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F-type lectin from the sea bass (*Dicentrarchus labrax*): Purification, cDNA cloning, tissue expression and localization, and opsonic activity

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ABSTRACT

Recently described biochemical and structural aspects of fucose-binding lectins from the European eel (*Anguilla anguilla*) and striped bass (*Morone saxatilis*) led to the identification of a novel lectin family (“F-type” lectins) characterized by a unique sequence motif and a characteristic structural fold. The F-type fold is shared not only with other members of this lectin family, but also with apparently unrelated proteins ranging from prokaryotes to vertebrates. Here we describe the purification, biochemical and molecular properties, and the opsonic activity of an F-type lectin (DIFBL) isolated from sea bass (*Dicentrarchus labrax*) serum. DIFBL exhibits two tandemly arranged carbohydrate-recognition domains that display the F-type sequence motif. *In situ hybridization* and immunohistochemical analysis revealed that DIFBL is specifically expressed and localized in hepatocytes and intestinal cells. Exposure of formalin-killed *Escherichia coli* to DIFBL enhanced their phagocytosis by *D. labrax* peritoneal macrophages relative to the unexposed controls, suggesting that DIFBL may function as an opsonin in plasma and intestinal mucus.

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1. Introduction

Lectins are multivalent proteins that recognize and bind carbohydrate moieties through specific domains (CRDs) [1] and participate in various biological processes [2–5], including innate and adaptive immune responses [6,7]. Because most lectins may display CRDs in combination with other domains, they not only recognize carbohydrates on the surface of potential pathogens, but also mediate several effector functions including agglutination, immobilization, and opsonization of microbial pathogens, and complement pathway and phagocyte activation [8]. Based on the presence of conserved amino acid sequence motifs in their CRDs, structural fold, and calcium requirements, animal lectins have been classified into several families, such as C-, P-, I-, and L-type lectins, galectins, pentraxins, and others [6,9]. Recently, a novel lectin family (F-type; fucose-binding) characterized by a unique CRD sequence motif and structural fold has been identified both in prokaryotes and eukaryotes, including invertebrates and vertebrates [10–12]. The F-type CRD can be associated with pentraxin, C-type lectin, or “sushi” domains yielding complex chimaeric proteins [9].

C-type, lectins, galectins, and rhamnose-binding lectins have been isolated from serum, skin mucus, and eggs from several fish species [13–16], and described as opsonic [6,17], or endowed with capacity to enhance respiratory burst and bactericidal activity of phagocytic cells [8,18–22].

F-type lectins have been identified and characterized in the serum from *Anguilla japonica* [13], *Anguilla anguilla* [12], *Morone saxatilis* [11], and *Sparus aurata* [23]. Although F-type lectins have been proposed to mediate role(s) as molecular recognition factors in innate immunity, the experimental evidence is fragmentary and the detailed mechanisms of their activity have not been elucidated [9]. Prior to the identification of the F-lectin family, a 34 kDa fucose-binding Ca²⁺-independent serum lectin had been purified from the sea bass (*Dicentrarchus labrax*) and named DIFBL [24]. More recently, and based on partial N-terminal amino acid sequence, DIFBL and a similar lectin (SauFBP) from the gilt head sea bream (*Sparus aurata*) were both identified as members of the F-type lectin family [23].

In this study we analyzed in further detail the molecular properties and phylogenetic relationships of DIFBL. The lectin subunit contains two tandemly arrayed, distinct CRDs that exhibit the sequence motif typical of the F-type lectins. *In situ hybridization* and immunohistochemical analysis revealed that DIFBL is expressed and localizes in hepatocytes and intestinal mucocyte globet cells. Further, in an *in vitro* assay with peritoneal

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macrophages, DIFBL displayed opsonic activity for formalin-killed *Escherichia coli*, suggesting that it may function in recognition and opsonization of potential microbial pathogens in the blood stream and intestinal mucus.

2. Material and methods

2.1. Chemicals, molecular biology reagents and bacterial strains

Unless otherwise specified, chemicals and molecular biology reagents were from Sigma-Aldrich (USA). The *E. coli* (ATCC 25922) strain was from Chrysope Technologies (LA).

2.2. Animals, collection of blood and tissue samples, and preparation of tissue extracts

Sea bass (*D. labrax*) ($n = 50$; approximately 250 g each) were provided by the Ittica Trappeto fish-farm (Trappeto, Palermo, Italy). Animals were anaesthetized in seawater containing 0.02% 3-aminobenzoic acid ethyl ester (MS-222 Sigma), and bled by heart puncture. The blood was allowed to clot at room temperature for 1 h, the serum separated by centrifugation at $800\times g$ (10 min, 4 °C), aliquoted, and stored at $-20\text{ }^{\circ}\text{C}$ until use.

Liver and intestine were excised from freshly killed fish and immediately frozen by immersion in liquid nitrogen. Frozen samples were ground into powder under liquid nitrogen, suspended in TBS (50 mM Tris-HCl, 0.15 M NaCl, pH 7.4), centrifuged at $15,000\times g$ and the supernatant stored at $-80\text{ }^{\circ}\text{C}$.

2.3. Purification of sea bass serum F-type lectin (DIFBL)

The DIFBL was isolated by an optimized two-step chromatography procedure. The first step consisted of a Sepharose CL6B affinity chromatography column eluted with 0.2 M galactose, as previously reported [24]. As monitored by absorbance at 280 nm, the elution profile yielded two peaks, of which the second displayed the highest hemagglutinating activity (titre: 256–512). In the second separation step, DIFBL was purified by loading the pooled active fractions from the second peak on a fucose-agarose affinity chromatography following the method reported by Honda et al. [13]. Briefly, the fractions of the Sepharose CL6B peak 2 (20 ml; about $1\text{--}2\text{ mg ml}^{-1}$ protein content) were pooled, dialyzed against TBS, centrifuged at $10,000\times g$ for 30 min, and passed through a 5 ml α -fucose-agarose column (Pierce). The column was washed with 1.0 M NaCl, followed by TBS (10 volumes) at a 0.2 ml/min flow rate. The elution of DIFBL was carried out with 20 ml of 50 mM α -fucose in TBS at the same flow rate, monitored by absorbance at 280 nm, and with 2 ml fractions collected. These were tested for hemagglutinating activity towards rabbit erythrocytes, and those that exhibited the highest activity were pooled and analyzed by electrophoresis.

2.4. Hemagglutination assay

Rabbit and sheep erythrocytes (RBC; supplied by Istituto Zooprofilattico della Sicilia) were washed three times with PBS, centrifuged at $500\times g$ for 10 min at 4 °C and suspended at 1% in TBS containing 0.1% (w/v) pig gelatin. A volume (25 μ l) of sea bass serum (1:10) or 25 μ l of the purified DIFBL preparation (250 μ g/ml) dialyzed in TBS were serially (2-fold) diluted with TBS-gelatin in 96-well round-bottom microtitre plates (Nunc, Denmark), and mixed with an equal volume of RBC suspension. The hemagglutinating titre (HT) was measured after 1 h incubation at 37 °C, and expressed as the reciprocal of the highest dilution showing clear agglutination.

2.5. Protein content estimation

Protein content was estimated according to the method of Bradford [25], using bovine serum albumin (BSA) as a standard.

2.6. Polyacrylamide gel electrophoresis

SDS-PAGE (10%) was carried out following the method of Laemmli [26], under reducing conditions (5% mercaptoethanol). To evaluate the molecular size, gels were calibrated with low range standard proteins (Bio-Rad, Richmond, CA). Proteins were stained with Coomassie brilliant Blue R-250.

