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A second form of collagenous lectin from the tunicate, *Styela plicata*

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Abstract

This study characterised a 90 kDa lectin from an invertebrate chordate, the tunicate *Styela plicata*. One- and two-dimensional electrophoresis showed that the apparent molecular weight of this protein is maintained under both reducing and non-reducing conditions, suggesting that its native form is a monomer. The 90 kDa lectin was localised within a single type of hemocyte (morula cells), but was secreted from those cells when tunicates were challenged with the inflammatory elicitor, zymosan. Functional studies showed that the 90 kDa protein binds to galactose-based sugars in a divalent cation-dependent manner. Amino acid composition analysis and N-terminal amino acid sequencing indicated that the 90 kDa lectin is related to a previously characterised, collagenous lectin from *S. plicata*, splic43. However, peptide mass fingerprinting identified numerous differences between the two proteins. This suggests that the 90 kDa molecule represents a novel protein that is involved in host defence.

Keywords: Tunicate; Ascidian; Collagenous lectin; Innate immunity; *Styela plicata*; Collectin; Ficolin

1. Introduction

Collagenous lectins (collectins and ficolins) play an important role in the innate immune responses (Epstein et al., 1996). Ficolins, like human p35, and the collectin, mannose-binding lectin (MBL), are pattern recognition molecules (Holmskov et al., 1994; Lu, 1997; Matsushita et al., 1990). They can attach to common carbohydrates on a variety of bacteria, viruses, fungi and protozoans (Lu, 1997; Sugimoto et al., 1998). The binding of p35 or MBL opsonises microbes for phagocytosis (Epstein et al., 1996; Matsushita et al., 1990). Both molecules can also initiate complement-mediated effector activities via mannose-binding lectin-associated serine proteases (MASPs; Lu et al., 1990; Matsushita and Fujita, 1992; Ohta et al., 1990; Petersen et al., 2000).

Collectins and ficolins have similar structures that incorporate collagen-like domains and carboxy-terminal carbohydrate binding elements. Their collagenous regions are characterised by the repeating amino acid motif, glycine–X–Y, where X and Y can be any amino acid but is often proline or hydroxyproline (Hakansson and Reid, 2000; Holmskov, 2000; Holmskov et al., 1994; Hoppe and Reid, 1994). Glycine–X–Y repeats are preceded at the amino terminus by a short (approximately 20 amino acid) "tail" domain that incorporates conserved cysteine residues involved in oligomerisation. In collectins, the carboxy-terminal region is a C-type lectin carbohydrate recognition domain (CRD) specific for either glucose/mannose- or galactose-based sugars. Ficolins, on the other hand, have a carboxy-terminal fibrinogen-like domain that can bind a variety of carbohydrates, most often *N*-acetyl-D-glucosamine (GluNAc) (Matsushita and Fujita, 2002; Matsushita et al., 1990; Ohashi and Erickson, 1998; Sugimoto et al., 1998).

Recent evidence suggests that the role of collagenous lectins in innate immunity has a long evolutionary history. We have

Abbreviations: CAPS, cyclohexylaminopropane sulfonic acid; CRD, carbohydrate recognition domain; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; FSW, sterile filtered seawater; galNAc, *N*-acetyl-D-galactosamide; GBS, goat blocking serum; gluNAc, *N*-acetyl-D-glucosamide; MALDI, matrix assisted laser desorption ionisation; MASP, mannose-binding lectin-associated serine protease; MBL, mannose-binding lectin; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonylfluoride; PVDF, polyvinyl difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; splic43, 43 kDa collagenous lectin from *Styela plicata*; TBS, Tris-buffered saline.

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shown that the tunicate, *Styela plicata*, expresses a galactose-specific lectin designated splic43 that is similar to collectins and ficolins in both its structure and function (Nair et al., 2000). Splic43 has a tail domain of 20 amino acids, including one of the critically conserved cysteines found in collectins and ficolins. It also contains a collagenous region with typical glycine–X–Y amino acid repeats. Under native conditions, splic43 polypeptides combine to form homodimers, homotrimers and higher order oligomers comparable to those found among ficolins and collectins. Splic43 is also a powerful opsonin and contributes to the activation of a protein in *S. plicata* that resembles the central component of the mammal complement cascade, C3.

Similar lectins have been identified in other tunicates. A close relative of *S. plicata*, *Halocynthia roretzi*, has four ficolin-like genes encoding typical 20 amino acid tail regions, carboxy-terminal fibrinogen-like domains and collagenous regions with 5 glycine–X–Y repeats (Kenjo et al., 2001). A number of genes that resemble ficolins and collectins are also evident in the genome of another tunicate, *Ciona intestinalis* (Azumi et al., 2003).

