Mar. Biotechnol. 5, 45–57, 2003
DOI: 10.1007/s10126-002-0049-2

MARINE BIOTECHNOLOGY

Characterization of the Adhesive from Cuvierian Tubules of the Sea Cucumber *Holothuria forskali* (Echinodermata, Holothuroidea)

Sonia DeMoor,[1] J. Herbert Waite,[2] Michel Jangoux,[1,3] and Patrick Flammang[1,*]

[1]*Marine Biology Laboratory, University of Mons-Hainaut, 6 Avenue du Champ de Mars, B-7000 Mons, Belgium*
[2]*Marine Science Institute and Molecular, Cellular and Developmental Biology Department, University of California, Santa Barbara, CA 93106, U.S.A.*
[3]*Marine Biology Laboratory, Free University of Brussels, B-1050 Brussels, Belgium*

Abstract: Sea cucumbers possess a peculiar specialized defense system: the so-called Cuvierian tubules. The system is mobilized when the animal is mechanically stimulated, resulting in the discharge of a few white filaments, the tubules. Their great adhesivity, combined with their high tensile strength, allows Cuvierian tubules to entangle and immobilize potential predators. The cellular origin and composition of the Cuvierian tubule adhesive were investigated in the species *Holothuria forskali* by studying prints left on the substratum after mechanical detachment of the tubule. Polyclonal antibodies raised against tubule print material were used to locate the origin of tubule print constituents in the tubules. Extensive immunoreactivity was detected in the secretory granules of mesothelial granular cells, suggesting that their secretions make up the bulk of the adhesive material. Tubule print material consists of 60% proteins and 40% carbohydrates, a composition that is unique among the adhesive secretions of marine invertebrates. Although it is highly insoluble, a small fraction of this material can be extracted using denaturing buffers. Electrophoretic analysis of the extracts revealed that it contains about 10 proteins with apparent molecular masses ranging from 17 to 220 kDa and with closely related amino acid compositions, rich in acidic and in small side-chain amino acids. The adhesive from the Cuvierian tubules of *H. forskali* shares these characteristics with many marine bioadhesives and structural biomaterials.

Key words: Defense system, underwater adhesion, protein-based material, immunocytochemistry, polyacrylamide gel electrophoresis.

INTRODUCTION

The fact that many marine invertebrates produce adhesives that act in the presence of water has aroused increasing scientific and technological attention because such adhesives are sorely needed for applications in underwater construction and in medicine and dentistry (Strausberg and Link, 1990; Taylor and Waite, 1997). Most studies of invertebrate adhesive systems have focused on the characterization of the permanent adhesives from sessile organisms such as mussels, barnacles, or tube-dwelling worms (e.g., see Naldrett and Kaplan, 1997; Taylor and Waite, 1997; Kamino et al., 2000). This is because in these organisms the adhesives are secreted

Received January 28, 2002; accepted March 12, 2002
Corresponding author: telephone +32-65-373439; fax +32 65 373434; e-mail: Patrick.Flammang@umh.ac.be

as a fluid and then gradually solidify to form a cement possessing high adhesive and cohesive strengths (Walker, 1987). However, the adhesive biochemistry of sessile invertebrates has proved to be complex: not only do the adhesives consist of a blend of several different proteins (Taylor and Waite, 1997; Kamino et al., 2000; Waite and Qin, 2001), but their hardening relies on enzymatic modifications of the secreted proteins (Dougherty, 1997; Hansen et al., 1998). In the search for simpler bioadhesion models, other adhesive systems from marine invertebrates are therefore also worth investigating. One of these systems is the defensive reaction occurring in some species of holothuroid echinoderms (sea cucumbers), which involves the so-called Cuvierian tubules (Flammang, 1996; Hamel and Mercier, 2000).

Several species of holothuroids, all belonging exclusively to the family Holothuriidae, possess this peculiar specialized defense system. It is mobilized when the animal is mechanically stimulated, resulting in the discharge of a few white filaments, the tubules. In seawater the expelled tubules lengthen considerably and become sticky upon contact with any object (VandenSpiegel and Jangoux, 1987). The great adhesivity of the outer tubule epithelium, combined with the high tensile strength of their collagenous core, makes Cuvierian tubules very efficient at entangling and immobilizing most potential predators (VandenSpiegel and Jangoux, 1987; Hamel and Mercier, 2000).

The adhesive properties of Cuvierian tubules are remarkable in several respects. Their tenacity (defined as force per unit area of attachment) measured on paraffin wax, (Zahn et al., 1973) is superior to that of mussel byssus on this particular substratum (Young and Crisp, 1982). Moreover, their adhesive bonds are formed in less than 10 seconds (Zahn et al., 1973). Paradoxically, no biochemical study has been done on the adhesive of Cuvierian tubules, the only published work dealing with collagen (Bailey et al., 1982). All the information available on the nature and composition of the adhesive material thus comes from histochemical studies and from studies of the influence of various reagents on the tenacity. Histochemical tests performed on the Cuvierian tubules of *Holothuria leucospilota* (Endean, 1957) and *H. forskali* (VandenSpiegel and Jangoux, 1987) indicated that the material enclosed in the secretory granules of the adhesive cells of the outer epithelium (i.e., the mesothelium) tends to be proteinaceous. Tests on the influence of various reagents on the tenacity of Cuvierian tubules of *H. forskali* have given convergent results, tubule adhesion being reduced by several proteolytic enzymes (Müller et al., 1972; Zahn et al., 1973).

The aim of this work was to study the composition and cellular origin of prints from the Cuvierian tubules of *Holothuria forskali*. Tubule prints are patches of material left on a substratum after a tubule has adhered to it and then been mechanically detached. The prints thus represent a starting material that is presumably considerably enriched in adhesive secretions. The present study provides the chemical composition of tubule print material (TPM) as well as the initial characterization of the major proteins present in this material. Moreover, the cellular origin of tubule print constituents in the tubules was investigated by immunohistochemistry and immunocytochemistry using new antisera raised against TPM.

Materials and Methods

Individuals of *Holothuria forskali* (Delle Chiaje, 1823) were collected at depths ranging from 10 to 20 m by scuba diving in Banuyls-sur-Mer (Pyrénées-Orientales, France). They were transported to the Marine Biology Laboratory of the University of Mons-Hainaut, where they were kept in a marine aquarium with closed circulation (14°C, 35‰ salinity).

Collection of Tubule Print Material

Discharge of the Cuvierian tubules was induced mechanically by pinching the dorsal integument of sea cucumbers with forceps. The expelled tubules (usually between 10 and 15) were collected in clean glass petri dishes, 15 cm in diameter, filled with artificial seawater. After the tubules adhered firmly on the bottom of the petri dishes, their collagenous cores were detached manually using fine forceps. The petri dishes were then thoroughly rinsed in distilled water and freeze-dried. The lyophilized TPM was scraped off using a glass knife and stored at −20°C.

