Mar Biotechnol (2009) 11:686–698
DOI 10.1007/s10126-009-9182-5

ORIGINAL ARTICLE

First Insights into the Biochemistry of Tube Foot Adhesive from the Sea Urchin *Paracentrotus lividus* (Echinoidea, Echinodermata)

R. Santos · G. da Costa · C. Franco · P. Gomes-Alves · P. Flammang · A. V. Coelho

Received: 13 August 2008 / Accepted: 18 January 2009 / Published online: 17 February 2009

Abstract Sea urchins are common inhabitants of wave-swept shores. To withstand the action of waves, they rely on highly specialized independent adhesive organs, the adoral tube feet. The latter are extremely well-designed for temporary adhesion being composed by two functional subunits: (1) an apical disc that produces an adhesive secretion to fasten the sea urchin to the substratum, as well as a deadhesive secretion to allow the animal to move and (2) a stem that bears the tensions placed on the animal by hydrodynamism. Despite their technological potential for the development of new biomimetic underwater adhesives, very little is known about the biochemical composition of sea urchin adhesives. A characterization of sea urchin adhesives is presented using footprints. The latter contain inorganic residues (45.5%), proteins (6.4%), neutral sugars (1.2%), and lipids (2.5%). Moreover, the amino acid composition of the soluble protein fraction revealed a bias toward six amino acids: glycine, alanine, valine, serine, threonine, and asparagine/aspartic acid, which comprise 56.8% of the total residues. In addition, it also presents higher levels of proline (6.8%) and half-cystine (2.6%) than average eukaryotic proteins. Footprint insolubility was partially overcome using strong denaturing and reducing buffers, enabling the visualization of 13 proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The conjugation of mass spectrometry with homology–database search allowed the identification of six proteins: alpha and beta tubulin, actin, and histones H2B, H3, H2A, and H4, whose location and function in the adhesive are discussed but require further investigation. For the remaining unidentified proteins, five de novo-generated peptide sequences were found that were not present in the available protein databases, suggesting that they might be novel or modified proteins.

Keywords Marine adhesion · Sea urchin · Tube feet footprints · Protein-based material · Mass spectrometry

Electronic supplementary material The online version of this article (doi:10.1007/s10126-009-9182-5) contains supplementary material, which is available to authorized users.

R. Santos (✉) · G. da Costa · C. Franco · A. V. Coelho
Instituto de Tecnologia Química e Biológica,
Universidade Nova de Lisboa,
Oeiras, Portugal
e-mail: romana_santos@yahoo.com

P. Gomes-Alves
Departamento de Genética,
Instituto Nacional de Saúde Dr. Ricardo Jorge,
Lisbon, Portugal

P. Flammang
Laboratoire de Biologie Marine,
Université de Mons-Hainaut,
Mons, Belgium

A. V. Coelho
Departamento de Química,
Universidade de Évora,
Évora, Portugal

Introduction

Inspired by biomimetic applications, there has been a growing interest in the molecular mechanisms of adhesive bonding in marine organisms because of the efficiency of their adhesives in aqueous media. Therefore, studying bioadhesion might lead to the design of new synthetic adhesives for wet environments (e.g., for surgery and dentistry). In addition, as many of these bioadhesives are used by fouling organisms, elucidation of their structure

and function could also lead to the development of environmentally friendly, nontoxic coatings to prevent biofouling.

Underwater attachment is a quite complex phenomenon, involving a number of subfunctions such as water displacement from the substratum, spreading of the adhesive, coupling to a variety of substrata, curing to stiffen and toughen the adhesive, and protection from microbial degradation (Waite 1987). The design of biomimetic adhesives thus requires a full understanding of all this functional properties.

Despite their technological potential, a considerable dearth of information remains regarding the biochemical composition of marine adhesives. Most studies have focused on the characterization of permanent adhesives characteristic of sessile organisms staying at the same place throughout their adult life such as mussels, tube-dwelling polychaetes, and barnacles. These permanent cements consist of multiprotein complexes, usually secreted as a fluid that undertakes progressive hardening due to intermolecular cross-linking. In mussels and tubeworms, cross-linking is achieved through covalent bonds involving a posttranslationally modified amino acid, 3,4-dihydroxy phenylalanine or DOPA, while in barnacles it is due to intermolecular noncovalent interactions (e.g., hydrophobic or electrostatic interactions) (for review, see Kamino 2008; Sagert et al. 2006; Silverman and Roberto 2007). Comparatively, nonpermanent adhesives that allow the organisms to attach and also to move have received much less attention. They are typically more hydrated and consist of a mixture of proteins and polysaccharides. This is the case of the best characterized nonpermanent adhesive, the limpet adhesive, that forms a hydrogel in which proteins are linked to polysaccharides through noncovalent bonds (for review, see Smith 2006). Although a few publications on the biochemistry of other nonpermanent adhesives from flatworms, sea stars, and sea cucumbers have enlarged our knowledge (Flammang et al. 1998; Hamwood et al. 2002; DeMoor et al. 2003; Li and Graham 2007), there is still a considerable gap relative to permanent adhesives.

The properties of sea urchin temporary adhesive are remarkable for several reasons. Tube feet (1) have a high adhesive strength (force per unit area) ranging from 0.09 to 0.54 MPa (Santos et al. 2005; Santos and Flammang 2006, 2008), in the range of values measured in other marine invertebrates (0.1–0.5 and 0.5–1 MPa for nonpermament and permanent adhesives, respectively; Smith 2006) and matching the technological requirements for underwater synthetic adhesives (0.2–0.7 MPa; Waite 2002); (2) attach efficiently to substrata with various chemistries and roughness (Santos et al. 2005; Santos and Flammang 2006); and (3) have highly specialized epidermal adhesive areas made up of different secretory cells that release separately adhesive and deadhesive secretions, thus enabling repeated attachment–detachment cycles (Santos and Flammang 2006).

To our knowledge, no study has been done on the biochemistry of sea urchin tube foot adhesive. The only available information on the nature and composition of this adhesive comes from histochemical studies showing that echinoid footprints (circular prints of secreted adhesive that remain on the substratum after detachment) stain for acid mucopolysaccharides but not for proteins (Flammang and Jangoux 1993). Therefore, the aim of the present work was to increase our knowledge on sea urchin temporary adhesive through the study of the biochemistry of the tube feet footprints. The gross biochemical composition of the footprints as well as the initial characterization and identification of their major proteins was achieved by combining classical biochemical techniques with modern tools, such as mass spectrometry and homology–database search.

Materials and Methods

Animal and Sample Collection

Sea urchins from the species *Paracentrotus lividus* (Lamark 1816) were collected at low tide on the west coast of Portugal (Estoril, Cascais) After collection, the animals were transported to the "Vasco da Gama Aquarium" (Algés, Oeiras) and kept in open-circuit tanks at 15°C and 33‰. The adhesive material (AM) was collected by placing sea urchins in small plastic aquariums (3 L) filled with artificial seawater (Crystal Sea, Marine Enterprises International, Baltimore, MD, USA). These aquaria were covered internally with removable glass plates to which animals were allowed to attach. After a few hours, these glass plates, covered with hundreds of footprints, were removed, rinsed with distilled water, and scrapped with disposable scalpels. The obtained material was extensively washed with Milli-Q water, dried by vacuum centrifugation, and stored at −20°C until further use.

Chemical and Biochemical SAM Composition

All analyses were performed on duplicate samples (1.0 to 1.5 mg each) of freeze-dried adhesive material (AM) weighed on a micro analytical balance (Sartorius AG, Goettingen). For inorganic residue analysis, samples were ashed in a muffle furnace for 3 h at 550°C and reweighted. The mass of residual ash, expressed as a percentage of the sample mass, was taken as the total inorganic residue.

