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Chymotrypsin gene expression during the intermolt cycle in the shrimp *Penaeus vannamei* (Crustacea; Decapoda)

A. Van Wormhoudt, D. Sellos, A. Donval[a], S. Plaire-Goux and G. Le Moullac

Collège de France; Laboratoire de Biologie Marine, F-29900 Concarneau and [a]Université de Bretagne occidentale; Faculté des Sciences, F-29200 Brest (France)
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Abstract. In *Penaeus vannamei*, chymotrypsin is present as two isoenzymes in the hepatopancreas. The enzyme has been localized in F-cells by immunocytochemistry using a specific antibody. By in situ hybridization, with a 510 pb cDNA probe encoding for the first 170 amino acids of the shrimp chymotrypsin, mRNA was localized in the same cells. Gene expression was followed during the intermolt cycle by measuring changes in specific activity in crude extracts, and by the estimation of mRNA levels by Northern blots using the same probe. The increase in specific activity in premolt is preceded in early premolt by an increase in the amount of chymotrypsin mRNA. A second increase is observed in postmolt, suggesting a different mode of regulation of gene expression
Key words. Crustaceans; prawns; hepatopancreas; chymotrypsin; northern-blot; in situ hybridization.

Many changes in the constituents of the hepatopancreas during the intermolt cycle in Crustaceans have been measured, including amounts of RNA and DNA[1], digestive enzymes[2,3] and total proteins[4]. For digestive enzymes, two maxima were detected in premolt and intermolt[3], depending on seasonal conditions. The minimum is generally reached for all constituents in the Do stage, the transition period from the intermolt stages to the premolt stages, which is very important for the regulation of gene expression in the hepatopancreas as well as the hypodermis[5]. Chymotrypsin from the hepatopancreas of the shrimp *Penaeus vannamei* is one of the most active proteases in this species[6]. Two different chymotrypsin cDNAs were recently isolated from a hepatopancreas cDNA library and sequenced[7]. They represent around 1% of the clones present in the cDNA library. Our purpose was to investigate enzyme synthesis in the hepatopancreas of the shrimp *P. vannamei* during the intermolt, by measuring changes in the specific activity of chymotrypsin, and by identifying changes in gene expression by hybridization with a chymotrypsin cDNA probe. Cellular localization of chymotrypsin and of chymotrypsin mRNA were compared using a specific antibody and in situ hybridization with the cDNA probe, respectively.

Materials and methods

Animals. Shrimps (*Penaeus vannamei*) were obtained from IFREMER (French Polynesia): the hepatopancreas was removed by dissection, immediately frozen in liquid nitrogen and stored at −80 °C until used. Determination of the molting stages was made according to the method of Drach & Tchernigotzeff[8].

Biochemical methods. Chymotrypsin was measured on crude hepatopancreas extracts using the method of Delmar et al.[9] with SAPPNA as the substrate. Crude extracts were made from freeze-dried hepatopancreas, homogenized in a 10 mM Phosphate buffer pH = 7.5 at 4 °C with an Ultraturrax. Extracts were centrifuged for 20 min at 55,000 *g* at 4 °C to remove lipids. The supernatants were collected. A unit of specific activity was defined as the liberation of one µmole of p-nitroanilide in one minute per mg of protein. Proteins were measured according to Lowry et al[10].

Purification of the enzyme was made as previously described[7]. Protein purity was checked by electrophoresis in the Davis system[11] on acrylamide gels after silver staining. Polyacrylamide gels were fixed in 20% ethanol/10% acetic acid, then rinsed with water (UHQ ELGA, Villeurbanne, France). They were soaked in 0,2 g/l sodium hydrosulfite and stained with silver (0,1% in 0,15% formaldehyde). After washing, the gels were developed with potassium carbonate (0,8%; 5% thiosulfate; 0,1% formaldehyde). The reaction was stopped by adding acetic acid. Western blotting was performed after transfer of a crude extract of hepatopancreas protein (100 µg), separated by electrophoresis in the Davis system, on an Immobilon-P membrane with a BIOLYON apparatus (Dardilly, France). Immunochemical staining was performed using a specific antibody against purified shrimp chymotrypsin, according to the same method as was used in immunocytochemistry. This antibody was produced in a guinea pig by the classical boosting method with a total of 300 µg of pure enzyme in 6 injections.

