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EPITHELIAL CYTOLOGY AND FUNCTION IN THE DIGESTIVE GLAND OF *THENUS ORIENTALIS* (DECAPODA: SCYLLARIDAE)

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ABSTRACT

The morphology and epithelial cytology of the digestive gland of the slipper lobster *Thenus orientalis* is described. The primary ducts possess extensive musculature which indicate that they may move fluid into and out of the glands. Immunohistochemical localization of trypsin demonstrated that it is synthesized and secreted solely by the F-cells in the digestive gland. Trypsin is absent from tissue in the oral region, proventriculus, and hindgut, but is present in digestive fluid throughout the alimentary tract, indicating that it has a role in extracellular digestion. Substantial accumulations of lipid in the cytoplasm of R-cells indicate a role in the absorption and storage of digestion products. Residual bodies in the apical cytoplasm are consistent with metal-storing granular inclusions and supranuclear vacuoles in the R-cells of other decapods. Based on the presence of an apical complex of pinocytotic vesicles and subapical vacuoles, an absorptive role for B-cells is evident. The presence of trypsin in the central vacuole is consistent with a digestive role, although our results cannot distinguish whether the enzyme is endogenous (lysosomal versus digestive) or was absorbed from the tubule lumen for excretion.

The decapod digestive gland occupies much of the cephalothoracic cavity and is connected to the ventroposterior region of the pyloric stomach by two small primary ducts. Each duct branches into numerous tubules which comprise the gland. Tubule epithelial cells are differentiated into 4 types: E (embryonic), R (resorptive), B (blisterlike), and F (fibrillar), according to the schema of Jacobs (1928). Their distribution along the tubules was originally thought to be E-cells in the distal tips, young R- and F-cells in the adjacent cell-differentiation zone, mature R-, F-, and B-cells in the midtubular region, and R- and F-cells in the proximal region (Gibson and Barker, 1979; Dall and Moriarty, 1983). However, young B-cells have recently been identified in the differentiation zone of the fresh-water crayfish *Astacus astacus* Linnaeus by Vogt (1993, 1994). This supports a 3-cell lineage, where E-cells give rise independently to F-, B-, and R-cells. This concept is now widely accepted. Cell function remains controversial, due in part to their proteolytic autolysis immediately after death or trauma. This has resulted in reliance on histological and cytological techniques, which, because of the limitations of cell fixation, can be difficult to interpret. Rapid autolysis may also modify intracellular proteins resulting in the misidentification of histochemical data. Recent application of advanced techniques, such as X-ray microanalysis, immunolocalization, and Percoll density-gradient centrifugation (Al-Mohanna and Nott, 1987; Vogt *et al.*, 1989; Toullec *et al.*, 1992) have helped to clarify some cell functions.

From ultrastructural work, it is widely accepted that R-cells are involved in absorption of diffusible metabolites and nutrients and the storage of lipid and glycogen (Al-Mohanna and Nott, 1987; Icely and Nott, 1992). These reserves can be mobilized to provide energy during periods of starvation, molting, and reproduction (Vogt, 1994, 1996). X-ray microanalysis has revealed that they can detoxify heavy metals by their accumulation in an insoluble form in the cytoplasm before excretion from the body (Hopkin and Nott, 1979; Lyon and Simkiss, 1984). Accumulation depends on the type of metal and route of administration (Vogt and Quinitio, 1994). Copper, in particular, is accumulated in high quantities from both hemocyanin metabolism and the environment. The functions of B- and F-cells are not so clear. Based on tracer experiments and cytological features during a feeding cycle, Al-Mohanna and Nott (1986, 1989) believed that the B-cells of *Penaeus semisulcatus* de Haan were nutrient-absorbing cells involved in intracellular digestion. They were able to identify pinocytotic vesicles and the sequential formation of subapical vacuoles, digestive bodies, and the large central vacuole. The presence of intact B-cells in feces of the shore crab *Carcinus maenas*

(Linnaeus) provided evidence of metabolic waste-product excretion from intracellular digestion (Hopkin and Nott, 1980). In contrast, Vogt (1993, 1994) proposed that B-cells are involved in the absorption of remnants of digestion from the tubule lumen for degradation and eventual excretion. He concluded that the subapical vacuoles and central vacuole identified by Al-Mohanna and Nott (1986) are, in fact, lysosomes which facilitate the breakdown process. Although tracer experiments have revealed that the apical complex of B-cells is capable of distinguishing between inert and biological material (Al-Mohanna and Nott, 1986), the precise nature of material being absorbed and possibly degraded remains controversial.

