Proteomics
Proteomics and Systems Biology
www.proteomics-journal.com

Investigating Byssogenesis with Proteomic Analysis of Byssus, Foot, and Mantle in Mytilus Mussels by LC-MS/MS

*Maxime Sansoucy, Réjean Tremblay, Emily Carrington, Isabelle Marcotte, and Lekha Sleno**

Mussel byssus represents a fascinating class of biological materials with a unique capacity to adhere onto virtually any solid surface. Proteins expressed in byssus, the byssal-producing organ (foot) as well as mantle tissue from *Mytilus edulis* or *Mytilus californianus* are analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The mantle is used as a control tissue to pinpoint unique proteins from the foot samples potentially involved in byssogenesis. This work represents an important step towards identifying biologically important proteins expressed in foot, as well as extending knowledge on the byssus proteome. Considering the minimal proteomics data of the studied species, this data also contributes to a more complete description of *M. edulis* and *M. californianus* proteomes.

1. Introduction

Byssus threads represent a biological material with unique flexibility, self-healing, and underwater adhesion properties.[1–3] They can also adapt to different environmental factors.[4,5] In turbulent waters, for example, mussels modify the secondary structure of their byssal proteins to increase abrasion endurance.[6] This fibrous material is synthesized and secreted from the animal's foot gland, a granulo-muscular tissue. It consists of a stern, a prepepsinized collagen core, and a plaque made primarily of foot proteins, many responsible for underwater attachment. Though byssus has been the subject of many studies, byssal assembly remains a poorly described process.

These intriguing properties have motivated structural investigations addressing the protein composition of byssus for over 30 years. A limited number of proteins have been identified in the byssus, including mussel foot proteins (mfps), precollagens, and matrix thread proteins;[7] however, many more proteins are involved in the biofabrication process and its regulation. Mass spectrometry has been invaluable for the determination of proteins in byssus and other mussel tissues over the years.[8–11] An important limitation, however, exists for these species, due to the lack of complete protein databases. Several transcriptomic studies have been performed to describe putative proteins in mussel, however, with only indirect proof of the proteins' importance.

In this study, LC-MS/MS was applied to identify proteins in the foot organ potentially important during byssogenesis. Mussel foot and mantle tissues from *Mytilus edulis* and *Mytilus californianus* were digested with two complementary enzymes prior to LC-MS/MS analysis. Byssus samples from both species were also analyzed. This contribution is a unique look at the mussel proteome from two closely related species, while presenting a list of foot-specific proteins potentially involved in byssus fabrication and regulation.

2. Experimental Section

M. edulis mussels (Prince Edward Island, Canada) were shipped live on ice, tissues dissected, flash frozen, and lyophilized. *M. californianus* (Friday Harbor, WA) tissues were prepared similarly prior to being shipped for analysis. For each sample type, three biological replicates were digested with trypsin and three biological replicates were also digested with pepsin. Ammonium bicarbonate buffer (100 mM, pH 8) was added to dried tissues prior to homogenization, and resulting extracts divided into 200 µL aliquots (3–5 mg tissue each). Reductive alkylation was performed prior to trypsin (pH 8) or pepsin (pH 2) digestions. Samples were digested overnight (20 µg protease) at 37 °C, desalted using OASIS HLB cartridges (Waters, Milford, MA, 30 mg 1 cc^{-1}), and dried. For byssus samples (containing threads and plaques), 50 µL 7 M urea and 2 M thiourea was added to ground samples (1–2 mg), sonicated and heated at 50 °C (20 min). Trypsin and pepsin (25 µg protease) digestions were performed as above. Resuspended extracts (100 µL 10% acetonitrile (ACN)) were injected (30 µL) onto an Aeris PEPTIDE XB-C18 100 × 2.1 mm column at 40 °C (Phenomenex, Torrance, CA), with water

