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Isolation and characterization of a fish F-type lectin from gilt head bream (*Sparus aurata*) serum

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Abstract

A novel fucose-binding lectin, designated SauFBP32, was purified by affinity chromatography on fucose–agarose, from the serum of the gilt head bream *Sparus aurata*. Electrophoretic mobility of the subunit revealed apparent molecular weights of 35 and 30 kDa under reducing and non-reducing conditions, respectively. Size exclusion analysis suggests that the native lectin is a monomer under the selected experimental conditions. Agglutinating activity towards rabbit erythrocytes was not significantly modified by addition of calcium or EDTA; activity was optimal at 37 °C, retained partial activity by treatment at 70 °C, and was fully inactivated at 90 °C. On western blot analysis, SauFBP showed intense cross-reactivity with antibodies specific for a sea bass (*Dicentrarchus labrax*) fucose-binding lectin. In addition, the similarity of the N-terminal sequence and a partial coding domain to teleost F-type lectins suggests that SauFBP32 is a member of this emerging family of lectins.

Keywords: F-type lectin; *Sparus aurata*; *Dicentrarchus labrax*; Teleost; Serum hemagglutinins

1. Introduction

Lectins are typically multivalent proteins that recognize and bind specific carbohydrate moieties through carbohydrate recognition domains (CRDs) [1,2]. In vertebrates, the role of lectins as mediators of self and non-self recognition in early development and innate immunity has been well documented [2–5]. The presence of multiple CRDs in combination with other protein domains, enable not only the recognition of carbohydrates on the surface of potential pathogens, but also effector functions including agglutination, immobilization and opsonization of potential pathogens, and activation of the complement pathway [6–8]. Based on their primary structure, requirement of divalent cations, and structural fold, animal lectins have been classified into several families, such as C-, P-, and I-types, galectins, and pentraxins [6,9]. Lectins have been isolated from serum, plasma, mucus and eggs from several fish species but their biological function(s) have only been partially elucidated [10–13].

The presence of terminal L-fucose (6-deoxy-L-galactose) as non-reducing terminal residue on various glycoproteins and glycolipids is a key moiety mediating many cellular interactions [14]. Expression of fucose-containing antigens has been observed to dramatically increase during inflammation [14]. Lectins that recognize fucose have been detected in tissues and fluids of vertebrate and invertebrate species [16]. In mammals, most fucose-binding lectins identified so far belong to the C-type family. Recently, a new family of lectins specific for fucose (F-type lectins) was described with members present in both prokaryotes and eukaryotes, including invertebrates and

Abbreviations: SauFBP, *S. aurata* Fucose-binding protein; DlaFBP, *D. Labrax* Fucose-binding protein; MsaFBP, *M. saxatilis* Fucose-binding protein; AAA, European eel agglutinin; 2-ME, 2-β-mercaptoethanol; CRD, carbohydrate recognition domain; HT, hemagglutinating titre; HA, Hemagglutinating activity; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis (β-aminoethylether) *N,N,N′,N′*-tetraacetic acid; FBP, fucose-binding protein; Gal, D-galactose; Glc, D-glucose; NBT/BCIP, nitroblue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate; ORF, open reading frame; PVDF, polyvinylidene difluoride; HPLC, High pressure liquid chromatography; RACE, rapid amplification of cDNA ends; RBC, red blood cells; RT-PCR, reverse transcription/ polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography

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vertebrates [15]. This lectin family shares a characteristic sequence motif in the CRD, and a novel structural fold [18]. In teleost fish, well-characterized members of this family are the serum lectins from the Japanese eel *Anguilla japonica* [16], sea bass *Dicentrarchus labrax* [17], European eel (*A. anguilla*) [18], and the striped bass *Morone saxatilis* fucose-binding protein [15].

In this paper we report the identification, purification and characterization of a fucose-binding lectin from serum of the gilt head bream (*Sparus aurata*). The similarity of the N-terminal sequence and partial coding sequence to the F-type lectins confirms that it belongs to this recently recognized lectin family.