Electrophoretic Analysis

Weighed quantities of freeze-dried TPM were suspended in reducing sample buffer at a ratio of 100 µl of buffer per 1 mg of TPM, and homogenized in a 1-ml ground glass tissue-grinding tube and pestle (Kontes Scientific Glassware/Instruments, Vineland, N.J.). The reducing sample buffer was composed of 0.063 M Tris-HCl (pH 8.8), 2% sodium dodecyl sulfate (SDS), 1.6 M urea, and 0.1 M dithiothreitol (DTT). The homogenized suspension was heated 10 minutes at 95°C and, after cooling, centrifuged at 16,000 *g* for

10 minutes. The supernatant was collected and kept at −20°C until used. The pellet was reextracted with the same buffer at a ratio of 40 µl of buffer per 1 mg of TPM and centrifuged, and the supernatant was pooled with the one obtained from the first extraction. Other buffers were also tested for their ability to solubilize TPM. Factors that were varied included pH and the presence of denaturing reagents such as urea, SDS, and DTT.

Polyacrylamide gel electrophoresis (PAGE) was used to assess the number and mobility of extracted proteins. Fractions of the TPM extract were electrophoresed on mini slab gels (Mini-Protean II Cell, Bio-Rad, Hercules, Calif.) using the Tricine-SDS-PAGE discontinuous system (Schägger and Von Jagow, 1987). Gels were stained with Bio-Safe Coomasie G-250 Stain (Bio-Rad).

Tubule print proteins were also analyzed by 2-dimensional electrophoresis. Isoelectric focusing (IEF) was used as the first dimension, using precast immobilized pH gradient (IPG) strips, followed by SDS-PAGE as the second dimension. Samples of TPM were extracted in a sample buffer containing 8 M urea, 2% CHAPS, 2% IPG buffer, and 20 mM DTT. Soluble proteins were applied to IPG strips (Immobiline DryStrip Gels, Amersham Pharmacia Biotech, Piscataway, N.J.), with a 3 to 10 pH gradient and focused for 360 minutes at 3500 V (2 mA). Afterward, proteins were run on a 12.5% SDS polyacrylamide gel for 300 minutes at 200 V (60 mA). Finally, the gels were stained with Coomassie blue G-250.

Analytical Methods

All analyses were performed on triplicate samples (1 mg each) of freeze-dried TPM. For inorganic residue analysis, samples were ashed in a muffle furnace for 3 hours at 550°C. The mass of the residual ash, expressed as a percentage of the sample mass, was taken as the total inorganic residue.

The lipid determination was based on the method of Holland and Gabbott (1971), using tripalmitine as the standard. Neutral sugars were determined by the phenol-sulfuric method, as described by Dubois et al. (1956). D-Galactose was used as the standard.

For amino acid analysis, samples were suspended in 6 M HCl with 5% phenol and hydrolyzed under vacuum in sealed tubes for 24 hours at 110°C. Amino acid concentrations were measured on a Beckman 120C or a Beckman 6300 amino acid analyzer (Beckman Instruments, Fullerton, Calif.). Total protein was estimated by adding the masses of the individual amino acids as calculated by amino acid analysis and expressing the total mass of the amino acids as a percentage of the dry mass of the sample.

Proteins separated by electrophoresis were blotted onto polyvinylidene difluoride (PVDF) membranes (Sequi-blot, Bio-Rad) using 90 mM Tris-borate, 2.5 mM EDTA, 0.1% SDS, and 25% methanol as transfer buffer. Running conditions were 200-mA constant current for 90 minutes. After the transfer, the membranes were stained with 0.025% Coomassie Blue R-250 in 40% methanol and destained with 50% methanol. The most prominent bands were excised, further destained with 100% methanol, and allowed to dry. Proteins were hydrolyzed as described above, and their amino acid compositions were determined on a Beckman 6300 Autoanalyzer (Beckman Instruments).

Antiserum Production and Characterization

Polyclonal antisera to the TPM were raised in 2 female rabbits (R3 and R4). The immunization schedule was as follows. About 1 mg of TPM was suspended in 0.75 ml of phosphate-buffered saline (PBS) and emulsified with an equal volume of complete Freund's adjuvant (Difco, Detroit, Mich.). The rabbits were then injected subcutaneously at several sites in the back, with a total volume of 0.5 ml of the emulsion to R3 and 1 ml to R4. Four boosts were prepared identically but with incomplete Freund's adjuvant (Difco) and administered, respectively, 30, 60, 90, and 120 days after the first injection. Antisera were harvested 3 times: 14 days after the second boost (AS1), 25 days after the third boost (AS2), and 35 days after the fourth boost (AS3). Preimmune sera (PS) were obtained by bleeding the rabbits just before the first injection of TPM.

An enzyme-linked immunosorbent assay (ELISA) was used for the comparison of specific antibody titers between the different antisera and preimmune sera. The TPM was digested with trypsin (Sigma, St Louis, Mo.) under the following conditions. About 1 mg of TPM was suspended in 2 ml of 0.1 M sodium bicarbonate buffer, pH 8.2, at a protease-protein ratio of approximately 1:100, under constant stirring at 22° to 24°C for 180 minutes. The progress of the digestion was monitored at 30-minute intervals by centrifuging the suspension at 3000 *g* for 5 minutes and measuring the absorbance of the supernatant at 280 nm. The digestion was terminated by centrifuging the suspension at 3000 *g* for 15 minutes and freezing the supernatant. The ELISA was carried out according to the following procedure at 37°C. The soluble fraction of the trypsinised TPM, diluted 300 times in 0.1 M carbonate-bicarbonate buffer, pH 9.2, was loaded (200 µl

per well) into 96-well microtitration plates (Nunc Maxisorb Immunoplates, Rochester, N.Y.), and incubated overnight. The plates were washed several times with 300 µl of PBS containing 0.05% Tween 20 and 0.1% bovine serum albumin (PBS-Tween-BSA1) per well. Each antiserum (200 µl) and the corresponding preimmune serum (200 µl) (serial dilution in PBS) were then added and incubated for 1 hour. After a second series of washes with PBS-Tween-BSA1, 200 µl of alkaline-phosphatase-conjugated sheep antirabbit immunoglobulins (Roche Molecular Biochemicals, Basel, Switzerland), diluted 1:1000 in the same buffer, were added and incubated for an additional hour. Then the plates were washed thoroughly with PBS-Tween-BSA1 and incubated for 10 minutes with 10 mM diethanolamine buffer (pH 9.5) containing 0.5 mM $MgCl_2$. Finally, 200 µl of the enzyme substrate (a 0.1% solution of *p*-nitrophenyl phosphate in 10 mM diethanolamine buffer, pH 9.5, 0.5 mM $MgCl_2$) was added. After 15 minutes at room temperature, the reaction was stopped by the addition of 0.4 M EDTA and the plates were read at 405 nm using a Titertek Multiskan plate reader.