2.7. Preparation of anti-DIFBL antisera

As previously described [24], bands identified in the SDS-PAGE gels as the purified DIFBL were excised and pooled, suspended in distilled water, and passed repeatedly through a syringe. Anti-DIFBL antibodies were raised in rabbits by Medprobe (Norway), with a coarse suspension of the gel pool containing DIFBL (50 μ g) as the antigen. To control for antibody specificity, the antiserum was absorbed with the purified lectin. Briefly, 500 μ l of anti-DIFBL antiserum were mixed with 100 μ l of the affinity chromatography Fraction II (50 μ g protein content), incubated overnight at 4 °C, and centrifuged at $27,000\times g$ for 1 h at 4 °C. Specificity of the anti-DIFBL antibodies was validated by comparing the activity of the diluted (5:1 in PBS: Na_2HPO_4 1 M, NaH_2PO_4 1 M, NaCl 1.5 M, pH 7.4) antiserum with the adsorbed one.

2.8. Western blot analysis

SDS-PAGE gels were soaked in transfer buffer (20 mM Tris, 192 mM glycine, 10% methanol, pH 8.8) for 10 min and proteins transferred for 75 min at 0.8 mA/cm² to nitrocellulose sheet in a semi dry blotting bath (Bio-Rad, USA). The filter was soaked in blocking buffer (PBS containing 3% BSA and 1% Tween 20) for 1.5 h. After washing with PBS-T the nitrocellulose sheet was incubated with anti-DIFBL antiserum (1:800 in PBS) for 1 h, then washed 4 times with blocking buffer, and incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit sheep IgG (Sigma; 1:15,000 in blocking buffer). After washing with PBS (4 times for 15 min), the filter was treated with 3 ml of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system.

2.9. Isolation of total RNA from liver, PCR amplification, and cDNA cloning

Total RNA was isolated from liver by using an RNAqueousTM-Midi kit purification system (Ambion), and reverse-transcribed with the Ready to Go T-primed first-strand kit using random primers (Amersham-Pharmacia Biotech). Amplification was performed by using 1 mM of the following degenerate primers, designed on the basis of sequences: P1.DFBP1.F (5'-dCAAAGCTTTAYA AACTAYAARAACGTNGC-3'); P2.DFBP3.R (5'-dTCGAATTCGTNACGATRTANGGCTC-3'). PCR amplification was carried out in a MJ Research DNA PTC-100 thermal cycler as follows: after a denaturing step at 94 °C for 3 min, the primers were annealed at 37 °C for 30 s, then 35 amplification cycles (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min) and a final elongation at 72 °C for 10 min were carried out. A single band of 136 bp in size was visible in agarose-gel electrophoresis (not shown). This product was ligated into the pCR 4-TOPO (TA cloning Kit, Invitrogen) according to the manufacturer's instructions. Plasmid DNA was isolated from recombinant bacterial clones using NucleoSpin extraction kit (Macherey-Nagel Sarl, Hoerd, France), and

sequenced at CRIBI (Univ of Padova-Italy) as a service. The cDNA sequences were completed by 5'- and 3' RACE using the Marathon RACE kit (Clontech), with the internal specific primers described above.

2.10. Expression analysis of DIFBL in several tissues

To determine whether the F-lectin was expressed in kidney, spleen, ovary, gills, intestine and heart tissues, total RNA was isolated by using a RNAqueous™-Midi Kit purification system (Ambion) and reverse-transcribed by the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen). The following primers were designed by using the liver cDNA sequence: upper primer, 5'd-TCTGTGAAGTGGAGGTTTAT-3'; lower primer, 5'd-AGGGTCAGG TACTCTTCTTT-3'. PCR amplification was carried out as follows: 94 °C 1 min, 52 °C 1 min, 72 °C 1 min for 30 cycles. A single band of 390 bp was visible in agarose-gel electrophoresis. The amplicon was purified and sequenced at CRIBI (Biotechnology Center of the University of Padua, Italy, <http://bmr.cribi.unipd.it>, ABI PRISM-DNA sequencer, Applied Biosystems). A similarity search was performed using the FASTA program (<http://www.ebi.ac.uk/Tools/fasta/>). The signal peptide has been determined by signalP algorithm <http://www.cbs.dtu.dk/services/SignalP/>. Proteins from the above mentioned tissues were prepared by adding liquid nitrogen to the dissected organs in a mortar, the frozen tissues ground to a fine powder with a pestle for 10–15 sec, and 1 ml of RIPA buffer (25 mM PBS -Tween 20 buffer pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) was added. The sample was centrifuged at 27,000 × g for 30 min at 4 °C, the resulting pellet was removed, and the supernatant used as crude extract or frozen at -80 °C.

2.11. Phylogenetic analysis

The deduced amino acid sequences were submitted to multiple alignments using the Clustal W v. 1.81 program [27]. Alignment of protein sequences was done using CLUSTAL X v.1.83 [28] (<ftp://ftp-igbmc.u-strasbg.fr/pub/>) and similarity shaded with GeneDoc v.2.6.002 (www.psc.edu/biomed/genedoc/) and Bioedit version 5.0.9. A phylogenetic tree was constructed by the Neighbour-Joining method (NJ), considering 1000 bootstrap hits.

Calculations of theoretical protein characteristics from the deduced peptide sequence were performed with ProtParam (www.exPASy.ch). The putative tertiary structure of each CRD from DIFBL was modeled using the crystallographic structure of the *A. anguilla* F-type lectin as a template [12]. The polypeptide sequence of each CRD was aligned to the *A. anguilla* F-type lectin template structure (Protein Data Bank accession code 1K12) using Cn3D 4.1 produced by NCBI.

2.12. In situ hybridization

In situ hybridization was performed on tissue sections (7 µm) according to Alonso et al. [29]. Antisense (AS) and sense (S) probes were synthesized based on the DIFBL cDNA clone. Digoxigenin-labeling was performed using the digoxigenin-UTP *in vitro* transcription kit (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions. The sections were washed twice in PBS-T (1 M Na₂HPO₄, 1 M NaH₂PO₄, 1.5 M NaCl, pH 7.4 0.1% Tween 20), permeabilized with 1 µl ml⁻¹ proteinase K (Sigma-Aldrich) in PBS-T, and the reaction blocked by 2 mg ml⁻¹ glycine in PBS-T. After washing with PBS-T, the sections were post-fixed for 30 min with 4% formaldehyde in PBS-T, treated with hybridization solution (50% formamide, 50 µg ml⁻¹ heparin, 500 yeast tRNA, 0.1% Tween 20 and 5x sodium chloride/sodium citrate solution (SSC): 0.15 M NaCl/

0.05 M sodium citrate, pH 7) at 42 °C for 1 h, followed by 15% AS probe in hybridization solution at 42 °C overnight. After washing with PBS-T at 42 °C (10 min) and then with 0.3% SSC 20x containing 1% Tween 20, the sections were incubated for 30 min at 20 °C with 2% horse serum in PBS-T, and then treated for 1 h at 20 °C with anti-digoxigenin-Fab-Ab (Roche Diagnostics, Meylan, France) diluted 1:100 in the horse serum-PBS-T solution. Finally, the sections were washed twice in PBS-T and incubated with BCIP/NBT for 2 h in the dark. Controls consisted of tissue sections processed similarly, using the corresponding sense RNA (1 µg ml⁻¹). Three individual fishes were examined for both experimental and control sections.

