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SHORT COMMUNICATION

Identification and preliminary characterization of a Ca^{2+}-dependent hemagglutinin in the celomic fluid of *Sipunculus nudus*

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Abstract

A soluble agglutinin was purified by affinity chromatography of the celomic fluid of the marine worm *Sipunculus nudus*. This agglutinin requires metal cations for its activity and is specific for derivatives of D-galactose. It resulted lightly thermostable, with a pH optimum around 7.5. On SDS-PAGE, it was resolved in two bands, of 33 and 31 kDa in reducing conditions and 29 and 26 kDa in non-reducing conditions. This behavior is probably due to the presence of disulfide bridges between cysteine residues, which are required for the correct functioning of the hemagglutinin, as β-mercaptoethanol completely abolish the agglutinating activity of cell-free celomic fluid. The purified lectin can influence *in vitro* phagocytosis of yeast by celomic leukocytes: in the presence of the molecule, ingestion of foreign particles results significantly decreased and yeast cells agglutinate and forms rosettes around the celomocytes. This suggests a role of the molecule in immunosurveillance.

Key Words: *Sipunculus*; invertebrates; celomic fluid; hemagglutinin; Ca^{2+}-dependent lectin

Introduction

Lectins are carbohydrate-binding proteins widely distributed in living organisms, animals included (Barondes, 1981; Yeaton, 1981a). Animal lectins fulfill a variety of functions (Yoshizaki, 1990; Drickamer and Taylor, 1993; Gabius, 1997; Dodd and Drickamer, 2001) and many of them act as recognition molecules within the immune system, implicated in direct first-line defense against pathogens, cell trafficking and immune regulation (Yeaton, 1981b; Yoshizaki, 1990; Drickamer and Taylor, 1993; Gabius, 1997). As regards the last point, lectins appear to participate in the tagging and exclusion of foreign organisms by invertebrate immunocytes, which are covered with different carbohydrate receptors (Yeaton, 1981b; Yoshizaki, 1990; Gabius, 1997; Kilpatrick, 2002). In the last two decades, the study of animal lectins has greatly increased and today we know that lectin activity in animals is found in association with a wide variety of primary structures which enable us to distinguish at least 12 families of sugar-binding proteins and a series of "orphan" lectins belonging to either some unknown lectin family or well-established protein families with the majority of the members unable to bind sugars. In addition, many animal lectins also bind structures other than carbohydrates via protein-protein, protein-lipid or protein-nucleic acid interactions (Gabius, 1997; Dodd and Drickamer, 2001; Kilpatrick, 2002; Loris, 2002).

Sipunculans are marine worms devoid of circulatory system and with a large celomic cavity filled with celomic fluid containing various types of celomocytes, the majority of which are represented by hemerythrocytes, nucleated cells containing the red pigment hemerythrin (Valembois and Boiledieu, 1980; Dybas, 1981). In addition, the celomic fluid contains wandering ciliated urn cell complexes, which secrete mucus able to trap foreign particles or cells (Bang and Bang, 1976, 1980; Dybas, 1976; Nicosia and Sowinski, 1995), and leukocytes involved in defense responses against non-self materials. Leukocytes are classically classified as granulocytes and hyalinocytes on the basis of the presence or absence of acidophilic or basophilic cytoplasmic granules (Dybas, 1981). Both granulocytes and hyalinocytes can engulf non-self material (Brown and Winterbottom, 1969; Matozzo *et al.*, 2001) and phagocytosis represents the main cell-mediated defense mechanisms against microbes in these organisms. Both the cell types share a similar content of hydrolytic lysosomal enzymes and can produce superoxide anions as a consequence of the activation of a phagocytosis-associated respiratory burst. In addition, they contain lysozyme which can be released as a

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consequence of a bacterial challenge (Matozzo *et al.*, 2001). As regards other humoral immune responses, the presence of various factors, such as lysins, agglutinins, opsonins, and antibacterial molecules has been reported in the celomic fluid of various sipunculan species (Bang, 1966; Weinheimer *et al.*, 1970; Evans *et al.*, 1973; Matozzo *et al.*, 2001).

However, up to now, no clear characterization of soluble molecules involved in immune responses has been carried out. With the aim of contributing to fill this gap, a search for soluble molecules with hemagglutinating activities in the celomic fluid of *Sipunculus nudus* was undertaken. This reports presents some preliminary results on the identification and partial characterization of a Ca^{2+}-dependent lectin with hemagglutinating activity from the celomic fluid of *S. nudus* with specificity for D-galactosides and able to influence phagocytosis.

