ISJ 16: 15-24, 2019 ISSN 1824-307X

RESEARCH REPORT

Sabella spallanzanii mucus contain a galactose-binding lectin able to agglutinate bacteria. Purification and characterization

M Cammarata[1*,#], G Benenati[2,#], M Dara[1], MG Parisi[1], D Piazzese[1], F Falco[3], L Stabili[4,5]

[#]*Equal contribution*

[1]*Department of Heart and Marine Science DISTEM, Marine Immunobiology Laboratory, University of Palermo, , Italy*
[2]*Department of Biological, Chemical and Pharmaceutical Sciences and Technologies STEBICEF, University of Palermo, Italy*
[3]*Istituto per le Risorse Biologiche e le Biotecnologie Marine (IRBM), Units of Capo Granitola Italy*
[4]*Istituto per l'Ambiente Marino Costiero, U.O.S. di Taranto, CNR, Taranto, Italy*
[5]*Department of Biological and Environmental Sciences and Technologies, University of Salento, Lecce, Italy*

Accepted March 04, 2019

Abstract

Lectins are present in almost all living organisms and are involved in several biological processes, including immune responses. In the present study, a calcium dependent galactose-binding lectin exhibiting an apparent MW of 43 kDa has been characterized and purified from the mucus of the polychaete *Sabella spallanzanii* by using both affinity chromatography and high-pressure liquid chromatographic methods. Its agglutinating activity towards rabbit erythrocytes was significantly modified by the addition of calcium or EDTA. The activity was optimal at temperature values comprised between 4 and 18 °C, maintain a 50% of activity between 20 and 37 °C, was significant deleted after exposure at 50 °C, and was depleted at 90 °C. The *S. spallanzanii* Galactose-Binding Lectin (*SsGBL*) was able to agglutinate bacteria and to preferentially recognize Gram-negative bacteria. The strongest agglutinating activity was observed towards *Vibrio alginolyticus* and *Escherichia coli*, by contrast mucus agglutinated in a lesser extent both *Aeromonas hydrophyla* and the Gram-positive *Micrococcus lysodeikticus* thus suggesting its involvement in host pathogen interactions.

Key Words: mucus; hemagglutinin; bacteria; galactose-binding lectin; *S. spallanzanii*

Introduction

Lectins are multifamily proteins present in almost all living organisms and due to their carbohydrate binding ability are involved in several biological processes (Kaltner and Stieltorfer, 1998; Kilpatrick, 2002), including development, cell adhesion, glycoproteins interactions (Kaltner and Stierstorfer, 1998; Kilpatrick, 2002), and immune responses (Liao *et al*., 1994; Arason, 1996). Lectins and sugars constitute an evolutionary conserved recognition system, involved in innate immunity, able to mediate several effector functions. These activities include agglutination, immobilization and opsonization towards microbial pathogens and complement activation, by either recognition of glycans exposed on potential pathogens either immunoregulation binding to carbohydrates on immunocompetent cells surfaces (Turner, 1996; Kilpatrick, 2002; Loris, 2002; Fujita *et al*., 2004; Sharon and Lis, 2004; Vasta *et al*., 2004).

Lectins have been classified into various structural families such as C-type lectin, galectin and R-type lectin. The lectins are distinguished on the basis of conserved amino acid sequence motifs in their carbohydrate recognition domain (CRD), structural folds and calcium requirements (Aranson, 1996; Turner, 1996; Fujita *et al*., 2004; Sharon and Lis, 2004; Vasta *et al*., 2004).

Galactose-binding lectins have been documented both in vertebrates and in invertebrates, and their involvement in humoral

Corresponding author:
Matteo Cammarata
Department of Heart and Marine Science DISTEM
Marine Immunobiology Laboratory
University of Palermo
Viale delle Scienze Ed. 16, Palermo, Italy
E-mail: matteo.cammarata@unipa.it

immunological processes is well described (Arason *et al*., 1996; Vasta *et al*., 2004). Moreover, many of them are able to bind β-galactoside carbohydrates other than monosaccharide galactose (Hirabayashi *et al*., 2002; Vasta *et al*., 2004). Much evidence exists about the presence of galactose-binding lectins in marine invertebrates. In phylum Annelida, a 29 kDa galactose-binding lectin was characterized in the earthworm *Lumbricus terrestris* (class Oligochaeta) (Hirabayashi *et al*., 1998). The primary structure of the earthworm lectin belongs to R-type lectin family that is involving ricin B-chain. On the other hand, annelid lectins that recognize galactose and others sugars were isolated from various marine worms including polychaeta and oligochaeta. Each lectin has different characteristics on carbohydrate-binding specificities, metal requirement and primary structure. Isolation, physicochemical properties, and, in some cases, biological activity and primary structure of such lectins have been described. Amphitritin, a Ca^{2+}-independent N-acetyl D-galactosamine-binding lectin with molecular mass of 30 kDa was the first hemagglutinin isolated from a sea worm *Amphitrite ornata* (Garte and Rissel, 1976). A 30 kDa β-galactose-specific lectin was isolated and characterized from the sea worm *Chaetopterus variopedatus* (Mikheyskaya *et al*., 1995). Curiously, this lectin revealed cytopathic effect induced by human immunodeficiency virus (Wang *et al.,* 2006). D-galactose-binding lectins (33-35 kDa) were isolated from body walls of echiuroid (*Urechis unicinctus*; oligochaeta) and marine worms (*Neanthes japonica* and *Marphysa sanguinea*) (Ozeki *et al*., 1997). Another 32 kDa D-galactose-binding lectin isolated from the marine worm *Perinereis nuntia* was shown to have QxW sequence in the polypeptides (Kawsar *et al*., 2009). This sequence motif was seen in R-type lectin family.

Marine duster worm, *Sabella spallanzanii* (phylum Annelida, family Sabellidae) is a representative tube worm in the Mediterranean bay. Its glandular epithelium secreting mucus often appears conspicuous and forming the so called "ventral shield". Mucus production, as in many invertebrates, constitutes a key factor determining the ability of many polychaete species to survive in their environment (Beckwith, 1999; Smith, 2002; Davies and Ogawa, 2011). As reported by Storch (1988) mucus intervenes in fertilization and egg protection, consolidates the tunnel wall of burrowing polychaetes and may also play a role in the absorption of metabolites (Mouneyrac *et al*., 2003; Mastrodonato *et al*., 2005, Dales, 1961; Stabili *et al*., 2009). Their defensive functions, such as cytotoxicity and lysozyme-like activity, (Canicattì *et al*., 1992; Stabili *et al*., 2009; Giangrande *et al*., 2014) inhibit *in vitro* the growth of *Vibrio anguillarum*, *Vibrio harveyi*, *Pseudomonas aeruginosa* and *Candida albicans* (Stabili *et al*., 2011).

