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Primary structure and opsonic activity of an F-lectin from serum of the gilt head bream *Sparus aurata* (Pisces, Sparidae)

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Primary structure and opsonic activity of an F-lectin from serum of the gilt head bream *Sparus aurata* (Pisces, Sparidae)

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Abstract

The recently described fucose-binding agglutinin from the European eel revealed a novel lectin fold (the 'F-type' fold) that is shared with other carbohydrate-binding proteins and proteins from prokaryotes to vertebrates clustered under the newly established F-type lectin (FTL) family. We previously reported the purification and biochemical characterization of a fucose-binding protein (FBP) isolated from serum of the gilt head bream (*Sparus aurata*, SauFBP). In the present article, the complete coding sequence of SauFBP revealed that it is a member of the FTL family, consisting of two tandem carbohydrate recognition domains (CRD) that display the F-type sequence motif. *In vitro* opsonization assays showed that the isolated SauFBP binds to formalin-killed *Escherichia coli* and enhances their phagocytosis by peritoneal macrophages.

Keywords: F-lectin, *Sparus aurata*, teleost, hemagglutinins, opsonin

Introduction

The roles of lectin–carbohydrate interactions in self/non-self recognition in early development and innate immunity of vertebrates has been well documented (Kuhlman et al. 1989; Cooper et al. 1994; Matsushita et al. 1996; Tino & Wright 1996; Vasta & Ahmed 2008; Vasta 2009). Lectins specifically bind to soluble or cell surface-associated carbohydrate ligands through a variety of polar and non-polar interactions, mediated by selected amino acid residues housed in the carbohydrate recognition domain (CRD). These interactions may lead to precipitation of soluble glycans, and agglutination/immobilization of cells. In some ('chimeric' or 'mosaic') lectins, the combination of one or multiple CRDs with distinct functional domains enables additional effector functions including opsonization and phagocytosis, and activation of the complement pathway (Turner 1996; Fujita et al. 2004; Sharon & Lis 2004; Vasta et al. 2004). Based on their CRD sequence motifs and cations requirement, several animal lectin families have been categorized, such as C-, I-, X-, and P-type lectins, galectins, and pentraxins (Arason 1996;

Turner 1996; Sharon & Lis 2004; Vasta et al. 2004; reviewed in Vasta & Ahmed 2008, and Vasta et al. 2011). Recently, based on the identification of a novel CRD sequence motif and a unique structural fold, the F-type lectin family was identified both in prokaryotes and in fluids and tissues of invertebrates and vertebrates (Bianchet et al. 2002; Odom & Vasta 2006). Some of its members had been previously characterized as fucose-binding proteins (FBPs) in different contexts (Saito et al. 1997; Listinsky et al. 1998; Honda et al. 2000).

Members from most lectin families described in mammals, including C- and X-type lectins, and galectins have been isolated from fish serum, skin mucus and other tissues (Listinsky et al. 1998; Honda et al. 2000; Russel & Lumsden 2005; Odom & Vasta 2006). Furthermore, some lectin families unique to fish, such as the rhamnose-binding lectins, have also been identified in eggs and embryos (Watanabe et al. 2009). Lectins can exert opsonic activity (Arason et al. 1994; Arason 1996; Vasta et al. 2004; Salerno et al. 2009) or enhance respiratory burst and bactericidal activity of phagocytic

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cells (Yano 1996; Listinsky et al. 1998; Ottinger et al. 1999; Russel & Lumsden 2005).

F-type lectins (FTLs) were initially identified and characterized as such in the serum from fish (Bianchet et al. 2002; Odom & Vasta 2006). While the European eel (*Anguilla anguilla*) agglutinin (AAA; Odom & Vasta 2006) possesses a single CRD, those characterized in the striped bass (*Morone saxatilis*) (MsFBP32 and MsFBP32 II; Odom & Vasta 2006) and sea bass (*Dicentrarchus labrax*) (DIFBP; Salerno et al. 2009) exhibit two tandemly arrayed CRDs (N- and C-terminal CRDs). The structure of MsFBP32 in complex with L-fucose revealed a trimeric organization with two globular opposite halves containing, respectively, the N-CRDs and the C-CRDs (Bianchet et al. 2010). We proposed that fish F-lectins mediate immune defense responses both in the blood stream (Bianchet et al. 2002, 2010; Odom & Vasta 2006) and intestinal mucus (Cammarata et al. 2001, 2007; Salerno et al. 2009).