Materials and Methods

Animals

Specimens of *Sipunculus nudus* were collected by hydraulic dredging in the sandy bottom (5-6 m depth) of the west coast of the Northern Adriatic Sea, off the lagoon of Venice (Italy). They were transferred in 15 l aquaria containing abundant sand on the bottom and filtered (5 µm filter) seawater (FSW) at a temperature of 19 °C.

Celomic fluid collection

Celomic fluid (CF) was collected from the celomic cavity with a 1 ml plastic syringe. CFs from 10 animals were pooled and centrifuged at 780x*g* for 10 min and the supernatant was referred as cell-free celomic fluid (CFCF).

Hemagglutination (HA) assay

Rabbit erythrocytes were washed three times by centrifugation at 500x*g* for 10 min in Tris-buffered saline (TBS: Tris-HCl 50 mM, NaCl 150 mM, pH 7.4) and incubated for 30 min at 37 °C in 0.1 mg/ml trypsin in TBS (Ballarin *et al.*, 1999). They were then washed again and resuspended in TBS containing 0.2 % gelatin to get a 1 % (v/v) solution. Fifty µl of CFCF were serially diluted two-fold with TBS in the wells of U-bottomed microtiter plates and an equal volume of erythrocyte suspension was added to each well; FSW was used in controls. TBS containing 5 mM EGTA or 5 mM $CaCl_2$ (TBS-Ca) was also used to assess the Ca^{2+}-dependency of the reaction. In another experimental series, CFCF was incubated for 30 min with 1 % rabbit erythrocyte to control the specificity of the interaction: the suspension was then centrifuged at 780x*g* for 10 min and the erythrocyte-absorbed supernatant was collected and used in the HA assay. Plates were gently shaken, incubated for 1 h at 37 °C and then kept at 4 °C. The HA titer (HT), i.e., the reciprocal of the highest dilution giving positive HA was then evaluated.

HA inhibition assay

The following sugars were assayed for their effects on the agglutination of trypsinised erythrocytes: D-galactose, D-glucose, D-galactosamine, D-glucosamine, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-α-D-glucopyranoside, 2-deoxy-D-galactose, D-mannose, D-fucose, L-rhamnose, D-melibiose, D-sucrose, D-lactulose, D-lactose. They were purchased from Sigma (St Louis, MO, USA) and added to TBS-Ca to yield 400 mM storage solutions. A total of 25 ml of CFCF were then added to an equal volume of two-fold serial dilutions of carbohydrates in the wells of U-bottomed microtiter plates which were incubated for 30 min at 37 °C. Erythrocytes were then added and, after a further incubation of 60 min at 37°C, the lowest carbohydrate concentrations able to inhibit agglutination were evaluated (modified from Parrinello and Canicattì, 1982).

Effects of periodate and β-mercaptoethanol on hemagglutinating activity

To evaluate the importance of hemagglutinin-conjugated carbohydrates on HA, CFCF was incubated for 2 h at 4 °C with an equal volume of 0.08 M sodium meta-periodate in 0.2 M citrate buffer, pH 5.4 acid in order to oxidize sugars (Millar and Ratcliffe, 1987). The mixture was then dialyzed against TBS-Ca for 3 h to remove periodate and used in HA assay.

The importance of disulphide bridges in hemagglutinating activity was assessed by incubating CFCF with 20 mM of β-mercaptoethanol. The mixture was then dialyzed as described above and used in HA assay.

Lectin purification and characterization

Affinity chromatography of CFCF on acid-treated Sepharose CL-6B (Pharmacia, Uppsala, Sweden) was carried out as described by Parrinello and Canicattì (1982, 1983). The column (7 x 1.6 cm) was previously equilibrated with phosphate-buffered saline (PBS: 0.8 % NaCl, 0.02 % KCl, 0.02 % KH_2PO_4, 0.115 % Na_2HPO_4, pH 7.2) containing 5 mM $CaCl_2$, loaded with 40 ml of CFCF, and washed with a solution of NaCl 1 M and $CaCl_2$ 5 mM. The flow rate was kept constant at 20 ml/h and 2-ml fractions were collected, the absorbance of which was measured, at 280 nm, with a Kontron Uvikon 930 UV-Vis spectrophotometer. When absorbance resulted stable, at values close to zero, the column was eluted with 0.2 M D-galactose in 0.1 M NaCl. A single absorbance peak was usually obtained after elution with D-galactose, and fractions corresponding to the peak were collected, dialyzed overnight at 4 °C against distilled water, lyophilized with a Savant vacuum centrifuge, and stored at -20 °C until use. Protein concentration was evaluated according to Bradford (1976) using bovine serum albumin as standard.

