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Comparative Biochemistry and Physiology Part B 133 (2002) 383–394

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Influence of molting and starvation on the synthesis of proteolytic enzymes in the midgut gland of the white shrimp *Penaeus vannamei*

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Received 3 April 2002; received in revised form 26 July 2002; accepted 23 August 2002

Abstract

We investigated the effect of starvation as a stimulant of the digestive system on digestive proteinase activities in the white shrimp *Penaeus vannamei*. The starved organisms were sampled periodically according to the molting stage and compared with a continuously fed group. Molting stage was included as an independent variable. Most analyzed variables, except for trypsin, were more affected by starvation than by molting, indicating that starvation is a stimulant that masks the effect of molting and showing that food or alimentary stress is more conspicuous than physiological ones. We found that starvation is a stimulant that surpasses the effect of molting, and because it affects the activity of digestive proteinases, studies of starving organisms in combination with tools of molecular biology, can be a helpful working model in the understanding of mechanisms of regulation of digestive enzyme activity. In the starved organisms, trypsin and chymotrypsin activities were similar, suggesting dependence of one to the other. Changes in proteolytic activities and the number of protein bands in electrophoresis showed evidence of synthesis regulation in the midgut gland of white shrimp.
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Keywords: Chymotrypsin; Midgut gland; Molting; Proteolytic enzymes; Starvation; Trypsin

1. Introduction

Digestion is a complex physiological process that depends on molecular activation, recognition and hydrolysis of food at specific times and places. Reports on regulation of invertebrate trypsin and chymotrypsin activities are abundant, especially those related to insects (Noriega and Wells, 1999; Zhu and Baker, 1999). However, in crustaceans only some mechanisms of enzyme synthesis have been proposed. In penaeids, it has been observed that enzymatic adaptation depends on factors such as: the morphological changes related to ontogeny (Lovett and Felder, 1989; Lemos et al., 1999); metabolic rates (Rosas et al., 1995); circadian rhythms (Hernández-Cortés et al., 1999); physiological processes, such as molting and digestion (Emmerson, 1980; Dall et al., 1990; Lemos et al., 1999); and feeding habits and behavior (Lemos et al., 1999). Trypsin and chymotrypsin are the most abundant proteolytic enzymes in the midgut gland of shrimp (Dall et al., 1990), and are responsible for more than 60% of total protein digestion in the midgut gland of penaeids (Galgani et al., 1984; Galgani, 1985; Tsai et al., 1986). Thus, the synthesis and regulation of these enzymes is important for understanding the biology of the organism and for improving feeding methods for aquafarming.

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PII: S1096-4959(02)00163-X

Studies on the white shrimp *Penaeus vannamei* have shown variations in digestive enzyme activity according to factors such organism size, ingested protein quantity and temperature (Galgani, 1985; Le Moullac and van Wormhoudt, 1994). Such flexibility allows the organism to grow and survive (Al-Mohanna and Nott, 1989; Rodríguez et al., 1994). Some regulatory mechanisms of enzyme synthesis have been suggested (Giorgi et al., 1985; Corring et al., 1989; Le Huerou-Luron et al., 1993; Lhoste et al., 1994; Le Moullac and van Wormhoudt, 1994; Rodríguez et al., 1994; Péres et al., 1998). Enzymatic adaptation is the process of adjusting digestive enzyme activity when organisms are exposed to food stress such inadequate food, or food containing anti-nutritional factors (García-Carreño and Hernández, 1996).

There are several reports about changes of activity of proteolytic enzymes in crustaceans and its association with molt, gender, age and environmental parameters. However, none of them studied the combined effect of the permanently molting and starvation as food stressor. We are interested in a working model to study mechanisms of regulation of the digestive enzyme activities, mostly affecting the gene expression. Because in some other invertebrate model like mosquito *Aedes aegypti* food stress has being helpful to understand the mechanisms of regulation of the trypsin gene expression, we would like to know if the effect of starvation and molting could be segregated in shrimp. In this study, we evaluated the effects of starvation and molting as stimulants of the digestive system to get some clues about possible mechanisms of synthesis regulation of trypsin and chymotrypsin activity in the midgut gland of white shrimp *Penaeus vannamei.*

2. Materials and methods

2.1. Starvation and molting stage

This assay was designed to evaluate the proteolytic activity in the midgut gland at different molting stages in combination with different starvation times. Shrimp (600) (7.5 ± 1.0 g) of *Penaeus vannamei* from CIBNOR facilities were maintained under controlled laboratory conditions (28 °C, 34 ppt salinity, and 7.3 mg/l dissolved oxygen). During the 15-day acclimatization period, shrimp were kept in 12 plastic tanks of 1000-l capacity each, containing 50 shrimp. Daily exchange of marine water was 70% of the total volume. Organisms were fed twice, 08.30 and 16.00h with a commercial feed for shrimp, containing 35% protein. Feces and uneaten feed were discarded before the next feeding. At the end of the acclimation, two main groups were kept apart; one, the control group, was kept feeding as previously and the second was starved. Both groups were sampled at 2, 24, 72 and 120 h, in triplicate. Five organisms of each replicate were selected by each of the following four molting stages by setogenesis (Chan et al., 1988): A/B = postmolt; C = intermolt; ED = early premolt; and LD = late premolt. Each specimen was individually weighed and decapitated. The midgut gland was dissected and processed in the laboratory as described below. The hepatosomatic index was calculated with the formula: (%) = (midgut gland weight $\times$ 100)/total body weight) and values were transformed (arc $\sin^2$) for statistical analysis (Jussila, 1999).

