Journal of Proteomics 128 (2015) 83–91

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

An integrated transcriptomic and proteomic analysis of sea star epidermal secretions identifies proteins involved in defense and adhesion

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ARTICLE INFO

Article history:
Received 19 May 2015
Accepted 2 July 2015
Available online 11 July 2015

Keywords:
Sea stars
Adhesive secretions
Mucus
Proteomes
Adhesive proteins
Transcriptomics

ABSTRACT

Sea stars rely on epidermal secretions to cope with their benthic life. Their integument produces a mucus, which represents the first barrier against invaders; and their tube feet produce adhesive secretions to pry open mussels and attach strongly but temporarily to rocks. In this study, we combined high-throughput sequencing of expressed mRNA and mass-spectrometry-based identification of proteins to establish the first proteome of mucous and adhesive secretions from the sea star *Asterias rubens*. We show that the two secretions differ significantly, the major adhesive proteins being only present in trace amounts in the mucus secretion. Except for 41 proteins which were present in both secretions, a total of 34 and 244 proteins were identified as specific of adhesive secretions and mucus, respectively. We discuss the role of some of these proteins in the adhesion of sea stars as well as in their protection against oxygen reactive species and microorganisms. In addition, 58% of the proteins identified in adhesive secretions did not present significant similarity to other known proteins, revealing a list of potential novel sea star adhesive proteins uncharacterized so far. The panel of proteins identified in this study offers unprecedented opportunities for the development of sea star-inspired biomimetic materials.

1. Introduction

The integument is the organ that forms the interface between an animal and its environment. It comprises the epidermis and its derivatives (shells, cuticles, setae and secretions of various types). In sea stars, various types of glands have been described in the body wall epidermis, from which the secretions are generally referred to as mucous secretions, or simply mucus [1–4]. These secretions constitute the first molecular barrier against external aggressions and act as a cleaning mechanism by agglutinating debris which are then moved away by the cilia [1,5]. In some species, they can also play a role in deterring fouling organisms or predators [6,7]. At the level of the distal part of the tube feet (Fig. 1), which are the external organs of the sea star water-vascular system, epidermal secretions are specialized for adhesion and are called adhesive secretions [8,9]. These secretions are used for dynamic attachment to the substratum during locomotion, vis-à-vis a static sustained attachment to withstand the action of waves, and to grip and pry open a mussel during feeding. They are produced by specialized gland cells, the so-called adhesive cells. These cells co-exist with de-adhesive cells which secrete a de-adhesive material, allowing tube foot detachment and leaving the adhesive secretion bound to the substratum in the form of a footprint [9,10]. Biochemical analyses showed that the organic fraction of both types of sea star epidermal secretions (mucous and adhesive) is mainly made up of proteins [6,11]. The study of mucus protein composition has been limited to the species *Marthasterias glacialis* and *Porania pulvillus* and highlighted the presence of lysozyme-like, protease and hemolytic activities and of high molecular weight glycoproteins [6,7,12]. To the best of our knowledge, none of these proteins has been identified and fully characterized so far. The characterization of tube foot adhesive secretions has been the subject of more studies, driven by the fact that these adhesives are effective in aqueous environments and, therefore, present a strong potential to inspire water-resistant materials for applications in underwater construction (e.g. for navies) or in the field of medicine and dentistry [13,14]. The complete sequence of one sea star adhesive protein, Sfp1, has recently been obtained in *Asterias rubens* [14]. This large protein of 426 kDa displays specific protein-, carbohydrate- and metal-binding domains that could contribute to the cohesion of the adhesive footprint as well as to adhesive interactions between the footprint and the cuticle covering the tube foot epithelium [14]. Other potential novel adhesive proteins have been highlighted but no complete sequence is available yet for them [15].

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http://dx.doi.org/10.1016/j.jprot.2015.07.002

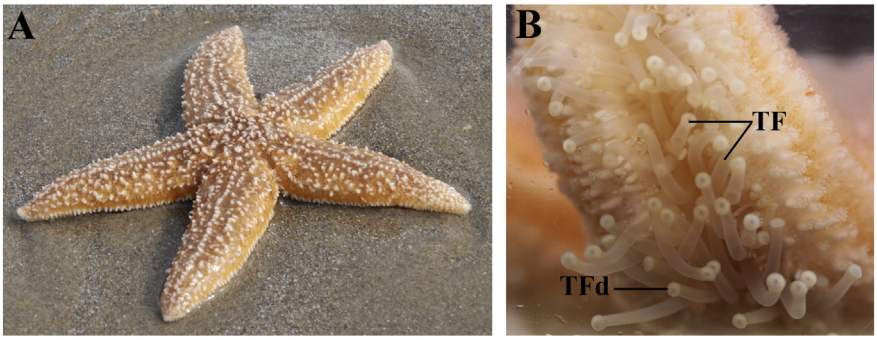


Fig. 1. General view of a sea star of the species *Asterias rubens* (A) and underside view of one of the five arms showing the tube feet (TF), of which the distal part (TFd) is used for adhesion to surfaces.

The paucity of information available in databases regarding protein sequences of non-model species such as sea stars is unambiguously an obstacle limiting the ability to study associated proteomes. Moreover, adhesive secretions are notoriously difficult to solubilize and characterize, and the retrieval of a protein sequence using a traditional approach through cDNA cloning and sequencing can take years. In recent years, the combined use of transcriptomics and proteomics has emerged as the best way leading to the identification of novel proteins and retrieval of their complete sequences [16,17]. In this study, we combined high-throughput sequencing of expressed mRNA (transcriptome analysis) and mass-spectrometry-based identification of proteins to establish the first proteome of mucous and adhesive secretions from the sea star *A. rubens*. This approach allowed not only to describe the protein content of both secretions, but also to highlight novel (i.e., with no similarity in databases) proteins. Specific proteins are discussed in terms of potential roles in adhesion and protection against invaders. The mucus proteome is also compared with mucosal secretions from other species including humans, revealing striking similarities. The large panel of proteins identified in this study offers unprecedented opportunities for the development of sea star-inspired materials.

