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Amino acid and metal content of *Crassostrea gigas* shell infested by *Polydora* sp. in the prismatic layer insoluble matrix and blister membrane

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Abstract

The spionid polychaete *Polydora* sp. can live and reproduce inside the oysters' shell, excavating a U-shaped burrow the central portion of which is filled with detritus and particles of dissolved shell. The oyster responds by secreting an organic layer, confining the worm, and later covering it with calcitostracum. This process creates a characteristic "mud blister". *Crassostrea gigas* were analysed for the organic matrix amino acid content and 14 metals of the whole shell of normal oysters and severely *Polydora* infested ones. Amino acid, DOPA and FT-Raman analyses were also performed on isolated membranes from the characteristic mud blister and on prismatic layer insoluble matrix (PLIM) of infested oysters, with the aim of detecting any differences between the two organic matrices. Membranes and PLIM composition differ from whole normal shell organic matrix by having lower aspartate and glycine and higher levels of virtually all hydrophobic amino acids, especially alanine. There are no significant differences between membranes and PLIM in the ratio of charged to non-polar amino acids, respectively 0.54 and 0.59. L-DOPA content is also similar in the two matrices with 0.45 (±0.18) and 0.57 (±0.30) ng.mg^{-1} protein for PLIM and membranes respectively. The FT-Raman spectra of membranes is very similar to that of PLIM, suggesting that they have identical composition. Zinc, iron and manganese are significantly higher in infested than in normal shells. Infested oysters can provide an excellent opportunity for investigating normal biological calcification processes.

Keywords: *Crassostrea gigas*, *Polydora* sp., shell organic matrix, amino acids, metals.

Composition en acides aminés et en métaux de la coquille de Crassostrea gigas *infestée par* Polydora *sp. dans la couche prismatique de la matrice insoluble et dans la membrane des ampoules ou chambres vaseuses.*

Résumé

Le polychète spionidé *Polydora* sp. peut vivre et se reproduire à l'intérieur des coquilles d'huître, creusant un tube en forme de U, la partie centrale est remplie de détritus et de particules dissoutes de coquilles. L'huître répond en secrétant une couche organique, confinant et finalement couvrant le ver de calcitostracum. Ce mécanisme crée « une ampoule vaseuse » caractéristique. Sont analysés les acides aminés et 14 métaux de coquilles d'huîtres *Crassostrea gigas*, normales et sévèrement infestées par *Polydora*. Les acides aminés, L-dihydroxyphénylalanine (DOPA) et les spectres-Raman sont aussi analysés,

des membranes des ampoules vaseuses et de la couche insoluble prismatique (PLIM) des huîtres infestées. La composition des membranes et celle de PLIM diffèrent de celles d'huîtres non infestées par des niveaux plus faibles en aspartate et en glycine, par des niveaux plus élevés en acides aminés hydrophobes, l'alanine en particulier. Il n'y a pas de différences significatives entre les membranes et PLIM dans la proportion d'acides aminés non polaires, 0,54 et 0,59 respectivement. Le contenu en L-DOPA est similaire entre les deux matrices avec 0,45 (±0,18) et 0,57 (±0,30) $ng.mg^{-1}$ protéine pour PLIM et les membranes respectivement. Le spectre TF-Raman de la membrane est très semblable à celui de la couche insoluble PLIM, supposant qu'elles ont la même composition. Zinc, fer et manganèse sont significativement plus élevés chez les huîtres infestées. Les huîtres infestées peuvent être l'occasion d'études des mécanismes de calcification biologique.

Mots-clés : *Crassostrea gigas*, *Polydora* sp., matrice organique de la coquille, métaux, acides aminés.

INTRODUCTION

The spionid polychaetes of the genus *Polydora* have a world-wide distribution (Laukner, 1983) and are found in a wide variety of substrata, from soft clays or mud to hard calcareous material. Oysters are affected when planktonic larvae settle onto the inner shell surface at its growing margin. The worm basically excavates a U-shaped burrow, the central portion of which is filled with detritus and particles of dissolved shell with two openings to the outside (Zottoli and Carriker, 1974).

The oyster responds to the penetrating activity by sealing off the perforation with a membrane of organic matrix and later covering it with calcitostracum. This process creates a characteristic "mud blister". The area covered by such blisters does, in some cases, extend to more than half of the shell inside.