Total lipids and neutral sugars content were quantified by spectrophotometric methods with, respectively, the

chloroform/sulfuric acid method using tripalmitin as a standard (Marsh and Weinstein 1966) and the phenol/sulfuric acid method using D-glucose as a standard (Dubois et al. 1956). The total amount of protein present in the footprint material was estimated using amino acid analysis. For this, the samples were suspended in 6 M HCl with 1% phenol and hydrolyzed under vacuum in sealed tubes for 24 h at 110°C. Amino acid concentrations were measured on an amino acid analyzer (Waters Alliance System, Milford, MA, USA). Then, the masses of individual amino acids were added and the total mass of the amino acids was expressed as a percentage of the mass of the sample. In addition, the protein content of the soluble fraction (soluble adhesive material—SAM) was also quantified with a commercial kit using bovine serum albumin as a standard (2D Quant Kit, GE Healthcare, Buckinghamshire).

Protein Extraction and Separation by 1D SDS-PAGE

AM (1 mg in dry weight) was suspended in 1 mL 10% trichloroacetic acid, 0.07% β-mercaptoethanol (*w*/*v*) for 1 h at 4°C to precipitate the proteins, then washed three times with 1 mL of cold (≈−20°C) 0.07% β-mercaptoethanol in acetone (*v*/*v*), and finally vacuum dried. The obtained protein pellet was solubilized in 60 μL of 2% sodium dodecyl sulfate (SDS), 0.5 M dithiothreitol (DTT) in 63.2 mM Tris–HCl pH 6.8. The homogenized suspension was heated for 3 h at 60°C and, after cooling, centrifuged at 6,200×*g* for 35 min at 20°C. The supernatant (SAM) was fractioned in 15 μL aliquots. The remaining pellet (insoluble adhesive material) was washed several times with Milli-Q water, freeze-dried, and weighed on a micro analytical balance. To assess the efficiency of the extraction protocol, footprints left by sea urchins on microscope glass slides were subjected to the same solubilization buffer and their integrity was checked, after staining, with a light microscope.

For gel electrophoresis, 5 μL of sample buffer (2% SDS, 20% glycerol, 5% β-mercaptoethanol (*w*/*v*/*v*) in 62.5 mM Tris–HCl pH 6.8) were added to each 15 μL supernatant (SAM) fractions and the resulting solution was heated for 5 min at 95°C. Protein separation was achieved using 8% or 15% polyacrylamide gels with 3.5% stacking gels. Electrophoresis was carried out using the mini Protean II gel system (Bio-Rad) at a constant voltage of 150 V. The separated proteins were visualized by staining overnight with a very sensitive Colloidal Coomassie Brilliant Blue (Candiano et al. 2004). Protein separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (8% and 15% polyacrylamide) was repeated for three footprint collection events in three different gels, in order to ensure enough replication for subsequent protein identification.

Protein Disulfide Bond Detection by 2D Diagonal SDS-PAGE

The detection of disulfide bonds was done by 2D diagonal SDS-PAGE as described by Winger et al. (2007). SAM proteins were prepared as described above, with the exception that, for the first dimension, proteins were solubilized under reducing and nonreducing conditions, i.e., with and without DTT, prior to protein separation by SDS-PAGE in 12.5% polyacrylamide gels. Following separation, lanes were excised and incubated in 0.5 M DTT for 1 h at room temperature with stirring. Then, the reduced lanes were sealed with agarose on top of 12.5% polyacrylamide gels, in order to run the second dimension.

In-Gel Digestion

Protein bands were manually excised from the gels using a disposable scalpel, washed in Milli-Q H_2O, and distained in 50% acetonitrile (ACN) and subsequently 100% ACN. Disulfide bonds were reduced with 10 mM DTT and alkylated with 50 mM iodoacetamide. The dried gel pieces were swollen in a 50-mM NH_4HCO_3 digestion buffer containing 6.7 ng/μL of trypsin (modified porcine trypsin, sequencing grade; Promega, Madison, WI, USA) on an ice bath. After 30 min, the supernatant was removed and discarded, 20 μL of 50 mM NH_4HCO_3 was added to the gel pieces and digestion was allowed to proceed at 37°C overnight. After digestion, the remaining supernatant was removed and stored at −20°C until use (Kussmann and Roepstorff 2000).

MALDI-MS/MS

Protein digests were desalted and concentrated as previously described (Gobom et al. 1999; Larsen et al. 2002). Home-made microcolumns were made by packing POROS R2 chromatographic resin (PerSeptive Biosystems, Foster City, CA, USA) or graphite powder (activated charcoal; Sigma-Aldrich, St. Louis, MO, USA) in a constricted GELoader tip (Eppendorf, AG, Hamburg). A syringe was used to force liquid through the columns by applying gentle air pressure. The columns were equilibrated with 20 μL of 2% trifluoroacetic acid (TFA) and the peptide digests were added first to R2 microcolumns and the flow through transferred directly to graphite microcolumns. Then, the columns were washed with 20 μL of 2% TFA and the peptides were eluted with 0.8 μL of α-cyano-4-hydroxycinnamic acid solution (CHCA, Sigma-Aldrich, St. Louis, MO, USA; 10 mg/μL in 70% ACN, 0.1% TFA) directly onto the MALDI target.

Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) spectra were acquired on an Applied Biosystems

4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, Foster City, CA, USA) in both MS and MS/MS mode. Positively charged ions were analyzed in positive reflectron mode and the collision gas used for fragmentation was atmospheric air. Each MS spectrum was obtained with a total of 1,000 laser shots accumulations (eight subspectra consisting of 125 laser shots each) and was externally calibrated using six spots of the standard mixture (Calibration Mixture 2, Applied Biosystem, Foster City, CA, USA). Five *s*/*n* best precursors from each MS spectrum were selected for MS/MS analysis. For MS/MS spectra, a maximum of 5,200 laser shots were accumulated, each subspectrum consisting of 65 shots (maximum of 80 subspectra). Raw data were generated by the 4000 Series Explorer Software v3.0 RC1 (Applied Biosystems, Foster City, CA, USA) and all contaminant *m*/*z* peaks originating from human keratin, trypsin autodigestion, or matrix were included in the exclusion list used to generate the peptide mass list used in database search.

The interpretation of the combined MS+MS/MS data was carried out using the GPS Explorer software (Version 3.5, Applied Biosystems, Foster City, CA, USA). Peptide mass maps and sequences obtained were searched against the National Center for Biotechnology Information (NCBI) database with no taxonomic restriction (6,572,387 entries, June 6, 2008) and against the purple sea urchin *Strongylocentrotus purpuratus* database (42,420 entries; ftp://ftp.ncbi.nih.gov/genomes/Strongylocentrotus_purpuratus/protein/protein.fa.gz) using an in-house MASCOT server (Version 2.0). The search was performed using monoisotopic peptide masses and the following criteria: one missed cleavage, $p<0.05$ significance threshold, 50 ppm peptide mass tolerance, 0.25 Da fragment mass tolerance, carbamidomethylation of cysteine as fixed modification, and methionine oxidation as variable modification. Significant hits were visually inspected to eliminate false positives.

MS/MS spectra of the unidentified proteins were further analyzed by the DeNovo Explorer™ software (Version 3.5, Applied Biosystems, Foster City, CA, USA) using the following settings: trypsin as enzyme, carbamidomethylation of cysteine as fixed modification, methionine oxidation as variable modification, 0.2 Da fragment tolerance. This software automatically generates candidate sequences, assigning them a score between 0 and 100, which is an indication of the degree of matching between the theoretical fragmentation pattern and the fragmentation spectra of the SAM peptides. In order to minimize randomness, we only considered peptides with scores higher than 70 and with at least two spectra with identical candidate sequence for the same gel band in two replicate gels. These de novo-derived sequences were submitted to Basic Local Alignment Search Tool (BLAST) searches at http://blast.ncbi.nlm.nih.gov/Blast.cgi, using the following settings: nonredundant protein sequence database, taxonomy restricted to *S. purpuratus* (taxid: 7668), and blastp as algorithm.

