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Purification and characterization of a humoral opsonin, with specificity for D-galactose, in the colonial ascidian *Botryllus schlosseri*

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Abstract

A humoral agglutinin from the hemolysate of the colonial ascidian *Botryllus schlosseri* was purified by affinity chromatography. This agglutinin does not require metal cations for its activity and is specific for derivatives of D-galactose. On SDS-PAGE analysis, it was resolved in two bands, of 17 and 19 kDa in reducing conditions and 15 and 16 kDa in non-reducing conditions. This behavior is due to the establishment of disulfide bridges between the thiols of cysteine, well-represented in the molecule as revealed by amino acid analysis. The latter also indicated high percentages of hydrophilic residues, probably involved in sugar recognition. The lectin is an opsonin, as it increases both the phagocytic index and the number of phagocytized yeast cells. The hypothesis that this *Botryllus* agglutinin belongs to the galectin family of lectins is discussed. © 1999 Elsevier Science Inc. All rights reserved.

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1. Introduction

Widely distributed among plants and animals, lectins are proteins able to recognize and bind specific glycoconjugates. Most of them have agglutinating activity towards vertebrate erythrocytes and other animal cells, due to the presence of multiple carbohydrate recognition domains which bind to cell surface sugars.

Several humoral and cellular lectins have been reported in invertebrates, with different specificities, sizes and physico-chemical characteristics [21]. Although their biological role has been elucidated only in a few cases [11,27,28], their involvement in immune defence processes, such as phagocytosis, encapsulation, hemolymph clotting and cytotoxicity, has been suggested for most of them [34].

As far as ascidians are concerned, lectins (both soluble and cell-bound) have been described in *Enterogona* and *Pleurogona*: they show different carbohydrate specificities, metal ion requirements and molecular sizes [23]. Humoral lectins have been revealed by exploiting their hemagglutinating activity [7,8,29], and for some of them it has been possible to assign a biological role. A 14-kDa D-galactose-specific lectin is required for the correct morphogenesis of palleal buds in *Polyandrocarpa misekiensis* [17]. Involvement in non-self recognition and defence reactions has been postulated for the lactose-specific DCL-I lectin of *Didemnum candidum*, showing mitogenic activity [35] and for the 120-kDa lectin of *Halocynthia roretzi*, able to bind gram (+) and gram (−) bacteria by recognizing surface lipopolysaccharides [1]. An opsonic role has been demonstrated for the lactose-binding lectin HA-2 from *Botrylloides leachi* [7] and for a lectin of *Styela clava* which recognizes galactose residues [18]: the latter can also stimulate cell proliferation and has chemotactic properties [19].

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Non-self recognition has been particularly investigated in botryllid ascidians, especially in relation to colony specificity, in which unidentified humoral factors are involved in allorecognition [3,29,31,32]. The presence of a membrane lectin, specific for mannosides, mediating the recognition of yeast cells by phagocytes has been demonstrated in the compound ascidian *Botryllus schlosseri*, and the presence of humoral factors affecting phagocytosis was also suggested on the basis of the effects of yeast pre-incubation in blood plasma [2]. In the same species the presence of humoral hemagglutinins was reported more than a decade ago [8,36] but until now no information about the biochemistry of these factors, their biological functions and cellular origin has been available.

This report presents results on the purification and biophysical characterization of a humoral agglutinin from the hemolysate of *B. schlosseri* and defines a biological role for this molecule in immune defence.

2. Material and methods

2.1. Animals

Wild colonies of *B. schlosseri* from the lagoon of Venice, Italy, were used. They were kept in aerated aquaria, attached to glass slides, and fed with Liquifry Marine (Liquifry Co., Dorking, England, UK) and algae.