2. Material and methods

2.1. Animal and sample collections

Individuals of *A. rubens* Linnaeus, 1758 were collected intertidally in Audresselles (Pas-de-Calais, France). They were kept in a marine aquarium with closed circulation (13 °C, 33 psu) and were fed mussels (*Mytilus edulis* Linnaeus, 1758).

Sea star footprints were obtained by allowing individuals to walk across and/or attach to the bottom of cleaned glass Petri dishes filled with filtered sea water (0.22 μm pore size). The sea stars and the sea water were renewed every 2 h for about 8 h. The Petri dishes were then thoroughly rinsed with ultra-pure water to remove salt and as much as possible non-adhesive material and placed in a freeze dryer. The lyophilised footprint material was then scraped off using a razor blade and stored at −20 °C [11].

To collect mucus produced by the integument of sea stars, three individuals were placed upside down in glass Petri dishes and stressed by shaking. Mucus was collected with a glass pipette between the rows of tube feet and on the bottom of the Petri dishes. It was then freeze-dried and stored at −20 °C.

2.2. Illumina sequencing, assembly and analysis of tube foot transcriptome

Sample preparation and sequencing were performed at the GIGA-Genomics facility (Liège, Belgium). Total RNA was extracted from 100 mg tube feet using TRIzol (Life Technologies, Carlsbad, CA). RNA quality was assessed using the Experion Automated Electrophoresis System with RNA StdSens chips (Bio-Rad, Hercules, CA). Illumina Truseq RNA Sample Preparation kit (San Diego, CA) was used to prepare a library from 1 μg of total RNA. Polyadenylated RNA was purified with polyT-coated magnetic beads, chemically fragmented to a length of 80 to 380 nt, reverse-transcribed using random hexamers, and end-repaired and adaptor-ligated according to the manufacturer's protocol (Illumina). Finally, the ligated library fragments were enriched by PCR following Illumina's protocol and purified using magnetic beads (Agencourt Ampure XP beads, Beckman Coulter, Beverly, MA). The Library was validated on Bioanalyser DNA 1000 chip and quantified by qPCR with the KAPA library quantification kit (Kapa Biosystems, Wilmington, MA). Sequencing was performed on a Genome Analyser II (Illumina) in paired-end 2 × 75 base protocol. The Trinity software suite [18] was used with default parameters to reconstruct the transcriptome. Completeness of the assembly was estimated using the Core Eukaryotic Genes Mapping Approach (CEGMA) pipeline [19]. To evaluate the depth of coverage, the "alignReads.pl" script of the Trinity package [20] using bowtie [21] was applied to map the reads against the assembled transcripts.

2.3. Protein extraction and mass spectrometry analysis

Proteins were extracted from 4 independent samples of mucus (~10 mg each) and 4 independent samples of adhesive footprints (~1 mg each) in a 1.5 M Tris–HCl buffer (pH 8.5) containing 7 M guanidine hydrochloride (GuHCl), 20 mM ethylenediaminetetraacetate (EDTA) and 0.5 M dithiothreitol (DTT), and incubated for 1 h at 60 °C under agitation. The sulfhydryl groups of the proteins were then carbamidomethylated with iodoacetamide used in a 2.5-fold excess (w/w) to DTT in the dark at room temperature for 20 min. The reaction was stopped by adding mercaptoethanol (βMSH) in the same quantity as iodoacetamide. The suspension was centrifuged at 13,000 rpm for 15 min at 4 °C and the supernatant was collected. Protein concentration was measured using the Non-Interfering Protein Assay Kit (Calbiochem, Darmstadt, Germany) with bovine serum albumin as a protein standard. For each sample, 50 μg of proteins was precipitated in 80% acetone overnight at −20 °C. After 15 min centrifugation at 13,000 rpm and acetone evaporation, the resulting pellet was submitted to overnight enzymatic digestion using modified porcine trypsin at an enzyme/substrate ratio of 1/50 at 37 °C in 25 mM NH_4HCO_3. The reaction was stopped by adding formic acid to a final concentration of 0.1% (v/v).

Tryptic peptides were analyzed by reverse-phase HPLC–ESI-MS/MS using an Eksigent Ultra Plus nano-LC 2D HPLC system connected to a quadrupole time-of-flight Triple TOF 5600 mass spectrometer (AB SCIEX, Concord, ON). Briefly, after injection, peptide mixtures were transferred onto the analytical C18-nanocapillary HPLC column (C18 Acclaim PepMap100, 75 μm I.D. × 25 cm, 3 μm particle size, 100 Å pore size, Dionex, Sunnyvale, CA) and eluted at a flow rate of 300 nl/min using the following gradient: 2–35% solvent B in A (from 0 to 12 min), 35–90% solvent B in A (from 12 to 14 min), 90% solvent B in A (from 14 to 19 min), 90–2% solvent B in A (from 19 to 20 min) and 2% solvent

B in A (from 20 to 50 min), with a total runtime of 50 min including mobile phase equilibration. Solvents were prepared as follows, mobile phase A: 2% acetonitrile/98% of 0.1% formic acid (v/v) in water and mobile phase B: 98% acetonitrile/2% of 0.1% formic acid (v/v) in water. MS data acquisition was performed with a Triple TOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON) and a pulled quartz tip as the emitter (New Objectives). Ionization was obtained with an ion spray voltage of 2.6 kV, curtain gas set at 15 psi and ion source gas at 4 psi. For data-dependent acquisition, survey scans were acquired in 250 ms and as many as 50 product ion scans were collected if exceeding a threshold of 100 counts per second and with a 2+ to 4+ charge-state. Total cycle time was fixed to 1.85 s. A rolling collision energy was used with a collision energy spread of ±10 eV. Peaklists were created using Mascot Distiller 2.3.2 using default parameters and exported as mgf files. The mass spectrometry proteomics raw data as well as search results (see below) have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [22] with the dataset identifier PXD001607 and http://dx.doi.org/10.6019/PXD001607.