To investigate the carbohydrate content of the antigenic determinants, the soluble fraction of the trypsinized TPM was mildly oxidized with 10 mM periodate in 50 mM sodium acetate buffer, pH 4.5, for 1 hour at room temperature in the dark (Woodward et al., 1985). It was subsequently tested by ELISA as described above using antisera diluted 1:1000.

Immunohistochemistry and Immunocytochemistry

For light microscopy, 2 different kinds of fixative were used. Cuvierian tubules were fixed either in Bouin's fluid (Gabe, 1968) overnight at 22° to 24°C and stored in 70% ethanol, or in 4% paraformaldehyde in 0.1 M cacodylate buffer containing 0.05% $CaCl_2$ for 3 hours at 4°C and washed overnight in 0.2 M cacodylate buffer. Both sets of tubules were then dehydrated in graded ethanol, embedded using a routine method in paraffin wax, sectioned at 8 µm, and mounted on clean glass slides. A few sections were stained with Alcian blue (pH 2.6) coupled with Mayer's hemalum and phloxine (Gabe, 1968); the others were subjected to an indirect immunofluorescence method according to the following protocol. Tubule sections were permeabilized in PBS with 0.25% Triton X-100 for 1 hour and preincubed for 30 minutes in 10% normal swine serum (DAKO A/S, Glostrup, Denmark) in PBS. Primary antisera, diluted 1:500 in PBS containing 1% Tween 20 and 3% BSA (PBS-Tween-BSA2), were then applied for 1 hour at room temperature. After several washes in PBS, the sections were incubated for 1 hour in fluorescein-isothiocyanate-conjugated (FITC) swine antirabbit immunoglobulins (DAKO A/S) diluted 1:50 in PBS-Tween-BSA2. Following a final wash in PBS, they were mounted in Vectashield mounting medium (Vector, Burlingame, Calif.) and observed with a Leica TCS 4D confocal laser scanning microscope.

Table 1. Biochemical Composition of the Cuvierian Tubule prints of *Holothuria forskali*

Chemical	%
Protein	59
Lipid	0
Neutral sugars	39
Inorganic residue	11

For transmission electron microscopy, Cuvierian tubules were fixed for 3 hours at 4°C in 3% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.8; adjusted to 1030 mOsm with NaCl). Then they were rinsed in cacodylate buffer and postfixed for 1 hour in 1% OsO_4 in the same buffer. Another set of tubules was also fixed with paraformaldehyde as described for light microscopy. Both sets of tubules then were dehydrated in graded ethanol and embedded in Spurr's resin. Ultrathin sections (about 90 nm) were cut with a Leica UCT ultramicrotome equipped with a diamond knife and collected on gold grids. The tubule sections were blocked with 10% normal goat serum (BioCell, Cardiff, U.K.) in PBS for 30 minutes and incubated overnight at 4°C with the primary antisera diluted 1:1000 in PBS-Tween-BSA2. After rinsing in PBS, the sections were immunogold-stained for 1 hour at room temperature in goat antirabbit immunoglobulins conjugated to 15-nm gold particles (Sigma) diluted 1:100 in PBS-Tween-BSA2. Following several washes in PBS, and finally in distilled water, they were further stained with aqueous uranyl acetate and lead citrate and observed using a Zeiss LEO 906E transmission electron microscope.

For both light and transmission electron microscopy, 3 types of control were carried out: substitution of the primary antiserum with PBS-Tween-BSA2, substitution of the primary antiserum with the corresponding preimmune sera diluted 1:500 in PBS-Tween-BSA2, and saturation of the antibodies by an excess of antigen. For this last control, about 1 mg of TPM was suspended in 1 ml of the antisera diluted 1:500 in PBS-Tween-BSA2 and stirred for 1 hour at room temperature. This suspension was then centrifuged at

Table 2. Amino acid Compositions of Tubule Prints from the Cuvierian Tubules of the Sea Cucumber *Holothuria forskali* and of Selected Proteins Extracted from This Material*

Amino acid	TPM	20 kDa	33 kDa	45 kDa	56 kDa	63 kDa	95 kDa
Asx	78	79	89	89	87	91	93
Thr	87	74	79	74	67	70	67
Ser	60	77	72	70	67	67	65
Glx	91	106	118	131	134	126	129
Pro	55	44	46	70	76	77	75
Gly	266	224	182	167	155	157	160
Ala	88	76	71	76	67	70	69
Cys/2	14	ND	ND	ND	ND	ND	ND
Val	38	45	55	57	68	66	62
Met	10	11	7	8	12	7	8
Ile	28	35	40	35	39	37	39
Leu	37	57	62	55	59	59	58
Tyr	20	31	35	33	34	34	34
Phe	20	31	37	31	30	33	33
His	26	25	22	20	19	19	22
Lys	31	33	34	32	37	37	36
Arg	50	53	53	52	51	52	51

*Values are in residues per thousands. ND indicates not determined.

16,000 *g* for 10 minutes, and the supernatant was collected and used for the immunolabeling.

Results

Biochemical Composition of the Tubule Prints

Results of the chemical analysis of the TPM are presented in Table 1. Tubule prints were made up essentially of organic material, the inorganic residue (ash) amounting only to 11% dry weight. Total protein content calculated from the amino acid analysis was 59% dry weight. The carbohydrate content was 39% dry weight. No lipid was detected. The amino acid composition of the TPM is given in Table 2. It is mostly distinguished by the high proportion of glycine residues, which represent more than one quarter of all the residues. In a few samples, hydroxyproline was detected and accounted for up to 3.40% of the amino acid residues. It was absent, however, in most samples.

Characterization of the Proteins Extracted from the Tubule Prints

Among the conditions that were tested to extract TPM, protein solubilization was improved in basic rather than acidic buffers. Urea, SDS, and reducing agents increased the extraction of tubule print proteins, especially when they were used together. Buffers that increased extraction did so by extracting more of the same proteins; i.e., the banding pattern in Tricine-SDS-PAGE did not change. Tubule print proteins were therefore best extracted using the reducing sample buffer. However, even in this buffer, less than 10% of the TPM was solubilized. The Tricine-SDS-PAGE analysis revealed that the extracts contained about 10 different proteins (Figure 1). Their apparent molecular masses were approximately 17, 20, 33, 45, 56, 63, 95, 110, 120, 140, and 220 kDa. There was some variability between the different extracts, and only a few samples contained the 10 proteins. Two proteins were especially inconsistent in extraction, the 33-kDa and the 220-kDa proteins. There was also a component that was too large to enter the running gel.

