

## TRYPSIN SYNTHESIS AND STORAGE AS ZYMOGEN IN THE MIDGUT GLAND OF THE SHRIMP *LITOPENAEUS VANNAMEI*

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### ABSTRACT

An immunological approach was used to elucidate whether trypsin is synthesized and stored as trypsinogen in the midgut gland of the shrimp *Litopenaeus vannamei*. Two peptides were constructed using sequences deduced from known shrimp genes: trypsinogen activation peptide and an internal sequence. These peptides were used as haptens to elicit antibodies in rabbits. Specific antibodies were used to detect trypsinogen by Western blot and in histological sections of the midgut gland. Trypsinogen was found by Western blot and was localized into the midgut gland B cells by using immunohistology. In fed shrimp, trypsinogen associated with food particles was found in the lumen of the midgut gland tubules as well. Our results show that regulation of shrimp trypsin activity is similar to that of frequent feeder species, in which trypsin is stored as a zymogen, waiting for secretion and activation.

Digestive proteases play a fundamental role in food digestion to obtain building blocks for autologous protein synthesis and to store chemical energy. Knowledge about digestive protease activity regulation would help to understand how digestion is linked to synthesis, maintenance, and repair of tissue. Trypsins are the most important enzymes in digestion of food protein (Tidwell and Allan, 2001). Regulation of trypsin activity is diverse in the animal kingdom. One regulation mechanism is the synthesis and storage of inactive proenzymes called zymogens (Martin *et al.*, 1982). In vertebrates, hydrolysis of trypsinogen is part of a cascade reaction, in which duodenase, enteropeptidase, trypsinogen, and other zymogens participate (Light and Janska, 1989).

Vertebrate digestive proteases respond to a hormonal signal triggered by feeding (Hara *et al.*, 1999). Pepsins are secreted into the stomach, and trypsin, chymotrypsin, and other proteases are secreted into the small intestine through the pancreatic duct. Trypsinogen, the inactive form of trypsin, is stored in pancreatic cell granules of these organisms (Fukuoka *et al.*, 1986) and is secreted and mixed with food passing into the midgut gland (Corring *et al.*, 1989). Synthesis and storage of trypsinogen helps to maintain the integrity of secretory cells and to execute quick secretion and activation

(Martin *et al.*, 1982). Hydrolytic posttranslational removal of the trypsin activation peptide near the N-terminus of the molecule and subsequent conformational changes yield a fully functional enzyme (Light and Janska, 1989).

Crustaceans synthesize digestive enzymes in an organ known as the midgut gland or hepatopancreas. There is a considerable amount of information available related to the physiology of digestion in crustaceans, the midgut gland itself, and the assimilation and storage of nutrients (Gibson and Barker, 1979; Dall, 1992; Babu and Manjulatha, 1995). Crustaceans, like vertebrates, possess orthologue proteinases such as trypsin (Hernández-Cortés *et al.*, 1999a), chymotrypsin (Hernández-Cortés *et al.*, 1997), carboxypeptidase (García-Carreño and Haard, 1993), and leucine amino peptidase (García-Carreño *et al.*, 1994). Besides these enzymes, crustaceans possess unique proteases, such as astacine (Zwilling *et al.* 1981) and one that hydrolyses collagen (Tsu and Craik, 1996).

Decapod trypsin was first detected in the midgut gland of the shrimp *Penaeus setiferus* (Linnaeus, 1767) (= *Litopenaeus setiferus*, see Pérez Farfante and Kensley, 1997) by Gates and Travis (1969). Since then, several studies have characterized trypsins in decapods such as the shrimps *Penaeus monodon* (Fabricius, 1798) (see Lu *et al.*, 1990) and *Litopenaeus vannamei*

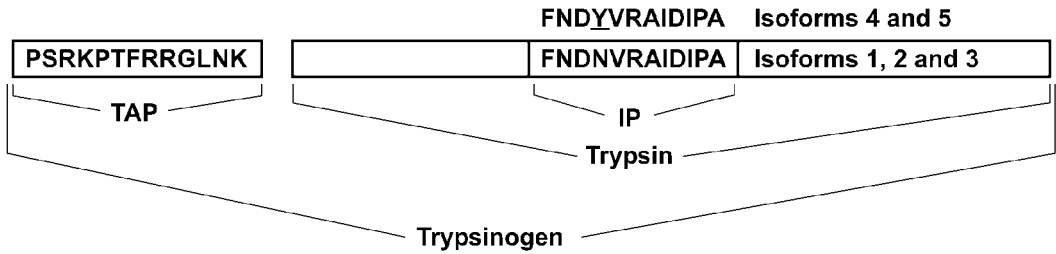


Fig. 1. Representation of the peptides used as antigens. The sequences, positions, and names are illustrated. Differences among the isoform sequences are also indicated.