2. Materials and methods

2.1. Reagents and bacterial strains

If not otherwise specified carbohydrates and reagents were purchased from Sigma (USA) *E. coli* (ATCC 25922) strain was from Chrysope Tecnologies (La). Virulent strains of *Vibrio alginolyticus* were isolated from infected fish or supplied by Istituto Zooprofilattico Sperimentale (IZS), Palermo.

2.2. Animals and serum preparation

Fish were provided by Ittica Trappeto fish-farm (Trappeto, Palermo, Italy), anesthetized in sea water 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, and the serum was separated by centrifugation (10 min, 800×*g*, 4 °C), divided in aliquots and stored at −20 °C until use.

2.3. Hemagglutination assay

Rabbit and sheep erythrocytes (RBC, supplied by IZS) were washed three times with phosphate buffered saline (PBS, pH 7.4, 0.1% (w/v) pig gelatin), suspended at 1% in TBS 0.1% gelatin, and used in a microtitre plate hemagglutination assay (HA) in which 25 µl of serially diluted serum or purified lectin (250 µg/ml) were mixed with an equal volume of RBC suspension. Serial dilutions (2-fold) of the TBS-dialyzed and diluted serum (1:10) were performed with TBS containing 0.1% gelatin. The hemagglutinating titre (HT) was evaluated after 1 h incubation at 37 °C, and expressed as the reciprocal of the highest serum dilution showing clear agglutination.

2.4. Physical and chemical treatments

To examine divalent cation requirements for lectin binding, $CaCl_2$ or $MgCl_2$ was added in the HA medium to obtain 5–10 mM final concentration. EDTA (10 mM) or EGTA (10 mM) were used to examine the effect of Ca^{++}/Mg^{++} depletion on the lectin activity. To examine the thermal stability of the protein, the purified lectin (250 µg/ml) was incubated at 18 °C, 37 °C, 50 °C, 60 °C, 70 °C and 90 °C for 20 min and cooled down for 10 min on ice before the HA. Susceptibility of the lectin to freeze–thaw was examined by carrying out the HA on samples maintained at −20 °C for 2 months and thawed at room temperature.

2.5. Purification of serum fucose-binding lectin

Serum (2–5 ml) was diluted 10 times with TBS (50 mM Tris HCl, 0.15 M NaCl, pH 7.4) and filtered through a 0.22 µm filter (Millex GV, Millipore), applied to a 5 ml column of L-fucose–agarose, and washed with TBS (10 volumes), following Honda et al. [16]. The column was eluted with 200 mM L-fucose in TBS and absorbance at 280 nm was measured for the collected fractions (volume/fraction). Fractions deemed to contain protein were pooled and dialyzed against TBS.

2.6. Bacterial suspensions

Bacteria cultured in tryptic soy broth containing 3% (w/v) NaCl at 25 °C, 120 rpm were harvested at the stage of exponential growth. The density of bacteria was estimated from serial dilutions plated on tryptic soy agar/3% (w/v) NaCl and counting colony forming units. To kill bacteria, formaldehyde was added to the stock suspension to give 2% final concentration and shaken overnight at 21 °C, 120 rpm. After centrifugation (6000×*g*, 15 min, 4 °C) the killed bacteria were washed three times with sterile phosphate buffered saline (PBS) pH 7.2, suspended 1×10^9 cells/ml in PBS containing 0.1% (w/v) gelatin and kept at 4 °C until use.

2.7. Bacterial agglutination assay

The assay was carried out in standard 96 wells microtitre plates. Activity of serum and purified fucolectin (30–250 µg/ml) was assayed after serial dilutions in PBS 0.1% gelatin; the same volume (25 µl) of bacterial suspension containing $n\times10^7$ cells was added to each well. As a control, bacteria were incubated with PBS 0.1% gelatin.

The plates were then covered and incubated for 24 h at room temperature. Bacterial agglutination was assessed under a phase contrast microscope. The agglutination titer was reported as the highest dilution in which agglutinates were clearly visible.

2.8. Protein estimation

Protein quantitation was performed according to Bradford [1976], using bovine serum albumin (BSA, ranging from 0.1 mg/ml to 15 mg/ml) as a standard.