The characterisation of collagenous lectins in tunicates is significant because it helps to resolve the early evolution of the complement system. Tunicates are invertebrate members of the Chordate evolutionary lineage and so they occupy a critical position in the phylogeny of the immune system. Even though tunicates and other invertebrates have defensive proteins analogous to C3, they do not express the immunoglobulin antibodies that are primarily responsible for C3 activation in vertebrates (Al-Shariff et al., 1998; Clow et al., 2004; Nonaka et al., 1998; Raftos et al., 2002). The identification in tunicates of collagenous lectins like splic43 that can activate C3-like proteins helps to explain how the evolutionary diversification of complement-based defence systems was driven in the absence of bona fide antibodies.

In this study, we characterise an additional, 90 kDa lectin from *S. plicata*. Even though it is closely related to splic43, the 90 kDa molecule is shown to be a novel protein involved in host defence responses.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma Aldrich (Sydney, NSW) unless otherwise noted.

2.2. Anti-splic43 antibody

A 20 amino acid synthetic peptide based on the N-terminal sequence of splic43 (SNELMVCRRADTNTEVIIG; Nair et al., 2000) was synthesized, conjugated to keyhole limpet hemocyanin as a carrier and used to immunise New Zealand White rabbits by Bethyl Laboratories (Michigan, USA). Anti-splic43 antibodies were affinity purified from rabbit serum using the immunising peptide conjugated to CNBr-activated Sepharose 4B.

2.3. Tunicates

S. plicata were collected from boat moorings, pontoons or netting at Clifton Gardens or Birkenhead Point, New South Wales (NSW) with the permission of the NSW Department of Primary Industries (Marine Research Permit # 95067). They were maintained in aerated aquaria filled with seawater (15 °C) until required for experimentation (up to 8 days).

2.4. Hemolymph and tissue preparation

Tunicates were bled from incisions in the buccal syphon and the exuding hemolymph was harvested on ice. Hemocytes were counted before being centrifuged at 3000 ×*g* for 5 min (4 °C) or 12,000 ×*g* for 10 s (room temperature). The hemolmyph supernatant (designated "serum") was collected before the hemocytes were resuspended in 10 mM Tris (pH 8.5) containing phenylmethylsulfonylfluoride (PMSF; final concentration 2 mM) and 1% v/v Triton-X 100 or 1% v/v Nonidet P-40. Most often, serum samples were prepared by pooling material from up to twenty tunicates. In some cases though, samples from individual tunicates were analysed independently. Hemocyte lysates were allowed to stand on ice for 10 min, before being centrifuged (3000 ×*g*, 10 min, 4 °C or 12,000 ×*g*, 10 s, room temperature) to remove cell debris.

Pharyngeal and hepatopancreas tissues were dissected from tunicates and suspended in either 2 mL 10 mM Tris (pH 8.5) or filtered seawater (FSW; 0.45 μm filter). They were then diced into 9 mm^3 portions and washed 3 times in FSW. The tissue was disrupted in a Sorvall rotary blade homogeniser (Heraeus, Armonk, NY) before the addition of 2 mM PMSF and either 1% v/v Triton-X 100 or 1% v/v Nonidet P-40. Homogenates were centrifuged (3000 ×*g*, 10 min, 4 °C or 12,000 ×*g*, 10 s, room temperature) so that the resulting supernatants (designated "neat tissue homogenates") could be collected.

2.5. Carbohydrate affinity precipitation

To determine whether anti-splic43 reactive proteins exhibited specific carbohydrate binding activities, 1 mL aliquots of pooled serum were incubated (4 h, 4 °C, constant rotation) with 50 μL 4% beaded agarose conjugated to either *N*-acetyl-D-galactosamide (galNAc), *N*-acetyl-D-glucosamide (gluNAc), D-mannose or D-glucose. The agarose beads were then washed 5 times by centrifugation through 1 mL FSW, before being resuspended in SDS-PAGE sample buffer, electrophoresed and silver stained as described below.

2.6. Carbohydrate affinity chromatography

Anti-splic43 reactive proteins were affinity purified from 50 mL pooled serum or hepatopancreas homogenates. The serum and hepatopancreas samples were filtered (0.45 μm; Sarotorius, Goettingen, Germany) before being passed twice through an Econoflow 10 mL chromatography column (BioRad, Regent's Park, NSW) packed with 1 mL 4% beaded agarose conjugated with galNAc. The beads were then washed

with at least 50 mL FSW before 5 mL ethylendiamine tetraacetic acid (EDTA; 20 mM in 10 mM Tris, pH 8.0) was added to elute the bound proteins. Two hundred microliter fractions were collected after the addition of EDTA. The total protein content of each fraction was determined using the Bradford assay (Total Protein Determination Kit, BioRad).

