < 0.05$). Each value represents mean \pm S.E. ($n = 9$).

only when shrimp were fed 30% protein to enable the shrimp to hydrolyze more protein. Therefore, this third paralogue trypsin seems to be induced once a threshold is reached, and is switched off far beyond this optimal protein concentration. It remains to be investigated whether this paralogue trypsin is responsible for the greater activity observed in the experiments as well as the mechanism regulating its synthesis.

Our study confirmed the correlation between trypsin and chymotrypsin activities found by Muhlia-Almazán and García-Carreño (2002)

(Figs. 1 and 2). This agrees with generally observed correlations in some insect species. Trypsin and chymotrypsin activation studies suggest that both enzymes are components of a cascade, and their activity is modified after feeding, as observed in *Anopheles gambiae* (Muller et al., 1995; Vizioli et al., 2001).

We observed a general increase of relative mRNA concentrations of trypsin and L21, as protein in food increased probably indicating a general positive effect on transcription. When the relative mRNA concentrations of trypsin are nor-

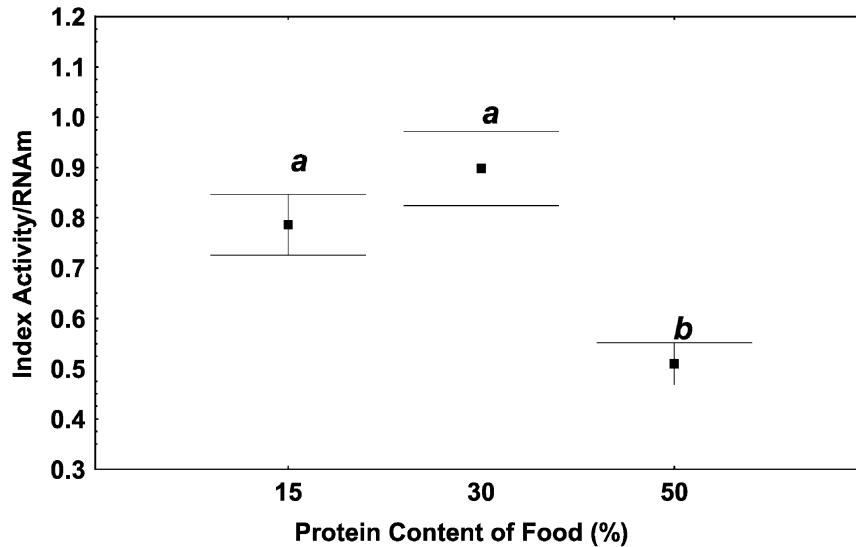


Fig. 7. Trypsin activity/trypsin/L21 relative mRNA concentration index. Different italic letters indicate statistical differences between groups ($P < 0.05$). Each value represents mean \pm S.E. ($n = 9$).

malized against a constitutively expressed gene, as a ribosomal protein, an increase to 50% protein in food resulted in a decrease of the trypsin/L21 mRNAs concentration in shrimp, almost to the same level as the 30% group, while the highest value was observed in the midgut gland of shrimp fed 30% protein (Fig. 6b). Changes in the relative mRNA concentrations of trypsin agree with the results of Klein et al. (1996), those authors suggested that trypsin genes of the midgut gland of *P. vannamei* are regulated at transcriptional level.

Correlation between trypsin activity/mRNA concentration and protein in food (Fig. 7), suggested that there is at least partially, a transcription regulation of the gene, and that once an adequate number of synthesized transcripts have been produced, the transcription process declines, regardless of the increase in protein content of food, with a concomitant decrease in enzyme activity. In shrimp, trypsin synthesis appears to be proportional to the protein concentration in food up to a certain level, this contrasts with the induction of trypsin transcription by blood meal in the mosquito *A. aegypti* (Noriega et al., 1994), increasing the concentration of trypsin mRNA.