2.9. Polyacrylamide gel electrophoresis

SDS-PAGE was performed on a 10% gel according to Laemmli [19]. Reducing conditions were obtained by treating the sample with 5% mercaptoethanol. Proteins were stained with Coomassie brilliant Blue R250. To evaluate the molecular size, gels were calibrated with low range SDS-PAGE standard proteins (Bio-Rad, Richmond, CA).

2.10. Analytical size exclusion chromatography (SEC)

The molecular weight of native SauFBP32 was determined by SEC. From a 1 mg/ml solution of SauFBP, 200 µl was loaded onto a Superose 12 HR 10/30 connected to an AKTA Purifier HPLC (Amersham Biosciences). The column was pre-equilibrated in TBS-Ca (50 mM Tris–HCl, 100 mM NaCl, 10 mM $CaCl_2$, pH 7.4) and monitored at 280 nm. Calibration of the column was performed using an LMW gel filtration calibration kit (Amersham Biosciences). The molecular weight for SauFBP32 was interpolated from a 3rd order polynomial curve (Microsoft Office Excel) fitted to the results.

2.11. Immunoblotting analysis

SDS-PAGE gels were soaked in transfer buffer (20 mM Tris, 150 mM glycine, pH 8.8) for 10 min and proteins transferred to nitrocellulose for 1 h at 210 mA in transfer buffer. The filter was soaked in blocking buffer (PBS containing 2% (w/v) BSA and 0.05% (v/v) Triton X-100) for 2 h, incubated with a rabbit anti-*Dicentrarchus labrax* F-type lectin antiserum (1:800) for 1 h, 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). Finally, the filter was washed with PBS (4×15 min) and developed with 3 ml of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma).

2.12. Carbohydrate specificity

Hemagglutinating activity was assayed with rabbit RBC in the presence of saccharides as potential inhibitors of lectin binding. Inhibition experiments were carried out using decreasing concentrations (starting from 100 mM, in TBS pH 7.4; 5 mM $CaCl_2$; pH 7.4) of monosaccharides (L-fucose, D-arabinose,

Table 1
Hemagglutinating ($titer^{-1}$) activity of *S. aurata* serum and the purified lectin (50 μg/ml) towards human, rabbit and sheep erythrocytes and bacteria

Target	Serum	Isolated fraction
Human A	–	–
Human B	–	–
Human AB	–	–
Human O	–	–
Rabbit	256	32–128
Sheep	–	–
E. coli	32	–
Vibrio alginolyticus	64	–

D-galactose, D-glucose, D-mannose, *N*-acetyl-galactosamine, and *N*-acetyl-glucosamine), oligosaccharides (maltose, lactose, D-raffinose, cellobiose), and mannan (from *Saccharomyces cerevisiae*).

2.13. N-terminal sequencing

The purified lectin from SDS-PAGE gels run under reducing conditions was electrophoretically transferred (4 μg/well) to Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), at 160 mA for 1 h. The membrane was stained with an aqueous solution of Coomassie blue R-250, extensively rinsed with distilled water, and the band corresponding to the isolated protein was excised. N-terminal and internal aminoacid sequences were determined by automated Edman degradation at the Microchemical and Proteomics Facility, Emory University, Atlanta, GA.

2.14. Isolation of total RNA from liver, synthesis of first strand cDNA and cDNA cloning

Total RNAs from *Dicentrarchus labrax* and *Sparus aurata* were isolated from liver by using a RNAqueousTM-Midi Kit purification system (Ambion) and reverse transcribed with a Ready-to-Go T-primed first-strand using random primers (Amersham Biosciences). Amplification was performed using 1 mM of the degenerate primers, DFBP1.F and DFBP3.R [15]. Thermal cycling was performed with an MJ Research DNA engine PTC-100 and the following parameters were used: after a 94 °C for 3 min. denaturation step, primers were annealed at 37 °C for 30 s after which, 35 amplification cycles were performed (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. A single band of 133 bp in size was visible on agarose. This product was ligated into the pCR 4-TOPO (Invitrogen) according to the manufacturer's instructions and plasmid DNA was isolated from recombinant bacterial clones using NucleoSpin extraction kit (Macherey-Nagel Sarl, Hoerdt, France) and sequenced by CRIBI (University of Padova-Italy). Sequence alignment to other F-lectins was produced with Clustal X v.1.83 (ftp://ftp-igbmc.u-strasbg.fr/pub/) and illustrated with GeneDoc v.2.6.002 (www.psc.edu/biomed/genedoc/).