2.4. Bioinformatic analyses of proteomic sequence data

MS/MS data were searched for protein candidates against the six open reading frames (ORFs) of the tube foot transcriptome using MASCOT 2.2.07 (Matrix Sciences, Boston, USA). The peptide mass tolerance was set to ±20 ppm, and fragment mass tolerance was set to ±0.05 Da. Carbamidomethyl cysteine was set as fixed modification, and oxidized methionine and deamidated asparagine were set as variable modifications. The maximum expectation value for accepting individual peptide ion scores [−10 × Log(p)] was set to ≤0.01, where p is the probability that the observed match is a random event. The FDR was estimated at peptide level using the decoy database option of MASCOT and the emPAI (exponentially modified Protein Abundance Index) was calculated to give an evaluation of absolute protein abundance in the samples [23]. This index was normalized across samples by calculating the following percentage:

$$\text{Normalized emPAI} = (\text{emPAI}_{\text{protein}}/\text{emPAI}_{\text{total}}) \times 100$$

where $\text{emPAI}_{\text{protein}}$ and $\text{emPAI}_{\text{total}}$ are, respectively, the emPAI for a given protein and the sum of the emPAI of all the identified proteins within a sample.

For mucus proteome, MS/MS results which were not assigned to any sequence from the tube foot transcriptome (unmatched MS/MS spectra) were used for an additional search against the entire NCBI nr database (release 61). Search outputs resulting from this analysis were further manually curated to eliminate proteins orthologous to previous identifications.

The lists of transcripts resulting from the MASCOT search for all mucus and footprint samples were used for prediction of all putative ORFs (>200 nucleotides; translation between Start and Stop codons) using GetORF [24]. The ORFs were then used for a BLASTp search against the NCBI nr database (release 61) using a minimal E-value set to 1×10^{-6} to identify transcripts with sequence similarity to known proteins. The results were collated in Excel. It is noteworthy that with this method, transcripts shorter than 200 nucleotides or which did not present any Start or Stop codons were not used in the BLAST search and were considered as "non-annotated". Transcripts for which more than one ORF gave rise to an annotation in NCBI nr were manually inspected and the ORF with the longest length and/or presenting the best bit score and E-value was retained while the others were discarded. The new list of ORFs with annotations was imported into the Blast2GO program [25] which was used to predict gene ontology (GO) using the default settings. Go annotations were completed using InterProScan searches, which were performed using default parameters against all available protein domain databases. Protein secretion was predicted using SignalP 4.1 [26] and SecretomeP 2.0 [27] for classical and nonclassical secretory pathways, respectively.

3. Results and discussion

3.1. The tube foot transcriptome

A total of 78,475,660 reads, with a length of 76 bp, were produced for the tube feet through Illumina paired-end sequencing. The raw sequencing data have been deposited in the NCBI Sequence Read Archive with accession number SRP050362. The statistics of data output and de novo assemblies are summarized in Fig. 2A. The reads were assembled into 97,945 transcripts with an average length of 736 bp (ranging from 201 bp to 47,888 bp) and an N50 of 1140 bp.

To assess the quality of the transcriptome, we looked at (i) transcript length distribution, (ii) transcript coverage, and (iii) CEGMA analysis. The transcript length distribution after assembly (Fig. 2B) shows a decreasing number of transcripts with increasing transcript length. A relative abundance of short transcripts up to 500 bp is obvious, a situation also seen in other transcriptome assemblies [e.g. 28]. However, a notable number of long and very long transcripts are also present in the transcriptome (Fig. 2B). For assessing transcript coverage, we mapped all sequenced reads against assembled transcripts (Fig. 2C). The results show that about half of the transcripts are covered by more than 100 reads, several of them even reaching more than 100,000 mapped reads. CEGMA explores the presence of a set of 248 highly conserved core eukaryotic genes (CEGs) which occur in all eukaryotes [19,29]. These genes are classified into four groups according to their average degree of conservation. Group 1 comprises the least conserved genes, with the conservation degree increasing in subsequent groups through to group 4. Furthermore, the CEGMA pipeline provides details about the occurrence of complete or partial CEG homologues in the transcriptome. CEGs are considered as "complete" when the alignment length exceeds 70% of the protein length. Analysis of our transcriptome assembly identified a total of 200 (81%) out of the 248 CEGs as "complete" and 226 (91%) out of the 248 CEGs as "partial" (Fig. 2D). In the ultra-conserved group 4, 63 (98%) and 64 (97%) out of the 65 CEGS were recovered as "complete" and "partial", respectively. The percentage of completeness decreases towards group one genes for both, complete and partial CEGs. These results are similar to those obtained from other published de novo assembled transcriptomes [e.g., 30,31]. In summary, our analyses confirm that the tube foot transcriptome generated here is a valuable source for downstream applications such as e.g. data mining for mass spectrometry.

3.2. Proteome analysis of mucous and adhesive secretions

3.2.1. Proteome versus transcriptome

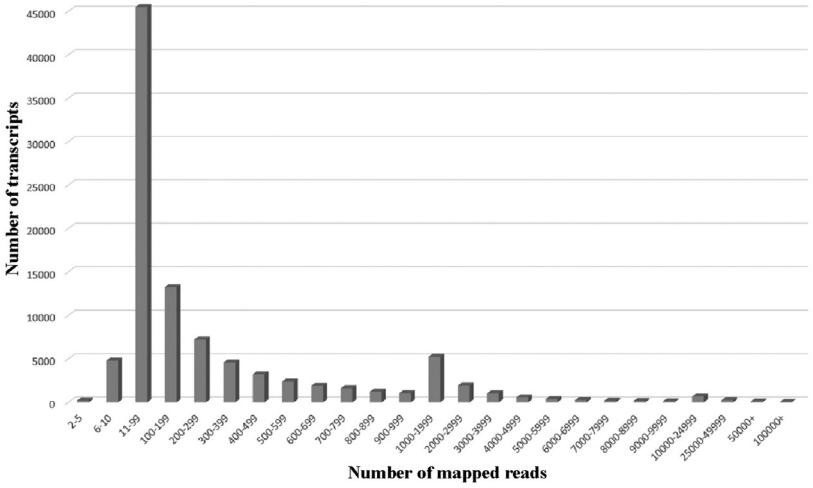
Proteins were extracted from 4 independent samples of mucus and adhesive footprints and, for each sample, the same quantity of proteins was subjected to in-solution trypsin digestion. Resultant peptides were analyzed by mass spectrometry and MS/MS data were searched against the translated tube foot transcriptome (complete information on all peptide and protein identifications are presented in Supplemental Table S1). Doing so, a total of 208, 150, 156 and 156 proteins were identified in the 4 footprint samples, respectively (FDR at peptide level < 1.20%), while the analysis of the 4 mucus samples led to the identification of, respectively, 559, 545, 523, and 615 proteins (FDR at peptide level < 1.40%) (Supplemental Table S2).