M. Sansoucy, Prof. I. Marcotte, Prof. L. Sleno
Chemistry Department
Université du Québec à Montréal
P.O. Box 8888, Downtown Station, Montréal, Québec H3C 3P8, Canada
E-mail: sleno.lekha@uqam.ca
Prof. R. Tremblay
Institut des Sciences de la Mer
Université du Québec à Rimouski
310 allée des Ursulines Rimouski, Québec G5L 3A1, Canada
Prof. E. Carrington
Department of Biology and Friday Harbor Laboratories
University of Washington
620 University Road Friday Harbor, WA 98250, USA

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/pmic.202000014

DOI: 10.1002/pmic.202000014

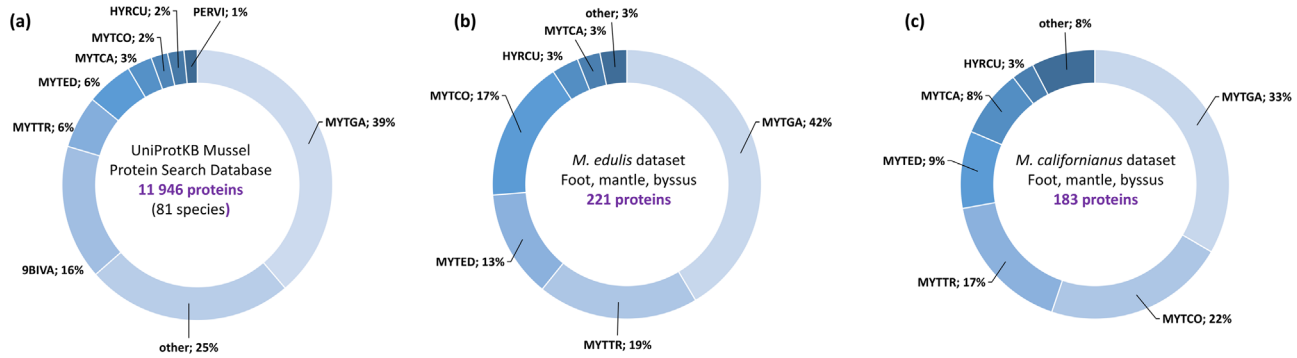

Figure 1. Relative species distribution of proteins, from search database (a), as well as from MYTED (b) and MYTCA (c) datasets combining foot, mantle, and byssus analyses.

and acetonitrile, both containing 0.1% formic acid. A Nexera UHPLC system (Shimadzu, Columbia, MD) was operated at 300 µL min^{-1}. After a 3 min hold at 5% ACN, the organic composition was increased to 30% at 50 min, 50% at 55 min, then to 90% within 1 min. TOF-MS and MS/MS spectra were acquired on a hybrid quadrupole time-of-flight TripleTOF 5600 mass spectrometer (AB Sciex, Concord, ON, Canada) in positive electrospray mode at 500 °C, with ionspray voltage of 5000 V, declustering potential 60 V, nebulizer and drying gases 50 psi. TOF-MS (m/z 120–1250), followed by MS/MS acquisition (m/z 80–1500), used dynamic background subtraction and information-dependant acquisition on the 15 most intense ions, with collision energy of 30 ± 10 V and a total cycle time of 1.05 s. LC-MS/MS data for trypsin and pepsin for each biological replicate were searched separately against a protein database from UniprotKB/Swiss-Prot (released on April 2020), using ProteinPilot software (version 5.0.2), from 81 species (**Figure 1**a). Search results were then combined and processed in Scaffold-Q+ (v4.9.0, Proteome Software). Proteins with a minimum of two peptides at 1% false discovery rate (FDR), and seen in a minimum of two biological replicates, were considered. Data have been deposited to the ProteomeXchange Consortium via the PRIDE[12] partner repository with the dataset identifier 10.6019/PXD021023.

3. Results and Discussion

The results presented here were compiled from triplicate tryptic and peptic digests of byssus, foot, and mantle tissues from *M. edulis* and *M. californianus*. All listed proteins were identified with a minimum of two peptides at 1% FDR and found in at least two biological replicates.