SDS-PAGE (12 % separating gel) of purified lectin was performed according to Laemmli (1970). Samples of lyophilized lectin were diluted to 1.0 mg/ml in sample buffer (0.5 M Tris-HCl, pH 6.8, 10 % glycerol, 10 % SDS, 0.5 % bromophenol blue with or without 5 % β-mercaptoethanol, for reducing and non-reducing conditions, respectively. Proteins treated with β-mercaptoethanol were also boiled for 5 min. Gels were calibrated with low molecular

weight marker proteins (BioRad Laboratories, Hercules, CA, USA), run at a constant current of 18 mA/gel for approximately 3.5 h and stained with Coomassie blue.

Effects of temperature and pH on hemagglutinating activity

To study the effects of temperature on hemagglutinating activity, the *Sipunculus* agglutinin (SA), at a concentration of 2.0 mg/ml in distilled water, was incubated for 30 min at 4, 25, 37, 60 and 80 °C, and then used in the HA assay as previously described. The stability of the lectin, at the above concentration, was tested by assaying its agglutinating activity, in TBS-Ca, after incubation at room temperature for 0, 30, 60, 90, 120 and 180 min.

The effect of pH was evaluated using the following buffers in the HA assay, in the presence of 5 mM $CaCl_2$: 0.2 M Tris-maleate (pH 6.0, 6.6, 7.0, 7.6, 8.0), 0.2 M glycine-NaOH (pH 8.6, 9.0, 9.6, 10.0). Data are expressed as mean ± SD.

Phagocytosis assay

Sixty µl of CF, collected as described above, were placed in the centre of culture chambers, prepared as described by Ballarin *et al.* (1994) and cells were left to adhere to coverslips for 30 min at room temperature (RT). After adhesion, slides were repeatedly washed by dipping in a large volume of FSW in order to remove hemerythrocytes, which do not adhere to glass, and the remaining celomocytes were incubated with 60 ml of a suspension of yeast cell (yeast: hemocyte ratio = 10 : 1) in FSW in the presence or in the absence of 0.5 mg/ml of SA in FSW. In another series of experiments, yeast was previously incubated with SA (0.5 mg/ml in FSW) for 30 min before the assay. Slides were then washed in FWS to remove uningested yeast and cells were fixed for 30 min at 4°C in a solution of 1 % glutaraldehyde and 1 % sucrose in FSW, and stained with 10 % Giemsa for 5 min. At least 300 hemocytes per coverslip were observed under a Leitz Dialux 22 light microscope, in ten optical fields, at a magnification of 1250x, to determine the percentage of hemocytes with ingested yeast cells. Each experiment was repeated three times with three different celomocyte pools. Data are expressed as mean ± SD and were compared using the χ^2 test.

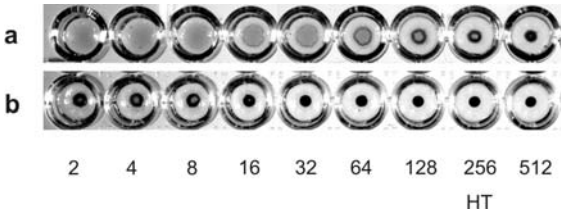

Fig. 1 Presence and absence of hemagglutinating activity of *S. nudus* CFCF incubated in FSW (**a**) or FSW containing 5 mM EGTA (**b**). HT, hemagglutination titer

Results

CFCF shows hemagglutinating activity

CFCF can agglutinate trypsinized rabbit erythrocytes (HT: 64; Fig. 1a). HA was almost absent in erythrocyte-absorbed CFCF. It was inhibited in presence of various monosaccharides and disaccharides at various concentrations. Galactose resulted the most powerful inhibiting sugar, showing the lowest minimum effective concentration, followed by other galactosides and galactose-containing disaccharides (Table 1).