During the comparison of MS/MS data with the tube foot transcriptome, the Mascot research server generated the emPAI, which provides an estimation of the abundance of a protein in a sample. In our study, this index was normalized in order to allow valid comparisons between different samples. For each protein, the sum of normalized emPAI values was calculated for the four replicates of the same

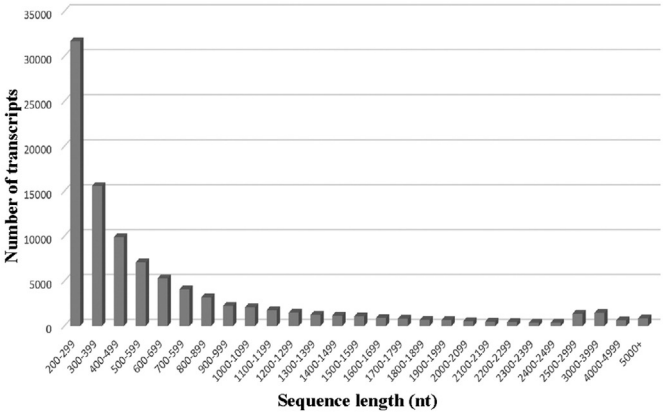
A

Output result	
Raw reads	78,475,660
GC percentage (%)	39.5
High quality reads	
Q20	70,377,296
Q30	65,420,318
Assembly result	
Transcript number	97,945
Total size (bp)	72,061,298
Transcript N50 length (bp)	1,140
Transcript mean length (bp)	736
Transcript largest length (bp)	47,888
GC percentage (%)	40.9

C



B



D

Groups	# Complete CEGs present		# Partial CEGs present	
	Obs/Exp	% Completeness	Obs/Exp	% Completeness
Group 4	63/65	96.92	64/65	98.46
Group 3	49/61	80.33	57/61	93.44
Group 2	44/56	78.57	52/56	92.86
Group 1	44/66	66.67	53/66	80.30
Total	200	80.65	226	91.13

Fig. 2. Overview of *A. rubens* tube foot transcriptome analysis. A, Statistical summary of transcript sequence output and assembly results. B, Assembled transcript length distribution. C, Distribution of the number of reads mapped to assembled transcripts. D, Prediction of the 248 CEGMA core eukaryotic genes (CEGs) in the transcriptome. Groups 1–4 are CEG groups with increasing degree of conservation from group 1 to group 4 as classified in [29]. Obs: observed, Exp: expected.

epidermal secretion and the proteins were classified according to the values obtained (Tables 1 and 2, Supplemental Table S2). Among the most abundant footprint proteins, most are missing or are present only in trace amounts in the mucus samples (Table 1, Supplemental Table S2), meaning that these proteins are specific to the footprints and would be involved in sea star adhesion. The trace amounts found in the mucus samples would therefore correspond to contamination of mucous material with adhesive secretion. The 4 most abundant mucus proteins, on the other hand, are present in similar quantities in adhesive footprints, although not in all the samples, highlighting some potential common features between the two epidermal secretions and/or contamination during the sample collection (Table 2, Supplemental Table S2). Indeed, it is likely that sea stars produce some mucus quantities during adhesive footprint collection, leading to some contaminations of the samples.

3.2.2. Functional annotation of identified proteins

As a first step, we merged the lists of transcript IDs coding for proteins identified in the different samples of the two epidermal secretions in order to work with a dataset as complete as possible. The 1014 transcripts of the generated list were predicted for ORFs which were then used for a BLASTp search against the NCBI nr database. Results are presented in Supplemental Table S2. To validate the use of tube foot transcriptome as a database to characterize the mucus proteome, MS/MS data with no assigned transcript IDs in the tube foot

Table 1
Major proteins identified in adhesive footprints.

	Adhesive footprints				Mucus			
Transcript ID	1	2	3	4	1	2	3	4
comp199_c0_seq5	9.24	10.62	9.04	7.01		0.20	0.70	0.19
comp43_c4_seq1	6.33	5.85	5.94	5.53	0.02	0.09	0.06	0.03
comp1698_c0_seq2	5.63	4.86	4.14	4.31				
comp6449_c0_seq1	3.92	3.56	4.16	5.82	0.06		0.05	
comp73892_c0_seq1	3.35	3.10	4.34	2.75				
comp33_c8_seq14	3.80	2.44	4.92	2.16				
comp17_c0_seq1	2.91	3.34	3.29	3.32		0.07	0.05	
comp9623_c0_seq4	1.71	4.18	3.56	2.31				
comp9623_c0_seq5	2.83	2.25	1.92	3.82			0.00	
comp3731_c0_seq4	1.66	3.60	3.06	2.24	2.01	1.49	4.29	2.60
comp133_c0_seq4	1.42	4.13	1.84	1.92				
comp1476_c0_seq6	3.49		1.94	3.19				
comp9623_c0_seq1	1.33	2.03	3.25	1.80	0.19	0.10	0.59	0.20
comp1476_c0_seq9	2.50	3.80		1.78				
comp1476_c0_seq3	1.85	0.48	1.57	3.60				
comp67793_c0_seq1	0.54	0.82	1.71	3.37	1.55	4.27	2.41	2.52
comp3966_c0_seq1	1.57	1.48	1.26	2.11				
comp56_c1_seq2	1.28	1.31	2.18	1.36	0.51	1.23	1.11	0.49
comp47861_c0_seq1	2.31		2.10	1.45	1.96	3.52	3.19	1.38
comp15624_c0_seq2	1.06	1.38	1.18	1.22			0.10	0.05

Values are normalized emPAI calculated as detailed in the "Material and methods" section, for 4 replicates of adhesive footprints and mucus. For each protein, the sum of normalized emPAI values was calculated for the 4 replicates of the same epidermal secretion and the proteins were classified according to the values obtained. Only the 20 most abundant footprint proteins are presented. Normalized emPAI values ranging from 0.00 to 2.00 are highlighted in light gray, values ranging from 2.01 to 4.00 are highlighted in middle gray, and values above 4.00 are highlighted in dark gray.

