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Comparative Biochemistry and Physiology, Part A 156 (2010) 495–501

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

Identification and molecular characterization of a mucosal lectin (MeML) from the blue mussel *Mytilus edulis* and its potential role in particle capture

Emmanuelle Pales Espinosa *, Mickael Perrigault, Bassem Allam

School of Marine and Atmospheric Sciences, State University of New York, Stony Brook, NY 11794, USA

ARTICLE INFO

Article history:
Received 4 March 2010
Received in revised form 2 April 2010
Accepted 6 April 2010
Available online 11 April 2010

Keywords:
Suspension feeding bivalve
Mollusk
Mucocyte
Lectin
Real-time PCR
Gene expression

ABSTRACT

Molecular recognition of food particles has been suspected to play an important role in particle selection in suspension feeding bivalves. Lectins are a group of sugar-binding proteins that are widely involved in biological recognition. They have been reported in mucus covering bivalves feeding organs and were recently shown to mediate particle sorting in these animals. In this study, we report a novel putative C-type lectin from the blue mussel *Mytilus edulis*. The cDNA of this lectin (hereby designated MeML for *M. edulis* mucocyte lectin) is 459 bp long encoding a 152-residue protein. MeML presents a signal peptide and a single carbohydrate recognition domain (CRD) which contains a QPS (Gln, Pro, and Ser) motif and two putative conserved sites, WND and ENC, for calcium binding. MeML was expressed in mucocytes lining the epithelium of pallial organs (gills, labial palps and mantle) and intestine, and its expression was significantly up-regulated following starvation. MeML transcript was not detected in other tissues including hemocytes. MeML is suspected to play a role in the capture of food particle which further support the involvement of this lectin in particle selection mechanism.

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1. Introduction

In the aquatic environment, communication between organisms is often based on molecular language. In numerous physiological processes (defense, reproduction, and predation) marine organisms interact with each other using thousands of organic metabolites belonging to diverse chemical groups (Faulkner, 2002; Hay, 1996 and references therein). Among molecules known for non-self recognition and cell-to-cell interactions, lectins are a large and diverse group of sugar-binding proteins that specifically and reversibly bind to glycans covering living cells (Sharon and Lis, 2004; Vasta, 2009). Their functions are diverse and they have been described to play a role in the defense mechanism (Kenjo et al., 2001; Tasumi and Vasta, 2007), in reproduction (Springer et al., 2008), in parasitism (Hager and Carruthers, 2008; Stevens et al., 2006), and in symbiosis (Bulgheresi et al., 2006; Nyholm and McFall-Ngai, 2004; Wood-Charlson et al., 2006). They can assist the organism by immobilizing particles through agglutination (Fisher and Dinuzzo, 1991; Pales Espinosa et al., 2009) and encapsulation (Koizumi et al., 1999) or can initiate a cascade of events leading, for example, to host colonization (Nyholm and McFall-Ngai, 2004) or to limit pathogen infection (Holmskov et al., 2003).

Lectins are ubiquitously distributed in nature as they are found in viruses, bacteria, fungi, plants, invertebrates and vertebrates (Sharon and Lis, 2004; Vasta and Ahmed, 2008). In bivalves, lectins have been mostly described in hemolymph (Zhang et al., 2009), associated or not with hemocyte membranes (Tasumi and Vasta, 2007), and linked to the defense mechanism (Fisher and Dinuzzo, 1991; Minamikawa et al., 2004; Tripp, 1992; Zhang et al., 2009). In some rare cases, bivalve lectins have been found to be potentially involved in other functions. For example, in *Codakia orbicularis*, a clam known for its symbiotic relationship with a sulfide-oxidizing chemoautotrophic bacteria (Frenkiel and Moueza, 1995), the lectin "codakine" has been found to be the predominant protein in the gill (Gourdine and Smith-Ravin, 2002) leading Gourdine et al. (2007) to propose its involvement in the mediation of symbiosis. Additionally, the presence of lectins have been suspected (Fisher, 1992) and recently demonstrated (Pales Espinosa et al., 2008) in mucus covering pallial organs (gills, labial palps) in the oyster *Crassostrea virginica* (Pales Espinosa et al., 2009, Pales Espinosa et al., 2010) and the mussel *Mytilus edulis* (Pales Espinosa et al., submitted).

Suspension feeding bivalves are well known to be able to select among particles (Cognie et al., 2003; Newell and Jordan, 1983; Pales Espinosa et al., 2008; Ward and Shumway, 2004). Although some aspects of the selection process have been elucidated, the actual mechanism(s) by which particles of poor quality are rejected into pseudofeces while those of higher quality are ingested remain unclear. Among theories advanced in the literature, some studies support the idea that bivalves use chemical cues to discriminate among particles (Beninger and Decottignies, 2005; Beninger et al., 2004; Kiorboe and Mohlenberg, 1981; Newell and Jordan, 1983; Pales Espinosa et al., 2007; Shumway et al., 1985; Ward and Targett, 1989). More recently, our results in oysters (Pales Espinosa et al., 2009, Pales

* Corresponding author. Tel.: +1 631 632 8694; fax: +1 631 632 8915.
E-mail address: Emmanuelle.Palesespinosa@stonybrook.edu (E. Pales Espinosa).