Previously, we reported the purification of sea bass (DIFBP) and gilt head bream (SauFBP32) serum FBPs (Cammarata et al. 2001, 2007; Salerno et al. 2009), and that SauFBP32 displays epitopes recognized by anti-DIFBP specific antibodies. Further, based on the DIFBP cDNA sequence, we showed that it is a bona fide F-lectin, shares carbohydrate specificity and biochemical properties with other well characterized F-lectins (Salerno et al. 2009), is expressed in larval and juvenile tissues and is contained in eggs (Parisi et al. 2010). Finally, using degenerate primers designed on the F-lectin conserved sequence, we amplified a SauFBP32 partial sequence with high similarity to F-type lectins.

In the present study, we further characterized the SauFBP transcript as encoding an authentic F-lectin with two CRDs, and demonstrate its opsonic properties for formalin-killed *Escherichia coli* to peritoneal macrophages.

Materials and methods

Reagents and bacterial strains

Unless otherwise specified, carbohydrates and reagents were purchased from Sigma (USA).

Escherichia coli (ATCC 25922) strain was from Chrysope Technologies (LA, USA). For the phagocytosis assay, the formalin-killed bacteria were washed three times with sterile phosphate-buffered saline (PBS) (6 mM KH₂PO₄, 0.11 mM Na₂HPO₄, 30 mM NaCl, pH 7.4), suspended in carbonate buffer to get a concentration of 1×10⁹ cell/ml (0.1 M Na₂CO₃, 0.1 M NaHCO₃, pH 9.5) containing 0.1 mg/ml fluorescein isothiocyanate (FITC) and incubated for 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 (PBS–Ca), and suspended in PBS–Ca to a concentration of 1×10⁸ cell/ml.

Animals, and preparation of serum and organ extracts

Fish were from the Ittica Trappeto fish-farm (Trappeto, Palermo, Italy), anesthetized in seawater containing 0.02% of 3-aminobenzoic acid ethyl ester (MS-222 Sigma), and bled by heart puncture. After 1 h at room temperature the serum was separated by centrifugation at 800g for 10 min at 4°C, aliquoted and stored at –20°C until use. Liver and gut were excised from freshly euthanized fish, frozen in liquid nitrogen, ground into powder with a mortar and pestle, suspended in tris-buffered saline (TBS): 50 mM Tris–HCl, 0.15 M NaCl, pH 7.4, and centrifuged at 15,000g (30 min, 4°C). The protein concentration of the extracts was assessed as indicated below.

Hemagglutination assay

Rabbit and sheep erythrocytes (RBC (red blood cells); kindly supplied by Istituto Zooprofilattico della Sicilia) were washed three times in PBS, centrifuged (500g for 10 min at 4°C) and suspended in TBS to reach the concentration of 1% containing 0.1% (w/v) pig gelatin. A volume (25 µl) of sea bream serum (1:10) or 25 µl of the purified SauFBL preparation (250 µg/ml; dialyzed in TBS) were serially (twofold) diluted with TBS–gelatin in 96-well round-bottom microtiter plates (Nunc, Denmark), and mixed (v/v) with the RBC suspension. After 1 h incubation at 37°C, the hemagglutinating titer (HT) was recorded as the reciprocal of the highest dilution showing clear agglutination.

Purification of serum fucose-binding lectin

SauFBL was purified by affinity chromatography following Cammarata et al. (2007). Briefly, the serum (2–5 ml), was diluted 10-fold in TBS, filtered through a 0.22-µm filter (Millex GV, Millipore), applied to an L-fucose–agarose column (1.6×5 cm, 5 ml) equilibrated with TBS, and washed with TBS (10 vols). The column was eluted with 200 mM L-fucose in TBS and the absorbance at 280 nm of the collected fractions (vol/fraction) recorded. Fractions deemed to contain protein were pooled and dialyzed against TBS.

Bacterial suspensions

Bacteria were cultured in tryptic soy broth containing 3% (w/v) NaCl at 25°C, with continuous shaking

(120 rpm) in a Gallenkamp incubator, and harvested at the exponential growth phase. The optical density of the bacterial culture was estimated, serial dilutions plated on tryptic soy agar/3% (w/v) NaCl, and the colony forming units counted. Bacteria were fixed by adding formaldehyde to the stock suspension to reach 2% final concentration and incubating overnight at 21°C, with shaking at 120 rpm. After centrifugation (6000g for 15 min at 4°C) fixed bacteria were washed (3 times) with sterile PBS, pH 7.2, suspended at 10⁹ cells/ml in PBS containing 0.1% (w/v) gelatin, and stored at 4°C until use.