Table 2
Major proteins identified in mucus.

	Mucus				Adhesive footprints			
Transcript ID	1	2	3	4	1	2	3	4
comp67793_c0_seq1	1.55	4.27	2.41	2.52	0.54	0.82	1.71	3.37
comp3731_c0_seq4	2.01	1.49	4.29	2.60	1.66	3.60	3.06	2.24
comp47861_c0_seq1	1.96	3.52	3.19	1.38	2.31		2.10	1.45
comp174_c1_seq2	2.46	1.63	2.05	2.14	1.79	2.39		
comp7351_c0_seq1	1.61	0.97	1.71	1.21	0.08	0.12	0.13	
comp6752_c0_seq1	1.03	1.33	2.28	0.76			0.23	
comp70054_c0_seq1	1.33	0.73		3.32				
comp47030_c0_seq1	1.87	0.84	0.76	1.80	0.34		0.44	0.45
comp6752_c0_seq4	0.71	1.32	1.99	0.69				0.00
comp24506_c0_seq1	1.12	0.80	1.28	0.96		0.12	0.33	
comp174_c1_seq1	2.27		1.71				1.44	1.07
comp5_c1_seq1	0.94	1.20	0.94	0.65	0.56	0.34	1.16	1.09
comp63668_c0_seq1	0.65	1.18	1.07	0.62	0.44		0.57	1.40
comp46169_c0_seq1	1.16	0.53	0.84	0.88	0.17	0.26	0.22	0.48
comp67906_c0_seq1	0.85	0.23	0.45	1.87				
comp686_c1_seq1	0.78	1.13	0.66	0.81	0.57	0.36	0.46	0.66
comp56_c1_seq2	0.51	1.23	1.11	0.49	1.28	1.31	2.18	1.36
comp2566_c0_seq1	0.65	0.94	0.85	0.89				
comp76367_c0_seq1	0.77	0.78	0.29	1.38				
comp1164_c0_seq1	0.99	0.78	0.36	1.06	0.91		0.51	0.53

Values are normalized emPAI calculated as detailed in the "Material and methods" section, for 4 replicates of mucus and adhesive footprints. For each protein, the sum of normalized emPAI values was calculated for the 4 replicates of the same epidermal secretion and the proteins were classified according to the values obtained. Only the 20 most abundant mucus proteins are presented. Normalized emPAI values ranging from 0.00 to 2.00 are highlighted in light gray, values ranging from 2.01 to 4.00 are highlighted in middle gray, and values above 4.00 are highlighted in dark gray.

transcriptome for this secretion were used directly for a search in the NCBI nr database. Indeed, the mucus we collected could contain proteins produced not only by the tube feet but also by the sea star aboral integument. Adhesive proteins, on the other hand, are only encoded by mRNA synthesized in the tube feet [11]. Given that this new search led to the identification of less than 4% of additional proteins in comparison to the direct analysis against the tube foot transcriptome (data not shown), we considered that the approach used was appropriate to characterize the mucus proteome.

For further analyses, in order to reduce the FDR of our protein dataset, only proteins identified in all 4 biological replicates (i.e., 4 footprint samples or 4 mucus samples) were taken into account.

3.2.2.1. Footprint proteins. To search for proteins with a potential function in sea star adhesion, we specifically focussed on proteins present in the 4 footprint samples and not present in all the mucus samples. These criteria allowed to take into account abundant adhesive proteins which could be present in trace amounts in mucus samples due to contaminations, while excluding proteins common to both secretions (see below). Applying these stringent criteria, 34 proteins were considered as specific to footprints. All of them were identified at least once with 2 peptides or more, thus ensuring a very low FDR in this dataset. The 34 footprint proteins have been classified according to the normalized emPAI values as explained above, and the top BLAST hits in NCBI nr are presented in Table 3 (for the 20 most abundant proteins) and Supplemental Table S3a. On the 34 proteins, 20 were provided with an annotation in the nr database, 25% of them matching to the sea urchin *Strongylocentrotus purpuratus*, which is the only echinoderm species for which the whole sequenced and annotated genomes [32] are available in public databases, 15% matching to the acorn worm *Saccoglossus kowalevskii*, and the remaining matching to other species (including the sea star *Solaster dawsoni*). Based on SignalP and SecretomeP analyses, 80% of the annotated proteins were inferred to contain a secretion signal (Supplemental Table S3a).

Some of the annotated proteins present a strong potential to play a role in sea star adhesion (Fig. 3, Supplemental Fig. S1). Comp5429_c0_seq1 codes for a protein for which the first BLAST hit is Repellent, a protein which was identified in the predator sea star *S. dawsoni*, and which was shown to induce escape response in another sea star species [33]. This protein has not been characterized so far and its function in *S. dawsoni* is not known yet. Significant similarity was also

Table 3
Top BLAST hits of the major footprint proteins in NCBI nr.