3. Results

3.1. Hemagglutinating activity (HA) of the Sparus aurata serum

The diluted serum from *Sparus aurata* (1.0–2.4 mg/ml of protein) agglutinated rabbit erythrocytes but not sheep erythrocytes, human A and B and 0 groups and bacteria (Table 1). Hemagglutinating activity of the serum samples was maintained after 2 months at −20 °C, reduced by 50% and 70% at 60 °C and 70 °C for 30 min, respectively, and abolished at 90 °C. HA at pH 7–9 yielded the highest HT, whereas at pH lower than 7 or higher than 9 HT were reduced in 50–60%. Addition of $CaCl_2$ or $MgCl_2$ (5 mM) or EDTA or EGTA (10 mM, either in the presence or absence of $CaCl_2$ or $MgCl_2$) to individual sera (HA ranging from 64 to 512) did not significantly affect the HA.

3.2. Carbohydrate specificity of the serum HA

As shown in Table 2, 25 mM L-fucose and 100 mM D-galactose (monosaccharides) and 200 mM melibiose and 100 mM lactulose fully inhibit hemagglutination by serum. However, no significant inhibition was observed when the monosaccharides glucose and N-acetyl-glucosamine and the oligosaccharides arabinose, cellobiose, lactose, maltose, D-mannose, N-acetyl-galactosamine and D-raffinose were assayed up to a final concentration of 200 mM.

3.3. Lectin purification and characterization

A typical affinity purification profile on L-fucose–agarose is shown in Fig. 1. The eluted protein represented approximately 0.1–0.5% of the total serum protein loaded onto the column. HT of the separated fractions (28–36, Fig. 1) assayed against rabbit RBC ranged from 1:64 to 1:518, and the activity was abolished by addition of L-fucose (Table 2). Electrophoresis on SDS-PAGE under non-reducing conditions revealed a single 30.0±2.2 kDa component, whereas under reducing conditions the apparent mass was 35.5±1.2 kDa (Fig 2A). An antiserum against a *Dicentrarchus labrax* F-type lectin cross-reacted with the purified *S. aurata* lectin (Fig. 2B). A cross-reacting component of similar electrophoretic mobility was identified by Western blot in *S. aurata* whole serum. Similarly, the molecular weight for native lectin determined by SEC under the experimental conditions tested was 31.5 kDa (Fig. 3).

3.4. Aminoacid sequence of the isolated lectin

The protein identified by SDS-PAGE under non-reducing conditions was transferred to PVDF membrane and sequenced. The determined N-terminal sequence was YTYQNVALRG-KATQSYRxG (Table 3; *x*=no aminoacid identified in this cycle).

Table 2
Inhibition of hemagglutination activity of the *Sparus aurata* serum or isolated lectin with RE by various sugars

Inhibitor	Minimum concentration (mM) of sugar required for 100% inhibition of HA reaction	
	Serum	Isolated lectin
L-fucose	25 mM	12.5 mM
D-galactose	100 mM	25.0 mM
Lactulose	100 mM	25.0 mM
Melibiose	200 mM	25.0 mM

Arabinose, cellobiose, D-glucose, Lactose, Maltose, Mannan, D-mannose N-Ac-galactosamine N-Ac-glucosamine, D-raffinose; LPS (from *E. coli*, *P. aeruginosa*, and *S. marcescens*)=no inhibition for 200 mM of sugar concentration and 1 mg/ml for LPS.