LC-ESI-MS/MS

Protein digests were also analyzed by liquid chromatography electrospray ionization (LC-ESI) linear ion trap-MS/MS using a Surveyor LC system coupled to a linear ion trap mass spectrometer model LTQ (Thermo-Finnigan, San Jose, CA, USA). Peptides were concentrated and desalted on a RP precolumn (0.18×30 mm, BioBasic18, Thermo Electron, San Jose, CA, USA) and online eluted on an analytical RP column (0.18×150 mm, BioBasic18, Thermo Electron, San Jose, CA, USA). Peptides were eluted using 33-min gradients from 5% to 60% solvent B (solvent A: 0.1% formic acid, 5% acetonitrile; solvent B: 0.1% formic acid, 80% acetonitrile) at a flow rate of 2 μL/min. The linear ion trap was operated in data-dependent ZoomScan and MS/MS switching mode using the three most intense precursors detected in a survey scan from *m*/*z* 450 to 1,600. Singly charged ions were excluded for MS/MS analysis. ZoomScan settings were: 200 ms maximum injection time, 3,000 ions as zoom target parameter, and three microscans. Normalized collision energy was set to 35%, and dynamic exclusion was applied during 10-s periods to avoid fragmenting of the same ion more than twice.

Peptide MS/MS data were evaluated using Bioworks™ 3.3.1 software. Searches were performed against an indexed UniRef100 database with no taxonomic restriction (5,888,655 entries; http://www.uniprot.org). The following constraints were used for the searches: two missed cleavages, 2 Da tolerance for precursor ions, 1 Da tolerance for MS/MS fragments ions, carbamidomethylation of cysteine as fixed modification, and methionine oxidation as variable modification. Only protein identifications with two or more distinct peptides, a $p<0.01$, and Xcorr thresholds of at least 1.5/2.0/2.5 for singly/doubly/triply charged peptides were accepted. Protein identifications were further validated by visual inspections of the MS/MS spectra.

Results

Biochemical Composition

The analysis of *P. lividus* footprint material revealed that it contains a significant amount of inorganic residues (45.5%) and a considerably lower amount of proteins (6.4%), neutral sugars (1.2%), and lipids (2.5%) (Fig. 1). Moreover, the quantification of the protein content of the SAM by a commercial kit revealed that it contains approximately 4.9% of protein, indicating that around 1.5% of the total

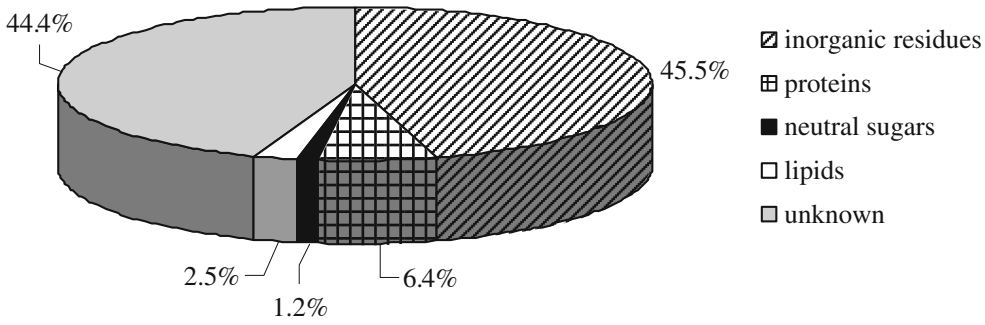

Fig. 1 Pie diagram illustrating the biochemical composition of the footprint material of *P. lividus*

protein content of the footprint material is insoluble in the buffers tested in the present study.

The whole footprint adhesive material presented slightly more nonpolar (57.4%) than polar amino acids (42.6%), the latter being composed of equivalent amounts of both charged (20.2%) and uncharged residues (22.4%) (Table 1). It is also interesting to notice that the adhesive material of *P. lividus* has a bias toward six amino acids: Gly, Ala, Val, Ser, Thr, and Glx, which comprise 56.8% of the total residues. In addition, it also presents higher levels of Pro (6.8%) and Cys/2 (2.6%) than average eukaryotic proteins. No DOPA residues were found in the footprint material.

Protein Extraction

Buffer composition optimization included the use of denaturing agents such as SDS, urea, or guanidine hydrochloride, reducing agents such as DTT or β-mercaptoethanol, acids such as acetic acid, and calcium chelators such as ethylenediaminetetraacetic acid. Among the conditions tested, proteins were best solubilized in the presence of important amounts of the anionic detergent SDS and the reducing agent DTT. Still, complete solubilization could not be achieved and a small pellet persisted after centrifugation, corresponding, on average, to 20% of the whole footprint material (in dry weight). Quantification of the amount of protein extracted also indicated that about 77% of the SAM proteins were extracted whereas about 23% remained insoluble. Prior washing steps to remove excess salt and contaminants significantly improved the subsequent protein separation by SDS-PAGE.

The effect of the extraction buffer was tested on whole footprints deposited on microscope glass slides. Most footprints were removed from the slides with this treatment while they were unaffected when the extraction buffer was replaced by distilled water (Fig. 2).

Protein Separation by 1D SDS-PAGE and 2D Diagonal SDS-PAGE

One-dimension gel electrophoresis revealed that SAM separate into 13 protein bands with apparent molecular masses ranging from 10 to 200 kDa, forming two groups, one with eight bands ranging from 40 to 100 kDa and another with five bands ranging from 10 to 20 kDa (Fig. 3). Due to the low resolution achieved after separation in 12.5% polyacrylamide gels, it was difficult to excise the bands for further analysis. The use of 8% and 15% polyacrylamide gels allowed a better separation of high- and low-molecular-weight proteins, respectively. In this way, the obtained protein separation pattern was reproducible among different footprint collection events and technical replicates.

SAM proteins were also separated by two-dimension diagonal gel electrophoresis under nonreducing and reducing conditions, in the first dimension, the latter used as a control. In the absence of DTT, a lower amount of protein was separated due to the formation of agglomerates that failed to enter the gel, accumulating in upper part of the lane (Fig. 4a). However, when the nonreduced proteins were exposed to DTT and separated in a second dimension, three spots showed altered electrophoretic mobility which

Table 1 Amino acid composition of the adhesive footprints from the sea urchin *P. lividus*

Amino acid	Footprints
ASX[a]	48
THR	74
SER	86
GLX[a]	74
PRO	68
GLY	147
ALA	98
CYS/2[b]	26
VAL	89
MET	19
ILEU	50
LEU	72
TYR	38
PHE	31
LYS	27
HIS	13
ARG	40

Values are in residues per thousand

[a] Deamidation during acid hydrolysis of the footprint material eliminates the distinction between Asn and Asp, the same happening with Glu and Gln; therefore, these two amino acid pairs are presented together as Asx and Glx, respectively

[b] Reported values are for half-cystine, cysteine being destroyed by the acid hydrolysis of the footprint material