Protein estimation

Protein content was estimated according to the method of Bradford (1976), using bovine serum albumin (from 0.1 mg/ml to 15 mg/ml) as a standard.

Polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl sulfate (SDS)–PAGE (10%) was performed according to the method of Laemmli (1970). For PAGE under reducing conditions, samples were treated with 5% mercaptoethanol. Proteins were stained with Coomassie Brilliant Blue R250. To estimate the molecular size of the bands of interest, gels were calibrated with low-range SDS–PAGE standard proteins (Bio-Rad, Richmond, CA).

Isolation of total RNA from liver and cDNA cloning

Total RNA was isolated from the liver by using the RNAqueous™-Midi Kit purification system (Ambion), and reverse-transcribed by the Kit Ready to Go T-primed first-strand using random primers (Amersham-Pharmacia Biotech). Amplification of the SauFBP32 cDNA sequence was performed using the degenerate primers (1 mM) DFBP1.F (5'-dCAAAGCTTTAYAACTAYAAARAACGTNGC-3') and DFBP3.R (5'-dTCGAATTCGTNACGATRTANGGCTC-3'). Amplification was performed following Cammarata et al. (2007) in an MJ Research DNA PTC 200 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, 72°C for 1 min) were carried out, and a final elongation at 72°C for 10 min was performed. The single 136-bp band visible on agarose-gel electrophoresis was excised, isolated, and ligated with pCR 4-TOPO (TA cloning Kit, Invitrogen) according to the manufacturer's instructions. To elucidate the entire cDNA sequence

of SauFBL, 3' and 5' RACE-PCR (rapid amplification of cDNA ends-polymerase chain reaction) was carried out from the 136-bp amplicon using specific primers based on the amplicon sequence. Plasmid DNA was isolated from recombinant bacterial clones using NucleoSpin extraction kit (Macherey-Nagel Sarl, Hoerd, France), and sequenced by CRIBI (University of Padova, Italy) (Cammarata et al. 2007). The cDNA sequences were completed using marathon RACE kit (Clontech) with internal specific primers. Sequence alignment to F-lectin family members was performed with CLUSTAL × v.1.83 (<ftp://ftp-igbmc.u-strasbg.fr/pub/>) and illustrated with GeneDoc v.2.6.002 (www.psc.edu/biomed/genedoc/). The signal peptide was predicted with the SignalP 3.0 (Bendtsen et al. 2004). The protein structural models were developed with SWISS-MODEL and the Swiss-PdbViewer (Guex & Peitsch 1997; Schwede et al. 2003; Arnold et al. 2006).

Phylogenetic analysis

The deduced amino acid sequence of SauFBP cDNA was subjected to multiple alignments using the CLUSTAL W program (Thompson et al. 1994), and the final sequence alignment was done using CLUSTAL X v.1.81 (Thompson et al. 1997), and similarity shaded with GeneDoc v.2.6.002 and Bioedit version 5.0. A phylogenetic tree was constructed by the Neighbor-Joining method (NJ) after 1000 bootstrap iterations.

Phagocytosis assay

Peritoneal cells were harvested by injecting 10 ml L-15 medium containing 100 U/ml penicillin/streptomycin and 10 U/ml heparin into the peritoneal cavity of anesthetized fish. After massaging the body cavity for 10 min, the exudate containing the suspended peritoneal cells was collected with a sterile syringe (22-gauge needle), and centrifuged at 500g for 10 min. The cells, suspended in 1 ml L-15 medium, were counted with a hemocytometer. The cell suspension was adjusted to 1×10⁷ cell/ml, and cell viability recorded by Trypan blue exclusion test. Macrophages from the peritoneal exudate (1×10⁷/ml), fluorescent bacteria (1×10⁸/ml), and purified SauFBL (10 µg/ml in PBS–Ca) (or PBS–Ca alone as a control) were mixed (v/v) in a microtube and incubated for 30 min at 18°C. Subsequently, Trypan blue (2 mg/ml in 0.15 M NaCl, 0.02 M citrate buffer, pH 4.4, containing Crystal violet 2 mg/ml in PBS, pH 7.4) was added to each tube to quench the fluorescence of the non-ingested particles (Hed 1986). The fluorescent ingested particles were

observed under a UV light microscope equipped with a Nomarsky differential interference contrast optic (Diaplan, Leica, Wetzlar, Germany). The phagocytic rate (PR) was calculated for the experimentals and controls as the percent of cells showing internalized bacteria, and the phagocytic index (PI) as the average of ingested bacteria upon the amount of counted phagocytes.