2.13. Immunohistochemical and immunocytochemical methods

Liver and intestine samples (approximately 300 mg) were fixed in Bouin's for 24 h, rinsed in ethanol 75% and embedded in paraffin. Serial sections (5 µm) were treated for 30 min with PBS containing 0.2% Triton X-100 and 0.1% Tween 20 (PBS-T). To prevent non-specific antibody binding, the sections were incubated with 3% BSA in PBS for 1.5 h at room temperature. After two washings in PBS-T, the sections were incubated overnight with anti-DIFBL antibodies (1:800 in PBS-T containing 0.1% BSA), and, after washing, the sections incubated for 1.5 h at room temperature with sheep anti-rabbit IgG alkaline phosphatase conjugate as the secondary antibody (1:15,000 in PBS-T 0.1% BSA). After washing in PBS-T the sections were treated with the substrate mixture (3 ml of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, BCIP/NBT) and the reaction blocked in distilled water. To validate the antibody specificity, the following controls were performed: (1) The anti-DIFBL antiserum was replaced with pre-immune rabbit serum; (2) the primary (anti-DIFBL) antibodies were replaced with PBS; 3. anti-DIFBL antiserum was replaced with the absorbed anti-DIFBL antiserum.

Histological sections were stained with Mallory stain (0.5% of aniline blue; 2% orange G; 2% oxalic acid in distilled water) for 30 min [30], or with Gomori trichromic stain [Chromotrope 2R (0.6 g), Fast green FCF (0.3 g), phosphotungstic acid (0.8 g), glacial acetic acid (1 ml), 100 ml DW] for 30 min [31].

2.14. Bacterial suspensions

Bacteria were grown to log phase in tryptic soy broth (TSB) containing 3% NaCl at 25 °C, with continuous shaking (120 rpm) in a Gallenkamp incubator. Cell numbers were estimated by absorbance at 600 nm. The relationship between absorbance and cell number had previously been determined by plate count, and cultures were diluted to a final 10% bacterial stock suspension. Bacteria were fixed by adding formaldehyde to the bacterial stock suspension to a 2% final concentration, and the mixture shaken (120 rpm) overnight at 21 °C. After centrifugation at 6000 × g for 15 min (4 °C), the formalin-killed bacteria were washed three times with sterile PBS, suspended in PBS containing 0.1% (w/v) gelatin to obtain 1 × 10⁹ cells ml⁻¹, and stored at 4 °C until use.

For the phagocytosis assay, the formalin-killed bacteria were washed three times with sterile PBS, suspended 1 × 10⁹ ml⁻¹ in carbonate buffer (0.1 M Na₂CO₃, 0.1 M NaHCO₃, pH 9.5) containing 0.1 mg ml⁻¹ FITC and incubated 30 min at 37 °C with gentle shaking. FITC-treated formalin-killed bacteria were washed three times in NaCl 0.9% and twice in PBS containing 2 mM CaCl₂, and suspended (1 × 10⁸ ml⁻¹) in PBS-Ca.

2.15. Collection of peritoneal macrophages

Peritoneal cavity cells (PCC) were harvested by injecting 10 ml L-15 medium [L-15 medium (Leibovitz) without L-glutamine, liquid,

sterile-filtered, cell culture tested; Sigma] containing 100 units ml⁻¹ penicillin/streptomycin and 10 units ml⁻¹ heparin into the peritoneal cavity. The body cavity was massaged for 10 min, and the medium containing the peritoneal cells was withdrawn with a sterile syringe and centrifuged at 500×g for 10 min. The cell suspension was adjusted to 1 × 10⁷ cells ml⁻¹ in L-15 medium, and cell viability was evaluated by the trypan blue exclusion test.

2.16. Phagocytosis assay

FITC-treated formalin-killed bacteria (1 × 10⁸ ml⁻¹) were mixed in a microtube with purified F-lectin (5, 10, 25 µg ml⁻¹) in PBS Ca²⁺,

incubated at 18 °C for 60 min, and washed twice with the same buffer. In the controls the purified lectin was replaced with PBS Ca²⁺. The opsonized bacteria (100 µl) were incubated with an equal volume of PCCs (1 × 10⁷ ml⁻¹) for 30 min at 18 °C. Fluorescence of the non-phagocytosed bacteria, was quenched by adding trypan blue (2 mg ml⁻¹) in 0.02 M citrate buffer pH 4.4, containing 0.15 M NaCl and 2 mg ml⁻¹ crystal violet in PBS [32]. The phagocytosed fluorescent bacteria were observed under a UV light microscope equipped with Nomarsky differential interference contrast optic (Diaplan, Leica, Wetzlar, Germany). The phagocytic rate (PR) was determined as the percent of cells showing internalized bacteria, and the phagocytic index (PI) as the average of ingested bacteria relative to the total phagocyte number.

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1                               ATGTCTCAAAGAGGCTCGGTACAGTAACTCACTGG
1                               m r h s v v
36 ACTCCAGGGATAAAAGATCTGTTCTAACCAGGAAGCAGGAATAATGAGGCACAGTGTGGT
7   f l l l l l l g a g s a Y N Y K N L A L 8
96 ATTTCTGTGTGCTGCTCCTCTTAGGGGCGGGTTCAGCTTACAACCTATAAAAATCTGGCCCTT
27   R G K A T Q S A R Y V H T F G A A Y S A 28
156 GCGAGGAAAAGCGACTCAGTCGCGCACGTTATGTGCACACATTTGGAGCTGCCTACAGCCG
47   I D G N R D P V F D A G S C T H S A E Q 48
216 TATTGATGGAAACCGTGACCCCTGTCTTCGATGCTGGATCGTGCACCCACAGTGTGAACA
67   T N P W W R V D L L E P Y I V T S I I I 68
276 GACCAACCCCTGGTGGAGAGTTGACCTGCTGGAGCCCTACATCGTCACCTCCATCATCAT
87   T N R E D C C A D R L N G A E I H I G N 88
336 CACCAACAGAGAAGACTGCTGTGCAGACAGGCTCAACGGGGCTGAGATTACATCGGAAA
107  S L L D G G A A N P R V G V I S H I P A 108
396 CTCTTTACTAGACGGTGGTGTGCAAACCAAGGGTTGGTGTAAATTTCTCATATCCCTGC
127  G I S H T F S F T E R L E G R Y L T V L 128
456 AGGGATCTCACATACTTTCAGTTTCACTGAACGTCTGGAGGGACGTTACTTGACTGTGCT
147  I P G T N K F L T L C E V E V Y G Y R A 148
516 TATACCTGGTACAAACAAGTTTCTTACACTCTGTGAAGTGGAGGTTTATGGGTACCGAGC
167  P T G E N L A L Q G K A T Q S S L Y E F 168
576 CCCAACTGGAGAGAACCTGGCCCTCCAAGGAAAAGCCACACAGTCTTCATTGTATGAATT
187  G I A Y N A I D G N Q A N N W I K A S C 188
636 TGGTATTGCCTATAATGCCATTGACGGGAATCAAGCCAACAATTTGGATAAAGGCCTCCTG
207  S H T N K E M N P W W R L D L R K T H R 208
696 CAGTCACACAAACAAGGAGATGAACCCCTGGTGGCGACTGGATCTGCGCAAAACCCACAG
227  V F S V K V T N R D S Y E Q R I N G A E 228
756 AGTGTTTTCTGTTAAGGTAACCAACCGAGATTTCATATGAACAACGAATCAATGGAGCCGA
247  I R I G D S L D N N G N N Q R C A V I 248
816 GATCCGAATTGGAGATTCCTCGACAACAACGGCAACAACAATCAGAGGTGTGTGTGAT
267  T S I P A S A T T E F Q C N G M D G R Y 268
876 CACAAGCATCCCAGCAAGTGTACTACTGAATTCAGTGTAAATGGGATGGATGGCCGCTA
287  V N I V I P G K E E Y L T L C E V E V Y 288
936 TGTTAACATTGTTATCCCTGGAAAAGAAGGTACCTGACCCCTGTGTGAGGTGGAGGTGTA
307  G S V L D * 293
996 TGGCTCTGTCTGGATTAGGTGTCAATACTAATACTGTTGAATGTACGCAAAACAAACAA
1056 AATAGTAGATTAAGCTAAAATTTGTTTCCATTCAAATAAGACTTATCCAATAAAATTAC
1116 ATCACGAAATGCAAAAAAAAAAAAAA

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Fig. 1. The complete cDNA and deduced protein sequence of DIFBL. The cleaved signal peptide is indicated by lowercase amino acids and is negatively enumerated. The sequence of the N-terminal peptide is underlined with a single line. The in-frame stop codon is marked with an asterisk. The polyadenylation motif is double underlined. The two CRD are highlighted in grey (Accession number EU877448).

