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Novel mannose binding natterin-like protein in the skin mucus of Atlantic cod (*Gadus morhua*)

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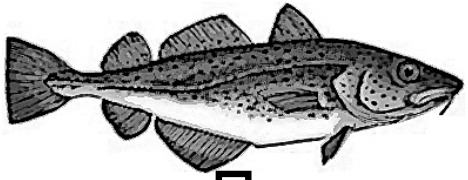


Atlantic cod skin mucus

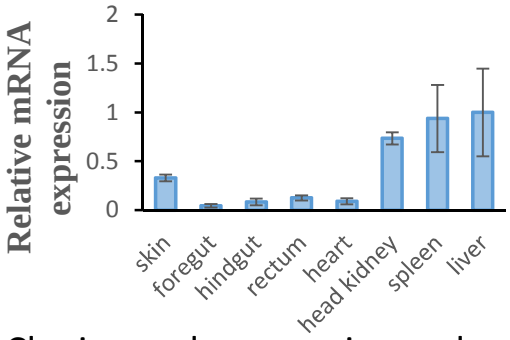
Mannose affinity chromatography

Size-exclusion chromatography

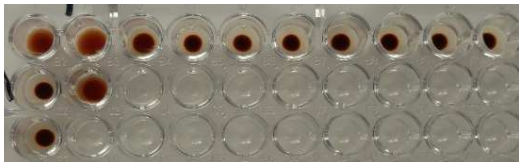
Isolated protein

SDS-PAGE and LC-MSMS

Natterin-like protein identified



Cloning and sequencing and primer design.
Tissue distribution of *nlp* transcripts analyzed



Nlp gives Ca^{++} depended and mannose inhibitable hemagglutination

Title: Novel mannose binding natterin-like protein in the skin mucus of Atlantic cod (*Gadus morhua*).

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Abstract

This study presents the first report of purification of natterin-like protein (Nlp) in a non-venomous fish. The peptide identities of purified cod Nlp were confirmed through LC-MSMS and matched to a cod expressed sequence tag (EST). A partial cod *nlp* nucleotide sequence was amplified and sequenced based on this EST. Multiple sequence alignment of cod Nlp showed considerable homology with other teleost Nlps and the presence of an N-terminal jacalin-like lectin domain coupled with a C-terminal toxin domain. *nlp* expression was higher in skin, head kidney, liver and spleen than in other tissues studied. Hemaggluttination of horse red blood cells by Nlp was calcium dependent and inhibited by mannose. A *Vibrio anguillarum* bath challenge however, did not alter the expression of cod *nlp* transcripts in the skin and gills. Further functional characterization is required to establish the significance of this unique protein in Atlantic cod and other teleosts.

Keywords

Lectin; isolation; natterin-like; innate immunology; hemagglutination; tissue distribution; qPCR; Atlantic cod; mucosal immunology; gill; skin; *Vibrio anguillarum.*

Highlights

- A mannose binding lectin was isolated from Atlantic cod skin
- The lectin was identified as natterin-like protein with a jacaline-like lectin domain
- Hemagglutination by Nlp was inhibited by mannose.
- The expression of cod *nlp* did not change during a *Vibrio anguillarum* bath challenge

1. Introduction

Lectins are proteins which possess carbohydrate-binding characteristics, and are non-enzymatic in nature [1]. Although initially purified and characterized from plants, a wide array of lectins from microorganisms to vertebrates, including those from teleosts and other aquatic animals, have been reported [2,3]. Lectin families are defined based on their carbohydrate ligands and divalent cation requirement for binding. Several types of lectins, including different forms of mannose-binding lectins (MBL), have been identified in fish skin mucus [4]. MBLs belong to the collectin family of C-type lectins that binds to mannose moieties present in microbial pathogens and opsonize them, thereby activating phagocytosis and/or the complement pathway [5]. Other functions include direct or indirect roles in agglutinating microorganisms, in scavenging cellular debris, in cell-cell communication, as inflammatory mediators and mitogens of immune cells [6].

Lectins have a single carbohydrate recognition domain (CRD) or a repeat of CRDs. Lectins with multiple carbohydrate ligands and/ or with unique molecular architecture include among others, the ubiquitous collectin and selectin groups within the C-type lectin family [6]. There are several such lectin types identified in fish as well as in aquatic invertebrates. For example, intelectins with a fibrinogen related domain (FReD) identified from the skin mucus of *Silurus asotus* (Amur catfish) [7]. A novel lectin with domain features identical to mammalian plasma kallikrein was isolated from skin mucus of flathead (*Platycephalus indicus*) [8]. An unique lectin was identified in periwinkle (*Littorina littorea*), which has an N-terminal F-type lectin domain followed by several EGF (epidermal growth factor) domains [9]. Orthologues of mammalian lectin type siglecs have also been reported in fish genomes. They are sialic acid-binding lectins with an Ig (Immunoglobulin) fold [10].