In the present study, we further investigated the defensive role of *S. spallanzanii* mucus and we reported the identification, purification and characterization of a novel galactose-binding lectin with agglutinating activity against rabbit red blood cells and several bacteria. This lectin was isolated by both affinity chromatography and high-pressure liquid chromatographic methods. Results are discussed in the light of elucidating the involvement of mucus in prevention of pathogenic microorganism proliferation.

Material and Methods

Chemicals, molecular biology reagents

Unless otherwise specified, chemicals and reagents were from Sigma-Aldrich (USA).

Animals, mucus collection and preparation

Sampling was undertaken in the harbor of Brindisi (Southern Adriatic Sea, Italy) using SCUBA equipment (depth range = 5-15 m). About 200 adult specimens of *Sabella spallanzanii* were collected and transferred to the laboratory. In order to stimulate the secretion of the mucus, all the individuals have been removed from the tube where they lived and kept for 30 min in a Petri dish. Within the secreted mucus, we checked for trapped material by microscopical observations, whilst we excluded any contamination of other excretion products by pH measurements. Secreted mucus was collected and centrifuged at 12000 x*g* for 30 min at 4 °C and stored at -80 °C until used. It was ten folds diluted in tris-buffered saline (TBS) and filtered through 0.2 µm pore size before performing affinity chromatography.

Hemagglutination assay

Rabbit and sheep red blood cells (RaRBC and SRBC, supplied by Istituto Zooprofilattico della Sicilia) were washed three times in phosphate buffered saline (PBS), centrifuged at 500 x*g* for 10 min at 4 °C and suspended at 1% in PBS containing 0.1% (w/v) gelatin. A volume (25 µl) of *S. spallanzanii* mucus or 25 µl of the dialyzed purified *S. spallanzanii* Galactose-Binding Lectin (SsGBL) were serially (2-fold) diluted in PBS-gelatin in 96-well round-bottom microtiter plates (Denmark), and an equal volume of erythrocytes suspension was added. The hemagglutinating titer (HT) was measured after 1 hour incubation at 37 °C and expressed as the reciprocal of the highest dilution showing clear agglutination (Ballarin *et al*., 2008).

Physical and chemical characterization

To examine divalent cation requirement for mucus hemagglutination activity (HA), $CaCl_2$ and $MgCl_2$ were added to the assay medium to obtain 3 mM each one final concentration. EDTA (10 mM) or EGTA (10 mM) were used to examine the effect of Ca^{2+} versus Mg^{2+} depletion. To examine the thermolability, mucus samples were incubated at 4, 10, 18, 37, 50, 70, and 90 °C for 20 min and cooled down for 10 min on ice before testing the HA.

Carbohydrate specificity

Hemagglutinating activity was assayed against RaRBC in the presence of serially diluted saccharides as potential inhibitors (Ballarin *et al*., 2008). A volume (25 µl) of *S. spallanzanii* mucus or 25 µl of the purified *S. spallanzanii* Galactose-Binding Lectin (SsGBL) and 25 µl of a serially

diluted sugar. Finally, an equal volume of erythrocytes suspension was added and after 1 hour incubation at 37 °C the HT was evaluated. Inhibition experiments were carried out using decreasing concentrations (starting from 130 mM in PBS pH 7.4, 3 mM $CaCl_2$, 1% gelatin) of monosaccharides (L-fucose, L-rhamnose, D-galactose, D-glucose, D-mannose, N-Acetylglucosamine) and disaccharides (Lactose, and Lactulose).

The same procedure was performed in the inhibition experiments having bacteria as target.

Bacterial suspensions and agglutination

In order to evaluate the hemagglutinating activity the following bacterial strains were employed: *Vibrio alginolyticus*, *Escherichia coli*, *Aeromonas hydrophyla, Staphylococcus aureus* and *Micrococcus lysodeikticus*. 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. Log phase was estimated by absorbance at 600 nm. The correspondence between cell number and spectrophotometric absorbance have been determined by serial dilution plate count method. Bacteria were killed with heat incubating them at 121 °C, for 20 min, at 1 atm.

For the agglutination assay, they were washed three times in sterile PBS, suspended in PBS containing 0.1% (w/v) gelatin to obtain $1x10^7$ bacteria/ml and dispensed in 96 wells plate. Plates were incubated at 18 °C over night.

Lectin purification

Lectin was isolated by a two-steps chromatography procedure. The first consisted of a galactose-agarose affinity chromatography column with elution with 0.1 M galactose in TBS, 3 mM $CaCl_2$, as previously reported (Salerno *et al.*, 2009). The elution step was monitored by absorbance at 280 nm and protein concentration in collected fractions was evaluated through Bradford method (1976). After dialysis in TBS, 3 mM $CaCl_2$, these were tested for hemagglutinating activity towards rabbit erythrocytes, and those that exhibited the highest activity were pooled and analysed by SDS-PAGE (Laemmli, 1970).

In the second step, the collected fractions from the chromatographic procedure exerting hemagglutinating activity were applied to a High-Pressure Liquid Chromatography Size Exclusion Column BioSuite 250–10 µm SEC 7.5 x 300 mm Waters, 350 psi pressure, 280/254 nm (mAU) and analysed by HPLC method. Phosphorylase b (97kDa), bovine serum albumin (BSA, 67kDa), enolase (46.7kDa), myoglobin A (18.7kDa) and RNaseA (13.7kDa) were used as calibration standards.

Affinity column purified fractions were then applied to High Pressure Liquid Chromatography Size Exclusion Column BioSuite 250–10 µm SEC 7.5 x 300 mm Waters, 350 psi pressure, 280/254 nm (mAU). Phosphorylase b (97kDa), bovine serum albumin (BSA, 67kDa), enolase (46.7kDa), myoglobin A (18.7kDa) and RNase A (13.7kDa) were used as calibration standards (Fig. 3B).