Table 1 Effects of different sugars in hemagglutinating activity of *S. nudus* CFCF

Sugar	Minimum effective concentration (mM)
D-Galactose	50
D-Glucose	200
D-Galactosamine	> 400
D-Glucosamine	> 400
N-Acetyl-D-galactosamine	—
N-Acetyl-D-glucosamine	—
Methyl-α-D-galactopyranoside	100
Methyl-α-D-glucopyranoside	200
Methyl-β-D-galactopyranoside	> 400
2-Deoxy-D-galactose	400
D-Fucose	—
L-Rhamnose	—
D-Mannose	—
D-Sucrose	—
D-Raffinose	—
D-Lactulose	200
D-Lactose	200
D-Melibiose	400

CFCF, cell-free celomic fluid

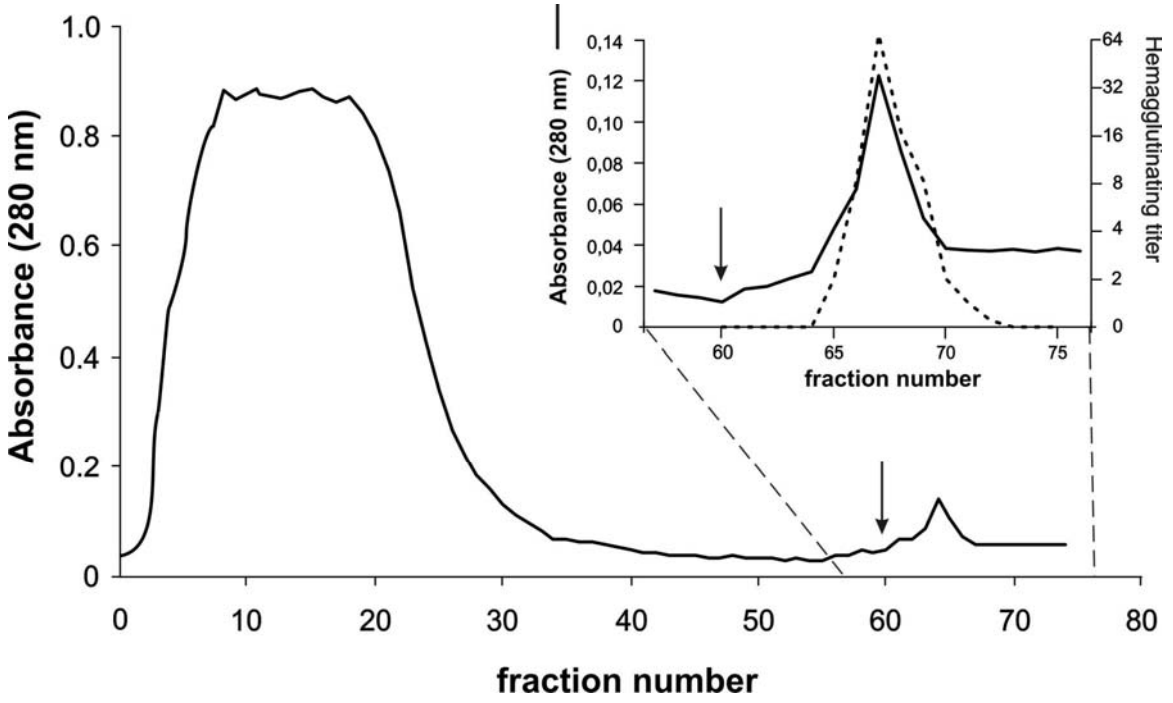

Fig. 2 Affinity chromatography of *S. nudus* CFCF on acid-treated Sepharose CL-6B. Arrows indicate addition of galactose.

The hemagglutinating activity required bivalent cations, as indicated by the absence of HA in the presence of EGTA (Fig. 1b). Treatment with periodate and β-mercaptoethanol completely abolished the ability of CFCF to agglutinate rabbit erythrocytes.

Hemagglutinin purification and physico-chemical characterization of the lectin

Sugar specificity was exploited to purify soluble SA by affinity chromatography on acid-treated Sepharose CL-6B. We obtained a single peak showing hemagglutinating activity towards trypsinized rabbit erythrocytes (Fig. 2).

When SA was incubated at RT, its HT remained stable at the value of 64 after 60 min, but dropped to 16 after 120 min and conserved this residual activity in the following 4 h. The agglutinin resulted lightly thermostable as the HT remained stable at the value of 64 after 30 min exposure at temperatures ranging from 4 to 25 °C; decreased to 8 after 30 min at 37 °C and retained residual detectable activity (HT: 2) after 30 min at 80 °C (Fig. 3a). The lectin was stable within pH ranging from 7.0 to 9.5, with maximum activity around 7.5 (Fig. 3b).