Carbohydrate affinity chromatography has been the preferred method for purification of lectins that can bind to specific carbohydrate ligands and/or matrix [11]. We have reported the presence of galectin-1 in Atlantic cod skin mucus, purified it using lactose affinity chromatography, and shown that the molecule gave hemagglutination and agglutinate pathogenic bacteria [12]. We have also identified a 13 kDa mannose-binding lectin in Atlantic cod skin mucus based on proteomic studies [13]. In this

study, we report the purification of a novel lectin with a unique primary structure from the skin mucus of Atlantic cod.

2. Materials and methods

2.1 Fish and mucus collection

Adult Atlantic cod weighing 2-3 kg, obtained from Cod Juveniles AS (Bodø, Norway), were used in this study. The fish were anesthetized with MS-222 (70 mg L^{-1}) and killed with a blow to the head. Skin mucus samples were collected from 50 fish using sterile glass microscope slides. The mucus samples were pooled together, transferred to sterile 50 ml tubes and immediately stored at -80°C for later use. Skin tissue samples for RNA extraction for cloning and tissue distribution work were collected and snap frozen in liquid nitrogen from fish reared at the indoor facilities of University of Nordland (now renamed Nord University), Norway. All animal handling procedures were performed according to guidelines set by Animal Research Authority in Norway.

2.2 Mannose affinity chromatography and gel filtration

Prior to analysis, skin mucus samples were thawed on ice, diluted 1:10 with binding buffer (20mM Tris-HCl, pH 7.5, 0.5M NaCl, 10 mM $CaCl_2$, 10 mM MgCl2) and homogenized with a VDI 12 hand-held homogenizer (VWR, Norway). The diluted mucus was centrifuged twice at 3000 *g* to remove cell debris and was batch bound to 10 mL mannose coupled sepharose (GALAB Technologies, Germany) at 4°C for 1 h. Batch bound slurry was washed 3 times with binding buffer and loaded on to the chromatography column (Bio-Rad, CA, USA) and manually eluted using elution buffer (one step elution; binding buffer + 0.5 M mannose). The protein fractions (1 mL each) were monitored (absorbance, 280 nm) and collected using Biologic LP fraction collector (Bio-Rad). Affinity purified, pooled and concentrated fractions were further separated by gel filtration on Sephacryl S-200 HR (GE Healthcare, UK) column. Flow rate in this column was maintained at 0.5 mL min^{-1} and 2 mL fractions were collected. The eluted fractions corresponding to a single peak in the chart were pooled and concentrated using nanosep 3 kDa cutoff columns (Pall corporation, WA, USA). Isolated protein was mixed with Laemmli sample buffer and was loaded on to 12 % SDS-PAGE gels. Gels were stained with colloidal Coomassie Blue stain (0.08%

Coomassie Blue G-250, 1.6% ortho-phosphoric acid and 8% ammonium sulphate in buffered 20% methanol) and images were captured using the ChemiDOC™ XRS imaging system (Bio-Rad). The protein band (corresponding to ≈ 35 kDa) was analysed by LC-MSMS (Liquid chromatography-mass spectrometry) as described elsewhere [13]. The pkl files obtained after LC-MSMS were analysed using MASCOT (http://www.matrixscience.com/) with the following settings: enzyme: trypsin with one missed cleavage, fixed modification: carbamidomethyl of cysteine and variable modification: oxidation of methionine, peptide charge: 2+ and 3+, peptide tolerance: 100 ppm and MS/MS ion tolerance: 0.1 Da. The taxonomic class searched was *Actinopterygii* (ray finned fishes).

2.3 Bioinformatic analysis and cloning of natterin

Based on the peptide matches identified through LC-MSMS, nucleotide sequences corresponding to the peptide sequences flanking the matched EST were used to design primers, perform PCR and amplify the putative partial nucleotide sequence of the identified protein. Briefly, cDNA for the PCR was prepared from the total RNA extracted from the skin, as described elsewhere [14]. The amplicon was then cloned in to the pCR4-TOPO vector using TA TOPO cloning kit (ThermoFisher Scientific, MA, USA) and the DNA was extracted and sequenced. The partial nucleotide sequence was translated and was used as the query for the BLASTp algorithm at NCBI database to retrieve similar protein sequences, which were aligned using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/). The domain features of cod and other teleost proteins were predicted using SMART (http://smart.embl-heidelberg.de) or NCBI CDD (conserved domain database). The primers used for cloning the partial *cod nlp* cDNA were codNatF (CGCACATGACTGAATGGC) and codNatR (GAGTAGGGGAGGTCAATGACT)

2.4 Hemagglutination assay.

The hemagglutination assay was done with defibrinated horse erythrocytes. Erythrocytes (ThermoFisher Scientific, MA, USA) were trypsinated, fixed in glutaraldehyde, and diluted to 4 % as reported earlier [12] with some modification. In short, erythrocytes were washed three times in TBS I buffer (Tris buffered saline; 20 mM Tris-HCl, 150 mM NaCl, pH 7.6), treated with 0.1 % trypsin EDTA in TBS-I for 1 h at room temperature, and extensively washed in TBS-I, and fixed in 1 %

glutaraldehyde in TBS-I for 1 h. Fixed erythrocytes were washed twice in 0.1 M glycine in TBS-I, washed extensively in TBS-I, and diluted to 4 % erythrocyte concentration.