F-cells have extensive rough endoplasmic reticulum, mitochondria, Golgi apparatus, and Golgi vesicles adjacent to the apical brush border. Histochemical staining with mercuric bromophenol blue, indicated that they are involved in the synthesis and secretion of protein (Dall and Moriarty, 1983; Al-Mohanna *et al.*, 1985; Vogt, 1985). Immunohistochemical localization of α-amylase in the F-cells of *Palaemon serratus* Pennant (see Malcoste *et al.*, 1983) and the protease astacin in the F-cells of *A. astacus* (see Vogt *et al.*, 1989) have confirmed that these cells are sites of enzyme synthesis. Van Wormhoudt *et al.* (1995) have since localized chymotrypsin in the F-cells of *Penaeus vannamei* Boone by immunocytochemistry and in situ hybridization using a c-DNA probe. These findings are consistent with detection of α-amylase activity in F-cell homogenates separated by Percoll density-gradient centrifugation (Toullec *et al.*, 1992). F-cells have also been recently found to be involved in the detoxification of iron (Vogt and Quinitio, 1994).

This paper describes the structure and function of tubule epithelial cells (R-, F-, B-) of the digestive gland in the commercially important slipper lobster *Thenus orientalis* Lund. Immunolocalization of purified trypsin of *T. orientalis* was used to examine the role of F-cells in enzyme synthesis and secretion. Other possible sites of trypsin production in the alimentary tract were similarly investigated.

Materials and Methods

Animals, Histology, and Histochemistry.—Adult *T. orientalis* were collected by otter trawling in Cleveland Bay, Townsville, Australia (19°15′S, 146°50′E). They were transferred to recirculating sea-water holding tanks (1.5 × 1 m) and fed every second day. Between 6 and 12 h after feeding, each of 12 intermolt *T. orientalis* were chilled on ice for 20 min prior to carapace removal using bone cutters. This attention to molt stage and feeding status was important, since they influence epithelial cytology, activity, and frequency (Al-Mohanna *et al.*, 1985; Al-Mohanna and Nott, 1989; Icely and Nott, 1992). Digestive glands were removed and fixed for 48 h in marine Bouin's fluid (Winsor, 1984), processed routinely for paraffin wax infiltration, and sections (6 μm) were stained with hematoxylin-eosin and Mallory-Heidenhain. Protein localization in the digestive gland and associated tissues was investigated using mercuric bromophenol blue.

Cytology.—Small pieces (1 mm^3) of digestive gland were fixed in 4% glutaraldehyde in Millipore-filtered sea water (MFSW) for 1 h at 20°C. Tissue was washed in 0.1 M cacodylate buffer pH 7.1, postfixed in 1% osmium tetroxide for 30 min at 20°C, and dehydrated in graded ethanols before embedding in resin. Sections were cut on an LKB Nova Ultramicrotome, mounted on hexagonal 200 copper grids, and stained with acidified, saturated uranyl acetate in 50% ethanol for 7 min followed by 1 min in lead citrate. Sections were examined on a JEOL 2000 FX transmission electron microscope at 80 kV.

Immunohistochemistry.—Trypsin of *T. orientalis* was isolated and purified from digestive gland extract by ion exchange chromatography and gel filtration. Antisera were prepared in male Quackenbush mice following the method of Johnston *et al.* (1995). Specificity and concentration of antitrypsin antiserum of *T. orientalis* were determined by Western Blotting of SDS polyacrylamide gels using a series of antisera dilutions: 1:1,000, 1:5,000, 1:10,000 against crude digestive gland extract of *T. orientalis*. Gels were blotted at 25 V for 2 h at 4°C onto nitrocellulose and the membranes were probed with antitrypsin primary antibodies of *T. orientalis* for 1 h. A 1:5,000 dilution of rabbit antimouse antibodies was then conjugated to horseradish peroxidase (HRP) for 1 h. Immunoreactive polypeptides were visualized using 3 mM 3,3′-diaminobenzidine (DAB) and H_2O_2 (Harlow and Lane, 1988).

