



Proteomic characterization of mucosal secretions in the eastern oyster, *Crassostrea virginica*



Emmanuelle Pales Espinosa^{a,*}, Antonius Koller^b, Bassem Allam^a

^a School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, NY 11794-5000, United States

^b Proteomics Center, Stony Brook University Medical Center, Stony Brook, NY 11794-8691, United States

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ABSTRACT

The soft body surface of marine invertebrates is covered by a layer of mucus, a slippery gel secreted by mucocytes lining epithelia. The functions of this gel are diverse including locomotion, cleansing, food particles processing and defense against physicochemical injuries and infectious agents. In oysters, mucus covering pallial organs has been demonstrated to have a major importance in the processing of food particles and in the interactions with waterborne pathogens. Given the limited information available on mucus in bivalves and the apparent wide spectra of activity of bioactive molecules present in this matrix, the characterization of these mucosal secretions has become a research priority. In this study, mucus was separately collected from the mantle, gills and labial palps of the eastern oyster (*Crassostrea virginica*) and analyzed by liquid chromatography and tandem mass spectrometry. Results showed the presence of a wide variety of molecules involved in host–microbe interactions, including putative adhesion molecules (e.g. c-type lectins) confirming that transcripts previously identified in epithelial cells are translated into proteins secreted in mucus. Mucus composition was different among samples collected from different organs. These results generate a reference map for *C. virginica* pallial mucus to better characterize the various physiological functions of mucosal secretions.

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1. Introduction

The body surface of marine invertebrates is often covered by a layer of mucus, a slippery gel secreted by mucocytes lining epithelia [1–4]. It is made of mucins, water, electrolytes, epithelial and blood cells and a wide range of bioactive molecules produced by these and mucus-secreting cells [5]. The consistency, viscosity and elasticity of mucus are generally attributed to polymers within the gel and to the physical entanglement of these polymers with other molecules [6–8]. These polymers are mucoproteins associated with carbohydrates, such as high molecular weight mucins and mucin-like glycoproteins [9] that are heavily glycosylated (up to 90% of carbohydrate) and present short carbohydrate chains [10–12] whose charges are slightly negative. In addition to large mucin type glycoproteins, mucus matrices have been found to also contain repetitive highly sulfated polysaccharides [13] and a wide variety of bioactive molecules [14–16]. Based on the biochemical diversity of molecules contained in vertebrate and invertebrate mucus, it is not surprising that these gels are involved in various functions as speculated by Auld [17] as early as 1920.

The major role of mucus in invertebrates is to serve as a protector of epithelial cells against physicochemical injuries [18–21] or infectious agents [22,23]. In addition, mucus is also used in many other activities

including locomotion [24,25], adhesion [26], cleansing of body surface [27], and nutrition [28,29]. If the roles of mucus in invertebrates are particularly studied [2,30], information on its composition is limited, probably due to its complexity, seasonal variation [31] and the limitation of analytical methods. In particular, the exact nature of glycoconjugate matrices and associated carbohydrates (mucin-type glycoproteins and polysaccharides, see review by [2]) are poorly known [13]. Despite these limitations, some studies have identified or suspected the presence in invertebrate mucus of lysozymes [1,32,33], terpenoids [34], antimicrobial peptides [22], antioxidants [14], proteases [1,35], agglutinins [36] and lectins [15,37,38].

In the eastern oyster, *Crassostrea virginica*, mucus is abundantly secreted and contributes to the processing of waterborne particles. As all suspension-feeding bivalves, *C. virginica* use their gill to pump water into their pallial (i.e. shell) cavity to capture, process and transport food particles [39–41]. Particles captured on gills are transferred to a pair of sorting organs surrounding the mouth called the labial palps, and from there are either rejected as pseudofeces through a specialized area on the mantle, or directed to the mouth and ingested [42]. In addition, *C. virginica* as many other bivalves, was found to be able to differentiate between nutritious and detrital particles (see the review by [29]). Interestingly, mucus is involved in all of these steps. Particles directed as pseudofeces are embedded in mucus and rejected back to the environment as masses of mucoid substances entangling live unwanted cells, debris and abiotic material of low nutritional

* Corresponding author.