The data was searched with a database including 81 mussel species, since complete protein databases are not yet available for *M. edulis* and *M. californianus*. This database consisted mainly of putative proteins from transcriptomic analysis, with a very small proportion (about 1%) having previous experimental evidence. Figure 1a shows the proportion of proteins by species in the search database. By comparing the distribution of species for identified proteins in *M. edulis* (MYTED, Figure 1b) and *M. californianus* (MYTCA, Figure 1c), a good sequence homology from *M. edulis* and *M. californianus* is seen with *M. coruscus*, *M. galloprovincialis*, and *M. trossulus* proteins. It is striking that, of all proteins detected, only 13% (MYTED) and 8% (MYTCA) were attributed to their respective species. This is, of course, a consequence of incomplete databases for such species. Without searching protein sequences from other species, most proteins would not have been detected. This LC-MS/MS data thus serves to confirm overlapping regions within proteins from different species.

The comparison of proteins identified is shown in Venn diagrams (**Figure 2**). There were 73 and 58 "foot-specific" proteins found in the MYTED and MYTCA datasets, using the mantle as a control tissue. From byssus analyses, 37 and 50 proteins were confidently identified from MYTED and MYTCA, respectively, with 30 proteins in common. The foot-specific and byssus proteins were investigated further, since the aim of this work was to unveil proteins potentially involved in byssus fabrication and regulation. The details of each complete protein list from the three tissues can be found in Tables S1 and S2, Supporting Information, along with the number of confident peptides and sequence coverage for each. Table S3, Supporting Information, compiles the 62 foot-specific proteins compiled using the mantle from both species as a control tissue, with additional information such as molecular weight, pI, % abundances for specific amino acids, GRAVY scores and gene ontology (GO) annotations. For the byssus and foot-specific proteins, the largest proportion did not have any associated GO terms, followed by proteins with involving binding and metal-ion binding. This is not surprising, since it is well known that byssus has very strong adhesive properties and metal binding capacities.

The main goal of this study was to identify proteins from the mussel's foot potentially implicated in byssogenesis. Many proteins known to be main constituents of byssus itself were detected, as well as several previously implicated in the formation of byssus.[13] **Table 1** lists thread-related proteins found in foot and byssus, including specific enzymes known to be byssal-related, such as glycosyl-hydrolase, peroxidase, tyrosinase, and procollagen-proline dioxygenase. The three precollagens (D, NG, P), as well as thread matrix and protease inhibitor proteins are also listed. Ten distinct mfps are included, five of which were detected in both species.

Mussel foot proteins are well known for their crucial roles in determining the unique adhesive properties of byssus. Mfp-2, -4, -6, -10, and -12 were detected in foot and/or byssus samples of both species. Mfp-3, in three of its variant forms, was detected in *M. californianus* byssus samples. These proteins were identified based on sequences from MYTED and MYTCA for