3. Results

3.1. DIFBL cDNAs cloning and sequence analysis

By using degenerate P1, P2 primers designed on the basis of *M. saxatilis* sequences a 136 bp long amplicon was obtained. To elucidate the sequence of the entire transcript 3' and 5' RACE-PCR using the specific primers P3 and P4 designed from the initial 136 bp amplicon was carried out. The 1142 bp cDNA product presented the complete cDNA sequence with an open reading frame encoding 312 amino acids (Fig. 1). Nucleotide sequence analysis showed that a 77-nucleotide 5'-UTR preceded the putative translation start site, and a 93-nucleotide 3'-UTR spanned from the stop codon up to the polyadenylation site resulting in a 1128-nucleotide transcript excluding the poly(A) tail. The deduced protein sequence (Fig. 1) was 293 residues long. The cleavage site of the 18-residue signal sequence at the N-terminal was predicted by the SignalP algorithm to reside between Ala¹⁸ and Tyr¹⁹. Therefore, the calculated molecular mass of the DIFBL is 34.4 kDa (ProtParam; www.expasy.ch), with a theoretical isoelectric point of 5.84.

A comparative sequence analysis (Fig. 2) revealed the presence of two CRDs spanning from Asn⁵ to Gly¹⁴³ (N-CRD) and from Asn¹⁵³ to Gly²⁸⁷ (C-CRD) connected by a nine amino acid linker peptide.

3.2. Phylogenetic analysis and CRD comparison

BLAST analysis revealed that the DIFBL deduced amino acid sequence presents close homologies with vertebrate F-lectins depending on their CRD organization. In the phylogenetic tree (Fig. 2), DIFBL clustered with the other binary (two CRDs) teleost FBPs. A second cluster includes F-lectins from *Anguilla* species and *Tetraodon nigroviridis*, which are characterized by a single CRD, whereas F-lectins containing more than two CRDs form a third cluster. Finally, a cluster of heterogeneous proteins, containing

significant sequence similarity to F-type CRDs from bacteria, insects, sea urchin, and *Xenopus* was identified.

Detailed phylogenetic analysis of the CRD in binary fish F-lectins (Fig. 3) showed highly conserved sequences in their N-CRDs or C-CRDs, being the N-CRDs closer to the *Anguilla* CRD cluster. Conserved Cys form two disulphide bounds (Cys⁴²-Cys¹³⁹ and Cys⁹⁷-Cys¹¹³), whereas an additional bond (Cys⁷⁴-Cys⁷⁵) is present in the *A. anguilla* F-type lectin. Conserved amino acids involved in sugar binding (His⁴⁴, Arg⁷⁰, Arg⁷⁷) were present in both N-CRDs and C-CRDs (Fig. 4).

3.3. SDS-PAGE analysis of the DIFBL purified fractions

Fig. 5 shows a typical fucose-agarose affinity chromatography purification profile of 20 ml of pooled fractions from Sepharose chromatography (peak 2, 3–5 mg ml⁻¹ protein; 256–512 HA). The purified agglutinating fraction (peak 2, 30–32 fractions pooled) contained 131 ± 44 µg DIFBL ml⁻¹ (n = 4) with a 512–1024 HT. The specific activity (HT × volume/protein content: 11,035 for the DIFBL pool; 1076 for Sepharose CL6B active fraction; 64 for whole serum) of the DIFBL pool was 160 to 180-fold higher than that of whole serum, and SDS-PAGE under reducing conditions revealed a single 34 kDa component (DIFBL, Fig. 6, lane 1). The fractions (6–12) from peak 1 collected before the column was eluted with L-fucose, showed no hemagglutinating activity. SDS-PAGE under reducing conditions of peak 2 from the first Sepharose chromatography step revealed an intense 34 kDa band and a minor 70 kDa component. Both protein bands were eluted from the gel. The N-terminal sequence of the 70 kDa (APAEEKVK) protein was 100% identical to the putative N-terminal sequence of transferrin from the teleost *Acanthopagrus schlegelii*.

When the active fractions from the first and second separation steps were analyzed by SDS-PAGE under non-reducing conditions, the mobility of the 34 kDa band increased to an equivalent mass of 30 kDa (Fig. 6, lane 2), suggesting the presence of disulfide bonds in DIFBL.

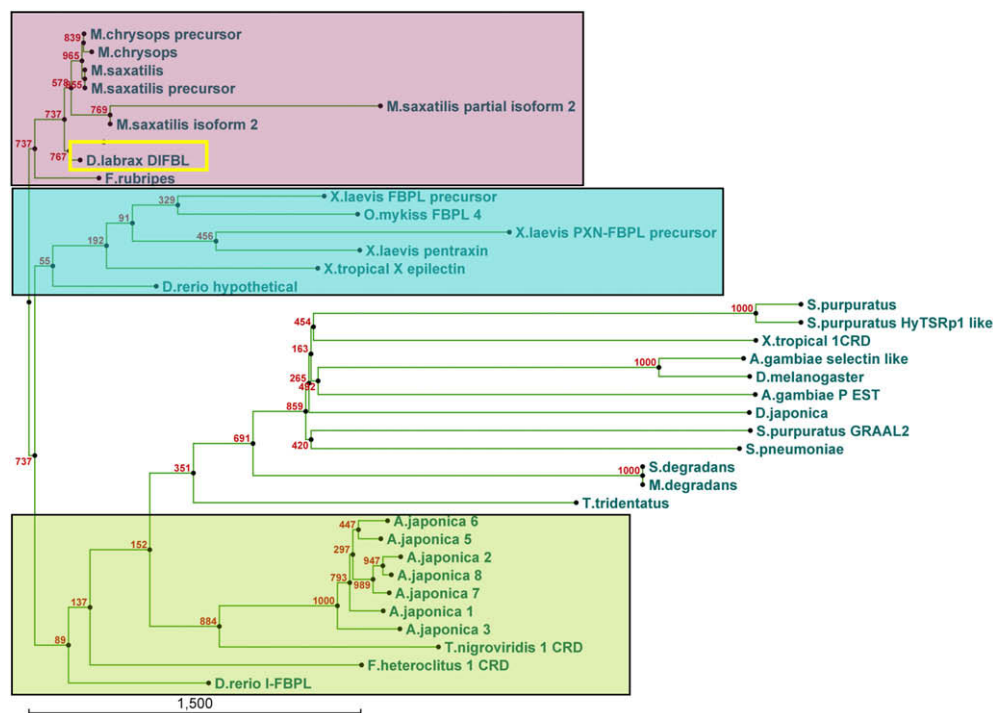


Fig. 2. Phylogenetic analysis of F-type lectins: Multiple alignment of full ORFs. The phylogram was created from neighbour-joining analysis using Clustal_X v1.83. Distances were corrected for multiple substitutions and gap positions were excluded. Bootstrap values are percentages from 1000 iterations. Scale bar measures substitutions per site.