2.2. Short-term starvation

A second assay was designed to evaluate midgut gland proteolytic activities during a 48-h period after ingestion. Three hundred shrimp were selected by size (8.0 ± 1.0 g) at intermolt stage. During the 15 days of acclimatization, organisms were maintained as described above, and placed in six plastic 1000-l tanks (50 shrimp per tank). Organisms were fed once a day with commercial food (35% protein). Illumination was 12 h a day, turning lights off at 18.00 h. Once acclimatized (19.00 h), two groups were keep apart. One was fed as previously and the second starved. Organisms of both groups were sampled at different time. After dissection, midgut glands were weighed and frozen in liquid nitrogen. Then the enzymatic extract was obtained from frozen midgut glands and the enzymatic analysis was performed. Samples were analyzed individually.

2.3. Enzyme activity evaluation

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, the enzyme extract, was stored at -20 °C until used. Total soluble protein content of each enzyme extract was evaluated using the method described by Bradford (1976). Bovine serum albumin was used as the protein standard.

Total proteinase activity of the extracts was measured by the degree of hydrolysis of 1% azocasein as the substrate in 50 mM Tris–HCl buffer, pH 7.5 at 25 °C. The reaction was stopped and the unhydrolyzed casein precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10%. The amount of hydrolyzed casein was measured at 366 nm by spectrophotometry (García-Carreño, 1992). Trypsin and chymotrypsin activities were evaluated by the rate of hydrolysis of synthetic substrates. Trypsin activity was measured using Nα-Benzoyl-dl-Arginine-*p*-nitroanilide (BAPNA) as substrate. BAPNA (1 mM) was dissolved in 1 ml of dimethylsulfoxide (DMSO) and made to 100 ml with Tris–HCl buffer, pH 7.5, containing 20 mM $CaCl_2$. Triplicates of each midgut gland extract (5 μl) were added to 750 μl of substrate solution at 37 °C, and the change of absorbance at 410 nm were recorded during 10 min. Chymotrypsin activity was measured using *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAPNA) as substrate in 0.1 M Tris–HCl buffer, pH 7.5 and 20 mM $CaCl_2$. Triplicates of midgut gland extracts (5 μl) were mixed with 750 μl of substrate solution and the absorbance at 410 nm was recorded during 3 min, according to the method described by García-Carreño et al. (1994). Each assay included blanks and commercial enzymes (1 mg ml^{-1}) as internal controls.

Total proteinase, trypsin and chymotrypsin activity units were expressed as the change in absorbance min^{-1} mg^{-1} of protein of the enzymes used in the assays (ΔAbs min^{-1} mg^{-1} $protein^{-1}$). A calculation was made of the number of activity units in the whole midgut gland according to the 1:4 v/v diluted extract obtained from each shrimp.

Composition of proteins in the enzyme extracts was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gels according to Laemmli (1970). Enzyme preparations, 0.02 mg of protein, of each individual extract (one volume of sample with one volume of loading buffer) were loaded per lane, in a temperature controlled (4 °C) electrophoresis device. Molecular weight standards (4 μl) were included in each plate. Electrophoresis was done at a constant current of 15 mA per gel. After electrophoresis, gels were stained for protein with a solution containing 40% ethanol, 10% acetic acid and 0.1% Coomassie brilliant blue R-250. After 24 h of staining, gels were distained with the same solution without the Coomassie dye.

In order to evaluate enzymes activity by substrate-PAGE (García-Carreño et al., 1993), aliquots containing 7 mU of activity (not boiled with the sample buffer), were loaded in 12% polyacrylamide gels. After separation of proteins, gels were immersed in 3% casein in 50 mM Tris–HCl buffer, pH 7.5, for 30 min at 5 °C, to allow the substrate to diffuse into gel at low enzyme activity. Afterwards, temperature was raised to 25 °C for 90 min. Then gels were washed with distilled water and stained as above. Protein and activity bands corresponding to trypsin and chymotrypsin enzymes were individually compared between specimens exposed to different treatments.

To determine the class and specificity of the enzyme, enzyme extracts were incubated with different specific protease inhibitors (García-Carreño and Haard, 1993). Phenylmethylsulfonyl fluoride (PMSF) was used as inhibitor of proteinases belonging to the serine class. Na-*p*-Tosyl-Lysine chloro-methyl ketone (TLCK) was used as specific inhibitor of trypsin. Solutions of 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 (inhibitor/extract) and incubated for 1 h at 25 °C. Distilled water was used instead of inhibitors in controls. Then, the inhibitor-enzyme mixture was diluted with sample buffer and loaded into 12% acrylamide gels.

Normal distribution and homogeneity of variances were analyzed; also no differences between replicas were evaluated for both assays. In the first assay statistical differences among treatments were analyzed by two-way analysis of variance to test the effects of both factors, molting and starvation. Effects were evaluated separately and then the interaction of both was analyzed. Statistical analysis of the second assay was done using a one-way analysis of variance. Tukey multiple comparison test was used to define differences among treatments (Statistica V. 6.0). Differences are reported as statistically significant when $P < 0.05$ (Zar, 1984).