Enzyme localization was undertaken using an immunohistochemical technique modified from Foster (1988). The digestive glands of recently fed individuals were cut into 2-mm thin pieces and fixed for 24 h in marine Bouin's fluid (Winsor, 1984). After fixation, the glands were washed for 12 h in 70% ethanol and processed routinely for paraffin wax infiltration. Sections (6 μm) were dewaxed and incubated at room temperature for 1 h in trypsin antiserum of *T. orientalis* diluted 1:200 with 10% sheep sera in 0.1 M phosphate-buffered saline (PBS), pH 7.0. After three 5 min washes in PBS, sections were incubated for 1 h in rabbit antimouse antibodies conjugated to horseradish peroxidase (secondary (2°) antibody) diluted 1:200 in 10% sheep sera in PBS. The sections were then washed again in PBS (3 5-min washes) and developed in 0.05% DAB in 50 mM Tris-HCl pH 7.5 in the presence of 1 μl H_2O_2 ml^{-1} for approximately 10 min (Harlow and Lane, 1988). Sections were counterstained in hematoxylin-eosin (5 min) and blued using Scotts tap-water substitute (2 min).

The following controls tested the specificity of the primary (1°) antibody (trypsin antiserum of *T. orientalis*) to ensure that staining was not due to nonspecific reactions (false positives): (i) 2° antibody + DAB only, to

detect any nonspecific binding of rabbit antimouse antibodies to the section; (ii) DAB only, to detect any endogenous HRP activity in the section; (iii) negative control tissue—adductor muscle of *T orientalis*; and (iv) antigen/antibody control, to verify that no nonspecific binding of trypsin antiserum of *T. orientalis* occurs. That is, the trypsin antiserum of *T. orientalis* is only specific for the antigen trypsin.

Immunolocalization was also applied to other regions of the alimentary tract, including the oral region (membranous lobe; see Johnston, 1994), the proventriculus, and the hindgut.

Results

The digestive gland of *T. orientalis* has two lobes, both connected to the ventroposterior region of the pyloric stomach by a small primary duct (Fig. 1). Each duct is composed of elongated, densely arranged epithelial cells on a basal lamina (Fig. 2). Cells are of one type, with a granular cytoplasm and apical brush border, and stain positive for protein with mercuric bromophenol blue (Fig. 2). Beneath the basal lamina are bands of longitudinal striated muscle and bundles of circular muscle which extend the length of the primary duct.

Four epithelial cells: E, R, F, and B, were identified in the gland tubules with R- and F-cells the most and least abundant, respectively (Fig. 3). F-cells are characterized by a densely granular cytoplasm (Fig. 3), extensive rough endoplasmic reticulum (rER), and numerous mitochondria (Fig. 4). They give a strongly positive response with mercuric bromophenol blue, indicating large quantities of protein. Mature R-cells are characterized by lipid deposits (1–10 μm) throughout their cytoplasm (Fig. 5). Numerous mitochondria are present in the apical cytoplasm (Fig. 5). Golgi apparatus, rER, and smooth endoplasmic reticulum (sER) are also evident in the proximal cytoplasm. Yellow-staining granules occur in the apical cytoplasm in optical sections stained with Mallory-Heidenhain. In electron micrographs, they are identified as intensely osmiophilic residual bodies with electron-dense granular inclusions, ranging in size from 0.8–6 μm (Figs. 5, 6). B-cells are characterized by an apical complex. Invagination of the apical membrane forms large numbers of pinocytotic vesicles in the apical cytoplasm (Fig. 7, 7A). These coalesce to form larger subapical vacuoles that fuse with the single swollen central vacuole (Fig. 7).