(Boone, 1931) (Klein *et al.*, 1996) and the crayfish *Pacifastacus leniusculus* (Dana, 1852) (Hernández-Cortés *et al.*, 1999a). Molecular techniques have been used to describe gene cDNAs of enzymes involved in digestion (Van Wormhoudt *et al.*, 1995; Klein *et al.*, 1996; Hernández-Cortés *et al.*, 1999a). However, the information is limited because it does not provide insight on system physiology. These studies decoded the cDNA sequence of *Litopenaeus vannamei* trypsin (Klein *et al.*, 1996) and detected a small peptide between the functional trypsin sequence and the signal peptide. In *L. vannamei*, this peptide is composed of 14 amino acids and seems to be the trypsin activation peptide, which implies that the enzyme is synthesized as a zymogen. However, the trypsinogen molecule has never been identified.

In other invertebrates, such as the mosquito *Aedes aegypti* (Linnaeus, 1767), the amino acid sequence predicted from the trypsin cDNA suggests the presence of a trypsinogen activation peptide. However, there is no zymogen storage (Noriega and Wells, 1999), indicating that if the enzyme is translated as a zymogen, it is not stored, and secretion and activation take place simultaneously.

The aim of the present work was to obtain evidence, using immunohistological and Western blot techniques, that trypsin is stored as trypsinogen in the midgut gland of the shrimp *L. vannamei*.

## MATERIALS AND METHODS

### Preparation of Haptens

Two peptides were synthesized (ResGen, Huntsville, Alabama, U.S.A.) according to the amino acid sequence predicted from the trypsinogen nucleotide (Klein *et al.*, 1996): putative trypsinogen activation peptide (TAP), comprised of 13 of the 14 amino acids, and an internal peptide (IP) that included 12 amino acids from an internal sequence corresponding to amino acid residues 135–146 of the longest sequenced cDNA encoding the putative

trypsinogen of the *L. vannamei* midgut gland. Sequences were taken from SWISSPROT access code [TRYP\_PENVA] or EMBL nucleotide sequence database accession number Y15039, Y15040, and Y15041. Figure 1 shows the location and composition of the chosen peptide sequences. Localization on the surface of the molecule and high antigenic character were criteria for choosing the IP (von Heijne, 1987). Amino acid residues comprising the IP formed an exposed loop that was deduced by comparison with structures of bovine [TRYC\_bovine], bacterial [TRYP\_Strgr], and fungal [TRYP\_Fusox] trypsins (Rypniewski *et al.*, 1994). Sequence and three-dimensional structure similarities were detected with the Cn3D freeware program (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) (Hogue, 1997).

To increase antigenicity, peptides were coupled to a carrier molecule (Dyrberg and Oldstone, 1986). Each peptide (7 M) was incubated for 2 h with 10% glutaraldehyde and 5% glycerol and dialyzed against phosphate buffer solution (PBS): 140 mM NaCl, 2.5 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2. Each peptide solution was mixed with a solution of 0.2 M bovine serum albumin (BSA) (Harlow and Lane, 1988). Theoretically, the peptide-protein ratio yielded a complex of 30–35 antigenic determinants per molecule of carrier. Electrophoresis showed greater molecular weight of the peptide-albumin complex, confirming coupling efficacy (Laemmli, 1970).

### Antibody Production

Antibodies were elicited from two 3.5-kg male albino rabbits used for each peptide BSA complex. The animals were kept at the Centro de Investigaciones Biológicas del Noroeste animal facilities and were fed and watered *ad libitum*. The TAP-BSA-complex or IP-BSA complex (1 mg mL<sup>-1</sup>) in PBS was injected intradermally in the back of different animals at three-week intervals. Antibodies against the BSA carrier were also elicited as a control. Blood samples were taken before the first immunization (normal serum) and 10 days after each boost. Blood was collected from the ear marginal vein and stored at 10°C overnight. Serum was separated from the blood clot and centrifuged for 20 min at 2000 G. The serum was collected and labeled as anti-TAP, anti-IP, or control antibodies. Titration was carried out by ELISA, using horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG as the second antibody (Sigma Chemical Co., St. Louis, Missouri, U.S.A.). Antibodies were tested for specificity, using homologous (BSA, midgut gland extract, TAP, and IP peptides) and heterologous (crossed TAP and IP peptides, and bovine chymotrypsin) antigens.

