

### 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).