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doi:10.1016/j.cbpa.2010.04.004

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Espinosa et al., 2010) and mussels (Pales Espinosa et al., submitted) showed that particle selection in bivalves is mediated by interactions between lectins present in mucus covering feeding organs and carbohydrates associated with the surface of suspended food particles. Although our previous studies represent, to the best of our knowledge, the first indications for the involvement of lectins in the feeding mechanism of metazoans, carbohydrate–lectin interactions have already been shown to be involved in the feeding mechanisms of predatory protozoans. For example, previous studies have demonstrated the involvement of mannose-binding lectins as a feeding receptor for recognizing preys in the marine dinoflagellate *Oxyrrhis marina* (Wootton et al., 2007) and in the amoeba *Acanthamoebe castellanii* (Allen and Dawidowicz, 1990).

In this study, we screened public EST (Expressed Sequence Tag) databases and used a diverse set of molecular techniques to identify lectin candidates that are produced in the feeding organs of the blue mussel, *M. edulis*. These investigations allowed the identification of a secretory lectin (hereby designated MeML for *M. edulis* mucocyte lectin) that is specifically produced in mucocytes lining mussel feeding organs (gills, labial palps). The full lectin sequence is presented and the expression of this molecule in response to starvation was investigated. Results highlight the potential involvement of this lectin in particle capture processes.

2. Materials and methods

2.1. Animals

Adult (60 to 70 mm in length) blue mussels, *M. edulis*, were collected from Long Island Sound (Port Jefferson, NY, USA). Their external shell surface was scrubbed to remove mud and marine life. Mussels were then randomly subdivided into 3 different groups. The first 2 groups were immediately used for RNA extraction/cDNA amplification or *in situ* hybridization analysis. The last group was acclimated in the lab before being used in the starvation study (see below).

2.2. RNA extraction

Eight mussels were bled from the posterior adductor muscle using a syringe fitted with an 18-gauge needle. Hemolymph was pooled from eight mussels, centrifuged at 800×*g* for 10 min at 4 °C. Supernatants were discarded and total RNA was immediately extracted from hemocyte pellets using TRI Reagent® (MRC, Cincinnati, OH, USA). Additionally, digestive gland, gills and labial palps were separately dissected for RNA extraction following the same procedure. RNA samples were pooled from all eight individuals and used for cDNA amplification (Section 2.4).

2.3. Homology screening and primers design

Public *M. edulis* EST database available at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) was searched using the sequence of C-type lectin-1 from the Pacific oyster *Crassostrea gigas* lectin (GenBank accession no. BAF75353). This sequence was used because its transcripts were shown to be strongly expressed in the feeding organs of the Pacific oyster (Pales Espinosa et al., unpublished) and since the database did not contain any *Mytilus* sp. sequences specifically identified as C-type lectins other than the vitelline coat lysins. *M. edulis* ESTs homolog to *C. gigas* lectin-1 with *E*-values less than 10^{-5} were considered significant. Specific primers were designed on 13 potential candidates identified in *M. edulis* using this approach (Table 1).

2.4. cDNA amplification

cDNA was generated from extracted RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and was used as template with each set of primers listed in Table 1. The PCR reaction was carried out in an Eppendorf Mastercycler (ep gradient S) using GoTaq® DNA Polymerase (Promega) for 10 min of initial denaturation, followed by 40 cycles of denaturation (95 °C, 30 s), annealing (50 °C, 30 s), and extension (72 °C, 1 min), with an additional 10 min primer extension after the final cycle. PCR products were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide.

2.5. Sequence analysis

The cDNA and deduced amino acid sequences were analyzed by BLAST program (NCBI, http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi) and protein motif features were predicted using the Prosite software (www.expasy.org/prosite/) and http://www.cbs.dtu.dk/services/SignalP/. Multiple alignments were performed using CLUSTAL W2 (http://www.ebi.ac.uk/Tools/clustalw2/).

2.6. RNA labeling

One cDNA product (342 bp) that fulfilled our selection criteria (see Results section) was ligated into pGEM-T Easy Vector (Promega, Madisson, WI) which was used to transform *E-coli* DH5α bacteria (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. Positive clones were selected on lysogeny broth

Table 1
Primers used in this study for the amplification of 13 expressed sequence tags identified in *Mytilus edulis* (MeESTs). The last 2 lines provide sequence information for the primers used in the quantitative real-time PCR. Primers used to amplify MeML are in bold.