## Midgut Gland Samples

Farmed *Litopenaeus vannamei* in intermolt state (Chan *et al.*, 1988) and weighing 25 g, were kept in 1500 L circular flat-bottom tanks at 28°C, 30‰ salinity, and oxygen saturation. Organisms were fed twice a day at 8:00 and 17:00 with PIASA feed: 40% protein, 7% lipid, 3% fiber, 12% water, 11% ash, and 27% free nitrogen extract. Organisms were starved for 24 h prior to trypsinogen testing by Western blot. Organisms were decapitated, and the midgut glands were excised and homogenized in acetone 2:1 (v:w) at 4°C to precipitate proteins. The precipitate was dissolved in 20 mM ethylenediamine-tetraacetic acid (EDTA) in PBS, denatured in Laemmli sample buffer, and analyzed immediately. For immunohistology, one group of 40 organisms was starved 24 h, and a second group of 40 was killed 3 h after feeding.

## Electrophoresis and Immunoblotting

Protein from 30 midgut gland extracts was analyzed by discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% acrylamide (Laemmli, 1970). Twenty µg of protein from the 10-µL midgut gland extracts was mixed (1:2) (v:v) with Laemmli loading buffer (125 mM Tris HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue) and heated for 2 min at 95°C. Electrophoresis was carried out for 2 h at 20 mA and 4°C. Protein was visualized by staining with Coomassie Brilliant Blue R-250. Protein was also transferred onto a polyvinylidene fluoride membrane for Western blotting (Towbin *et al.*, 1979). Transfer efficiency was evaluated by Ponceau staining. Membranes were blocked with skim milk (PBS, 0.2% Tween 20, 5% skim milk, pH 7.2) and incubated with anti-TAP or anti-IP diluted 1:3000 in PBS-Tween (PBS, 0.2% Tween 20, pH 7.2). Antibodies blocked with their antigens were used as controls. Membranes were washed with PBS-Tween three times for 20 min and incubated with anti-rabbit-IgG-HRP diluted 1:5000 in PBS-Tween. Specific peptides were detected using 3,3'-diaminobenzidine 0.6 mg/mL in PBS, at pH 7.4 with 0.03% NiCl<sub>2</sub> and 0.2% H<sub>2</sub>O<sub>2</sub> as the substrate for HRP (Harlow and Lane, 1988).

## Immunohistochemistry

Midgut glands of 40 fed and 40 starved organisms were dissected and fixed for 36 h in formaldehyde diluted to 3.7% with seawater. Fixed tissues were dehydrated in increasing concentrations of ethanol at room temperature and embedded in paraffin. Serial cuts of 3 µm were obtained from each midgut gland. Slices were mounted on glass slides and dried for 16 h at room temperature. After paraffin removal with xylol and passage through another dehydration series, sections were treated in serial order to obtain images of cells stained with hematoxylin-eosin, anti-TAP, and anti-IP antibodies diluted 1:200 in PBS for 2 h at room temperature. Controls were incubated with normal rabbit serum, with first antibody omitted or blocked with its antigen (Harlow and Lane, 1988). After thorough rinsing with PBS, sections were incubated with anti-rabbit-HRP, diluted 1:3000 in PBS. Finally, sections were washed with PBS. Antibody-reactive molecules were indicated by chromogenic reaction of HRP (Harlow and Lane, 1988). Photos were taken with a light microscope at 20× magnification (BX41 Olympus). Images were obtained with a 1.4 megapixel CoolSNAP-Pro cooled CCD camera, utilizing Image-Proplus 4.1 Olympus software.

## RESULTS

Based on results of the ELISA technique (Harlow and Lane, 1988), antibodies elicited against TAP and IP had titers of 1:3200 against the synthesized peptides. No cross-reaction was observed when IP antibodies were tested with synthesized TAP or when TAP-antibodies were tested against synthesized IP. Control sera reacted negatively against both synthesized IP and TAP. Both TAP and IP-antibodies reacted negatively when tested with bovine chymotrypsin.

The SDS-PAGE of midgut gland extract showed a complex protein composition, related to both size and concentration (Fig. 2, Lane I). Western blots for the same extract displayed three bands (M<sub>r</sub> 29.7, 30.2, and 32.9) when anti-TAP antibodies were used (Fig. 2, Lane II). Two proteins were displayed when using anti-IP antibodies against the crude extract (Fig. 2, Lane III). The molecular mass of the two proteins was the same as those of the two heavier proteins displayed by the anti-TAP antibodies (30.2 and 32.9 kDa). The anti-TAP antibodies detected no protein when extracts were not prepared by precipitation with acetone. No signal was observed when blots were treated with control sera or with antibodies previously incubated with synthesized peptides.

The different cell types were identified by histology when samples were stained with hematoxylin and eosin (Figs. 3A, 4A). The figures show transverse 3-µm paraffin serial sections of midgut glands. Cell type was classified according to Bell and Lightner (1988). The B cells contained one very large vacuole, typically having a highly convex lumen surface, and the nucleus was displaced peripherally toward the basal region of the cell. The F cells had a prominent basal nucleus and were fibrous in appearance, with strongly basophilic cytoplasm.