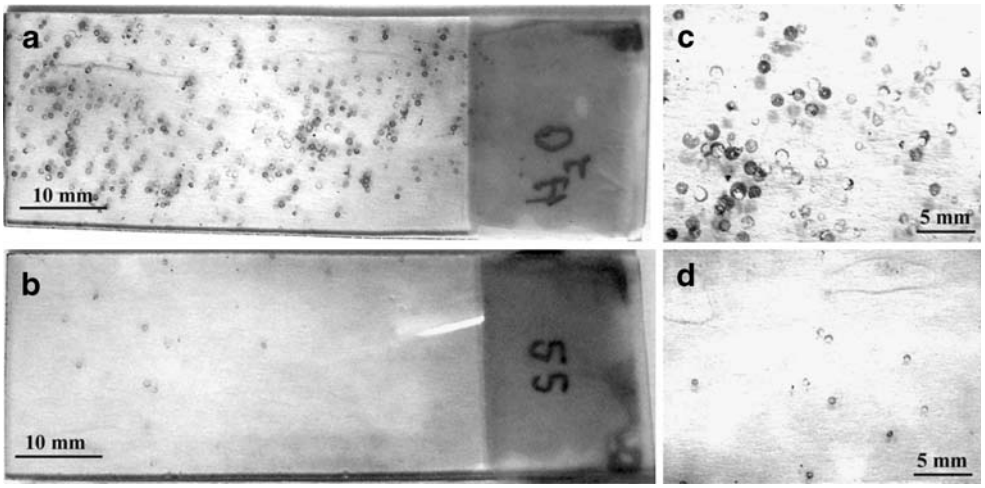

Fig. 2 Tube feet footprints remaining on microscope slides after treatment with the extraction buffer (**b**, **d**) and with distilled water (**a**, **s**); see text for details. After treatment, the footprints were stained with a 0.05% aqueous solution of the cationic dye crystal violet

was not observed in the presence of DTT (Fig. 4b). Proteins unresponsive to the reducing agent disposed themselves in a diagonal line. These results suggest that at least two proteins of the footprint material present disulfide bonds. A spot around 20 kDa in the second dimension (below the diagonal) is consistent with a monomer reduced from a dimer of approximately 40 kDa with intermolecular S–S bridges in the first dimension. As for the two spots above the diagonal line, around 50 and 60 kDa, they are more consistent with the presence of intramolecular disulfide bonds which reduction retarded its displacement in the second dimension. These two spots can correspond to two proteins or to two reduction states of the same protein.

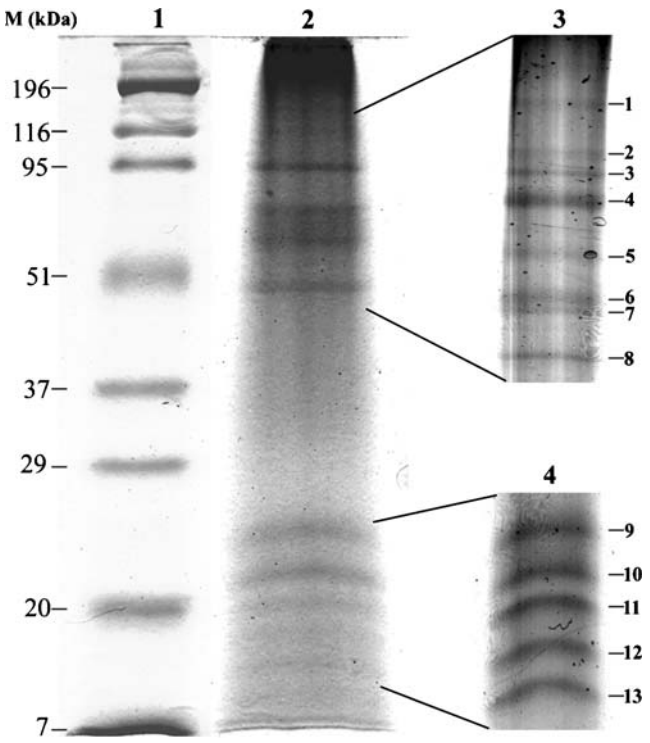

Fig. 3 Proteins from the footprint material of *P. lividus*. *Lane 1* molecular mass markers, *lane 2*, *3*, and *4* proteins separated in 12.5%, 8%, and 15% polyacrylamide gels, respectively. *Numbers on the right side of lanes 3 and 4* indicate the number given to the analyzed gel bands

Protein Identification by Mass Spectrometry

Among the 13 gel bands separated by SDS-PAGE, six could be identified by MALDI-TOF/TOF (Table 2 and Supplemental Table 1 of the Electronic supplementary

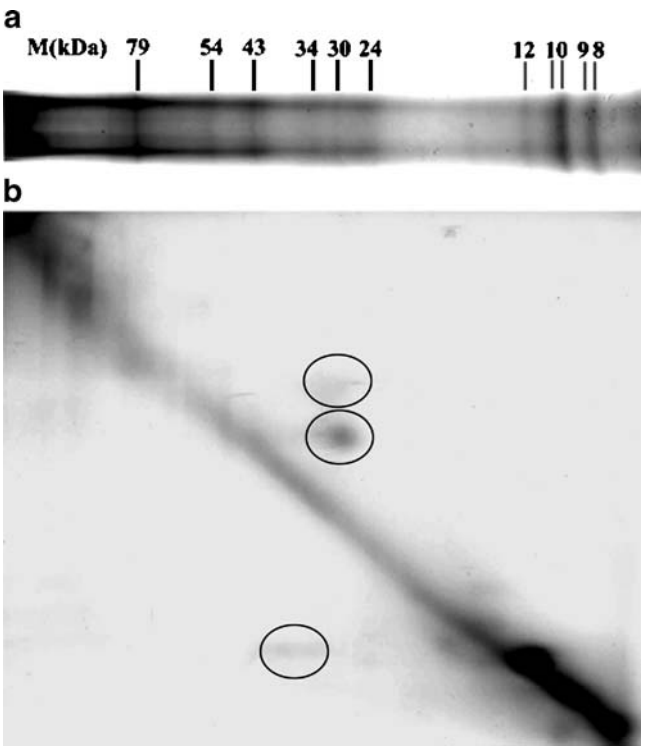

Fig. 4 2D diagonal SDS-PAGE analysis of footprint material of *P. lividus*. Footprint proteins were extracted in nonreducing conditions (**a**) prior to first dimension separation by SDS-PAGE. The excised lane was reduced in 0.5 M DTT prior to the second dimension by SDS-PAGE (**b**). Proteins containing intramolecular or intermolecular disulfide bonds separate in the second dimension above or below the diagonal, respectively (*circled spots*)

Table 2 Proteins of the footprints from the sea urchin *P. lividus* identified by MALDI-TOF/TOF-MS of in-gel tryptic digests of the bands indicated in Fig. 2

Gel band	Protein name	Species	Accession number[a]	Protein molecular masses (kDa)[b]		Protein		Peptide		Number of distinct peptides[c]	Sequence coverage[d]
				Observed	Expected	Score[e]	CI (%)[f]	Score[e]	CI (%)[f]		
7	Hypothetical protein	*Strongylocentrotus purpuratus*	gi|72065171	49	50	226	100	142	100	10	22.1
	Tubulin beta chain	*Paracentrotus lividus*	gi|135489	49	51	226	100	142	100	10	22.1
	Similar to tubulin alpha 2 isoform 2	*Strongylocentrotus purpuratus*	gi|115843650	49	50	158	100	131	100	5	17.9
	Tubulin alpha chain	*Paracentrotus lividus*	gi|135400	49	50	158	100	131	100	5	17.9
8	Muscle actin	*Strongylocentrotus purpuratus*	gi|187282496	39	41	415	100	326	100	11	37.5
	Actin, cytoskeletal	*Heliocidaris erythrogramma*	gi|1703136	39	42	415	100	326	100	11	37.5
9	Histone H2B-1, sperm-specific	*Strongylocentrotus purpuratus*	gi|47551059	19	15	135	100	103	100	5	32.1
	Histone H2B-2, sperm	*Parechinus angulosus*	gi|108885304	19	16	92	99.5	59	99.5	5	31.3
11	Histone H3	*Strongylocentrotus purpuratus*	gi|72088352	14	15	191	100	163	100	5	25.0
12	Histone H2A-3	*Strongylocentrotus purpuratus*	gi|115933975	12	13	148	100	131	100	3	18.4
	Histone H2A, sperm	*Lytechinus pictus*	gi|121977	12	12	152	100	131	100	3	20.5
13	Similar to histone H4	*Strongylocentrotus purpuratus*	gi|115916202	10	11	425	100	322	100	9	58.2
	Histone H4	*Paracentrotus lividus*	gi|3287225	10	11	425	100	322	100	9	59.4