All the proteins extracted with the IEF sample buffer were acidic. Among those that could be identified unequivocally, the proteins of 33, 45, 63, and 110 kDa had IEF points of about 3, 4.5, 5.5, and 5, respectively.

The proteins that were analyzed (Figure 1) had similar amino acid compositions (Table 2). They were all rich in glycine (16–22%) and in acidic residues (19–22%). The method of Marchalonis and Weltman (1971) gave relatedness values (SΔQ) ranging from 1 to 75 for the comparisons among the different proteins; any value lower than

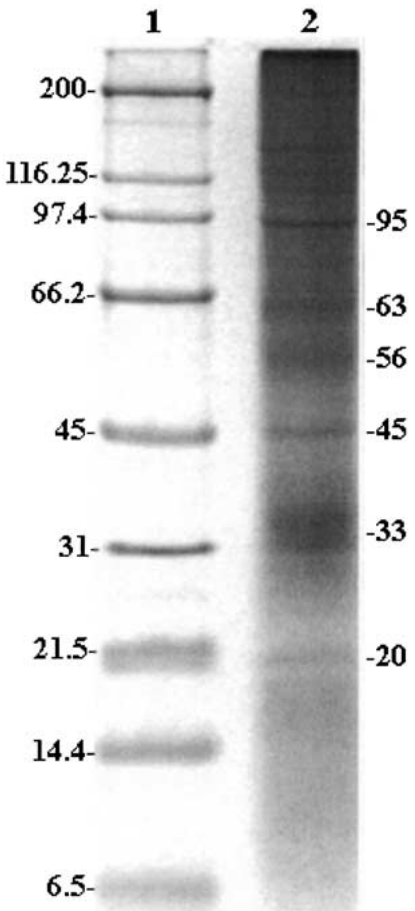

Figure 1. Proteins from the Cuvierian tubules of *Holothuria forskali*. Lane 1, molecular weight markers; Lane 2, proteins from tubule prints extracted with the reducing sample buffer. Numbers on the right side of lane 2 indicate the apparent molecular weights of the proteins for which an amino acid composition has been obtained. The samples were separated by SDS-PAGE (10% polyacrylamide gel and a Tris-Tricine buffer system). The gel was stained with Coomassie Blue G-250 after electrophoresis.

100 suggests relatedness. Close relatedness between the most prominent tubule print proteins was also corroborated by the occurrence in each amino acid analysis of 2 undetermined peaks, one of which could correspond to phosphoserine. These 2 peaks together accounted for 2% to 4% of the total amino acids.

Antiserum Characterization

The high insolubility of the freeze-dried Cuvierian tubule prints in water and nondenaturing buffers is incompatible with quantitative assays for specific interactions. Therefore, to estimate the antibody titer in each serum, TPM was digested with trypsin to allow the collection of a soluble fraction that could be used in an ELISA. Trypsin was used because it had previously been shown to have a strong inhibitory effect on Cuvierian tubule adhesion (Müller et al., 1972). Moreover, owing to the specificity of trypsin for cleavage after lysine and arginine residues, it was expected that the peptides generated would be large enough to be recognized by the specific antibodies. This was found to be the case, and dilution curves were constructed (Figure 2, A). The levels of the specific antibodies were always much higher in the antisera than in the preimmune sera. Among the antisera, however, no differences were noted in these levels either between the 2 rabbits (R3 and R4) or between the 3 successively collected antisera (AS1, AS2, and AS3) (Figure 2, A).

The pretreatment of the trypsin-digested TPM with sodium periodate reduced the immunoreactivity by 15% to 25% compared with untreated samples, depending on the antiserum considered (Figure 2, B). These data suggest that the antigenic determinants of the tubule prints are a combination of protein and polysaccharide moieties, but with a proteinaceous predominance. However, it is noteworthy that, for both rabbits, the ratio of antibodies directed against protein epitopes to antibodies directed against carbohydrate epitopes increased from the first bleed (AS1) to the last one (AS3) (Figure 2, B).

Immunohistochemistry

The presence and location of tubule print constituents in the Cuvierian tubules of *Holothuria forskali* was investigated by immunofluorescent labeling using light microscopy. Whatever the fixative used, there was a strong and reproducible immunolabeling of the tubules with the antisera, whereas there was no labeling with the preimmune sera and with the antisera pretreated with excess antigen.

Quiescent Cuvierian tubules are hollow organs consisting of a narrow central lumen surrounded by a thick wall (VandenSpiegel and Jangoux, 1987). The tubule wall is made up of an outer mesothelium and an inner epithelium encompassing a thick connective tissue sheath (Figure 3, A). This sheath includes muscle fibers that separate it into a thick, collagen-rich inner layer and a much thinner outer layer. The mesothelium is the tissue layer involved in the adhesive process. In quiescent tubules, it is highly folded along the long axis of the tubule. It is in this tissue layer that the immunoreactivity was the strongest, the whole epithelium being extensively labeled (Table 3; Figure 3, B).