The 80 shrimp analyzed presented reactive molecules with both anti-peptide antibodies only in secretory B cells of both fed and starved organisms (Figs. 3, 4). The figures show equivalent areas of serial cuts, although the pictures displayed (B, C, and D) are slightly displaced. When animals were fed (Fig. 4), it was possible to recognize feed in the lumen. Some B cells in both corresponding positions were stained with the antibodies (Figs. 4B, C), and the feed was also reactive. Controls with anti-serum blocked by the respective antigen (IP or TAP) showed no positive result (Figs. 3D, 4D).

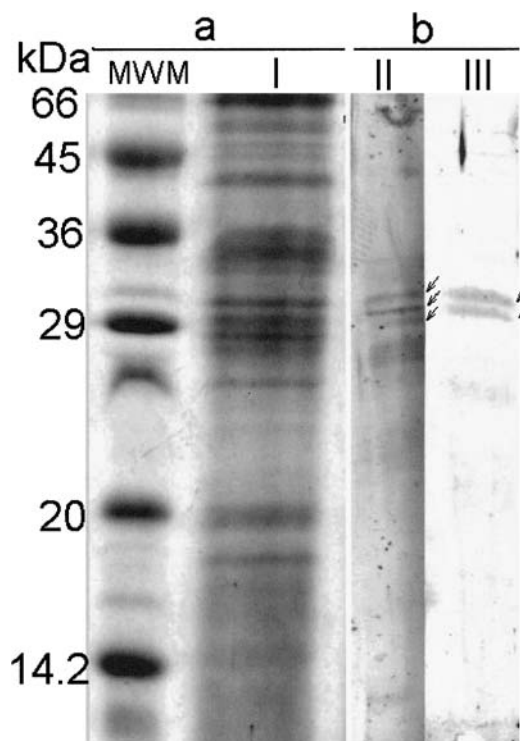


Fig. 2. SDS-PAGE (a) and Western blot (b) of proteins from *L. vannamei* midgut gland. MWM, molecular weight marker; I, Coomassie stained; II, TAP antibody labeled; III, IP antibody labeled. Arrows indicate the different bands.

Granules, containing aggregated molecules (Fig. 5), were darker than the cytoplasm background. Similar cells in different sections of the midgut gland were labeled with both treatments.

#### DISCUSSION

Results showed that the *L. vannamei* midgut gland synthesizes and stores trypsinogen to keep trypsin inactive while stored and ready to be secreted and activated. Evidence of trypsinogen storage in the penaeid midgut gland is provided by Western blot analysis and immunohistology. As shown in Fig. 1, anti-TAP antibodies specifically detected trypsinogen only, but anti-IP detected both trypsin and trypsinogen. The criterion to identify a true trypsinogen molecule was that antibodies had no cross-reaction, and that the antibody against the trypsinogen activation peptide detected the same molecule as the internal peptide antibody elsewhere in the Western blot (molecular weight) or the immunohistology (cells stained). Antibodies generated by the two peptides were

specific and were suitable for distinguishing two different sequences in the trypsin molecule, one of them specific to the inactive form of trypsin. Trypsinogen purification was not possible (Klein *et al.*, 1996), but eliciting antibodies from synthetic peptide was an option.

Detection of *L. vannamei* trypsinogen is difficult because it is activated quickly, possibly by itself (Van Wormhoudt *et al.*, 1995). To prevent possible activation during homogenization, protein from the midgut gland was precipitated with acetone, and EDTA, reducing agents, and heat were used. These treatments eliminated enzyme activity by chelating calcium ions needed for trypsin stabilization (Rypniewski *et al.*, 1994), breaking disulfide bonds, and denaturing protein.

The trypsin activation peptide sequence is different among species. In vertebrates, the peptide is small, around six amino acid residues long, and highly acidic because of the Asp content (Light and Janska, 1989). The enzyme enteropeptidase is responsible for cleavage and activation of trypsinogen because of its high and precise affinity for the peptide bond linking the trypsinogen activation peptide and the enzyme forming the trypsinogen (Light and Janska, 1989). In *L. vannamei*, the activation peptide is larger, with 14 amino acid residues, and the composition suggests that the activation enzyme is trypsin itself, in contrast to vertebrate trypsinogen that protects itself from activation (Light and Janska, 1989; Van Wormhoudt *et al.*, 1995; Klein *et al.*, 1996).

By comparing the pattern of the total protein stained with Coomassie with that of proteins labeled with antibodies by Western blot, it was observed that trypsin sequences obtained by Klein *et al.* (1996) were transcribed in the midgut gland of *L. vannamei*. Substrate gel electrophoresis analysis and inhibition (Lemos *et al.*, 2000) showed that at least three different trypsins were present, as demonstrated by Le Moullac *et al.* (1996).