Transcript ID	Present in N mucus samples	Query length (aa)	Description	Species	Accession number	% identity	Alignment length (aa)	Hit length (aa)	E-value	Bit score
comp199_c0_seq5	3		NA							
comp1698_c0_seq2	0		NA							
comp6449_c0_seq1	2	386	Hyalin domain-containing protein, partial	*Gillisia* sp. CBA322	WP_1232488	44.94	89	120	1E−11	70.9
comp73892_c0_seq1	0		NA							
comp33_c8_seq14	0		NA							
comp17_c0_seq1	2	1152	Hypothetical protein CBG5362	*Caenorhabditis briggsae*	XP_2634766	35.75	372	1336	5E−36	158
comp9623_c0_seq4	0		NA							
comp9623_c0_seq5	1		NA							
comp133_c0_seq4	0		NA							
comp1476_c0_seq3	0		NA							
comp3966_c0_seq1	0	234	Hypothetical protein BRAFLDRAFT_123266	*Branchiostoma floridae*	XP_267695	35.65	115	469	7E−08	61.2
comp15624_c0_seq2	2		NA							
comp5429_c0_seq1	0	291	Repellent	*Solaster dawsoni*	BAL43194	59.27	248	276	3E−92	286
comp1476_c0_seq11	0		NA							
comp7802_c0_seq2	0		NA							
comp362_c0_seq1	1	1562	Hypothetical protein CAPTEDRAFT_22865	*Capitella teleta*	ELT94944	35.94	1508	1669	0	818
comp2480_c0_seq2	0	66	Endo-1,4-beta-xylanase	*Kineococcus radiotolerans* SRS3216	YP_13682	44.83	58	912	2E−06	53.1
comp4570_c0_seq1	0	470	Farnesoic acid O-methyltransferase-like[a]	*Saccoglossus kowalevskii*	XP_2742382	26.1	295	507	2E−15	87.8
comp7100_c0_seq1	0	405	Farnesoic acid O-methyltransferase	*Eriocheir sinensis*	ACX33	24.53	265	276	9E−12	73.9
comp33_c8_seq19	0		NA							

NA: non-annotated.
Only the 20 most abundant proteins present in the four footprint samples and not all mucus samples are presented.
[a] Sequences annotated in the database as "predicted".

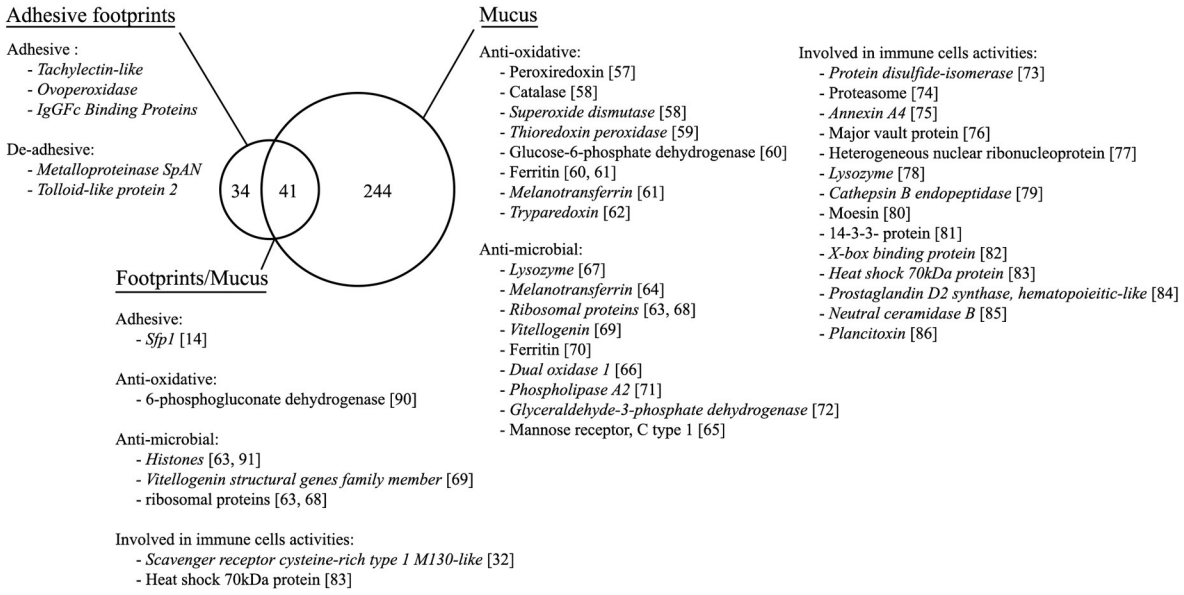


Fig. 3. Venn diagram depicting overlap between adhesive footprint and mucus proteomes and presenting proteins with potential relevant function. For adhesive footprints, only proteins present in the 4 footprint samples and not all mucus samples were considered; for mucus, only proteins present in the 4 mucus samples and not all footprint samples were considered; and proteins present in the 4 samples of both secretions are in the overlapping category. Proteins in italics were predicted to be secreted using SignalP and SecretomeP analysis [14, 32, 57-86, 90, 91].

found between the protein coded by this transcript and tachylectin-like proteins (47% identity, E-value 7.00E−62, bit score 209). These proteins are lectins which, by their ability to bind to various carbohydrate components of bacterial cell walls, play a role in the innate immunity of various organisms [e.g., 34–37]. The properties of such a protein make it a good candidate as a component of footprint homogeneous priming film [see 10], where it would promote adhesion to the biofilm present on the surface of the substratum.

The protein coded by comp15560_c0_seq1 presents significant similarity with ovoperoxidase from sea urchins. In these organisms, this protein is secreted by eggs and catalyzes the formation of di-tyrosine residues between polypeptides of the fertilization envelope in order to harden it [38]. Ovoperoxidase is related to other heme-dependant peroxidases which favor tyrosine as a substrate [39]. The secretion of an enzyme with similar activity in sea star adhesive footprints would allow the formation of cross-links between the adhesive proteins, thereby improving footprint cohesion. In mussels and tubeworms, other marine organisms relying on adhesion, this function is provided by tyrosinase enzymes, which convert tyrosine into 3,4-dihydroxyphenylalanine (DOPA), a residue involved in the formation of cross-links between the different mussel and tube worm adhesive proteins [40,41].

Two transcripts (comp1654_c0_seq1 and comp736_c1_seq1) code for proteins similar to the IgGFc binding protein. In human, this mucin-like protein is characterized by a high molecular weight, many predicted glycosylated sites and a high cysteine content [42], characteristics also encountered in the proteins coded by the two transcripts (data not shown). In addition to intra- and inter-disulfide bonds, it has been proposed that the cysteines could be involved in the oligomerization of IgGFc binding proteins, as described for vWF [42,43]. In sea star adhesive footprints, the two mucin-like proteins could be involved in the formation of structural networks through their potential ability to oligomerize and/or cross-link to other adhesive molecules.