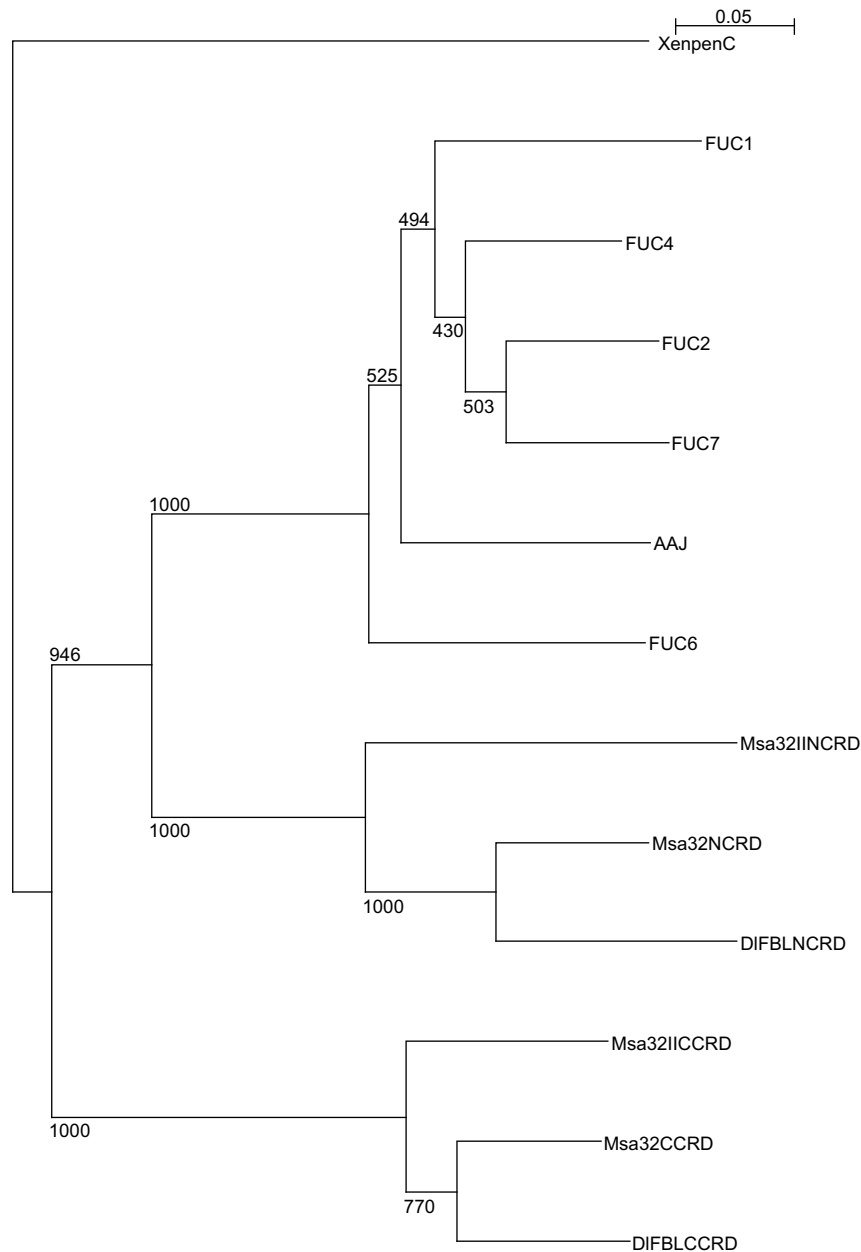


Fig. 3. Analysis of genetic distance between FBPL domains. The phylogram was created from neighbour-joining analysis using Clustal_X v.1.8.1. Distances were corrected for multiple substitutions and gap positions were excluded. Bootstrap values are percentages from 1000 iterations. Organism abbreviations: DI, *Dicentrarchus labrax*; Msa, *Morone saxatilis* (striped bass); AAJ, *Anguilla japonica* (Japanese eel); Xenpen, *Xenopus tropicalis* (diploid clawed frog); FUC, *A. anguilla* fucolectin 1–7.

3.4. Immunoblotting of serum and tissue homogenate supernatants

Western blot analysis (Fig. 6) of the isolated fraction showed that anti-DIFBL antibodies reacted equally well with both the 34 kDa band (Fig. 6, lane 3) observed under reducing conditions, and the 30 kDa band found under non-reducing conditions (Fig. 6, lane 4).

The 34 kDa DIFBL was identified in protein extract from liver, intestine, head kidney, spleen, ovary, gill and heart. However, differences in the band density indicated that the protein was mainly contained in the liver and intestine, whereas a thin band was visible in the head kidney, spleen, ovary, gill, and heart preparations (Fig. 7b). The specificity of the anti-DIFBL antiserum was validated by testing the pre-immune rabbit serum and the antiserum absorbed with purified DIFBL. No bands were observed with either antisera (Fig. 6, lanes 7 and 8, respectively). In addition, no

bands were observed in the control where the primary (anti-DIFBL) antibody was omitted.

3.5. Immunohistochemical analysis

Anti-DIFBL antibodies identified DIFBL epitopes in liver parenchymal cells (Fig. 8a), and intestinal mucocyte goblet cells (Fig. 8d arrows) interposed between the absorbent cells of the intestinal columnar epithelium. The specificity of the antibody binding was confirmed by replacing the primary antibody with pre-immune rabbit serum or omitting the primary antibody (Fig. 8 b, e), and by treating sections with the absorbed antiserum (Not shown). In either control no stained cells were observed (Fig. 8 b, e). No antibody binding was observed in head kidney, spleen, heart, gill and ovary histological sections.

