The Journal of Experimental Biology 203, 3065–3076 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB2669

IMMUNOLOCALIZATION OF Dpfp1, A BYSSAL PROTEIN OF THE ZEBRA MUSSEL *DREISSENA POLYMORPHA*

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Accepted 18 July; published on WWW 26 September 2000

Summary

The zebra mussel is one of only a few freshwater bivalves known to produce a byssus. This fibrous, proteinaceous and highly cross-linked structure allows the mussel to attach to a variety of substrata and contributes to its notoriety as a major freshwater biofouling species. We have successfully expressed a full-length version of *Dreissena polymorpha* foot protein 1 (Dpfp1), a putative byssal thread precursor, and have used the recombinant protein as an antigen for polyclonal antibody production. Antisera obtained from rabbits immunized with recombinant Dpfp1 recognize the protein in western blots of extracts from foot tissue and byssal threads. On the basis of this evidence, we conclude that Dpfp1 is a byssal precursor protein manufactured and stored in the foot of the mussel. Immunohistochemical localization of Dpfp1 suggests that the protein is localized in secretory granules in a large gland surrounding the ventral groove of the foot. Only a subset of these glandular cells stockpiles the protein, implying that the zebra mussel foot is a complex organ capable of several distinct secretory activities involved in byssal thread formation. The uniform distribution of Dpfp1-containing cells suggests that the protein is a significant load-bearing component of zebra mussel byssal threads, although a more rigorous test of this hypothesis awaits ultrastructural localization of the protein in mature byssal threads.

Key words: biological attachment, biofouling, *Dreissena polymorpha* foot protein 1, *Dreissena polymorpha*, byssal protein, byssus, immunolocalization, zebra mussel.

Introduction

The zebra mussel, *Dreissena polymorpha* (Pallas), has earned a reputation as a major biofouling species in the Great Lakes and in the lakes and rivers of over one-third of the North American continent (Johnson and Padilla, 1996). Zebra mussels are capable of attaching themselves in large numbers to a wide variety of substrata using a bundle of thread-like structures collectively termed a byssus. Millions of dollars are spent annually to remove attached mussels from ship hulls, navigation buoys and water intake pipes (Roberts, 1990). Consequently, a significant interest has emerged in understanding the biochemistry of attachment in this exotic species. The pivotal role played by the byssus in mussel attachment has focused our research efforts on the protein components of this structure, with the goal of identifying vulnerable byssal thread components.

Few details are known about the mechanism of byssal thread formation, although it does not appear to involve a spinning process such as that used for arthropod silks. In the best-studied case, that of *Mytilus edulis*, byssal proteins are synthesized and stockpiled in the foot, a muscular and glandular organ located on the ventral side of the animal. The foot of *M. edulis* is a complex organ subdivided into various glands, each responsible for a different aspect of byssal thread synthesis (Waite, 1992). During thread formation, these glands secrete the granular thread precursors into a longitudinal cleft at the base of the foot, known as the ventral or pedal groove. Rhythmic muscular contractions of the foot mix and shape the secreted precursors, and the newly formed thread is carefully deposited onto the substratum.

The byssus of *D. polymorpha*, while unique in its amino acid composition (Rzepecki and Waite, 1993a), resembles byssi of other marine bivalves in containing the post-translationally modified amino acid 3,4-dihydroxyphenylalanine (Dopa) (Papov et al., 1995; Rzepecki et al., 1991, 1992; Waite, 1986; Waite et al., 1989; Waite and Tanzer, 1981). The chemical maturation of byssal threads is closely linked to the formation of intermolecular cross-links derived from oxidized Dopa (McDowell et al., 1999). Rzepecki and Waite (1993b) have isolated and partially characterized soluble Dopa-containing proteins from the foot of the zebra mussel and have demonstrated the presence of Dopa in tissue surrounding the ventral groove (Rzepecki and Waite, 1993a). Anderson and Waite (1998) determined the complete primary sequence of one of these proteins, *Dreissena polymorpha* foot protein 1 (Dpfp1), and demonstrated that transcripts for this protein occur only in the foot. Circumstantial evidence has suggested

that Dpfp1 is present in the byssus, but the rapid maturation and highly intractable nature of byssal proteins have made it impossible to establish its presence directly in this structure. Moreover, little is known about the distribution of the foot tissue responsible for the synthesis and storage of Dpfp1.

In this study, we begin to address these concerns by raising polyclonal antibodies to a recombinant version of Dpfp1. Results obtained with these antibodies support the hypothesis that Dpfp1 is a byssal precursor protein that is stockpiled in secretory vesicles in a subset of glandular cells surrounding the ventral groove and distal depression. These results suggest that the process of byssal thread synthesis in *D. polymorpha* is similar to that in *M. edulis* and provide new insights into the process of byssal thread formation in this important freshwater biofouling species.

Table 1. *Sequence of primers used in this study*

Primer	Sequence
Gene-specific	
Dp1.5′UT(+)	ATACTTCAGAGCATC
Dp1.NTerm(+)	GGGAGCTCTGATTGGAC
Dp1.GSP2(–)	TTGTTGTATAGTTCGGAATTTTAG
Dp1.3′UT(–)	TGATCGTATTTATTTATTAGTATG
Miscellaneous	
PolyT-LD(–)	AGAGAGATTTTTTTTTTTTTTTTVN
AAP	GGCCACGCGTCGACTAGTACGGG…
	…IIGGGIIGGGIIG
SP6	GATTTAGGTGACACTATAG

(+) and (–) indicate forward and reverse primers, respectively.
V indicates A, G or C; N indicates A, G, C or T; I, inosine.

Materials and methods

All polymerase chain reactions (PCRs) were performed on a RoboCycler 40 (Stratagene; La Jolla, CA, USA) using 2.5 units of Taq2000 polymerase (Stratagene; La Jolla, CA, USA) in amplification buffer (10 mmol l^{-1} Tris, pH 8.8, 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 0.25 mmol l^{-1} each deoxyribonucleotide triphosphate, dNTP, and 0.001 % gelatin). All cycling reactions began with an initial 5 min thermal denaturation at 95 °C. Primers were used at a concentration of 20 pmol each, and the sequences of all primers are listed in Table 1.

PCR of Dpfp1 cDNA

Zebra mussel *Dreissena polymorpha* (Pallas) foot tissue used for reverse transcription-PCR (RT-PCR) was excised, immediately frozen in liquid nitrogen, and ground in a mortar that had been chilled to –80 °C. The tissue was homogenized in a hand-held glass homogenizer (Kontes; Vineland, NJ, USA), and total RNA was extracted using the method of Chomczynski and Sacchi (1987) as modified by Coyne (1997). Total RNA (800 ng) was incubated with polyT-LD(–) primer (80 ng) in diethylpyrocarbonate (DEPC)-treated water for 10 min at 70 °C and subsequently chilled on ice for 1 min. Total RNA was reverse-transcribed for 50 min at 42 °C using 200 units of SuperScript II Reverse Transcriptase (Life Technologies; Bethesda, MD, USA) in RT buffer (20 mmol l^{-1} Tris-HCl, pH 8.4, 50 mmol l^{-1} KCl, 2.5 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} dithiothreitol, DTT, 400 µmol l^{-1} each dNTP). The reaction was terminated by incubation at 70 °C for 50 min. RNA was degraded with a combination of RNAase H and RNAase T1 for 30 min at 37 °C, and cDNA was purified from primer and residual nucleotides according to the methods of Vogelstein and Gillespie (1979), as implemented in the 5′ RACE system, V2.0 (Life Technologies; Bethesda, MD, USA). After thermal denaturation of the purified cDNA at 94 °C for 3 min, an oligo-(dC) tail was added to the 5′ end of the cDNA by incubating with 15 units of terminal deoxynucleotide transferase (Life Technologies; Bethesda, MD, USA) for 10 min at 37 °C in a TdT buffer (10 mmol l^{-1} Tris, pH 8.4, 25 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} dCTP). The reaction was terminated by incubating the sample at 65 °C for 10 min.