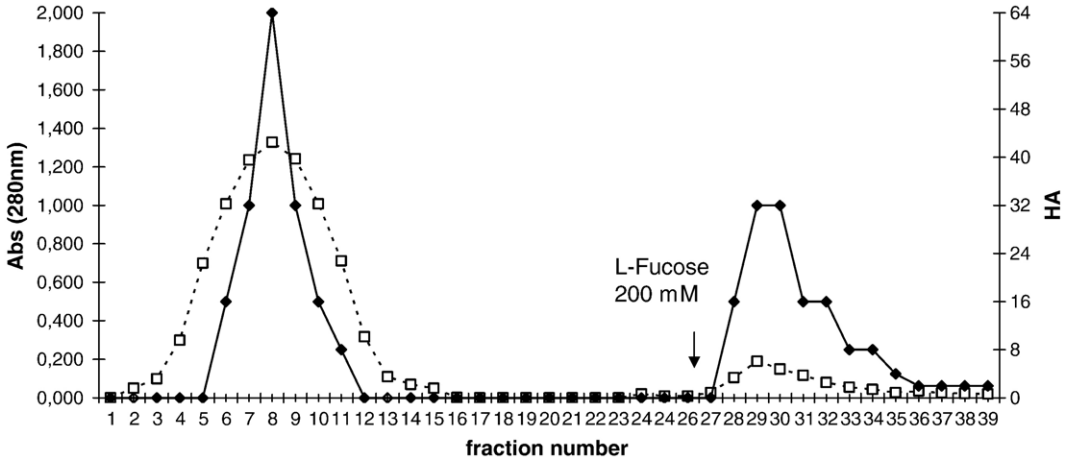

Fig. 1. Affinity chromatography of *S. aurata* serum on a fucose–agarose column absorbance at 280 nm (□); Hemagglutinating activity (♦). Peak II eluted with L-fucose 200 mM.

In addition, the purified protein sample was digested with trypsin and the fragments separated by HPLC. The peak obtained after HPLC separation revealing the highest absorbance at 280 nm was sequenced producing two similar sequences: XGAHNITVLLG and XGEANGTVLLG. A GenBank search with the N-terminal and internal peptides revealed identities at several positions with F-type lectin family members (Table 3).

3.5. Isolation of amplified fragment and sequences analysis

By using degenerate primers an amplicon (136 bp) was obtained from *Sparus aurata* and *Dicentrarchus labrax* cDNAs. The deduced aminoacid sequence from the partial gene sequence from both species show a strong similarity to the F-lectins CRD region (Fig. 4).

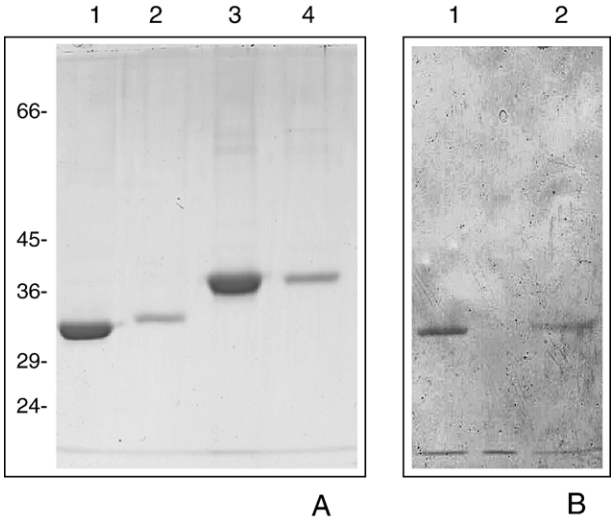

Fig. 2. (A) SDS-PAGE of *S. aurata* and *D. labrax* purified lectins. Lanes: 1, purified *D. labrax* lectin in non-reducing conditions; 2, purified *S. aurata* lectin in non-reducing conditions; 3, purified *D. labrax* lectin in reducing conditions; 4, purified *S. aurata* lectin in reducing conditions . Molecular mass of markers to the left. (B) Immunoblotting of. *aurata* and *D. labrax* purified lectins subjected to SDS-PAGE in reducing conditions and immunoblotted with anti-*D. labrax* fucolectin antibodies. Lanes: 1, purified *D. labrax* lectin in non-reducing conditions; 2, purified *S. aurata* lectin in non-reducing conditions.