The mass of denatured trypsins detected by Western blot analysis was similar to that estimated for the trypsin pool (30 kDa) by Klein *et al.* (1996). Three protein bands were detected with anti-TAP, and two protein bands of sizes close to those of the two heavier proteins detected with anti-TAP were detected with anti-IP antibodies. Differences in band number could be related to differences in trypsin isoforms among IP sequences (Klein *et al.*,

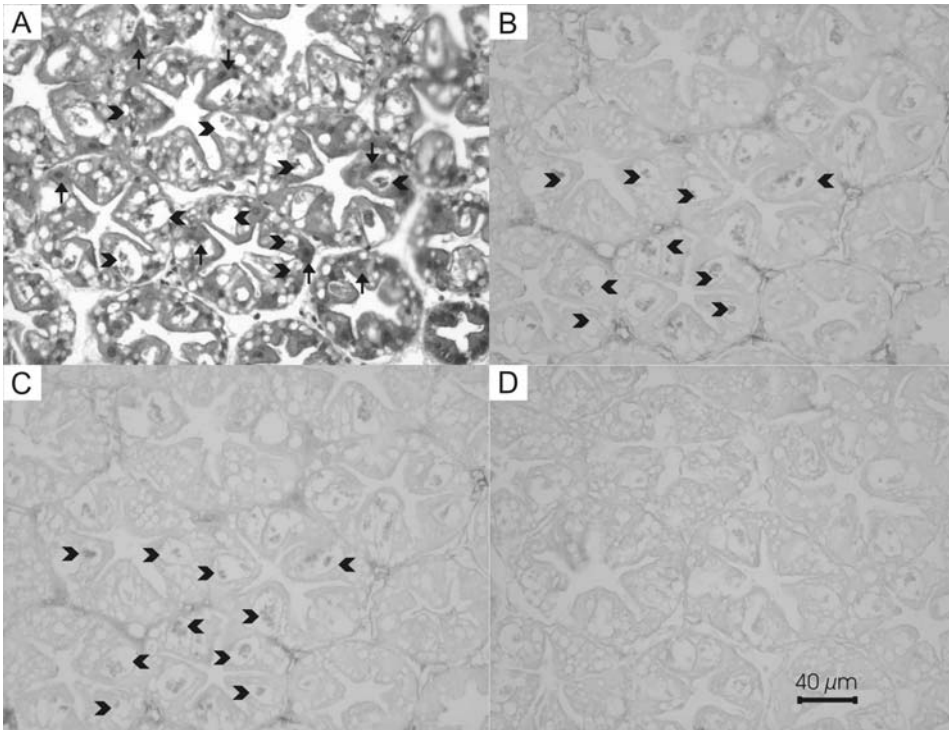


Fig. 3. Serial cuts of the proximal region of *L. vannamei* midgut gland. Samples were taken from unfed organisms. (A) Hematoxylin and eosin, B cells (▲) and F cells (▲) are indicated. (B) Section treated with anti-TAP antibodies. (C) Section treated with anti-IP antibodies. Positive B cells are indicated. (D) Section treated with anti-IP antibodies blocked with its antigen.

1996). The TAP sequence was conserved in the five trypsinogen forms; therefore, all of them react with anti-TAP antibodies. Another possibility is that in the denatured condition, two of the three bands matched the same  $M_r$ , so two bands were visible and not three. The anti-TAP antibody detected the same bands as anti-IP, which means that the trypsin activation peptide was part of the amino-acid sequence of the trypsin in these bands, i.e., the trypsinogen.

Immunohistochemistry analysis showed that trypsinogen detected in tubules mixed with food in fed *L. vannamei* was stored in granules in the B cells. No signal of the antibodies used in this study was found in E, R, or F cells in *L. vannamei* midgut gland tissue sections. Cells labeled with anti-TAP and anti-IP antibodies were B type. Gibson and Baker (1979) reported that B cells were associated with protease activity and have secretory function (Ceccaldi, 1989). Probes for the mRNA digestive enzymes amylase, cathepsin-L, chitinase-1, and trypsin in *P. monodon* (see Lehnert and Johnson, 2002), and chymotrypsin in *L. vannamei* (Le Moullac *et al.*, 1996)

were localized in the cytoplasm of F cells. No digestive enzyme gene expression was observed in *P. monodon* B cells (Lehnert and Johnson, 2002), and it was suggested that transcription and storage of digestive enzymes might not be related. Because the mRNA of digestive enzymes was found in F cells, and enzyme activity was in B cells, questions about the mechanism explaining the phenomenon should be addressed. Synthesis and storage of proteases as inactive precursors in the midgut gland have numerous advantages for an organism. The risk of tissue damage by proteolytic enzymes is reduced, and proenzymes are ready for activation when needed. Transverse sections of the midgut gland proximal region showed that B cells were not homogeneously distributed in the tubules, that food was not homogeneously distributed in the midgut gland tubules, and that B cells appeared in tubules filled with food or empty. This means that trypsinogen is not secreted totally from a single cell, and it appears to be secreted partially as an effect of ingestion.