2.2. Blood plasma, blood cell lysate and hemolysate (HL) preparation

Blood was collected with a glass micropipette by puncturing the tunic marginal vessels of colonies previously blotted dry. The supernatant obtained after blood centrifugation at $780 \times g$ for 15 min was referred to as blood plasma. Hemocytes were obtained from blood collected from colonies previously rinsed in filtered sea water (FSW) containing 10 mM L-cysteine (Sigma), pH 7.5, to prevent clotting, and then centrifuged at $780 \times g$ for 15 min; pellets were finally resuspended in FSW to give a concentration of 15×10^6 cells/ml. Both hemocytes and whole blood were sonicated at 0°C in a Braun Labsonic U sonifier at 50% duty cycles for 5 min and subsequently centrifuged at $12\,000 \times g$ for 20 min at 4°C to get blood cell lysate and HL, respectively. Protein concentration was determined according to [5] using bovine serum albumin as standard.

2.3. Erythrocytes

Human blood of groups A, B, AB and 0 and blood from rabbit, sheep, cow, guinea pig, turkey and duck in Alsever solution (0.42% NaCl, 0.8% Na-citrate dihydrate, 0.045% citric acid monohydrate, 2.05% D-glucose, pH 7.2) was used. Erythrocytes were washed three times before use by centrifugation at $500 \times g$ for 10 min in Tris-buffered saline (TBS: Tris–HCl 50 mM, NaCl 150 mM, pH 8.0); alternatively, after washing they were incubated with 0.1 mg/ml trypsin in TBS for 30 min at 37°C, washed again three times in TBS and then used in hemagglutination assays.

2.4. Hemagglutination (HA) assay

A total of 25 μl of either blood plasma, blood cell lysate, HL or purified lectin (2.0 mg/ml) were serially diluted two-fold with TBS in the wells of U-bottomed microtiter plates, and an equal volume of 1% erythrocyte suspension in TBS containing 0.2% gelatin was added to each well. Plates were gently shaken, incubated for 1 hr at 37°C and then taken to 4°C. The hemagglutination titre was defined as the reciprocal of the highest dilution giving positive hemagglutination. Trypsinized rabbit erythrocytes (RE) were routinely used in the HA assay of the purified agglutinin (see below).

HL was dialysed overnight against distilled water and $CaCl_2$ and $MgCl_2$ (both at 10, 20, 40 and 80 mM concentrations) or EGTA at 1, 2 and 4 mM concentrations was added to TBS in HA assay to determine the effects of divalent cations on lectin agglutinating activity.

HL was also absorbed with equal volumes of packed trypsinized RE for 1 h and the supernatant resulting from centrifugation at $500 \times g$ was used in the HA assay.

2.5. Yeast agglutination assay

Ordinary baker's yeast was thoroughly washed in TBS and cells were incubated with a solution (final concentration: 2.0 mg/ml) of purified lectin in TBS at room temperature for 30 min. Aliquots of the suspension were then carefully collected, placed on glass slides, and observed under a Leitz Dialux 22 light microscope to evaluate yeast agglutination.

2.6. HA inhibition assay

The following sugars were assayed for their effects on the agglutination of trypsinized erythrocytes: D-sorbitol, D-glucose, D-galactose, D-mannose, D-glucosamine, D-galactosamine, D-mannosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, methyl-α-D-glucopyranoside, methyl-α-D-mannopyranoside, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, 2-deoxy-D-galactose, D-fucose, *p*-nitrophenyl-α-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, D-sucrose,

D-lactose, D-lactulose, D-melibiose and D-raffinose. They were purchased from Sigma and added to TBS to yield 0.4 M storage solutions. A total of 25 μl of HL were then added to an equal volume of two-fold serial dilutions of carbohydrates in the wells of U-bottomed microtiter plates and incubated for 30 min at 37°C. Hemocytes were then added and, after a further 60 min of incubation at 37°C, the lowest carbohydrate concentrations able to inhibit agglutination were evaluated (modified according to [24]).

