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Anticoagulant sulfated glycosaminoglycans in the tissues of the primitive chordate *Styela plicata* (Tunicata)

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We performed a biochemical and histochemical study of sulfated glycosaminoglycans in the tissues of the ascidian *Styela plicata*. A highly sulfated dermatan sulfate and a heparin-like polymer, identified by incubation with specific lyases, occur at different concentrations in intestine, heart, pharynx, and cloak. Dermatan sulfate prevails in the pharynx, whereas the heparin-like polymer abounds in the intestine. Staining of tissues sections with the cationic dye 1,9-dimethylmethylene blue before and after incubation with specific lyases revealed that the dermatan sulfate occurs in the extracellular matrix, while the heparin-like polymer is located within cytoplasmic granules of cells in the lumen of intestine and pharynx. The dermatan sulfate has a similar disaccharide composition in all tissues studied, whereas the heparin-like polymer differs in sulfate content. A direct relationship between sulfate content of the heparin-like polymer and antithrombin activity was observed. Analysis of the repeating disaccharide units of the heparin-like polymer indicates the presence of relatively high amounts of the disulfated disaccharide namely ΔUA-1→4-GlcN(SO_4)-($6SO_4$), which may suggest the occurrence in ascidians of regulatory biosynthetic mechanisms different from those observed for heparin in mammals.

Key words: sulfated glycosaminoglycans/dermatan sulfate/heparin/anticoagulant/ascidian tissues

Introduction

Glycosaminoglycans are complex polysaccharides based on repeating disaccharide units of hexuronic acid (α-L-iduronic acid or β-D-glucuronic acid) and hexosamine (α-D-glucosamine or β-D-galactosamine). The heterogeneity of these polymers results from variations in the degree of sulfation and occurrence of two types of hexuronic acid (Conrad, 1998). The glycosaminoglycans have a ubiquitous distribution in living organisms occurring in virtually all organs and tissues (Mathews, 1975; Cassaro and Dietrich, 1977). They are present in the extracellular matrix, at the cell surface, and also in cytoplasmic granules (Yurt *et al.*, 1977a,b; Ruoslahti, 1996), and can interact with several factors such as, morphogens, growth factors, cytokines, enzymes, and surface proteins of microorganisms (Kjellén and Lindahl, 1991; Rostand and Esko, 1997; Conrad, 1998; Wodarz and Nusse, 1998).

Sulfated glycosaminoglycans with unique structures have been reported in the visceras of several species of ascidians. A highly sulfated dermatan sulfate-like polymer, composed by the unusual di-sulfated disaccharide α-L-IdoA ($2SO_4$)-1→3-GalNAc($6SO_4$) was isolated from the visceras of the ascidian *Ascidia nigra* (Pavão *et al.*, 1995). Similarly, an oversulfated dermatan sulfate composed by disaccharide repeating units of α-L-IdoA($2SO_4$)-1→3-GalNAc($4SO_4$) was recently purified from the visceras of the ascidian *Halocynthia pyriformis* and *Styela plicata* (Pavão *et al.*, 1998). In addition, heparan sulfate-/heparin-like polymers have also been detected in the visceras (Pavão *et al.*, 1994) and recently isolated from the test cells of *S.plicata* (Cavalcante *et al.*, 1999). We now expand the study of these molecules to the tissue level as an approach to understand their biological roles. Here we report a biochemical and histochemical analysis of the distribution of these unique glycosaminoglycans among the tissues of the ascidian *S.plicata*. Evidence of the occurrence of a heparin-like polymer in this animal is also presented.

Results

Analysis of the glycans from the ascidian tissues

In order to determine the tissue distribution of sulfated glycosaminoglycans in ascidian body, tissues from different organs, intestine, heart, pharynx, and cloak were subjected to papain extraction and the glycans were analyzed by agarose gel electrophoresis (Figure 1). The glycans from intestine and heart display the same electrophoretic pattern, migrating as two main metachromatic bands. The low mobility band migrates as standard heparan sulfate, while the second major band migrates between standard heparan sulfate and dermatan sulfate (Figure 1). The glycans from pharynx display an array of metachromatic bands with a less defined migration, while the glycans from the cloak possess a main metachromatic band migrating between heparan sulfate and dermatan sulfate, and a less intense band migrating as heparan sulfate (Figure 1).

A qualitative analysis of the sulfated glycans extracted from each tissue was carried out by agarose gel electrophoresis before and after degradation of the glycans with specific lyases (Figure 1). The low mobility band of the glycans from

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intestine, heart, pharynx, and cloak, which has the same migration of heparan sulfate, resists degradation with chondroitin ABC lyase (Figure 1A) and heparan sulfate lyase (Figure 1C), but is totally degraded after incubation with heparin lyase, except for the glycans from the pharynx, which is only partially degraded (Figure 1B). The band migrating between standard heparan sulfate and dermatan sulfate of the glycans from intestine, heart, pharynx, and cloak is resistant to heparin lyase (Figure 1 B) and chondroitin AC lyase (Figure 1C), but is completely degraded by chondroitin ABC lyase (Figure 1 A). A minor metachromatic band, migrating as chondroitin sulfate standard, which resists degradation with both chondroitinase ABC and heparin-lyase, as well as DNase are also observed in the glycans from the intestine, heart, and pharynx (Figure 1). The chemical nature of this material remains to be determined.