4. Discussion

In this study, a 35 kDa fucose-binding lectin from serum of the gilt head bream *Sparus aurata* was isolated and biochemically characterized. Recently, a novel family of fucose-binding lectins was identified by Odom and Vasta [15]. These lectins possess characteristic L-fucose-binding and calcium-binding sequence motifs, and a unique lectin fold [18]. The F-lectin sequence motif has a wide phylogenetic distribution being found in eubacteria, molluscs, arthropods, echinoderms, fish, and amphibians, but appears to be absent from protozoa, fungi, nematodes, ascidians and amniotes such as birds and mammals [15]. In some species, the F-type carbohydrate-binding domain may be associated with other structural domains including pentraxin, other lectin domains such as, C-type lectin, or complement control "sushi" domains to yield complex chimaeric proteins, such as the *Drosophila melanogaster furrowed* and the CG9095

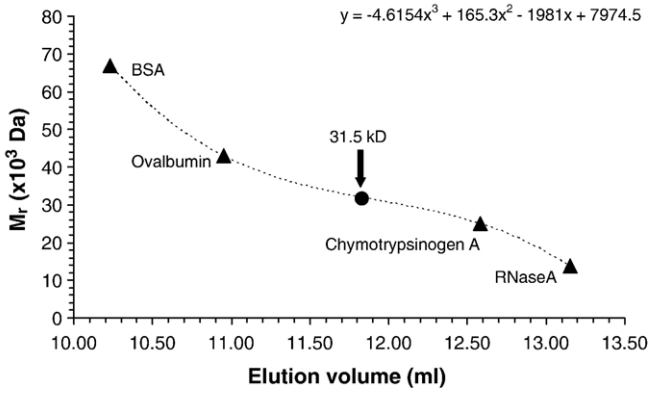

Fig. 3. Native size estimation of SauFBP by chromatography on Superose 12. The arrow points to the elution position of SauFBP. The calibration standards (triangles) are: bovine serum albumin (BSA, 67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and RNaseA (13.7 kDa).

Table 3
Alignment of *Sparus aurata* fucolectin amino-terminal sequence with teleostean F-type lectin sequences

	AP	
SAU	:	YTYQNVALRGKATQSVR:
Msa-FBPII	12:	YTYKNVALRGKATQSAR:
Dla	18:	YNYKNLALRGKATQSAR:
Msa-FBP32	19:	YNYKNVALRGKATQSAR:
AAA	38:	YTQENVAVRGKATQSAQ:
fuc5	38:	YTQENVAVRGKATQSAL:
Fuc4	24:	YVEENVALRGRATQSAQ:
Omy	25:	DELVNVALRGKAAQSST:
Fhe	36:	QNYPNVALRGKATQAQR:

AP: aminoacid starting position.
Organism abbreviations: Sau, *Sparus aurata* (sea bream); Dla, *Dicentrarchus labrax* (sea bass); Msa, *Morone saxatilis* (striped bass); AAA, *Anguilla anguilla* (European eel); *Oncorhynchus mykiss* (rainbow trout) *Fundulus heteroclitus* (killifish) Fuc#, *Anguilla japonica*. Invariant (100%) residues are shaded black; conserved residues, as defined by the Blosum62 similarity matrix, in ≥80 of sequences are shaded gray with white lettering; conservatively substituted residues in ≥60% of sequences are shaded grey with black lettering. Alignment was produced with Clustal X v.1.83 (ftp://ftp-igbmc.u-strasbg.fr/pub/) and illustrated with GeneDoc v.2.6.002 (www.psc.edu/biomed/genedoc/).

protein [15]. Interestingly, a common observation in this lectin family is the expansion of tandem CRD repeats which appears to be the exclusive mode of diversification evident in teleost species.