2.7. Lectin purification

Affinity chromatography of *Botryllus* HL on acid-treated sepharose CL-6B (Pharmacia) was carried out as described by Parrinello and Canicattì [24]. The column (15 × 1.5 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), loaded with 40 ml of HL, and washed with NaCl 1 M. The flow rate was kept constant at 20 ml/h and 2-ml fractions were collected, the absorbance of which at 280 nm was measured on a Kontron Uvikon 930 UV/Vis spectrophotometer. When absorbance reached stability at values close to zero, the column was eluted with 0.2 M D-galactose in 0.1 M NaCl. Both non-absorbed and D-galactose eluted fractions, after dialysis for 12 h against distilled water, were assayed for hemagglutinating activity towards trypsinized rabbit erythrocytes. A single absorbance peak was usually obtained after elution with D-galactose, and fractions corresponding to the peak were collected, dialysed overnight at 4°C against distilled water, lyophilized with a Savant vacuum centrifuge, and stored at −20°C until use.

2.8. Effects of temperature and pH on hemagglutinating activity

To study the effects of temperature on hemagglutinating activity, the purified lectin was diluted at a concentration of 2.0 mg/ml, 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 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: 0.2 M Na-acetate (pH 4.6, 5.0, 5.6), 0.2 M phosphate buffer (pH 6.0, 6.6, 7.0, 7.6, 8.0), 0.2 M glycine buffer (pH 8.6, 9.0, 9.6, 10.0).

2.9. Amino acid composition

For amino acid analysis, samples were dialysed, oxidized with performic acid and hydrolysed with 6 M HCl for 24 h, according to [15]. Previous experiments had shown that hydrolysis was complete after such a period. Samples were assayed on a Beckman 6300 amino acid analyser. The most likely set of residues and the range of molecular weights of the chain were obtained by comparing three amino acid analyses. Data were processed according to [9].

2.10. Electrophoresis and staining for carbohydrates

SDS-PAGE (10% separating gel) of purified lectin was performed according to [20]. Samples of lyophilized lectin were adjusted to 2.0 mg/ml with distilled water and then diluted with an equal volume of SDS sample buffer (0.5 M Tris–HCl, pH 6.8, 10% glycerol, 10% SDS, 0.5% bromophenol blue), with or without 5% β-mercaptoethanol, and boiled for 3 min. Gels were calibrated with low molecular weight marker proteins (Sigma) for determination of lectin molecular weight and run at a constant current of 18 mA/gel for approximately 3.5 h. Protein bands were visualized with Coomassie brilliant blue R-250.

Staining for carbohydrates was also applied: in this case, after electrophoresis, the gel was fixed in 7.5 acetic acid for 1 h, rinsed three times in distilled water, oxidized in 0.2% periodic acid for 45 min, rinsed in tap water, and stained with Shiff's reagent for 45 min. The gel was finally washed in warm running tap water for 5 min (modified after [6]). In addition, proteins were also transferred from gels to 0.45 μm Electran (BDH) nitrocellulose membranes according to [33], using a Bio-Rad Trans-Blot cell. After blotting membranes were immersed in PBS containing 5% milk powder for 1 h and incubated with horse radish peroxidase-labeled concanavalin A (HRP-ConA; Sigma) at the final concentration of 10 μg/ml for 2 h. They were then extensively washed with PBS and stained with a solution of 0.5 mg/ml of 3,3′-diaminobenzidine (DAB; Sigma) in PBS, containing 0.01% H_2O_2 (modified after [10]).

2.11. Hemocyte cultures and phagocytosis assay

Blood cells were collected with a glass micropipette after puncturing, with a fine tungsten needle, the marginal vessels of colonies previously rinsed in filtered sea water (FSW) containing 10 mM L-cysteine to prevent clotting. They were centrifuged at 780 × *g* for 10 min and resuspended in FSW at a final concentration of 10^7 cells/ml. A total of 60 μl of hemocyte suspension were placed in the center of the wells, made by glueing teflon rings (15 mm internal diameter, 1 mm thick) on siliconized glass slides. Washed coverslips were gently pressed down over the teflon rings, previously smeared with vaseline, to touch the drop of cell suspension. Culture slides were kept upside-down for 30 min to allow cells to settle and adhere. Dirty FSW was then