The correlation between trypsin activity, mRNA concentration in the three protein feed groups, and the additional trypsin band observed in the 30% protein feed group supports the hypothesis that changes in trypsin relative mRNA concentrations

are determinant for trypsin activity. However, we cannot rule out other contributing factors in trypsin regulation, as previously suggested by Noriega et al. (1994). Positive correlations between trypsin activity and trypsin mRNA (evaluated by the dot-blot method) was also reported by van Wormhoudt et al. (1996), but no difference in the number of expressed isoforms was observed by these authors.

The use of housekeeping genes as internal controls relies on the premise that they exhibit a constant basal transcription rate in all nucleated cell types and are independent of other physiological influences (Thellin et al., 1999). Ribosomal proteins are commonly used as standards because of the low variation in their mRNA levels. Several studies of insects have reported using successfully these proteins as standards (Salazar et al., 1993; Dimopoulos et al., 1998; Barillas-Mury et al., 1999). For crustaceans, in addition to cell-cycle changes, high variability of nutritional conditions and molting processes has to be considered because these parameters are difficult to control between organisms (Sánchez-Paz, 2001).

Regulatory mechanisms related to changes in trypsin mRNA concentration and trypsin activity suggest a close relationship to the feeding habits of the organism. Many studies on vertebrates and invertebrates demonstrated a significant correlation between feeding habits and the magnitude by which digestive processes are regulated (Secor,

2001). Blood-feeding insects, such as the mosquito *A. aegypti* (Noriega and Wells, 1999), which is an infrequent feeder, shows a significantly up-and-down regulatory response of digestive processes. This is even more pronounced by the amount of blood or prey taken up, which may surpass the feeder's weight and has to be digested after a single ingestion. Shrimp, which are frequent-feeding organisms, show a modest regulatory response. This suggests that activation or inactivation of trypsin genes in shrimp suppose a benefit, probably related to a low, average cost of energy expenditure, as proposed by Secor (2001).

Acknowledgments

Funding was provided by Consejo Nacional de Ciencia y Tecnología (CONACYT grant 28257B) to FLGC and a scholarship to AMA. At CIBNOR, we thank M. Angeles Navarrete for technical assistance, Norma Hernandez S., Ricardo Vazquez J., and Arturo Sierra for critical review of the data, and the editing staff improving the English text.