The *S. aurata* serum lectin described herein, designated SauFBP32, binds preferentially to fucosyl residues. Although less efficiently, galactose and oligosaccharides containing non-reducing terminal galactosyl residues such as melibiose [D-gal 1–4glc] and lactulose also behaved as inhibitors of lectin agglutination to rabbit RBC. As reported for other F-type lectins [15–17], the fucose-binding properties of the *S. aurata* lectin enabled its isolation on a fucose–agarose column in a single affinity chromatography step. The lectin shows good thermal stability and does not require calcium for binding to cells. Calcium appears to serve a role in stabilizing the fold in F-lectins [18] and is not affected by chelation. However for tachylectin 4 [20], chelation abrogates RBC agglutination, which may stem from the formation of larger order assemblies [6]. In addition, the electrophoretic mobility of the *S. aurata* lectin under reducing or non-reducing conditions, (35 kDa and 30 kDa, respectively) is similar to that observed for MsaFBP32. With regards to this, increased size of 35 kDa is probably due to polypeptide linearization as an effect of intrachain bridge reduction. Furthermore, its hydrodynamic behavior suggests that under the experimental conditions tested the native protein is present as a monomer, like MsaFBP32 [15]. This result is in contrast to the crystal structures resolved for the European eel agglutinin AAA [18], and *M. saxatilis* MsaFBP32 [Bianchet et al, in preparation], which revealed a trimeric organization in both proteins. It is possible that these proteins exhibit concentration-dependent oligomerization, as observed in sedimentation equilibrium experiments for the lectin from the ascidian *Didemnum candidum* [21].

The similarity of the N-terminal sequence and internal sequence of the SauFBP32 to teleostean F-lectin family members confirms it is an F-type lectin. In addition similarity in size suggests SauFBP32, MsaFBP32 and DlaFBP32 are homologous. Furthermore, immunological cross-reactivity of this lectin with the *D. labrax* F-type lectin, as revealed by immunoblotting, further supports that the isolated *S. aurata* lectin belongs to the F-type lectin family. It is noteworthy that two similar yet distinct internal peptide sequences were identified in the tryptic digest from a single peak. This suggests that probably each peptide came from the tandem CRDs. In teleost fish, the presence of multiple F-lectin isoforms, such as in *A. japonica* [16], *A. anguilla* [18], and *M. saxatilis* [15], and their inducibility upon inflammatory challenge, together with the observation that fucose and fucose derivatives such as colitose, may be present on the surface of microbial pathogens, suggest a role as recognition factors in innate immune functions [14,19].

Finally in the present paper we used a cDNA identification approach with conserved F-lectin sequence traits (*M. saxatilis*) to pull out amplicon sequences. Degenerate primers designed from the conserved domains gave a fragment with the expected size for *D. labrax* and *S. aurata* cDNA. The translated aa sequence revealed strong similarity with F-lectin family CRDs. Analysis of the functional aspects of the *S aurata* F-type lectin in innate immunity are in now progress.

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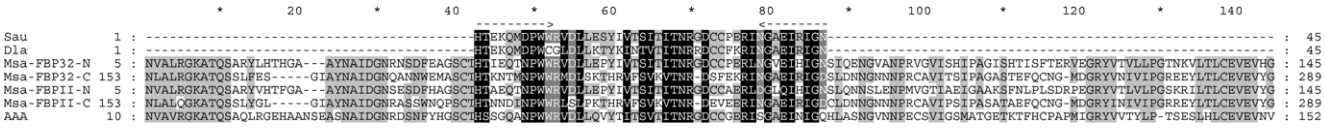

Fig. 4. Alignment of the deduced aminoacid sequences to characterized teleostean F-type lectins. Residues encoded by the degenerate primers are indicated by the overlying arrows. Organism abbreviations: Sau, *Sparus aurata*; Dla, *Dicentrarchus labrax*; Msa, *Morone saxatilis* (striped bass); AAA, *Anguilla anguilla* (European eel). N and C refers to the tandem CRDs present in the striped bass lectins. Invariant residues (100%) are shaded black; conserved residues, as defined by the Blosum62 similarity matrix, in ≥80 of sequences are shaded gray with white lettering; conservatively substituted residues in ≥60% of sequences are shaded grey with black lettering. Alignment was produced with Clustal X v.1.83 (ftp://ftp-igbmc.u-strasbg.fr/pub/) and illustrated with GeneDoc v.2.6.002 (www.psc.edu/biomed/genedoc/).

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