The tailed cDNA was amplified using the AAP and polyT-LD(–) primers for 25 cycles under the following conditions: 95 °C for 0.5 min, 54 °C for 0.5 min, 72 °C for 2 min. A sample (10 %) of this reaction was then re-amplified using the primers Dp1.5′UT(+) and Dp1.3′UT(–). Amplification conditions were as described above, except that an annealing temperature of 40 °C was employed and the amplification was conducted for a total of 40 rounds.

The resulting amplification products were cloned into a pGEM-T vector (Promega; Madison, WI, USA) and transformed into *Escherichia coli* JM109 cells. The sequences from three insert-bearing vectors were determined using nested deletions, as described by Sambrook et al. (1989) and utilizing reagents from the Double-Stranded Nested Deletion Kit (Pharmacia; Piscataway, NJ, USA). One of these vectors, pDp1.16, was selected for subsequent construction of a maltose-binding fusion protein.

Construction of the expression vector pMBP_Dp1.16

cDNA encoding the sequence for mature Dpfp1 was amplified using pDp1.16 as a template and Dp1NTerm(+) and SP6 as primers. The template was amplified for 30 cycles, as described above, using an annealing temperature of 50 °C. Protruding 3′ adenine nucleotides were removed from the phenol/chloroform-extracted amplification product using T4 polymerase (New England Biolabs; Beverly, MA, USA), and a cohesive 3′ end was generated by digesting the amplification product with *Pst*I followed by gel purification. The target expression vector, pMAL-c2 (New England Biolabs; Beverly, MA, USA), was digested with *Pst*I and *Xmn*I, treated with calf intestinal alkaline phosphatase (New England Biolabs; Beverly, MA, USA) and phenol/chloroform-extracted. The previously prepared amplification product was unidirectionally cloned into the digested expression vector and transformed into competent *E. coli* CAG-597 cells (New England Biolabs; Beverly, MA, USA). Proper in-frame insertion of the Dp1.16

transcript was confirmed by sequencing the linker region of three separately purified clones using a gene-specific primer Dp1.GSP2(–).

Expression and purification of recombinant Dpfp1

MBP_Dp1.16 was expressed and purified using a modification of the methods of Riggs (1992). Briefly, CAG-597 cells containing the Dpfp1 fusion-protein gene were grown at 30 °C in LB medium supplemented with glucose (2 mg ml^{-1}) and ampicillin (100 μg ml^{-1}). After reaching an absorbance at 600 nm of 0.5, the cells were induced with 1 mmol l^{-1} isopropyl-β-D-thiogalactoside (IPTG) for 6 h at 30 °C. The induced cells were subsequently pelleted, resuspended in three volumes of chilled (4 °C) column buffer (20 mmol l^{-1} Tris-HCl, pH 8.0, 100 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA) and frozen at –20 °C overnight. The frozen cells were brought to room temperature (18–22 °C), incubated with lysozyme (0.5 mg ml^{-1} of supernatant) for 30 min and sonicated. The lysed cells were pelleted, and the protein content of the supernatant was adjusted to 2.5 mg ml^{-1} with column buffer. The supernatant was loaded onto a column packed with amylose resin (New England Biolabs; Beverly, MA, USA), washed overnight with 12 column volumes of column buffer at 4 °C and eluted in column buffer supplemented with 10 mmol l^{-1} maltose. Column fractions containing the fusion protein (MBP_Dp1.16) were pooled and concentrated to 1 mg ml^{-1} using a Centriplus-10 concentrator (Amicon; Beverly, MA, USA).

Concentrated fusion protein was cleaved by an 18 h incubation at room temperature with the factor Xa protease (New England Biolabs; Beverly, MA, USA) in column buffer at an enzyme:protein ratio of 1:100 (w/w). The buffer containing the cleaved protein was centrifuged at 18 000 ***g*** for 10 min, the supernatant was reserved, and the pellet was resuspended in an equal volume of 8 mol l^{-1} urea. Both the supernatant and resuspended pellet were separately purified using a reversed-phase high-performance liquid chromatography (HPLC) system equipped with a C-8 column (250 mm×4.6 mm; Brownlee RP-300) using a linear elution gradient (acetonitrile/milli-Q water, both containing 0.1 % trifluoroacetic acid, TFA). The purity of recombinant Dpfp1 (Dp1.16) was assessed using SDS–PAGE (Laemmli, 1970), and fractions containing the majority of eluted protein were pooled.

Characterization

After affinity purification, the identity of the uncleaved fusion protein was confirmed using mass spectrometric fingerprinting. Recombinant protein (40 pmol) was digested with endoprotease Lys-C (Boehringer Manheim; Indianapolis, IN, USA) in a buffer composed of 10 mmol l^{-1} Tris-HCl, pH 7.3, and 2 μmol l^{-1} $CaCl_2$ (1 μmol l^{-1} final protein concentration). An enzyme:protein ratio of 1:20 (w/w) was used, and digestion was allowed to proceed for 2 h at 37 °C. The digested protein, in buffer, was mixed 1:5 (v/v) with 40 % acetonitrile/0.1 % TFA saturated with the matrix sinapinic acid. A volume containing 2 pmol of digested protein was then spotted onto a sample plate and allowed to dry in air. The masses of the peptides were determined by matrix-assisted laser desorption-ionization mass spectrometry time of flight (MALDI-TOF) using a PerSeptive Biosystems Voyager DE mass spectrometer (PerSeptive Biosystems; Foster City, CA, USA) in delayed extraction mode. Analysis of positive ions was conducted *in vacuo* (1×10^{-5} Pa) with an acceleration voltage of 25 000 V, 90 % grid voltage and a guidewire setting of 0.1 %. The spectra were generated by averaging data collected from 112 pulses of a 337 nm laser (2300 arbitrary power setting). The instrument has a typical mass resolution of 0.01 % and was externally calibrated with gramicidin A (mono-isotopic mass 1142.5 Da) and bovine insulin (mono-isotopic mass 5734.6 Da).

The N-terminal sequence of Dp1.16 was determined using a Porton Instruments microsequencer (model 2090) operating under standard Edman degradation conditions and program 38 (Waite, 1991).

Antiserum production

Polyclonal antisera were raised in two female New Zealand white rabbits (R1, R2) against the factor-Xa-cleaved and purified recombinant protein (Dp1.16). Rabbits were injected subcutaneously and intramuscularly with 200 μg of recombinant protein (100 μg per location) in complete Freund's adjuvant followed by boosts performed at 21 and 53 days after the first injection using 250 μg of protein (125 μg per location) in incomplete Freund's adjuvant. A final intravenous boost was administered 91 days after the first injection with 70 μg of antigen in 0.85 % saline solution. Antisera were collected from each rabbit 7 days after the second boost (B2) and 10 days after the third boost (B3). Pre-immune serum (PI) was collected prior to antigen injection for use in control experiments.