Various reports have described the boring mechanism of *Polydora* sp. and there are many theories related to this process (Dorsett, 1961; Haigler, 1969; Travis and Gonsalves, 1969; Zottoli and Carriker, 1974; Sato-Okoshi and Okoshi, 1993). However, the biochemical composition of the membrane, or if this is a periostracum-like material, has not yet been fully clarified. Periostracum is secreted in the periostracal groove of the mantle edge. The newly formed periostracum is extruded distally as a multilayered entity. Its innermost layers become continuous with the outer covering of the valve at the forming edge of the shell (Saleuddin and Petit, 1983). The secretion and extrusion of new periostracum from the periostracal groove is accompanied by sclerotization of the periostracal protein. Although they are very abundant in nature, scleroproteins are among the least understood of all proteins. The most persistent properties of the versatile scleroproteins are their insolubility in vitro and their resistance to proteinases (trypsin and pepsin) and various hydrolytic solvents (Waite, 1983). This stability is attributed to two factors: (a) special arrangement of amino acids (conformation) and (b) covalent cross-linking of the primary valence chains (Linderstrom-Lang and Duspiva, 1936). In molluscs, in addition to collagens, there occur a variety of scleroproteins presumed to be stabilized by quinone tanning (Meenakshi *et al.*, 1969; Bubel, 1973; Brown, 1975; Jones and Saleuddin, 1978).

An immediate precursor to quinone is 3,4-dihydroxyphenylalanine (DOPA). This has been found in the periostracum of various bivalve molluscs (Degens *et al.*, 1967; Waite, 1977; Waite and Andersen, 1978) and in the dark fraction of insoluble organic matrix of *Crassostrea virginica* shell (Wheeler *et al.*, 1988).

In this study, we analysed the amino acid and metal content of normal *Crassostrea gigas* shells and others heavily infested by *Polydora* sp. Our aim is to compare the prismatic layer insoluble matrix (PLIM) with the organic matrix of the isolated membrane secreted by the oysters, as defence against the worm penetration. We report here amino acid, L-DOPA and FT-Raman analyses performed on both matrices.

MATERIAL AND METHODS

Live specimens of the Japanese oyster *Crassostrea gigas* were collected from two earth fish ponds in Mondego River estuary (Portugal). Some of the shells were heavily infested by *Polydora* sp. (type IV, according to Catherine *et al.*, 1990) and some of them were normal. The shells were removed, cleaned of any adhering organisms and detritus, rinsed briefly in 5% NaOH and scrubbed in running tap water to remove residual organic matter. Infested shells were fractured with scalpel and hammer in order to free every piece from the mud accumulated in the holes made by the spionid. After drying, the shells were ground to a powder.

To dissolve the mineral, the powdered shells were suspended in 10% acetic acid. The organic matrix was then separated by centrifugation and repeatedly washed to remove most residual acid. The pellet was then freeze dried and lyophilized. In some of the infested shells, it was possible to isolate the organic matrix membranes and treat them separately. Unfragmented shells were also decalcified and the dark frac-

tion of the insoluble matrix was physically removed with a spatula for analysis (Wheeler *et al.*, 1988).

Amino acid determinations on lyophilized fractions, PLIM and membranes were performed by hydrolysis in 6 N HCl and 5% v/v phenol under vacuum for 24 h at 110°C and analysed on a LKB 4151 Alpha Plus®AminoAcid Analyser. The protein concentrations were determined using the Lowry *et al.* (1951) method and bovine serum albumin as a standard.

To analyse L-3,4-dihydroxyphenylalanine (L-DOPA), tissue samples were weighed (approx. 4.5 mg for membranes and 50 mg for PLIM) and left to stand in 1 ml of 0.2 M perchloric acid for 24 h at 4°C. The homogenate was centrifuged and the supernatant decanted. Aliquots were extracted with alumina at pH 8.4. Elution of alumina from Millipore (MFI) microfilters were effected with 0.1 M perchloric acid. A sample of the eluate was analysed by HPLC with electrochemical detection (BAS model 304).

Metal analyses were accomplished on aliquots of powdered oyster shell (normal and infested) and were carefully digested in nitric acid and analysed by atomic absorption spectrophotometry (AAS) in a Perkin-Elmer (Model 5000). Sodium, calcium and potassium were analysed by flame photometry.

Organic compounds binding groups from PLIM and membranes were detected by FT-Raman spectrometry. Fourier Transform Raman spectra were obtained on a Perkin Elmer 2000 NIR FT-Raman spectrometer using a near infrared Nd3+ YAG laser at the excitation source. All samples were run at a spectral resolution of 8 cm^{-1}. The operating laser power was 200 mW with 100 scans.