3. Results

3.1. Starvation and molting stage

The body and midgut gland weight; hepatosomatic index; soluble protein in the midgut gland extract; and total proteinolytic activity were not significantly different ($P > 0.05$) in the control of

Table 1
Effect of starvation and molting stage on body and midgut gland weight of *Penaeus vannamei*

Starvation	Body weight (g)			Midgut gland weight (g)		
time	Molting stages			Molting stages		
(h)	A/B	C	ED	A/B	C	ED
2	8.2±0.8 *1a*	8.2±0.8 *1a*	7.8±0.8 *1a*	0.32±0.07 *1a*	0.32±0.04 *1a*	0.32±0.04 *1a*
24	7.3±0.8 *1ab*	7.1±0.8 *1ab*	7.1±0.8 *1a*	0.24±0.03 *1b*	0.26±0.04 *1ab*	0.27±0.03 *1a*
72	7.2±0.9 *1ab*	6.8±0.8 *1b*	6.9±0.7 *1a*	0.22±0.05 1*bc*	0.22±0.04 *1b*	0.21±0.05 *1b*
120	6.5±0.8 *1b*	7.1±0.8 *1ab*	7.0±0.8 *1a*	0.18±0.03 *1c*	0.20±0.06 *1b*	0.18±0.04 *1b*

Each value represents mean±*s* of triplicate determinations. Different italic letters show statistical differences by starvation between groups of the same molting stage ($P<0.05$). Different italic numbers show statistical differences by molting stage between groups of the same starvation time ($P<0.05$).

continuously fed organisms ($N=5$), or in the starved group ($N=5$). Molting did not affect these variables in both control and starved groups. Soluble protein in the midgut gland was not significantly different in both groups. The continuously fed group had 15 mg/midgut gland and the starved group 16 mg/midgut gland. If total proteinolytic, trypsin and chymotrypsin activities were not significantly different in the continuously fed and in the starved groups along the study, they were different between groups. Total proteinolytic activity was 30 U/midgut gland, while the variable was 14 U/midgut gland in the starved group. Trypsin activity was 23 and 13 U/midgut gland in the fed and starved organisms. Chymotrypsin was 60 and 35 U/midgut gland in the fed and starved groups.

Starvation affected the body weight significantly at postmolt (A/B) and intermolt (C) stages ($P<0.05$; Table 1). Midgut gland weight was not affected significantly by molting stage ($P>0.05$). However, as starvation progressed, midgut gland weight had a steep decrease in postmolt (A/B), intermolt (C) and early premolt (ED) stages after 120 h of starvation ($P<0.05$; Table 1). The hepatosomatic index, which is a body and midgut gland ratio index (Jussila, 1999), mirrored the results of body and midgut gland weight. No effect of molting stage was observed ($P>0.05$), but a significant decrease was observed in starved organisms at A/B, C and ED molting stages, as starvation time increased ($P<0.05$; Fig. 1). Soluble protein data showed a wide range of deviation especially at intermolt stage as showed in Table 2.

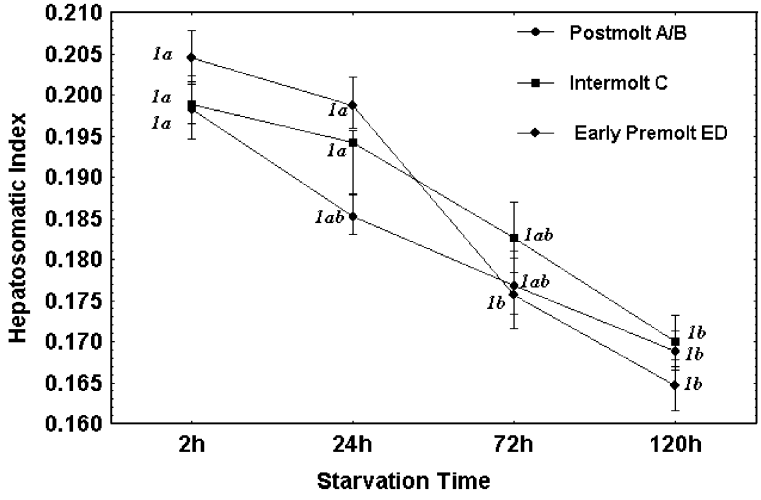

Fig. 1. The effect of starvation and molting stage on hepatosomatic index of *Penaeus vannamei*. *Note*: different italic letters show statistical differences by starvation time between groups of the same molting stage ($P<0.05$). Different italic numbers show statistical differences by molting stage between groups of the same starvation time ($P<0.05$).

Table 2
Effect of starvation and molting stage on protein content of the midgut gland of *Penaeus vannamei*[a]

Starvation time (h)	Molting stage A/B	C	ED
2	8.93±2.5 *1a*	11.45±5.7 *1a*	10.39±1.2 *1a*
24	7.19±1.5 *1a*	9.45±3.2 *1ab*	9.54±2.5 *1ab*
72	7.47±1.4 *1a*	7.18±1.8 *1b*	7.76±2.4 *1ab*
120	7.68±3.5 *1a*	7.46±3.9 *1b*	6.77±1.6 *1b*

Different italic letters show statistical differences by starvation time between groups of the same molting stage ($P<0.05$). Different italic numbers show statistical differences by molting stage between groups of the same starvation time ($P<0.05$).

[a] Protein (mg/midgut gland). Each value represents mean±*s* of triplicate determinations.

