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# Humoral opsonins of the tunicate, *Pyura stolonifera*

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### Abstract

This study characterizes humoral opsonins from the tunicate, *Pyura stolonifera*. The predominant opsonic components in *P. stolonifera* hemolymph were found to be calcium-dependent lectins with broad carbohydrate specificities. The opsonic lectins were purified by carbohydrate affinity chromatography which eluted a complex pattern of proteins ranging in molecular mass from 80 to >200 kDa. Reducing and two dimensional SDS–PAGE indicated that the diversity of mature lectins evident under non-reducing conditions resulted from the differential oligomerization of two polypeptide sub-units (35 and 22 kDa). In addition to lectin-mediated opsonic activity, hemolymph was also found to contain proteolytically activated opsonins. These data suggest that multiple, possibly interactive opsonic systems co-exist in *P. stolonifera*. © 2001 Elsevier Science Ltd. All rights reserved.

**Keywords:** Tunicate; Ascidian; Lectin; Phagocytosis; Complement; Opsonization; *Pyura stolonifera*

### 1. Introduction

Immunological recognition systems based on rearranging antibodies seem to be restricted to the jawed vertebrates (gnathostomes). All other animals (the invertebrates and agnathans) rely on non-immunoglobulin recognition molecules to initiate cellular defensive processes [1–4]. In crustaceans, for instance, the prophenoloxidase (proPO) cascade, which cata-

lyzes the oxidation of phenols to quinones during melanization reactions, can elicit phagocytosis and cellular activation [5]. In defensive contexts, proPO is activated by a proteolytic cascade responding to tissue damage or the recognition of microbial cell wall constituents such as LPS and peptidoglycan. Intermediates in the proPO cascade that lead to melanin formation, and melanin itself, are believed to be fungistatic [6], whilst other components, notably  $\beta$ -1,3-glucan binding proteins, act as humoral opsonins [7].

Proteolytic systems with opsonic activity have also been identified in the deuterostome relatives of jawed vertebrates (agnathans, tunicates and echinoderms). Homologues of complement component C3 have recently been identified in LPS-activated coelomocytes of the sea urchin, *Strongylocentrotus purpuratus*, and in the hemolymph of lampreys, hagfish and tunicates [8–12]. These complement-like proteins from deuterostomes have the capacity to bind foreign

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**Abbreviations:** BSA, bovine serum albumin; CRD, carbohydrate recognition domain; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; FITC, fluorescein isothiocyanate; galNAC, N-acetyl-D-galactosamine; gluNAC, N-acetyl-D-glucosamine; FSW, sterile filtered seawater; MAC, marine anti-coagulant; PBS, phosphate buffered saline; proPO, prophenoloxidase; PSI, phagocytic stimulation index; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

targets and initiate downstream effector responses including phagocytosis [11,13].

In addition to these proteolytic opsonic systems, carbohydrate-binding lectins have often been associated with opsonization in invertebrates [14,15]. Opsonic humoral lectins have been identified in mollusks, arthropods and tunicates [1,16–22]. In the solitary tunicate, *Styela plicata*, the opsonic activity of hemolymph (blood equivalent) is primarily mediated by a galactose-specific lectin that is structurally related to vertebrate collectins [23,24]. Like its vertebrate counterparts, the tunicate collectin is an oligomer of 46 kDa sub-units that are comprised of multiple functional domains including a collagenous region and a calcium-dependent carbohydrate recognition domain (CRD).

Even though opsonic lectins have been identified in a number of animal groups, they show little molecular uniformity between species apart from the expression of homologous CRDs [18,20–23,25,26]. This structural diversity has led Weis and Drickamer [26] to conclude that the ability of lectins to opsonize carbohydrate antigens has evolved independently on many occasions resulting in a variety of protein structural frameworks.

In this study, we characterize humoral opsonins from the solitary tunicate, *Pyura stolonifera*. The data suggest that lectins with broad carbohydrate specificities and highly oligomeric structures are primarily responsible for opsonization. However, evidence is presented which implies that a proteolytic system also contributes to opsonic activity.