The hemagglutination assay was performed in U bottomed microtiter plates (VWR, Norway). In each test well 20 µl of protein sample (Nlp), 20 µl of 4% erythrocyte suspension, 20 µl of 0.5 % BSA-TBS I and 20 µl TBS II (TBS I + 10 mM CaCl2, pH-7.6) were added. To find the hemagglutination titre, 2-fold serial dilutions of the protein sample (undiluted 401 µg/ml) were used. The final concentrations of Nlp was hence 100 µg/ml in the first well. In control well, the protein sample was replaced with 20 µl TBS II. To check mannose dependency, TBS I was replaced with 20 µl of 0.5 M mannose in TBS-I, and to check calcium dependency 10 mM EDTA in TBS I replaced TBS-II.

For visualization of agglutination in light microscopy erythrocytes was mixed with TBS-I (control), TBS-I and Nlp or TBS-I with EDTA and Nlp, respectively, and inspected within 5 minutes.

2.5 Vibrio anguillarum bath challenge

Juvenile Atlantic cod of average size 90 g procured from a commercial hatchery and held at the Pathogen-challenge facilities of the Institute of Marine Research, Bergen, Norway were used for the experiment. This study, conducted in cooperation with the University of Nordland, was approved by the Norwegian Animal Research Authority (http://www.FDU.no). Twenty-four fish each were introduced in to two 500 L experimental tanks and were fed on laboratory prepared fishmeal-based feed. A suspension of *V. anguillarum* (strain H610) at 1.6x 10^7 cfu mL^{-1} was inoculated into one tank, after lowering the water level. The control tank was mock-challenged with culture media (tryptic soy broth with 1.5% NaCl). The water level was brought back to the normal level in both tanks after 1 h exposure. Gill and skin samples from 6 fish each for initial (0h), 4 h, 24 h and 48 h time points were collected and snap frozen in liquid nitrogen before being stored at -80°C.

2.6 qPCR and statistical analysis

Natterin mRNA transcript levels were evaluated using quantitative realtime PCR. For the challenge experiment total RNA extraction was carried out as described before

[15]. Total RNA (500 ng per reaction) was reverse transcribed using Quantitect RT kit (Qiagen, Venlo, The Netherlands). Quantitative PCR was carried out using a LightCycer 96 (Roche) and FastStart Universal SYBR Green Master mix (Roche). Natterin primers used for q-PCR were codNat_qF2 (GGCTCCGACATGGACTGTAT) and codNat_qR2 (TTTGTTTACCTGGGGTGTATAC). These primer sets have been designed around intron-exon boundaries. Three reference genes namely, ubiquitin (*ubi*) [16], elongation factor 1 alpha (*ef1a*) [17] and cyclophilin A (*cyca*) [18] were used for calculating the normalization factor. No-template and non-reverse transcribed controls were included for each primer set. The thermal profile for qPCR was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. The specificity of PCR amplifications was determined by melting curve analysis and further confirmed by Sanger sequencing.

For *nlp* tissue distribution analysis cDNA prepared from mRNA isolated from skin, gills, foregut (middle of first half of gut), hindgut (middle of second half of gut), rectum (rectal chamber), muscle, liver, spleen and head kidney were used. The samples were the same as used previously [13]. PCR was run with preincubation (95˚C for 10 min), two step amplification (95˚C for 10s, 60˚C for 30s), and melting (95˚C for 10s, 65˚C for 60s, 95˚C for 1s). The total number of cycles was 45. Bestkeeper [15] was used to assess the stability of the housekeeping genes *ubi*, *ef1a* and *cyca* (table 1 and 2). The geometric mean of all the genes was used for relative expression of natterin among tissues. Delta Ct method was used for analysis and one-way analysis of variance (ANOVA) was done followed by post-hoc Tukey HSD (Honestly Significant Difference) test.