SauFBP recovery from the bacterial surface

At the end of bacteria opsonization, the lectin was removed from the opsonized *E. coli* pellet by fucose elution. The bacteria (1×10^7 cell/ml) were washed by centrifugation (4000g for 10 min at 4°C) with TBS. To retrieve the lectin, the bacteria were incubated for 20 min in a rotating shaker with 75 μ l of TBS containing 100 mM of L-fucose at 21°C. The supernatant was separated from the bacterial pellet by centrifugation (4000g for 10 min at 4°C) and, finally, centrifuged in a micro-concentrators Corning Spin-X UF (MWCO 5K) to remove the fucose. The recovered SauFBP was subjected to SDS-PAGE under reducing condition and analyzed in Western blotting.

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 (Biorad, USA). The filter was soaked in blocking buffer (PBS containing 3% bovine serum albumin (BSA) and 1% Tween 20) for 1.5 h. After washing with PBS-T, the nitrocellulose sheet was incubated with anti-D/FBL-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.

Results

cDNA sequence and primary structure analysis

The 1149-bp 3' and 5' RACE-PCR cDNA product contained the complete SauFBL cDNA sequence (Figure 1) with a 1137 open reading frame encoding the 333 amino acids. The putative translation start site is 90 bp downstream of the 5'-UTR (untranslated region), and a 34-nucleotide 3'-UTR spans from stop

codon to the polyadenylation site resulting in a 1137-bp transcript, without including the poly (A) tail.

The deduced mature protein sequence (Figure 1) is 315 amino acids long, with a calculated molecular mass of 36.57 kDa. The cleavage site of the 18-residue signal peptide at the N-terminal resides between Ala18 and Tyr19. Sequence comparison with the AAA F-type CRD revealed about 50% identity spanning from Asn 5 to Gly 144 (N-CRD) and from Asn155 to Gly287 (C-CRD) connected by a 10 amino acid linker.

Sequence comparison and phylogenetic analysis

Basic Local Alignment Search Tool (BLAST) analysis showed that the SauFBL deduced amino acid sequence presents close homologies with vertebrate binary F-lectins. Detailed sequence analysis of binary fish F-lectin CRDs (Figure 2) showed highly conserved sequences in their N-CRDs or C-CRDs, the N-CRDs being closer to the *Anguilla* CRD cluster. Conserved Cys can form two disulfide bonds (Cys42–Cys139 and Cys97–Cys113), whereas an additional bond between the two neighbouring residues Cys74 and Cys75 in N-CRD can be formed as in AAA, and participate in ligand binding via a hydrophobic interaction with the sugar. The canonical triad of basic amino acids involved in sugar binding (His44, Arg70, Arg77) are present in both SauFBL N-CRD and C-CRD (Figure 3). Moreover, the SauFBL32 N- and C-CRD structural analysis showed that the similarity to MsFBP32 (83.57%, of identity E value 7.53e-138) concerns the internal CRD sequence (Figure 4, labeled in green) except for a C-terminal elongation that falls out of the CRD (Figure 4, labeled in red).

Effect of SauFBP on phagocytic activity of peritoneal cavity sea bream leukocytes