RESULTS

The most abundant amino acids in all four matrices are aspartate, serine, glycine and alanine (*table* 1). Generally, the level of the different amino acids in the infested shell is between those from normal shell and membranes. Membranes and PLIM have much lower contents of aspartate (93 and 90 residues per thousand) and glycine (201 and 229 residues per thousand) while

Table 1. – *Crassostrea gigas* amino acid composition of shell matrix fraction (in residues per thousand).

Amino acids	Total organic matrix			
	Membranes	PLIM	Normal shell	Infested shell
Aspartic acid[+] Asparagine	93	90	150	122
Threonine	33	33	25	26
Serine	110	88	119	111
Glutamic acid[+] Glutamine	62	62	56	52
Proline	59	87	55	51
Glycine	201	229	304	276
Analine	193	122	71	133
Cystine	6	12	10	9
Valine	33	43	24	27
Methionine	7	5	17	7
Isoleucine	22	24	18	20
Leucine	41	46	31	34
Tyrosine	43	51	45	52
Phenylalanine	41	42	25	33
Histidine	13	16	8	10
Lysine	18	19	18	16
Arginine	27	32	22	22
Total uncharged [a]	192	184	199	198
Total charged [b]	213	219	254	222
Total non-polar [c]	396	369	241	305
Hydophobicity index [d]	401	418	320	242
Ratio of charged to non-polar residues	0.54	0.59	1.4	0.73

[a] Sum of Thr, Ser, Cys and Tyr residues per thousand.

[b] Sum of Asp, Glu, His, Lys and Arg residues per thousand.

[c] Sum of Pro, Ala, Val, Met, Iso, Leu and Phe residues per thousand.

[d] $HI = \sum n_i s_i$, where n_i is the given concentration of an amino acid in a protein in residues per thousand, and s_i is the free energy change (kcal/mol) of that amino acid in being transferred from ethanol to water at 25.1°C (Waite, 1983). The s values used here are taken from Nozaki and Tanford (1971) and Levitt (1975).

alanine is much higher (193 and 12 residues per thousand) than in normal shell where the contents for these amino acids are 150, 304 and 71 residues per thousand, respectively.

There are significant differences ($p<0.01$, $n=6$) in serine, proline, alanine, cystine, valine, tyrosine and histidine levels between membranes and PLIM (*fig.* 1). However, these differences are not reflected in the ratio of charged to non-polar groups because these are not significantly different between the two matrices (*table* 1). In contrast, normal shell has a higher ratio (1.4) of charged to non-polar residues than membranes (0.54) and PLIM (0.59). There are no differences between the four matrices for the total uncharged amino acids. The ratio of charged to non-polar amino acids and the hydrophobicity index was calculated considering all asparagine and glutamine as the carboxyl amino acids aspartate and glutamate. The hydrophobicity index is very similar between membranes and PLIM, 401 and 418 respectively, being much higher than in normal shell matrix (242).

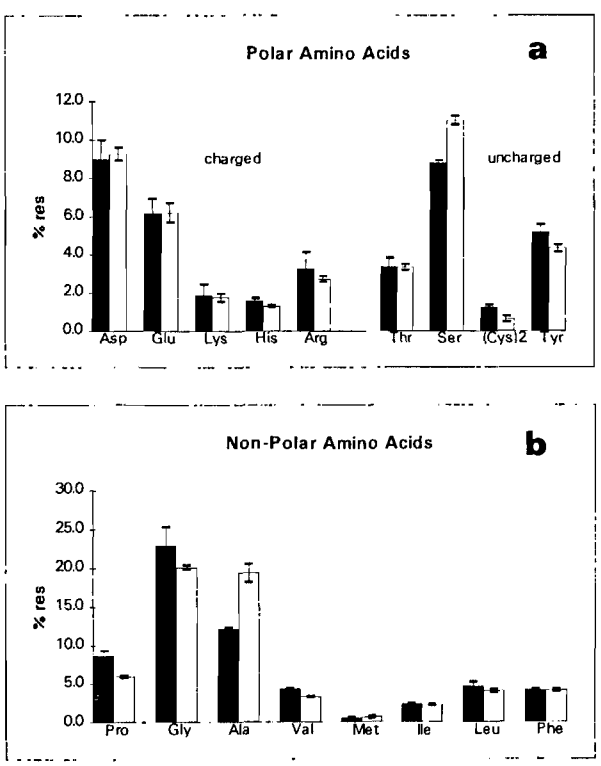

Figure 1. – Amino acid composition of periostracum □ and membranes ■ in percentage of residues, grouped by polarity: (a) polar and (b) non-polar amino acids.