3. Results and discussion

The mannose affinity chromatography of Atlantic cod skin mucus (in the presence of divalent cations) and subsequent SDS-PAGE analysis showed 3 protein bands; a ~13 kDa band, a 35 kDa band and a heavy molecular weight band. We were primarily interested in the major 35 kDa protein, since we thought it could be cod intelectin. However, mass spectrometric analysis of the ≈ 35 kDa band (Fig. 1 C and D) obtained after mannose elution (Fig 1. A) and gel filtration (Fig 1 B) indicated that the protein belonged to a group of natterin-like proteins (Nlp) (NCBI dbEST Accession:

GW854606). Primers designed based on the matched cod EST (Fig 2) amplified the target 523 bp nucleotide sequence which was shown to be Nlp using BLAST searches. The partial cod *nlp* sequence was submitted to GenBank (GenBank : KP242020). Like other teleost Nlps, cod Nlp although a partial sequence, showed the presence of N-terminal jacalin-like domain and C-terminal aerolysin/ *Clostridum perfringens* like toxin domain (Fig. 2). Natterins were earlier identified as a novel family of proteins from the venom of *Thalassophyrne nattereri* [19], a member of the venomous stone fish family Batrachoididae. Further bioassays have indicated that natterins have kininogenase activity and can cause nociception and edema in mice, confirming their role as stone fish toxins [19]. Nlp (PL-toxin I and II) exhibiting similar activity were also purified from the skin mucus of Oriental stinging catfish (*Plotosus lineatus*) [20]. A natterin-like gene cloned from lamprey (*Lampetra japonica*) blood was found to have pore-forming aerolysin-like domain in addition to an N-terminal lectin domain [21].

Jacalins originally belong to a group of galactose-binding lectins found within the plant family Moracea, but several mannose-binding lectins that share the jacalin-like domain have also been identified [22]. Jacalins have been reported to specifically interact with T cell subsets [23] as well as being associated with plant stress [22].

Atlantic cod *nlp* is expressed in all tissues analysed (Fig. 3); the expression was high in skin, head kidney, liver and spleen. Isolated natterin-like protein gave hemagglutination (Fig 4) in the presence of calcium (Fig. 4B and 4D). The hemagglutination was inhibited with mannose and partly inhibited with EDTA (Fig 4C and 4D), indicating that natterin binds to mannose on red blood cells in a calcium dependent manner. We have previously used a lactose affinity column to isolate galectin-1 from Atlantic cod [12], natterin-like protein was not found, suggesting that Nlp cannot bind lactose.

We were interested to understand the modulation of the cod *nlp* in the event of a bacterial infection. A *V. anguillarum* bath challenge however, failed to identify a significant differential expression on the mucosal surfaces (skin and gills; Fig 5) of cod during the early phase of infection. The infection in the challenge experiment was ascertained by confirmation of high levels of *il1b* transcripts in the gills 48 h post challenge [14]. The results suggest that the lectin might be constitutively expressed,

rather than upregulated during infection. The mechanism of Nlp secretion in to the mucosal mileu is not known, there is no evidence of a signal peptide in the available fish Nlp sequences but nonclassical secretion of protein with jacalin-like domains are observed in some instances [24], and could explain the presence of Nlp in cod skin mucus.

This is the first report on purification of an Nlp using mannose affinity chromatography, showing the mannose-binding activity of cod Nlp and possibly other fish Nlps as well. The mannose-binding activity, coupled with the pore-forming domain of the natterin-like proteins [25], suggests that cod Nlp is a lectin with potential toxin function. There are examples of proteins with an architecture involving the combination of a lectin domain with a pore forming toxin domain like the toxic perivitellin protein Pc PV2 from aquatic apple snail *Pomacea canaliculata* [26]. The presence of Nlp in cod skin mucus is interesting because Atlantic cod is not associated with any venom apparatus or known toxic effects. Interestingly, the presence of an aerolysin domain is reported in a wide range of eukaryotic organisms in combination with other functional domains. Their occurrence could be due to horizontal gene transfer events from bacteria to eukaryotes and their persistence could indicate functional significance to the host [27], in eg. protection against parasites. Further functional analysis of the purified cod Nlp is important to identify the role of these proteins in cod innate immune defense, especially on the mucosal surfaces.

Acknowledgements

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Figure legends

Fig 1 Purification of cod natterin-like protein. A) Crude, ten-fold diluted skin mucus was batch bound to mannose-sepharose in the presence of Ca^{2+}/ Mg^{2+} and one-step eluted with 0.5M mannose. B) The fraction corresponding to single largest elution peak after mannose elution was passed through a gel filtration column (sephacryl S-200HR) at a flow rate of 0.5 mL min^{-1} C) SDS-PAGE gel of representative fractions corresponding to the single largest peak during mannose elution D) SDS-PAGE gel corresponding to representative fraction of the single largest peak in gel filtration. M stands for marker. Black arrow indicates the band corresponding to cod Nlp.

Fig 2 Alignment of partial cod Nlp amino acid sequences with the sequences of teleost Nlps. *G. morhua* (GenBank: AKO698112), *Salmo salar* (GenBank: NP_001134309), *Oncorhynchus mykiss* (GenBank: CDQ92460), *Esox lucius* (GenBank: XP_010866454), *Danio rerio* (GenBank: XP_ 005166416), *Lethenteron camtschaticum* (GenBank: AFX60113). LC-MSMS generated peptides that matched cod Nlp are indicated in red. The forward and reverse primers on the cod Nlp sequence are in bold red and underlined. Dashed line on the top of the alignment indicates partial span of the jacalin-like lectin domain and solid line indicates the toxin like domain (Epsilon-toxin domain/ aerolysin). Domain predictions are based on NCBI's Conserved Domain Database (CDD). Symbols below the alignment: * single, fully conserved residue. : (colon) groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. . (period) groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix (as explained in the ClustalW / MUSCLE documentation.