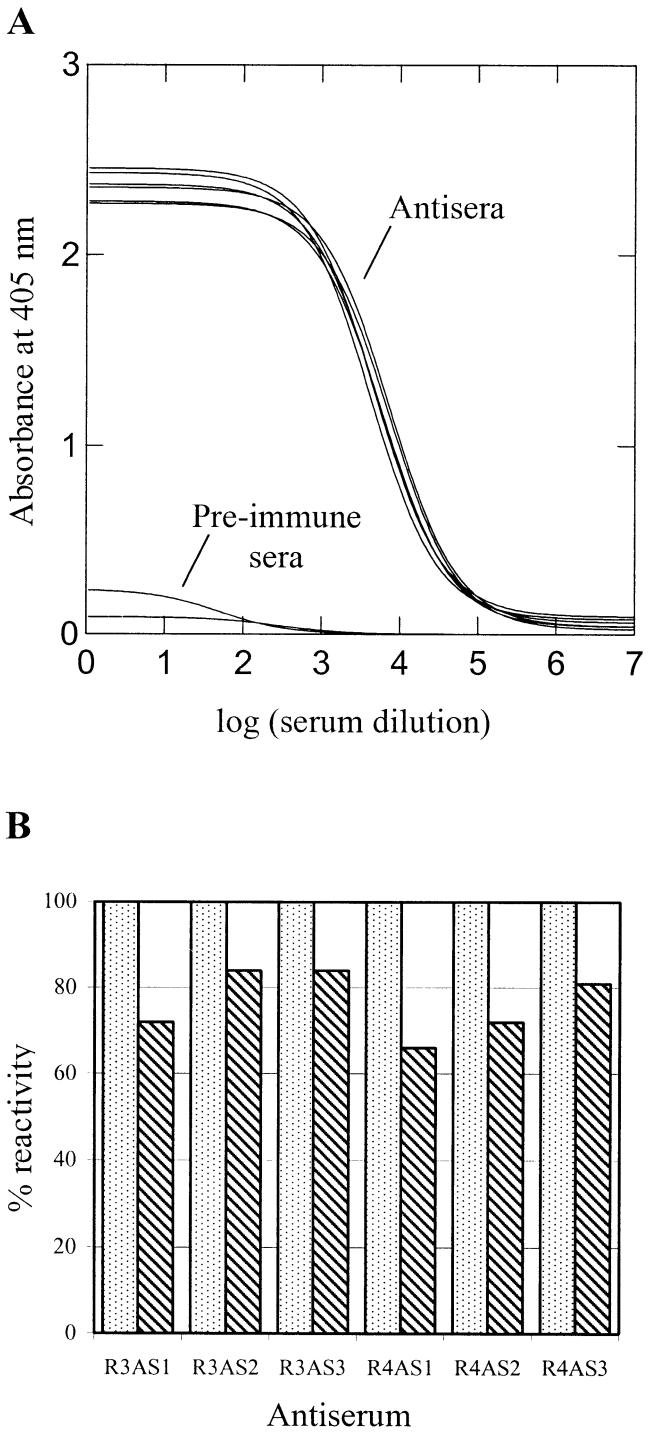

Figure 2. Characterization of the polyclonal antibodies raised against the tubule prints from the Cuvierian tubules of *Holothuria forskali*. **A:** Binding in ELISA of the different preimmune sera and antisera with peptides generated by trypsin digestion of the TPM (see text). **B:** Reactivity tests of the different antisera with peptides generated by trypsin digestion of the TPM and pretreated by oxidation with sodium periodate. The immunoreactivity of the pretreated antigens (striped bars) was compared with that of untreated antigens (dotted bars), which was considered to be 100%. R3AS1, R3AS2, and R3AS3 are the first, second, and third antisera collected in rabbit R3; R4AS1, R4AS2, and R4AS3, in rabbit R4.

Immunoreactive structures were also observed in the muscle layer, the inner connective tissue layer, and the inner epithelium, but the fluorescent labeling was much weaker than in the mesothelium (Table 3; Figure 3, B).

Immunocytochemistry

To locate more precisely the tubule print constituents in the Cuvierian tubules of *H. forskali*, immunogold labeling and transmission electron microscopy were used. As in light microscopy, the antisera were strongly immunoreactive (Table 3), all labeling the same structures, and there was no labeling with the preimmune sera and with the antisera pretreated with excess antigen. In transmission electron microscopy, however, the fixative used greatly influenced the immunolabeling pattern observed.

The mesothelium of the tubule is a pseudostratified epithelium made up of 2 cell layers, an apical layer of peritoneocytes and a basal layer of granular cells (Figure 4, A) (for a more detailed description of Cuvierian tubule ultrastructure in *H. forskali*, see VandenSpiegel and Jangoux, 1987). Together both cell layers form conspicuous transversal folds that penetrate the underlying connective tissue. Peritoneocytes are T-shaped, with the basal portion of the T penetrating deeply into the tubule connective tissue. Their apical parts enclose conspicuous mucous granules averaging 2 to 4 μm in diameter. Granular cells form extensive penetrations into the connective tissue compartment. These penetrations are V-shaped, with the V surrounding the basal processes of the peritoneocytes. Granular cells are filled with densely packed proteinaceous granules (1 to 2 μm in diameter). The granules of both peritoneocytes and granular cells were strongly immunoreactive (Table 3; Figure 4, B and C, respectively). In the latter, however, the immunolabeling appeared to be fixation-dependent, being totally suppressed when the tubules were fixed in glutaraldehyde and postfixed in osmium tetroxide. In addition to the immunoreactivity of the cell secretory granules, weak to moderate immunogold labeling was also observed on the apical plasma membrane (Table 3; Figure 4, B) of the peritoneocytes as well as on the basal lamina lining the mesothelium.

Longitudinal and circular myocytes occur under the tips of the mesothelial folds. They are characterized by a conspicuous myofibril, which occupies most of the volume of the cytoplasm (Figure 4, D). Myocytes are clustered in bundles of 2 to 5 cells surrounded by a basal lamina. In the muscle layer, the only immunoreactivity detected was a