Immunocytochemical localization of chymotrypsin. After hydration in Tris buffer solution (TBS) (50 mM Tris,

200 mM NaCl, pH 7.6) sections were treated with 0.3% H_2O_2 to eliminate endogenous peroxidases. For the blocking reaction, slides were placed for 60 min in a 1.5% solution of 1% of normal rabbit serum (NRS), then rinsed in TBS. Incubation with guinea pig antiserum was carried out overnight at 4 °C in TBS containing 1% NRS and 1/500 anti chymotrypsin serum. The anti-guinea pig immunoglobulin peroxidase conjugate (Sigma, St. Louis, MO) was used diluted 1/500 in 1% NRS at room temperature for 1 h. 3,3'-diaminobenzidine-4-HCl (DAB) (Sigma Immunochemical) was used as a substrate. Slides were mounted after dehydration in EUKITT (Labonord, Villeneuve d'Asq, France).

In situ hybridization. Hepatopancreases were fixed in Bouin-Holland fluid, and embedded in paraffin. 5 µm sections were collected on slides coated with poly-L-lysine (Sigma). After 30 min in 30 mM sodium citrate and 300 mM sodium chloride pH = 7 (2 × SSC), sections were treated for 1 h in the hybridization buffer containing 50% formamide (10 ml), 4 × SSC (4 ml), Denhart solution (0.8 ml), dextran sulfate (2 g) and ultrasonicated salmon sperm (400 µg). The probe was an amplified cDNA cloned fragment encoding the first 170 residues of the major variant of chymotrypsin. This cDNA differs from the less-represented variant cDNA by 33 nucleotide changes in a total of 510 bases. Accordingly, this probe hybridizes to both chymotrypsin mRNAs. The labeled probe was diluted in the hybridization buffer and applied to sections for 48 h at 40 °C in a moist chamber. Slides were rinsed in 2 × SSC after hybridization. Visualization of the probe was performed using a non-radioactive DNA detection kit (Boehringer Mannheim/Indianapolis, IN). The hybrids were detected by enzyme-linked immunoassay using an antibody conjugate (anti digoxigenin alkaline phosphatase conjugate). The colour reaction was obtained after incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt.

RNA characterization. Total RNA was isolated according to the method of Chomczynski and Sacchi[12] using acid-guanidinium thiocyanate-phenol-chloroform extraction.

The same cloned cDNA fragment, encoding the first 170 amino acids of the shrimp chymotrypsin, was used to probe RNA, which was fractionated by denaturing electrophoresis on 1,5% agarose gels in phosphate buffer. Transfer to nylon membranes (Hybond N^+ Amersham, France) was performed as described by the manufacturer. The filter was prehybridized in 50% formamide in the presence of 1 M NaCl, 1% SDS and 0.1 µg/ml yeast RNA at 42 °C during 4 h, and then hybridized overnight at 42 °C in the same solution with the probe randomly labelled with p32 dATP (10^6 cpm/ml, specific activity: 10^9 cpm/µg DNA).

The filters were washed twice in 2 × SSC during 5 min at room temperature, then twice in 2SSC containing 1% SDS, for 30 min at 65 °C, and finally twice in 2 × SSC at room temperature for 15 min. The membranes were autoradiographed for two days using hyperfilm (Amersham) and an intensifying screen. Hepatopancreases from five animals for each molting stage were used and the results subjected to statistical analysis (student's test).

Results

Chymotrypsin was present in *Penaeus vannamei* hepatopancreas in two forms (fig. 1a). These two isoenzymes were previously purified and isolated[7]. They are major constituents of the protein in crude extracts (fig. 1b), as shown by Coomassie staining, using the bands visualized by western blotting as reference (fig. 1c).

Localization of chymotrypsin in cells in the hepatopancreas is shown in figure 2a + 2b. It appears as granules in the cytoplasm of one type of cell. In some cells (arrow) a larger amount of the enzyme was found close to the lumen of the tubule. By in situ hybridization,