SauFBP was isolated from *Sparus aurata* serum by using an affinity column of L-fucose-agarose, following Cammarata et al. (2007). The molecular weight and purity were analyzed by PAGE electrophoresis (10%) under reducing and non-reducing conditions. In three distinct experiments, the phagocytic activity of peritoneal macrophages for control bacteria, and bacteria pre-exposed to purified SauFBP was examined under light and fluorescence microscope. FITC-labeled bacteria that were pre-exposed to SauFBP were internalized at significantly higher rates ($16.9 \pm 6.9\%$; 450 cells/experiment) than the unexposed control FITC-labeled bacteria ($7.4 \pm 2.1\%$; 450 cells/experiment). This opsonic effect reached the highest level when bacteria were pretreated

```

1          ACTGTCAACAACGAGGCCCAAAGAGTTAAGAAGCTCCGGC
1          m k h
39  TGCTCATCAGAACACAGGCAAATCATTTTTCTAACCAGGAATCAGGAATAATGAAGCAC

4      t g v f i l l l f f g s c s a Y T Y Q N
99  ACTGGTGTATTTATTTTGTGCTCTTCTTCGGGTCATGCTCAGCTTATACTTACCAAAT

24      V A L R G K A T Q S Y R Y E P S N Y G A
159  GTGGCCCTACGAGGCAAAGCAACTCAGTCATACCGCTATGAGCCTAGTAATTATGGGGCT

44      A Y N A I D G N R E T D F K A G S C T H
219  GCATACAATGCCATTGATGGAACCGCGAGACTGATTTTAAAGCCGGTTCATGCACCCAT

64      T A E Q T N P W W R V D L L E S Y I V T
279  ACCGCTGAACAAACCAACCCCTGGTGGAGAGTTGACCTGCTGGAGTCCACATCGTCACC

84      S V T I T N R G D C C P E R I N G A E I
339  TCCGTCACCATCACCAACAGAGGAGACTGCTGTCCAGAAAGGATCAACGGAGCTGAGATC

104     R I G N S L L D G G A A N P R V G V I S
399  CGCATCGGAAACTCTTTACTAGACGGTGGTGTGCAAACCCAAGGGTGGTGTAAATTCT

124     H I P A G I S H T F S F T E R L G G R Y
459  CATATCCCTGCAGGGATCTCACATACTTTCAGTTTCACTGAACGTCTGGGGGGACGTTAC

144     V T V L I P G T N K F L T L C E V E V Y
519  GTGACTGTGCTTATACCTGGTACAAACAAGTTTCTTACACTCTGTGAAGTGGAGGTTTAT

164     G Y R A P T G E N L A L Q G K A T Q S S
579  GGGTACCGAGCCCCAACTGGAGAGAACCTGGCCCTCCAAGGAAAAGCCACACAGTCTTCA

184     L Y E F G I A Y N A I D G N Q A N N W I
639  TTGTATGAATTTGGTATTGCCTATAATGCCATTGACGGGAATCAAGCCAACAATTGGATA

204     K A S C S H T N K E M N P W W R L D L R
699  AAGGCCTCCTGCAGTCACACAAACAAGGAGATGAACCCCTGGTGGCGACTGGATCTGCGC

224     K T H R V F S V K V T N R D S C E Q R I
759  AAAACCCACAGAGTGTTTTCTGTTAAGGTAACCAACCGAGATTCATGTGAACAACGAATC

244     N G A E I R I G D S L D N N G N N N Q R
819  AATGGAGCCGAGATCCGAATTGGAGATTCCTCGACAACAACGGCAACAACAATCAGAGG

264     C A V I T S I P A S A T T E F Q C N G M
879  TGTGCTGTGATCACAAGCATCCAGCAAGTGTACTACTGAATTCCAGTGTAAATGGGATG

284     D G R Y V N I V I P G K E E Y L T L C E
939  GATGGCCGCTATGTTAACATTGTTATCCCTGGAAAAGAAGAGTACCTGACCCTGTGTGAG

304     V G G V W L C P G L G V N T N T A E C T
999  GTGGGAGGTGTATGGCTCTGTCCTGGATTAGGTGTCAATACTAATACTGCTGAATGTACG

324     Q T K Q N S R L S *
1059  CAAACAAAACAAAATAGTAGATTAAGCTAAAATTTGTTTCCATTCAAATAAGACTTATC