Peptide mass (MS) and fragmentation (MS/MS) data were used to search against the purple sea urchin and NCBI databases

[a] First and second accession numbers of the identified protein in the Sea Urchin and the NCBI database, respectively, with one exception in which the same protein was identified in both databases

[b] Identified protein molecular mass

[c] Peptide whose sequence differs in at least 1 amino acid residue

[d] Percentage of the identified protein sequence covered by the matched peptides

[e] *Mowse* scoring algorithm

[f] Confidence index that the protein/peptide match was not random

material). Graphite microcolumns generally produced equivalent or better identification scores in comparison to reverse-phase columns. The former recovered more small hydrophilic peptides than the latter (Larsen et al. 2002), which allowed us to improve both the MS and MS/MS score and to increase sequence coverage (data not shown). Therefore, only the best identification scores, obtained with one of the two microcolumns tested, were included in Table 2. Six proteins were successfully identified in the two databases used with significant scores, a considerable number of distinct peptides, and high sequence coverage. Moreover, it is interesting to notice that, whenever a positive match was obtained in the purple sea urchin (*S. purpuratus*) predicted protein database, it was confirmed by a match with a homologous protein from another sea urchin species deposited in the NCBI database, although no taxonomic restriction was applied.

Gel band 7 gave three possible identifications: a hypothetical protein and beta or alpha tubulin. *S. purpuratus* hypothetical protein probably corresponds to a beta tubulin because it shares 97% of common amino acids with *P. lividus* tubulin beta chain. These problems are not unusual, especially in databases that are still being annotated and have not yet been curated. Although higher scores and better sequence coverage were obtained for beta tubulin, specific alpha tubulin tryptic peptides were also found in the same band. In fact, both chains have around a similar mass, 50 kDa, which matches the apparent molecular mass observed in SDS-PAGE. As for gel band 8, it was identified either as muscle actin (41 kDa) or as cytoskeletal actin (42 kDa), also in accordance with the band position in the gel. As for gel bands 9, 11, 12, and 13, they were successfully identified as histones H2B, H3, H2A, and H4, respectively. These histones have molecular weights of about 16, 15, 13, and 11 kDa, respectively, which again correspond to their position in the gel. The remaining bands could not be identified despite the quality of the obtained MS and MS/MS spectra.

An additional analysis of digests by LC-MS/MS (Table 3) did not increase the number of identified proteins, but in fact allowed for confirmation of four out of six proteins identified by MALDI-MS/MS. Significant identifications were obtained in both the purple sea urchin and the UniRef100 database; however, in some cases, the homologies found in the UniRef100 were not from sea urchin species but other organisms, such as nematodes or protozoans. Although a lower number of peptides were identified by LC-MS/MS, these peptides were longer, thus ensuring reasonable sequence coverage. Similarly to MALDI-MS/MS, the remaining proteins could not be identified despite the quality of the obtained chromatography and MS/MS spectra.

Unfortunately, it was not possible to identify the proteins that presented an altered electrophoretic mobility in the 2D diagonal SDS-PAGE (Fig. 4) due to the reduced amount of protein in these spots.

Table 3 Proteins of the footprint material from the sea urchin *P. lividus* identified by LC-ESI-MS/MS of in-gel tryptic digests of the bands indicated in Fig. 2

Gel band	Protein name	Species	Accession number[a]	Protein molecular weight (kDa)[b]		Protein score[c]	Number of distinct peptides[d]	Sequence coverage[e]
				Observed	Expected			
7	Similar to beta tubulin	*Strongylocentrotus purpuratus*	gi|115975114	49	50	20.20	2	8.74
	Tubulin beta-1 chain	*Caenorhabditis briggsae*	gi|2501421	49	49	20.23	2	8.84
	Similar to tubulin alpha chain	*Strongylocentrotus purpuratus*	gi|115951336	49	52	20.25	2	6.18
8	Muscle actin	*Strongylocentrotus purpuratus*	gi|187282496	39	41	40.32	4	19.95
	Cytoplasmic actin CyII	*Heliocidaris erythrogramma*	gi|74892236	39	40	10.32	1	8.31
9	Histone H2B-1, sperm-specific	*Strongylocentrotus purpuratus*	gi|47551059	19	15	20.22	1	10.71
	Histone H2B-1, sperm	*Parechinus angulosus*	gi|108885302	19	16	20.25	1	10.34
13	H4 histone protein	*Strongylocentrotus purpuratus*	gi|47551061	10	11	50.20	4	40.78
	Histone H4, major	*Tetrahymena pyriformis*	gi|51317342	10	11	30.19	2	11.70

Peptide mass (MS) and fragmentation (MS/MS) data were used to search against the purple sea urchin and UniRef100 databases

[a] First and second accession numbers of the identified protein in the Sea Urchin and the NCBI database, respectively, with one exception in which the protein was only identified in the Sea Urchin database.

[b] Identified protein molecular mass

[c] Sequest XCorr score

[d] Peptide whose sequence differs in at least one amino acid residue

[e] Percentage of the identified protein sequence covered by the matched peptide

Table 4 Peptide de novo-generated sequences representative of the unidentified proteins of the footprints from the sea urchin *P. lividus*

Gel band	Protein molecular weight (kDa)[a]	Peptide mass	Sequence[b]	Replicates[c]	Score[d]
1	129	–	–	–	–
2	104	1,053.48	RQ*MLYAN*R	2	85–92
3	93	1,170.47	SSGM*N*TSKQ*GR	2	87–91
4	81	1,022.49	KPGYI/LAM*AR	2	91–94
5	64	1,022.48	KPGYI/LAM*AR	4	87–91
6	51	–	–	–	–
10	16	1,070.46	KEGWQ*M*XK	3	76–78
		1,267.61	HAM*DPN*L(297)R	4	75–80

Asterisks represent modified amino acids, either oxidation of methionine or deamidation of asparagine or glutamine. Values between brackets correspond to gaps in the peptide sequence that can be filled with various isobaric combinations of amino acid residues

[a] Molecular weight observed in the gel

[b] Candidate sequenced obtained with DeNovo Explorer

[c] Number of spectra of the same peptide with the same candidate sequence

[d] DeNovo Explorer score

MALDI-MS/MS spectra of the unidentified protein bands were further used to automatically generate de novo sequences in an attempt to get more information on these proteins. This approach generates sequences based on the "best fit" to the MS/MS data taking into account the complexity of the spectra and the numerous possible *a*, *b*, *c*, *x*, *y*, *z* ion combinations (Supplemental Figure 1 of the Electronic supplementary material). Although several different peptides were fragmented for each gel band, only the most representative are presented (Table 4). For two of the gel bands, 1 and 6, no candidate peptide sequences could be assigned. As for the remaining bands, 2–5 and 10, at least one putative peptide sequenced with a significant score could be assigned. Curiously, for gel bands 4 and 5, the same representative peptide was found which might be explained by a combination of gel streaking and band contiguousness. These five de novo-derived sequences were submitted to BLAST search against the *S. purpuratus* predicted protein database, but no significant match was obtained. Some partial homologies were found, but with proteins with unmatched molecular weights or with low expectation values (data not shown). Therefore, all the different approaches used to identify the remaining seven proteins were unsuccessful, strongly suggesting that these proteins are either novel or highly modified proteins.