2.7. Electrophoresis

Proteins were separated by Tris–glycine or Tris–tricine SDS-PAGE using either pre-cast Tris–tricine 4–20% gradient gels (BioRad) or Tris–glycine gels comprising lower resolving layers of 10% acrylamide and upper 4% stacking layers. When required, samples were reduced by adding 10 mg/mL dithiothreitol (DTT), 5% w/v 2-mercaptoethanol or 10 mg/mL sodium dithionite. Molecular weight markers (Kaleidoscope Prestained Standards, BioRad) were used to determine the relative molecular weights of unknown proteins. Gels were either silver stained by the method of Blum et al. (1987), stained with Coomassie blue (0.1% w/v Coomassie Brilliant Blue R-250, 50% v/v methanol, 10% acetic acid) or Western blotted as described below.

2.8. Western blotting and immunostaining

Proteins were transferred from SDS-PAGE gels to nitrocellulose or polyvinyl difluoride (PVDF) membranes (BioRad) using the semi-dry method of Kyhse-Anderson (1984). Gels were blotted at 12 V for 50 min.

After blotting, membranes were blocked overnight in Tris-buffered saline (TBS; 20 mM Tris, 150 mM NaCl, pH 7.5) containing 5% w/v skimmed milk (Blotto; Diploma, Sydney, NSW). Blots were then incubated for 4 h in anti-splic43 (1:2000 v/v in TBS) followed by 2 h in alkaline phosphatase-conjugated monoclonal anti-rabbit IgG (1:10,000 v/v in TBS) with 3 washes in TBS containing 0.1% v/v Tween-20 (TBS–Tween) after each incubation. Immunostained bands were visualised with 18.75 mg/mL nitro blue tetrazolium chloride and 9.4 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (Roche, Castle Hill, NSW) in alkaline phosphatase buffer (0.1 M Tris–HCl, 0.05 M $MgCl_2$ and 0.1 M NaCl, pH 9.5).

2.9. Peptide mass fingerprinting, amino acid composition analysis and N-terminal amino acid sequencing

Carbohydrate affinity chromatography fractions were subjected to SDS-PAGE and stained with Coomassie blue. Individual proteins of 43 and 90 kDa were excised from the gel and subjected to N-terminal amino acid sequencing or peptide mass fingerprinting by the Australian Proteome Analysis Facility (Macquarie University, NSW). For peptide mass fingerprinting, excised bands underwent 16 h in gel tryptic digest at 37 °C, before the resulting peptides were analysed by matrix assisted laser desorption ionisation (MALDI) mass spectrometry. Peptides of identical molecular mass to common contaminants (trypsin and human keratin) were excluded from the data.

The amino acid composition of the 90 kDa protein was determined after affinity purified fractions had been Western blotted to polyvinyl difluoride (PVDF) membranes (BioRad) using cyclohexylaminopropane sulfonic acid (CAPS) transfer buffer (10 mM CAPS, pH 11, 10% v/v methanol) according to the method of Aebersold et al. (1987). The 90 kDa protein was excised from membranes and subjected to amino acid composition analysis by the Australian Proteome Analysis Facility (North Ryde, NSW) or to N-terminal amino acid sequencing by the Macquarie University Centre for Analytical Biochemistry. The statistical significance of differences in the amino acid composition of the 90 kDa protein and splic43 was determined by contingency Chi-squared analysis using SPSS v.11 for Windows (Microsoft, Sydney, NSW).

2.10. Immunohistochemistry

Hepatopancreas tissue was cut into 9 mm^3 portions and fixed with 4% paraformaldehyde in 0.4 M phosphate buffer (pH 7.2) at 4 °C for 24 h. The tissue portions were then washed (3 × 10 min in FSW), embedded in paraffin and sectioned (5 μm sections). The sections were adhered to poly-L-lysine coated microscope slides, deparaffinised and rehydrated. All subsequent incubations were carried out at room temperature and slides were rinsed (3 × 3 min) with phosphate buffered saline (PBS; 130 mM NaCl, 2 mM KCl, 10 mM $NaHPO_4$, 1.7 mM KH_2PO_4, pH 7.2) between each step, unless stated otherwise. Endogenous peroxidase activity was inhibited by incubation with hydrogen peroxide (0.3% v/v in methanol; 20 min). Non-specific reactivity was then blocked with PBS containing 10% v/v normal goat serum (PBS-GBS; 20 min). PBS-GBS was removed and, without rinsing, sections were incubated with anti-splic43 (1:100 in PBS) for 1 h at 37 °C. The slides were then incubated with biotinylated goat anti-rabbit IgG (1/200; 30 min), followed by Vectastain Elite ABC Reagent (Vector Laboratories, Burlingame, CA, 30 min). Sections were stained with 3′-diaminobenzidine (DAB)/H_2O_2 solution (Liquid DAB Substrate-Chromagen system, Dako Laboratories, Botany, NSW) before being counterstained in haematoxylin for 30 s. Slides were then dehydrated, cleared and mounted. Cells in hepatopancreas sections were considered to be positive for anti-splic43 antigens if they displayed a brown colouration. Cells that were negative for antigen remained transparent with blue nuclei. Negative controls (no primary antibody) were included in all experiments.