1119  CAATAAAATTACATCACGAAAAAAAAAAAAA

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Figure 1. The complete cDNA and deduced protein sequence of SauFBP (GenBank accession number JN107560). The cleaved signal peptide is indicated by lowercase amino acids. Chemically sequenced 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 gray (N-CRD is light gray and the C-CRD dark gray).

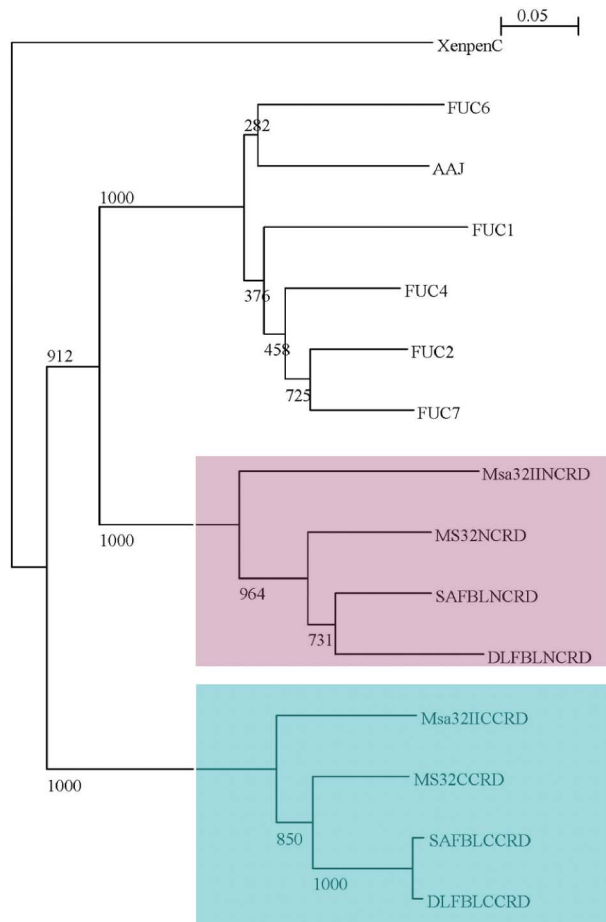


Figure 2. Analysis of genetic distance between FBP CRD domains. The phylogram was created from NJ analysis using CLUSTAL_X v.1.81. Distances were corrected for multiple substitutions and gap positions were excluded. Bootstrap values are percentages from 1000 iterations. The pink box contains the FBP N-CRDs whereas the blue box contains the C-CRDs.

with 10 $\mu\text{g/ml}$ ($p < 0.001$). The phagocytosis index increased accordingly, from 2.0 ± 0.8 bacteria/cell (untreated bacteria) to 3.9 ± 2.2 bacteria/cell (opsonized bacteria) ($p < 0.005$; 450 cells/experiment).

When fucose (0.1 M, final concentration) was added in the pre-treatment FITC-labeled bacteria–SauFBP suspension, the SauFBP32 opsonizing effect was significantly reduced to approximately the control values ($7.7 \pm 1.8\%$; 450 cells/experiment) and the supernatant from the centrifuged bacteria contained a Western blot-detectable SauFBP (Figure 5, lane 2).

Discussion

We previously reported the purification by agarose–fucose affinity chromatography and characterization of the 32-kDa lectin SauFBL32 from the serum of the gilt head bream *Sparus aurata*. SauFBL32

shared some biochemical properties and carbohydrate specificity with members of the recently established F-type family of fucose-binding lectins, first identified by Odom and Vasta (2006). This lectin family possesses characteristic L-fucose-binding and calcium-binding sequence motifs, and a unique lectin structural fold (Bianchet et al. 2002). The F-lectin sequence motif has been identified in single or multiple tandemly arrayed CRDs in eubacteria, invertebrates, fishes and amphibians (Odom & Vasta 2006). Interestingly, a common feature of this lectin family is the mode of expansion and diversification of tandem CRD repeats which appears to be the exclusive of some teleost lineages, such as the four-tandem CRD F-lectins that are unique to the salmoniformes (Vasta et al. 2004).

N-terminal amino acid sequence and the fucosyl binding specificity suggested that SauFBL could be a member of the F-type lectin family (Cammarata et al. 2007). Further, thermal stability and Ca^{2+} -independent binding properties of SauFBL were similar to AAA (Bianchet et al. 2002). Moreover, like the striped bass MsFBP32 (Odom & Vasta 2006), the native SauFBL32 behaved as a 30-kDa and 31.5-kDa monomer in SDS–PAGE under non-reducing conditions and size exclusion chromatography, respectively. Further, the decrease in electrophoretic mobility (35 kDa) under reducing conditions suggested the presence of intramolecular disulfide bonds in the native SauFBL32, also observed in AAA and MsFBP32.