Discussion

Biochemical Composition of Sea Urchin Footprint Material

Wave-swept shores are among the most stressful environments on earth. Organisms that inhabit these areas had to develop different mechanisms to attach themselves to the substratum in order to withstand the action of waves. Sea urchins do so by employing a multitude of independent adhesive organs, the adoral tube feet, which are extremely well-designed for temporary adhesion. They possess an enlarged and flattened apical disc that produces an adhesive secretion to fasten the sea urchin to the substratum as well as a deadhesive secretion to allow voluntary detachment. In addition, this disc is connected to an extensible stem that bears the tensions placed on the animal by hydrodynamic forces (Flammang and Jangoux 1993; Flammang 1996). The adhesive secretion is delivered through the disc cuticle onto the tube foot distal surface where they form a thin film that binds the disc to the substratum (Flammang et al. 2005). Deadhesive secretions are released within the cuticle where they are believed to cause the discard of its outermost layer, the so-called fuzzy coat, due to their enzymatic activity. Thus, after detachment, most of the adhesive material remains strongly attached to the substratum as a footprint (Flammang and Jangoux 1993; Flammang 1996; Flammang et al. 1998). Since very little is known about the biochemical composition of both secretions, as it is the case for nonpermanent adhesives in general, there is an urgent need to fill this gap. That was the motivation for the present study, which constitutes the first report on the biochemical composition of the tube feet footprints of a sea urchin species.

Inorganic residues apart (45.5%), the footprints are made up of proteins (6.4%), neutral carbohydrates (1.2%), and lipids (2.5%). At present, data on the biochemical composition of echinoderm adhesive footprints are only available for one sea star species, *Asterias rubens* (Flammang et al. 1998). In this species, footprints also contain a significant amount of inorganic residues (40%), similar values of neutral sugars (3%) and lipids (5.6%) but a much higher

amount of proteins (20.6%). Other carbohydrates such as amino sugars (1.5%) and uronic acids (3.5%) were also found in *A. rubens* footprints. However, the same authors questioned the presence of lipids in SAM, arguing that it might be due to the presence of membranes from granules secreted by the adhesive cells and that would have been incorporated into the footprint. Results on the composition of footprints of sea urchins and sea stars are in accordance with previous studies using various dyes in which sea urchin footprints stained for acid mucopolysaccharides but not for proteins (Flammang and Jangoux 1993) whereas sea star footprints stained for both biomolecules (Flammang et al. 1994). This might indicate that the unknown fraction of sea urchin footprints might be partly composed of amino sugars and uronic acids that were not quantified in this study. Since adhesive secretion appears to make up the bulk of the footprint material, the biochemical composition given in Table 1 probably reflects the composition of this secretion. However, as pointed out by Flammang et al. (1998), interpretation of the biochemical composition must take into account the fact that, in addition to adhesive material, footprints also seem to contain cuticular material. The high proportion of inorganic material found in echinoderm footprints is also present in the adhesives from marsh periwinkle snails and limpets (Smith et al. 1999; Smith and Morin 2002), and these authors suggest that it presumably results from dried salts left over when seawater within the adhesive evaporates. The cooccurrence of proteins and carbohydrates also seems to be a common trait among nonpermanent adhesives of marine invertebrates, being observed, besides sea stars and sea urchins, in sea cucumbers (DeMoor et al. 2003), snails (Smith and Morin 2002) and limpets (Smith 2006). These protein–carbohydrate complexes typically form highly hydrated adhesives with viscoelastic properties (Flammang et al. 1998; Smith 2006) which contrast with the rigid permanent proteinaceous adhesive cements.

The protein moiety of *P. lividus* footprint adhesive material was further characterized in terms of amino acid composition, showing that it contains slightly more nonpolar (57%) than polar (43%) residues, the latter being composed of equivalent amounts of both charged (20%) and uncharged 23%) polar residues. There is a clear predominance of six amino acids: Gly, Ala, Val, Ser Thr, and Glx that together constitute more than half of the total residues, as well as a higher amount of Pro and Cys/2 than in average eukaryotic proteins. In comparison, the footprints of *A. rubens* contain slightly more polar (55%) than nonpolar (45%) residues and, among polar residues, more charged (34%) than uncharged residues (21%). Like in sea urchin, sea star adhesive material have high levels of Gly and Cys/2, but also presents higher amounts of Glx and Asx (Flammang et al. 1998).

Cysteine residues may be involved in intermolecular disulfide bonds reinforcing the cohesive strength of the adhesive and contributing to the insolubility of marine adhesives (Flammang et al. 1998; Flammang 2006). In mussels, a cystine-rich protein (mefp-2) is believed to be responsible for the sponge-like matrix formation in byssal plaques (Rzepecki et al. 1992). Alternatively, they may form intramolecular disulfide bonds, holding proteins in the specific shape required for interaction with their neighbors, as is the case in barnacle (Kamino 2006) or snail adhesives (Smith 2006). These intramolecular bonds, therefore, also contribute to the cohesiveness and insolubility of the adhesive but in a more indirect way. Any of these two types of disulfide bonds may occur in echinoderm adhesives since, in both sea urchins and sea stars, considerable amounts of Cys were found. In sea urchins, this is further corroborated by the identification of three proteins of the footprint material, presenting a shift in mobility in 2D diagonal SDS-PAGE.

The absence of DOPA from the footprint material indicates that contrary to the cements of mussels and polychaetes (Jensen and Morse 1988; Waite and Tanzer 1981), in sea urchin, DOPA cross-links do not seem to contribute for the adhesive cohesiveness and insolubility.

Small side chain amino acids (Ser, Gly, Ala, Pro) are also characteristic of both permanent and nonpermanent marine adhesives (for review, see Flammang 2006), being also present in large quantities in elastomeric proteins that can withstand significant deformation without rupture (Tatham and Shewry 2000). Therefore, these amino acids may also presumably account for the high cohesive strength of marine adhesives. Moreover, the predominance of the amino acids Ser and Thr on one hand and Val on the other hand in the marine adhesives has been interpreted as being useful in coupling with diverse foreign substrata via hydrogen bonding or electrostatic interactions and via hydrophobic interactions, respectively. In fact, during the initial process of underwater attachment, adhesive proteins have to bind to a substratum to which water molecules are adsorbed. Therefore, this weak boundary water layer has to be removed prior to attachment and spreading of the adhesive on the substratum surface. The importance of the hydroxyl group on the Ser and Thr residues for this priming process has been postulated in barnacles, polychaetes, and mussels (Kamino et al. 1996; Jensen and Morse 1988; Waite 1987). As for charged and polar amino acids, they may be involved in adhesive interactions with the substratum through hydrogen and ionic bonding, and therefore, contribute to the high adhesive strength of marine bioadhesives (Waite 1987). There seems to be a higher contribution of these amino acids in sea star adhesion (Asx, 11.8%; Glx, 10.2%; Flammang et al. 1998) compared to sea urchins (Asx, 4.8%; Glx, 7.4%).