Regulation of enzymes involved in protein

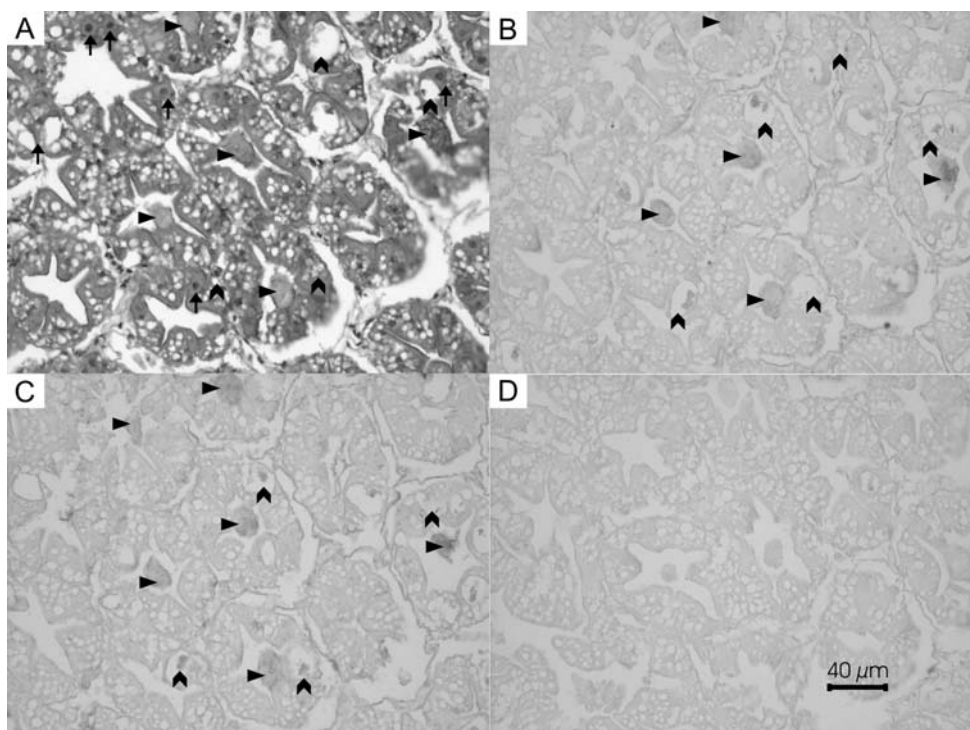


Fig. 4. Serial cuts of the proximal region of *L. vannamei* midgut gland. Samples were taken from fed organisms. (A) Hematoxylin and eosin stain, B cells (▲), F cells (◆) and feed (●) in tubules are indicated. (B) Section treated with anti-TAP. (C) Section treated with anti-IP. Positive B cells and feed in lumen are indicated. (D) Section treated with anti-TAP blocked with its antigen.

digestion is species-specific (Secor and Diamond, 2000). One of the best-studied models of protease regulation is the mosquito *Aedes aegypti* (see Noriega and Wells, 1999). This bloodsucker has an early and a late trypsin that are not stored as zymogens. The mosquito stores mRNA for the early trypsin, which is translated and secreted upon feeding. The late trypsin gene is transcribed in response to the quality and quantity of the feed. The early trypsin controls synthesis of the late trypsin. Transcription of trypsin by feeding is also present in other mosquitoes, such as *Anopheles gambiae* Giles, 1902 (see Muller *et al.*, 1993). In contrast, methods of digestive enzyme activity regulation in organisms that feed and digest once or several times a day include post-transcriptional control, which implies storage, secretion, and activation of zymogens (Secor, 2001).

Shrimp are frequent feeders, and eating follows a circadian pattern (Hernández-Cortes *et al.*, 1999b) to which this type of trypsinogen regulation fits well (Secor, 2001). Organisms that store zymogens have the advantage of faster response

to feeding stimulus over sporadic feeders that have to transcribe or translate to get active enzymes. In *L. vannamei*, a circadian cycle with feeding increasing a few hours after dusk was observed (Hernández-Cortes *et al.*, 1999b). However, whether feeding rhythms influence regulation and secretion of trypsin in penaeids remains unknown.

The results of this study give evidence that in a crustacean such as shrimp, a stage of digestive enzyme activity regulation involving the synthesis and storage of trypsinogens in granules inside the secretory B cells of the midgut gland occurs. This post-translational regulation appears to be linked to feeding habits (frequent *versus* sporadic) more than phylogenetic relationships. The rates of trypsinogen synthesis and secretion, and the trypsinogen activation mechanism, remain to be elucidated.