2.11. Induction of inflammatory responses in vivo

Inflammatory reactions were induced by injecting tunicates with the yeast cell wall extract, zymosan A (Green et al., 2003). Tunicates were weighed to determine correct titer of zymosan for injection. Zymosan doses were based on previous calculations, which indicated that hemolymph constitutes approximately 20% of wet body weight. To inject zymosan, sterile scalpel blades were used to shave away a section of tunic to reveal the underlying mantle surface. One milliliter syringes fitted with 26 1/2 G needles were then used to inject, into the

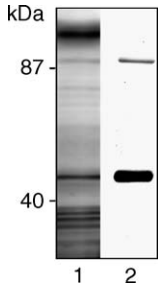

Fig. 1. Coomassie blue stained SDS-PAGE gel (1), and anti-splic43 stained Western blot (2) of DTT-reduced, pooled serum. The positions of molecular mass markers (kDa) are shown on the left.

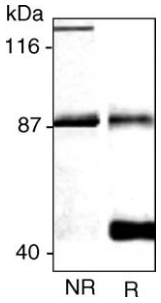

Fig. 3. Anti-splic43 stained, one-dimensional Western blot of non-reduced (1) and DTT-reduced (2) pooled serum. The positions of molecular mass markers (kDa) are shown on the left.

body wall immediately under the surface of the mantle, either FSW (controls) or sufficient zymosan (prepared in FSW) to yield a final concentration in hemolymph of 2 µg/mL. The needle was kept in place for 5 min to allow the wound to seal by muscle contraction.

Hemolymph was extracted from tunicates 0, 1, 2 and 4 days after injection and serum was Western blotted and immunostained with anti-splic43. Immunostained Western blots were dried and a digital image recorded so that the relative intensity of anti-splic43 reactive bands could be determined by image analysis. The intensity of staining in all blots was standardized by including known concentrations of anti-splic43 reactive proteins that had been purified from serum by affinity chromatography (see Section 2.5: Carbohydrate affinity precipitation). Anti-splic43-positive bands in these affinity purified preparations were arbitrarily allocated a relative intensity of 1.0. The statistical significance of differences between relative intensities were determined by Student's *t*-test. Differences were deemed to be significant if $p < 0.05$.

3. Results

3.1. A 90 kDa protein cross-reacts with anti-splic43

Western blotting of DTT-reduced serum showed that anti-splic43 recognised a reduced polypeptide of approximately 90 kDa, as well as splic43 (Fig. 1). Control blots, which omitted anti-splic43 did not stain either of these polypeptides (data not shown).

The 90 kDa molecule, but not splic43, was identified in the hepatopancreas, pharynx and hemocytes (Fig. 2). Splic43 was found exclusively in the serum. Serum from some tunicates contained only splic43, even though serum pooled from a number of different individuals also contained the 90 kDa molecule. Seven times more splic43 was evident in pooled serum when compared to the 90 kDa molecule.

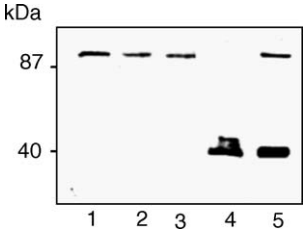

Fig. 2. Anti-splic43 stained Western blots of hepatopancreas (1), pharynx (2), hemocytes (3), serum from an individual tunicate (4), or pooled serum (5). The positions of molecular mass markers (kDa) are shown on the left.

Both one- and two-dimensional electrophoresis indicated that the native form of the 90 kDa protein is a monomer. After one-dimensional electrophoresis of non-reduced serum, two bands of 90 kDa and approximately 135 kDa were stained by anti-splic43 (Fig. 3). Under reducing conditions, bands could be detected at 90 and 43 kDa. Similar results were obtained when samples were reduced with 2-mercaptoethanol or sodium dithionite instead of DTT (data not shown). Two-dimensional electrophoresis of hepatopancreas lysates showed that the predominant 90 kDa band identified by anti-splic43 under non-reducing conditions comprised two components at 90 and 43 kDa when reduced (Fig. 4).