discarded and replaced with an equal volume of a suspension of yeast in FSW (yeast:hemocyte ratio = 10:1). In experimental series, yeast was resuspended in lectin-containing FSW (final concentration: 2.0 mg/ml). In other experimental series, yeast was previously incubated for 30 with the purified lectin (final concentration: 2.0 mg/ml). Cultures were kept upside-down for 30 and 60 min. Hemocyte monolayers were then washed by dipping the coverslips several times in FSW, fixed in a solution of 1% glutaraldehyde and 1% sucrose in FSW at 4°C for 30 min, and stained with 10% Giemsa for 10 min. The coverslips were finally mounted on glass slides with an aqueous medium (Acquovitrex, Carlo Erba), and cells were observed under a Leitz Dialux 22 light microscope.

Hemocytes were counted at a magnification of 1250 ×, at least 200 cells per coverslip in ten fields, and the phagocytic index, i.e. the percentage of hemocytes with ingested particles, was determined.

2.12. Statistical analysis

Each experiment was repeated at least three times. Values are given as means ± SD. Phagocytosis indexes were compared using the X^2 test. Student's t-test was used for agglutinating titers.

3. Results

3.1. Agglutinating activity of B. schlosseri HL

As previously reported [8,36], *Botryllus* HL can agglutinate mammalian erythrocytes. Agglutination of rabbit erythrocytes (RE) was higher than that of the other mammalian species tested, and their agglutination titres increased when trypsinized cells were used. No agglutination of red blood cells from the two avian species was observed (Table 1).

Hemagglutinating activity was maintained after dialysis against distilled water and was not affected by the addition of divalent cations or EGTA in the incubation medium. Following absorption with RE, the HA titre completely disappeared.

The Ca^{2+}-independent hemagglutinating activity was also present in blood plasma (HA titre: 32–64) and in blood cell lysate (HA titre: 64–128).

3.2. Carbohydrate specificity of Botryllus hemagglutinin

Hemagglutinating activity towards trypsinized RE was inhibited by the presence of derivatives of D-galactose in the incubation medium (Table 2). The C6 hydroxyl seems to be important for correct sugar–lectin interactions, as its absence in fucose resulted in normal HA. The absence of the C2 hydroxyl does not alter the inhibitory power of sugars, which is influenced by the steric hindrance of replacement groups. Among the oligosaccharides, only galactose derivatives can inhibit RE agglutination, lactose having the highest inhibitory power, blocking HA at a concentration of 25 mM.

Table 1
Hemagglutinating activity of *Botryllus* hemolysate towards erythrocytes of various mammalian and avian species, either untreated or incubated for 30 min at 30°C with 0.1 mg/ml trypsin

Erythrocytes	Agglutinating titre	
	Treatment	
	Untreated	Trypsin (0.1 mg/ml)
Human A	2–4	2–4
Human B	2–4	2–4
Human 0	2–4	2–4
Human AB	2–4	2–4
Guinea pig	–	–
Rabbit	16–32	128–256
Sheep	4–8	–
Cow	2–4	2–4
Turkey	–	–
Duck	–	–

Table 2
Effect of different sugars on hemagglutinating activity of *B. schlosseri* hemolysate

Sugar	Minimum effective concentration (mM)
D-Glucose	–
D-Glucosamine	–
N-Acetyl-D-glucosamine	–
Methyl-α-D-glucopyranoside	–
D-Mannose	–
D-Mannosamine	–
Methyl-α-D-mannopyranoside	–
D-Fucose	–
D-Galactose	50
2-Deoxy-D-galactose	50
D-Galactosamine	200
N-Acetyl-D-galactosamine	–
Methyl-α-D-galactopyranoside	100
Methyl-β-D-galactopyranoside	50
p-Nitrophenyl-α-D-galactopyranoside	100
p-Nitrophenyl-β-D-galactopyranoside	50
D-Sucrose	–
D-Lactose	25
D-Lactulose	50
D-Melibiose	100
D-Raffinose	50
D-Sorbitol	–