After SDS-PAGE, of the affinity-purified material, two bands were obtained with apparent molecular weight of 33 and 30 kDa and 29 and 26 kDa under reducing and non-reducing conditions, respectively (Fig. 4).

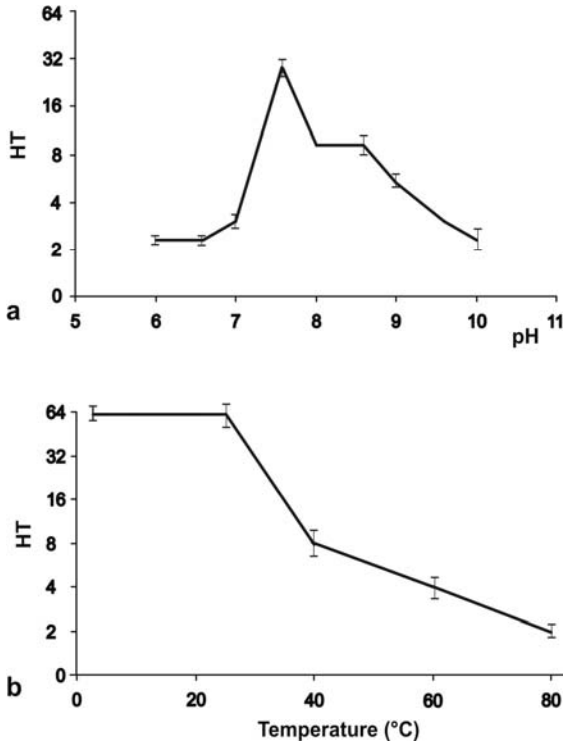

Fig. 3 Effects of pH (**a**) and temperature (**b**) on hemagglutinating activity of *S. nudus* CFCF. HT, hemagglutination titer

Purified lectin can influence phagocytosis

Most of the *S. nudus* leukocytes can phagocytose foreign cells or particles (Fig. 5a, Table 2). Incubation of celomocytes and yeast with the purified lectin resulted in a significant decrease of *in vitro* yeast phagocytosis (Table 2). Yeast cells appeared agglutinated and frequently form rosettes or clumps adherent to celomocyte surface without being ingested (Figs 5b, c). When yeast was preincubated with the purified lectin, washed and incubated with celomocytes in FSW, no significant variation in the fraction of phagocytozing cells with respect to controls was observed (Table 2).

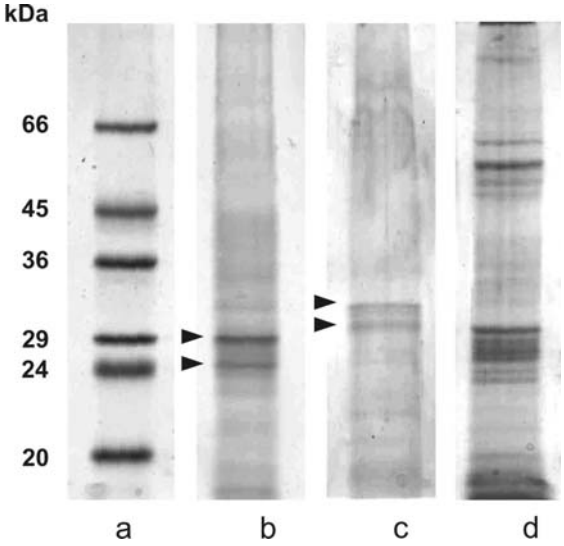

Fig. 4 SDS-PAGE of *S. nudus* purified lectin (**b**, **c**) and CFCF (**d**). Lane *a*) molecular weight markers; lanes **b** and **c**) non reducing and reducing conditions, respectively.

Discussion

In the present study, we have identified a lectin with agglutinating properties in the celomic fluid of *S. nudus*. D-galactose shows the highest inhibiting power and the molecule resulted specific for derivatives of D-galactose, which share the C4 hydroxyl in β position, whereas limited or no effects on HA were observed in the presence of glucosides, mannose, and rhamnose. The C6 hydroxyl is important for sugar-lectin interaction as its absence in fucose resulted in the lack of inhibition. The inhibition of agglutination is influenced by the addition of a methyl group to C1 hydroxyl: it leads to a light decreases if in position α (in methyl-α-D-galactopyranoside), but to a higher decrease when in position β (in methyl-β-D-galactopyranoside). The absence of C2 hydroxyl (in 2-deoxy-D-galactose) reduces the inhibitory power which is further decreased by its substitution as in D-galactosamine and N.acetyl-D-galactosamine: in this case the degree of inhibition depends on the steric hindrance, the latter compound being less effective than the former. Among disaccharides, those containing galactose, i.e., lactulose and lactose, show inhibition of HA although to a less extent than the monosaccharide.