## 2. Materials and methods

### 2.1. Collection of hemocytes and serum

*P. stolonifera* were collected from Balmoral Beach, NSW, Australia. They were maintained in aerated aquaria filled with seawater (15°C) for up to 6 days. Tunicates were bled by severing major hemolymph sinuses at the base of the tunic. Serum was obtained by collecting hemolymph in chilled microcentrifuge tubes, followed immediately by centrifugation (16,000 × g, 10 s) to remove hemocytes. To harvest hemocytes for opsonization assays, 5 ml hemolymph was collected in chilled polyethylene tubes containing

5 ml marine anti-coagulant (MAC; sodium chloride 26.3 g/l, trisodium citrate 4.4 g/l, citric acid 2.73 g/l, ethylenediamine tetraacetic acid 3.72 g/l, pH 7.0). The hemocyte suspensions were then centrifuged (200 × g, 5 min), the supernatants removed and the cell pellets resuspended in filtered seawater (FSW, 0.45 µm filter, Millipore, Pleasanton, CA) to yield 2 × 10<sup>6</sup> cells/ml.

### 2.2. Biotinylation of serum proteins and neoglycoproteins

NHS-LC-Biotin (Pearce; 200 mg/ml; 10 µl per 2.5 ml of serum or neoglycoprotein) was added to 20 ml serum or 5 ml neoglycoprotein solutions (1 mg/ml of bovine serum albumin conjugated fucose, glucose, galactose, N-acetyl-D-galactosamine or lactose; Sigma Chemicals, St Louis, MO), and incubated at room temperature for 4 h, with occasional gentle mixing. Ammonium chloride was then added (8 mM final concentration) for 10 min, followed by Tris-HCl (pH 8; 10 mM final concentration) to inhibit the biotinylation reaction. Biotinylated proteins were stored aliquoted at –20°C.

### 2.3. Opsonization assay

The opsonization assay used here was based on those of Anderson and Mora [27], Hed [28] and Thornqvist et al. [7]. Bakers Yeast type II (*Saccharomyces cerevisiae*; Sigma Chemicals) labeled with fluorescein isothiocyanate (FITC) were used as targets for opsonization. To prepare these target cells, washed yeast (10<sup>9</sup> cells/ml) were incubated with FITC solution (0.1 mg/ml) for 30 min at 37°C, mixing gently every 10 min. The FITC-stained yeast were then washed five times with phosphate buffered saline (PBS; sodium chloride 8.0 g/l, potassium chloride 0.2 g/l, sodium hydrogen orthophosphate 1.44 g/l, potassium dihydrogen orthophosphate 0.24 g/l, pH 7.2) and stored at –20°C.

For opsonization, 10<sup>7</sup> FITC-yeast cells were incubated with serum or affinity chromatography fractions for 60 min (room temperature, shaking). Yeast were then washed by centrifugation (16,000 × g, 10 s) three times with FSW (1 ml per wash) before being resuspended in 500 µl FSW. In some cases, serum or affinity chromatography fractions were pre-incubated with a range of potential opsonin inhibitors (EDTA,

carbohydrates or protease inhibitors) for 15 min before being added to yeast. The protease inhibitors used were: phenylethanolamine-N-methyltransferase (PMSF; Sigma) and 4-(2-aminoethyl)-benzenesulfonyl-flouride, hydrochloride (Pefabloc, Boehringer Mannheim).

After opsonization, 250  $\mu$ l yeast suspensions were added to polystyrene flow cytometry tubes (Becton Dickinson, Burlingame, CA) containing 250  $\mu$ l hemocyte suspensions ( $2 \times 10^6$  cells/ml in FSW). This mixture gave a hemocyte to yeast (effector: target) ratio of 1:2.5. The hemocytes and yeast were co-incubated for 20 min at room temperature before phagocytosis was terminated by adding sodium azide to a final concentration 0.1% w/v.