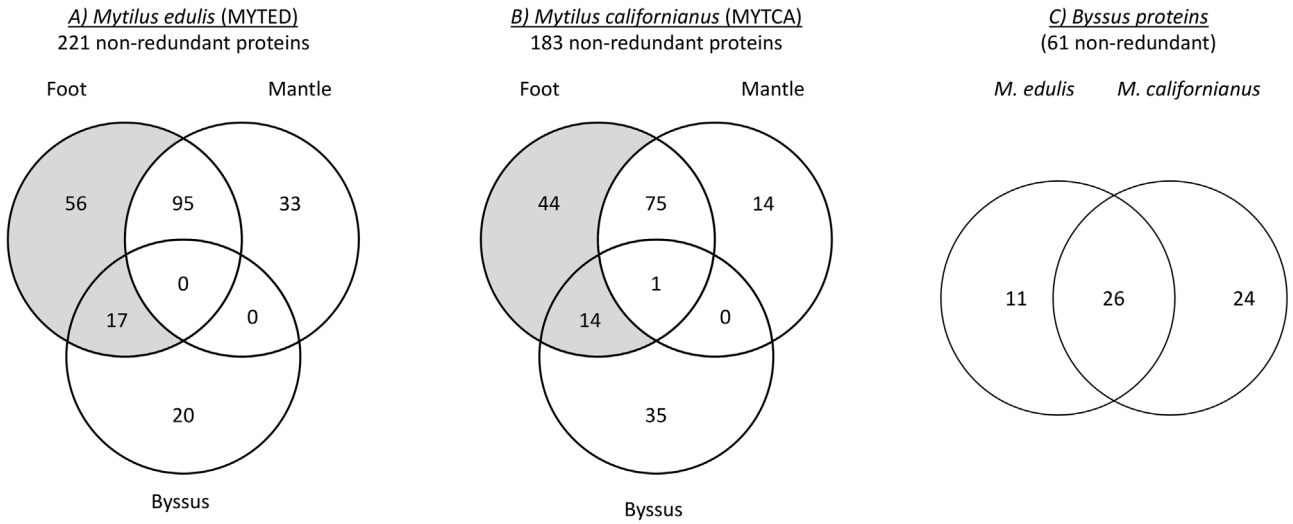

Figure 2. Number of confidently identified proteins detected in foot, mantle, and byssus from *Mytilus edulis* (A) and *Mytilus californianus* (B). Proteins considered as foot-specific are shown in shaded regions in A and B found in byssus samples were compared between the two species (C).

Table 1. Thread-related proteins found in foot and byssus samples from *Mytilus edulis* and *Mytilus californianus*.

Foot		Byssus	
Byssal calumenin-like 1	**Precollagen (D, P, NG)**	**Byssal glycosyl-hydrolase-like 2**	Foot protein 3, 11, 17
Byssal glycosyl-hydrolase-like 2	**Procollagen-proline dioxygenase**	**Byssal peroxidase-like** 1, **2**, 4	**Mussel byssus collagen-like 3**
Byssal peroxidase-like 2	**Protease inhibitor-like 1**	**Byssal protease inhibitor-like 1**	**Precollagen (D, P, NG)**
Byssal protease inhibitor-like 1	**Proximal thread matrix 1, 1a**	Byssal protein 1, 2, 3	**Protease inhibitor-like 1, D1, D2**
Byssal tyrosinase-like 1, **2**	**TSP_1 domain containing 1**	**Byssal tyrosinase-like 1, 2**	**Proximal thread matrix 1, 1a**
Collagen-like 5, 6, 7		Collagen chain alpha 1	**Thread matrix 2A**
Foot protein 2		**Collagen-like** 4, **5, 6, 7**	**TSP_1 domain containing 1**
Foot protein 6, 10, 12, 18, 19		**Foot protein 2, 4, 6, 10, 12**	**Tyrosinase 5**

Proteins in bold were detected in both species.

mfp-2, and MYTCA for mfp-3, -4, and -6 (variants 1/2). Variant 3 of mfp-6 (from MYTCO) was found in both species, whereas variants 1 and 2 were detected only in *M. californianus*. Figure S1, Supporting Information, shows the amino acid sequences of all detected mussel foot proteins, and indicates which regions were confidently identified in MYTED and MYTCA samples.

Six *M. californianus* mfps recently reported in a transcriptomic study[7] were detected here for the first time. These proteins were mfp-10, -11, -12, -17, -18, and -19. Mfp-10 and -12 were detected in both species, whereas mfp-11, -17, -18, and -19 were exclusively detected in *M. californianus*.

In addition to the byssal precollagens (distal, proximal, and non-gradient), two enzyme classes known to impact byssus adhesion and plasticity were found. The first enzyme class is responsible for the formation of dihydroxyphenylalanine (DOPA) and includes byssal tyrosinase-like proteins 1 and 2 (from MYTCO) and tyrosinase 5 (from PERVI). This post-translational modification is argued to have a determinant role in adhesion and structural properties.[14–17] Also, three byssal peroxidase-like proteins, previously identified from transcriptomic data in *M. corsucus*, were found in our dataset. These proteins are believed to protect the threads against reactive oxygen species, which may initiate adverse reactions involving DOPA related intra- or intermolecular crosslinking between byssus proteins.[18] Crosslinking in byssus is believed to negatively affect thread adhesion and elasticity, however, this still needs further study.[13,19]

The present study also includes 14 "uncharacterized" proteins obtained from transcriptomic evidence in *M. coruscus, M. galloprovincialis, and M. trossulus* (see Tables S1 and S3, Supporting Information). The sequences of these proteins were submitted to a BLAST search (https://www.uniprot.org/blast/), and analogous proteins are presented in Table S4, Supporting Information, with the percentage sequence homology for each.