GenBank references	EST/primer names	Primer sequence (5′->3′)	
		Forward	Reverse
AM879299	MeEST1	TGTGCATCCACCAGAACTGT	CTCCAATCACCCTCCTTGAA
AM878884	MeEST2	CTTTCCGCAGTTCTGGTTCT	TTCTTCAAGTCTGATGCCCC
AM879361	MeEST3	TGGGCTTAGCGTCAATCATC	TCCCATATCCGCTGTACCAT
AM877827	MeEST4	TTCACCAAGTTGTCCAGCAG	ACCATAGTTGTTGGGTTCGC
AM877735	MeEST5	GCCATGATGAGTCAATCCG	TACCATGTCTGTCCGTGGAA
AM879358	MeEST6	CATCTGCTTACCAATGGGCT	GTCCTCCAATCTGCTTGCAT
AM878668	MeEST7	GATGGGTCTTCATGGTGGAG	CCACAGTTTTCCGATCTTCC
AM878044	MeEST8	GACGCTCTATCTAATGCCGC	TTACTGTTGCACGGATGGTC
AM880418	MeEST9	GCAACCACGACGTCACTAAA	TGTATGGCTCCGCAGTTACA
AM878921	MeEST10	GAAATCTGCTGGGTGTCCAT	CGAGTCATTGGTCCATCTCC
AM878056	**MeEST11**	**GGCATCATGGTTATCGTGAC**	**CACCATGTCGATCATTCCAC**
AM878233	MeEST12	CTTGGGCATAACGAAAGAGC	ATTTCCTGTTCCTCCCGAAG
AM878451	MeEST13	CACCCGTTCTTCTCGTTTGT	GAAAGTCCAACGCCAATCTC
AM878056	**MeEST 11 for qPCR**	**ATGCTCAATTGGCTGGCATCATGG**	**ATCCGGGAATCTTCGATGTCTTGC**
	18S ribosomal RNA	CTGGTTAATTCCGATAACGAACGAGACTCTA	TGCTCAATCTCGTGTGGCTAAACGCCACTTG

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(LB) agar (with 100 µg L^{-1} ampicillin, final concentration). Vector containing the cDNA insert was extracted from the bacteria using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) and insert orientation was determined by sequencing. Purified vector containing the cDNA insert was linearized using the restriction enzyme SpeI and SacII separately (sense and anti-sense) and purified using the DNA purification kit (Promega) according to manufacturer's recommendations. Digoxigenin-UTP (DIG) labeled sense and anti-sense RNA was produced from linearized plasmid using the DIG RNA labeling Kit (SP6/T7) (Roche Applied Science, Indianapolis, IN, USA).

2.7. In situ hybridization (ISH)

Mussel tissues were fixed in 10% formalin for 48 h before being dehydrated in an ascending ethanol series, embedded in paraffin blocks and cut in serial sections (5 µm thickness). Four consecutive sections were processed for standard hematoxylin-eosin staining (1 section) or for *in situ* hybridization (3 sections). The latter sections were deparaffinized in xylene, rehydrated through a descending ethanol series, and equilibrated in diethylpyrocarbonate (DEPC)-treated water. The sections were then digested with Proteinase K in PBS (50 µg mL^{-1}) and permeabilized with Triton X-100 in PBS (0.1%). Sections were then post-fixed in 4% paraformaldehyde in DEPC-treated water, rinsed with PBS containing active DEPC (0.1%) and processed for ISH according to the protocols described by Braissant and Wahli (1998). Two serial sections were separately hybridized with the anti-sense (test) or sense (negative control) probes (600 ng mL^{-1}). No probe was added to the third serial section which was used as another negative control slide. Following incubation (overnight at 60 °C), slides were added with an anti-digoxigenin antibody coupled with alkaline phosphatase (Roche Applied Science, Indianapolis, IN, USA). Positive reactions were revealed using nitroblue tetrazolium (NBT), BCIP (5-bromo-4-chloro-3-indolyl phosphate) and levamisol. Slides were finally counter-stained with fast red solution and sections were dehydrated, mounted with glass coverslips, and observed under a brightfield microscope.

2.8. Effect of starvation on MeML–mRNA expression

Thirty mussels were acclimated in the laboratory for a minimum of 1 week (salinity of 28, 15 °C) where they were fed daily (15% dry weight) using fresh cultures of *Isochrysis sp.*, *Tetraselmis maculata* and DT's Live Marine Phytoplankton (Sycamore, IL) (Pales Espinosa and Allam, 2006). After 1 week, animals were randomly divided in two equal groups and kept under the same conditions of salinity and temperature. One group was fed the same diet as described above and the other group was unfed. After 5 days, mussels were sacrificed; gills and palps were dissected, flash-frozen and conserved at −80 °C until processing for RNA extraction. The expression of MeML transcripts in pallial tissues was measured using real-time PCR. Total RNA was extracted from tissues and single-strand cDNA was synthesized as described above. One set of gene-specific primers was used to amplify a product of 181 bp and 18S ribosomal RNA was used as housekeeping gene (Table 1, qPCR). Real-time PCR assay was carried out in an Eppendorf RealPlex cycler with 6 µL of 1:15 diluted cDNA. The amplifications were performed in a 20 µL reaction volume containing 1× Brilliant II SYBR green qPCR Master mix (Stratagene) and 100 nM of each primers. Thermal profile for real-time PCR assay was an initial denaturation step at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 30 s, annealing and extension at 60 °C for 1 min. Each run was followed by a melting curve program for quality control. PCR efficiency (E) was determined for each primer pair by determining the slope of standard curves obtained from serial dilution analysis of cDNA. The comparative CT method (2-ΔΔCT method) was used to determine the expression level of MeML among tissues (Livak and Schmittgen, 2001). Data obtained from real-time PCR analysis were subjected to t-test (SigmaStat, version 3.1). Differences were considered significant at $p<0.05$.

