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

Matteo Cammarata [a], Gigliola Benenati [a], Eric W. Odom [b], Giuseppina Salerno [a], Aiti Vizzini [a], Gerardo R. Vasta [b], Nicolò Parrinello [a,*]

[a] *Marine Immunobiology Laboratory, Department of Animal Biology, University of Palermo, Via Archirafi 18, Palermo, Italy*
[b] *Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 E. Pratt Street, Baltimore, MD 21202, USA*

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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

* Corresponding author. Tel.: +39 091 6230150; fax: +39 091 6230144.
E-mail address: nicpar@unipa.it (N. Parrinello).

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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,