Fig 3 Relative levels of cod *nlp* transcripts in tissues of Atlantic cod expressed relative to liver. Quantification was done by q-PCR and values are expressed as mean ± SEM (n=3). Bars with no common letter are significantly different ($p<0.05$) by one-way ANOVA and post-hoc Tukey HSD (Honestly Significant Difference) test.

Fig 4. Hemagglutination assay with natterin-like protein. (A**)**. Red blood cells in TBS-I with $CaCl_2$ (control), (B). Red blood cells in TBS-I with $CaCl_2$ with Nlp, (C). Red blood cells with Nlp and EDTA. (D). For the plate analysis of agglutination red blood cells in the presence of TBS-I with $CaCl_2$ and BSA were used. Final concentration of Nlp was 100 µg/ml in A1, wells A2 till A10 are two-fold serially diluted. B1 is negative control without Nlp. B2 is 100 µg/ml Nlp with EDTA. C1 is 100 µg/ml Nlp with EDTA.

Fig 5 Relative levels of cod *nlp* transcripts in the skin (A) and gill (B) tissues of cod. Quantification was done by q-PCR and values are expressed as mean ± SEM (n = 6). Initial samples (black bars) refer to zero hour samples.

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Table 1 PCR efficiency of the primers

Gene name	PCR efficiency	R^2
cycA	89.24 %	0.9999
elfalfa1	90.25%	0.9989
ubiquitin	89.91%	0.9991
natterin	87.60%	0.9976

Table 2 Results of Bestkeeper analysis of reference genes

	cycA	*Elfalfa 1*	*ubi*
coefficient of correlation [r]	0,928	0,963	0,974
coefficient of determination [r^2]	0,861	0,927	0,949
intercept [crossing point]	-2,093	0,429	1,482
slope [crossing point]	1,201	0,891	0,926
SE [crossing point]	±0.427	±0.221	±0.19
p-value	0,001	0,001	0,001
Power [x-fold]	2,30	1,85	1,90