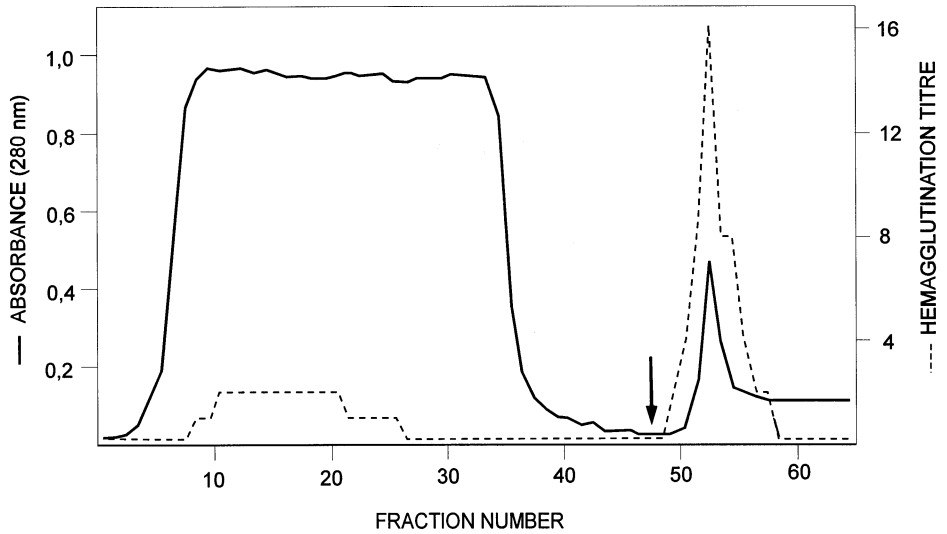

Fig. 1. Affinity chromatography of *B. schlosseri* hemolysate on acid-treated sepharose CL-6B. Arrow indicates addition of galactose.

3.3. Lectin purification

Sugar specificity was exploited to purify a soluble *Botryllus* hemagglutinin by affinity chromatography on acid-treated sepharose CL-6B. After loading with HL and extensive washing of the column, a soluble protein, seen as a single peak at 280 nm, was eluted with D-galactose (Fig. 1). Once dialysed and concentrated by lyophilization, it was easily able to agglutinate RE (up to an HA titre of 512 at 2.0 mg/ml) and yeast cells (Fig. 2).

Low agglutinating activity was observed in the break-through peak fractions, the hemagglutinating titres of which considerably increased (from 2 to 32) when 10 mM $CaCl_2$ was added in the HA assay.

3.4. Physico-chemical characterization

The HA titre of the purified lectin decreased from the value of 128 after 30 min incubation at 4°C to 32 and 16 when incubated at 20–40°C and 16°C, respectively. A HA titre of 8 was still maintained after 30 min incubation at 80°C. The HA titre at room temperature decreased progressively with the pre-incubation time: from the value of 256–512 registered in the absence of pre-incubation, a value of 32 was observed after 3 h of incubation. The protein was stable within pH ranging from 5 to 8, with maximum activity around 8.

The agglutinin has the amino acid composition reported in Table 3. It shows similarities with other deuterostome lectins with specificity for D-galactose in having relatively high quantities of amino acids with hydrophilic residues, particularly Asx and Glx, and low contents of basic amino acids (Table 4).

After SDS-PAGE of the purified lectin, two bands were obtained with apparent molecular weights of 17 and 19 kDa in reducing conditions and 15 and 16 kDa in non-reducing conditions, respectively (Fig. 3). In no cases the two bands stained for carbohydrates, neither after gel treatment with Schiff's reagent, nor following western blot staining with HRP-ConA and DAB.