There are relevant biochemical structural and functional similarities between SauFBL and the two binary tandem CRD F-type lectins isolated from striped bass (MsFBP32) (Odom & Vasta 2006), and sea bass (DIFBP) (Salerno et al. 2009). The complete SauFBL cDNA sequence revealed that this lectin, like MsFBP32 and DIFBP, possesses two tandemly arrayed CRDs. The SauFBL cDNA presents an open reading frame encoding 333 amino acid residues, including an 18-amino acid signal sequence at the N-terminus. Thus, the deduced size of 35 kDa for the mature protein is in agreement with the experimental data from SDS–PAGE under reducing conditions of the purified protein (Cammarata et al. 2007). Sequence analysis demonstrated that SauFBL is a binary tandem domain F-type lectin with the N- and C-CRDs connected by a 10 amino acid peptide linker. Furthermore, BLAST analysis revealed sequence homologies with other vertebrate F-lectin CRDs, and the phylogenetic tree shows that SauFBL clusters with other teleost binary F-type lectins, clearly distinguishable from the clusters comprising F-lectins containing a single CRD (eels), or more than two CRDs. Both

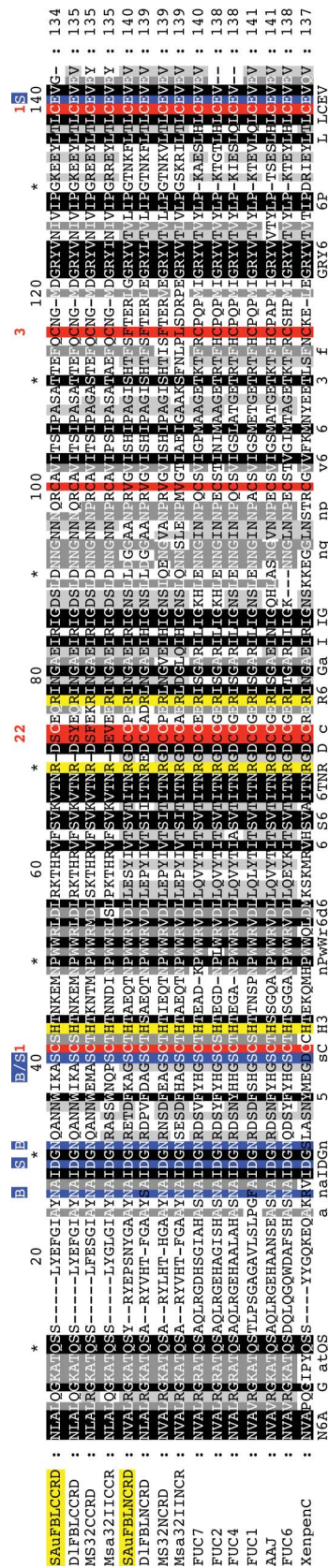


Figure 3. Conservation of the functionally relevant amino acid residues of SauFBL within the F-type lectin family. Residues that interact with the calcium ion through their side chain oxygen are highlighted in blue. Half-cysteines highlighted in red below the alignment indicates disulfide bridge partners. Sugar-binding residues are highlighted in yellow. Organism abbreviations: Msa, *Morone saxatilis* (striped bass); FUC, *Anguilla japonica* (Japanese eel); Xenpen, *Xenopus tropicalis* (diploid clawed frog); DL, *Dicentrarchus labrax*. Alignment was produced with CLUSTAL_X v.1.81 and illustrated with GeneDoc v.2.6.002. Invariant residues are shaded black; conserved residues, as defined by the Blossum62 similarity matrix, in $\geq 80\%$ of sequences are shaded gray 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 Blossum 62 matrix similarity groups.

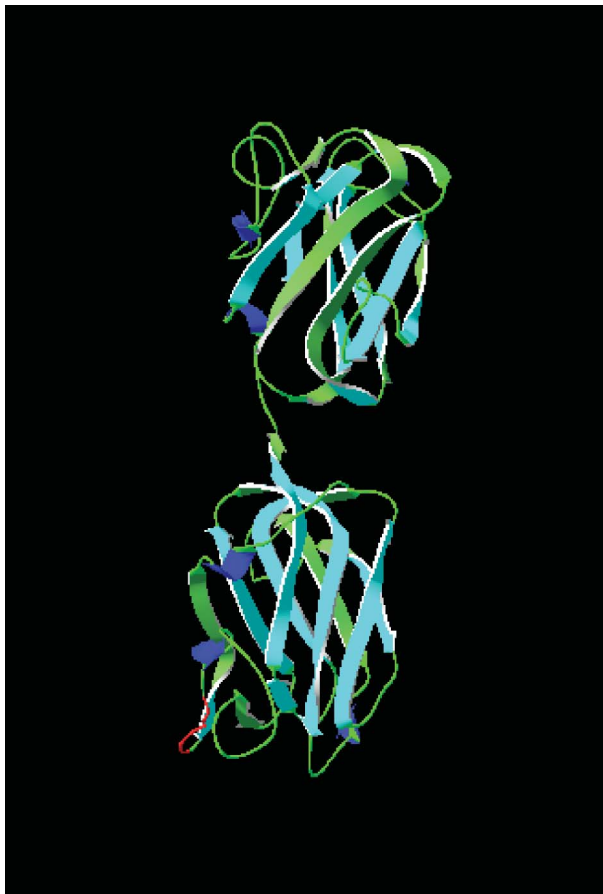


Figure 4. Homology modeling and exon-domain relationship. The N- and C-CRD of SaFBL were modeled on the template of MsFBL32 (swissprot 3cqoB). The homologies are shown in green, the difference stained red.