Table 1 Range of hemagglutinating activity (titer^{-1}) of *S. spallanzani* mucus and the purified lectin (25 µg/ml) towards various erythrocytes and bacteria

Erythrocytes	Mucus	Isolated fraction
Rabbit Red Blood Cells	512-1024	32-128
Sheep Red Blood Cells	0-2	-
Escherichia coli	128- 256	16-32
Vibrio alginolyticus	128-512	16-32
Aeromonas hydrophila	64-128	8-16
Staphilococcus aureus	NA	NA
Micrococcus lysodeikticus	32-64	8-16

Protein content estimation

Protein content was estimated according to the Bradford method using BSA as a standard. Undiluted mucus showed a protein content of about 0.6 mg/ml while the best eluted chromatographic fraction had a protein concentration of about 0.2 mg/ml.

Polyacrylamide gel electrophoresis

SDS-PAGE (16%) was carried under reducing (5% mercaptoethanol) and non-reducing conditions. To evaluate the molecular size, gels were calibrated with low molecular weight (6.5-66 kDa) standard proteins. Proteins were stained with Coomassie brillant Blue R250.

Results

Mucus hemagglutinating activity

Mucus agglutinating activity was tested towards both erythrocytes and bacteria. It showed almost no activity when sheep erythrocytes were used as target cells (HA titer = 2). Otherwise, this matrix had a strong agglutinating activity when rabbit red blood cells were used in the test showing an average agglutination titer of 512 (Table 1). This activity was calcium dependent because it was strongly affected by calcium depletion when 10 mM EDTA or 10 Mm EGTA and 3 mM magnesium were added to the hemagglutination assay medium.

RaRBC hemagglutinating activity thermolability was tested performing the assay after 20 min pre-incubation of mucus at different temperatures. The optimum of the activity was recorded when temperature ranged between 4 and 18 °C and decreased after 20 min pre-incubation temperature ranging from 37 to 90 °C (Fig. 1). Almost no loss of biological activity was detected after two months storage of samples at -80 °C.

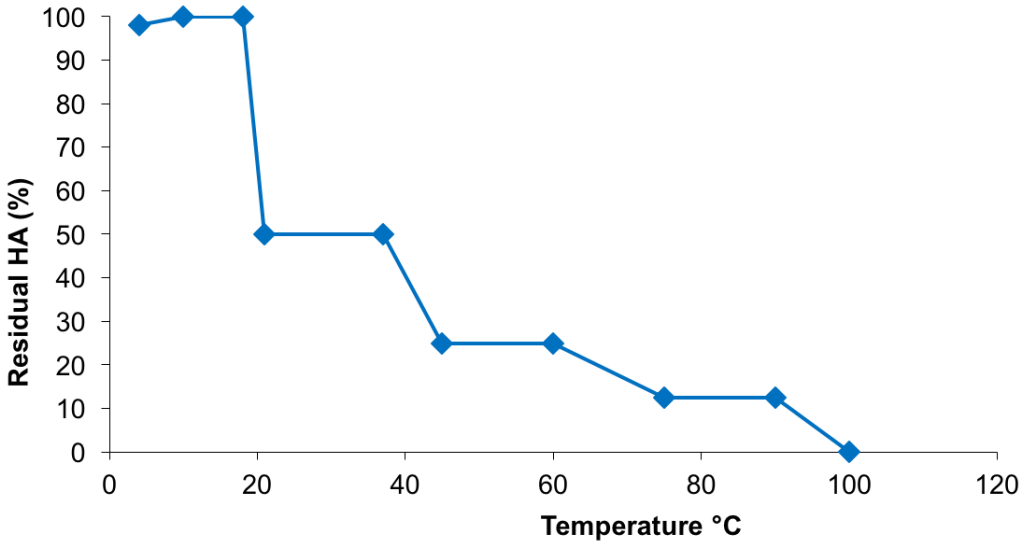

Fig. 1 *S. spallanzanii* mucus preincubated 20 min at different temperature was tested towards RaRBC. It to have an optimum agglutination temperature ranging between 10 °C and 18 °C and a reduced but not completely deleted activity even after 20 min pre-incubation step from 37 °C up to 90 °C

Mucus was able to preferentially recognize Gram-negative bacteria; indeed, the strongest agglutinating activity was observed towards *V. alginolyticus* and *E. coli*, by contrast mucus agglutinated in a lesser extent *A. hydrophyla* and the Gram-positive *M. lysodeikticus* and did not agglutinate the Gram-positive *S. aureus* (Table 1).

Carbohydrates inhibition test was performed by adding several carbohydrates in decreasing concentrations (final concentration ranging from 130 to 4 mM) to the assay medium. Galactose and at lesser extent fucose revealed to have inhibition activity even at the lowest concentration (8 mM Galactose, 16 mM Fucose) used in the hemagglutination assay (Table 2).

SsGBL purification and characterization

The *SsGBL* has been purified starting from a 20 ml diluted collected mucus applied on galactose-agarose column. The profile of the affinity purification is shown in Figure 2. In a typical isolation, the eluted fractions, having a protein concentration ranging from 0.08 to 0.18 mg/ml, represented approximately 10-30% of the total mucus protein content loaded onto the column (0.61 mg/ml). The recovery in terms of hemagglutinating activity was about 25% and the 3% after HPLC elution (Table 3).

The galactose eluted fractions, having the highest protein concentration, showed similar average hemagglutinating activity towards RaRBC that ranged from 32 to 64 (Table 2). The action of the active fractions was calcium dependent because it appeared magnified when the medium contained 3 mM calcium and was heavily affected by the addition of 10 mM EDTA or 10 mM EGTA, 3 mM magnesium, as already observed for mucus extracts.

Electrophoresis analysis on SDS-PAGE revealed that the purified lectin consisted of a single component with an apparent molecular weight of 45 kDa, under reducing and non-reducing conditions (Figure 3A inset) suggesting a monomeric organization of the effector responsible of the hemagglutinating activity. The eluted fraction from affinity chromatography was applied to a HPLC size exclusion column and the obtained profile is shown in Fig. 3A. From the HPLC size exclusion step the purified lectin seems to have an approximate molecular weight of 43 kDa (Fig. 3B).