E-mail address: Emmanuelle.PalesEspinosa@stonybrook.edu (E. Pales Espinosa).

value. Those directed to the mouth are ingested in a cohesive mucus string [43]. In addition, mucus has been suspected to mediate particle selection [44]. Recently, lectins have been identified in mucus covering *C. virginica* feeding organs [15,45] and were found to interact with carbohydrates associated with microalgae cell surface and to mediate food particle sorting [15,45–47].

Bivalve mucus has also been found to promote or inhibit the growth of diverse microorganisms. For example, the mucus contained in the biodeposits (i.e. pseudofeces and feces) of the oyster *Crassostrea gigas* was shown to stimulate microalgae growth [48]. Similarly, Allam et al. [49] showed that while mucus collected from oyster pallial organs (mantle in particular) enhanced the proliferation of *Perkinsus marinus* (a lethal parasite of the eastern oyster *C. virginica*), mucus collected from the digestive gland was inhibitory. Interestingly, pallial mucus of the noncompatible host *C. gigas* (Pacific oyster) was strongly inhibitory suggesting that *P. marinus* host specificity may begin in the mucus. The *in vivo* virulence of *P. marinus* was also significantly enhanced when the parasite was exposed to pallial mucus from *C. virginica* [50]. Mortality was significantly higher (up to 10 fold) in oysters injected with parasite cultures supplemented with pallial mucus as compared to oysters injected with parasite cells supplemented with digestive mucus or unsupplemented cultures.

Given the limited information available on the biochemical composition of pallial mucus in bivalves and the apparent wide spectra of activity of molecules present in this matrix, the identification of the proteomic makeup of these secretions has become a research priority. In this study, mucus was collected from the principal pallial organs (i.e. mantle, gills and labial palps) of *C. virginica* and analyzed by tandem mass spectrometry to create a proteome reference map for the eastern oyster pallial mucus. Results were analyzed with a particular focus on molecules involved in adhesion and interaction with waterborne microbes. To our knowledge, this work represents the first comprehensive proteomic analysis of mucus in bivalves (see [51] for review).

2. Material and methods

2.1. Mucus collection

Adults *C. virginica* (85–90 mm in length, $n = 9$) were obtained from a commercial source (Frank M. Flower and Sons Oyster Company, Oyster Bay, New York, USA) in April 2012 (temperature = 7 °C, salinity = 28). Animals were carefully opened and tissues were abundantly rinsed with artificial seawater (ASW28, salinity of 28). Mucus from pallial organs (i.e. gills, mantle and labial palps) was separately collected following the general procedures described by Pales Espinosa et al. [15]. Briefly, mucus was carefully collected using small sterile pieces of cotton-balls. Cotton-balls were then immersed in 5 to 10 ml of ice-cold ASW. Tubes containing cotton-balls were placed at 4 °C for 1 h on a rotating shaker. The resulting fluids (i.e. 27 samples) were centrifuged (3000 g, 30 min, 4 °C), filter sterilized (0.22 µm syringe filters) and maintained at 4 °C until use, typically within the following hour. A 25 µl aliquot of each fluid was used to determine protein concentrations with a Pierce BCA protein assay reagent kit (Pierce, Rockford, Illinois, USA) as per manufacturer's recommendations. Fluids were then diluted with ASW28 to a protein concentration of 2 mg·ml⁻¹. Fluids from each pallial organ (i.e. gills, mantle or labial palps) were then pooled (equal volume) in order to obtain 3 pools made from 3 oysters each.