Some footprint proteins appear to be annotated on the basis of the presence of functional domains such as hyalin, EGF, and discoidin domains (e.g., the proteins coded by the transcripts comp6449_c0_seq1, comp1172_c0_seq1, comp199_c0_seq1, Supplemental Fig. S1). These domains are known from other studies to mediate protein–protein, protein–carbohydrate, or protein–metal interactions [44–46]. Such domains could therefore provide cohesive and adhesive interactions between sea star footprint proteins and other glycans and/or proteins present in the adhesive material and in the outermost layer of the cuticle covering the tube foot epidermis, respectively [11,14,47]. Indeed, some of them have already been highlighted in Sfp1, the first adhesive protein characterized so far in sea stars [14].

Finally, two proteins identified in the sea star footprints are similar to enzymes presenting a metalloendopeptidase activity (metalloproteinase SpAN and tolloid-like protein 2; [48]). These proteases, by degrading adhesive proteins, could be involved in the detachment process of the tube feet, as proposed by Flammang and co-workers [11].

Interestingly, most of the non-annotated (NA) proteins are among the most abundant footprint proteins (Table 3, Supplemental Table S3a) and could correspond to novel sea star adhesive proteins uncharacterized so far. Moreover, 25% of the proteins with an annotation in NCBI nr correspond to “hypothetical” or “uncharacterized” proteins which could also enter this category.

3.2.2.2. Mucous proteins. To identify proteins specific to mucus, we selected proteins present in the 4 mucus samples and not present in all the footprint samples. Applying these stringent criteria, 244 proteins were considered as specific to mucous, only 9 of which being identified based on a unique peptide. Those identifications were manually validated based on the presence in at least one of the MS/MS spectra of a series of 6 or more consecutive fragment ions as well as inclusion of all top 5 ions in the y or b ion series. On the 244 selected proteins, 216 were provided with an annotation in the nr database (Table 4, Supplemental Table S3b). Among them, 59% were matched to *S. purpuratus* (38%), *S. kowalevskii* (15%), and *Branchiostoma floridae* (6%). Among the remaining matching species were the sea stars *A. rubens*, *Patiria pectinifera*, *Patiria miniata*, *Parvulastra exigua*, *Asterias forbesi*, and *Acanthaster planci*. GO classification was used to obtain a primary overview of how the mucus proteome is associated with its cellular components, molecular functions, and biological processes (Supplemental Fig. S2). Among the 216 proteins with a BLAST result, 185 were assigned to one or several GO terms. For the category “cellular components”, predominant terms were “cell” and “organelle” (67 and 47 proteins respectively); for the category “molecular function”, they were “binding” and “catalytic activity” (110 and 81 proteins respectively);

Table 4
Top BLAST hits of the major mucus proteins in NCBI nr.

Transcript ID	Present in N footprint samples	Query length (aa)	Description	Species	Accession number	% identity	Alignment length (aa)	Hit length (aa)	E-value	Bit score
comp47861_c0_seq1	3	104	Uncharacterized protein LOC100888806[a]	*Strongylocentrotus purpuratus*	XP_003729826	41.24	97	876	1E−14	79
comp174_c1_seq2	2	372	Tubulin, beta 2C-like	*Saccoglossus kowalevskii*	NP_001171814	99.73	372	447	0	783
comp7351_c0_seq1	3	1596	Vitellogenin	*Parvulastra exigua*	AFH56436	65.72	1581	1592	0	2202
comp6752_c0_seq1	1	162	Hypothetical protein[a]	*Saccoglossus kowalevskii*	XP_002739378	51.88	160	381	2E−53	184
comp47030_c0_seq1	3		NA							
comp6752_c0_seq4	1	77	Hypothetical protein[a]	*Salinispora pacifica*	WP_018735955	44.87	78	260	4E−16	79
comp24506_c0_seq1	2		NA							
comp63668_c0_seq1	3		NA							
comp67906_c0_seq1	0		NA							
comp2566_c0_seq1	0	151	Superoxide dismutase [Cu–Zn]-like[a]	*Strongylocentrotus purpuratus*	XP_784574	73.51	151	153	2E−69	217
comp76367_c0_seq1	0		NA							
comp1164_c0_seq1	3	124	Uncharacterized protein LOC752895[a]	*Strongylocentrotus purpuratus*	XP_001180492	30.23	129	129	2E−07	55.1
comp6264_c0_seq1	3		NA							
comp686_c1_seq6	3	519	Filamin-C isoform 3[a]	*Strongylocentrotus purpuratus*	XP_003729507	68.21	519	2288	0	710
comp1201_c0_seq2	2	176	Plancitoxin-1	*Acanthaster planci*	Q75WF2	46.24	173	358	9E−47	166
comp22224_c0_seq1	3		NA							
comp249_c0_seq1	3	373	Arginine kinase-like[a]	*Strongylocentrotus purpuratus*	XP_786080	69.62	372	417	0	547
comp1383_c0_seq1	3	508	Fascin	*Danio rerio*	NP_001070028	37.93	522	491	2E−95	309
comp214_c0_seq5	0	107	Calmodulin	*Salmo salar*	ACI68592	96.26	107	135	1E−66	207
comp15288_c0_seq3	1		NA							

NA: non-annotated.
Only the 20 most abundant proteins present in the four mucus samples and not all footprint samples are presented.
[a] Sequences annotated in the database as "predicted".

and for the category "biological process", they were "metabolic process" and "cellular process" (100 and 96 proteins respectively). Although GO annotation classified less than 1% of annotated mucus proteins as having an extracellular localization, analysis of protein sequences using SignalP and SecretomeP increased this number to 47% (Supplemental Table S3b). Annotated proteins were also compared with other published human and invertebrate mucus proteomes [49–55] (see Supplemental Table S4). Interestingly, 96 sea star mucus proteins grouped into 56 related proteins appeared to be similar to proteins identified in these proteomes. Taken together, 2%–55% of sea star mucus proteins are respectively shared with human olfactory cleft mucus (9%), cervical mucus (10%), nasal mucus (2%), and airway epithelial secretions (3%), and with the fish *Gadus morhua* skin mucus (5%), the sea anemone *Stichodactyla duerdeni* mucus (6%), and the planarian *Schmidtea mediterranea* mucus (55%). To the best of our knowledge, this is the second report of the conservation of mucus proteins across species [54].