Membranes L-3,4-dihydroxyphenylalanine (DOPA) content is higher than PLIM, although these values are not significantly different ($p<0.01$) (*table* 2).

Table 2. – L-3,4-dihydroxyphenylalanine (DOPA) content of prismatic layer insoluble matrix and membranes from *Crassostrea gigas* infested by *Polydora* sp.

	DOPA ($ng.mg^{-1}$ protein)
Membranes	0.40
	1.00
	0.35
	0.53
Average (±SD)	0.57 (±0.30)
Periostracum	0.66
	0.32
	0.63
	0.38
	0.28
Average (±SD)	0.45 (±0.18)

Figure 2 shows the FT-Raman spectra of three samples of PLIM and membranes, respectively. The FT-Raman spectra of both matrices are very similar, suggesting they have identical composition. The distinguishing characteristics are the vibration modes at 1 087 cm^{-1} and 2 927 cm^{-1} which tend to be more intense and sharp in the periostracum matrix. The band at 1 087.5 cm^{-1} is possibly associated with the carbonate vibration due to the presence of calcium carbonate as a consequence of deficient demineralization of the shell.

The first results in what concerns metal ion content in normal and infested shells show that iron, zinc and manganese are significantly higher ($p<0.01$) in infested (6.8±2.4, 168.0±93.7 and 53.5±17.9 $\mu g.g^{-1}$ respectively) than in normal shells (5.0±2.0, 56.6±30.9 and 28.4±9.8 $\mu g.g^{-1}$ respectively) (*table* 3). Metal analyses in PLIM and membranes verify the origin of these differences.

Table 3. – Means (±SD) for chemical concentrations $\mu g.g^{-1}$ measured in normal ($n=27$) and infested ($n=45$) *Crassostrea gigas* shells.

Element	Normal $n=27$	Infested $n=45$
Cu	6.9±1.4 ($n=25$)	8.0±1.0 ($n=41$)
Zn	5.0±2.0	6.8±2.4
Fe	56.6±30.9	168.0±93.7
Li	3.1±0.2	3.3±0.4
Mn	28.4±9.8	53.5±17.9
Cd	3.7±0.2	3.6±0.3
Co	12.7±1.3	12.5±1.3
Ni	23.8±8.8	21.5±6.4
Pb	46.1±3.6	45.0±5.5
Cr	4.7±0.9	4.9±0.8
Sr	726±90	857±76 ($n=42$)
Ca[a]	390±17	378±59
Na[a]	5.7±1.5	5.8±1.5
Mg[a]	1.8±0.4	2.0±0.4 ($n=44$)
K	128±77	121±32 ($n=38$)