The observed dependency on divalent cations suggests that the identified lectin belongs to the C-lectin family. This is further supported by: i) the nature of the protein which carries glycoconjugates required for the interaction with ligands, as indicated by the absence of HA after treatment with periodate. This excludes its belonging to galectin family, that includes the majority of Ca^{2+}-independent lectins, none of which are glycoproteins (Kasai and Hirabayashi, 1996); ii) the absence of any inhibitory effect on HA of rhamnose and melibiose. This indicates that SA is not a member of rhamnose-binding lectins, another family of sugar-binding proteins which do not require divalent cations for their activity (Jimbo et al., 2007; Terada *et al.*, 2007; Gasparini *et al.*, 2008).

Two bands were present in the electrophoretic pattern of the affinity-purified material. Similar patterns were reported for other purified lectins (Parrinello and Arizza, 1989; Arizza *et al.*, 1991; Gasparini *et al.*, 2008) and they can be interpreted as the consequence of the presence, in the pooled samples, of lightly different isoforms of the same molecule. The presence of different isoforms in the pooled hemolysate has been recently demonstrated

Table 2 Effect of the preincubation and incubation of yeast cells in the affinity-purified lectin on yeast phagocytosis by *Sipunculus* leukocytes

Preincubation medium	Incubation medium	Percentage of phagocytosing cells
FSW	FSW (control)	54.8 ± 8.9
FSW	SA	39.5 ± 5.3 **
SA	FSW	47.1 ± 5.6

FSW, seawater; SA, *Sipunculus* agglutinin
** = $p < 0.01$

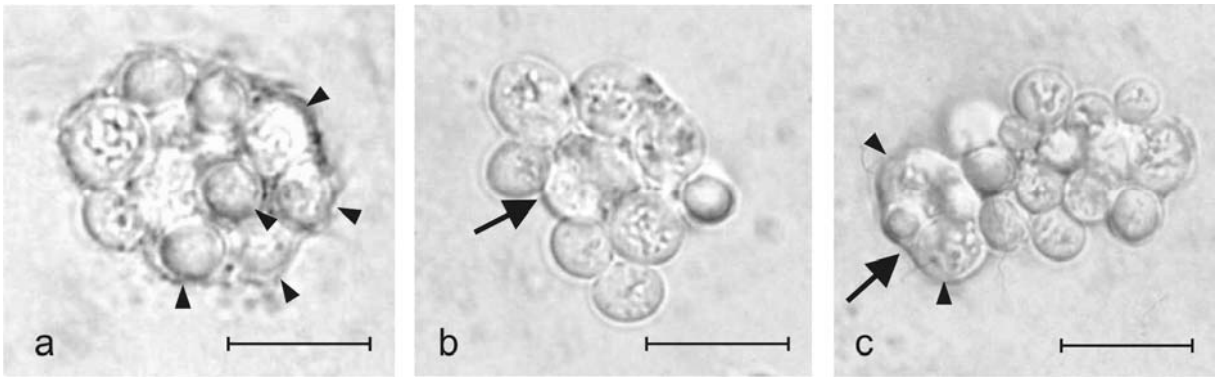

Fig. 5 a) phagocytes filled with ingested yeast cells (arrowheads); **b**) yeast cells clumped around a leukocyte (arrow) forming a rosette; **c**) agglutinated yeast cells in the proximity of a leukocyte (arrow) with ingested yeast cells (arrowheads). Bar = 10 µm.

in the compound ascidian *Botryllus schlosseri* (Gasparini *et al.*, 2008). The apparent molecular weight of the two protein bands of 29 and 26 kDa under non-reducing and 33 and 31 kDa under reducing conditions suggests the presence of intramolecular disulphide bridges which keep the proteins in a highly folded form in non-reducing conditions so that they can move more rapidly in PAGE. This is supported by the observation that β-mercaptoethanol completely abolish the agglutinating capability of CFCF, which stress the importance of the disulphide bonds for the correct functioning of the hemagglutinin. In addition, similar electrophoretic behavior has been reported for various ascidian lectins (Parrinello and Arizza, 1988; Cammarata *et al.*, 2007; Gasparini *et al.*, 2008).