The percentage of tunicate cells that had phagocytosed FITC-yeast was determined using a FACScan flow cytometer (Becton Dickinson). Gates were set in forward vs  $90^\circ$  light scatter plots to exclude the vast majority of non-ingested yeast. The fluorescence of any remaining non-ingested yeast was quenched by adding 25  $\mu$ l trypan blue (10 mg/ml) 1 min prior to analysis. Hemocytes that had ingested FITC-yeast were then identified in green fluorescence histograms (Fig. 1(A)). Data were calculated as phagocytic stimulation indexes (PSI), which represented the percentage of hemocytes that had ingested opsonized FITC-yeast divided by the mean percentage of hemocytes that had ingested non-opsonized FITC yeast (i.e. yeast incubated in FSW only). In some cases, data are shown as a percentage inhibition of opsonization, which was calculated as the PSI for FITC-yeast opsonized in the presence of potential inhibitors divided by the mean PSI of FITC-yeast opsonized with serum in the absence of potential inhibitors.

#### 2.4. Carbohydrate binding assay

To gauge the ability of *P. stolonifera* proteins to bind carbohydrates, serum (100  $\mu$ l per well) was aliquoted into 96 well flat-bottomed EIA/RIA plates (Costar, Cambridge, MA) and incubated overnight at  $4^\circ\text{C}$ . The wells were then washed with FSW and blocked with bovine serum albumin (BSA, 5% w/v in FSW) for 1 h at room temperature. The wells were washed again with FSW before biotinylated neoglycoproteins were added (100  $\mu$ l per well). Plates were incubated with neoglycoproteins for 2 h at room

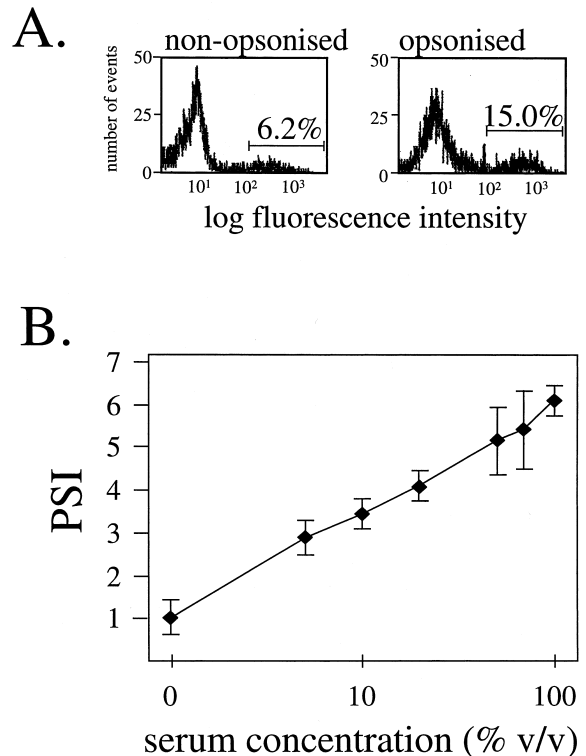


Fig. 1. Opsonic activity of *P. stolonifera* serum. (A) Log fluorescence histograms of a typical opsonization assay using FITC-yeast that had been incubated with FSW (non-opsonized) or 20% v/v serum (opsonized). The regions of the histogram representing FITC-yeast that had been ingested by *P. stolonifera* hemocytes are shown by bars with the percentage of cells in that region shown above, (B) The effects of opsonization with increasing concentrations of *P. stolonifera* serum on phagocytic stimulation indexes (PSI). Bars represent SEM,  $n \geq 4$ .

temperature and then washed three times with FSW. Extravidin alkaline phosphatase (1:5000 dilution, 100  $\mu$ l) was then added to each well for 1 h, followed by 100  $\mu$ l p-nitrophenyl phosphate (Sigma Chemicals; 1 mg/ml in 0.1 M carbonate buffer). Absorbance was measured at 410 nm after 10 min.

#### 2.5. Carbohydrate affinity chromatography of *P. stolonifera* serum

One hundred milliliters of serum was filtered (0.45  $\mu$ m) and then passed twice over 1 ml 4% beaded agarose conjugated with either N-acetyl-D-glucosamine (gluNAc) or N-acetyl-D-galactosamine (galNAc)

(Sigma Chemicals) in an Econoflow 10 ml column (BioRad, Sydney, NSW). Serum components that had not bound to the columns (designated 'flowthrough') were collected after the second passage. The columns were then washed with at least 50 ml FSW before 5 ml ethylenediamine tetraacetic acid (EDTA; 20 mM in 10 mM Tris, pH 7) was added. Two hundred microliter fractions were collected in microcentrifuge tubes after the addition of EDTA. The total protein content of fractions was determined by the Bradford assay (BioRad).