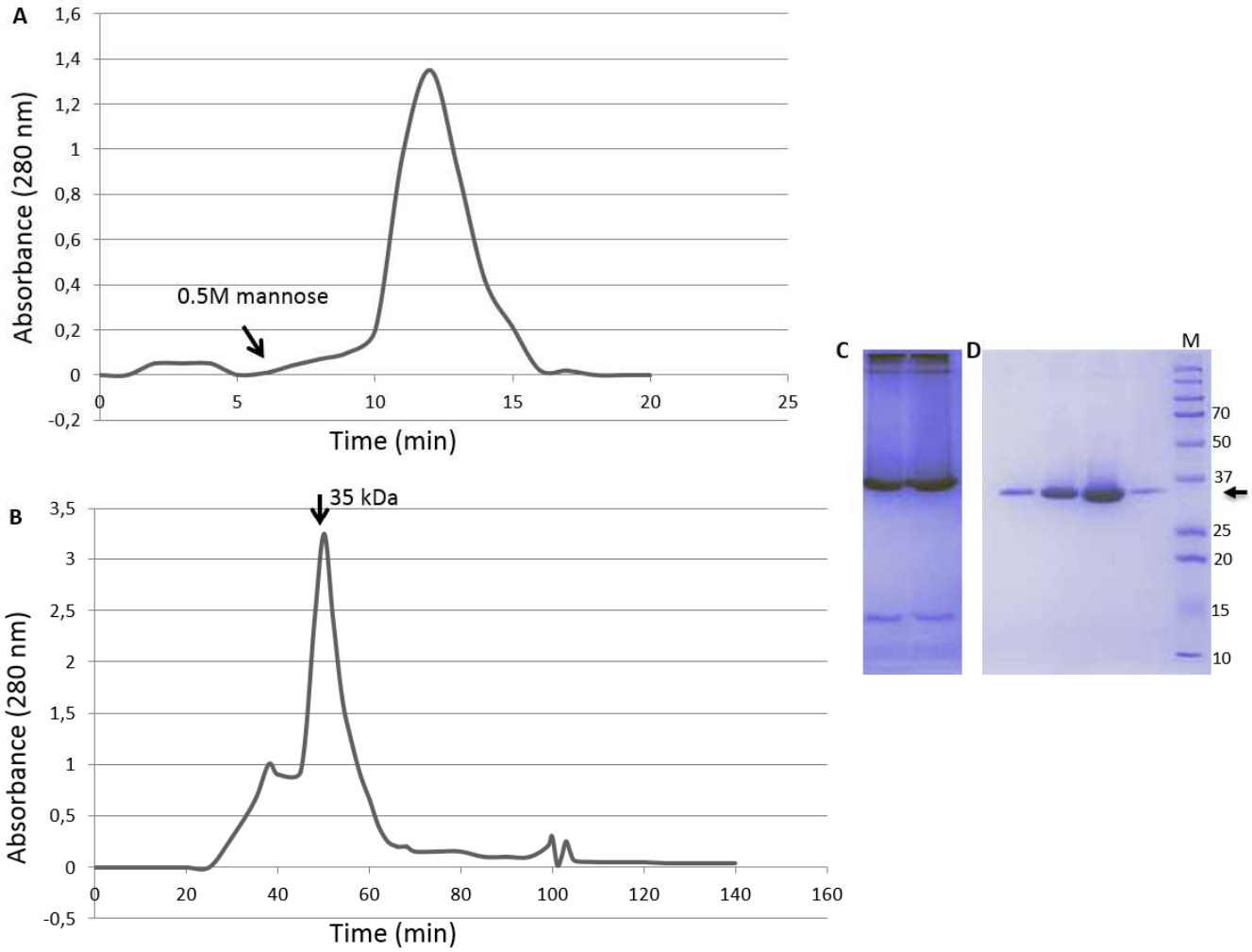

A
Absorbance (280 nm)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
-0,2
0.5M mannose
0
5
10
15
20
25
Time (min)
B
35 kDa
Absorbance (280 nm)
3,5
3
2,5
2
1,5
1
0,5
0
-0,5
0
20
40
60
80
100
120
140
160
Time (min)
C
D
M
70
50
37
25
20
15
10