Extraction and purification of Dpfp1

Dpfp1 was extracted and purified from zebra mussel foot tissue according to the methods of Rzepecki and Waite (1993b) and will henceforth be referred to as 'foot-derived' Dpfp1. Mussels used for these experiments were obtained from the Niagara River, NY, USA, and shipped live and on ice *via* overnight delivery from the State University of New York at Buffalo. *D. polymorpha* were separated from *D. bugensis* on the basis of differences in shell morphology (May and Marsden, 1992). Tissue used in this study was obtained by severing the animal's adductor muscles with a razor blade and amputating the foot with a pair of tweezers.

Crude tissue extracts

Crude foot tissue extracts were prepared by purifying excised feet to the S1 stage (Rzepecki and Waite, 1993b). The remaining non-foot soft tissue (i.e. visceral mass, gill tissue, mantle, etc.) was extracted in an identical manner for use as a negative control. Total protein concentrations were quantified according to the methods of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Thread extracts

Threads collected for this study were obtained from adult *D. polymorpha* using a method similar to that described by Rzepecki and Waite (1993a). The collected threads were rinsed with deionized water and stored in 8 mol l^{-1} urea/5 % acetic acid at −20 °C. Pooled threads were thawed and homogenized in a ground-glass tissue grinder (Kontes; Vineland, NJ, USA). A small quantity of washed and ignited sand (Mallinkrodt; St Louis, MO, USA) was added to aid homogenization. After homogenization, the sample was centrifuged at 14 000 ***g*** for 10 min, and the supernatant was reserved. Total protein concentrations were quantified according to the method of Bradford (1976) using BSA as a standard.

Antiserum characterization

The specificity of anti-Dp1.16 antibodies was established using western blots of foot tissue, non-foot tissue and HPLC-purified foot-derived Dpfp1. Duplicate acid-urea polyacrylamide gels (Panyim and Chalkley, 1969) were run, each containing 1 μg of foot and non-foot tissue extracts and 2 μg of HPLC-purified foot-derived Dpfp1. The proteins were electrophoretically transferred in a Genie transfer unit (Idea Scientific; Minneapolis, MN, USA) for 90 min at 200 mA constant current onto Immobilon-P membranes (Millipore; Bedford, MA, USA) in a buffer consisting of 0.7 % acetic acid. The transferred proteins were washed for 5 min in phosphate-buffered saline (PBS; 10 mmol l^{-1} phosphate, pH 7.5, 2.5 % NaCl), and the membrane was blocked for 30 min in blocking buffer (PBS + 0.5 % casein). The membrane was then incubated for 60 min with primary antiserum (R2B2 or R2PI), diluted 1:1000 in blocking buffer, washed three times with PBS (5 min per wash) and incubated for 60 min with secondary antibody (biotin-conjugated goat-anti-rabbit IgG, Vector Laboratories; Burlingame, CA, USA) diluted 1:100 in blocking buffer. The membrane was washed three times as described above and incubated with streptavidin–biotin–peroxidase (Vector Laboratories; Burlingame, CA, USA) for 30 min in PBS, washed three times as described above and developed for 5 min using 3,3′-diaminobenzidine enhanced with Ni and Co in peroxidase buffer (DAB Substrate Kit, Boehringer-Mannheim; Indianapolis, IN, USA). The above procedures were carried out at room temperature.

Zebra mussel foot tissue preparation

Excised tissue was fixed in 10 % phosphate-buffered formalin (Fisher Scientific; Pittsburgh, PA, USA) for 48–72 h. Specimens were dehydrated in a graded series of ethanol, cleared with xylene and embedded in paraffin wax. Sections (5 μm thick) were cut and mounted on poly-L-lysine coated slides. Specimens were rehydrated in a graded ethanol series and rinsed in running distilled water for 5 min.

Fluorescence-based detection

Rehydrated sections were digested with trypsin (1 mg ml^{-1}) for 10 min at 37 °C in 20 mmol l^{-1} Tris, pH 7.5, and 0.01 % $CaCl_2$. After a 5 min rinse with distilled water, the sections were blocked (PBS + 1.5 % normal goat serum, 5 % BSA) for 1 h at room temperature, and primary antiserum (R2B2) or pre-immune serum (R2PI), in blocking buffer, was added at a concentration of 1:500. The sections were incubated with primary antiserum overnight at 4 °C in a humidity chamber. After removal of non-specifically bound primary antibodies with three washes in PBS for 5 min each at room temperature, the sections were incubated at room temperature for 1 h with secondary antibody (Alexa568-conjugated goat anti-rabbit IgG; Molecular Probes; Eugene, OR, USA) in blocking buffer at a concentration of 1:200. The sections were washed in PBS, as described above, counter-stained with a nuclear stain (Syto13; Molecular Probes; Eugene, OR, USA), and prepared for viewing using an aqueous mounting media (SlowFade; Molecular Probes; Eugene, OR, USA). Sections were viewed on a Zeiss LSM 510 confocal microscope using a Kr/Ar laser and a FITC/Rhodamine filter set. Identical instrument settings were used to image sections incubated with anti-Dp1.16 and pre-immune serum.

Results

PCR of Dpfp1 cDNA

PCR amplification of RT-PCR-generated foot tissue cDNA with Dpfp1-specific primers resulted in bands of 1240, 1340 and 1390 base pairs (bp) (Fig. 1). The primary sequence of

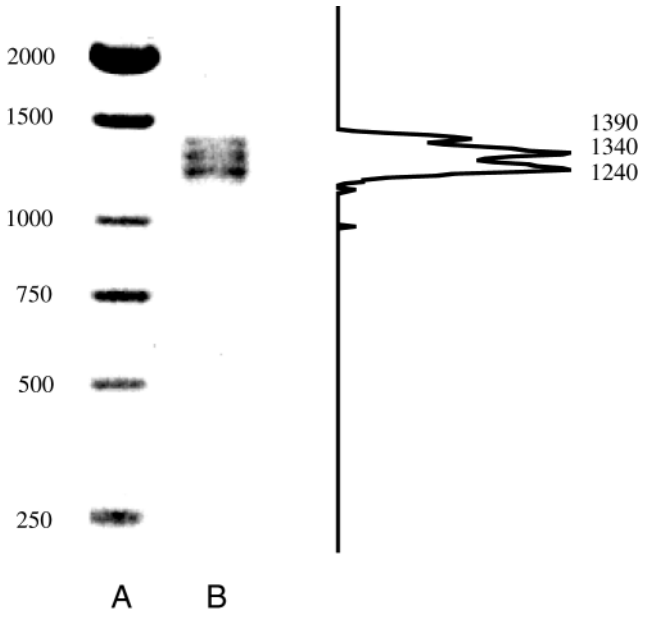

Fig. 1. Reverse transcription/polymerase chain reaction (RT-PCR) of zebra mussel foot tissue total RNA. Total RNA from zebra mussel foot tissues was reverse-transcribed and amplified using the gene-specific primers Dp1.NTerm(+) and Dp1.3′UT(−), as described in the text. Samples were run on a 1.5 % agarose/TAE (Sambrook et al., 1989) gel and stained with ethidium bromide. Lane A contains double-stranded DNA standards, and lane B contains the RT-PCR amplification products. The results of scanning densitometry of lane B are presented to the right of that lane. Numbers to the left and right of the figure indicate the size of standards and amplification products in base pairs.