### 2.6. Gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [29] using either pre-cast tris-tricine 4–20% gradient gels (BioRad) or tris-glycine gels (7.5% acrylamide gels with 4.5% acrylamide stacking layers). Kaleidoscope prestained standards (BioRad) were used to determine molecular weight. When required, dithiothreitol (DTT; 10 mg/ml) was added to sample buffers to provide reducing conditions.

For two-dimensional SDS-PAGE, proteins were electrophoresed in the first dimension under non-reducing conditions. Lanes were then cut from non-reducing gels and reduced in DTT (10 mg/ml in 10 mM Tris, pH 8.6) for 10 min. Reduced lanes were overlaid onto a 7.5% acrylamide gel without a stacking layer, and then re-electrophoresed. Gels were silver stained by the method of Blum et al. [30].

### 2.7. Statistical analysis

Statistical analyses were performed with the Microsoft Excel software package. Differences between mean values were analyzed using Student's two-tailed *t*-test where  $p < 0.05$  were considered significant.

## 3. Results

### 3.1. Opsonic activity of serum

*P. stolonifera* serum had strong, dose dependent opsonic activity (Fig. 1). Significantly enhanced phagocytosis, relative to non-opsonized controls ( $p < 0.05$ ), was evident when yeast were opsonized with as little as 5% v/v serum. Opsonization with

100% v/v serum enhanced phagocytic activity by six-fold when compared to non-opsonized controls ( $p < 0.05$ ).

### 3.2. Flow cytometric characteristics of phagocytic cells

Flow cytometry identified five distinct hemocyte populations based on their distinctive forward vs 90° (side) light scatter characteristics (Fig. 2(A)). Population 1 (29.8% of all hemocytes) was characterized by low 90° scatter and high forward scatter suggesting that it incorporated large agranular cells, whereas population 2 (11.3% of all hemocytes) had low 90° scatter and intermediate forward scatter indicative of mid-sized cells with limited internal complexity. Population 3 (13.9% of all hemocytes) exhibited low forward scatter and high 90° scatter consistent with small granular cells. Population 4 (13.8% of all hemocytes) had both high forward and 90° scatter of the type associated with large granular cells, whilst population 5 (31.2% of all hemocytes) contained cells with low forward and 90° scatter indicative of

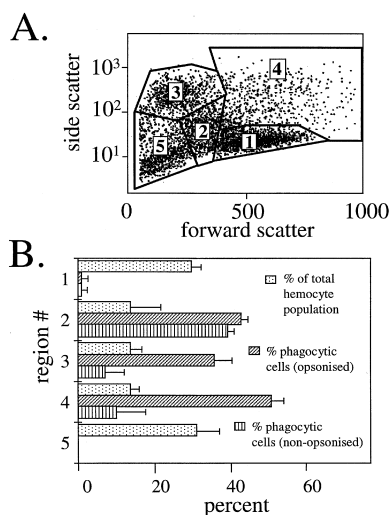


Fig. 2. Identification of phagocytic hemocyte populations. (A) A typical forward vs 90° (side) light scatter dot plot of hemocytes from *P. stolonifera*. Regions representing five distinct hemocyte sub-populations are shown. (B) The percentage of the total hemocyte population within each of the regions identified in (A), the percentage of cells within those regions that ingested FITC-yeast opsonized with 20% v/v serum and the percentage of cells within each region that ingested FITC-yeast that had been incubated in FSW (non-opsonized). Bars represent SEM,  $n = 4$ .