[a] ppt

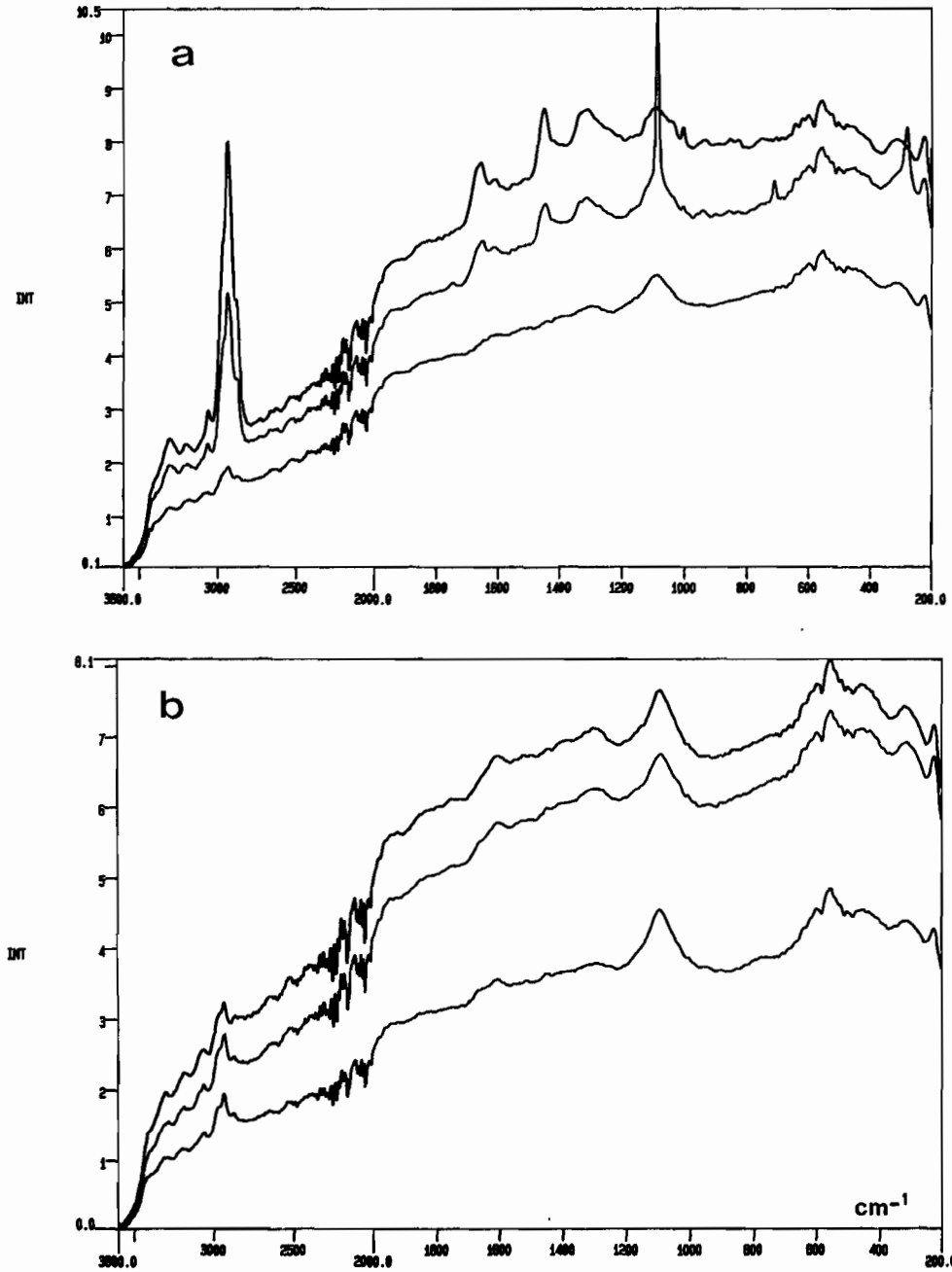

Figure 2. – Overlay of Raman spectra of (a) prismatic layer insoluble matrix and (b) mud blister membranes.

DISCUSSION

Periostracum plays a critical role in the life of the mollusc. Not only is it found in almost all species of the phylum, but it is also the first scleroprotein formed in the ontogeny of this group (Waite, 1983). A quinone-tanned periostracum seems to be the rule for gastropods and bivalves (Brown, 1975; Vovelle, 1974). Frequently authors, when referring to the *Crassostrea* genus, do not consider the existence of a periostracum (Galstoff, 1964; Wheeler *et al.*, 1988). The insoluble residue of the prismatic layer insoluble matrix (PLIM), in these invertebrates, presents similar characteristics with the so-called periostracum from other bivalves.

Protein is the major constituent of PLIM. We found values of 608 and 620 $\mu g.g^{-1}$ dry weight, respectively, for the protein content of PLIM and the organic matrix of membranes. Amino acid analyses show that there

are some quantitative differences in the organic matrix between the PLIM and the membranes. Nevertheless these differences are not reflected in total groups of amino acids. Membranes and PLIM composition differ significantly from whole normal shell organic matrix largely by having lower aspartate and glycine (calcium-binding groups) and higher levels of almost all hydrophobic amino acids, especially alanine. Our results are in agreement with Wheeler *et al.* (1988) and Krampitz *et al.* (1983) on the insoluble residue of the insoluble matrix of *Crassostrea virginica* and *Crassostrea gigas* respectively, further extracted with chemical agents or by mechanical methods. Despite the significant differences between proline, alanine and valine ($p<0.01$) for the membranes and the PLIM, there is a compensation among them so that the ratio of charged to non-polar residues is similar. Uncharged amino acids also present significant differences, but when considered as a group, we notice they compensate each other. This can mean that the function of uncharged and non-polar amino acids is determined by their place in the group rather than individually. On the contrary, charged amino acids do not present significant differences between PLIM and matrix proteins, when considered singly. Kawaguchi and Watabe (1993) report a ratio of charged to non-polar of 0.52, on the insoluble matrix of the prismatic layer of *Crassostrea virginica*. This is close to the values found in this work for the membranes (0.54) and the isolated PLIM (0.59).