The hemagglutinating activity of the purified fractions was maintained after 2 months at 20 °C, mildly affected when preincubated for 30 min at 50, 60 or 70 °C but reduced at 90 °C. Neither purified hemoagglutinin nor mucus showed agglutinating activity towards SRBC (Table 2).

Table 2 Inhibition of hemagglutination activity activity of the *S. spallanzanii* mucus or isolated lectin with RE by various sugars

Inhibitor	Minimum concentration (mM) of sugar required for 100% inhibition of HA reaction	
	Serum	Isolated lectin
D-galactose	25.0 mM	8.1 mM
L-fucose	100.0 mM	16.5 mM
D-Mannose	130 mM	NI
Rahmnose	130 mM	NI

Arabinose, cellobiose, D-glucose, Lactose, Lactulose, Maltose, Mannan, N-Ac-galactosamine N-Ac-glucosamine, D-raffinose; = no inhibition for 200 mM of sugar concentration

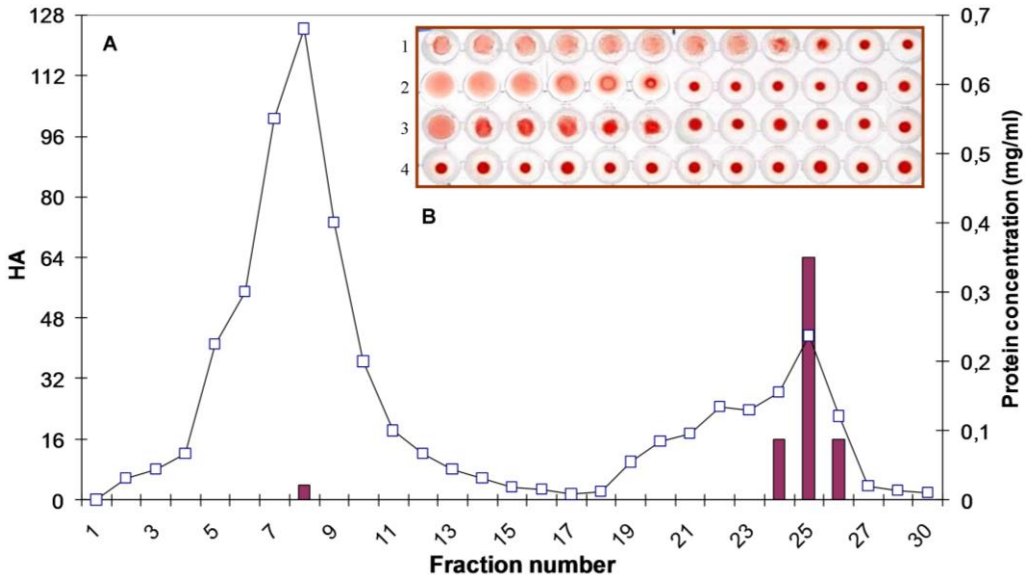

Fig. 2 Galactose-agarose affinity chromatography profile and eluted fractions from mucus. A: Diluted mucus was applied on a galactose-agarose column and elution step (1-20) performed with 100 mM galactose. The eluted fractions (11-14) show a protein concentration ranging from 0.08 to 0.18 mg/ml. B: Hemagglutinating activity (HA) against rabbit erythrocytes induced by: mucus (1), purified lectin fraction n. 26 (2), purified lectin fraction n. 27 (3), erythrocytes control (4)

SsGBL bacterial agglutination

The purified SsGBL agglutinated both Gram-positive and Gram-negative bacteria (Table 1, Fig. 4) and was inhibited by galactose (Table 2).

Therefore, the SsGBL binding specificity varied significantly depending on the bacterial target used. In fact, it strongly agglutinated *E. coli* and *V. alginolyticus*, in a lesser extent *A. hydrophyla and M. lysodeikticus* (Table 1) whilst no agglutination was found by using *S. aureus*.

Discussion

Comparative immunology is important to understand a fundamental aspect of immunology particular for the phylogenetic perspective (Ballarin and Cammarata 2016) and the study of Annelids immunology have been deeply contributed (Engelmann *et al.*, 2018). Lectins are important immunomediators in vertebrates and invertebrates (Kuhlman *et al.*, 1989; Cooper *et al.*, 1994; Matsushita *et al.*, 1996; Tino and Wright, 1996; Odom and Vasta, 2006; Vasta, 2009). Many carbohydrate binding proteins have been already described in Annelida, both in marine and terrestrial species. Kawsar *et al.*, (2010) purified a 32 kDa galactose-binding lectin from *P. nuntia* homogenates that showed hemagglutinating activity against human and rabbit erythrocytes. This lectin from *P. nuntia* revealed a clear antibacterial activity inhibiting the Gram-positive growth *in vitro*. Hirabayashi *et al.*, (1998) isolated a 29 kDa lectin from *L. terrestris* body extracts. These carbohydrates-binding molecules are highly specific for sugar moieties. On account of their capability to bind carbohydrates involved in attachment of potential pathogens to host, lectins can protect the animal preventing its invasion from pathogens. Lectins are also involved in cell agglutination, recognizing structures on pathogens surface, they can opsonize them and enhance host phagocytic activity or activate the complement pathway (Matsushita *et al.*, 1996; Cammarata *et al.*, 2014). Due to these properties, lectins evidenced in the

Table 3 Purification steps of *SsGBL*

Purification stage	Protein (mg)	HA titer	THA	Specific activity THA/PC	Purification (fold)	Yield %
Mucus	12.5	256	5120	410	1	100
Galactose-agarose	**0.33**	**64**	**1280**	**3879**	**9.46**	**25**
HPLC	**0.015**	**8**	**160**	**10600**	**26**	**3**

HA: hemagglutinating activity; THA: total hemagglutinating activity.