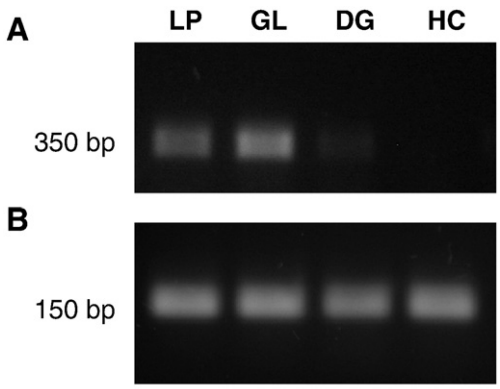

Fig. 1. Gene expression analysis of MeML by RT–PCR ($n=1$ pool of 8 oysters). Identical amounts of total RNA from labial palps (LP), gills (GL), digestive gland (DG) and hemocytes (HC) were reverse transcribed into cDNA. PCR amplifications were performed using MeML (MeEST11 in Table 1) specific primers (A). The expression of the housekeeping gene (18S) in each sample is presented in (B).

3. Results

3.1. Identification of MeML

Among the 13 tested ESTs, 3 candidates were not detected in any of the tested organs and 9 were homogenously expressed in all tissues including hemocytes. Only one candidate (EST 11) was expressed in the labial palps, gills and the digestive gland (weak signal) but not in hemocytes (Fig. 1). Further analysis of this EST revealed that it codes for a complete protein, hereby designated MeML [GenBank accession no. HM049926]. The complete sequence consisted of a 459 bp encoding for a predicted peptide of 152 amino acids (Fig. 2) with a theoretical isoelectric point of 7.63 and an estimated molecular weight of 18 kDa (http://au.expasy.org/tools/pi_tool.html).

3.2. Sequence comparison of MeML with other C-type lectins

The sequence analysis of MeML indicated some levels of homology (19–33% amino acid identity, Table 2) with previously described C-type

```
                atggtgtttgtttataagattactgctcttataattgtattctgc
1               M  V  F  V  Y  K  I  T  A  L  I  I  V  F  C
                ttatttgactatgcagcaacatacacatgctcaattggctggcat
16              L  F  D  Y  A  A  T  Y  T  C  S  I  G  W  H
                catggttatcgtgacaattgttactattttagtcgattcagtgcg
31              H  G  Y  R  D  N  C  Y  Y  F  S  R  F  S  A
                acgttttggagtgcaatgagtttctgcaaaacagttggtggaaaa
46              T  F  W  S  A  M  S  F  C  K  T  V  G  G  K
                ctgattgaaattgataactactgggaatttcaagtcctatccaga
61              L  I  E  I  D  N  Y  W  E  F  Q  V  L  S  R
                atggcaagacatcgaagattcccggatttttggattggcataaca
76              M  A  R  H  R  R  F  P  D  F  W  I  G  I  T
                gacatgtactccgaaggcgcctggcagaaagctacaacacaagaa
91              D  M  Y  S  E  G  A  W  Q  K  A  T  T  Q  E
                caacaaacgtatttcaactggtatgagtcacaacctagtaatagt
106             Q  Q  T  Y  F  N  W  Y  E  S  Q  P  S  N  S
                ggagggcatgaaaattgtgtggaggtgtatactaaacttggtatg
121             G  G  H  E  N  C  V  E  V  Y  T  K  L  G  M
                aagtggaatgatcgacatggtgatcaccgattgcgatttgtttgc
136             K  W  N  D  R  H  G  D  H  R  L  R  F  V  C
                gaaaagtga
151             E  K  *
```

Fig. 2. Nucleotide and deduced amino acid sequences of MeML. The putative signal peptide (grey), the motif of the CRD for ligand binding (white), the cysteine residues (dotted), and the putative calcium binding sites (striped) are boxed. * indicates the stop codon.

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Table 2
Lectins presenting similarities with MeML based on BLAST comparisons.