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-19                                mfsvvsfcllaagfgsslg   -1
  1 GSSDWTEKTSQSTIPTISGWSFFTTKSPLNPTLFTTKRPEYVTLSPVYPT   50
 51 KIPNYTTKPPVYPTKVPEYPTKDPTYPTFKTPEYPTKVPEYPTKVPTYPT  100
101 FQTPEYPTPTKYPVYPSQSPAYPTQYPEYPSQYPVYPDQYPVYPNQYPVK  150
151 QDHDPVYPPRSPLYGWRRPVYPKKTPVYPYLPLYPGYQPEYHRRPPVYPP  200
201 VYPYDPVEDKKPGPYDYDGPYDKNPGPYDYDGPYNKKPNPYGTDWQYDKK  250
251 TGPYVPIKPDDKKPNPYGTDWQYDKKTGPYVPDKSEDKKPGPYDYDGPYD  300
301 KNPGPYDSDGPYNKKPGPYDYDGPYDKNPGPYDYNGPYDKKPGPYDYDGP  350
351 YD↑IKPGPYDYDVPYDKKPDPYDTDGPYDKKTGPYVPDKPDDKKTDPYVPD 400
401 VPLEPPGPLGK                                        411
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Fig. 2. Deduced primary sequence of Dp1.16. These data were obtained from the results of double-stranded nested deletion sequencing of pDp1.16. Amino acid residues in lower case are part of the signal peptide and were not included in the recombinant protein. Amino acid residues in bold type differ from the previously deduced sequence for Dpfp1 (Anderson and Waite, 1998). ↑ indicates a deletion of 13 amino acid residues that does not occur in the previously deduced sequence.

Dp1.16 was obtained after the resulting amplification products had been cloned into pGEM-T vectors. Sequences corresponding to the three size variants reported above were obtained; however, only the sequence representing the 1340 bp amplification product was observed in more than one vector. Fig. 2 presents the deduced primary sequence of the insert contained in vector pDp1.16 and coding for Dp1.16. The nucleotide sequence for this insert was found in three separately sequenced clones and has been submitted to GenBank (Accession no. AF265353).

Expression and purification of recombinant Dpfp1

Fig. 3A follows the expression of fusion protein using SDS–PAGE. The fusion protein is visible as a strong band with an apparent molecular mass of 110 kDa; the intensity of staining increases throughout the IPTG induction. Fig. 3B follows the subsequent purification of Dp1.16 through the final HPLC purification of recombinant protein.

Fig. 4A presents a typical elution profile obtained after binding the supernatant from the crude, sonicated cellular extract to an amylose column and washing overnight in column buffer. The majority of the fusion protein eluted over a single column volume, although significant tailing was observed. Fig. 4B shows a representative reversed-phase HPLC elution profile after cleavage with factor Xa. Recombinant Dpfp1 eluted as a single peak at an acetonitrile concentration of 36 % with the maltose-binding protein eluting in the wash (not shown).

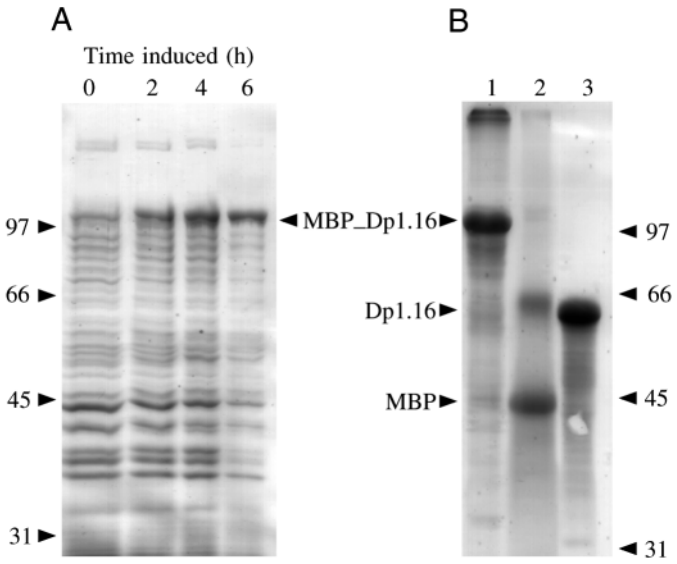


Fig. 3. SDS–PAGE of the expression and purification of MBP_Dp1.16 and Dp1.16. (A) Total protein extracts from various time points after to come in full (IPTG) induced fusion protein expression. Each lane contains the same number of cells. (B) Samples from various stages of the purification. Lane 1 contains fusion protein after elution from an amylose affinity column. Lane 2 contains fusion protein after cleavage with factor Xa. Lane 3 contains HPLC-purified Dp1.16. Numbers with arrowheads indicate the positions of molecular mass standards whose apparent molecular masses are given in kDa. A and B are 12.5 % SDS–PAGE gels stained with Coomassie Brilliant Blue R-250.

Characterization

Fig. 5 presents the results of mass spectrometric fingerprinting of MBP_Dp1.16. All major peaks in the spectrum could be mapped to MBP_Dp1.16. Table 2 compares the predicted masses of the observed peptides with their experimental values. Only a few relatively minor peaks could not be assigned to the fusion protein; these may represent peptides resulting from partial cleavage by endoprotease Lys-C or traces of contaminants in the digestion. Approximately 60 % of the primary sequence of Dp1.16 could be accounted for by the detected peptides. The N-terminal sequencing of the first 10 residues of Dp1.16 confirmed that they were identical to residues 1–10 in Fig. 2.

Extraction and purification of Dpfp1

Purification of Dpfp1 proceeded as described in Rzepecki and Waite (1993b) and resulted in a purified product with the expected apparent molecular mass of 76 kDa.

Antiserum characterization

Antisera obtained from rabbits immunized with recombinant Dpfp1 reacted to a single band in zebra mussel foot tissue extracts in western blots (Fig. 6). No significant differences were detected in antibodies raised in either rabbit (not shown). These antibodies recognized a major protein band in crude extracts of zebra mussel foot tissue that migrated with identical mobility to