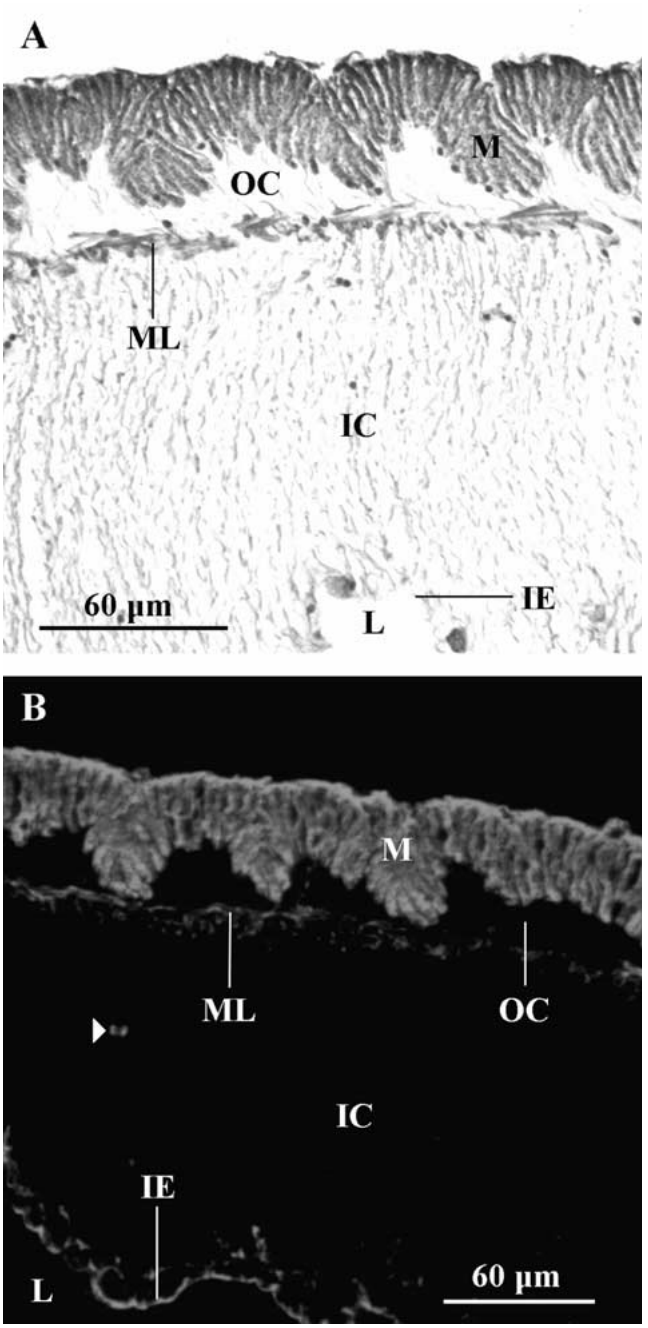

Figure 3. Histology of the Cuvierian tubules of *Holothuria forskali* and immunohistochemical location of tubule prints constituents. **A:** Longitudinal section through the wall of a tubule fixed in Bouin's fluid and stained with Alcian blue—Mayer's hemalum–phloxine. **B:** Longitudinal section through the wall of a tubule fixed in Bouin's fluid and immunolabeled with R4AS1 (the arrowhead indicates an immunoreactive cell within the connective tissue layer). IC indicates inner connective tissue layer; IE, inner epithelium; L, lumen; M, mesothelium; ML, muscle layer; OC outer connective tissue layer.

weak labeling of the myocyte plasma membrane and a moderate labeling of the basal lamina (Table 3; Figure 4, E).

The connective tissue layer contains mostly collagen fibers and processes from neurosecretory cells filled with electron-dense granules (Figure 4, F). These processes are always lined by a basal lamina. A moderate immunogold labeling was observed on the granules of neurosecretory cells and on the basal lamina surrounding these cells (Table 3; Figure 4, G). The collagen fibers were also weakly labeled.

The inner epithelium lines the narrow and convoluted lumen of the tubule. It is made up of a single cell type characterized by a finely granular cytoplasm seemingly lacking in organelles and by the occurrence of 1 to 3 basal processes penetrating deeply into the connective tissue (Figure 4, H). Each process encloses a cluster of large heterogeneous spherules containing both proteins and mucosubstances. Weak immunolabeling was observed in the spherules of the inner epithelial cells (Table 3; Figure 4, I), as well as in the whole cytoplasm (Table 3; Figure 4, J). As in the other tissue layers, the basal lamina was moderately immunoreactive.

Discussion

Individuals of *Holothuria forskali* respond to tactile stimulation by discharging 10 to 20 Cuvierian tubules. In seawater the expelled tubules elongate up to 20 times their original length and become very sticky upon contact with any object. Although the remarkable adhesivity of Cuvierian tubules has intrigued scientists for more than a century, almost nothing is known about their adhesive, except that it consists, at least partly, of proteins (Endean, 1957; Müller et al., 1972; Zahn et al., 1973; VandenSpiegel and Jangoux, 1987). The lack of a biochemical assay for adhesive substances makes it necessary to start the characterization of Cuvierian tubule adhesive with a material that is already considerably enriched in these substances, in this study we used the tubule prints, which consist of patches of material left on the substratum after mechanical detachment of the tubule collagenous core.

Origin of Tubule Print Constituents in Cuvierian Tubules

VandenSpiegel and Jangoux (1987) found that tubule elongation caused the unfolding of granular cells in the mesothelium. Upon contact with a surface, the granules of these cells were released and their contents were transformed into a sticky mass (Müller et al., 1972; Vanden-