Proteins from Sea Urchin Footprint Material

Similarly to previous studies on marine bioadhesives, the present work was hindered by the insolubility of the footprint material. Our experiments demonstrate that this material is highly insoluble even in the presence of strong solubilizing agents (insoluble fraction accounts for 20% of the total amount of adhesive material); protein insolubility being only overcome with the use of a significant amount of a reducing agent. Moreover, the analysis of footprint material using 2D nonreducing/reducing diagonal SDS-PAGE highlighted at least two proteins that had their mobility altered following the cleavage of disulfide bonds. Therefore, the solubility assays, the 2D diagonal SDS-PAGE results, and the significant amount of Cys found suggest that cohesion of sea urchin footprint material is partly due to disulfide cross-links. This links have also been described in barnacles as discussed above. However, even when using the strong reducing conditions reported for barnacles (0.5 M DTT at 60°C for 3 h; Kamino et al. 2000), the solubility of footprint material was not complete, suggesting the involvement of other type of bonds. It is also important to stress that some proteins were soluble in SDS in the absence of DTT. SDS is a strong denaturing agent, which abolishes almost all noncovalent bonds because it forms negatively charged complexes with proteins thereby neutralizing the charge of the proteins and keeping these apart by electrostatic repulsion. So, the solubility of part of the footprint proteins in SDS suggests the presence of noncovalent bonds such as hydrophobic and electrostatic interactions besides the disulfide bonds discussed above. The proteins of barnacle cement have also been reported to be cross-linked by noncovalent bonds (Wiegemann et al. 2006).

SDS-PAGE analysis of *P. lividus* footprint material revealed that the soluble fraction contains about 13 protein bands with molecular masses ranging from 10 to 200 kDa. This is in accordance with the general multiprotein nature of other marine invertebrate adhesives. Researchers on mussel byssus have so far identified nine proteins with several posttranslational modifications (for review, see Sagert et al. 2006), whereas barnacle cement is known to be made up of at least ten different proteins with no or limited posttranslational modifications (Kamino 2006). In nonpermanent adhesives, much less information is available, but some multiprotein complexes have been found in the adhesives of sea cucumbers, snails, and limpets (for review, see Flammang 2006; Smith 2006).

The innovative character of the present work is the use of mass spectrometry to identify the proteins present in the sea urchin footprint material, taking advantage of the recently sequenced genome and predicted protein database of a sea urchin species, *S. purpuratus* (Cameron et al. 2000; Sea Urchin Genome Sequencing Consortium 2006). Being aware that this database is still in process of annotation and verification, general databases were also used (NCBI and UniRef100), as well as two types of mass spectrometers (MALDI-TOF/TOF and LTQ) with different ionization modes and analyzers in order to increase the chances of obtaining positive and reliable protein identifications.

Among the 13 gel bands separated by SDS-PAGE, only six could be identified. In the 40–50 kDa region of the gel, two proteins could be identified: beta and alpha tubulin (both in band 7) and actin (band 8). Four more proteins were successfully identified in the lower mass region of the gel, between 10 and 20 kDa, as being histones H2B, H3, H2A, and H4 (bands 9, 11, 12, and 13, respectively). The identified proteins can be grouped into two groups: structural (tubulin and actin) and nuclear (histones) proteins, usually found in eukaryotic cells. Therefore, it can be hypothesized that their presence in the footprint material is not related with the adhesive itself but with the presence of cellular material of epidermal in the footprints. Nevertheless, the possibility that the identified proteins actually belong to sea urchin adhesive bulk should not be discarded. Indeed, structural proteins such as tubulin and actin have a flexible arrangement, being usually associated with tension-bearing functions, making them candidate proteins as cohesive elements in the adhesive matrix (Galli et al. 2005). As for histones, nowadays, there are evidences that they are not confined to the nucleus, being also found in the cytoplasm, cell surface, and extracellular environment (for review, see Perseghian and Luhrs 2006). A few studies have shown that nucleosome core histones, namely, H2A, H2B, H3, and H4 (Schmiedeke et al. 1989) as well as histone-like proteins (Kohnke-Godt and Gabius 1991; Henriquez et al. 2002), may have binding functions, attaching to basement membranes and cell surface proteoglycans. In addition, histones are natural polyelectrolytes that could provide polycations for complex coacervation in adhesive formation functioning for sea urchin as suggested for phosphoproteins in polychaetes (Stewart et al. 2004). Although complex coacervation remains to be demonstrated, its potential contribution to the process is undeniable because it would enable the secretion of the adhesive in a fluid but phase-separated form, obtaining an adhesive with higher density than seawater, good spreading due to low interfacial tension in water; and a two-step setting process, first by ion bridges, then by covalent cross-links (Sagert et al. 2006). Moreover, several histones and histone fragments have been proven to be important antimicrobial agents (e.g., Richards et al. 2001; Park et al. 1998). Hence, it can be hypothesized that the histones found in sea urchin footprint material might have a role as glycan linkers or in protecting the adhesive from microbial degradation. Interestingly, the only other "known" protein described in a

marine adhesive is a homolog of lysozyme in barnacle cement (Kamino 2006).

The remaining seven protein bands could not be identified (six gel bands in the 50- to 200-kDa regions and one band around the 16-kDa region) despite the quality of the obtained MS and MS/MS spectra. Further MALDI-MS/MS analyses led to the discovery of five peptide sequences for five out of the seven unidentified proteins. Interestingly, none of these de novo-generated peptides showed significant homologies with the predicted proteins present in the *S. purpuratus* database. This raises the possibility that these proteins or any homologs are not present in the databases used in this study, and thus constitute novel proteins. Although it may seem strange that no homology is found in the sea urchin genome database, this might be explained by the species-specific character of the composition of sea urchin adhesive. Indeed, antisera raised against SAM of one species do not cross-react with the adhesive of other species (Santos et al., unpublished observations). In addition, SAM proteins can be highly modified proteins which would alter both the predicted mass and fragmentation pattern of the resulting tryptic peptides, thus preventing significant matches. These results open new perspectives for future MS studies oriented to fragment more peptides for each unidentified protein and to obtain simpler spectra (namely, by derivatization) which would be more suitable for de novo sequencing and would allow easier manual inspection of the putative sequences. Moreover, for increased confidence in the accuracy of the derived sequences, it is also crucial to combine different de novo sequencing algorithms.

Acknowledgements The authors wish to acknowledge Dr. Paula Chicau for providing data from the Amino Acid Analysis Service at the Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa (Oeiras, Portugal), as well as the Aquário Vasco da Gama (Algés, Portugal) especially Dr. Fátima Gil and Miguel Cadete for sea urchin maintenance. Acknowledgements are extended to Dr. Déborah Penque from the Departamento de Genética, Instituto Nacional de Saúde Dr. Ricardo Jorge (Lisboa, Portugal) for the use of MALDI-TOF/TOF. This work was supported by the Portuguese Foundation for Science and Technology as a postdoctoral grant to R.S. (grant no. SFRH/BPD/21434/2005), Ph.D. grants to G.C. (SFRH/BD/14387/2003), C.F. (SFRH/BD/29799/2006), and P.A. (SFRH/BD/17744/2004), a project grant (PPCDT/DG/MAR/82012/2006) and in the frame of the National Re-equipment Program—National Network of Mass Spectrometry (REDE/1504/REM/2005). P.F. is a Senior Research Associate for the Fund for Scientific Research of Belgium (F.R.S.–FNRS). This study is a joint contribution of the "Centre Interuniversitaire de Biologie Marine" (CIBIM, Belgium) and the "Instituto de Tecnologia Química e Biológica" (ITQB, Portugal).