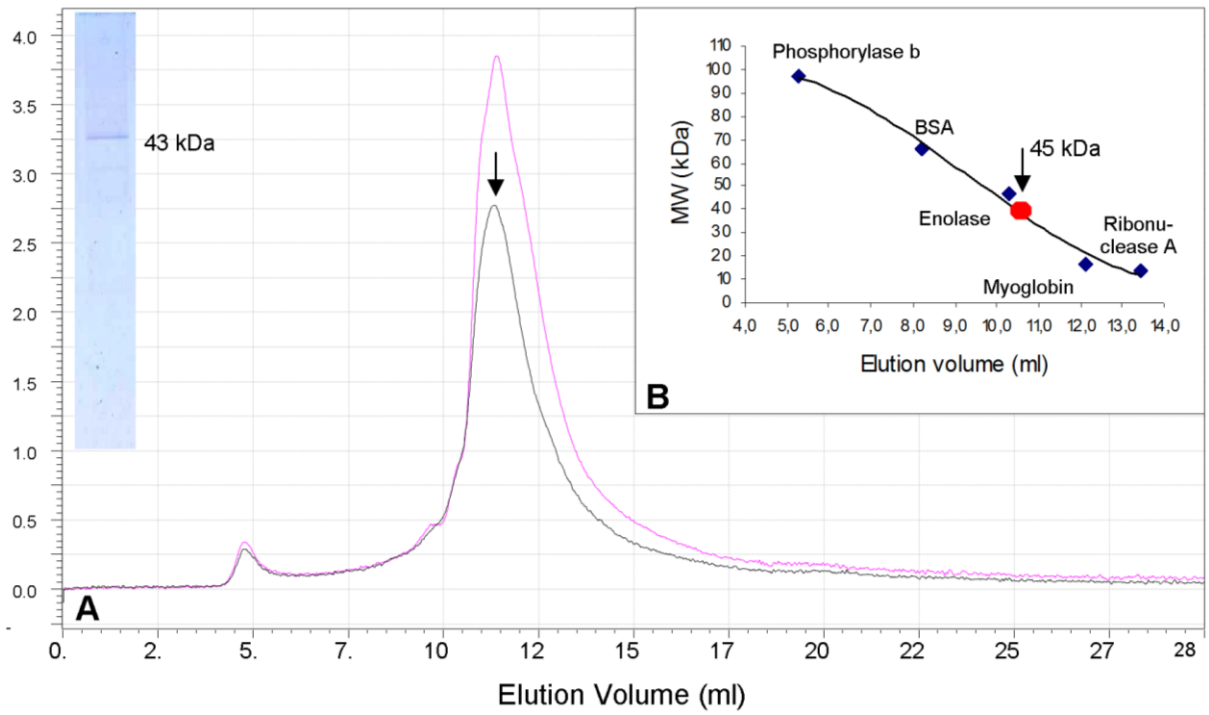

Fig. 3 (A) Affinity column purified fraction from galactose-agarose applied to a high-pressure liquid chromatography (HPLC) size exclusion column BioSuite 250-10 µm SEC 7,5 x 300 mm Waters, 350 psi pressure, 280 black line /254 red line nm (mAU). (A inset) SDS - PAGE (16%) of the purified lectins under reducing conditions; Standard proteins HPLC profile; Native size estimation of SsGBL by HPLC. The arrow points to the elution position of SsGBL. (B) Calibration standards (rhombus): phosphorylate b (97 kDa); bovine serum albumin (BSA, 67 kDa), enolase (46.7 kDa), myoglobin A (18.7 kDa) and RNaseA (13.7 kDa)

body surface mucus can be considered potential antimicrobial agents. Among Annellida *S. spallanzanii* is one of the best known and abundant Mediterranean sabellid. In this animal, a large amount of mucus is secreted when specimens are subjected to different stress conditions leading to suppose its involvement as protective compartment against microorganisms and/or epibiosis (Stabili *et al.*, 2011, 2014; Giangrande *et al.*, 2014) as already observed in other invertebrates (Denny *et al.*, 1989; Weis *et al.*, 1998; Smith *et al.*, 2010; Ogawa *et al.*, 2011, Stabili *et al.*, 2014). A defensive function in *S. spallanzanii* mucus was firstly suggested by Canicatti *et al.*, (1992) who evidenced a haemolytic activity in this matrix. Recently, Stabili *et al.* isolated a lysozyme-like activity and an *in vitro* antimicrobial activity in *S. spallanzanii* mucus towards some Gram-negative bacteria (Stabili *et al.*, 2009) clearly indicating the role of this compartment in defending the worms from bacterial attack serving as medium into which the antibacterial substances are exuded. Here a divalent-cation dependent lectin was newly discovered from mucus of sabellid, and we purified the SsGBL, a 43 kDa monomeric galactose-specific lectin from *S. spallanzanii* by using both affinity chromatography and high-pressure liquid chromatographic methods. Its agglutinating activity towards rabbit erythrocytes was significantly modified by the addition of calcium or EDTA. The activity shows good thermal stability, at temperature values comprised between 4°C and 37°C including the range of natural environment in which these annelid lives. Of particular interest, this lectin is a significant fraction (10-30%) of the soluble proteins of the *S. Spallanzani* mucus supporting an important functional role of this molecule.

Galactose-binding lectins (GBLs) have been discovered and isolated also from others Annelids. Hirabayashi *et al.* (1998) first isolated from the earthworm, *Lumbricus terrestris*, a galactose-binding lectin of 29 kDa inhibited by a wide range of galactose-containing saccharides. The lectin is composed of two homologous domains of 14.5 kDa showing 27% identity among other GBLs and contained multiple short conserved motifs, "Gly-X-X-X-Gln-X-Trp".

Another GBL with a molecular weight of 32 kDa was purified from the pacific annelid *Perinereis nuntia* ver. *vallata* by affinity chromatography showing a typical R lectin QxW sequence (Kwasar *et al.*, 2010).

In addition to the agglutinating activity against rabbit erythrocytes, the SsGBL bind both Gram-negative and Gram-positive bacteria; this ability to recognize and agglutinate exogenous target appears calcium dependent, according to the C-type lectins mode of action.