2.2. Electrophoresis

Plasma and extrapallial fluid of *C. virginica* contain a major protein designated dominin (Itoh et al., 2011), and our preliminary analyses showed relatively high abundance of dominin in pallial mucus as well. A pre-separation step on gel was therefore implemented to improve the resolution of our proteomic analysis and favor the detection of low abundance proteins. Mucus samples (25 µl) were mixed with 25 µl of

2× denaturing sample buffer, heated to 100 °C for 10 min and separated (20 µg per well) on a precast 12% Tris–Glycine gel (Jule Biotechnologies, Inc., Milford, CT). After electrophoresis, gels were stained using standard Coomassie blue protocol. Each gel lane was excised into 12 equal slices, de-stained, reduced, alkylated and digested with trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, USA) as described by Shevchenko et al. [52] with minor modifications. Special care was taken to prevent keratin contamination. Samples from different organs (i.e. gills, mantle or labial palps) were run on separate gels in order to avoid contamination and replicates were run on the same gel. Results were similar within each of the 3 groups.

2.3. Mass spectrometry and data analysis

The resulting concentrated peptide extract was diluted into a solution of 2% acetonitrile (ACN), 0.1% formic acid (FA) (buffer A) for analysis. Ten microliters of the peptide mixture were analyzed by automated microcapillary LC/MS-MS. Fused-silica capillaries (100 µm inner diameter (i.d.)) were pulled using a P-2000 CO2 laser puller (Sutter Instruments, Novato, CA, USA) to a 5 µm i.d. tip and packed with 10 cm of 5 µm Magic C18 material (Agilent Technologies, Santa Clara, CA, USA) using a pressure bomb. Ten microliters of the resulting 20 µl of concentrate were pressure-loaded onto a 10 cm 100 µm i.d. fused-silica capillary packed with 3 µm Magic C18 reverse phase (RP) particles (Michromer, USA) which have been pulled to a 5 µm i.d. tip using a P-2000 CO2 laser puller (Sutter Instruments). This column was then installed in-line with a Dionex 3000 HPLC pump running at 300 nL min⁻¹. Peptides were loaded with an auto-sampler directly onto the column and were eluted from the column by applying a 30 min gradient from 5% buffer B to 40% buffer B (98% ACN, 0.1% FA). The gradient was switched from 40% to 80% buffer B over 5 min and held constant for 3 min. Finally, the gradient was changed from 80% buffer B to 100% buffer A over 0.1 min, and then held constant at 100% buffer A for 15 min longer. The application of a 1.8 kV distal voltage was used to electro-spray the eluting peptides directly into an LTQ XL ion trap mass spectrometer equipped with a nano-liquid chromatography electrospray ionization source. Full MS spectra were recorded on the peptides over 400–2000 m/z, followed by five MS/MS fragmentation events on the five most intense ions. MS scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Finnigan, San Jose, CA, USA). MS/MS spectra were extracted from the RAW data file with ReAdW.exe (<http://sourceforge.net/projects/sashimi>). The resulting mzXML file contained all of the data for all MS/MS spectra and could subsequently be read by the analysis software.

All MS/MS samples were analyzed using X! Tandem (The GPM, thegpm.org; version CYCLONE 2013.02.01.1) set up to search a database (98,316 entries) created by combining published protein sequences from *C. virginica* and *C. gigas* on NCBI and Uniprot/Swissprot (26,612 proteins), longest open reading frames (ORFs) of expressed sequence tags (ESTs) databases from NCBI and marinegenomics.org created with DNA2pep [53] (22,518 proteins) and common contaminants (28 proteins). In addition, a Decoy database (all proteins in reverse order) was also added from this database with compass [54]. This database was searched with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 1.8 Da. Carbamidomethyl of cysteine was specified in X! Tandem as a fixed modification. Glu- > pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln- > pyro-Glu of the n-terminus, deamidated of asparagine and glutamine, oxidation of methionine and tryptophan and dioxidation of methionine and tryptophan were specified in X! Tandem as variable modifications.

Scaffold (version Scaffold_4.4.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 5.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were

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