Immunolocalization of trypsin of *T. orientalis* (Johnston *et al.*, 1995) established F-cells as the only sites of trypsin synthesis and secretion in the digestive gland of this species. F-cells in sections developed with 3,3′-diaminobenzidine (DAB) stained golden brown, indicating the presence of trypsin in the cytoplasm and microvilli (Fig. 8). This was supported by DAB/nickel-ammonium sulphate enhancement, which stained the F-cells blue/black, positive for trypsin. Comparison with almost identical digestive-gland sections stained with hematoxylin-eosin confirmed the identification of F-cells, since they characteristically stain purple with this stain (Figs. 8, 9). Trypsin is usually concentrated toward the apical membrane (Figs. 10, 11) and is secreted into the lumen. This was verified by the absence of trypsin basally and the intense positive staining of digestive fluid in the lumen (Fig. 10). Infrequent faint staining of the central vacuole contents of B-cells and small concentrations around the periphery and apical membrane indicated that only small concentrations of trypsin were present in these cells (Fig. 11).

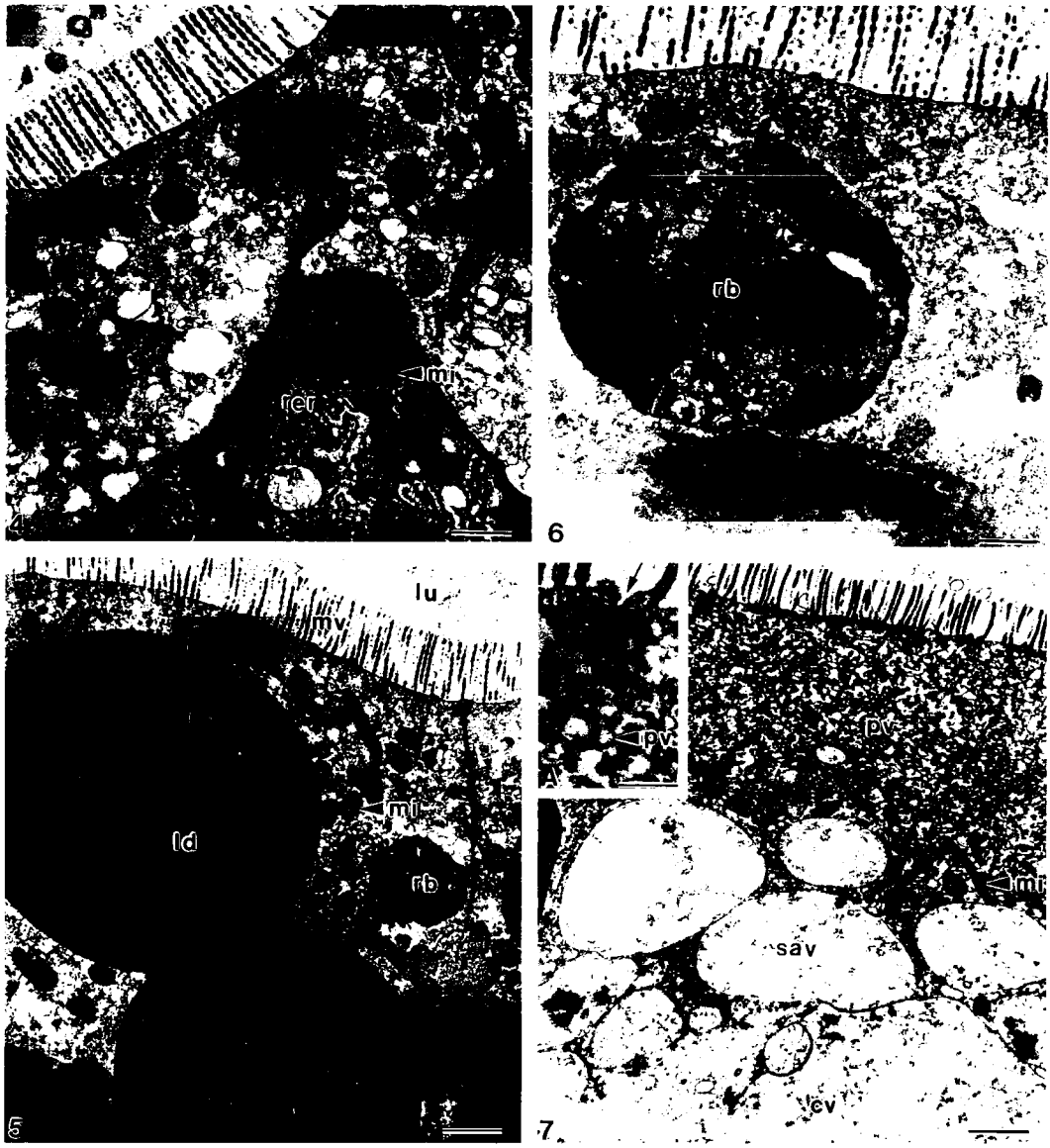
Trypsin was not present in the membranous lobe (oral region), nor in epithelial cells of the cardiac or pyloric stomachs and hindgut, but was present in the digestive fluid of all these regions.