3.2. The 90 kDa anti-splic43 protein is a novel form of collagenous lectin

Peptide mass fingerprinting identified 60 distinct tryptic peptides in digests of splic43 and the 90 kDa polypeptide (Table 1). Of these, only 5 peptides were common to both splic43 and the 90 kDa protein. Eight peptides were found exclusively in splic43, whilst the remaining 47 peptides came from the 90 kDa protein. A second series of digests under the same conditions yielded essentially identical results (data not shown).

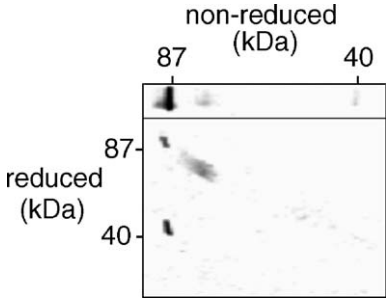

Fig. 4. Silver stained two-dimensional SDS-PAGE (non-reducing conditions, left to right; reducing conditions, top to bottom) of proteins isolated from hepatopancreas by galNAc affinity chromatography. A lane electrophoresed only in the first dimension (non-reducing) is also shown (top). The positions of molecular mass markers (kDa) are shown at the top (non-reducing) and left (reducing).

Table 1
Peptide mass (daltons, Da) fingerprints obtained by MALDI mass spectrometry of peptides generated by digesting splic43 and the 90 kDa protein with trypsin

splic43	Peptide mass (Da)			
	90 kDa			
974.296	**1028.393**	1307.708	1523.804	2064.205
1000.142	1050.303	1310.708	1532.779	2102.097
1017.255	1072.430	**1324.720**	1539.819	2120.102
1028.354	1082.625	1339.713	**1638.888**	2184.106
1251.745	1111.608	1379.752	1656.864	2201.159
1324.694	1118.608	1393.790	1670.878	2233.174
1427.888	1126.553	1407.749	1716.894	2286.136
1638.889	1157.604	**1427.909**	1757.957	2501.267
1761.952	1185.545	1458.749	1806.178	2548.288
1838.283	1203.652	1462.733	**1837.990**	2606.194
1980.012	1233.665	1472.742	1867.968	2621.239
2010.698	1267.732	1489.704	2012.016	2652.217
2249.183	1282.664	1515.698	2054.154	2668.197

Peptides shared between splic43 and the 90 kDa protein are identified by bold typeface. Peptides were deemed to be shared if they differed in mass by <1 Da.

Amino composition analysis showed that, like splic43, the 90 kDa protein has a high percentage of glycine (20.8%) and incorporates hydroxyproline (Table 2). Contingency χ^2 analysis indicated that there was no significant ($p>0.05$) difference between the amino acid compositions of splic43 and the 90 kDa protein.

N-terminal amino acid sequencing identified the first 10 residues of the 90 kDa protein (Fig. 5). This N-terminal sequence was identical to that of splic43 including a cysteine at residue 6 (Nair et al., 2000).

3.3. The 90 kDa protein is expressed by morula cells

Anti-splic43 immunostaining was evident in a single, morphologically distinct hemocyte type found in the hepatopancreas (Fig. 6). These hemocytes were identified as morula cells (sometimes called refractile vacuolated cells) according to the classification scheme of Radford et al. (1998). Anti-splic43 reactivity could not be detected in other hemocytes, or in endodermal hepatopancreatic cells. Within morula cells, anti-splic43 staining was restricted to the cytoplasm. It was not evident within the large vacuoles that characterise this cell type. Negative controls (slides incubated with secondary antibody but not anti-splic43) remained unstained (data not shown).

Table 2
Amino acid compositions (mol%) of splic43 (data from Nair et al., 2000) and the 90 kDa protein

Amino acid	splic43	90 kDa
Asx	9.5	8.1
Glx	8.9	8.7
Ser	12.1	10.2
His	2	1.7
Gly	16.6	20.8
Thr	6.1	5.5
Ala	8.7	7.8
Pro	5.4	8.7
Tyr	2.1	1.6
Arg	6.2	6.9
Val	5.6	4.9
Met	0.8	0.6
Ile	4.3	3.2
Leu	4.6	3.9
Phe	3.4	3.5
Lys	3.2	1.9
OH-Pro	2.4	3.5

OH-pro, hydroxyproline.