From this work, we have presented a list of foot-specific proteins potentially involved in byssogenesis. Of course, more detailed studies must be performed to clarify the specific roles of these proteins in byssus assembly. Hampered by a lack of complete proteomic databases for the studied species, many proteins were solely identified on the basis of overlapping regions from

related species. Once more complete protein databases become available for these species, this data could be further interrogated.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors would like to acknowledge the *Fonds de Recherche du Quebec - Nature et Technologies* (FQRNT) for the financial support of this research.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

byssus, foot, mantle, mussel, *Mytilus californianus*, *Mytilus edulis*, proteomics

Received: January 17, 2020
Revised: August 28, 2020
Published online: December 11, 2020

[1] X.-X. Qin, J. H. Waite, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 10517.
[2] M. J. Harrington, J. H. Waite, *J. Exp. Biol.* **2007**, *210*, 4307.
[3] C. N. Z. Schmitt, Y. Politi, A. Reinecke, M. J. Harrington, *Biomacromolecules* **2015**, *16*, 2852.
[4] S. L. Brazee, E. Carrington, *Biol. Bull.* **2006**, *211*, 263.
[5] M. O. Seguin-Heine, A. A. Lachance, B. Genard, B. Myrand, C. Pellerin, I. Marcotte, R. Tremblay, *Aquaculture* **2014**, *426–427*, 189.
[6] A. A. Arnold, F. Byette, M. O. Séguin-Heine, A. Leblanc, L. Sleno, R. Tremblay, C. Pellerin, I. Marcotte, *Biomacromolecules* **2013**, *14*, 132.
[7] D. G. DeMartini, J. M. Errico, S. Sjoestroem, A. Fenster, J. H. Waite, *J. Royal Soc. Interface* **2017**, *14*, 20170151.
[8] J. L. López, A. Marina, J. Vázquez, G. Alvarez, *Mar. Biol.* **2002**, *141*, 217.
[9] A. Gantayet, L. Ohana, E. D. Sone, *Biofouling* **2013**, *29*, 77.
[10] A. Gantayet, D. J. Rees, E. D. Sone, *Mar. Biotechnol.* **2014**, *16*, 144.
[11] S. Li, Z. Xia, Y. Chen, Y. Gao, A. Zhan, *Front. Physiol.* **2018**, *9*, 1.
[12] E. W. Deutsch, A. Csordas, Z. Sun, A. Jarnuczak, Y. Perez-Riverol, T. Ternent, D. S. Campbell, M. Bernal-Llinares, S. Okuda, S. Kawano, R. L. Moritz, J. J. Carver, M. Wang, Y. Ishihama, N. Bandeira, H. Hermjakob, J. A. Vizcaíno, *Nucleic Acids Res.* **2017**, *45*, D1100.
[13] J. H. Waite, *J. Exp. Biol.* **2017**, *220*, 517.
[14] M. J. Harrington, A. Masic, N. Holten-Andersen, J. H. Waite, P. Fratzl, *Science* **2010**, *328*, 216.
[15] W. Wei, J. Yu, C. Broomell, J. N. Israelachvili, J. H. Waite, *J. Am. Chem. Soc.* **2012**, *135*, 377.
[16] J. Yu, W. Wei, E. Danner, R. K. Ashley, J. N. Israelachvili, J. H. Waite, *Nat. Chem. Biol.* **2011**, *7*, 588.
[17] L. Petrone, A. Kumar, C. N. Sutanto, N. J. Patil, S. Kannan, A. Palaniappan, S. Amini, B. Zappone, C. Verma, A. Miserez, *Nat. Commun.* **2015**, *6*, 8737.
[18] H. Zhao, J. H. Waite, *Biochemistry* **2005**, *44*, 15915.
[19] J. Yu, W. Wei, M. S. Menyo, A. Masic, J. H. Waite, J. N. Israelachvili, *Biomacromolecules* **2013**, *14*, 1072.