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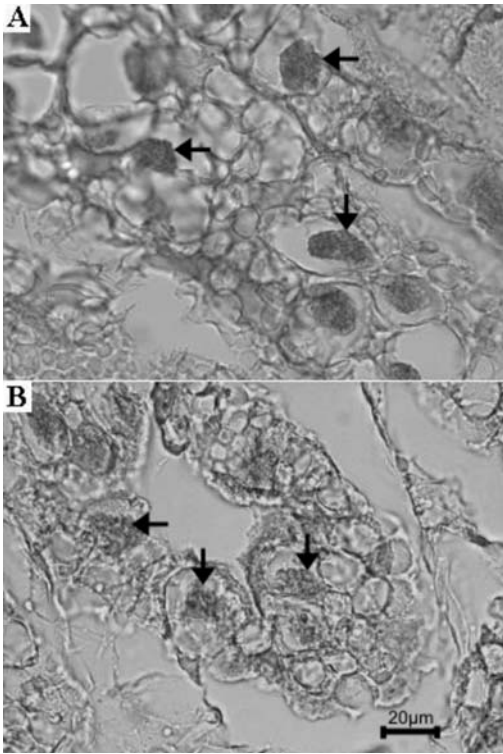


Fig 5. Immunohistochemistry of the midgut gland of *L. vannamei* showing positive granules inside of B cells. (A) Longitudinal section, anti-TAP was used for labeling. (B) Transverse section, anti-IP was used for labeling.

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#### LITERATURE CITED

- Babu, D. E., and C. Manjulatha. 1995. Zymogen secreting cells in the hepatopancreatic duct of *Pagurus bernhardus* (Linnaeus, 1758) and *Clibanarius longitarsus* (de Haan, 1849) (Anomura).—*Crustaceana* 68: 616–628.
- Bell, T. A., and D. V. Lightner, 1988. A Handbook of Normal Penaeid Shrimp Histology. World Aquaculture Society. University of Arizona, Tussan, Arizona. 112 pp.
- Ceccaldi, H. J. 1989. Anatomy and physiology of digestive tract of crustacean decapods reared in aquaculture.—*Advances in Tropical Aquaculture* 9: 243–259.
- Chan, S. M., S. M. Ranking, and L. L. Keeley. 1988. Characterization of the molt stages in *Penaeus vannamei*: setogenesis and hemolymph levels of total protein, ecdysteroids, and glucose.—*Biological Bulletin* (Woods Hole) 175: 185–192.
- Corring, T., C. Juste, and E. F. Lhoste. 1989. Nutritional regulation of pancreatic and biliary secretions.—*Nutrition Research Reviews* 2: 161–180.
- Dall, W. 1992. Feeding, digestion and assimilation in Penaeidae.—*Proceedings of the Aquaculture Nutrition Workshop*: 57–62.
- Dyrberg, T., and M. B. Oldstone. 1986. Peptides as antigens. Importance of orientation.—*Journal of Experimental Medicine* 164: 1344–1349.
- Fukuoka, S., H. Kawajiri, T. Fushiki, K. Takahashi, and K. Iwai. 1986. Localization of pancreatic enzyme secretion-stimulating activity and trypsin inhibitory activity in zymogen granule of the rat pancreas.—*Biochimica et Biophysica Acta* 884: 18–24.
- García-Carreño, F. L., and N. Haard. 1993. Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts.—*Journal of Food Biochemistry* 17: 97–113.
- , M. P. Hernandez-Cortes, and N. Haard. 1994. Enzymes with peptidase and proteinase activity from the digestive system of a freshwater and marine decapod.—*Journal of Agricultural and Food Chemistry* 42: 1456–1461.
- Gates, B. J., and J. Travis. 1969. Isolation and comparative properties of shrimp trypsin.—*Biochemistry* 9: 4483–4489.
- Gibson, R., and P. Barker. 1979. The decapod hepatopancreas.—*Oceanography and Marine Biology* 17: 285–346.
- Hara, H., N. Hashimoto, N. Akatsuka, and T. Kasai. 1999. Induction of pancreatic trypsin by dietary amino acids in rats: four trypsinogen isozymes and cholecystokinin messenger RNA.—*Journal of Nutrition Biochemistry* 11: 52–59.
- Harlow, E., and D. Lane. 1988. Antibodies, a Laboratory Manual. Cold Spring Harbor Laboratory, U.S.A. 726 pp.
- Heijne, G. von. 1987. Sequence Analysis in Molecular Biology. Treasure Trove or Trivial Pursuit. Academic Press, San Diego. 188 pp.
- Hernández-Cortes, P., L. Cerenius, F. García-Carreño, and K. Söderhäll. 1999a. Trypsin from *Pacifastacus leniusculus* hepatopancreas: purification and cDNA cloning of the synthesized zymogen.—*Biological Chemistry* 380: 499–501.
- , W. Quadros-Seiffert, M. A. Navarrete del Toro, G. Portillo, G. Colado, and F. L. García-Carreño. 1999b. Rate of ingestion and proteolytic activity in digestive system of juvenile white shrimp, *Penaeus vannamei*, during continual feeding.—*Journal of Applied Aquaculture* 9: 35–45.
- , J. R. Whitaker, and F. L. García-Carreño. 1997. Purification and characterization of chymotrypsin from *Penaeus vannamei* (Crustacea:Decapoda).—*Journal of Food Biochemistry* 21: 497–514.
- Hogue, W. V. 1997. Cn3d: a new generation of three-dimensional molecular structure viewer.—*Trends in Biochemical Sciences* 22: 314–316.
- Klein, B., G. Le Moullac, D. Sellos, and A. Van Wormhoudt. 1996. Molecular cloning and sequencing of trypsin cDNAs from *Penaeus vannamei* (Crustacea, Decapoda): use in assessing gene expression during the molt cycle.—*International Journal of Biochemistry and Cell Biology* 28: 551–563.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4.—*Nature* 227: 680–685.
- Lehnert, S. A., and S. E. Johnson. 2002. Expression of hemocyanin and digestive enzyme messenger RNAs in the hepatopancreas of the black tiger shrimp *Penaeus monodon*.—*Comparative Biochemistry and Physiology Part B* 133: 163–171.
- Lemos, D., J. M. Ezquerro, and F. L. García-Carreño. 2000. Protein digestion in penaeid shrimp: digestive proteinases, proteinase inhibitors and feed digestibility.—*Aquaculture* 186: 89–105.