Table 3
Effect of starvation and molting stage on total proteolytic activity of the midgut gland of *Penaeus vannamei*[a]

Starvation time (h)	Molting stage A/B	C	ED
2	11.48±5.2 *1a*	10.70±3.1 *1a*	13.03±5.0 *1a*
24	8.47±2.7 *1a*	11.97±3.0 *1a*	9.38±5.3 *1ab*
72	8.28±3.6 *1a*	9.00±2.5 *1a*	8.75±3.9 *1ab*
120	7.62±2.1 *1a*	10.65±5.3 *1a*	7.28±1.2 *1b*

Different italic letters show statistical differences by starvation time between groups of the same molting stage ($P<0.05$). Different italic numbers show statistical differences by molting stage between groups of the same starvation time ($P<0.05$).

[a] (Activity units/midgut gland). Each value represents mean±*s* of triplicate determinations.

Statistical analysis showed that protein content of the midgut gland did not show differences by molting stage ($P>0.05$), but a significant difference was observed for starvation ($P<0.05$). A decrease in protein content was observed in the midgut gland with the progress of starvation at the intermolt (C) and early premolt (ED) stages ($P<0.05$; Table 2). No interaction was observed of both effects ($P>0.05$).

No differences in total proteolytic activity of the midgut gland were observed between molting stages ($P>0.05$). However, a main effect with a significant decrease of total proteolytic activity was observed in the midgut gland with the progress of starvation for the early premolt (ED) stage ($P<0.05$; Table 3). No significant interaction of molting and starvation was found in proteolytic activity ($P>0.05$).

A significant interaction of main effects, molting stage and starvation was observed for trypsin activity ($P<0.05$; Fig. 2). Trypsin activity at early premolt (ED) and intermolt (C) was higher than postmolt (A/B) during the first 24 h of starvation and there were no differences after this period. At 2 h of starvation, a difference of 34% was observed between early premolt (ED) and intermolt (C) with postmolt (A/B). At 24 h of starvation, the difference increased to 54% between early premolt (ED) and postmolt (A/B) stages ($P<0.05$). A significant decrease in trypsin activity was

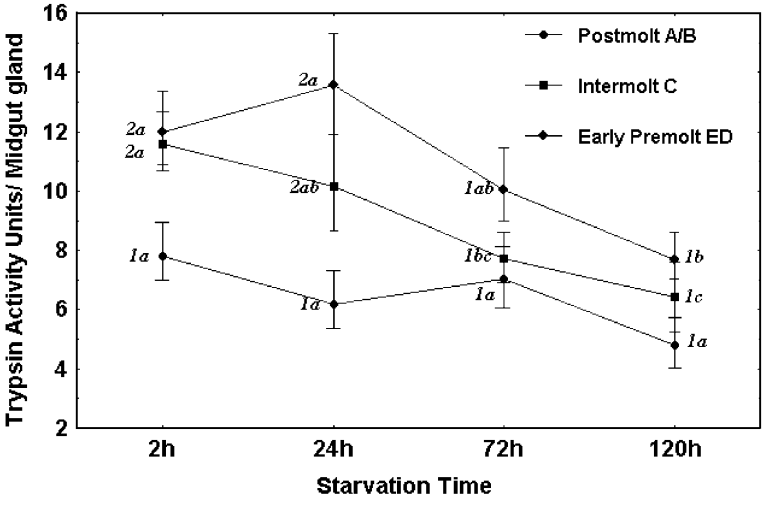

Fig. 2. The effect of starvation and molting stage on trypsin activity of the midgut gland of *Penaeus vannamei*. Different italic letters show statistical differences by starvation time between groups of the same molting stage ($P<0.05$). Different italic numbers show statistical differences by molting stage between groups of the same starvation time ($P<0.05$).

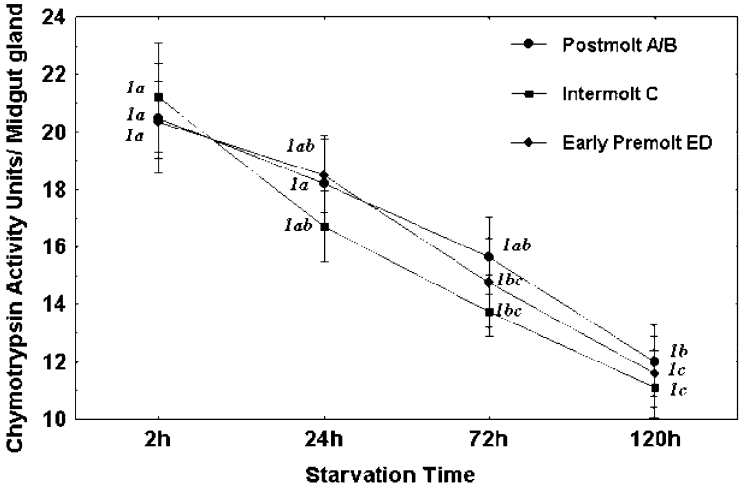

Fig. 3. The effect of starvation and molting stage on chymotrypsin activity of the midgut gland of *Penaeus vannamei*. Different italic letters show statistical differences by starvation time between groups of the same molting stage ($P<0.05$). Different italic numbers show statistical differences by molting stage between groups of the same starvation time ($P<0.05$).

observed for intermolt (C) and early premolt (ED) stages, after 24-h starvation ($P<0.05$). Chymotrypsin activity was not affected by the molting stage ($P>0.05$; Fig. 3). However, the progress of starvation severely affected the chymotrypsin activity, regardless of the molting stage. The mean chymotrypsin activity at 120 h of starvation was 44% lower than in specimens starved for 2 h ($P<0.05$).