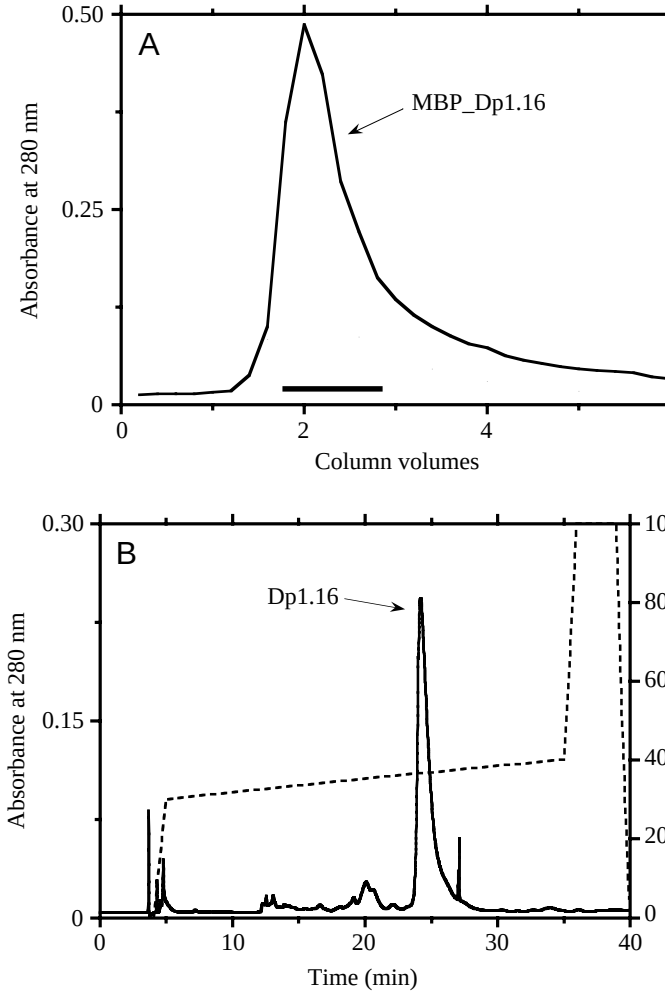


Fig. 4. Elution profiles for MBP_Dp1.16 and Dp1.16. (A) Amylose resin elution profile for MBP_Dp1.16. Protein elution was monitored by measuring the absorbance at 280 nm of column fractions. Fractions collected in the region denoted by the bar were pooled and subjected to further purification. (B) HPLC elution profile of Dp1.16 derived from the supernatant of the cleavage reaction. The solid line represents the absorbance at 280 nm, and the dashed line represents the acetonitrile gradient employed.

HPLC-purified, foot-derived Dpfp1 (Fig. 6A). No bands were detected by western analysis of foot tissue extracts when the pre-immune serum was employed or when non-foot tissue extracts were analyzed with either pre- or post-immune serum. Zebra mussel thread extracts contained two bands recognized by anti-Dp1.16 (Fig. 6B). The lower-mobility band migrated in approximately the same position as the band detected in crude extracts of foot tissue, while a second, broader band migrated with increased mobility. This higher-mobility band was not visible in crude extracts of foot tissue exposed to anti-Dp1.16. No signal was detected by western analysis of mussel byssal thread extracts when the pre-immune serum was employed.

Fluorescence-based detection

Portions of zebra mussel foot tissue were autofluorescent when excited at 488 and 568 nm. This autofluorescence was most intense in the region of the byssal gland and in the secretory products often found in the ventral groove. It was possible to detect positive labeling with anti-Dp1.16 at levels in excess of autofluorescence. To accomplish this, the excitation intensity at 568 nm was adjusted to minimize autofluorescence in the pre-immune samples. Identical conditions were then used to visualize anti-Dp1.16-exposed samples. Thus, only signal intensities significantly in excess of autofluorescence were recorded. In all cases, pre-immune controls showed no labeling.

Confocal images of longitudinal and transverse sections of zebra mussel foot tissue exposed to anti-Dp1.16 are presented in Fig. 8 (refer to Fig. 7 for the location and orientation of sections within the foot). Fig. 8A shows a longitudinal section of the foot near the ventral surface. Sections incubated with anti-Dp1.16 show that a secretion product lying in the ventral groove labels strongly with anti-Dp1.16 (red signal). Fig. 8B shows a transverse section of a zebra mussel foot including the ventral groove. The magnified area contains the base of the groove, and secretion product is visible in the cleft formed by the dorsal portion of the groove. Once again, clear labeling of the secretion product is evident with anti-Dp1.16, with no visible labeling in pre-immune controls. In these sections, the secretion product appears to have a granular consistency, and several spherical granules can be seen in the cleft of the groove that labels with anti-Dp1.16. These products are primarily concentrated in the region between the bulk of the secretion product and the cells forming the dorsal surface of the groove.

Fig. 8C shows a longitudinal section of the zebra mussel foot taken dorsal to the region containing the ventral groove. In this image, the region of the foot containing the byssal gland is magnified. Sections incubated with anti-Dp1.16 show faint labeling of granular material within the cytoplasm (arrow). The labeling is not uniform, and some cells appear to label more intensely than others.

Discussion

In this study we have expressed Dp1.16, a recombinant version of a byssal protein originally isolated from the zebra mussel (Rzepecki and Waite, 1993b), in sufficient quantities to serve as an antigen for the production of a polyclonal antibody. An antiserum obtained from one of two rabbits immunized with recombinant Dpfp1 was capable of recognizing the protein in western blots of extracts from foot tissue and byssal threads and failed to detect Dpfp1 in any other tissue. Immunohistochemical results employing this antiserum suggest that glandular tissue in the foot of the mussel is responsible for the synthesis and storage of Dpfp1. Finally, an extracellular secretion product was strongly and uniformly labeled with the antibody. The distribution of Dpfp1-positive cells within the foot and the uniform labeling of an apparent extracellular secretion product suggest that Dpfp1 acts as a load-bearing component of the byssal thread. Attempts to immunolocalize Dpfp1 in mature threads have, thus far, been unsuccessful, despite unequivocal detection of the protein in

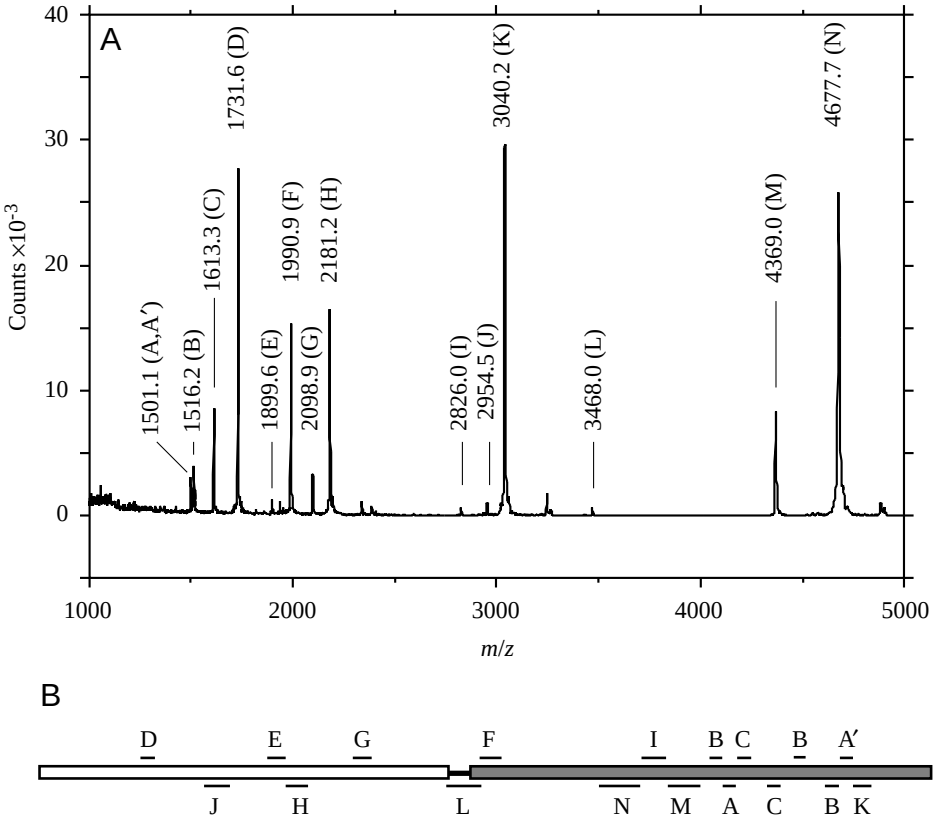


Fig. 5. Mass spectrometric fingerprinting of MBP_Dp1.16. (A) Peaks represent peptides generated from the cleavage of fusion protein with endoprotease Lys-C. $[M+H]^+$ values are given above each peak. Letters in parentheses can be used to map peptides to B and to Table 2. (B) Schematic representation of MBP_Dp1.16. The open rectangle represents the maltose-binding protein (MBP) sequence, the shaded rectangle represents the Dp1.16 sequence and the polylinker region is represented by a black line connecting these two regions. Lines above and below the diagram represent the location of peptides identified in A.