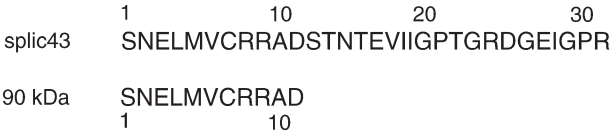

Fig. 5. Aligned N-terminal amino acid sequences for splic43 (Nair et al., 2000) and the 90 kDa protein.

3.4. The 90 kDa protein is a divalent cation-dependent, galactosyl specific lectin

Carbohydrate affinity precipitation using agarose-conjugated monosaccharides indicated that the 90 kDa protein specifically bound to galactose-based sugars. The 90 kDa polypeptide was found primarily in galNAc affinity precipitates (Fig. 7). None of the other carbohydrates tested (gluNAc, glucose or mannose) bound substantial quantities of the 90 kDa protein. Precipitation of the 90 kDa protein by galNAc-conjugated beads could be inhibited by co-incubation with 50 mM EDTA, suggesting that the interaction of the 90 kDa molecule with galNAc was divalent cation dependent.

The 90 kDa anti-splic43 reactive protein was subsequently purified by galNAc affinity chromatography (Fig. 8). Western blotting of reduced fractions from the galNAc affinity column

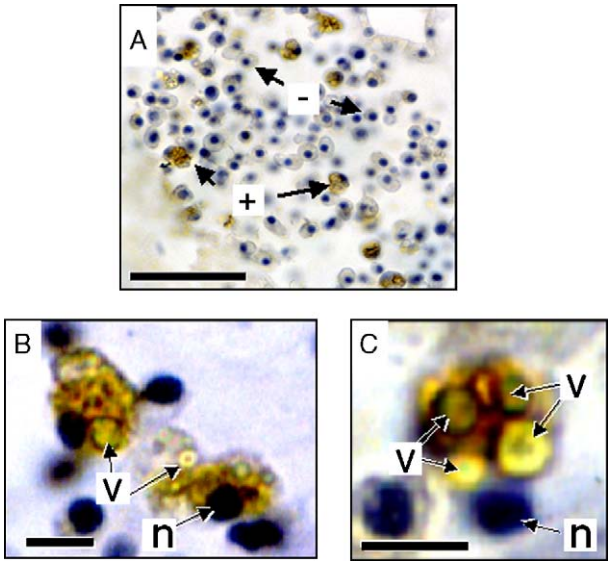

Fig. 6. Section of *S. plicata* hepatopancreas immunohistochemically stained with anti-splic43 antibody (1:100 v/v). (A) Low power micrograph showing anti-splic43-positive (+) and anti-splic43-negative hemocytes (−) (scale bar=100 μm). (B) and (C) High power micrographs of anti-splic43-positive morula cells (scale bars=10 μm). v, refractile vacuole; n, nucleus.

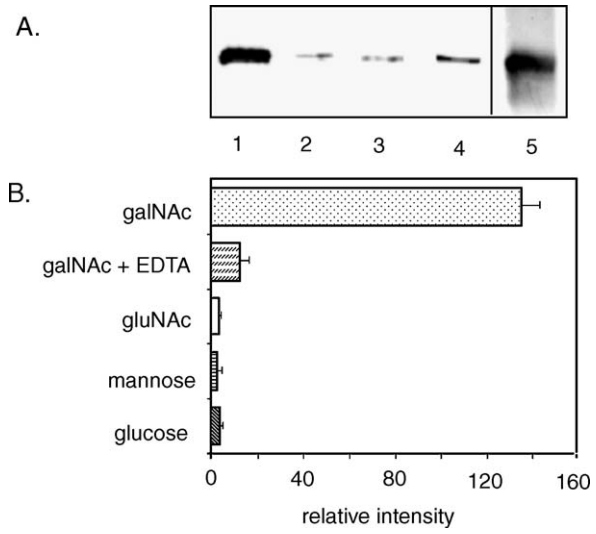

Fig. 7. Carbohydrate affinity precipitates of pooled serum. (A) Anti-splic43 stained Western blots of precipitates from *N*-acetyl-D-galactosamide (galNAc) (1), *N*-acetyl-D-glucosamide (gluNAc) (2), mannose (3) and glucose (4) conjugated agarose beads. The anti-splic43 stained 90 kDa protein from unfractionated serum is also shown (5). (B) Relative intensities of bands shown in (A) and of a affinity precipitation performed with galNAc beads in the presence of 50 mM EDTA.