All individual samples were analyzed by electrophoresis. Some differences were observed between organisms, but a common pattern in the number of paralogue trypsin and chymotrypsin was observed in organisms of the same molt stage and starvation time. Enzyme preparations were developed individually to find possible differences among molt stages over the time. We observed changes in the number of trypsin proteins and activity bands when molt stage and starvation time varied (Fig. 4). Active bands inhibited by TLCK and PMSF were considered trypsin and inhibited bands by PMSF are evidence of chymotrypsin activity (Fig. 5) (Lemos et al., 2000). Up to four paralogue trypsin bands were observed from each extract with molecular weights of 22.0, 20.7, 19.4 and 17.7 kDa. No correlation was observed between the number of bands, the molting stage and duration of starvation (Fig. 4). Two chymotrypsin protein and activity bands were observed with molecular weights between 24 and 30 kDa, and no changes in the chymotrypsin number of bands were observed (Fig. 4).

3.2. Short term starvation

In order to know about possible effects on proteinase activity at shorter starvation periods, a total of 132 samples were obtained from shrimp at intermolt (C). Starvation did not significantly affect the body weight up to 48 h ($P>0.05$). Midgut gland weight showed no differences but we observed two maximum values during the 48 h period, at 2 and 6 h of starvation. However, as time progressed, no differences were observed in the midgut gland weight between the 0.5 and 48 h ($P>0.05$). The hepatosomatic index showed no significant differences during the 48 h evaluated period ($P>0.05$; data not show).

The protein content of the midgut gland showed no differences between 0.5 and 48 h after the last ingestion ($P>0.05$), except for a maximum value observed at 6 h, which was observed to be significantly different to the minimum values observed after 12, 18 and 36 h ($P<0.05$; Fig. 6). Total proteolytic activity showed no differences between 0.5 and 48 h after the last ingestion ($P>0.05$). Similar patterns were observed when compared with those of protein content, a minimum value at 18 h was observed to be significantly different ($P<0.05$; Fig. 6).

Trypsin activity showed some significant variations during the 48-h trial ($P<0.05$). After the last ingestion, main differences were observed between two maximum values at 2 and 6 h, which were shown to be statistically different to two minimums observed at 12 and 18 h ($P<0.05$; Fig. 7). Chymotrypsin activity showed a similar pattern with some significant variations ($P<0.05$). A minimum value after 5 h was observed to be statistically different to two maximum values observed at 6 and 9 h after ingestion, followed by a steep decrease in activity (Fig. 7).

Electrophoretic analysis was done. Enzyme preparations were not pooled in order to find individual differences in the number of trypsin and chymotrypsin bands. We observed changes in the number of trypsin protein bands, which also showed a different number of activity bands with time. Two and three trypsin bands were observed in each extract, with molecular weights of 22.0, 20.7 and 19.4 kDa. Two chymotrypsin bands were observed with molecular weights of approximately 24 and 30 kDa (Fig. 8). No correlation was observed between the number of bands and duration of starvation.

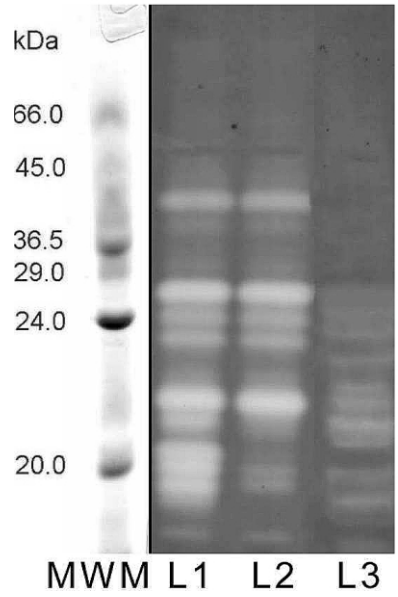

Fig. 5. SDS- PAGE of enzyme extracts of the midgut gland of white shrimp *Penaeus vannamei. Note*: Lane 1: control sample (midgut gland enzyme extract). Lane 2: TLCK incubated enzyme extract. Lane 3: PMSF incubated enzyme extract.