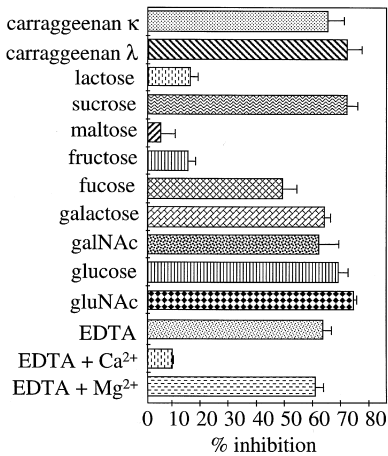


Fig. 3. Effects (% inhibition) of various mono- and poly-saccharides (all 20 mM), EDTA (20 mM), Ca<sup>2+</sup> (50 mM) and Mg<sup>2+</sup> (50 mM) on opsonization of FITC-yeast by 20% v/v *P. stolonifera* serum. Bars represent SEM,  $n = 4$ .

small cells or cellular debris with little internal complexity.

Independent interrogation of these populations in fluorescence histograms showed that the hemocyte populations designated 2, 3 and 4 ingested non-opsonized yeast (Fig. 2(B)). Opsonization with 20% v/v serum increased the percentage of phagocytic cells in populations 3 and 4 by five to six-fold when compared to non-opsonized controls ( $p < 0.05$ ). In contrast, opsonization had no effect on the percentage of phagocytic cells in population 2 ( $p > 0.05$  vs non-opsonized controls).

### 3.3. Effect of EDTA and carbohydrates on opsonization

Fig. 3 shows that the opsonic activity of *P. stolonifera* serum could be inhibited by EDTA and a variety of carbohydrates. In all cases, inhibition was dose dependent (data not shown) and had become asymptotic at the concentrations shown in Fig. 3. Incubation of serum with 20 mM EDTA decreased opsonic activity by 60% relative to yeast opsonized in the absence of EDTA. The inhibitory effect of EDTA could be abrogated by adding Ca<sup>2+</sup>, but not Mg<sup>2+</sup>, to the opsonizing mixture.

Opsonization was also inhibited when serum was pre-incubated with sucrose, fucose, galactose, glucose,

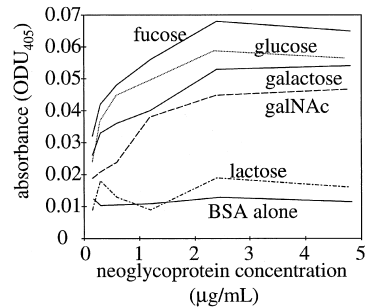


Fig. 4. Carbohydrate binding assays for *P. stolonifera* serum. The ability of various concentrations of biotinylated BSA-neoglycoproteins and biotinylated BSA alone to bind immobilized *P. stolonifera* serum are shown as spectrophometric absorbance (ODU at 405 nm).

galNAc and gluNAc, as well as the complex algal cell surface carbohydrates, carrageenan  $\kappa$  and  $\lambda$ . The inhibition of opsonization by these carbohydrates ranged from 48–73% relative to yeast opsonized in the absence of carbohydrates. Maltose, lactose and fructose failed to significantly inhibit opsonization ( $p > 0.05$  vs yeast opsonized without carbohydrates).

### 3.4. Binding of neoglycoproteins by *P. stolonifera* serum

The ability of *P. stolonifera* serum to bind biotinylated neoglycoproteins is depicted in Fig. 4. Four of the neoglycoproteins tested (BSA conjugates of fucose, glucose, galactose and galNAc) showed strong, dose dependent binding to *P. stolonifera* serum. Binding by all of these neoglycoproteins could be abrogated by pre-incubation with 20 mM EDTA (data not shown). In contrast, BSA-lactose did not yield binding that was significantly greater than that evident for BSA alone at any of the concentrations tested ( $p > 0.05$ ).

### 3.5. Affinity purification of opsonic lectins from *P. stolonifera* serum

N-acetyl-D-galactosamine and gluNAc binding lectins were purified from *P. stolonifera* serum by carbohydrate affinity chromatography. A single protein peak, which incorporated fractions 6–10, eluted from a galNAc affinity column (Fig. 5(A)). This peak incorporated up to 35 µg/ml protein (fractions 7 and 8) and corresponded precisely with a single peak of opsonic activity.