Based on literature search, we highlighted some of the annotated mucus proteins for their potential role in sea star defense (Fig. 3, Supplemental Fig. S1). They were classified as anti-oxidative, anti-microbial, and involved in immune cell activities. Being aquatic organisms, sea stars have to cope with a high variety of environmental reactive oxygen species (ROS) like hydroxyl radical (HO) and hydrogen peroxide (H_2O_2) which result mainly, but not only, from the photolysis of organic and inorganic matters [56]. The presence of antioxidant proteins in the mucus covering their integument was therefore expected. Enzymatic antioxidants such as peroxiredoxin, catalase and superoxide dismutase have already been described in various human and invertebrate mucus (see Supplemental Table S4). For instance, such enzymes are found in the mucus secreted by the polychaete *Laeonereis acuta* and act as an antioxidant defense system by intercepting and degrading ROS [58]. Non-enzymatic antioxidants such as ferritin and melanotransferrin were also highlighted in sea star mucus. These ion sequestering molecules could serve as cytoprotectants against metal-mediated oxidant damage [60,61].

Regarding proteins presenting a potential anti-microbial activity, they fall into four categories according to their mode of action: those hydrolyzing the bacterial cell wall (e.g., lysozyme), those inhibiting the growth of microorganisms (e.g., melanotransferrin, ribosomal proteins), those enhancing phagocytosis (e.g., mannose receptor), and those producing antioxidants (e.g., dual oxidase 1) [63–67]. The presence of anti-microbial proteins in the mucus covering the sea star integument constitutes obviously the first defense barrier against the penetration of invasive microorganisms.

Finally, a series of sea star mucus proteins exhibited similarity with proteins playing a role in the activity of immune cells in various organisms (Fig. 3). Interestingly, almost all these proteins were found in other mucus proteomes (see Supplemental Table S4). In echinoderms, two types of immune response have been described: a cellular response, mediated by coelomocytes, circulatory cells present in the perivisceral coelomic cavities, and a humoral response mediated by molecules present in the coelomic fluid [87,88]. Several of the immune cell-related proteins highlighted in this study were also found in sea urchin tube foot proteome [89]. In that case, their occurrence was attributed to the presence of coelomic fluid in the tube feet. In the present study, however, the way we collected mucus clearly prevented any contamination with coelomic fluid and coelomocytes. This therefore raises the possibility that another cell type – maybe mucocytes – present in the sea star integument would be involved in the immune response of sea stars.

3.2.2.3. Proteins common between adhesive footprints and mucus. Proteins present in all footprint and mucus samples were considered as common proteins between the two epidermal secretions (Fig. 3, Supplemental Table S3c). Based on normalized emPAI values, all proteins appear to be present in both secretions in similar abundances, except for the proteins coded by comp43_c4_seq1 and comp9623_c0_seq1 which are more abundant in adhesive footprints than in mucus (Supplemental Table S3c). The first transcript actually codes for Sfp1 [14] and the second most probably also codes for an adhesive protein since proteins coded by isoforms of this transcript appear in footprint specific proteins (Table 3). The small quantities of these proteins found in the mucus therefore highlight contaminations of the mucous secretion during its collection by these adhesive proteins. Among the 39 remaining proteins, 31 were annotated in the NCBI nr database. Of them, 58% matched *S. purpuratus* (42%), and *S. kowalevskii* (16%), and 52% were predicted to have an extracellular localization. While some proteins appear to

correspond to possible contaminant intracellular proteins (e.g., actin, myosin), others fall in the categories described above for mucus proteins, i.e., anti-oxidant (e.g., 6-phosphogluconate dehydrogenase), anti-microbial (e.g., histones) or involved in immune cell activities (e.g., heat shock proteins) [63,83,90] (Fig. 3). The presence of these proteins in adhesive footprints could therefore be the result of a cross-contamination during the collection of these samples.

4. Conclusions

The approach used in this study, combining transcriptome analysis and mass spectrometry-based identification of proteins, permitted to establish the first proteome of mucous and adhesive secretions from the sea star *A. rubens*. The use of tube foot transcriptome as first database constitutes a key element of this approach. Indeed, it allowed the identification of novel proteins uncharacterized so far and therefore absent from published databases. In addition, it permitted a more confident annotation of proteins presenting similarity in databases, as complete or partial ORFs were directly used for comparisons, instead of peptides.

The proteomes of the two secretions were compared in order to establish a list of proteins specific to each secretion. To the best of our knowledge, our study provides for the first time a complete list of proteins potentially involved in sea star adhesion as well as their relative abundance. This is supported by the fact that Sfp1, coded by transcript comp43_c0_seq1, was identified as the second most abundant protein in the list of footprint proteins. Interestingly, most of the proteins from this list appear to correspond to novel proteins uncharacterized so far. These results set the scene for future studies aimed at characterizing novel water resistant adhesive molecules. Both annotated and non-annotated proteins identified in this study offer unprecedented opportunities for the development of sea star-inspired biomaterials. Adhesive proteins could be used in numerous technological developments, including water-resistant biomedical adhesives (e.g., for surgery, or drug delivery) or biomaterials [e.g., 92,93]; while mucus proteins with anti-microbial properties could be used as non-toxic additive to pharmaceutical and nutraceutical formulations [e.g., 63].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.07.002.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

We thank Benoit Hennuy (GIGA-Genomics) for the sequencing of tube foot transcriptome, Thomas Ostermann for help with computer administration and bioinformatics, Nathan Puozzo for help in the design of Figures, and François Guillonneau for help in proteomic data conversion. This work was supported by the European Cooperation in Science and Technology action TD0906, by the Austrian Science Fund (FWF): [P 25404-B25], by the EU FP7-OCEAN Project "Low-toxic cost-efficient environment-friendly antifouling materials" (BYEFOULING) under grant agreement no. 612717, and by the Fund for Scientific Research of Belgium (F.R.S. - FNRS) under grant 'grand equipement' no. 2877824. E.H. is a postdoctoral researcher of the F.R.S. - FNRS. This study is a contribution of the Centre Interuniversitaire de Biologie Marine.

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