References

Cameron RA, Mahairas G, Rast JP, Martinez P, Biondi TR, Swartzell S, Wallace JC, Poustka AJ, Livingston BT, Wray GA, Ettensohn CA, Lehrach H, Britten RJ, Davidson EH, Hood L (2000) A sea urchin genome project: sequence scan, virtual map, and additional resources. Proc Natl Acad Sci U S A 97:9514–9518

Candiano G, Bruschi M, Musante L, Santicci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG (2004) Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25:1327–1333

DeMoor S, Waite H, Jangoux M, Patrick P (2003) Characterization of the adhesive from Cuvierian tubules of the sea cucumber *Holothuria forskali* (Echinodermata, Holothuroidea). Mar Biotechnol 5:45–57

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356

Flammang P (1996) Adhesion in echinoderms. In: Jangoux M, Lawrence JM (eds) Echinoderm studies. vol. 5. Balkema, Rotterdam, pp 1–60

Flammang P (2006) Adhesive secretions in echinoderms: an overview. In: Smith AM, Callow JA (eds) Biological adhesives. Springer, Berlin, pp 183–206

Flammang P, Jangoux M (1993) Functional morphology of coronal and peristomeal podia in *Sphaerechinus granularis* (Echinodermata Echinoida). Zoomorphology 113:47–60

Flammang P, Demeulenaere S, Jangoux M (1994) The role of podial secretions in adhesion in two species of sea stars (Echinodermata). Biol Bull 187:35–47

Flammang P, Michel A, Van Cauwenberge A, Alexandre H, Jangoux M (1998) A study of the temporary adhesion of the podia in the sea star *Asterias rubens* (Echinodermata, Asteroidea) through their footprints. J Exp Biol 201:2383–2395

Flammang P, Santos R, Haesaerts D (2005) Echinoderm adhesive secretions: from experimental characterization to biotechnological applications. In: Matranga V (ed) Progress in molecular and subcellular biology, marine molecular biotechnology. Echinodermata. Springer, Berlin, pp 199–218

Galli C, Guizzardi S, Passeri G, Macaluso GM, Scandroglio R (2005) Life on the wire: on tensegrity and force balance in cells. Acta Biomed 76:5–12

Gobom J, Nordhoff E, Mirgorodskaya E, Ekman R, Roepstorff P (1999) Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix assisted laser desorption/ionization mass spectrometry. J Mass Spectrom 34:105–116

Jensen RA, Morse DE (1988) The bioadhesive of *Phragmatopoma californica* tubes: a silk-like cement containing L-DOPA. J Comp Physiol B 158:317–324

Kamino K (2006) Barnacle underwater attachment. In: Smith AM, Callow JA (eds) Biological adhesives. Springer, Berlin, pp 145–166

Kamino K (2008) Underwater adhesive of marine organisms as the vital link between biological science and material science. Mar Biotechnol 10:111–121

Kamino K, Odo S, Maruyama T (1996) Cement proteins of the acorn barnacle, *Megabalanus rosa*. Biol Bull 190:403–409

Kamino K, Inoue K, Maruyama T, Takamatsu N, Harayama S, Shizuri Y (2000) Barnacle Cement Proteins. Importance of disulfide bonds in their insolubility. J Biol Chem 275:27360–27365

Kohnke-Godt B, Gabius HJ (1991) Heparin-binding lectin from human placenta: further characterization of ligand binding and structural properties and its relationship to histones and heparin-binding growth factors. Biochemistry 30:55–65

Kussmann M, Roepstorff P (2000) Sample preparation techniques for peptides and proteins analyzed by MALDI-MS. In: Chapman JR (ed) Methods in molecular biology, protein and peptide analysis: new mass spectrometric applications. Humana, Totowa, NJ

Hamwood TE, Cribb BW, Halliday JA, Kearn GC, Whittington ID (2002) Preliminary characterization and extraction of anterior

adhesive secretion in monogean (plathyelminth) parasites. Folia Parasitol 49:39–49

Henriquez JP, Casar JC, Fuentealba L, Carey DJ, Brandan E (2002) Extracellular matrix histone H1 binds to perlecan, is present in regenerating skeletal muscle and stimulates myoblast proliferation. J Cell Sci 115:2041–2051

Larsen MR, Cordwell SJ, Roepstorff P (2002) Grafite powder as an alternative or supplement to reversed-phase material for desalting and concentration of peptide mixtures prior to matrix-assisted laser desorption/ionization-mass spectrometry. Proteomics 2:1277–1287

Li D, Graham LD (2007) Epidermal secretions of terrestrial flatworms and slugs: *Lehmannia valentiana* mucus contains matrilin-like proteins. Comp Biochem Physiol B Biochem Mol Biol 148:231–244

Marsh JB, Weinstein DB (1966) Simple charring method for determination of lipids. J Lipid Res 7:574–576

Park IY, Park CB, Kim MS, Kim SC (1998) Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*. FEBS Lett 437:258–262

Parseghian MH, Lurhrs KA (2006) Beyond the walls of the nucleus: the role of histones in cellular signaling and innate immunity. Biochem Cell Biol 84:589–604

Richards RC, O'Neil DB, Thibault P, Ewart KV (2001) Histone H1: an antimicrobial protein of Atlantic salmon (*Salmo salar*). Biochem Biophys Res Commun 284:549–555

Rzepecki LM, Hansen KM, Waite JH (1992) Characterization of a cystine-rich polyphenolic protein family from the blue mussel *Mytilus edulis* L. Biol Bull 183:123–137

Santos R, Flammang P (2006) Morphology and tenacity of the tube foot disc of three common European sea urchin species: a comparative study. Biofouling 22:187–200

Santos R, Flammang P (2008) Estimation of the attachment strength of the shingle sea urchin, *Colobocentrotus atratus*, and comparison with three sympatric echinoids. Mar Biol 154:37–49

Santos R, Gorb S, Jamar V, Flammang P (2005) Adhesion of echinoderm tube feet to rough surfaces. J Exp Biol 208:2555–2567

Sagert J, Sun C, Waite JH (2006) Chemical subtleties of mussel amd polychaete holdfasts. In: Smith AM, Callow JA (eds) Biological adhesives. Springer, Berlin, pp 124–140

Sea Urchin Genome Sequencing Consortium (2006) The genome of the sea urchin *Strongylocentrotus purpuratus*. Science 314:941–952

Silverman HG, Roberto FF (2007) Understanding marine mussel adhesion. Mar Biotechnol 9:661–681

Schmiedeke TM, Stockl FW, Weber R, Sugisaki Y, Batsford SR, Vogt A (1989) Histones have high affinity for the glomerular basement membrane. Relevance for immune complex formation in lupus nephritis. J Exp Med 169:1879–1894

Smith AM (2006) The biochemistry and mechanics of gastropod adhesive gels. In: Smith AM, Callow JA (eds) Biological adhesives. Springer, Berlin, pp 167–182

Smith AM, Morin MC (2002) Biochemical differences between trail mucus and adhesive mucus from marsh periwinkle snails. Biol Bull 203:338–346

Smith AM, Quick TJ, St. Peter RL (1999) Differences in the composition of adhesive and non-adhesive mucus from the limpet *Lottia limatula*. Biol Bull 196:34–44

Stewart RJ, Weaver JC, Morse DE, Waite JH (2004) The tube cement of *Phragmatopoma californica*: a solid foam. J exp Biol 207:4727–4734

Tatham AS, Shewry PR (2000) Elastomeric proteins: biological roles, structures, and mechanisms. Trends Biochem Sci 25:567–571

Waite JH (1987) Nature's underwater adhesive specialist. Int J Adhes Adhes 7:9–14

Waite JH (2002) Adhesion à la moule. Integr Comp Biol 42:1172–1180

Waite JH, Tanzer ML (1981) Polyphenol substance of *Mytilus edulis*: novel adhesive containing L-DOPA and hydroxyproline. Science 212:1038–1040

Wiegemann M, Kowalik T, Hartwig A (2006) Noncovalent bonds are key mechanisms for the cohesion of barnacle (*Balanus crenatus*) adhesive proteins. Mar Biol 149:241–246

Winger AM, Taylor NL, Heazlewood JL, Day DA, Millar AH (2007) Identification of intra- and intermolecular disulphide bonding in plant mitochondrial proteome by diagonal gel. Proteomics 7:4158–4170