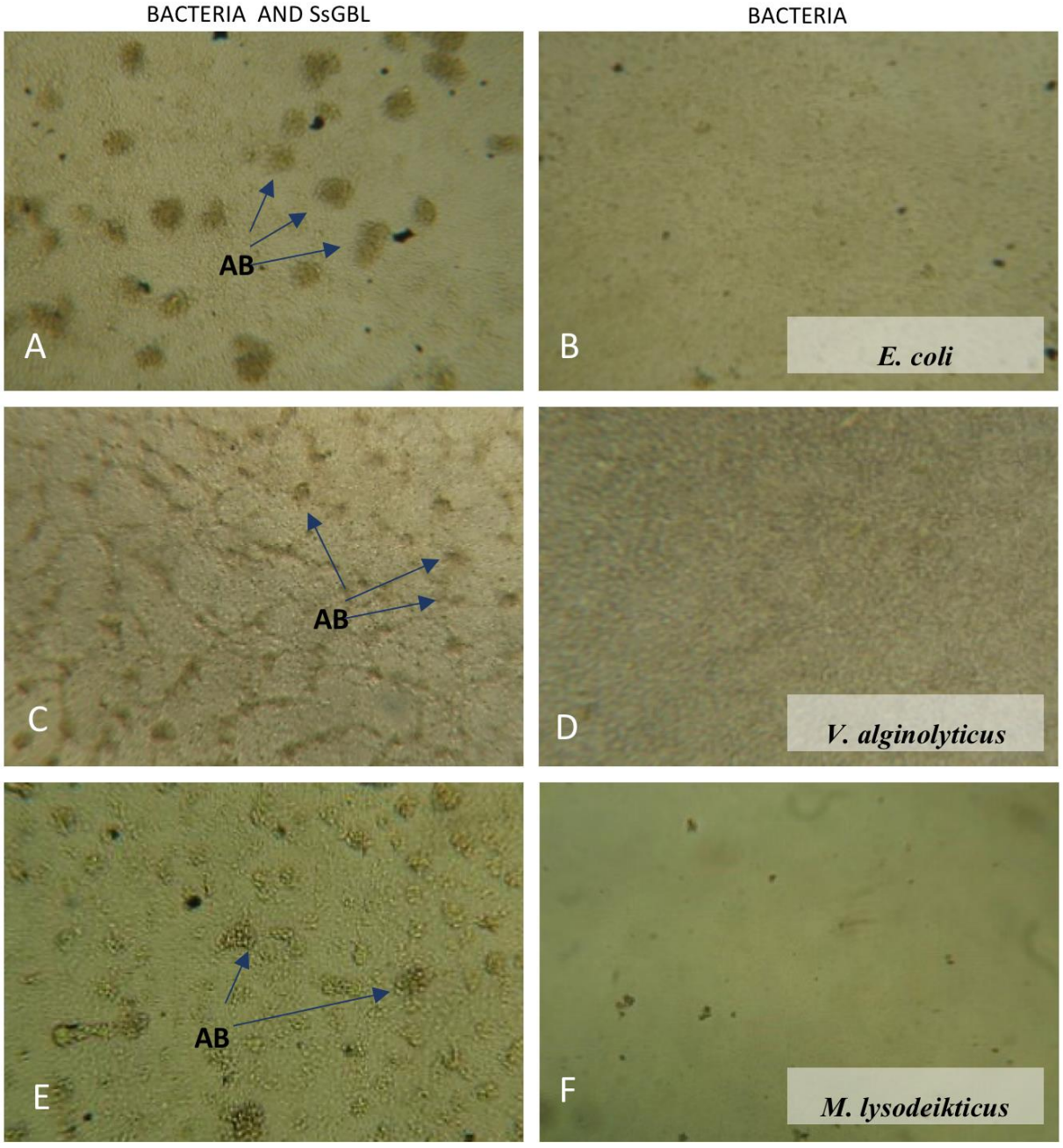

Fig. 4 The purified SsGBL displays the ability to agglutinate *E. coli* (A), *V. alginolyticus* (C) and *M. lysodeikticus* (E). *E. coli* (B), *V. alginolyticus* (D) and *M. lysodeikticus* (F) as negative control. BA: Bacterial agglutination

In particular, the strongest agglutinating activity was observed towards the Gram-negative *V. alginolyticus* and *E. coli* present in coastal areas and in the harbors in which *S. spallanzani* lives, by contrast mucus agglutinated in a lesser extent *A. hydrophyla* and the Gram-positive *M. lysodeikticus,* but not against *Staphylococcus aureus*. These data correspond to the findings already recorded for the total mucus, suggesting that the SsGBL could represent an important effector responsible for the mucus defense role.

The ability to recognize, bind and agglutinate bacteria has been well described among vertebrates in fish lectins (Bianchet *et al.*, 2002; Odom and Vasta, 2006; Vasta *et al.*, 2011), mammals (Cash *et al.*, 2006; Vaishnava *et al.*, 2011) and many invertebrates (Malagoli *et al.*, 2006) suggesting an antibacterial activity (Matsui *et al.*, 1994; Tateno *et al.*, 2002). Among these, galactose-binding lectins have been described both in invertebrates and fish skin mucus, with bacterial agglutination properties. The skin mucus galectin from Japanese eel exhibited agglutination of *Streptococcus difficile* and *E. coli* (Suzuki *et al.*, 2003). further confirming their protective role (Shiomi *et al.*, 1989; Mistry *et al.*, 2001; Suzuki *et al.*, 2003; Ogawa *et al.*, 2011). In

general galectins are parts of the fish defense system and mainly exist in organs and tissues that delineate the body from its surroundings, such as epidermal club cells of the skin, esophagus and gills (Nakamura *et al*., 2012).

In this paper, we propose that the SsGBL is involved in the mucus body defense role, probably it is able to prevent bacterial invasion through the ability to agglutinate some bacteria strains.

Our data suggest that the SsGBL could represent an important effector acting in contemporary presence with hemolytic factors and lysozyme as a first defense mechanism against potentially pathogens. Such synergic strategy is common and well known in invertebrates that lack acquired immunity and therefore reliant on mechanisms of innate immunity. This role is important taking into account that *S. spallanzanii* lives in eutrophic environments such as harbors where bacteria, including pathogens for humans, marine organisms, are abundant (Barg and Phillips, 1998). Although the worms may be aggressed by bacteria, they are able to survive bacterial attack.

Unfortunately, by using the classical Edman degradation technique, N-terminal sequencing seem to be blocked. Microsequence analyses carried out by MALDI spectra, despite showing strong peaks didn't give matches and the nLC-ESI MSMS only gave hits for keratin, and for the weaker sample a bit of BSA as well. The missing of significant match is plausible because the *S. spallanzanii* sequences are not well represented in the NCBI database and probably is not close enough in the NCBI database to give at least a match based on homology (data not showed).