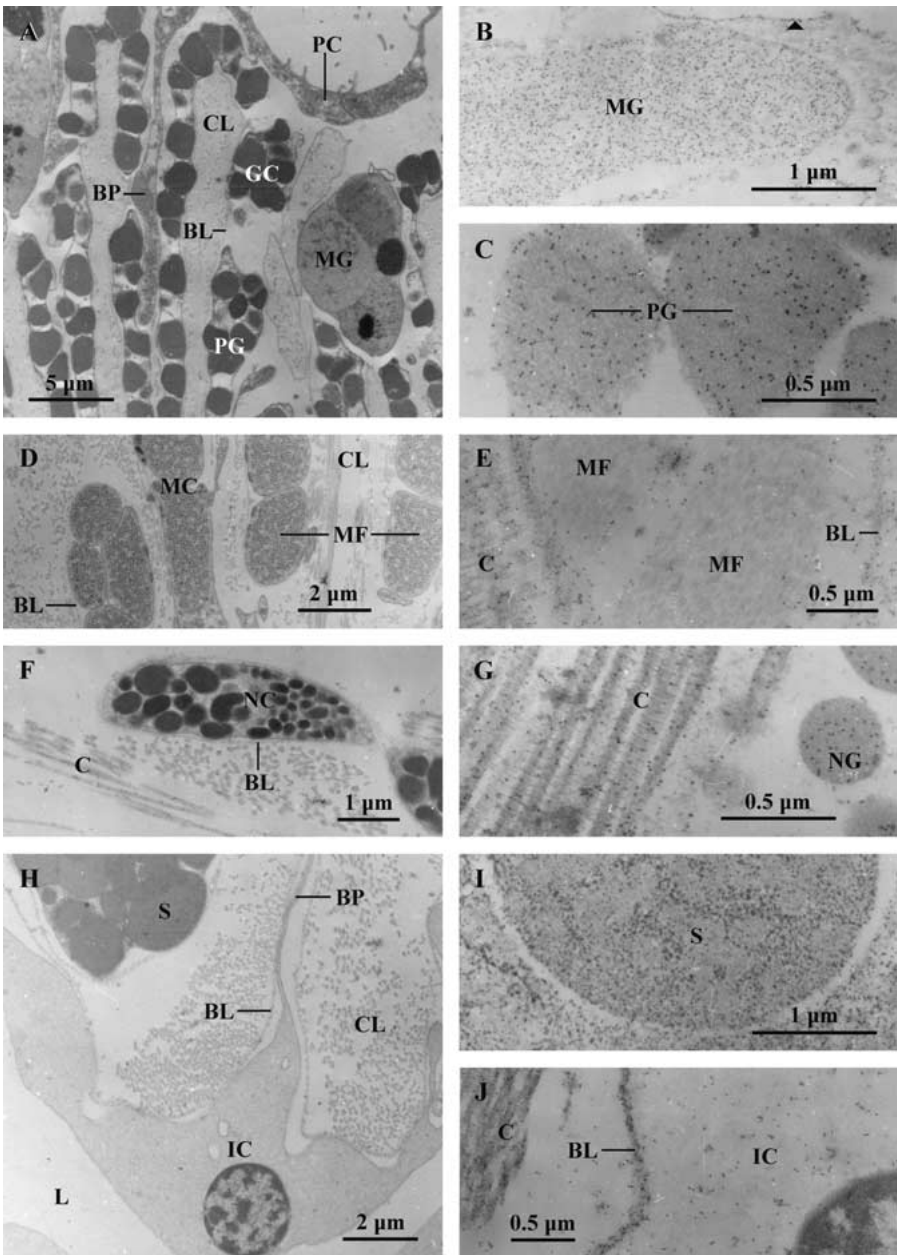

Figure 4. Ultrastructure of the Cuvierian tubules of *Holothuria forskali* and immunocytochemical location of tubule prints constituents. **A:** Longitudinal section through the mesothelium (fixed in glutaraldehyde–osmium tetroxide). **B**, **C:** Details of a peritoneocyte and a granular cell, respectively (fixed in paraformaldehyde and immunolabeled with R4AS3). The arrowhead panel B indicates the apical plasma membrane. **D**, **E:** Circular muscle layer (fixed in glutaraldehyde–osmium tetroxide, and fixed in paraformaldehyde and immunolabeled with R4AS3, respectively). **F**, **G:** Details of the inner connective tissue layer showing collagen fibers and a neurosecretory cell process (fixed in glutaraldehyde–osmium tetroxide, and fixed in paraformaldehyde and immunolabeled with R4AS3, respectively). **H:** Longitudinal section through the inner epithelium (fixed in glutaraldehyde–osmium tetroxide). **I**, **J:** Details of an inner epithelial cell showing a spherule and a portion of the cytoplasm, respectively (fixed in paraformaldehyde and immunolabeled with R4AS3). BL indicates basal lamina; BP, basal process; C, collagen; CL, connective tissue layer; GC, granular cell; IC, inner epithelial cell; L, tubule lumen; MC, myocyte; MF, myofibril; MG, mucous granule; NC, neurosecretory cell; NG, neurosecretory cell granule; PC, peritoneocyte; PG, proteinaceous granule; S, spherule.

Table 3. Summary of the immunoreactivity of Cuvierian tubule constituents

Tissue layer and structure	Immunoreactivity*
Mesothelium	
Proteinaceous granules	+++
of granular cells	+++
Mucous granules of peritoneocytes	++
Basal lamina	++
Muscle layer	
Myocytes	−
Basal lamina	++
Connective tissue layer	
Collagen	+/−
Granules of neurosecretory cells	+
Basal lamina of neurosecretory cells	++
Inner epithelium	
Spherules of inner epthelial cells	+
Cytoplasm of inner epthelial cells	+
Basal lamina	++

*Strong (+++), moderate (++), weak (+), and absent (−).

Spiegel and Jangoux, 1987). Therefore, these adhesive secretions presumably form the bulk of the TPM. However, tubule prints may also enclose other tubule constituents that are left on the substratum when the tubule collagenous core is peeled off. To address this issue, we have raised antisera against the TPM of *H. forskali* and used them to locate the origin of print constituents in the Cuvierian tubules by taking advantage of the polyclonal character of the antibodies generated. Indeed, being raised against tubule print as a whole, these antibodies may react with a wide variety of the substances originally present in the TPM. In sections of the tubules immunoreactive structures were observed in all tissue layers, but the labeling was several times stronger in the mesothelium. Most of it was localized in the proteinaceous granules of granular cells, demonstrating that their secretions make up the bulk of the TPM. An important immunoreactivity was also detected in the mucous granules of peritoneocytes. When tubules elongate, the peritoneocytes disintegrate and release their mucous granules into the outer medium (VandenSpiegel and Jangoux, 1987). Released mucus covers the tubule outer surface, acting supposedly as a protective coating that prevents elongating tubules from adhering to each other and to the holothuroid body. It is likely, therefore, that some of this mucus is present in the tubule prints.

A weak to moderate immunolabeling of the plasma membranes of the mesothelial cells as well as of the basal lamina lining the mesothelium indicates that constituents of these structures are incorporated in the tubule prints either during tubule elongation and attachment or during mechanical detachment. This incorporation partly explains the immunoreactivity observed in the other tissue layers of the tubules (i.e., the muscle layer, the connective tissue layer, and the inner epithelium), in which the basal laminae are always labeled. Occasionally, some collagen may be left in the tubule prints when the tubule is detached, hence the weak immunolabeling observed on the collagen fibers. This is corroborated by the occurrence of hydroxyproline in some of the amino acid analyses. The weak immunoreactivity of neurosecretory cells and of inner epithelial cells is, however, more difficult to explain. It is unlikely that constituents from these cells would be incorporated in tubule prints as both types of cells are located deep within the tubule tissues. Indeed, myocytes are located just beneath the mesothelium, and longitudinal ones are disintegrated during tubule elongation (VandenSpiegel and Jangoux, 1987), but they are not immunolabeled, indicating that their constituents are not incorporated into the tubule prints. The most likely explanation for the immunolabeling of the granules of neurosecretory cell and the cytoplasm and spherules of inner epithelial cells is that these structures share some common epitopes with tubule print constituents. Their immunoreactivity might thus represent adventitious cross-reactivity. Interestingly, the 3 structures have similar histochemical properties: they all are periodic acid Schiff stain positive, indicating that they enclose a saccharidic fraction (Guislain, 1953; VandenSpiegel and Jangoux, 1987; Heinzeller and Welsch, 1994). Moreover, their immunoreactivity is not fixation-dependent, contrary to that of the granular cells of the mesothelium, also suggesting carbohydrate epitopes (Hayat, 2000). Alternatively, it is also possible that a few compounds from the neurosecretory cells or the inner epithelial cells diffuse through the connective tissue layer and could be incorporated into the tubule prints.

Biochemical Composition of the Tubule Prints

Cuvierian tubule prints consist mainly of proteins and carbohydrates in a 3:2 ratio. A small inorganic fraction

(about 10%) is also present. Since adhesive secretions from granular cells appear to make up the bulk of the TPM, the biochemical composition given in Table 1 probably reflects that of these secretions. Its interpretation, however, must take into account the fact that, in addition to adhesive material, tubule prints seem to contain secretions from the peritoneocytes and, occasionally, connective tissue components including collagen. VandenSpiegel and Jangoux (1987) have published a detailed histochemical study of the Cuvierian tubules of *H. forskali*. Their results indicate that the secretory granules of granular cells are lipoproteic in nature. In this study, however, no lipids were detected in the TPM of the same species. This discrepancy may be explained by the fact that the histochemical stain they used (Sudan Black B) is known to bind to some proteins (Gabe, 1968). The carbohydrate fraction detected in the TPM may originate partly from the mucopolysaccharide secretions of the peritoneocytes (Guislain, 1953; VandenSpiegel and Jangoux, 1987). However, it is unlikely that these mucopolysaccharides account for the important neutral sugar fraction measured (about 40% dry weight of the TPM). Granular cell secretions would therefore enclose a carbohydrate fraction associated with their proteinaceous content.