References

- Al-Mohanna, S.Y., Nott, J.A., 1989. Functional cytology of the hepatopancreas of *Penaeus semisulcatus* (crustacea: decapoda) during the molting cycle. *Mar. Biol.* 101, 535–544.
- Barillas-Mury, C., Han, Y.S., Seeley, D., Kafatos, F.C., 1999. *Anopheles gambiae* Ag-STAT, a new insect member of the STAT family, is activated in response to bacterial infection. *EMBO J.* 18 (4), 959–967.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Chan, S.M., Rankin, S.M., Keeley, L.L., 1988. Characterization of the molt stages in *Penaeus vannamei*: setogenesis and haemolymph levels of total protein, ecdysteroids, and glucose. *Biol. Bull.* 175, 185–192.
- Dimopoulos, G., Seeley, D., Wolf, A., Kafatos, F.C., 1998. Malaria infection of the mosquito *Anopheles gambiae* activates immune-responsive genes during critical transition stages of the parasite life cycle. *EMBO J.* 17 (21), 6115–6123.
- Ezquerria, J.M., García-Carreño, F.L., Haard, N.F., 1997. Effects of feed diets on digestive proteases from the hepatopancreas of white shrimp *Penaeus vannamei*. *J. Food Biochem.* 21, 401–419.
- Fernandez-Gimenez, A.V., Garcia-Carreño, F.L., Navarrete del Toro, M.A., Fenucci, J.L., 2001. Digestive proteinases of red shrimp *Pleoticus muelleri* (decapoda, penaeoidea): partial characterization and relationship with molting. *Comp. Biochem. Physiol., Part B* 130, 331–338.
- Galgani, M.L., Benyamin, Y., Ceccaldi, H.J., 1984. Identification of digestive proteinases of *Penaeus kerathurus* (For-
skal): a comparison with *Penaeus japonicus*. *Comp. Biochem. Physiol., Part B* 78, 355–361.
- Galgani, F.G., 1985. Regulation de l'activité des proteases digestives de *Penaeus japonicus* Bate en relation avec la temperature. *J. Exp. Mar. Biol. Ecol.* 94, 11–18.
- García-Carreño, F.L., Dimes, L., Haard, N.F., 1993. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Anal. Biochem.* 214, 65–69.
- García-Carreño, F.L., Hernández, C.P., Haard, N., 1994. Enzymes with peptidase and proteinase activity from the digestive systems of a freshwater and a marine decapod. *J. Agric. Food Chem.* 42, 1456–1461.
- García-Carreño, F.L., Hernández, C.P., 1996. Enzimas del sistema digestivo del camarón I. Estado del arte y tendencias de investigación en digestión de proteínas. In: Calderón, J.V., Magallón, F., Andratta, E., Sánchez, R. (Eds.), Taller de Trabajo: La investigación científica en Peneidos de Iberoamérica. Ecuador, pp. 1–88.
- Hernandez, P., Quadros, W., Navarrete, A., Portillo, G., Colado, G., Garcia-Carreño, F.L., 1999. Rate of ingestion and proteolytic activity in digestive system during continuous feeding of juvenile shrimps. *J. Appl. Aquacult.* 9, 35–45.
- Klein, B., Le Moullac, G., Sellos, D., van Wormhoudt, A., 1996. Molecular cloning and sequencing of trypsin cDNAs from *Penaeus vannamei* (crustacea, decapoda): used in assessing gene expression during the moult cycle. *Int. J. Biochem. Cell Biol.* 28 (5), 551–563.
- Koshio, S., Teshima, S., Kanazawa, A., Watase, T., 1993. The effect of dietary protein content on growth, digestion efficiency and nitrogen excretion of juvenile kuruma prawns, *Penaeus japonicus*. *Aquaculture* 113, 101–114.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lee, P.G., Smith, L., Lawrence, A.L., 1984. Digestive protease of *Penaeus vannamei* Boone: relationship between enzyme activity, size and diet. *Aquaculture* 42, 225–239.
- Lemos, D., Hernández-Cortés, M.P., Navarrete, A., García-Carreño, F.L., Phan, V.N., 1999. Ontogenetic variation in digestive proteinase activity of larvae and postlarvae of the pink shrimp *Farfantepenaeus paulensis* (crustacea: decapoda: penaeidae). *Mar. Biol.* 135, 653–662.
- Lemos, D., Ezquerria, J.M., Garcia-Carreño, F.L., 2000. Protein digestion in penaeid shrimp: digestive proteinases, proteinase inhibitors and feeding digestibility. *Aquaculture* 186, 89–105.
- Le Moullac, G., van Wormhoudt, A., 1994. Adaptation of digestive enzymes to dietary protein, carbohydrate and fibre levels and influence of protein and carbohydrate quality in *Penaeus vannamei* larvae (crustacea, decapoda). *Aquat. Living Resour.* 7, 203–210.
- Le Moullac, G., Klein, B., Sellos, D., van Wormhoudt, A., 1996. Adaptation of trypsin, chymotrypsin and α -amylase to casein level and protein source in *Penaeus vannamei* (crustacea, decapoda). *J. Exp. Mar. Biol. Ecol.* 208, 107–125.
- Lhoste, E.F., Fislewicz, M., Gueugneau, A.M., Corring, T., 1994. Adaptation of exocrine pancreas to dietary proteins: effect of the nature of protein and rat strain on enzyme activities and messenger RNA levels. *J. Nutr. Biochem.* 5, 84–93.