Protein ID	Species	Fragment size	*E*-values (*E*), Identity (*I*) with amino acid (%)	Ligands	Sugar-binding site	References
MeML HM049926	*Mytilus edulis* (mussel) Pallial organs, intestine	152 aa	NA	Undefined	QPS	This study
P82596	*Haliotis laevigata* (abalone) Shell	155 aa	*E* = 2e-17 *I* = 44/130 (33%)	Galactose mannose/glucose	QPD	Mann et al. (2000)
AAX19697	*Codakia orbicularis* (clam) Gill extract	148 aa	*E* = 8e-16 *I* = 43/135 (31%)	Mannose	EPN	Gourdine et al. (2007)
BAB47156	*Anguilla japonica* (eel) Skin mucus	166 aa	*E* = 4e-15 *I* = 42/152 (27%)	Lactose	EPN	Tasumi et al.(2002)
ABB71672	*Chlamys farreri* (scallop) All body	130 aa	*E* = 1e-15 *I* = 41/156 (26%)	Mannose	EPD	Zheng et al. (2008)
ACO36046	*Pinctada fucata* (oyster) Undefined	168 aa	*E* = 3e-15 *I* = 39/153 (25%)	Undefined	QPD	Unpublished (GeneBank)
ACI69741	*Salmo salar* (salmon) Undefined	168 aa	*E* = 1e-11 *I* = 34/128 (26%)	Undefined	EPS	Unpublished (GeneBank)
BAF75353	*Crassostrea gigas* (oyster) Digestive gland	158 aa	*E* = 1e-08 *I* = 33/132 (25%)	Undefined	YPD	Yamaura et al. (2008)
AAX22004	*Laxus oneistus* (nematode) Cuticle	161 aa	*E* = 2e-07 *I* = 33/137 (24%)	Mannose	EPN	Bulgheresi et al. (2006)
EAT36508	*Aedes aegypti* (mosquito) Undefined	154 aa	*E* = 3e-06 *I* = 28/117 (23%)	Undefined	EPS	Unpublished (GeneBank)

lectins and similarity for specific characteristics of these proteins, including their calcium and carbohydrate binding residues (Table 2 and Fig. 3). Within MeML, the CRD (carbohydrate recognition domain) domain consists of 119 residues (Tyr^{33}–Glu^{151}) located in C-terminal of the protein (www.expasy.org/prosite/). Among the signature motifs for C-type lectins, MeML reveals three out of four consensus cysteine residues (Cys^{54}, Cys^{126}, Cys^{150}, lack of Cys^{142}) and two optional cysteine residues (Cys^{25}, Cys^{37}) that are expected to form disulfide bonds (Tasumi et al., 2002). Like most of the other C-type lectins, MeML presents a conserved WND (Trp^{137}, Asn^{138}, and Asp^{139}) residue that is considered to be the principal site for calcium binding (Drickamer, 1988, 1993). A secondary calcium binding site is also suspected with the conserved residue ENC (Glu^{124}, Asn^{125}, and Cys^{126}). Additionally, MeML shows a QPS (Gln^{116}, Pro^{117}, and Ser^{118}) motif, determinant of sugar specificity. This appears to be a unique feature since none of the homolog lectins from the other species presented a similar motif for the sugar-binding site. The first 22 amino acid residues from the N-terminus are mostly uncharged, hydrophobic and represent homology to signal peptides known to be present in secreted proteins (www.expasy.org/prosite/). No N-glycosylation site was detected in the MeML sequence.

3.3. Localization of MeML transcripts in mussel tissue

MeML mRNA was detected by *in situ* hybridization (anti-sense probe) in specific cells within the epithelial layer covering pallial organs: gills, the labial palps, and the mantle (Fig. 4). A weak signal was also observed in the epithelial layer of the intestine. No signal was found in the digestive tubules or in the gonad. *In situ* hybridization signals were also absent in the hemocytes, in agreement with PCR results (Fig. 1). More interestingly, examination of serial sections processed for *in situ* hybridization or stained with hematoxylin-eosin allowed a more precise localization of production sites and indicated that MeML transcripts are present in the mucocytes lining the epithelium of pallial organs and to a lesser extent in mucocytes associated with the epithelial layer of the intestine.

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M.edulis        AATY-----------TCSIGWHHGYRDNCYYFSRFSATFWSAMSFCK-TVGGK-----LI 62
H.laevigata     ---------------GCPLGFHQ-NRRSCYWFSTIKSSFAEAAGYCR-YLESH-----LA 38
C.gigas         VLS---VCLCD-----CGVGWAE-LNGECIYFSHDKHTWSDARTKCHNMNRSY-----LV 57
A.aegypti       FYQL-----------CCTHAQFN------FHIASEATNWFEASEYCHRMSMR------LA 53
A.japonica      VSHGAVLGLCEG---ACPEGWVE-HKNRCYLHVAEKKTWLDAELNCL-HHGGN-----LA 70
L.oneistus      TAAP------------AASGWLRSPQGTVVKLYRRSLSWSKADEFCKDVDGR------LV 57
                                  .  .                      *   *          *