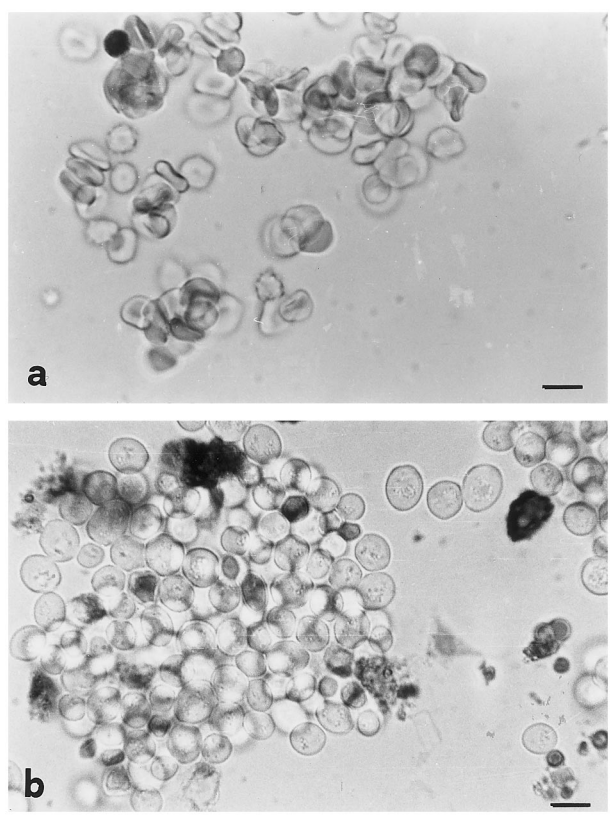

Fig. 2. Agglutination of rabbit erythrocytes (a) and yeast cells (b) by *Botryllus* purified lectin. Bar length: 10 μm for blood cells, 5 μm for yeast.

Table 3
Amino acid composition of *B. schlosseri* purified lectin

Amino acid	Residues per molecule	Nearest integer	Mol%
Cys[a]	10.67	11	6.9
Asx	20.50	20–21	13.3
Thr	14.30	14	9.3
Ser	13.75	14	8.9
Glx	15.62	16	10.1
Pro	8.47	8	5.5
Gly	12.87	13	8.3
Ala	10.01	10	6.5
Val	11.00	11	7.1
Ile	5.72	6	3.7
Leu	5.61	6	3.6
Met[b]	4.18	4	2.7
Tyr	3.74	4	2.4
Phe	3.50	3–4	2.3
His	2.86	3	1.9
Lys	5.94	6	3.7
Trp	N.D	N.D	N.D.
Arg	5.72	6	3.7
Total residues	155–157		
PM	16553–16815		

[a] Measured as cysteic acid.
[b] Measured as methionine sulfone.

3.5. Biological role

Pre-incubation of yeast with the purified lectin resulted in a significant ($P < 0.001$) increase in the phagocytic index after 30 and 60 min of incubation. The maximum number of phagocytized yeast cells also increased from four (untreated yeast) to seven (lectin-treated yeast) after 60 min of incubation. A similar significant ($P < 0.001$) increase in phagocytic activity was obtained resuspending yeast in lectin-containing FSW without previous incubation in the *Botryllus* agglutinin. In this latter case, no significant increase in the phagocytic index was observed when 0.2 M D-galactose was added to lectin-containing FSW (Table 5).

4. Discussion

The presence of humoral agglutinins in the colonial ascidian *B. schlosseri* was reported by Vasta et al. [36] in a study on the distribution of lectins in North American tunicates. Coombe et al. [8] confirmed previous data and demonstrated the presence of at least two hemagglutinins, one Ca^{2+}-dependent, with an apparent molecular weight greater than 150 kDa, and the other Ca^{2+}-independent, with an apparent molecular weight of around 67 kDa.