The significance of hydrophobicity in molluscan periostraca can be viewed in two ways: (1) periostraca precursors are initially secreted as a soluble though highly viscous substance and, by being hydrophobic, they tend to aggregate to avoid mixing with the water. The initial aggregation brings the molecules into proximal positions for the cross-linking that presumably follows; (2) periostracum is a barrier to water and the salts suspended in it, especially along the shell margin (Waite, 1983). A similar hydrophobicity index found in PLIM and membranes suggests an identical role of impermeabilization for the membranes.

Cross-linking of proteins is promoted by quinones. DOPA is an *o*-diphenol and readily forms quinones and semiquinones by photolysis, autoxidation and enzyme catalysis (Felix and Sealy, 1981). Periostracin (the precursor of periostracum) is secreted by the mantle border with a fixed amount of DOPA residues, the DOPA is oxidized, followed by sclerotization and a highly regimented type of scleroprotein is formed. Similar content of DOPA in membranes and PLIM suggests that DOPA proteins contribute fundamentally to the organism's ultimate defence, the mineralized shell. How these proteins are processed and mixed by the animal, remains unknown. Recognition that membranes have the same DOPA content as PLIM opens up a good perspective for comparative study of these mechanisms.

The presence of high levels of iron and manganese in the shell of marine and freshwater molluscs is a consistent observation in the literature (Segar *et al.*, 1971; Allen, 1960). Carriker *et al.* (1991) found higher levels of Fe, Mn and Zn in *Crassostrea virginica* prismatic shell layers than we did. Swinehart and Smith (1979) found, in the case of the freshwater molluscs *Anodonta californiensis* and *Unio novahollandae*, that Mn and Fe were concentrated locally at high levels (up to 13 and 40% respectively) in their periostraca. Swinehart and Smith (1979) also found that the most probable source for the Fe and Mn was the mud where the molluscs were living. We have noticed as well that oysters from muddy sites have much higher Fe and Mn levels in their shells than oysters from open sites. One explanation for this was that proteinaceous material can provide good complexing sites for metal ions. According to Swinehart and Smith (1979), the high concentrations of manganese and iron provide a defensive buffer against acid attack on the periostracum of the mollusc itself.

We also found higher concentration of Fe, Zn and Mn in infested than in normal shells, which can be due to the higher organic compound on infested shells caused by blister membranes (Almeida *et al.*, in press). Our preliminary analyses on the isolated membranes confirm that they have high levels of these three metals. The concentrations of metals in the proteinaceous material of these fractions can particularly be explained by the fact that *o*-diphenols and semiquinones are capable of chelating various metals with their vicinal aromatic hydroxyls. DOPA, for example, can form stable ligands with copper (II) (Boggess and Martin, 1975), nickel (II), zinc (II), (Gergely and Kiss, 1979; Felix and Sealy, 1981) and manganese (II) (Martius and Schwarzhans, 1978). This property of *o*-diphenols may contribute to sclerotization of molluscan proteins by fostering a passive mineralization resulting from a selective sequestration of metals from sea water (Waite, 1983).

Similar spectra of membranes and PLIM, with superimposed binding-group bands also support the idea that their secretion mechanisms and functional role are identical. However, in the PLIM spectra the vibration modes at 1 087.5 cm^{-1} tend to be more intense. This may suggest a higher concentration of the binding-group that corresponds to this band. A specific characterization of the most prominent bands is our aim in further research.

Results presented here suggest that the organic matrix of the membrane secreted by the oyster for the purpose of protection from *Polydora* sp. attack is similar to the PLIM material secreted by the oyster as an external protective layer. This means that the external mantle epithelium, that normally produces the calcitostracum, can alter its normal function when the shell is damaged in a place that cannot be reached by the mantle border. This is in agreement with Kawaguti and Ikemoto (1962),

Beedham (1965) and Tsujii (1976), who pointed out that during shell regeneration of the freshwater bivalves *Anodonta* and *Musculus senhousia*, the epithelial cells are sequentially transformed into periostracum and prismatic layer secreting-type cells, before resuming their normal activities.

The similarity of the two organic matrices also suggests that the defence reaction against *Polydora* sp. is not a specific mechanism, which means that probably the same protective material is secreted by the oyster against any other mechanical damage.

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