M.edulis        EIDNYWEFQVLSRMARHRR-----FPDFWIGITDMY-SEGAWQKATTQEQQT-----YFN 111
H.laevigata     IISNKDEDSFIRGYATRLG---E-AFNYWLGASDLN-IEGRWLWEGQRRMN------YTN 87
C.gigas         TIDNQPKADYINKFLSTLH-HFR-QVGYWLGGNDYI-VEGQWRWSETGTGLG----DFTQ 110
A.aegypti       VVNSEAKHNAVVNAAMATGLHHSGYFGVWLGATDLA-QTGIFTWRETGKRLE-----FTR 107
A.japonica      SEHSEDEHQFLKDLHKGSD-DP-----FWIGLSAVH-EGRSWLWSDGTSASAEG--DFSM 121
L.oneistus      VDRTADMHLFLIKNVLKEK-----KSRVWVGLRRTAPHSRTWIWSDGTRQNEDD-LDANL 111
                          :                  *:*          :

M.edulis        WYESQPSNSGG-HENCVEVYTKLGMK------WNDRHGD---HRLRFVCEK--------- 152
H.laevigata     WSPGQPDNAGG-IEHCLELRRDLGNY-----LWNDYQCQ---KPSHFICEKERIPYTNSL 138
C.gigas         WGPGYPDGNRT--HDCMLQVFNGETS-----MWIDSNCA---HPHYYICEHQAPTVST-- 158
A.aegypti       WAPGEPSEYG---ENCVMMAYWPSQG--FHWTWNDAYCS---SKYYAICELRYCI----- 154
A.japonica      WNPGEPNDAGG-KEDCVHDNYGGQKH------WNDIKCD---LLFPSICVLRMVE----- 166
L.oneistus      WLRDEPNDAAG-REECALFMYSPWEKAWG---LNDYLCRGGCRNYQFICEKWIR------ 161
```

Fig. 3. Multiple sequence alignment (ClustalW) of MeML with similar C-Type lectins from *Haliotis laevigata* (P82596), *Crassostrea gigas* (BAF75353), *Aedes aegypti* (EAT36508), *Anguilla japonica* (BAB47156), and *Laxus oneistus* (AAX22004). Amino acid residues that are 100% conserved are noted with a *, and similar amino acids are presented with one (.) or two (:) dots. The motif of the CRD for ligand binding (white) and the putative calcium binding sites (striped) are boxed.

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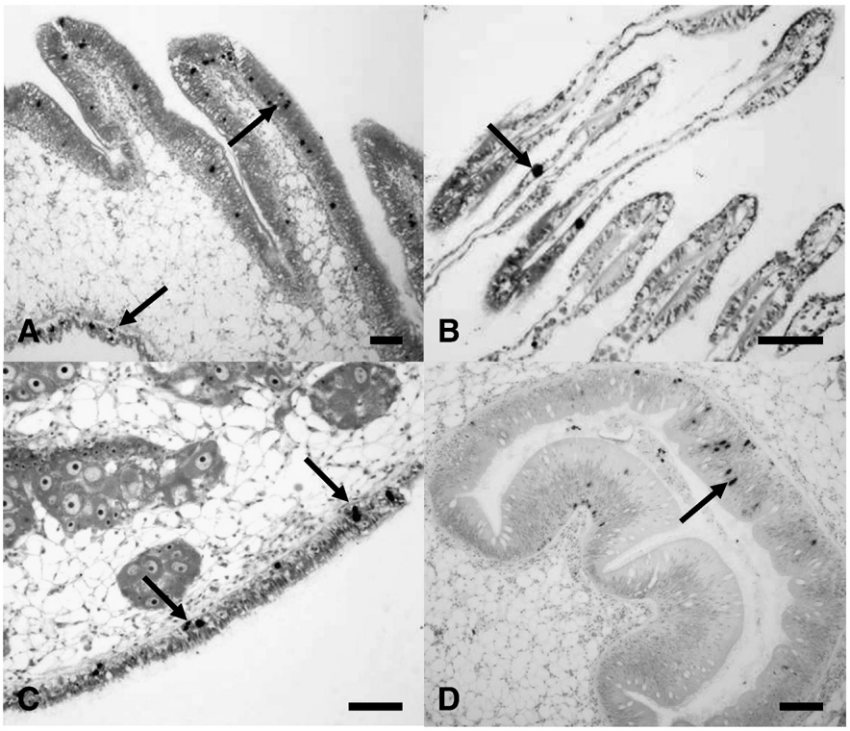

Fig. 4. *In situ* hybridization localization of MeML in labial palps (A), gills (B), mantle (C), and intestine (D) of *Mytilus edulis*. The arrows indicate positive cells for MeML transcripts. Scale bar = 50 µm.

3.4. MeML–mRNA expression after starvation treatment

Variation of MeML expression in gills and labials palps of mussels was investigated in response to starvation. Five days of starvation induced increase in MeML expression in gills (1207-fold, *t*-test, $p = 0.045$) and labial palps (768-fold, *t*-test, $p = 0.035$) of *M. edulis* compared to fed controls (Fig. 5). No significant variation in the expression of the housekeeping gene was observed between the two treatments. Although a higher level of MeML expression was detected in gills compared to palps in either fed or unfed mussels, no statistical difference was noticed between the two organs (paired *t*-test, $p = 0.25$).

4. Discussion

Most previously described lectins in marine invertebrates were identified in hemolymph or hemocytes and were suspected or found to be involved in the defense system against pathogens (Tasumi and Vasta, 2007; Vasta et al., 1984; Zheng et al., 2008). Given their diverse molecular structures and their specific interactions with carbohydrate moieties, lectins were also found in organs and tissues other than blood, and have been shown to play roles as diverse as the establishment of symbiosis (Bulgheresi et al., 2006) or the feeding process (Pales Espinosa et al., 2009). These reports of lectin involvement in processes other than immunity are however infrequent but are very important to broaden the actual knowledge on the biological functions of lectins. In the present study, we identified a secretory lectin (MeML) specifically produced in mucocytes associated with the epithelial layer covering the feeding organs of the blue mussel *M. edulis*. We further demonstrated over-expression of this lectin in response to starvation providing additional evidence for the involvement of MeML in the capture of food particles in mussels.

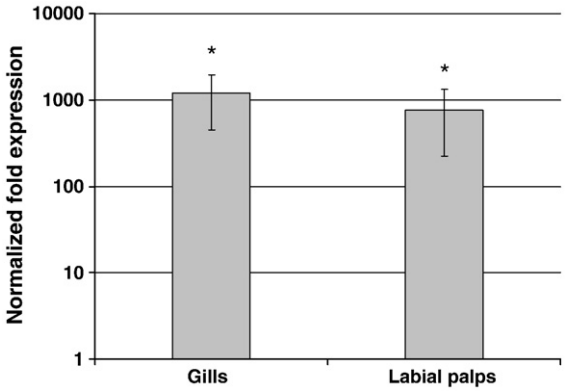

Fig. 5. Expression of MeML in gills and labial palps after starvation determined by quantitative real-time PCR. Expression levels were normalized to 18S RNA and presented as relative expression to controls (mean ± SD, $n = 10$ oysters/treatment). For both organs, * indicates significantly higher expression in unfed oysters compared to fed controls (*t*-test, $p < 0.05$).