In the present work, we identified a Ca^{2+}-independent humoral agglutinin in the HL of *Botryllus*. The presence of the Ca^{2+}-independent hemagglutinating activity in both blood plasma and blood cell lysate suggests that hemocytes themselves are involved in the synthesis and the secretion of the lectin. Our preliminary (unpublished) results support the above hypothesis. The agglutinin was specific for derivatives of

Table 4
Comparison of amino acid compositions of some deuterostome lectins with specificity for D-galactose derivatives

	Botryllus schlosseri	*Halocynthia roretzi* [38]	*Stichopus japonicus* [12]		*Anthocidaris crassispina* [22]
			SJL-I	SJL-II	
PM (kDa)	15–16	41	13	15	11.5
Amino acid	Mol%				
Cys	6.9	1.3	7.0	6.5	7.4
Asx	13.3	13.3	10.9	15.2	7.7
Thr	9.3	8.1	4.9	3.6	4.9
Ser	8.9	7.6	8.0	9.3	12.0
Gly	10.1	10.2	8.5	13.7	11.7
Pro	5.5	4.7	8.7	5.8	4.0
Gly	8.3	9.0	11.4	9.7	13.6
Ala	6.5	6.7	8.0	5.2	5.2
Val	7.1	4.8	4.6	3.0	7.5
Ile	3.7	6.3	2.6	3.5	0.4
Leu	3.6	4.0	4.0	6.1	5.3
Met	2.7	0.4	2.2	1.3	6.8
Tyr	2.4	5.5	6.6	6.0	2.8
Phe	2.3	2.8	5.7	3.9	4.7
His	1.9	5.0	3.0	1.0	0.0
Lys	3.8	4.8	2.5	2.4	7.0
Trp	N.D.	2.9	N.D.	N.D.	N.D.
Arg	3.7	2.7	1.6	3.8	4.6

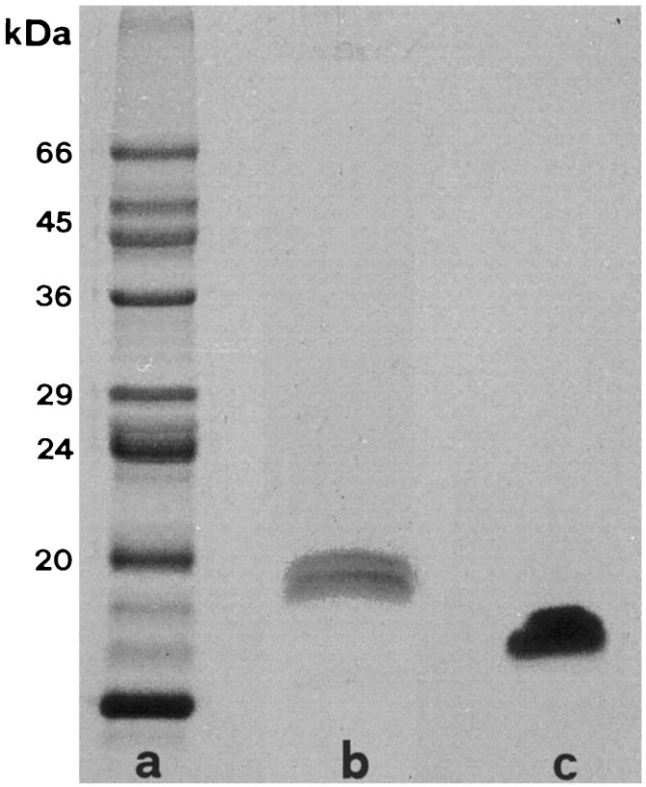

Fig. 3. SDS-PAGE of purified *B. schlosseri* lectin in reducing (lane b) and non-reducing (lane c) conditions. Lane a: molecular weights.

D-galactose, the highest inhibiting power being exerted by lactose.

We purified this lectin by affinity chromatography on acid-treated sepharose CL-6B in the absence of divalent cations in the elution buffer, and obtained a single protein peak after elution with D-galactose. After lyophilization and reconstitution in distilled water, the lectin showed high agglutinating activity towards RE and yeast cells. The higher HA titre observed using trypsinized RE is due to a better exposition of recognized sugars on erythrocyte surface after enzyme treatment, as already reported for other ascidian agglutinins [36].