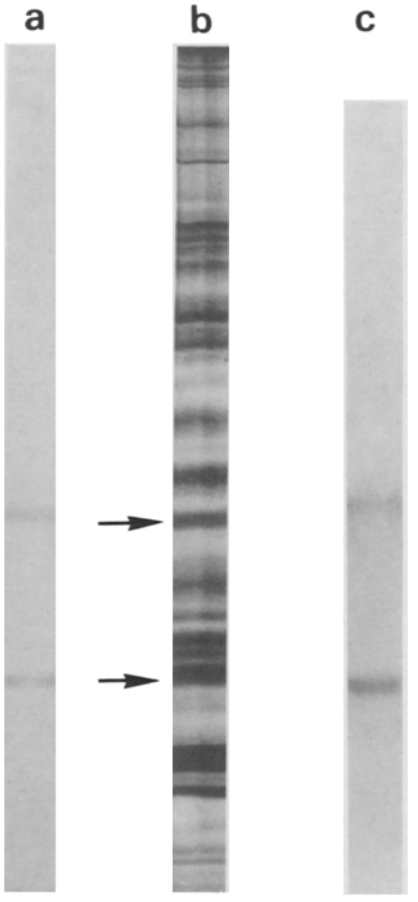

Figure 1. Electrophoresis of purified chymotrypsin in *Penaeus vannamei* hepatopancreas.
a Silver staining of purified chymotrypsin.
b Coomassie staining of hepatopancreas protein crude extract.
c Western blotting of crude extract with a specific guinea pig antichymotrypsin.