Discussion

The primary ducts of *T. orientalis* possess an extensive system of circular and longitudinal musculature beneath the basal lamina, indicating an active role in the movement of fluids in and out of the gland via peristaltic contraction. Duct epithelial cells are most likely absorptive, based on their microvillous border, although an investigation on the significance of this role is needed. General morphology and epithelial cytology of the digestive gland is consistent with that of other decapods (Al-Mohanna *et al.*, 1985; Caceci *et al.*, 1988; Icely and Nott, 1992; Brunet *et al.*, 1994; Vogt, 1994). F-cells contain considerable rough endoplasmic reticulum and stain positively for protein. This is consistent with immunohistochemical analyses of the digestive gland, which localized trypsin specifically in F-cells. This proved conclusively their role in the synthesis and secretion of this enzyme. These findings support those of Malcoste *et al.* (1983), Vogt *et al.* (1989), and Van Wormhoudt *et al.* (1995) who used immunohistochemistry, immunofluorescence, and in situ hybridization to detect α-amylase,

Figs. 1–3. Photomicrographs of the digestive gland and primary ducts of *Thenus orientalis*. Fig. 1. Transverse section (6 µm) through the ventroposterior part of the pyloric stomach, showing the location of the digestive gland primary ducts. Scale = 1 mm. dg = digestive gland; fp = filter press; pd = primary duct. Fig. 2. Epithelial cells of the primary duct, stained with mercuric bromophenol blue. Scale = 45 µm. cm = circular muscle; ct = connective tissue; lm = longitudinal muscle; lu = lumen; n = nucleus; sc = secretory cell. Fig. 3. Transverse section through a single digestive gland tubule, showing the types of epithelial cells (R, F, B). Note the obvious central vacuole in the B-cells. Scale = 50 µm. b = B-cell; ct = connective tissue; cv = central vacuole; f = F-cell; lu = lumen; n = nucleus; r = R-cell.

astacin, and chymotrypsin, respectively, in the F-cells of various decapods. The apical accumulation of trypsin, its release from the cell, and presence in the digestive fluid of the tubules, oral region (membranous lobe), proventriculus, and anterior hindgut, indicate that the F-cells of *T. orientalis* secrete this enzyme for extracellular digestion. Vogt *et al.* (1989) provided cytological evidence for the transport of protease vacuoles of *Astacus* from the dictyosomes to the cell apex, where they are discharged into the gland lumen by exocytosis. The absence of secretory granules from the F-cell cytoplasm, despite the presence of trypsin, suggests that they develop only a short time after feeding. This is consistent with observations on *P. semisulcatus,* in which F-cell granules were present only 30 min to 1 h after feeding (Al-Mohanna and Nott, 1987).



Figs. 4–7. Transmission electron micrographs of the digestive gland of *Thenus orientalis*. Fig. 4. Two R-cells and an F-cell. In the F-cell note the extensive rER and numerous mitochondria. Scale = 0.8 µm. mi = mitochondria; rer = rough endoplasmic reticulum. Fig. 5. R-cell with lipid deposits and residual body in the apical cytoplasm. Scale = 1.8 µm. ld = lipid deposit; lu = lumen; mi = mitochondria; mv = microvilli; rb = residual body. Fig. 6. R-cell with a residual body. Scale = 0.8 µm. rb = residual body. Fig. 7. Characteristic apical complex of a B-cell comprising pinocytotic vesicles, subapical vacuoles, and the large central vacuole. Note fusion of the subapical vacuoles with the central vacuole. Scale = 1.7 µm. A, (inset) invagination of the apical membrane (arrow) and numerous pinocytotic vesicles within the apical cytoplasm of a B-cell. Scale = 0.5 µm. cv = central vacuole; mi = mitochondria; pv = pinocytotic vesicle; sav = subapical vacuole.