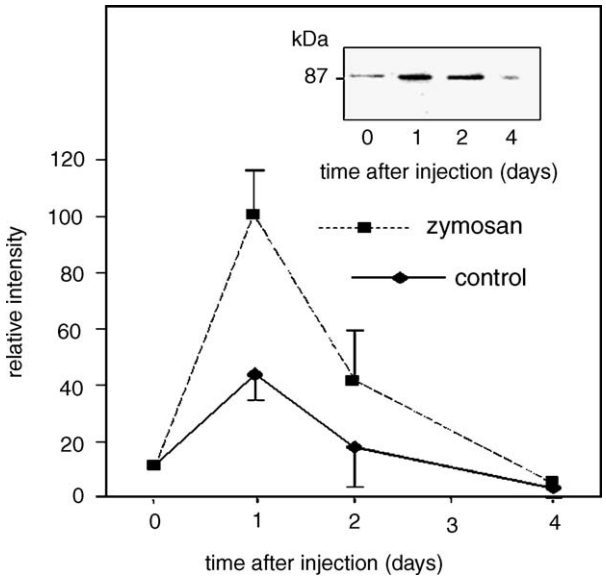

Fig. 9. Relative intensities of the 90 kDa band on anti-splic43 stained Western blots of serum collected from tunicates at various times after the injection of zymosan. Bars = SEM, $n \geq 3$. Inset: Anti-splic43 Western blot of serum collected at 0, 1, 2 and 4 days after zymosan injection. The positions of molecular mass markers are shown on the left (kDa).

revealed that fractions 5–8 contained both the 90 kDa protein and splic43 polypeptides. The highest titre of both polypeptides was evident in fraction 6.

3.5. Zymosan injection increases the concentration of the 90 kDa protein in serum

Fig. 9 shows that the serum concentration of the 90 kDa protein increased after tunicates were injected with the inflammatory agent, zymosan. Within one day of zymosan injection, the mean relative intensity of the 90 kDa protein was ten times greater than that of naive (day 0) tunicates ($p < 0.05$).

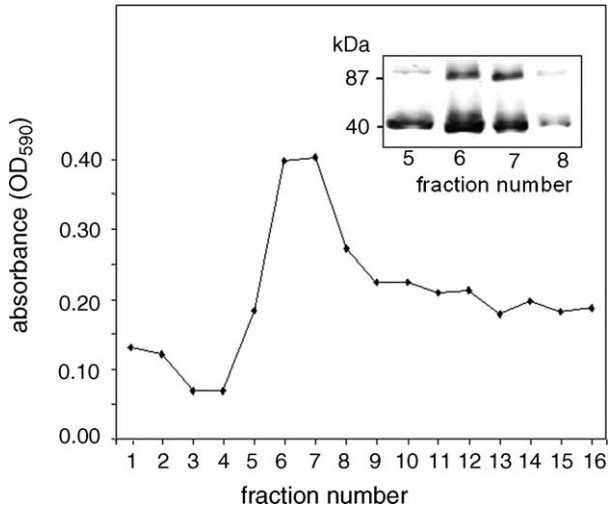

Fig. 8. Total protein concentrations (OD_{590}) in fractions of a hepatopancreas homogenate eluted from a galNAC affinity chromatography column with EDTA. Inset: Western blot of fractions 5–8 immunostained with anti-splic43. The positions of molecular mass markers are shown on the left (kDa).

The relative intensity of the 90 kDa band in serum began to decline within two days p.i., but remained significantly greater ($p < 0.05$) than that of non-injected (day 0) tunicates until 4 days p.i.

The concentration of the 90 kDa polypeptide also increased after the injection of FSW (Fig. 9). However, its concentration in FSW-injected (control) tunicates was significantly lower than that of zymosan challenged animals after 1 day p.i ($p < 0.05$), and decreased to an intensity that was indistinguishable from that of non-injected controls (day 0) within two days p.i. ($p > 0.05$).

4. Discussion

This study has characterised a novel 90 kDa collagenous lectin from the tunicate, *S. plicata*. The protein was first detected because of its serological cross-reactivity with the amino-terminal tail domain of a previously characterised lectin, splic43. Since the 90 kDa protein is approximately twice the molecular mass of splic43 polypeptides, we had previously thought it was either a non-reduced dimer of splic43 or a pre-processed form of that molecule incorporating two contiguous splic43 polypeptides (Nair et al., 2000). Even though the data reported here show that those assumptions were incorrect, they also reveal a close relationship between the 90 kDa protein and splic43. Like splic43, the 90 kDa protein binds to galNAc, but not to glucose/mannose carbohydrates. The galNAc binding activity of both splic43 and the 90 kDa protein can be inhibited by EDTA, and the two proteins co-eluted during galNAc affinity chromatography. This confirms that the 90 kDa protein is a divalent cation-dependent lectin with the same carbohydrate specificity as splic43.

splic43 and the 90 kDa protein also have similar amino acid compositions and identical N-terminal amino acid sequences. Both contain hydroxyproline, and high (approximately 20%) proportions of glycine. This is consistent with the presence of collagenous domains typified by a repeating motif of glycine–X–Y, in which the second and third positions can incorporate any amino acid but are often occupied by proline, or its rare variant, hydroxyproline.