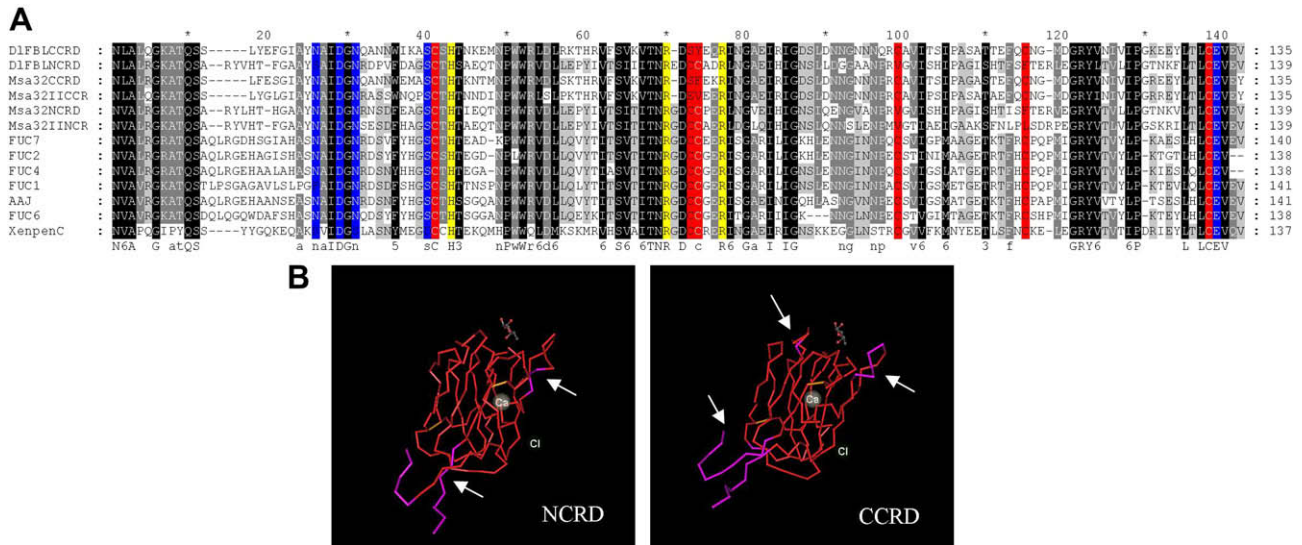


Fig. 4. Conservation of the functionally relevant amino acid residues of DIFBL within the F-type lectin family. A; Residues that interact with the calcium ion through their side chain oxygen are highlighted in blue. Half-cysteines are highlighted in red below the alignment indicates disulfide bridge partners. Sugar-binding residues are highlighted in yellow. Organism abbreviations: Msa, *Morone saxatilis* (striped bass); AAJ, *Anguilla japonica* (Japanese eel); Xenpen, *Xenopus tropicalis* (diploid clawed frog); Alignment was produced with Clustal_X v.1.81 (39) and illustrated with GeneDoc v.2.6.002 (81). Invariant residues are shaded black; conserved residues, as defined by the Blossum62 similarity matrix, in ≥ 80 of sequences are shaded grey with white lettering; conservatively substituted residues in $\geq 60\%$ of sequences are shaded grey with black lettering. Consensus is indicated on bottom row by lowercase letters for the most frequent residue and numerals indicating Blossum62 matrix similarity groups (i.e. 6:LIVM). B; Homology modelling and exon-domain relationship. The N and C-CRD of DIFBL were modelled on to the AAA structure. The homology are shown in red, the differences are highlighted in magenta and indicated by arrows.

3.6. DIFBL mRNA expression and in situ hybridization

RT-PCR analyses by using a specific primer pair identified the presence of DIFBL transcripts in several organs and tissues. Both liver and intestine expressed a 390 bp mRNA (Fig. 7a). A lower level of the transcript was expressed in the head kidney, spleen, ovary, whereas it was absent in gills and heart. The 390 bp bands purified from these organs, revealed a sequence identical to 34 kDa N-terminal sequence was in accordance with the amino acid sequence deduced from the DIFBL cDNA (data not shown).

In situ hybridization experiments were limited to liver and intestine, where the transcript was abundantly expressed (Fig. 9). The antisense riboprobe was found in the nucleus of liver parenchymal cells (Fig. 9a), intestinal columnar epithelium cells and mucocyte globet cells in the intestine (Fig. 9c). No signal was observed when the sense probe was used (Fig. 9b, d).

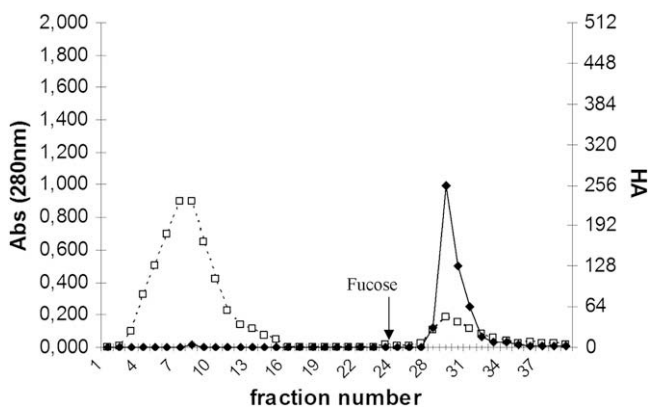


Fig. 5. Affinity chromatography of *D. labrax* pooled active fractions from a Sepharose CL6B on a fucose-agarose column absorbance at 280 nm (\square); Hemagglutinating activity (\blacklozenge).

3.7. Effect of DIFBL on phagocytic activity of peritoneal macrophages

The phagocytic activity of peritoneal macrophages increased significantly after bacteria were opsonized with the isolated DIFBL ($16.9 \pm 6.9\%$; 300 cells were counted in three distinct assays) (Fig. 10a), while the phagocytic index values were doubled (from 1.8 ± 0.9 up to 3.5 ± 2.1 $p < 0.005$). The opsonic effect of DIFBL was carbohydrate-specific as shown by opsonization-inhibition experiments. Galactose or glucose added in the phagocytosis mixture (25 mM final concentration) did not affect the opsonic effect of DIFBL, whereas the presence of 25 mM fucose reduced the phagocytosis activity ($7.7 \pm 1.8\%$ phagocytes) to levels similar to those for the non-opsonized bacteria of the control (Fig. 10a).

The opsonizing effect of DIFBL showed a dose-response profile, with increasing phagocytic activity levels when bacteria were pre-treated with 5 ($p < 0.001$) and $10 \mu\text{g ml}^{-1}$ ($p < 0.01$) DIFBL, and showing a moderate decrease relative to the latter at $25 \mu\text{g ml}^{-1}$ ($p < 0.001$) DIFBL (Fig. 10b). Internalization of the FITC-labelled bacteria by the peritoneal macrophages could be visualized by fluorescence microscopy (Fig. 10c, d).

4. Discussion

Lectins play important roles in the immune response of invertebrates and vertebrates either by recognizing exposed glycans of potential pathogens or by their immunoregulatory roles through the binding to carbohydrates on the surfaces of immunocompetent cells [2–4,33,34]. Despite the relatively weak binding affinities of the CRD for the carbohydrate ligands, high avidity is achieved through the cooperative binding interactions of the multiple CRDs displayed that result from (a) the bouquet- or cruciform-shaped oligomerization of the peptide subunits, (b) the presence of tandemly arrayed CRDs along the peptide subunits, or (c) both subunit oligomerization and tandemly arrayed CRDs, such as in the recently identified F-type lectin family. This lectin family, that received its

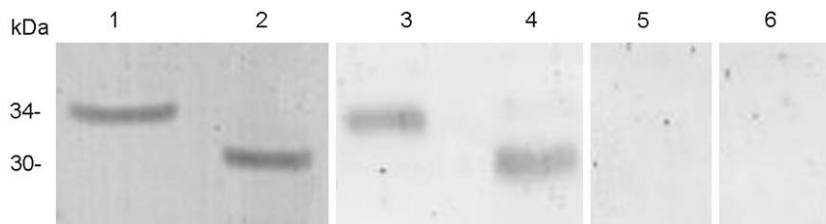


Fig. 6. SDS-PAGE and immunoblot of *D. labrax* purified lectins. SDS-PAGE analysis of DIFBL (2.5 μ g) in the presence (lane 1) or absence (lane 2) of reducing agent (NR) (2-mercaptoethanol) on a gel of 12.5% T stained with Coomassie Blue R-250. Immunoblotting of *D. labrax* serum subject to SDS-PAGE under reducing conditions and immunoblotted with anti-*D. labrax* F-lectin antibodies. Lane 3, serum in the presence of reducing agent; Lane 4, serum in the absence of reducing agent; lane 5, pre-immune rabbit serum; lane 6, antiserum absorbed with DIFBL fraction.

name for the nominal specificity for fucose of the first members identified, is characterized by the highest multiplicity of tandemly arrayed CRDs observed in any lectin peptide subunit described so far [11].