N- and C-CRD present highly conserved sequences characterized by Cys residues located at amino acid positions consistent with the formation of two intrachain disulfide bonds. The presence of intrachain disulfide bonds was supported by the differential electrophoretic mobilities under reducing (35 kDa) and non-reducing conditions (30 kDa). Binary CRD F-type lectins are shared by several teleost species, and are most likely a result of gene duplications that could have occurred independently in various perciform lineages (Vasta et al. 2004). In addition, the single-CRD F-lectins from eels, perhaps the ancestral state of this lectin family within the rayfined fish (Odom & Vasta 2006) suggests that binary homologues could be products of diversification through evolutionary gene duplications (Vasta et al. 2004). In this respect, the N-CRDs of the binary CRD F-type lectins show the highest similarity to the unique AAA CRD. The SauFBP32 primary sequence and CRDs organization confirms that it is a binary CRD F-type lectin,

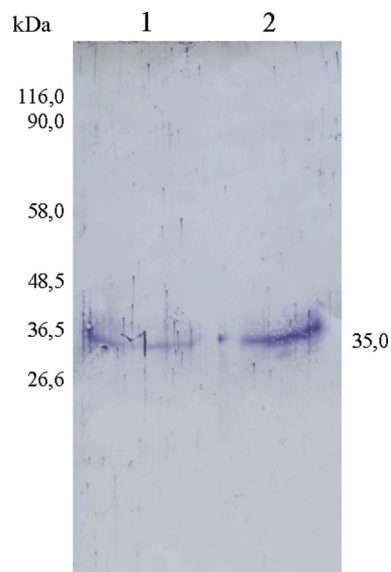


Figure 5. Western blot of the SauFBP eluted from the opsonised bacteria. Immunoblotting of *S. aurata* FBL eluted from the opsonized bacteria subject to SDS-PAGE under reducing conditions and immunoblotted with anti-*D. labrax* F-lectin antibodies. Lane 1: supernatant from the opsonized bacteria after L-fucose incubation; Lane 2: purified SauFBP.

and suggests that it is orthologous to MsaFBP and DIFBP.

Although the hydrodynamic and electrophoretic behavior of MsFBP32 suggested that the native form is a monomer, the recently resolved crystal structure of binary F-type lectin MsFBL32 revealed that its native quaternary structure is a trimer. In this MsFBL32 oligomeric structure the distinct sugar-binding faces (N- and C-CRDs) located at opposite ends of the dumbbell-shaped molecule with the ability to cross-link distinct glycans, perhaps on 'self' and 'non-self' surfaces (Bianchet et al. 2010). This would rationalize the observed opsonic properties of binary CRD F-lectins (Salerno et al. 2009). The hydrodynamic and electrophoretic behavior of SauFBP under the experimental conditions tested, also indicate a monomer as the native species. It is possible that like MsFBL32, under conditions of local high concentrations SauFBP also forms trimers endowed of a similar ability to bind different glycans on the surface of microbial pathogens and macrophages.

The monosaccharide L-fucose and fucose derivatives such as colitose are present as terminal sugars of a large variety of pro- and eukaryotic glycans (Staudacher et al. 1999), and are likely the natural ligands of F-type lectins (Bianchet et al. 2002). Therefore, the opsonic activity of the sea bass DIFBL (Salerno et al. 2009) and SAuFBL32, as revealed in this study, strongly suggests that fish binary CRD F-type lectin may have an active role as recognition

factors in innate immune functions by enhancing phagocytosis.

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