Despite the huge number of published papers on invertebrate lectins, their biological role is still a matter of debate. In most cases, they are thought to be involved in immune recognition, although only in few cases it has been clearly demonstrated. Our lectin is involved in cell-cell interactions between: i) yeast cells enabling their agglutination and hindering their phagocytosis; ii) celomocytes and yeast cells leading to the formation of rosettes. The formation of large aggregates of foreign cells, although preventing their phagocytosis, may stimulate their encapsulation and subsequent melanization by wandering celomocytes, leading to the final formation of brown bodies, present in the hemolymphatic or celomic cavities of many invertebrates (Hetzel, 1965; Valembois *et al.*, 1992, 1994; Jans *et al.*, 1996; Pagliara *et al.*, 2003). We have no direct evidences, at the moment, that this can occur in the case of yeast cells, but numerous brown bodies were frequently found in the celom of the animal used in the present experiments.

Future efforts are, therefore, required to better understand the role of the lectin, identify the source of the protein and complete its sequence for comparative and functional studies.

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References

Arizza V, Parrinello N, Schimmenti S. In vitro release of lectins by *Phallusia mamillata* hemocytes. Dev. Comp. Immunol. 15: 219-226, 1991.

Ballarin L, Cima F, Sabbadin A. Phagocytosis in the colonial ascidian *Botryllus schlosseri*. Dev. Comp. Immunol. 18: 467-481, 1994.

Ballarin L, Tonello C, Guidolin L, Sabbadin A. Purification and characterization of a humoral opsonin, with specificity for D-galactose, in the colonial ascidian *Botryllus schlosseri*. Comp. Biochem. Physiol. 123B: 115-123, 1999.

Bang FB. Serologic response in a marine worm, *Sipunculus nudus*. J. Immunol. 96: 960-972, 1966.

Bang BG, Bang FB. The mucous secretory apparatus of the free urn cell of *Sipunculus nudus*. Cah. Biol. Mar. 17: 423-432, 1976.

Bang BG, Bang FB. The urn cell complex of *Sipunculus nudus*: a model for study of mucus-stimulating substances. Biol. Bull. 159: 571-581, 1980.

Barondes SH. Lectins: their multiple endogenous cellular functions. Ann. Rev. Biochem. 50: 207-231, 1981.

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.

Brown AC, Winterbottom R. The fate of thorium dioxide injected into the coelom of the sipunculid, *Golfingia capensis*. J. Invertebr. Pathol. 13: 229-234, 1969.

Cammarata M, Benenati G, Odom EW, Salerno G, Vizzini A, Vasta GR, *et al*. Isolation and