Even though our knowledge about composition, production and roles of mucus in various marine invertebrates remains incomplete, our results contribute to the understanding of the mucus protective properties in the investigated polychaete (Stabili *et al*., 2009; 2011).

Acknowledgments

This work was supported by MC and MGP FtoFR (University of Palermo) and a research grant from the Italian Ministry of Education (PRIN 2010-2011 n. 20109XZEPR_007)

References

Aranson GJ. Lectins as defence molecules in vertebrates and invertebrates. Fish Shellfish Immunol. 6: 277-289, 1996.

Ballarin l, Cammarata M, Cima F, Grimaldi A, Lorenzon S, Malagoli M, *et al*. Immune-neuroendocrine biology of invertebrates: a collection of methods. Inv. Surv. J. 5: 192-215, 2008.

Ballarin L, Cammarata M. "Lessons in Immunity: from Single-Cell Organisms to Mammals", Elsevier, London, 2016.

Barg U, Phillips MJ. Environment and Sustainability. FAO Fisheries Circular No. 886FAO, Rome, FAO. (163 pp.). 1998.

Bianchet MA, Odom EW, Vasta GR, Amzel LM. A novel fucose recognition fold involved in innate immunity. Nature (Struct. Biol.) 9: 628-634, 2002.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding). Anal. Biochem. 72: 248-254, 1976.

Cammarata M, Parisi MG, Benenati G, Vasta G, Parrinello N. A rhamnose-binding lectin from sea bass (*Dicentrarchus labrax*) plasma agglutinates and opsonizes pathogenic bacteria. Dev. Comp. Immunol. 44: 332-34, 2014.

Salerno G, Parisi MG, Parrinello D, Benenati G, Vizzini A, Vazzana M, *et al*. F-type lectin from the sea bass (*Dicentrarchus labrax*): purification, cDNA cloning, tissue expression and localization, and opsonic activity. Fish Shellfish Immunol. 27: 143-153. 2009.

Canicattì C, Ville P, Pagliara P, Roch P. Hemolysins from the mucus of *Spirographis spallanzani* (Polychaeta: Sabellidae). Mar. Biol.114: 453-458, 1992.

Cash HL, Whitham CV, Behrendt CL, Hooper LV. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science 313: 1126-1130, 2006.

Cooper D, Butcher CM, Berndt MC, Vadas MA. P-selectin interacts with a beta 2-integrin to enhance phagocytosis. J. Immunol. 153: 3199–3209,1994.

Engelmann P, Bodó K, Najbauer J, Németh P. Annelida: Oligochaetes (segmented worms): earthworm immunity, quo vadis? Advances and new paradigms in the omics era pp135-149. In Advances in Comparative Immunology. Cooper, Edwin L. (Ed.) Springer International Publishing. 2018.

Davies MS, Beckwith P. Role of mucus trails and trail-following in the behavior and nutrition of the periwinkle *Littorina littorea*. Mar. Ecol. Prog. Ser. 179: 247-257, 1999.

Denny MW. Invertebrate mucous secretions: functional alternatives to vertebrate paradigms, in: E. Chantler, N.A. Ratcliffe (Eds.), Symposia of the Society for Experimental Biology, XLIII Mucus and related topics, Cambridge The Company of Biologists Limited, 337-366, 1989.

Fujita T, Matsushita M, Endo Y. The lectin-complement pathway – its role in innate immunity and evolution. Immunol. Rev. 198: 185-202, 2004.

Garte SJ, Rissell CS. Isolation and characterization of a hemagglutinin from *Amphitrite ornata*, a polychaetous annelid. Biochim. Biophys. Acta 439: 368-379, 1976.

Giangrande A, Licciano M, Schirosi R, Musco L, Stabili L. Chemical and structural defensive external strategies in six sabellid worms (Annelida). Mar. Ecol. 35: 36-45, 2014.

Hirabayashi J, Dutta SK, Kasai K. Novel galactose-binding proteins in Annelida. Characterization of 29-kDa tandem repeat-type lectins from the earthworm *Lumbricus terrestris*. J. Biol. Chem. 273(23): 14450-14460, 1998.

Hirabayashi J, Ubukata T, Kasai K. Purification and molecular characterization of a novel 16-kDa galectin from the nematode *Caenorhabditis elegans*. J. Biol. Chem. 271: 2497-505, 1996.

Hirabayashi J, Hashidate T, Arata Y, Nishi N, Nakamura T, Hirashima M, *et al*. Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. Biochim. Biophys. Acta. 1572: 232-254. 2002.

Kaltner H, Stieltorfer B. Animal lectins as cell adhesion molecules. Acta Anat. 161: 162-179, 1998.

Kawsar SMA, Mamun SMA, Rahman S, Yasumitsu H, Ozeki Y. Biological effects of a carbohydrate-binding protein from an Annelid, *Perinereis nuntia*, against human and phytopathogenic microorganisms. Int. J. of Biol. Biomolec. Agricultural, Food and Biotechnol. Engin. 4, 2010.

Kawsar SMA, Takeuchi T, Kasai K, Fujii Y, Matsumoto R, Yasumitsu H, *et al*. Glycan-binding profile of a D-galactose-binding lectin purified from the annelid *Perinereis nuntia* ver. *vallata*, Comp. Biochem. and Physiol. Part B: Biochem. and Molec. Biol. 152: 382-389. 2009.

Kilpatrick DC, Animal lectins: a historical introduction and overview. Biochim. Biophys. Acta. 1572: 187-197. 2002.

Kuhlman M, Joiner K, Ezekowitz RA. The human mannose-binding functions as an opsonin. J. Exp. Med. 169: 1733-1745, 1989.

Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685, 1970.

Liao DI, Kapadia G, Ahmed H, Vasta GR, Herzberg O. Structure of S-lectin, a developmentally regulated vertebrate beta-galactoside-binding protein. Proc. Nat. Acad. Sciences USA. 91: 1428-1432, 1994.

Loris R. Principles of structures of animal and plant lectins, Biochim. Biophys. Acta. 1572: 198-208, 2002.

Malagoli D, Sacchi S, Ottaviani E. Lectins and cytokines in celomatic invertebrates: two tales with the same end. Inv. Surv. J. 7: 1-10, 2010.