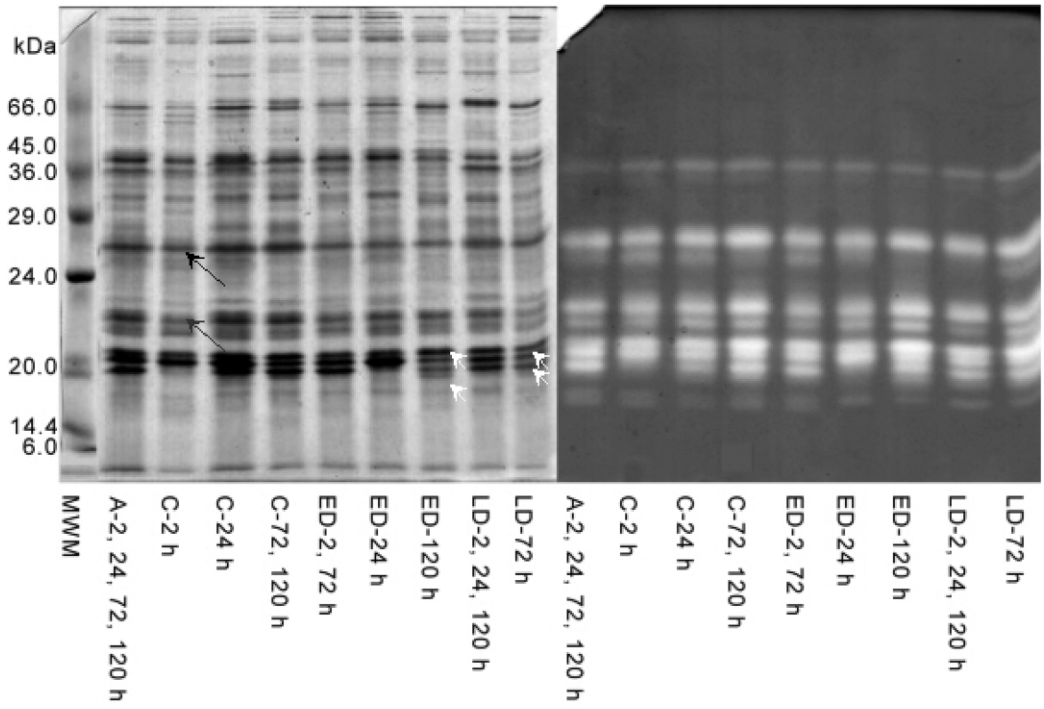

Fig. 4. SDS-PAGE of midgut gland enzyme extracts at different molting stages and starvation times. *Note*: letters indicate molting stage, numbers indicate time of starvation. White arrows show trypsin bands. Black arrows show chymotrypsin bands.

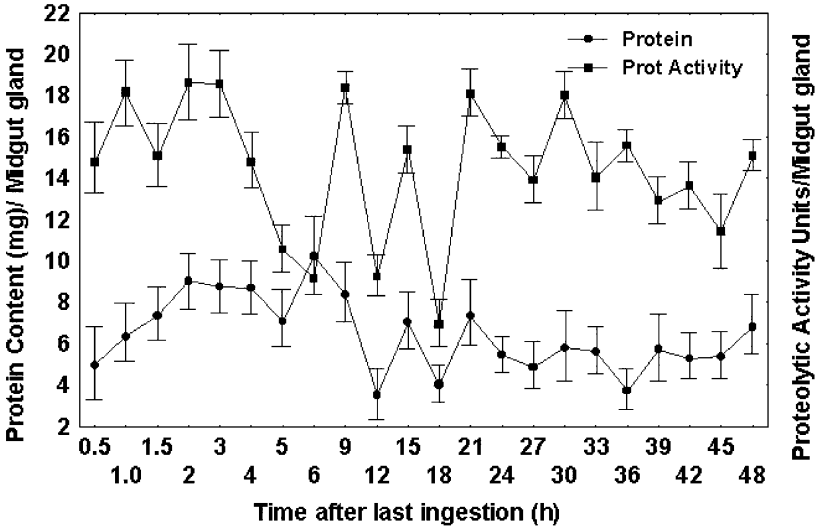

Fig. 6. Protein content and total proteolytic activity units of the midgut gland after ingestion. *Note*: different italic letters show statistical differences ($P<0.05$).

4. Discussion

In an effort to understand digestion biology of penaeid shrimp, the precise moment of proteolytic enzyme activation and the effects of molting, we designed and carried out two assays. During the shrimp life cycle, starvation occurs as a natural phenomenon when the organisms lose the old skeleton and are not able to handle food. However, these fasting periods of the molting cycle are never longer than 3–4 days, this is why we choose up to 120 h of treatment. Also, in trying to catch effects on the short run, we included a short term assay sampling more frequenty. Cuzon et al. (1980) and Barclay et al. (1983) observed in *Penaeus japonicus* and *P. esculentus* a decrease in the body and midgut gland weights of shrimp after prolonged starvation. We confirm these significant decreases in both variables mainly after 72 and 120 h of starvation on each molting stage. Total

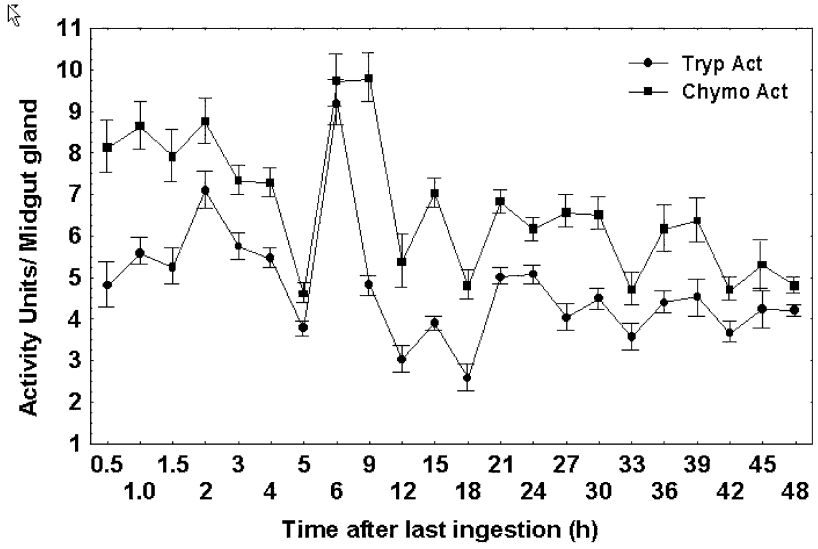

Fig. 7. Trypsin and chymotrypsin activity units of the midgut gland after ingestion. *Note*: different italic letters show statistical differences ($P<0.05$).