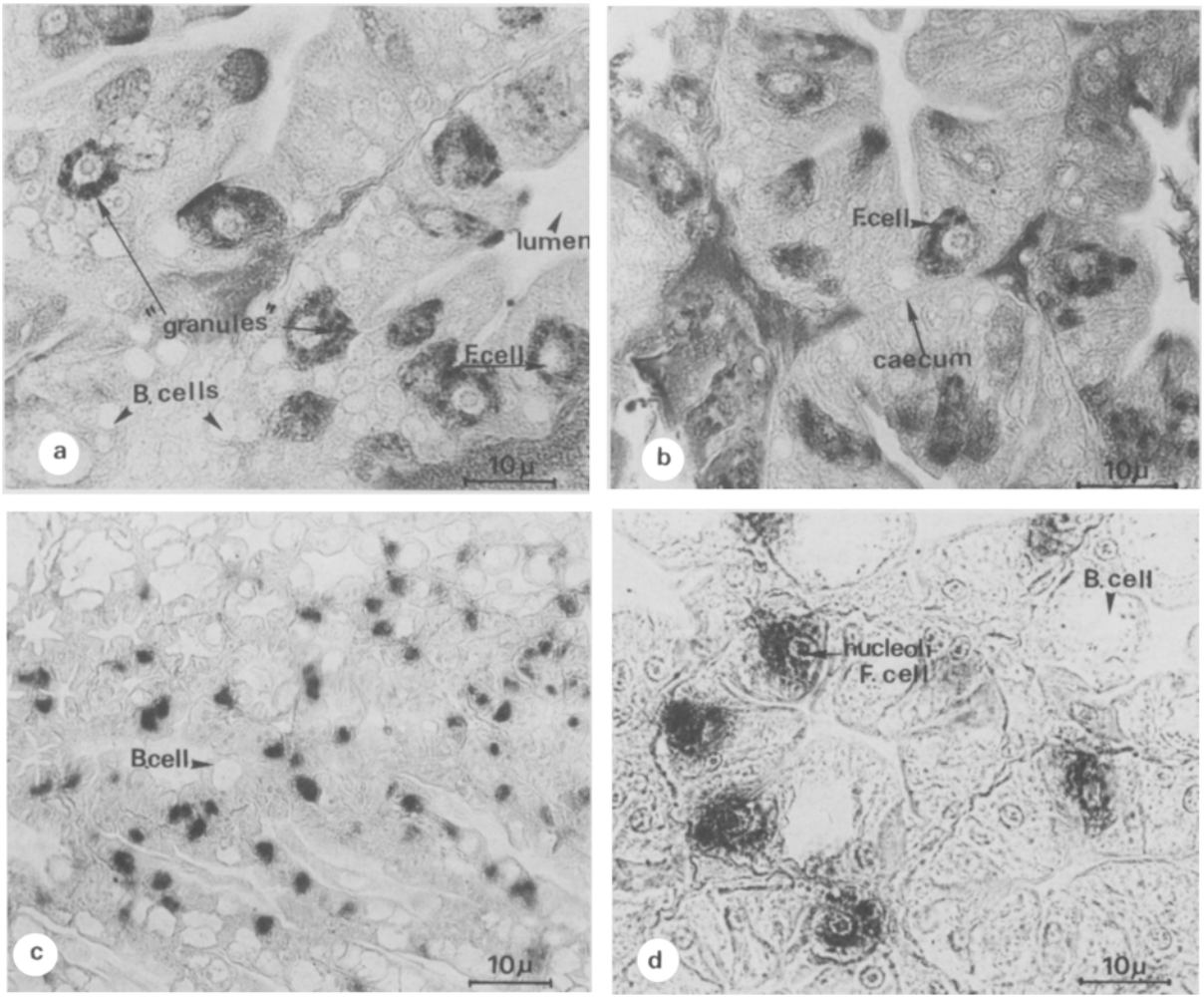

Figure 2. Immunocytochemical localization of chymotrypsin and in situ hybridization of chymotrypsin cDNA in *P. vannamei* hepatopancreas.
a Cross section of hepatopancreatic caeca showing granulated cells containing chymotrypsin (Arrow = nuclei).
b Transversal sections of hepatopancreatic caeca.
c In situ hybridization: only a few cells contained chymotrypsin mRNA.
d Detail of fig. c: B-cells (arrows indicate some of them) do not contain chymotrypsin mRNA.

only one cell type (type F) was found to contain chymotrypsin mRNA (fig. 2 c, d). A faint signal was also seen in nucleoli.

By northern blot hybridization, mRNA was found with a size of 1000 bp (fig. 3). Variations of chymotrypsin specific activities are shown in figure 4a. The maximum was reached at the end of premolt (stage D_2) and the minimum just after the molt (stage A). A second maximum was also observed at B. Northern blot analysis of total RNA extracted from hepatopancreas showed that chymotrypsin RNA transcripts increased in abundance in the early premolt (stage D_1'') and post molt (stage B: fig. 4b).

Discussion

In *Penaeus vannamei*, chymotrypsin synthesis was localized, both by antibody-immunocytochemistry and by in situ hybridization with a specific cDNA probe, in only one cell-type. These cells, which do not contain any vacuoles, are called F-cells. Localization of amylase in F-cells was previously demonstrated using a specific antibody[13]. No mRNA could be detected in other cells such as B-cells, which are characterized by the presence of numerous vacuoles. There is no evidence of release of digestive enzymes by holocrine secretion in B-cells, and no trace of any accumulation of newly synthesized digestive chymotrypsin in these vacuoles. These results confirm those obtained previously in *Palaemon serratus* where, after separation of cells on a Percoll gradient[14], amylase was found to be present only in F-cells.

Our results support the three cell-line concept originally proposed by Ogura[15]. This concept suggested that E-cells give rise independently to F-cells, B-cells and R-cells (absorption cells). This hypothesis was confirmed recently in *Penaeus monodon*[16], where B-cells seem to be

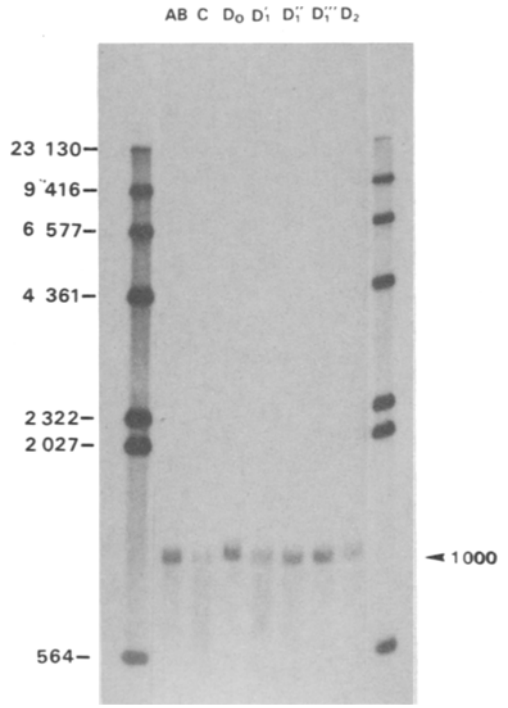

Figure 3. Northern blot of total mRNA: hybridization was realized with a cDNA probe coding for the first two thirds of the protein. Lambda Hind III fragments, end-labelled using the Klenow fragment with the four 32P $dNTP_s$, were used as size standards under the same conditions of electrophoresis (see 'Material and methods').