Table 2. *Expected and observed masses of MBP_Dp1.16 peptides*

Peptide	Expected $[M+H]^+$	Observed $[M+H]^+$	Sequence	Location
A	1500.5	1501.1	NPGPYDYDGPYNK	224–236
A′			NPGPYDYNGPYDK	328–340
B	1515.6	1516.2	KPGPYDYDGPYDK	211–223
				289–301
				315–327
C	1612.7	1613.3	KPNPYGTDWQYDK	237–249
				263–275
D	1730.9	1731.6	LYPFTWDAVRYNGK	MBP
E	1899.0	1899.6	HMNADTDYSIAEAAFNK	MBP
F	1990.2	1990.9	TSQSTIPTISGWSFFTTK	9–26
G	2098.3	2098.9	EFLENYLLTDEGLEAVNK	MBP
H	2180.4	2181.2	GETAMTINGPWAWSNIDTSK	MBP
I	2828.2	2826.0	QDHDPVYPPRSPLYGWRRPVYPK	151–173
J	2952.4	2954.5	SALMFNLQEPYFTWPLIAADGGYAFK	MBP
K	3039.2	3040.2	KPGPYDYDGPYDIKPGPYDYDVPYDK	341–366
L	3467.4	3468.0	DAQTNSSSNNNNNNNNNNLGIEGRGSSDWTEK	MBP–8
M	4367.9	4369.0	TPVYPYLPLYPGYQPEYHRRPPVYPPVYPYDPVEDK	175–210
N	4676.1	4677.7	YPVYPSQSPAYPTQYPEYPSQYPVYPDQYPVYPNQYPVK	112–150

Capital letters in the first column represent peptides from Fig. 5.
Masses are reported as mean isotopic masses, and the primary sequence of each peptide is given using the single-letter amino acid code.
The locations of Dp1.16 containing peptides are mapped to Fig. 2.
MBP indicates that all or a portion of the peptide can be mapped to the maltose-binding protein.

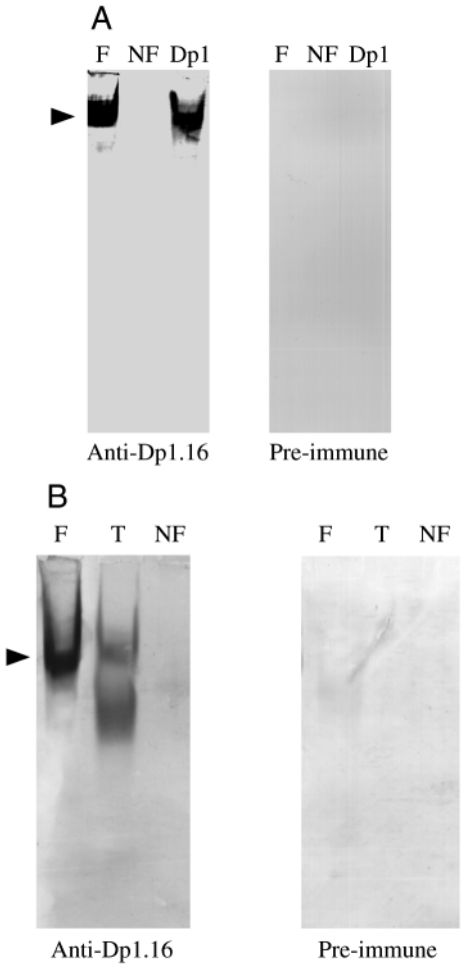


Fig. 6. Western blots of zebra mussel protein extracts exposed to anti-Dp1.16. Samples in the left-hand column were exposed to anti-Dp1.16, and those in the right-hand column are pre-immune controls. (A) F, 1 μg of zebra mussel foot extract; NF, 1 μg of non-foot tissue extract; Dp1, 2 μg of HPLC-purified Dpfp1. (B) T, 9 μg of byssal thread extract (8 mol l^{-1} urea/5 % acetic acid); F and NF, as in A. The arrowhead indicates the position of Dpfp1 in both panels.

nascent threads and in western blots of thread extracts. This suggests that the protein's epitopes become masked during the process of thread maturation.

Expression and purification of recombinant Dpfp1

Mass spectrometric fingerprinting and N-terminal sequencing of recombinant Dpfp1 confirm that the protein was successfully expressed. However, we also noticed that several minor, lower-molecular-mass bands were detected, first in gels of affinity-purified fusion protein and subsequently in HPLC-purified recombinant Dpfp1. We argue for several reasons that these minor bands are not *E. coli*-derived protein contaminants. First, these bands reacted positively to a polyclonal antibody specific to the maltose-binding protein in western blots of affinity-purified fusion protein, indicating that they contain a portion of the maltose-binding protein primary sequence. Second, the bands underwent a downward mobility shift after incubation with factor Xa, suggesting the presence of a recognition site for this protease. Third, N-terminal sequencing of HPLC-purified Dp1.16 detected a single N terminus. We are therefore confident that these bands originate from the recombinant fusion protein and may represent truncated expression products.

We noticed a tendency for recombinant Dpfp1 to precipitate after cleavage from its fusion partner. If this behavior reflects the *in situ* behavior of the foot-derived protein during synthesis of the byssal thread, it suggests an adaptation designed to promote efficient thread formation under water. To become efficiently incorporated into the byssus, byssal proteins must resist the tendency to diffuse. Aggregation or fibrillogenesis of byssal proteins may provide an effective mechanism to limit dispersion and encourage intermolecular contacts by forcing the byssal precursors together into an insoluble form. During the later stages of thread formation, such a mechanism would also encourage the formation of cross-links, which ultimately form the permanent cohesive and adhesive elements of the mature thread. The best-known examples of this are the fibrillogenic assembly of fibrin and type I collagen (Gelman et al., 1979; Hantgan and Hermans, 1979).

Foot and thread extracts

In a previous study, Rzepecki and Waite (1993b) demonstrated that a protein with a comparable electrophoretic mobility and similar staining characteristics to those of foot-derived Dpfp1 could be extracted from zebra mussel byssal threads. However, they were unable to extract sufficient quantities of the protein from the byssus to establish its identity unambiguously. We have shown that anti-Dp1.16 antibodies specifically recognize foot-derived Dpfp1 in western blots of foot tissue and thread extracts. However, it should be noted that western blots of zebra mussel thread extracts often contained a second band of higher mobility that migrated as a broad smear. It is unlikely that this band represents an unrelated byssal protein cross-reacting with the antibody, because western blots of tissue extracts exposed to anti-Dp1.16 never contained such a band. Rather, the band probably represents a degradation product, generated during the 24–48 h in which the threads were exposed to the environment prior to collection. The results presented here provide the first conclusive evidence that Dpfp1 is a component of the zebra mussel byssus.