Among the marine bioadhesives described so far, only those involved in the adhesion of whole organisms to a solid substratum have been studied biochemically. Three types of substratum adhesion are generally distinguished in marine invertebrates: (1) permanent adhesion involving the secretion of a cement (e.g., the attachment of barnacles on rocks); (2) transitory adhesion permitting simultaneous adhesion and movement along the substratum (e.g., the foot secretions of gastropod mollusks); and (3) temporary adhesion allowing an organism to attach strongly but momentarily to the substratum (e.g., the adhesion of echinoderm podia) (Walker, 1987; Tyler, 1988; Flammang, 1996; Whittington and Cribb, 2001). Permanent adhesives consist almost exclusively of proteins, while nonpermanent adhesives (both transitory and temporary) are made up of an association of proteins and carbohydrates, usually in a 2:1 ratio (Flammang et al., 1998; Whittington and Cribb, 2001). The composition of the TPM is reminiscent of that of nonpermanent adhesives by its association of proteins and carbohydrate. In the nonpermanent adhesives, however, the carbohydrate fraction is mostly in the form of acid and sulfated sugars and associated with an important inorganic fraction representing more than 40% of the adhesive material (Grenon and Walker, 1980; Flammang et al., 1998). Cuvierian tubule adhesive therefore appears to be unique among invertebrate adhesives in terms of its gross biochemical composition.

The amino acid composition of the protein fraction of TPM indicates that the adhesive is rich in small side-chain amino acids (more than 40%), especially glycine, and is predominantly made up of charged and polar amino acids (more than 70%). These characteristics are typical of the adhesives of many different marine invertebrates: e.g., platyhelminthes (Hamwood et al., 2002), mussels (Benedict and Waite, 1986), limpets (A.B. Smith et al., 1999), polychaetes (Jensen and Morse, 1988), barnacles (Kamino et al., 2000), and sea stars (Flammang et al., 1998). Charged and polar amino acids are probably involved in adhesive interactions with the substratum through hydrogen and ionic bonding (Waite, 1987). Small side-chain amino acids, on the other hand, are often found in large quantities in elastomeric proteins (Tatham and Shewry, 2000). These proteins are able to withstand significant deformation without rupture before returning to their original state when the stress is removed (B.L. Smith et al., 1999). The composition of holothuroid TPM therefore has all the characteristics of a strong and resistant underwater adhesive.

Proteins from Cuvierian Tubule Prints

The study of marine adhesive proteins is thwarted by their high insolubility in water (Waite, 1987). Our solubility experiments have demonstrated that Cuvierian tubule prints are also highly insoluble, even in strong denaturing buffers. Aggregation is due to the formation of crosslinks between the different proteins constituting the adhesive. These links have been characterized as di-DOPA bonds in mussels (McDowell et al., 1999; Burzio and Waite, 2000), and disulfide bonds in barnacles (Naldrett and Kaplan, 1997; Kamino et al., 2000). The former have not been detected in holothuroid TPM, but the latter are presumably involved in aggregate formation. Indeed, the addition of reducing agents to the extraction buffer improves TPM solubility. However, even when using the strong extraction conditions reported for barnacles (5% mercaptoethanol at 40°C for at least 10 hours, Naldrett and Kaplan, 1997; or 0.5 M DTT at 60°C for 1 hour, Kamino et al., 2000), TPM solubility was not significantly improved, and about 90% was still insoluble. This suggests that other types of crosslinks may be involved in Cuvierian tubule adhesive aggregation.

Electrophoretic analysis revealed that the soluble fraction from TPM contained about 10 proteins with apparent molecular weights ranging from 17 to 220 kDa. All these

proteins had similar amino acid compositions and thus appear to be closely related. Marine bioadhesives are generally made up of a complex blend of proteins. For example, mussel byssal plaques are composed of at least 5 different proteins (Taylor and Waite, 1997; Waite and Qin, 2001). However, except for the presence of the unusual amino acid DOPA in each of them, they are not related and all possess their own distinctive characteristics. Similarly, barnacle cement consists of at least 3 major proteins and 5 minor proteins, but they all have their own characteristic composition (Kamino et al., 2000; Kamino, 2001). Therefore, to the best of our knowledge, only holothuroid CT adhesive comprises so many closely related proteins. Some structural biomaterials, however, are known to enclose such protein families. This is the case for the nematode (Johnstone 1994) and insect cuticles (Andersen et al., 1995), and for insect egg chorion (Lecanidou et al., 1986).

All the proteins extracted from TPM were rich in glycine and acidic residues. These compositions contrast with histochemical tests performed on the Cuvierian tubules of *H. leucospilota* (Endean, 1957) which indicated that the material enclosed in the secretory granules of granular cells was strongly labeled with stains specific for the detection of tyrosine residues and of basic residues (lysine, histidine, and arginine). Tests on the influence of different reagents on the tenacity of Cuvierian tubules of *H. forskali* have given convergent results (Müller et al., 1972; Zahn et al., 1973). Indeed, tubule adhesion is reduced by several proteolytic enzymes. Among them, the most potent are, in decreasing order, carboxypeptidase A, chymotrypsine, and trypsine, demonstrating the presence of aromatic residues (phenylalanine, tyrosine, and tryptophan) and basic residues (lysine and arginine). This suggests that other proteins could be present in the insoluble fraction of the TPM. Future studies should attempt to identify and characterize the proteins from both the soluble and insoluble fractions of the tubule prints from *H. forskali*. The elucidation of their structure and physicochemical characteristics should provide the necessary basis for understanding the underwater adhesive mechanism of holothuroid Cuvierian tubules.

Acknowledgments

We thank Dr. A. Michel for his help with the preparation of the polyclonal antibodies, and G. Laurent and P. Postiau for technical assistance. P.F. is Research Associate of the National Fund for Scientific Research of Belgium (FNRS). This research was supported by the U.S. Office of Naval Research (grant N00014-99-1-0853) and by a FNRS grant to P.F. (grant 1.5.090.01, Crédit aux Chercheurs). This study is a contribution of the Centre Interuniversitaire de Biologie Marine (CIBIM).

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