characterization of a fish F-type lectin from gilt head bream (*Sparus aurata*) serum. Biochim. Biophys. Acta 1770: 150-155, 2007.
Dodd RB, Drickamer K. Lectin-like proteins in model organisms: implications for evolution of carbohydrate-binding activity. Glycobiology 11: 71R-79R, 2001.
Drickamer K, Taylor ME. Biology of animal lectins. Ann. Rev. Cell Biol. 9: 237-264, 1993.
Dybas L. 1976. A light and electron microscopic study of the ciliated urn of *Phascolosoma agassizii* (Sipunculida). Cell Tissue Res. 169: 67-75, 1993.
Dybas L. Sipunculans and echiuroids. In: Ratcliffe NA, Rowley AF (eds), Invertebrate blood cells, Vol. 1, Academic Press, New York, London, pp 161-188, 1981.
Evans EE, Cushing JE, Evans ML. Comparative immunology: Sipunculid bactericidal responses. Infect. Immun. 8: 355-359, 1973.
Gabius H-J. Animal lectins. Eur. J. Biochem. 243: 543-576, 1997.
Gasparini F, Franchi N, Spolaore B, Ballarin L. Novel rhamnose-binding lectins from the colonial ascidian *Botryllus schlosseri*. Dev. Comp. Immunol. 32: 1177-1191, 2008.
Hetzel HR. Studies on holothurian coelomocytes. II. The origin of coelomocytes and the formation of brown bodies. Biol. Bull. 128: 102-111, 1965.
Jans D, Dubois P, Jangoux M. Defensive mechanisms of holothuroids (Echinodermata): formation, role, and fate of intracoelomic brown bodies in the sea cucumber *Holothuria tubulosa.* Cell Tissue Res. 283: 99-106, 1996.
Jimbo M, Usui R, Sakai R, Muramoto K, Kamiya H. Purification, cloning and characterization of egg lectins from the teleost *Tribolodon brandti*. Comp. Biochem. Physiol. 147B: 164-171, 2007.
Kasai K, Hirabayashi J. Galectins: a family of animal lectins that decipher glycocodes. J. Biochem. 119: 1-8, 1996.
Kilpatrick CD. Animal lectins: a historical introduction and overview. Biochim. Biophys. Acta 1572: 187-197, 2002.
Laemmli UK. Cleavage of structural protein during the assembly of the head of the bacteriophage T4. Nature 277: 680-685, 1970.
Loris R. Principles of structures of animal and plant lectins. Biochim. Biophys. Acta 1572: 198-208, 2002.
Matozzo V, Cima F, Perin L, Ballarin L. Phagocytic and enzymatic activities of cells and urn cell complexes in the coelomic fluid of the marine worm *Sipunculus nudus* (Sipuncula). Ital. J. Zool. 68: 273-280, 2001.
Millar DA, Ratcliffe NA. Activity and preliminary characterization of a hemagglutinin from the hemichordate *Saccoglossus ruber*. Dev. Comp. Immunol. 11: 309-320, 1987.
Nicosia SV, Sowinski JM. Cytological analysis of the urn cell complex of *Sipunculus nudus* before and after serum-induced secretion. Biol. Bull. 188: 267-280, 1995.
Pagliara P, Candia Carnevali MD, Burighel P, Ballarin L. The spherule cells of *Holothuria polii* Delle Chiaie, 1823, (Aspidochirata, Holothuroidea) during brown body formation: an ultrastructural study. J. Submicrosc. Cytol. Pathol. 35: 295-301, 2003.
Parrinello N, Canicattì C. Carbohydrate binding specificity and purification by biospecific affinity chromatography of *Ascidia malaca* Traust hemagglutinins. Dev. Comp. Immunol. 6: 53-64, 1982.
Parrinello N, Canicattì C. Lactose-binding haemagglutinins from the ascidian *Phallusia mamillata* (Cuv.). Biol. Bull. 164: 124-135, 1983.
Parrinello N, Arizza V. D-Galactose binding lectins from the tunicate *Ascidia malaca*: subunit characterization and hemocyte surface distribution. Dev. Comp. Immunol. 12: 495-507, 1988.
Parrinello N, Arizza V. Sugar specific cellular lectins of *Phallusia mamillata* hemocytes: purification, characterization and evidence for cell surface localization. Dev. Comp. Immunol. 13: 113-121, 1989.
Terada T, Watanabe Y, Tateno H, Naganuma T, Ogawa T, Muramoto K, *et al.* Structural characterization of a rhamnose-binding glycoprotein (lectin) from Spanish mackerel (*Scomberomorous niphonius*) eggs. Biochim. Biophys. Acta 1770: 617-629, 2007.
Valembois P, Boiledieu D. Fine structure and functions of haemerythrocytes and leucocytes of *Sipunculus nudus*. J. Morphol. 163: 69-77, 1980.
Valembois P, Lassègues M, Roch P. Formation of brown bodies in the coelomic cavity of the earthworm *Eisenia fetida andrei* and attendant changes in shape and adhesive capacity of constitutive cells. Dev. Comp. Immunol. 16: 95-101, 1992.
Valembois P, Seymour J, Lassègues M. Evidence of lipofuscin and melanin in the brown body of the earthworm *Eisenia fetida andrei*. Cell Tissue Res. 277: 183-188, 1994.
Weinheimer PF, Acton RT, Cushing JE, Evans EE. Reactions of sipunculid coelomic fluid with erythrocytes. Life Sci. 9: 145-152, 1970.
Yeaton RW. Invertebrate lectins: I. Occurrence. Dev. Comp. Immunol. 5: 391-402, 1981a.
Yeaton RW. Invertebrate lectins: II. Diversity of specificity, biological synthesis and function in recognition. Dev. Comp. Immunol. 5: 535-545, 1981b.
Yoshizaki N. Functions and properties of animal lectins. Zool. Sci. 7: 581-591, 1990.