Western blots of zebra mussel tissues exposed to a Dpfp1-specific antibody localize the protein in the foot of the mussel and never in any other tissues, confirming that the foot is the sole site of synthesis for this protein. Results from several other studies support this hypothesis. Rzepecki and Waite (1993b) isolated soluble Dopa-containing byssal precursor proteins from zebra mussel feet, and the presence of Dopa, presumably associated with stored byssal precursor proteins, in foot tissue surrounding the ventral groove and distal depression was demonstrated (Rzepecki and Waite, 1993a). In addition, Dpfp1 mRNA transcripts were detected in foot tissue extracts but not in extracts from gill, mantle or adductor muscle (Anderson and

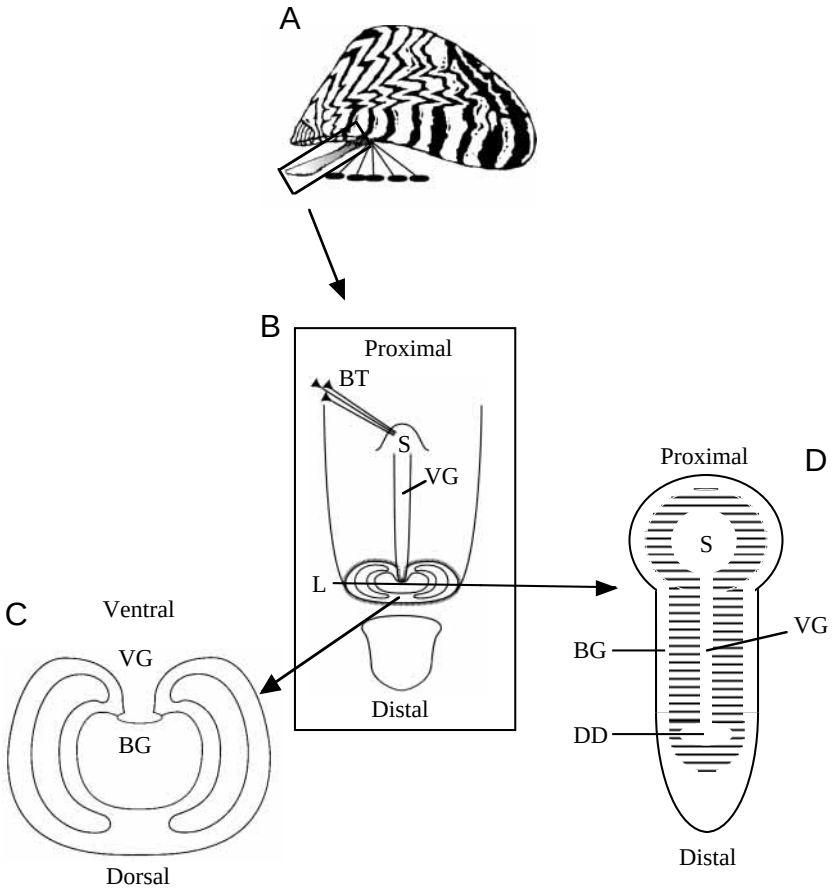


Fig. 7. Schematic representation of tissue section orientations (see Fig. 8). (A) A zebra mussel depositing byssal threads. The boxed appendage is the foot of the mussel. (B) Expanded view of the boxed region in A. The foot has been rotated to expose the ventral surface and transversely sectioned near the tip to expose the internal tissues. (C) Transverse section through the foot. (D) Longitudinal section through the foot along the plane formed by line L and the proximal–distal axis. BG, byssal gland; BT, byssal thread; DD, distal depression; S, stem-forming region; VG, ventral groove. B and C are adapted from Bonner and Rockhill (1994).

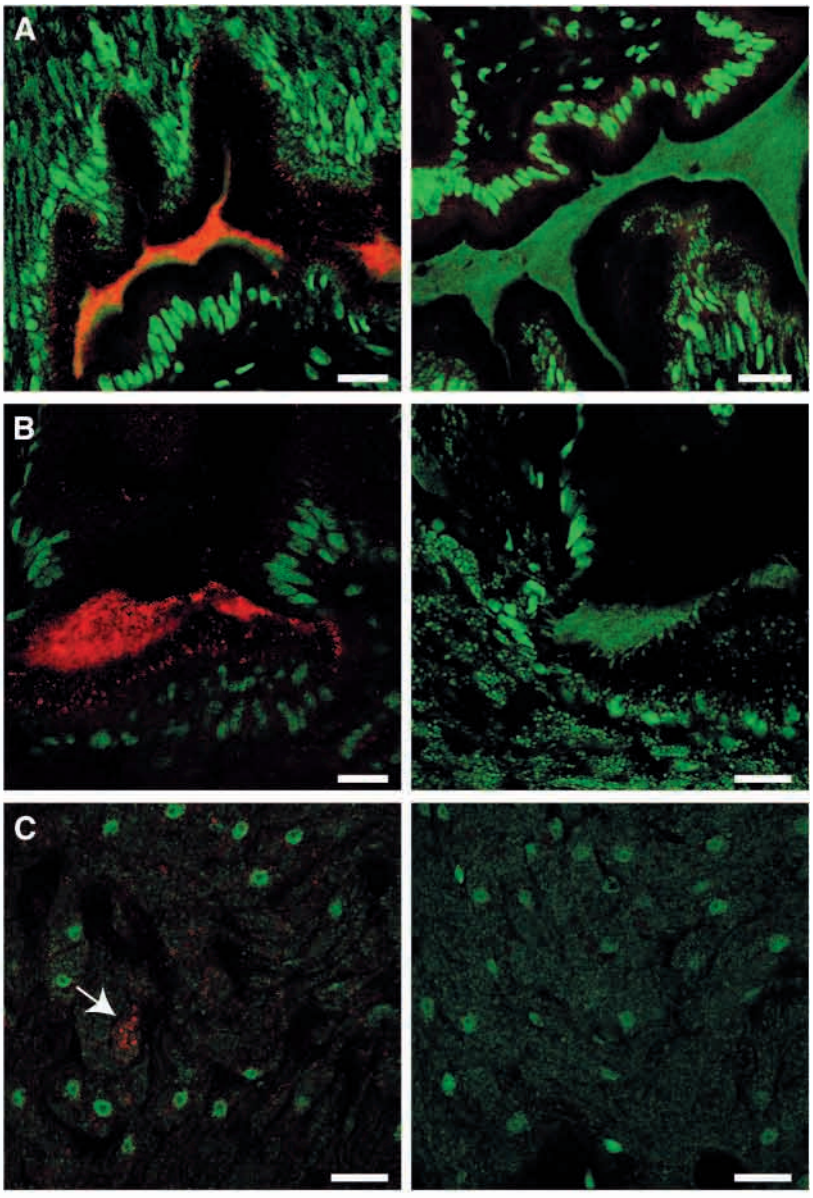
Waite, 1998). Collectively, these experiments strongly suggest that the foot is the only tissue responsible for the synthesis and subsequent storage of Dpfp1. The central role of the foot in byssal thread synthesis has been well established by studies in a variety of other bivalve taxa (Banu et al., 1980; Brown, 1952; Fontin et al., 1974; Gruffydd, 1978; Pujol, 1967; Shyamasundari and Rao, 1986).