- Muhlia-Almazán, A., García-Carreño, F.L., 2002. Influence of molting and starvation on the synthesis of proteolytic enzymes in the midgut gland of the white shrimp *Penaeus vannamei*. *Comp. Biochem. Physiol., Part B* 133 (3), 383–394.
- Muller, H.-M., Catteruccia, F., Vizioli, J., della Torre, A., Crisanti, A., 1995. Constitutive and blood-meal induced trypsin genes in *Anopheles gambiae*. *Exp. Parasitol.* 81, 371–385.
- Noriega, F.G., Barillas-Mury, C., Wells, M.A., 1994. Dietary control of late trypsin gene transcription in *Aedes aegypti*. *Insect Biochem. Mol.* 24 (6), 627–631.
- Noriega, F.G., Wells, M.A., 1999. A molecular view of trypsin synthesis in the midgut of *Aedes aegypti*. *J. Insect Physiol.* 45, 613–620.
- Olli, J.J., Hjelmeland, K., Kroghdahl, A., 1994. Soybean trypsin inhibitors in diets for Atlantic salmon (*Salmo salar*, L): effects on nutrient digestibilities and trypsin in pyloric caeca homogenate and intestinal content. *Comp. Biochem. Physiol., Part A* 109 (4), 923–928.
- Peres, A., Zambonino-Infante, J.L., Cahu, C., 1998. Dietary regulation of activities and mRNA levels of trypsin and amylase in sea bass (*Dicentrarchus labrax*) larvae. *Fish Physiol. Biochem.* 19, 145–152.
- Rodríguez, A., Le Vay, L., Mourente, G., Jones, D.A., 1994. Biochemical composition and digestive enzyme activity in larvae and postlarvae of *Penaeus japonicus* during herbivorous and carnivorous feeding. *Mar. Biol.* 118, 45–51.
- Salazar, C.E., Mills-Hamm, D., Kumar, V., Collins, F.H., 1993. Sequence of a cDNA from the mosquito *Anopheles gambiae* encoding a homologue of human ribosomal protein S7. *Nucleic Acids Res.* 21, 41–47.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York, USA.
- Sánchez-Paz, J.A., 2001. Regulación adaptativa del mRNA de tripsina del hepatopáncreas del camarón blanco (*Penaeus vannamei*) bajo condiciones de stress alimenticio. Master Thesis. Centro de Investigaciones Biológicas del Noroeste, S.C. La Paz, B.C.S., Mexico, p. 62.
- Secor, S.M., 2001. Regulation of digestive performance: a proposed adaptative response. *Comp. Biochem. Physiol., Part A* 128, 565–577.
- Southan, C., 2000. Assessing the protease and protease inhibitor content of the human genome. *J. Peptide Sci.* 6, 453–458.
- Thellin, O., Zorzi, W., Lakaye, B., et al., 1999. Housekeeping genes as internal standards: use and limits. *J. Biotechnol.* 75 (2–3), 291–295.
- van Wormhoudt, A., Le Moullac, G., Klein, B., Sellos, D., 1996. Caracterización de las tripsinas y amilasas de *Penaeus vannamei* (crustacea decapoda: adaptación a la composición del régimen alimenticio). In: Cruz-Suarez, E., Rique, M.D., Mendoza, A.R. (Eds.), *Avances en Nutrición Acuicóla III. Tercer Simposium Internacional de Nutrición Acuicóla*. Univ. Auton. Nuevo Leon, Mexico, 11–13 November.
- Vizioli, J., Catteruccia, F., della Torre, A., Reckmann, I., Muller, H.-M., 2001. Blood ingestion in the malaria mosquito *Anopheles gambiae*. Molecular cloning and biochemical characterization of two inducible chymotrypsins. *Eur. J. Biochem.* 268, 4027–4035.
- Yu, X.-Q., Kanost, M.R., 2000. Immulectin-2, a lipopolysaccharide-specific lectin from an insect, *Manduca sexta*, is induced in response to gram-negative bacteria. *J. Biol. Chem.* 275 (48), 37373–37381.
- Zambonino-Infante, J.L., Cahu, C., 1994. Development and response to a diet change of some digestive enzymes in sea bass. *Fish Physiol. Biochem.* 12, 399–408.
- Zar, J.H., 1984. *Biostatistical Analysis*. second ed. Prentice-Hall, Englewood Cliffs, NJ.