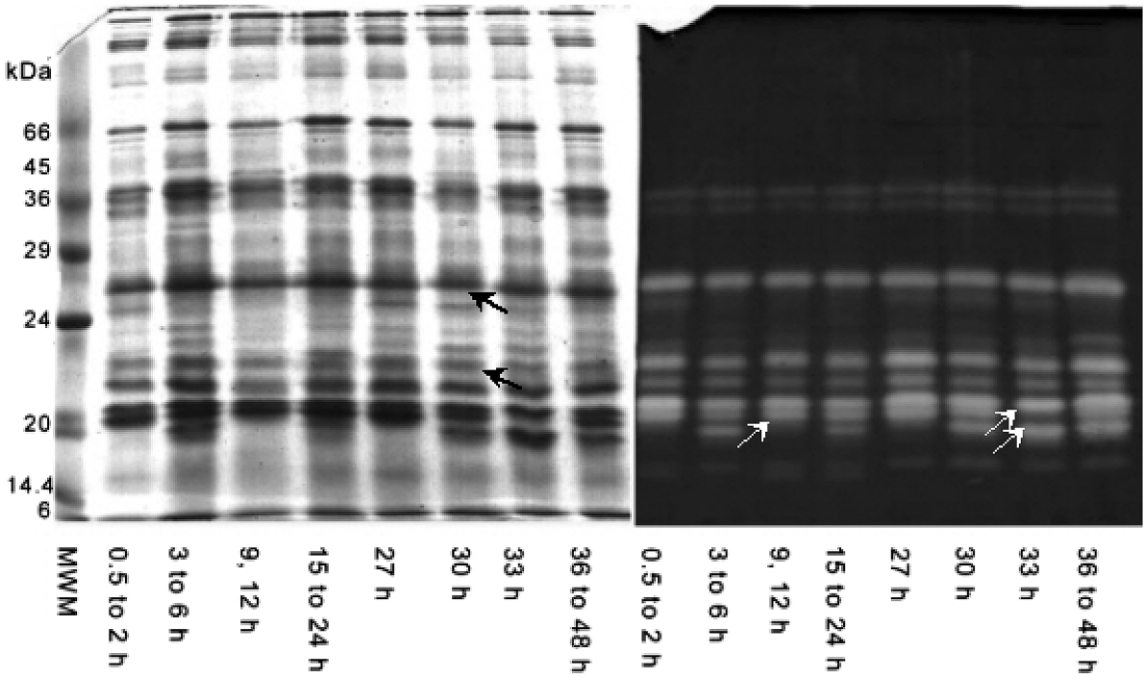

Fig. 8. SDS-PAGE of midgut gland enzyme extracts after ingestion. *Note*: numbers indicate time after last ingestion. White arrows show trypsin bands. Black arrows show chymotrypsin bands.

body weight is expected to decrease because the abdomen muscle protein is the main source of energy during starvation (Barclay et al., 1983). The midgut gland, which is also a storage organ, was more severely affected by starvation, and as a consequence, the hepatosomatic index of shrimp starved to 72 and 120 h decreased significantly regardless of the molting stage. These observations confirm the observations of Jussila (1999), who found no differences in hepatosomatic indices between intermolt and postmolt stages of *Cherax tenuimanus*. This means that nutritional status is independent of molting stage. Barclay et al. (1983) stated that the effect of starvation cannot be easily isolated from the effect of molting in organisms that molt frequently, such as penaeids. In this study, late premolt (LD) samples were difficult to find after 120 h of starvation. Few specimens at this stage were observed. The scarcity of shrimp at late premolt stage that were starved for 120 h agrees with the observations of Cuzon et al. (1980) that under prolonged starvation periods, the molting process subsides or even stops.

A decrease in the midgut gland weight during starvation implies a decrease in water and protein content, which are the major components of the organ, with protein as the energy source (Dall et al., 1990). Midgut gland weight and protein content are also reported to vary during the molting cycle (Al-Mohanna and Nott, 1989). In this study, protein content in the midgut gland was observed to decrease 72 and 120 h after starvation began, which confirms the findings of Cuzon et al. (1980) and Barclay et al. (1983). No differences were observed in protein content of the midgut during the starvation period between molting stages. However, those stages when specimens actively feed (C and ED) showed significant differences and higher values than at a no-feeding stage (A/B) at 2, 24 and 72 h of starvation. This means that organisms at intermolt (C) and early premolt (ED) stages started starvation with higher contents of protein than no-feeding stage organisms (A/B) (Table 2).

We observed that total proteolytic activity was reduced in shrimp starved for 120 h at early premolt stage, but no differences were observed between molting stages. Previous studies suggest that organisms at different molting stages showed different protein content and proteolytic activities in the midgut gland (Leung et al., 1990). However, in our evaluation of starvation, molting was a

negligible factor for protein and proteolytic activity. These observations may be explained as a consequence of a masking effect of starvation over molting, suggesting that the molting effect could be subdued by nutritional conditions.

Trypsin activity was significantly affected by the starvation and molting stage. Cuzon et al. (1980) reported that trypsin activity of shrimp declined during starvation. This could be explained as a decrease in the amount of the proteolytic enzymes and a consequent decline in their activity because of the absence of food or as in *Aedes aegypti* mosquito, because of shortage of amino acids to synthesize protein de novo. Decrease of trypsin activity during starvation is opposite to the increased amount of trypsin activity that Rodríguez et al. (1994) observed in *P. japonicus* when specimens were fed with food with low protein content. These observations suggest that trypsin activity can be affected by food quality as well as by lack of food. No differences were observed in trypsin activity at postmolt stage (A/B), which is consistent with no changes observed in protein content and proteolytic activity in the midgut gland of shrimp. This may suggest that at this molting stage, where no feeding activity is observed, protease synthesis mechanisms are diminished. Chymotrypsin activity was affected by starvation, but not by the molting stage. This enzyme was more influenced by food stress, with differences of 40% between 2 and 120 h of starvation, than trypsin, which showed activity differences of 35% between 2 and 120 h of starvation.