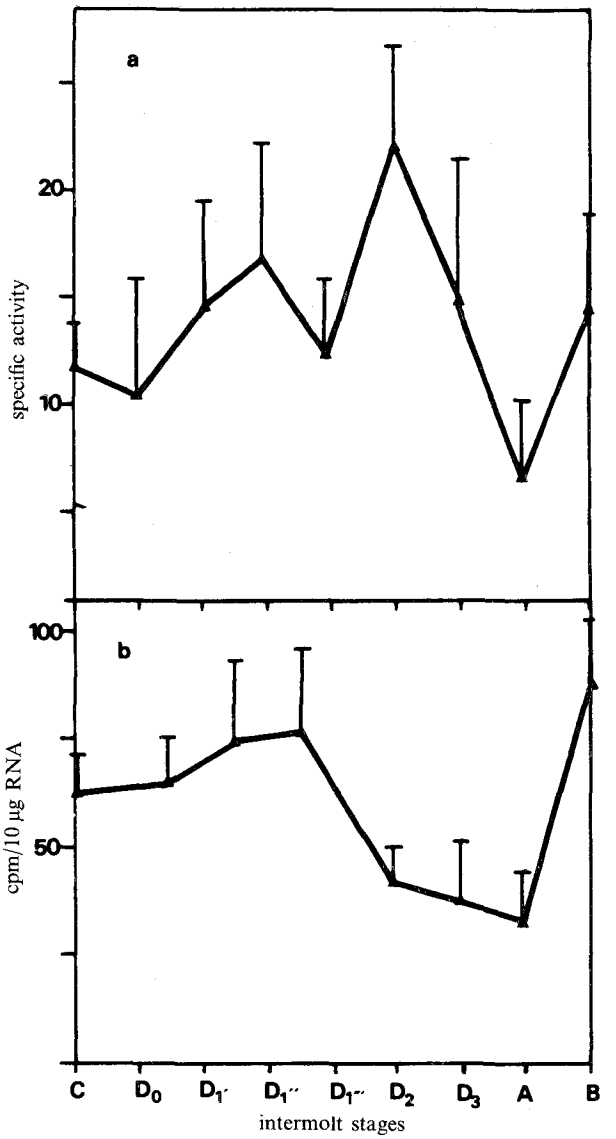

Figure 4. Specific activity of chymotrypsin and variation of mRNA during the molt cycle in *Penaeus vannamei* hepatopancreas.
a Specific activity was measured using SAPPNA as the substrate: 5 animals were used for each value.
b Northern blot: 10 µg of mRNA were transferred onto a nylon membrane and hybridized with a chymotrypsin cDNA probe. Autoradiographic films were exposed and after development, the regions on the nylon membranes corresponding to RNA were cut and counted.

derived directly from embryonic cells, and not from F-cells as suggested by Al Mohanna and Nott[17] for *Penaeus semisulcatus*.

In the hepatopancreas of *P. vannamei*, the variation of the amount of chymotrypsin mRNA follows the variations of protein synthesis described previously in the shrimps *Palaemon serratus* and *P. elegans* [4,18]. In *P. vannamei*, the maximum of specific activity of the chymotrypsin is shifted to the D_2 stage, probably owing to the mode of secretion of the enzyme (holocrine) and its high stability in the digestive juice. The enzyme is continuously secreted during the premolt, while its synthesis occurs at the beginning of premolt.

In *Palaemon serratus*, protein synthesis is minimal at the end of premolt, then begins to increase in the B stage and exhibits another increase in early premolt ($D_1' - D_1''$)[4]. This increase is generally interpreted as the consequence of the increase of ecdysteroids. 20-OH ecdysone is known to stimulate protein synthesis as well as RNA synthesis in the hepatopancreas[19–22]: a two-fold increase is generally observed[4], while changes in muscle protein are higher [23]. This variation can also be observed in mRNA expression. Crab actin mRNA is at a maximum in premolt and postmolt and is very low during intermolt[24], in contrast to shrimp chymotrypsin mRNA. In *P. vannamei* changes are smaller: the increase in mRNA in postmolt is similar to the increase observed in early premolt, but can hardly be due to the ecdysteroid effect, since the 20-OH ecdysone level is very low at this stage. It could be due to expression of preexisting mRNA, as suggested by Skinner[25]. According to this author, synthesis of new ribosomes occurred in premolt and preceded the new synthesis of RNA in stage B[22]. In vertebrates, the regulation of the concentration of mRNA for different digestive enzymes is well known[26], and long-term pancreatic stimulation results in transcriptional regulation. We are now studying regu-

lation of mRNA expression by crustacean secretagogue peptides[18], hormones and metabolites.

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