- Le Moullac, G. L., B. Klein, D. Sellos, and A. Van Wormhoudt. 1996. Adaptation of trypsin, chymotrypsin and  $\alpha$ -amylase to casein level and protein source in *Penaeus vannamei* (Crustacea Decapoda).—*Journal of Experimental Marine Biology and Ecology* 208: 107–125.
- Light, A., and H. Janska. 1989. Enterokinase (enteropeptidase): comparative aspects.—*Trends in Biochemical Sciences* 14: 110–112.
- Lu, P. J., H. C. Liu, and I. H. Tsai. 1990. The midgut trypsins of shrimp (*Penaeus monodon*). High efficiency toward native protein substrates including collagens.—*Biological Chemistry* 371: 851–859.
- Martin, D. W., V. W. Rodwell, and P. A. Mayer. 1982. *Bioquímica de Harper, El manual moderno S.A de C.V. Mex. Mex.* 633 pp.
- Muller, H. M., J. M. Crampton, A. della Torre, R. Sinden, and A. Crisanti. 1993. Members of a trypsin gene family in *Anopheles gambiae* are induced in the gut by blood meal.—*European Molecular Biology Organization Journal* 12: 2891–2900.
- Noriega, F. G., and M. A. Wells. 1999. A molecular view of trypsin synthesis in the midgut of *Aedes aegypti*.—*Journal of Insect Physiology* 45: 613–620.
- Pérez Farfante, I., and B. Kensley. 1997. *Penaeoid and Sergestoid Shrimps and Prawns of the World. Keys and Diagnoses for the Families and Genera. Memoires du Museum National d'Histoire Naturelle, Paris, France.* 233 pp.
- Rypniewski, W. R., A. Perrakis, C. E. Vorgias, and K. S. Wilson. 1994. Evolutionary divergence and conservation of trypsin.—*Protein Engineering* 7: 57–64.
- Secor, S. M. 2001. Regulation of digestive performance: a proposed adaptive response.—*Comparative Biochemistry and Physiology Part A* 128: 565–577.
- , and J. M. Diamond. 2000. Evolution of regulatory responses to feeding in snakes.—*Physiological and Biochemical Zoology* 73: 123–141.
- Tidwell, J. H., and G. L. Allan. 2001. Fish as food: aquaculture's contribution. Ecological and economic impacts and contributions of fish farming and capture fisheries.—*European Molecular Biology Organization Reports* 2: 958–963.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.—*Proceedings of the National Academy of Sciences of the United States of America* 76: 4350–4354.
- Tsu, C. A., and C. S. Craik. 1996. Substrate recognition by recombinant serine collagenase from *Uca pugilator*.—*Journal of Biological Chemistry* 271: 11563–11570.
- Wormhoudt, A. Van, D. Sellos, A. Donval, S. Plaire-Goux, and G. Le Moullac. 1995. Chymotrypsin gene expression during the intermolt cycle in the shrimp *Penaeus vannamei* (Crustacea Decapoda).—*Experientia* 51: 159–163.
- Zwilling, R., H. Doersam, H. Torff, and J. Rold. 1981. Low molecular mass protease: evidence for a new family of proteolytic enzymes.—*Federation of European Biochemical Societies Letters* 127: 75–78.

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