Matsui T, Ozeki Y, Suzuki M, Hino A, Titani K. Purification and characterization of two Ca^{++}-dependent lectins from coelomic plasma of sea cucumber, *Stichopus japonicus*. J. Biochem. 116: 1127-1133, 1994.

Matsushita M, Endo Y, Taira S, Sato Y, Fujita T, Ichikawa N, *et al*. A novel human serum lectin with collagen- and fbrinogen-like domains that functions as an. Opsonin. J. Biol. Chem. 271: 2448-2454, 1996.

Mikheyskaya LV, Evtushenko EV, Ovodova RG, Belogortseva NI, Ovodov YS, Isolation and characterization of new β-galactosespecific lectin from the sea worm *Chaetopterus variopedatus*. Carbohydr. Res. 2751: 193-200, 1995.

Mistry AC, Honda S, Hirose S. Structure, properties and enhanced expression of galactose-binding C-type lectins in mucous cells of gills from freshwater Japanese eels (*Anguilla japonica*). Biochem. J. 360: 107-115, 2001.

Mouneyrac C, Mastain O, Amiard JC, Amiard-Triquet C, Beaunier P, Jeantet AY, *et al*. Trace-metal detoxification and tolerance of the estuarine worm *Hediste diversicolor* chronically exposed in their environment. Mar. Biol. 143: 731-744, 2003.

Nakamura O, Watanabe M, Ogawa T, Muramoto K, Ogawa K, Tsutsui S, Kamiya H. Galectins in the abdominal cavity of the conger eel *Conger myriaster* participate in the cellular encapsulation of parasitic nematodes by host cells. Fish Shellfish Immunol. 33: 780-787, 2012.

Odom E, Vasta GR. Characterization of a binary tandem domain F-type lectin from striped bass (*Morone saxatilis*). J. Biol. Chem. 281: 1698-1713, 2006.

Ogawa T, Watanabe M, Naganuma T, Muramoto K. Diversified carbohydrate-binding lectins from marine resources. J. Amino Acids 838-914, 2011.

Ozeki Y, Tazawa E, Matsui T. d-galactoside-specific lectins from the body wall of an Echiuroid (*Urechis unicinctus*) and two annelids (*Neanthes japonica* and *Marphysa sanguinea*), Comp. Biochem. Physiol. Part B: Biochem. and Molec. Biol. 118: 1-6, 1997.

Sharon N, Lis H. History of lectins: from hemagglutinins to biological recognition molecules. Glycobiology. 14: 53-62, 2004.

Shiomi K, Uematsu H, Yamanaka H, Kikuchi T. Purification and characterization of a galactose-binding lectin from the skin mucus of the conger eel *Conger myriaster*. Comp. Biochem. Physiol. Part B: Biochem. and Molec. Biol. 92: 255-261, 1989.

Smith AM. The structure and function of adhesive gels from invertebrates. Integr. Comp. Biol. 42: 1164-1171, 2002.

Smith, VJ; Desbois AP, Dyrynda EA. Conventional and unconventional antimicrobials from fish, marine invertebrates and micro-algae. Mar. Drugs. 8: 1213-1262, 2010.

Stabili L, Schirosi R, Licciano M, Giangrande A. Role of *Myxicola infundibulum* (Polychaeta, Annelida) mucus: from bacterial control to nutritional home site. J. Exp. Mar. Biol. Ecol. 461: 344-349, 2014.

Stabili L, Schirosi R, Di Benedetto A, Merendino A, Villanova L, Giangrande A. First insights into the biochemistry of *Sabella spallanzanii* (Annelida: Polychaeta) mucus: a potentially unexplored resource for applicative purposes. J. Mar. Biol. Assoc. UK. 91: 199-208, 2011.

Stabili L, Schirosi R, Licciano M, Giangrande A. The mucus of *Sabella spallanzanii* (Annelida, Polychaeta): Its involvement in chemical defence and fertilization success. J. Exp. Mar. Biol. Ecol. 374: 144-149, 2009.

Storch V. The ultrastrucutre of Polychaeta. I Integument. In: Westheide, W. Hermans, C.O. (Eds.), Microfauna Marina, vol. 4. Verlag, Stuttgart, pp. 13-36, 1988.

Suzuki Y, Tasumi S, Tsutsui S, Okamoto M, Suetake H. Molecular diversity of skin mucus lectins in fish. Comp. Biochem. and Physiol. Part B: Biochem. and Molec. Biol. 136: 723-730, 2003.

Tateno H, Yamaguchi T, Ogawa T, Muramoto K, Watanabe T, Kamiya H. *et al*. Immunohistochemical localization of rhamnose-

binding lectins in the steelhead trout (*Oncorhychus mykiss*) Dev. Comp. Immunol. 26: 543-550, 2002.

Tino MJ, Wright JR. Surfactant protein A stimulates phagocytosis of specific pulmonary pathogens by alveolar macrophages. Am. J. Physiol. 270: 677-688, 1996.

Turner MW. Mannose-binding lectin: the pluripotent molecule of the innate immune system. Immunol. Today 17: 532-540, 1996.

Vaishnava S, Yamamoto M, Severson KM, Ruhn KA, Yu X, Koren O, *et al*. The antibacterial lectin RegIIIγ promotes the spatial segregation of microbiota and host in the intestine. Science 334: 255-258, 2011.

Vasta GR, Nita-Lazar M, Giomarelli B, Ahmed H, Du S, Cammarata M, *et al*. Structural and functional diversity of the lectin repertoire in fish: Relevance to innate and adaptive immunity. Devel. Comp. Immunol. 35: 1388-1399, 2011.

Vasta GR, Ahmed H, Odom EW. Structural and functional diversity of lectin repertoires in invertebrates, protochordates and ectothermic vertebrates. Curr. Op. in Structur. Biol. 14: 617-630, 2004.

Vasta GR. Roles of galectins in infection. Nat. Rev. Microbiol. 7: 424-438, 2009.

Wang JH, Kong J, Li W, Molchanova V, Chikalovets I, Belogortseva N, *et al*. A beta-galactose-specific lectin isolated from the marine worm *Chaetopterus variopedatus* possesses anti-HIV-1 activity. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 142: 111-117, 2006.

Weis WI, Taylor ME, Drickamer K. The C-type lectin superfamily in the immune system. Immunol. Rev. 163: 19-34, 1998.