Fig 1

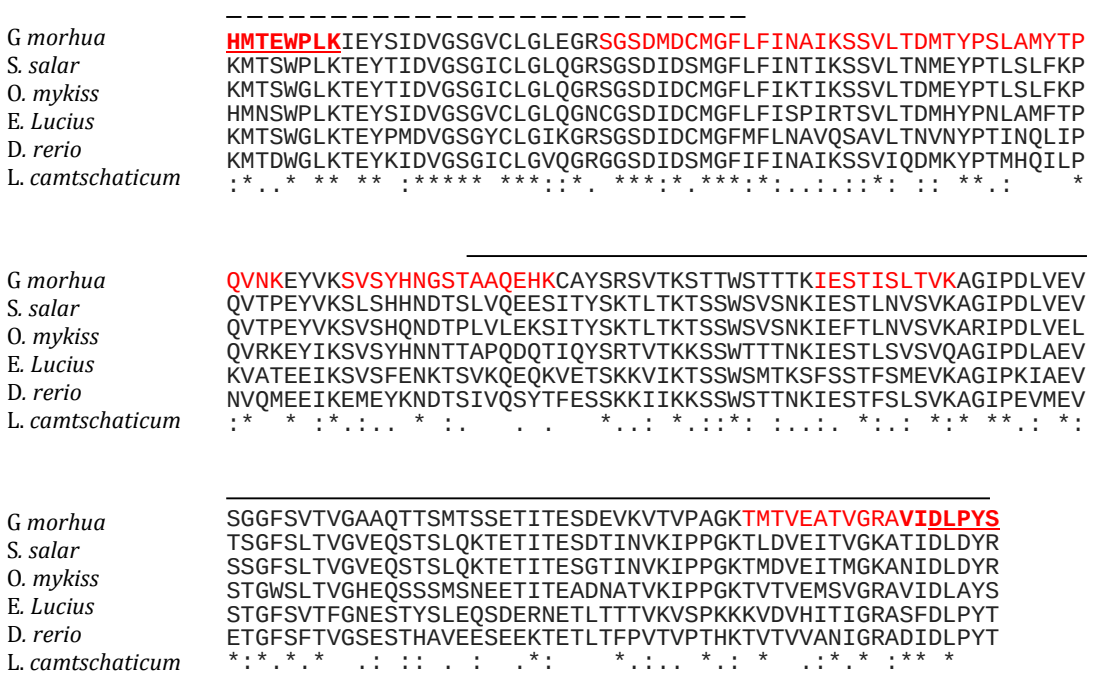


Fig 2

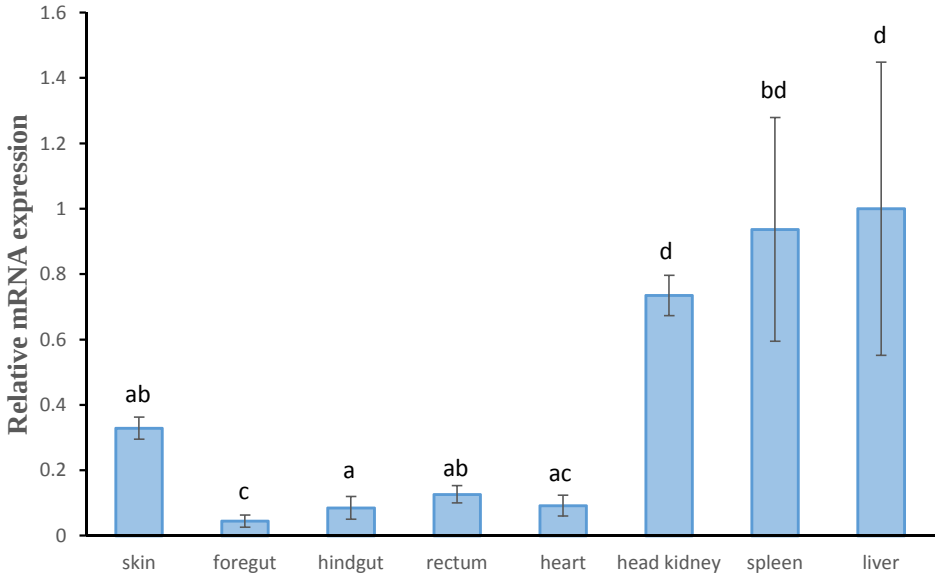
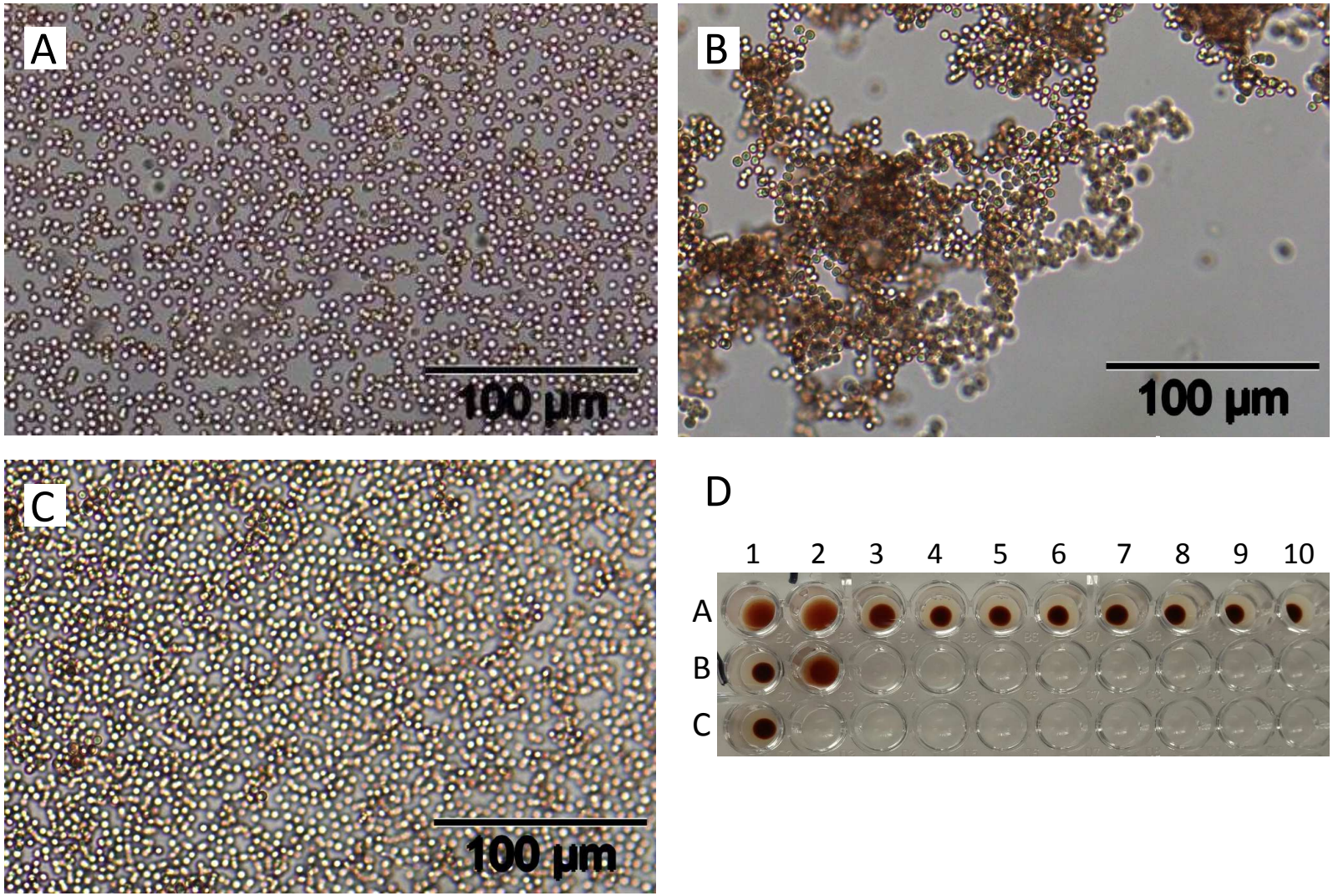