The absence of trypsin from other regions of the alimentary tract indicates that F-cells are the only sites of digestive enzyme synthesis in this species. This is supported by biochemical studies on other decapods, in which the digestive glands were identified as the sites of greatest digestive enzyme activity compared with all other regions of the alimentary tract (Tsai *et al.,* 1986; McClintock *et al.,* 1991).

Substantial lipid accumulations throughout the R-cell cytoplasm indicate their role in the

Figs. 8–11. Photomicrographs of the digestive gland of *Thenus orientalis*. Figs. 8, 9. Longitudinal serial sections (6 μm) of a digestive gland tubule, confirming that trypsin is localized only in F-cells (arrows). 8, Immunohistochemistry: the positive reaction of F-cells to trypsin with DAB. Note that the reaction product in the hemolymph space is due to capillary effects of the hemal space during dissection. Scale = 70 μm. 9. The characteristic densely staining F-cells with hematoxylin-eosin. Scale = 70 μm. Figs. 10, 11. Transverse section of a digestive gland tubule, illustrating the positively reacting F-cell cytoplasm, microvillous brush border, and digestive fluid with DAB. 10, Note the secretion of trypsin into the lumen from the F-cell in which trypsin is absent from its basal cytoplasm (arrow). Scale = 40 μm. 11, Note the apical accumulation of trypsin and the positively staining contents of the B-cell central vacuole. Reaction product in the hemolymph space is due to capillary effects of the hemal space during dissection. Scale = 40 μm. cv = central vacuole; df = digestive fluid; f = F-cell.

absorption and storage of digestion products, which is consistent with all decapods (Gibson and Barker, 1979; Caceci *et al.*, 1988; Brunet *et al.*, 1994). Nutrient absorption occurs via molecular transport across the apical membrane and is closely associated with an apical complex of sER tubules and mitochondria (Vogt, 1994). The basal tubule system of sER and rER in *T. orientalis* is likely to be involved in the delivery of nutrients to other organs via the hemolymph (Vogt, 1994). Residual bodies are consistent with the metal-storing supranuclear vacuoles of *P. semisulcatus* (see Al-Mohanna and Nott, 1987, 1989) and granular inclusions of the amphipod *Corophium volutator* (Pallas) (see Icely and Nott, 1980) and *A. astacus* (Vogt, 1994). This substantiates the role of R-cells of *T. orientalis* in the detoxification of metals by storing them in an insoluble form.

The presence of an apical complex of numerous pinocytotic vesicles, subapical vacuoles, and a large central vacuole (Fig. 7) indicates that the B-cells of *T. orientalis* are involved in absorption of fluid and small particles from the lumen of the digestive gland, a characteristic of all decapod B-cells (Lyon and Simkiss, 1984; Al-Mohanna and Nott, 1986; Vogt, 1993). Immunohistochemical detection of trypsin in the central vacuole of B-cells of *T. orientalis* suggests that an intracellular digestive role is likely. This role is further supported by the detection of digestive enzyme activity in other crustacean B-cells, including proteases in the amphipod *C. volutator* (see Icely and Nott, 1985), and α-amylase and trypsin in the crayfish *Orconectes rusticus* (Girard) (DeVillez and Fyler, 1986) and the crab *C. maenas* (see Loret and Devos, 1995). However, it is not clear whether these enzymes are endogenous to B-cells and, therefore, possibly lysosomal enzymes, or have been absorbed from the tubule lumen for excretion during the later stages of cell development. Vogt (1994, 1996) believed that B-cells clear the tubule lumen of remnants of digestion, including expended digestive enzymes. Further investigation on the nature of absorbed material and the presence of such enzymes in the B-cell cytoplasm and central vacuole are needed to clarify whether B-cells of *T. orientalis* have a digestive and/or excretory role. Nevertheless, the lack of an apical accumulation of trypsin in the B-cell and its confinement in the central vacuole refutes suggestions that B-cells are a source of enzymes for extracellular digestion (Loizzi, 1971; Gibson and Barker, 1979; Caceci *et al.*, 1988).

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