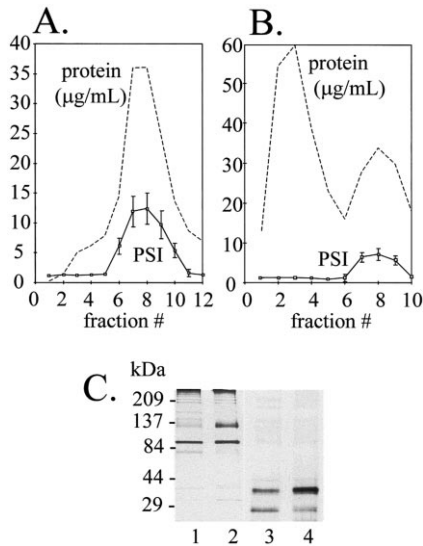


Fig. 5. Carbohydrate affinity chromatography of *P. stolonifera* serum. Fractionation of serum by (A) *N*-acetyl-D-galactosamine (galNAc) and (B) *N*-acetyl-D-glucosamine (gluNAc) conjugated to 4% beaded agarose, showing both the total protein content and opsonic activity (phagocytic stimulation indexes, PSI) of fractions collected after the addition of EDTA to the affinity columns. Bars represent standard deviations for triplicates of each fraction. (C) SDS-PAGE of fraction 8 from the galNAc (lanes 1 and 3) and gluNAc (lanes 2 and 4) columns. Lanes 1 and 2, non-reducing conditions; Lanes 3 and 4, reducing conditions. The position of molecular mass markers (kDa) is shown on the left.

Two discrete protein peaks were evident when serum was fractionated on a gluNAc affinity column (Fig. 5(B)). The first peak, incorporating fractions 2–5, had protein contents of up to 60 µg/ml (fraction 3). None of these fractions exhibited significant opsonic activity ( $p > 0.05$  vs non-opsonized yeast). In contrast, all fractions from the second peak (fractions 7–9), which incorporated up to 35 µg/ml protein (fraction 8), significantly increased opsonic activity relative to non-opsonized controls ( $p < 0.05$ ).

Fig. 5(C) shows that the peak opsonic fractions isolated by galNAc and gluNAc affinity chromatography (fraction 8 from both columns), contained a range of proteins when analyzed by non-reducing SDS-PAGE. The two columns eluted similar, but not identical, patterns of proteins ranging in molecular mass from 80 to >200 kDa. Predominant bands were evident at 90 and 130 kDa. Under reducing conditions, SDS-PAGE of fraction 8 from both the galNAc

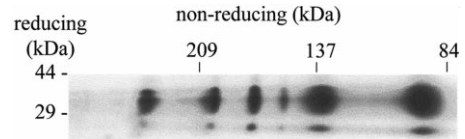


Fig. 6. Two-dimensional electrophoresis of galNAc affinity purified proteins from *P. stolonifera* serum. First dimension, non-reducing—left to right. Second dimension, reducing, top to bottom.

and gluNAc columns resolved two polypeptides of 35 and 22 kDa.

Two-dimensional electrophoresis of fraction 8 from the galNAc affinity column showed that all of the proteins identified by non-reducing SDS-PAGE were oligomers composed of the 35 and 22 kDa polypeptides identified by single dimension reducing SDS-PAGE (Fig. 6). In all cases the 35 kDa subunit was more intense than the 22 kDa polypeptide, indicating that the 35 kDa polypeptide may predominate in oligomers.

### 3.6. Additional opsonic factors in *P. stolonifera* serum

The carbohydrate binding proteins characterized above did not account for all of the opsonic activity in *P. stolonifera* serum. Fig. 7(A) shows that the flowthrough (i.e. non-bound components) from the galNAc affinity column retained opsonic activity. Although this opsonic activity was far lower than that for non-fractionated serum, significantly enhanced phagocytosis was evident when yeast cells were opsonized with 100% v/v flowthrough ( $p < 0.05$  vs non-opsonized controls). The opsonic activity retained in the flowthrough could be distinguished from that of carbohydrate affinity fractions by its sensitivity to protease inhibitors and EDTA. Opsonization by the flowthrough was inhibited in a dose dependent fashion by the serine protease inhibitor, Pefabloc (Fig. 5(B)). Similar inhibitory activity was evident when flowthrough was pre-incubated with another serine protease inhibitor, PMSF (data not shown). Incubation with 5 mM Pefabloc decreased the opsonic activity of flowthrough by 60% ( $p < 0.05$  vs yeast opsonized with flowthrough alone), but had no significant effect on the opsonic activity of fraction 8 from the galNAc affinity column ( $p > 0.05$  vs yeast opsonized with fraction 8 alone). Conversely, incubation with EDTA significantly reduced the opsonic activity of fraction 8 from the galNAc column, but had no