Relative mRNA expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ab
c
a
ab
ac
d
bd
d
skin
foregut
hindgut
rectum
heart
head kidney
spleen
liver

Figure 3


A
100 µm
B
100 µm
C
100 µm
D
1 2 3 4 5 6 7 8 9 10
A
B
C

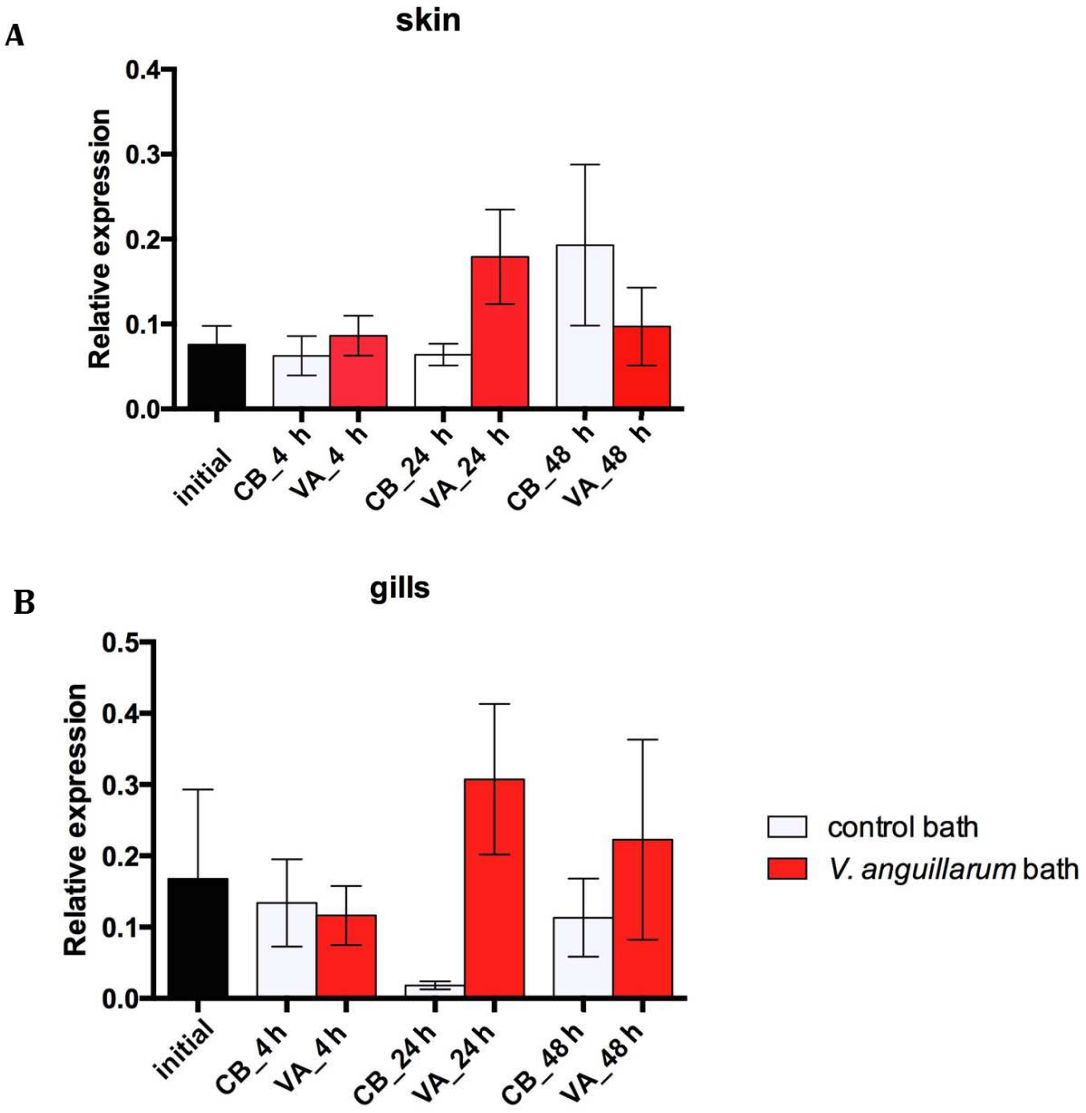

A
skin
Relative expression
0.4
0.3
0.2
0.1
0.0
initial
CB_4 h
VA_4 h
CB_24 h
VA_24 h
CB_48 h
VA_48 h
B
gills
Relative expression
0.5
0.4
0.3
0.2
0.1
0.0
initial
CB_4 h
VA_4 h
CB_24 h
VA_24 h
CB_48 h
VA_48 h
control bath
V. anguillarum bath

Figure 5

Highlights

- A mannose binding lectin was isolated from Atlantic cod skin
- The lectin was identified as natterin-like protein with a jacaline-like lectin domain
- Hemagglutination by Nlp was inhibited by mannose and partly with EDTA.
- The expression of cod nlp did not change during a Vibrio anguillarum bath challenge