Results of the short term assay showed that at 48 h of starvation, no changes were expected in the body and midgut gland weight of shrimp at intermolt stage, which is consistent with what we observed in the first assay where no significant changes were observed until 72 h of starvation. However, significant changes were observed in protein content of the midgut gland, with maximum at 6 h, which coincided with maximum values of trypsin and chymotrypsin activities at 6 h. Total proteolytic activity showed different variation patterns, with minimum values between 5 and 6 h, coinciding with one of the lowest values observed in trypsin and chymotrypsin activities at 5 h after beginning of starvation. In addition, decreases in protein content, total proteolytic activity, and trypsin and chymotrypsin activities were observed at 12 and 18 h. Trypsin activity showed maximum values at 2 and 6 h, which suggests the possible presence of at least two different genes encoding trypsin enzymes, as Noriega and Wells (1999) observed in the mosquito *Aedes aegypti*. Chymotrypsin activity reached maximum between 6 and 9 h. Correlation between trypsin and chymotrypsin activities suggests that responses after ingestion are dependent and sequential. Trypsin response appears earlier than chymotrypsin. Apparently, they are not regulated by the same mechanism in the midgut gland of shrimp. Correlation between these two enzymes is generally observed in vertebrates (Lhoste et al., 1994) and recent studies suggest that regulation mechanisms of both enzymes may be achieved by different processes (Lhoste et al., 1994). In juvenile *P. vannamei,* no correlation between trypsin and chymotrypsin activities was observed by Le Moullac et al. (1996).

The long term and short term assays showed a higher activity in chymotrypsin than in trypsin, which is in agreement with results reported in our previous work (Ezquerra and García-Carreño, 1997; Hernández-Cortés et al., 1999). This confirms the importance of chymotrypsin activity in midgut gland, which represents an important percentage of the total proteolytic activity in the midgut gland of shrimp (van Wormhoudt et al., 1995). Although no significant differences in enzyme activities were observed between molting stages, shrimp at no-feeding stages (postmolt and late premolt) showed consistently lower activities. Therefore, selection of molting stages in studies of enzymatic evaluations in shrimp is suggested. As stated by Dall et al. (1990), intermolt seems to be the best physiological stage for evaluation, comprising from 8 to 30% of the total molting cycle since the previous molt.

In the first and second assays, the statistical differences observed between starvation times; increase or decrease in proteolytic, trypsin and chymotrypsin activities; and the varying number of protein and activity bands observed in gels, suggest the existence of an enzymatic adaptation mechanism in the midgut gland of white shrimp, leading it to secrete more or less enzymes for digestion of food protein. This supports the idea that starvation, as a natural stimulus during molting, allows the organism to regulate enzyme synthesis rates.

The varying number of trypsin protein and activity bands observed in polyacrylamide gels is similar to those observed by Ezquerra and García-

Carreño (1997) (Figs. 4 and 7). Changes in the number of bands suggest that shrimp have four protein bands with trypsin activity, which are present or not, depending on the needs of the organism in food protein hydrolysis. Although we observed changes in chymotrypsin activity (Fig. 3) and changes in the intensity of chymotrypsin bands in polycrylamide gels, two bands with chymotrypsin activity were always present in midgut gland extracts. This supports the observations of van Wormhoudt et al. (1995) of two continuous iso-enzymes throughout the entire molting cycle and the possibility of different factors inducing changes in this enzyme. Many other studies on food quality and quantity, also reported no changes in the number of chymotrypsin bands in the same species (Le Moullac et al., 1996; Ezquerra and García-Carreño, 1997). Hence, different regulation mechanism of trypsin and chymotrypsin are likely. However, advanced research is required to understand this enzyme regulation mechanism to know why trypsin but not chymotrypsin is regulated and how.

Results obtained in this study agree with those reported by Sanchez-Paz (2001), who evaluated the trypsin mRNA concentrations in the midgut gland of *P. vannamei* under the same conditions as this study. Higher trypsin mRNA concentrations were observed after 24 h of starvation at early premolt (ED) and intermolt (C). We observed maximal trypsin activity after 24 h of starvation, at the early premolt stage. These observations agree with Klein et al. (1996) in *P. vannamei*, whose results showed a maximum of trypsin-encoding mRNA during premolt and a maximum of trypsin activity at the same molting stage. The phenomenon of trypsin mRNA synthesis has been demonstrated to be under hormonal control by ecdysteroids (van Wormhoudt et al., 1985). The similar pattern of change between trypsin activity and mRNA expression suggests that the regulation of protein synthesis is during transcription.

It is demonstrated that starvation is a stimulant that surpasses the effect of molting, and because it affects the activity of digestive proteinases, studies of starving organisms in combination with tools of molecular biology, it can be a helpful working model in the understanding of mechanisms of regulation of digestive enzyme activity. The study of mechanisms of regulation of the synthesis of trypsin is in progress.

Acknowledgments

Funding was provided by CONACyT (grant 28257-B) to FLGC and scholarship to AMA. Authors want to thank M. Angeles Navarrete and B.M. Paola Magallón S. for providing technical support and the critical review of the manuscript by Dr Illie Racotta and Ira Fogel who edited the English text.

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