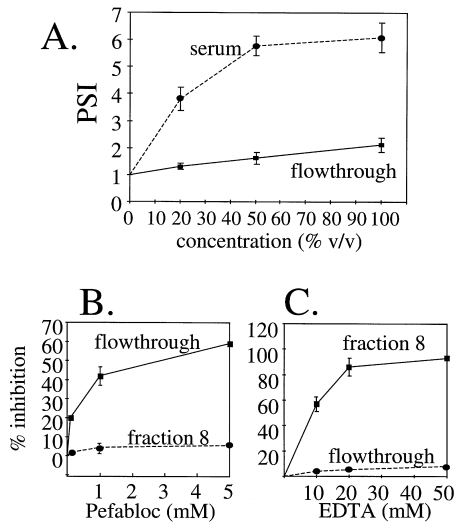


Fig. 7. (A) Comparison of opsonic activity (phagocytic stimulation index, PSI) in the flowthrough from the galNAc affinity column with that of non-fractionated *P. stolonifera* serum. (B) The effect (% inhibition) of various concentrations of the serine protease inhibitor, Pefabloc, on the opsonic activities of fraction 8 and the flowthrough from the galNAc column (Bars represent SEM,  $n = 4$ ). (C) The effect of various concentrations of EDTA on the opsonic activities of fraction 8 and the flowthrough from the galNAc column (Bars represent SEM,  $n = 4$ ).

significant effect on the ability of flowthrough to opsonize yeast ( $p > 0.05$  vs yeast opsonized with flowthrough alone).

#### 4. Discussion

This study has characterized humoral opsonins in the hemolymph of the tunicate, *P. stolonifera*. Opsonization with whole serum increased the phagocytosis of target cells by up to six-fold relative to non-opsonized controls. Since the target cells were washed extensively after opsonization, increased phagocytosis must have resulted from the binding or modification of target cell surfaces by humoral factors. It also appears that *P. stolonifera* hemocytes have specific surface receptors that can detect opsonin-bound targets and initiate their ingestion. Opsonization increased phagocytosis in only 2 of the 3 phagocytic sub-populations of hemocytes, implying that these sub-populations alone bear appropriate opsonin receptors.

Experiments in which opsonization was conducted in the presence of EDTA or carbohydrates indicated that

much of the opsonic activity in serum was mediated by  $\text{Ca}^{2+}$  dependent lectins. A range of mono- and di-saccharides (including both glucose- and galactose-based sugars), complex algal carbohydrates and EDTA could significantly inhibit opsonization, whereas  $\text{Ca}^{2+}$ , but not  $\text{Mg}^{2+}$ , could abrogate the inhibitory effect of EDTA. Moreover, carbohydrate binding studies using neoglycoproteins confirmed the presence of humoral factors with specificity for fucose, glucosides and galactosides in *P. stolonifera* serum.

The carbohydrate binding opsonins from *P. stolonifera* serum were purified by affinity chromatography using galNAc and gluNAc matrixes. In both cases, opsonic activity eluted as a single peak, although the gluNAc column also appeared to bind a non-opsonic lectin. SDS-PAGE analysis of the opsonic lectins showed that similar patterns of proteins were eluted from both the galNAc and gluNAc columns. Non-reducing SDS-PAGE resolved numerous proteins with a broad range of molecular masses. However, only two polypeptide sub-units with molecular masses of 35 and 22 kDa were identified in reducing SDS-PAGE.