The ability of anti-Dp1.16 specifically to recognize foot-derived Dpfp1 suggests that antibodies raised against recombinant antigens can be successfully employed in immunolocalization studies of byssal proteins. Should this be the case for other byssal proteins, such an approach will prove invaluable for the generation of specific antibodies to a wide variety of proteins involved in byssal thread synthesis. Previously, polyclonal antibodies raised against several foot-derived mytilid byssal precursors have been shown to cross-react with byssal proteins from the same species (Rzepecki et al., 1992) and with homologous proteins from other species (Waite et al., 1989). The source of the observed cross-reactivity has yet to be established, although the occurrence of shared epitopes among homologous proteins, possibly involving shared post-translational modifications, may play a role (Waite, 1992). Through careful selection of a cloning strategy and expression systems, researchers can produce antigens that exclude epitopes shared by other byssal proteins, thus increasing the chance of producing specific antibodies.

Immunohistochemical localization of Dpfp1

Dpfp1 was detected in a subset of byssal gland cells located along the ventral groove and covering the distal depression as well as in extracellular granules found in the ventral groove. Cells at the base of the groove often appeared to discharge portions of their apical cytoplasm into the dorsal area of the ventral groove, suggesting that they are exocrine in function. Bonner and Rockhill (1994) observed that zebra mussel byssal gland cells reacted positively in histochemical tests for tyrosine and phenols, consistent with the presence of Dopa-containing byssal precursor proteins such as Dpfp1. This distribution complements the observation that Dpfp1 can be extracted from byssal threads and plaques (Rzepecki and Waite, 1993b). In addition, the cell morphology of these byssal glands is reminiscent of that of the collagen-secreting cells in the foot of *M. edulis* (Pujol, 1967).

These observations are in good agreement with a model for byssal thread synthesis proposed for *M. edulis* (Waite, 1992). In this model, the granular thread precursors are secreted into the ventral groove, where they become part of the nascent byssal thread. During secretion, peristaltic contractions of the ventral groove act to break down the granules and mix the byssal components in a process analogous to the calendering of a thermoplastic resin. The prepackaging of byssal components into granules provides a mechanism that ensures precise control over the stoichiometry and mixing of secreted byssal proteins.



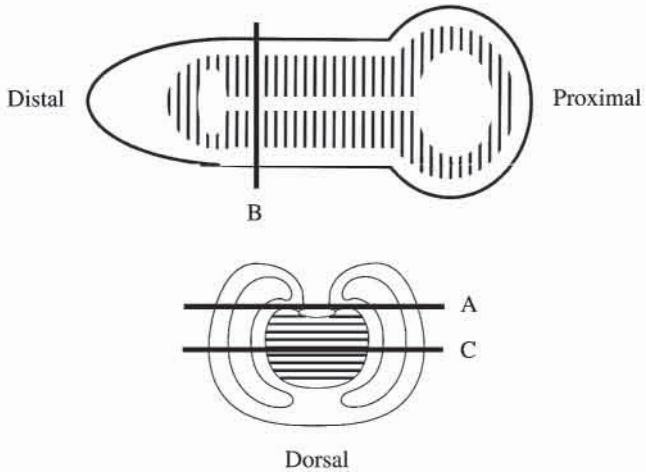


Fig. 8. Sections of zebra mussel foot exposed to anti-Dp1.16 and Syto13 counterstain as viewed under the confocal microscope. Sections in the left-hand column were exposed to anti-Dp1.16, and those in the right-hand column are pre-immune controls. Tissues in red are labeled with anti-Dp1.16, and those in green are counter-stained with Syto13. The orientation of sections is represented schematically below the panels, and all scale bars represent 20 μm. (A) Longitudinal section of the ventral groove with secretion product. (B) Transverse section through a zebra mussel foot exposing the ventral groove and secretion product. (C) Longitudinal section of a zebra mussel foot containing the byssal gland. Granular Dpfp1 precursor is highlighted by an arrow.

The observation that newly formed byssal threads in *Chlamys islandica* also contain granules (Gruffydd, 1978) suggests that this process may be widely adopted in byssate bivalves.

Previously, Bonner and Rockhill (1994) subdivided the zebra mussel byssal gland into distinct regions on the basis of differing reactions to tests for the presence of carbohydrates, quinones, tyrosine and catechol oxidase. In the present study, immunolabeling of byssal gland cells was not uniform; rather, labeling of Dpfp1 occurred in a subset of cells. This supports the hypothesis that zebra mussel foot tissue is composed of distinct cells types, each responsible for the synthesis of a different byssal component. The subdivision of the byssal gland into functionally distinct regions is not unique to *D. polymorpha*, but is well documented in a variety of bivalves. For example, histochemical and ultrastructural studies of the foot of *Mytilus* spp. identified at least four distinct glands responsible for different aspects of byssal thread synthesis (Brown, 1952; Ravindranath and Ramalingam, 1972; Smyth, 1954; Tamarin, 1975; Tamarin and Keller, 1972; Tamarin et al., 1976; Vitellaro-Zuccarello, 1980, 1981). Non-mytilid bivalves also appear to possess multiple byssal glands. Gruffydd (1978) demonstrated that the foot of the scallop *C. islandica* contains at least three glands, while Cranfield (1973) has shown that nine byssal glands occur in the pediveliger stage of the European oyster *Ostrea edulis*.

Byssal proteins contribute to the proper functioning of the mature byssus in three important ways. They may serves (i) as *bona fide* adhesives forming a bond between the plaque and substratum; (ii) as cohesive elements providing structural integrity to the byssus and helping to define its biomechanical properties; and (iii) as varnishes that protect the byssus from chemical and bacterial degradation. The observations that Dpfp1-containing cells occur over the ventral groove and distal depression (this study), combined with the earlier report that this protein can be extracted from byssal threads and plaques (Rzepecki and Waite, 1993b), suggests that Dpfp1 plays a role either as a cohesive component of the byssus or as a varnish. We conclude that Dpfp1 formed the bulk of a secretion product found in the ventral groove and was not confined along its periphery, as might be expected if the protein served as a varnish. If the secretion product accurately represents a stage in the process of byssal thread formation (i.e. is not an artifact of the preservation process), this suggests that Dpfp1 is an important load-bearing or cohesive component of byssal threads. Additional proof of this hypothesis awaits ultrastructural localization of Dpfp1 within the zebra mussel byssus using anti-Dp1.16.

The authors wish gratefully to acknowledge the assistance of several people who contributed to this effort: Alan Jordan for obtaining and shipping mussels, Kirk Czymmek for help with confocal microscopy, Hillary Reidy for the preparation of tissue sections, John McDonald for the use of sequencing facilities and Christine McDaniel for technical assistance in preparing the antibodies used in this study. We also thank Thomas Bonner for graciously providing the schematic diagrams of zebra mussel feet used in Fig. 6B,C. This research was supported by grants NIH 2 ROI DE10042 and ONR N00014-99-1-0774 to J.H.W. K.E.A. was supported in part by USPHS Grant T32-GM08550. cDNA sequences have been submitted to GenBank (Accession no. AF265353).

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