Our prior investigations in oysters (*C. virginica*) and mussels (*M. edulis*) demonstrated that mucus covering pallial organs (gills, palps) contains lectins that are involved in the mechanism of food particles selection (Pales Espinosa et al., 2009; Pales Espinosa et al., 2010, and submitted). The aim of the current study was the identification of lectins produced in the pallial organs of *M. edulis*. Therefore, given the expected specific function of our target lectin(s), we deliberately selected lectins specifically expressed in organs involved in the feeding process. Among the 13 tested candidates, only one EST matched our selection criteria. The corresponding lectin MeML (*M. edulis* Mucocyte Lectin) was detected in mucocytes lining the pallial organs, including gills, labial palps and mantle. The secretory nature of MeML was strongly suggested by the presence of a peptide signal at the N-terminus part of the protein (Blobel and Dobberstein, 1975; Hegde and Bernstein, 2006) and is consistent with its location in mucocytes, which secrete mucus covering pallial organs. MeML was also identified in mucocytes associated with the epithelial layer of the intestine but not in internal organs and tissues such as gonads or hemocytes supporting an involvement of MeML in specific function(s) related to pallial and digestive epithelia.

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Lectins have been described in mucus covering numerous fish species (Mistry et al., 2001; Nakamura et al., 2001; Tasumi et al., 2002; Tsutsui et al., 2007) and are produced by epithelial cells (Tsutsui et al., 2009) or, in some cases, in club cells (i.e. specialized secretory cells present in epidermis and mucosal epithelium lining the upper alimentary tract) before being secreted into mucus (Nakamura et al., 2001; Tasumi et al., 2002; Tsutsui et al., 2007). Fish mucus lectins have been suggested or shown to play a role in host defense against pathogens (Okamoto et al., 2005; Tasumi et al., 2002). Similarly, mucosal lectins have also been described in several invertebrate taxa, such as cnidarians (Kvennefors et al., 2008), nematodes (Bulgheresi et al., 2006), or mollusks (Fountain and Campbell, 1984; Furuta et al., 1995; Pales Espinosa et al., 2009) but few studies have clearly identified their origin and function. For example, a C-type lectin identified in the nematode *Laxus oneistus* was shown to be produced by the posterior glandular sensory organs underlying the animal cuticle and secreted onto the cuticle surface along with mucus. This mucosal lectin has been suggested to mediate symbiont acquisition (Bulgheresi et al., 2006).

Further analysis revealed that MeML presents several similarities with C-type lectins identified in the gills of the clam *C. orbicularis*, in the cuticle of the marine nematode *L. oneistus*, and in the skin mucus of anguilliformes such as the Japanese eel *Anguilla japonica* (Table 2 and Fig. 3). They all share similar architectures and have comparable size ranges (130 to 180 amino acids). More specifically, the CRD sequence of MeML includes two putative sites, WND and ENC, for calcium binding (Gourdine et al., 2007; Mullin et al., 1997) as well as a triplet (X-Pro-Y), known to be involved in carbohydrate binding (Drickamer, 1992; Kolatkar and Weis, 1996). For example, the QPD (Gln, Pro, and Asp) motif has a high affinity for galactose and similar residues especially when a W (Trp) is located in the vicinity of the motif (Drickamer, 1992; Kolatkar and Weis, 1996). In MeML, this triplet is QPS (Gln, Pro, and Ser). This motif is not present in the homolog lectins listed in Table 2 and Fig. 3 but has been described in the hypothetical protein of the purple sea urchin, *Strongylocentrotus purpuratus* [GenBank accession no. XP_784754], and in the EST sequence found in the mussel *Mytilus galloprovincialis* [FL499139] which also presents C-type lectin characteristics. To the best of our knowledge, the carbohydrate specificity of the QPS motif has not been described yet, but motifs close to QPD are known, however, to bind a variety of sugars with weak affinity (Childs et al., 1992). These observations suggest that MeML might have a wide recognition range allowing mucus to interact and bind carbohydrates covering the cell surface of a diverse group of microorganisms.

