TRYPSIN SYNTHESIS AND STORAGE AS ZYMGEN 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.*, 1992). 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-Cortes *et al.*, 1999a), chymotrypsin (Hernández-Cortes *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* (Linnaeus, 1767) (= *Litopenaeus vannamei*, 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*.
(Boone, 1931) (Klein et al., 1996) and the crayfish *Pacifastacus leniusculus* (Dana, 1852) (Hernández-Cortes et al., 1999a). Molecular techniques have been used to describe gene cDNAs of enzymes involved in digestion (Van Wormald et al., 1995; Klein et al., 1996; Hernández-Cortes 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 Na2HPO4, 2 mM KH2PO4, 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−1) 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.

![Fig. 1. Representation of the peptides used as antigens. The sequences, positions, and names are illustrated. Differences among the isoform sequences are also indicated.](image)
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:v) 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 immunohistochemistry, 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 (PBS, 0.2% Tween 20, pH 7.2). 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.

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 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 antisemum blocked by the respective antigen (IP or TAP) showed no positive result (Figs. 3D, 4D).
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 Mouillac *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-IP were detected with anti-IP antibodies. Differences in band number could be related to differences in trypsin isoforms among IP sequences (Klein *et al.*, 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 Mr, 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 (Cecaladi, 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 digestion is species-specific (Secor and Di-
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 appear 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.

**ACKNOWLEDGEMENTS**

The authors thank Taylor Morey for editing, and Manuel Diaz and Fernando Noriega for their constructive comments. Authors P. Hernández and J. C. Sainz thank Consejo
Nacional para la Ciencia y Tecnología (CONACyT) for grants J33750-N and 27371-B, and fellowship 153559.

LITERATURE CITED


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.