Table 5
Effects of pre-incubation in the purified *Botryllus* agglutinin on yeast phagocytosis

Medium of pre-incubation	Medium of incubation	Phagocytic index (incubation time)	
		30 min	60 min
FSW	FSW (control)	5.6 ± 1.1	12.3 ± 2.4
BA	FSW	9.6 ± 0.3[a]	21.9 ± 3.7[a]
FSW	BA	8.7 ± 0.5[a]	19.8 ± 2.1[a]
FSW	BA + D-gal	5.4 ± 0.7	10.9 ± 1.8

[a] Significant differences with respect to the controls: $P < 0.001$. BA, *Botryllus* agglutinin (2.0 mg/ml); D-gal, D-galactose (0.2 M).

The low agglutinating activity found in the unretained fractions is probably due to the humoral Ca^{2+}-dependent agglutinins already evidenced by Coombe et al. [8].

Amino acid analysis of the purified lectin showed a relative abundance of hydrophilic amino acids, probably involved in sugar recognition, as suggested by Hirabayashi and Kasai [13], and a similarity with other D-galactose-specific agglutinins of deuterostomes. Unfortunately, no functional relationships are possible, due to the lack of comparative data. The relative abundance in cysteine may explain the behavior of the two bands revealed by electrophoretic analysis of the D-galactose-eluted peak, whose apparent molecular weights change from 15 and 16 kDa to 17 and 19 kDa when passing from non-reducing to reducing conditions. This is due to the establishment of intramolecular disulfide bridges between the cysteine residues which shrink the subunits in non-reducing conditions so that they move more rapidly. Similar behavior has been reported for *Ascidia malaca* and *Phallusia mamillata* serum lectins [25,26].

On the basis of chromatographic profiles on gel filtration chromatography, Coombe et al. [8] calculated an apparent molecular weight of 67 kDa for the *B. schlosseri* Ca^{2+}-independent and D-galactose-specific agglutinin. This disagreement with our results, indicating the presence of two subunits with much lower molecular weights, suggests that the native form of the molecule is a tetramer with a couple of both 17- and 19-kDa subunits. The possibility that functional oligomers of D-galactose-binding proteins may form has already been reported in the sponge *Geodia cydonium* [37].

Invertebrate lectins are the subject of a vast production in the scientific literature and many of them have also been purified, but in most cases their biological role remains unknown. The few exceptions are some molluscan and tunicate lectins, for which an opsonic role has been demonstrated [7,11,18,27,28]. In Styelid ascidians, opsonins have been characterized in both solitary (*S. clava* [18]) and colonial (*B. leachi* [7]) species. In *B. leachi*, three hemagglutinins have been purified, only one of them (HA-2) being metal-independent, specific for D-galactose derivatives, and having opsonic activity [30]. On the basis of physico-chemical properties Coombe et al. [8] related the *Botrylloides* HA-2 to the Ca^{2+}-independent, D-galactose-binding *Botryllus* lectin and proposed an opsonic role for the latter. We have clearly demonstrated an opsonic role for the metal-independent *B. schlosseri* agglutinin, as it mediates interactions between yeast cells and phagocyte surfaces, thus enhancing both the phagocytic index and the number of ingested cells.

No stain for carbohydrates was revealed with either the PAS reaction or HRP-ConA in the bands obtained

after SDS-PAGE. The absence of glycosylation and the blocked N-termini (our unpublished observation), together with the above results (i.e. hydrophilicity, molecular weight, metal independence and sugar specificity) suggest that our opsonin is a member of the galectin family, belonging to the prototype group [4,16].

Until now galectins have been found in sponges [37], nematodes [14] and vertebrates [16], but they are believed to be widely distributed in the animal kingdom. Although they are assumed to play a role in several biological phenomena such as development, differentiation and immunity, no direct evidence is yet available except for the galectin of the sponge *G. cydonium*, which acts as an adhesion molecule involved in cell interactions during re-aggregation [37]. Since the presence of galectins in lower deuterostomes is expected [16], our data, if confirmed, demonstrate the presence of galectins in tunicates. Future efforts will be directed towards corroborating the above hypothesis.

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