Potential relationship between MeML and feeding functions was investigated. A distinctive feature of suspension feeding bivalves (such as mussels) is their ability to sort food particles among a large number of diverse types of suspended particles, using their gills and/or their labial palps. Through this mechanism, suspension feeders enhance the nutritive value of consumed particles by ingesting preferentially particles of interest while undesirable ones are rejected in pseudofeces before ingestion. This particle sorting process in bivalves is controlled by multiple physical, chemical, and biological factors and previous studies have demonstrated that changes in size, density, electrostatic charges or concentration of particles can affect particle selection (Barille et al., 1997; Cognie et al., 2001; Iglesias et al., 1996; Ward and Shumway, 2004). Recently, we demonstrated that mucus covering pallial organs (e.g. gills and labial palps) contains lectins that agglutinate several microalgae species and mediate food particle selection in suspension feeding bivalves (Pales Espinosa et al., 2009, 2010) including *M. edulis* (Pales Espinosa et al., submitted for publication). *M. edulis* is characterized by filibranch homorhabdic ctenidia (gill composed of single filament type). Bivalves with homorhabdic ctenidia are usually unable of qualitative selection on the gills and the labial palps represent, very often, the selection site for food particles (Beninger and StJean, 1997; Ward et al., 1998). The site of selection in *M. edulis* is restricted to the labial palps and the ctenidia play little role in particle selection and simply transport particulate matter to the palps for further processing (Ward et al., 1998). Results obtained in this study support that labial palps play a major role in the selection process in *M. edulis*, in agreement with previous *in vivo* studies (Ward et al., 1997, 1998). For instance, the results of the *in situ* hybridization showed higher numbers of MeML-positive mucocytes in the labial palps compared to the gills. PCR and real-time PCR, however, did not reveal any difference in MeML expression between gills and labial palps. Discrepancy between *in situ* hybridization and PCR results could be a consequence of the specific localization of MeML. For instance, transcripts were identified in mucocytes associated with epithelia (e.g. surface of gills and labial palps) but total RNA used for PCR was isolated from whole organs and surface/mass ratio (e.g. proportion of epithelia) is higher in gills compared to palps. Therefore, the use of standard housekeeping genes such as 18S for PCR standardization is problematic to compare lectin expression among organs because a large fraction of transcripts of the housekeeping gene may derive from sub-epithelial cells.

Despite this limitation, results of real-time PCR indicated a clear induction of the lectin in pallial tissues after 5 days of starvation. Increased levels of MeML in gills and labial palps of starved mussels may reflect an attempted increase in the efficiency of particle capture, further supporting the involvement of this lectin in particle selection mechanism. Our recent studies on a similar mucosal lectin identified in *C. virginica* (CvML) demonstrated that bacterial challenge does not induce any change in lectin expression in gills, labial palps, mantle and digestive gland whereas starvation clearly did (Jing et al., submitted for publication). These findings strongly support the role of these 2 bivalve mucosal lectins in particle capture/selection, but, at the same time, do not necessarily negate their passive involvement in immunity by immobilizing waterborne pathogens. As a matter of fact, mucosal lectins are able to bind nutritive and pathogenic microorganisms and could be involved in multiple mechanisms. For example, in the coral *Acropora millepora*, the millectin (a mannose-binding lectin) is suspected to be implicated in both symbiosis and defense mechanism since this molecule is able to bind coral symbiont and pathogens (Kvennefors et al., 2008).

In conclusion, a new putative C-type lectin (MeML) was identified in the blue mussel, *M. edulis*. MeML was specifically produced in the mucocytes lining the epithelium of the gills and the labial palps, which are both used by mussels to process food particles. The expression of this lectin was strongly regulated by starvation. These findings support the involvement of MeML in particle capture and/or selection in mussels. To our knowledge, MeML is the first lectin shown to be specifically produced by mucocytes in bivalves. Further studies are needed to determine the specific role of this lectin in particle processing and its possible participation in other physiological functions.

Acknowledgments

This work was funded in part by a grant from the National Science Foundation to EPE and BA (IOS-0718453).

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