In a previous report [24], we characterized a 34 kDa fucose-binding lectin from *D. labrax* serum (DIFBL) isolated through Sepharose CL6B affinity chromatography. In a subsequent study [23] we established that DIFBL and SauFBL, a lectin from the gilt head sea bream (*S. aurata*) of similar binding properties, were both members of the F-type lectin family as concluded from N-terminal amino acid sequence comparison. In the present study we further purified the 34 kDa lectin by a two-step chromatography procedure in which the pooled active fractions from a Sepharose CL6B column were loaded onto a α -fucose-agarose column. The second chromatography step enabled the separation of the 34 kDa protein from a 70 kDa component that co-eluted in the Sepharose 6B CL separation step [24]. In the present paper we show that the 70 kDa N-terminal sequence is identical to fish transferrin N-terminal amino acids (1–7 amino acids; Accession number: AY365052.1).

The complete DIFBL cDNA sequence revealed that this lectin, like the *M. saxatilis* F-lectins, possesses two tandemly arrayed CRDs. Odom and Vasta [11] isolated from serum and liver of the striped bass (*M. saxatilis*) two fucose-binding lectins of 30 and 32 kDa (MsaFBP32 and MsaFBP32II), each carrying two tandem CRDs that exhibit the F-type carbohydrate-recognition motif and the typical F-type structural fold established for the *A. anguilla* F-type lectin [12]. There are relevant biochemical and structural similarities between DIFBL and the two binary tandem CRD F-type lectins isolated from striped bass [11].

The DIFBL cDNA sequence consists of an open reading frame encoding 312 amino acid residues including 18-residue signal sequence at the N-terminal. The deduced size of 34 kDa for the mature protein is in agreement with subunit size of the lectin previously isolated from the serum through Sepharose CL6B column affinity chromatography [24]. The deduced amino acid sequence differed at the first position from the previously reported [24]. N-terminal sequence of the isolated 34 kDa lectin. With respect to that, a protein sequencing mistake at the first residue could be occurred.

Comparative sequence analysis revealed that DIFBL is a binary tandem domain F-type lectin with the N- and C-CRDs connected by

a nine amino acid peptide linker. BLAST analysis disclosed sequence homologies with vertebrate F-lectin CRDs. In the phylogenetic tree, DIFBL is included in a cluster of teleost binary F-type lectins, clearly distinguishable from those F-lectins with a single CRD or containing more than two CRDs. Both N-CRD and C-CRD showed highly conserved sequences characterized by Cys residues located at sequence positions consistent with the formation of two intrachain disulphide bonds. The presence of intrachain disulphide bonds was supported by the different electrophoretic mobilities of DIFBL under reducing (34 kDa) and non-reducing conditions (30 kDa). Binary CRD F-type lectins are present in several teleost species, and are most likely the result of gene duplications that took place independently in various perciform lineages [11]. In contrast with the single CRD F-lectins from eels, which may represent the ancestral state of this lectin family within the rayfined fish, the binary homologues may have diversified in the teleosts through gene duplications and speciation events, such as the four-tandem CRD F-lectins that are unique to the salmoniformes [11]. Although no studies were carried out so far concerning the potential oligomerization of the DIFBL 34 kDa peptide subunits, the formation of trimers, such as demonstrated in the *A. anguilla* F-lectin [12] or as proposed for the *M. saxatilis* lectins cannot be ruled out.

To examine the tissue localization of DIFBL, antibodies against the protein were raised, and their specificity validated by complete absorption of the antiserum activity with the purified DIFBL. Western blot analysis of crude serum and tissue extracts (liver, intestine, head kidney, spleen, ovary, gill, and heart), assayed with the specific antibodies only identified a 34 kDa band corresponding to the expected mobility of DIFBL. Differences in band intensities suggest that the lectin is mainly expressed in liver tissue, and to a lesser extent in intestine and head kidney, being very scarce in the gill, heart, spleen and ovary. These results are in accordance with the RT-PCR experiments, which revealed the highest transcript levels in liver, followed in decreasing order by intestine, head kidney, spleen, ovary, and transcripts absent in gill and heart. The liver appears to be the main site of DIFBL expression, and, consistently with observations on other F-lectins (11,13), it can also be expressed at lower levels in intestine, head kidney, spleen and ovary. To identify the liver and intestine cells which express DIFBL, in situ hybridization and immunohistochemistry analyses were

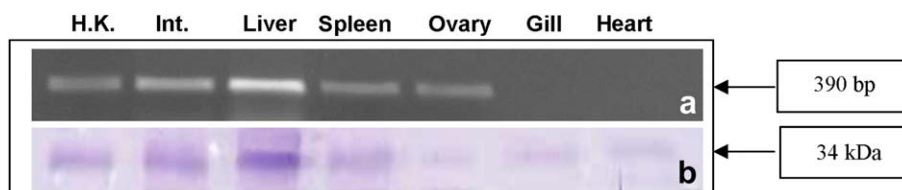


Fig. 7. Tissue distribution of *D. labrax* DIFBL. a) RT-PCR analyses of DIFBL cDNA; b) Immunoblotting of tissues extracts analyzed by SDS-PAGE under reducing conditions and immunoblotted with anti-DIFBL antibodies. H.K. = Head Kidney; Int. = intestine.

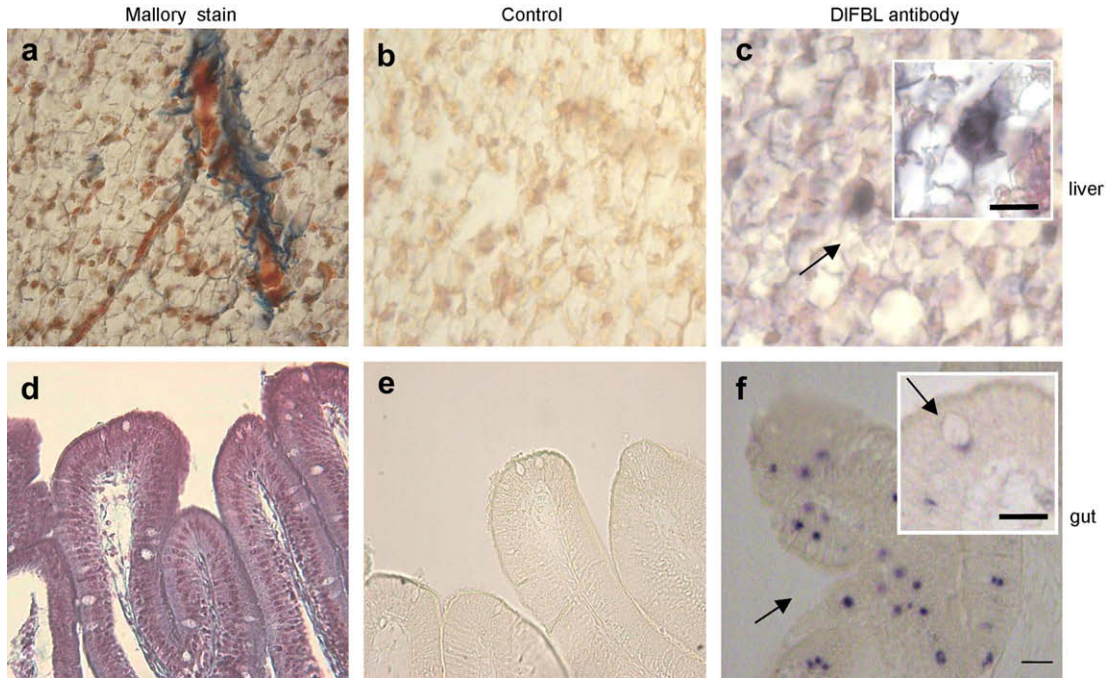


Fig. 8. Immunohistochemical localization of DIFBL in liver and gut of *Dicentrarchus labrax*. Adjacent sections in the liver and gut were immunostained with DIFBL antibody (b, c, e and f) and Mallory stain (a and d) as described in Section 2. Immunopositive cells were mainly observed among liver hepatocytes (arrows in c) and in mucocyte globet cells in the gut sections. No immunoreactivity was observed with DIFBL antibody preabsorbed with DIFBL (b and e). Bars: a–f, 20 μ m; c, f spots 10 μ m.