Two-dimensional electrophoresis confirmed that oligomerization of these two sub-units generated all of the mature proteins observed under non-reducing conditions. The differential intensity of the bands resolved by two-dimensional electrophoresis indicates that multiple 35 kDa polypeptides may interact with one or more 22 kDa sub-units to form mature oligomers. Certainly, the molecular weights of the predominant proteins identified in non-reducing SDS-PAGE (90 and 130 kDa) are compatible with this form of oligomerization ( $2 \times 35 \text{ kDa} + 1 \times 22 \text{ kDa} = 92 \text{ kDa}$ ;  $3 \times 35 \text{ kDa} + 1 \times 22 \text{ kDa} = 127 \text{ kDa}$ ).

The broad spectrum of carbohydrates that could inhibit opsonization by whole serum suggests that the *P. stolonifera* opsonins have far broader carbohydrate specificities than lectins from other tunicate species [1,16–18,23,24]. Most often, opsonization, or other lectin-mediated activities in tunicates are inhibited by narrow ranges of carbohydrates that usually reflect specificity for either galactose- or glucose/mannose-based sugars [1,16,18,23,25,31–34].

This propensity for discrimination between galactosyl and glucosyl/mannosyl sugars has been confirmed by binding site analyses of numerous vertebrate and invertebrate lectins [35]. High affinity interactions with calcium-dependent carbohydrate

recognition domains (CRDs) are usually discriminatory because sugars are bound via their 3' and 4' hydroxyls, which associate with  $\text{Ca}^{2+}$  and the acid and amide side chains of specific amino acids in the CRD [35, 36]. The binding of sugars such as mannose and glucose, which display equatorial 3'- and 4'-hydroxyl groups, is associated with a specific arrangement of acid and amide co-ordination ligands, whereas galactosyl sugars, which have equatorial 3'-hydroxyl and axial 4'-hydroxyl groups, are bound by a different arrangement of  $\text{Ca}^{2+}$  and amino acids. Hence, the specificity of human mannose binding lectin A (MBL-A) can be changed from mannose to galactose by two amino acid substitutions (Glu185 → Gln and Asn187 → Asp), and the galactose specificity of a defensive lectin (TC14) from the tunicate, *Polyandrocarpa misakiensis*, can be explained largely by the inclusion of Trp at residue 100 [36].

One explanation for the broader than usual specificity of the *P. stolonifera* opsonic lectins comes from their oligomeric structure. If the 35 and 22 kDa polypeptide sub-units of the *P. stolonifera* opsonins represent distinct lectins with different carbohydrate specificities, combinations of these sub-units in mature oligomers could produce proteins with the capacity to bind a broader than usual array of carbohydrates with high affinity.

Similarly complex lectin structures have been reported in other species. In the rainbow trout, *Oncorhynchus mykiss*, oligomerization of a  $\text{Ca}^{2+}$  dependent lectin was so complex when analyzed by non-reducing SDS-PAGE that it was designated 'ladder lectin' [37]. The mammalian collectin, conglutinin also comprises a 'ladder' of oligomerization states under native conditions that correspond to free polypeptides, dimers, trimers and higher order oligomers [38]. Such oligomerization, which clusters multiple CRDs, may increase the affinity and specificity of defensive lectins for complex patterns of oligosaccharide ligands found on microbial surfaces [26,38].

Even though the lectins characterized here contribute substantially to the opsonic activity of *P. stolonifera* serum, additional opsonins are also present. Opsonic factors with characteristics that were clearly distinct from those of affinity purified opsonic lectins, were detected in the flowthrough from carbohydrate affinity columns. Unlike the lectins, opsonins in the flowthrough could not be inhibited by EDTA, and so

were clearly not  $\text{Ca}^{2+}$  dependent. Conversely, the protease inhibitors Pefabloc and PMSF had no effect on the opsonic activity of carbohydrate affinity purified fractions, but inhibited opsonization by the flowthrough. In a subsequent publication we will report that this additional, protease dependent opsonic activity is mediated by a homologue of complement component C3.

The identification of both lectin-mediated and proteolytically activated opsonic systems in *P. stolonifera* suggests that this invertebrate may deploy a variety of potentially interactive recognition systems in its anti-pathogenic defenses.

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