Despite these similarities, a variety of reducing agents could not alter the apparent molecular mass of the 90 kDa molecule, indicating that it is not a dimer of splic43. Failure to reduce the 90 kDa protein also suggests that its native form is a monomer. This is rare amongst collagenous lectins, most of which form higher order homo-oligomers based on collagen-like trimers (Ohashi and Erickson, 1998; Sheriff et al., 1994; Weis and Drickamer, 1994). The 90 kDa molecule is also much larger than individual polypeptides from other collagenous lectins. Collectin polypeptides range in molecular mass from around 28 kDa for MBL to 65 kDa for conglutinin whilst ficolins are between 35 and 50 kDa (Matsushita et al., 1990; Sugimoto et al., 1998; Kenjo et al., 2001).

Differences between 90 kDa molecule and splic43 were also evident in their peptide mass fingerprints. If the 90 kDa protein did incorporate contiguous, pre-processed splic43 polypeptides, numerous peptides of identical molecular mass should have been present in the tryptic digests of splic43 and the 90 kDa molecule. However, splic43 and the 90 kDa protein yielded clearly distinct peptide mass fingerprints that shared few identical peptides. splic43 was digested into numerous peptides that were not present in the 90 kDa protein, indicating that the 90 kDa protein does not incorporate even a single complete splic43 polypeptide.

The substantial differences between the peptide mass fingerprints of splic43 and the 90 kDa protein cannot be explained by a failure to detect shared peptides, or by ineffective tryptic digestion. The MALDI-TOF system used here routinely captures more than 90% of peptides within the designated molecular weight range, so it is extremely unlikely that numerous shared peptides were not identified. Moreover, replication of the digests yielded essentially identical results to those described above, suggesting that on both occasions effective tryptic digestion had occurred.

Even though peptide mass fingerprinting clearly indicated that splic43 and the 90 kDa protein represent distinct proteins, it also identified similarities that may explain the serological cross-reactivity between 90 kDa protein and anti-splic43. Five tryptic peptides with molecular masses of 1028, 1325, 1428, 1639 and 1838 Da were common to both proteins. The predicted molecular mass of the splic43 tail domain, against which anti-splic43 was raised, is 2151 Da. It is possible that one of the peptides shared by splic43 and the 90 kDa protein is located within their identical tail domains. The fact that splic43 and the 90 kDa protein share at least some tryptic peptides and have identical N-terminal amino acid sequences suggests that the two proteins may be generated by the alternative splicing of the same mRNAs. Another, and maybe more likely explanation could be that the two molecules arose as a result of a recent gene duplication, where parts of the 90 kDa molecule were lost in the process.

In addition to the structural differences identified by peptide mass fingerprinting, splic43 and the 90 kDa protein also differ in their tissue localisation. In naive tunicates (i.e. those not exposed to inflammatory stimuli), splic43 is found only in serum, whilst the 90 kDa protein is often restricted to the hepatopancreas, pharynx and hemocytes. Immunohistochemistry indicated that within these tissues, the 90 kDa protein is expressed exclusively by a single type of hemocyte (morula cells).

It is equally clear, however, that the localisation of the 90 kDa protein is altered during inflammatory responses, indicating that the protein is involved in host defence responses. Even though, in previous in vivo experiments, we concluded that serum levels of the 90 kDa protein were not affected by inflammation (Green et al., 2003), we used far lower doses of inflammatory elicitor (zymosan) in the current study to show that inflammatory activation leads to the rapid appearance of the 90 kDa protein in serum. This is most likely to result from the active exocytosis of the 90 kDa protein. Morula cells, which express the 90 kDa protein, are known to respond rapidly to inflammatory stimuli. Their frequency in hemolymph increases soon after zymosan injection (Green et al., 2003), and they have the capacity to exocytose defence-related molecules in response to inflammatory antigens (Raftos et al., 2004).

The secretion of the 90 kDa protein during inflammatory reactions may also explain why some tunicates have naturally high levels of the 90 kDa protein in their serum even though they had not been injected with inflammatory elicitors. It is likely that these animals were already undergoing inflammatory responses to infection or injury when they were collected from the wild.

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