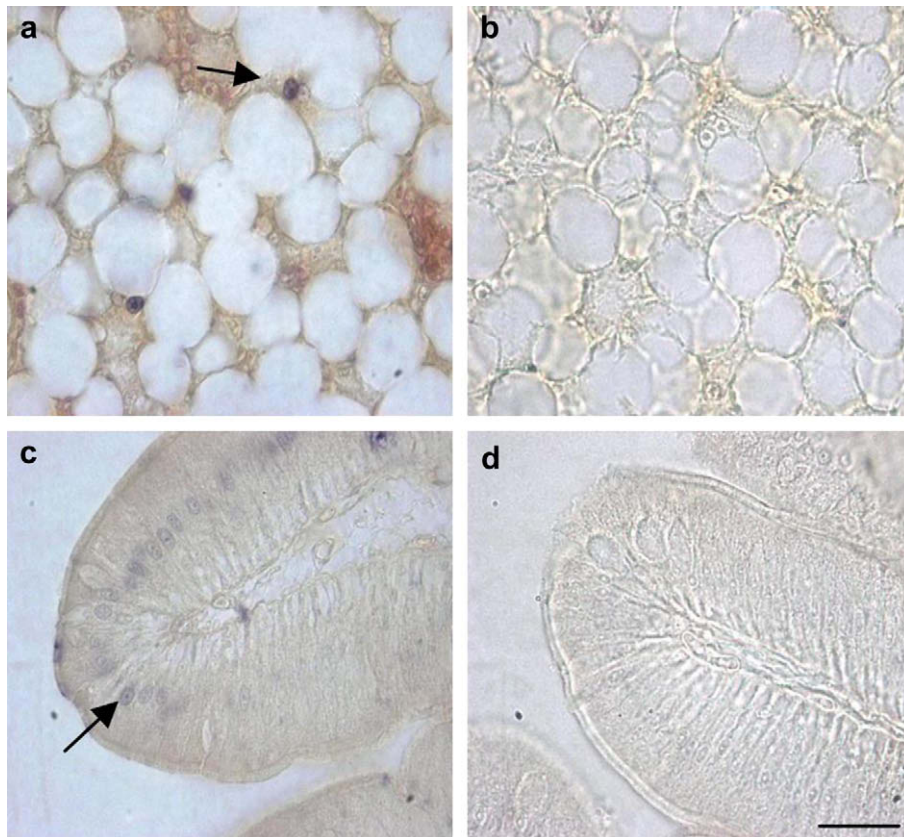


Fig. 9. Location of DIFBL mRNA in liver and gut. *In situ* hybridization with single-strand type DIFBL DIG_ribo probe: (a) liver with antisense DIFBL DIG_ribo probe; (b) control: liver with sense DIFBL DIG_ribo probe; (c) intestine with antisense DIFBL DIG_ribo probe; (d) control: intestine with sense DIFBL DIG_ribo probe; (a and b): bar 5 μ m; (c and d): 10 μ m.

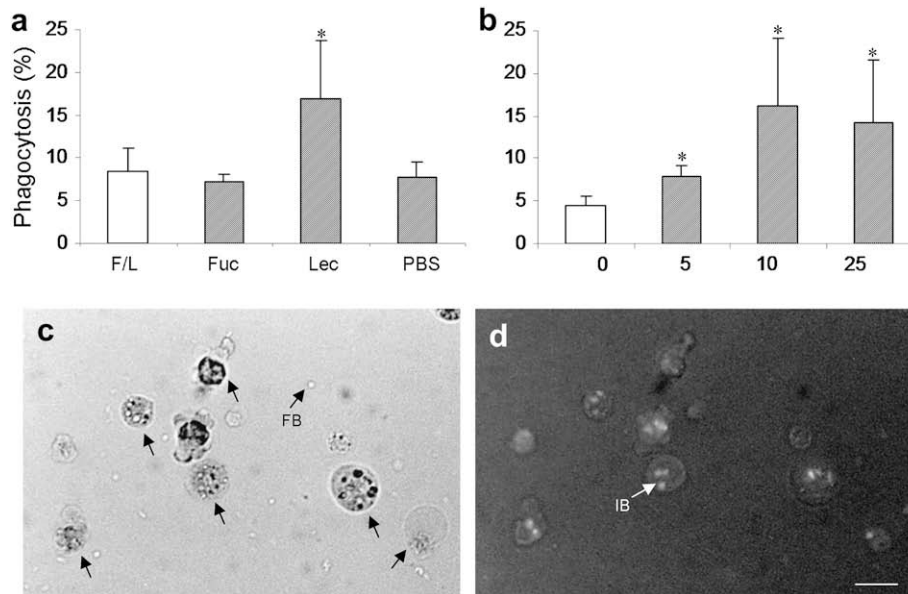


Fig. 10. Specificity and dose-response effect of the opsonic activity of DIFBL. Opsonic effect of DIFBL on sea bass peritoneal cavity leucocyte phagocytosis against *E. coli* (ATCC 25922) (a); effect of different doses of DIFBL on phagocytosis (b). Values are given as means \pm (N. 7 per group). Asterisk (*) denotes significant difference from control (0 mg ml⁻¹) at $p < 0.05$. Lec: Bacteria were opsonized with 10 μ g ml⁻¹ of DIFBL; bacteria Fuc: the bacteria were treated with 25 mM fucose; F/L: Bacteria were opsonized with 10 μ g ml⁻¹ of DIFBL in the presence of 25 mM fucose. Peritoneal cavity phagocyte (arrows) after FITC stained and opsonized bacteria; c; under light microscope; d under fluorescence microscope. IB: ingested bacteria; FB: Free bacteria. bar 20 μ m.

carried out. In agreement with the expression of fucose-binding lectins from *A. anguilla* [13], *M. saxatilis* and *Oncorhynchus mykiss* F-lectin [11,22], DIFBL transcripts were located in the nucleus of liver parenchymal cells and intestinal mucocytes of the columnar epithelium, whereas immunohistochemical analysis revealed that the DIFBL protein was located in the cytoplasm. Preliminary immunoblot results (work in progress) indicated that the DIFBL expression may be enhanced by challenging fish with intraperitoneal injection of bacteria (*Vibrio alginolyticus*).

The F-type lectin family received its name from the preferred binding to L-fucose of most of the members characterized so far [11,12]. The monosaccharide L-fucose is present as a non-reducing terminal sugar of a large variety of pro- and eukaryotic glycans [35]. It has been recently proposed that free L-fucose can be released into the human intestinal lumen through the hydrolytic activity of members of the indigenous microbial flora, as well as potential microbial pathogens, [36]. It is noteworthy that the presence of free L-fucose upregulated gene expression and secretion of their encoded proteins that are involved in both the innate and adaptive immune responses in which an active role of a fucose-binding lectin cannot be excluded [36]. In this regard, L-fucose-specific opsonizing capacity of DIFBL for bacteria targeted for phagocytosis by peritoneal macrophages suggests that this F-lectin may mediate immune defence responses both in the intestinal mucus and the blood stream.

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