A quantitative analysis of the distribution of sulfated glycosaminoglycans in ascidian tissues is shown in Table I. Uronic acid–containing glycans were detected in all tissues studied. They occur in higher amounts in the intestine and cloak, representing about 0.3% of the dry weight, when compared to the heart and pharynx, where they account for about 0.1% of the dry weight of the tissue. Dermatan sulfate and a heparin-like polymer are present in all tissues. Dermatan sulfate occurs mainly in the pharynx, whereas the heparin-like polymer prevails in the intestine (Table I).

Disaccharide composition of the dermatan sulfate and heparin-like polymer from the ascidian tissues

The glycans from the various tissues were exhaustively degraded with chondroitin ABC lyase or a mixture of heparan sulfate and heparin lyases, and the disaccharides formed were separated by gel filtration HPLC and strong anion-exchange HPLC.

The disaccharides formed by the action of chondroitin ABC lyase on the glycans from the different tissues are shown in Figure 2 and in Table II. The method used allowed us to separate the following standard disaccharides: ΔUA-1→3-GalNAc(6SO_4), ΔUA-1→3-GalNAc(4SO_4), ΔUA(2SO_4)-1→3-GalNAc(6SO_4), ΔUA-1→3-GalNAc(4SO_4)(6SO_4) and ΔUA(2SO_4)-1→3-GalNAc(4SO_4) (Figure 2A). The disaccharide composition of the dermatan sulfate from intestine, heart, pharynx, and cloak is very similar, yielding about 75% of the unusual disaccharide ΔUA(2SO_4)-1→3-GalNAc(4SO_4) and 25% of the ΔUA-1→3-GalNAc(4SO_4) (Figure 2B and Table II).

Degradation of the glycans from the intestine, heart, and pharynx with a mixture of heparan sulfate and heparin lyases produces mainly (over 60%) of the trisulfated disaccharide ΔUA(2SO_4)-1→4-GlcN(SO_4)(6SO_4), and smaller amounts of the disulfated disaccharide ΔUA-1→4-GlcN(SO_4)(6SO_4) (Figure 2D, Table III). On the contrary, the glycans from the cloak yield mainly (about 60%) the disulfated disaccharide ΔUA1→4GlcN(SO_4)(6SO_4) and smaller amounts of the trisulfated

Table I. Distribution of the sulfated glycosaminoglycans among various tissues of the ascidian *Styela plicata.*

Tissue	Concentration in each tissue (μg of uronic acid/mg of dry weight)[a]	Distribution of glycosaminoglycans among various tissue (%)[b]	
		DS	Heparin-like
Intestine	2.6	7.3	46
Heart	1.1	10.3	23.3
Pharynx	1.6	63.0	10.5
Cloak	3.2	19.4	20.2

[a]The content of uronic acid in the glycans extracted from each tissue was estimated by the carbazole reaction (Bitter and Muir, 1962).
[b]The percentage of the glycosaminoglycans in the tissues was estimated by subtracting the densitometric units (OD/mm^2) obtained by densitometry of the metachromatic bands on agarose gel electrophoresis before and after degradation with specific glycosidases.

Table II. Disaccharide composition of the dermatan sulfate polymer from different ascidian tissues

Position of elution[a]	Disaccharide	T_R[b]	Proportion of disaccharides[c]			
			Intestine	Heart	Pharynx	Cloak
1	ΔUA-1→3-GalNAc(6SO_4)	26.2	<1	<1	<1	<1
2	ΔUA-1→3-GalNAc(4SO_4)	27.2	24.0	25.0	26.3	28.4
3	ΔUA(2SO_4)-1→3-GalNAc(6SO_4)	31.7	<1	<1	<1	<1
4	ΔUA-1→3-GalNAc(4SO_4)(6SO_4)	32.6	<1	<1	<1	<1
5	ΔUA(2SO_4)-1→3-GalNAc(4SO_4)	33.9	76.0	75.0	73.7	71.6

[a]Standard peak number in order of elution from a Spherisorb-SAX column.
[b]Retention time of the disaccharides on a Spherisorb-SAX column.
[c]The areas under the peaks corresponding to the disaccharides from the Spherisorb-SAX column, were integrated to obtain the disaccharide proportions.

Table III. Disaccharide composition of the heparin-like polymer from different ascidian tissues

Position of elution[a]	Disaccharide	T_R[b]	Proportion of disaccharides[c]			
			Intestine	Heart	Pharynx	Cloak
1	ΔUA-1→4-GlcNAc($6SO_4$)	21.7	<1	<1	<1	<1
2	ΔUA($2SO_4$)-1→4-GlcNAc	22.8	<1	<1	<1	<1
3	ΔUA-1→4-GlcN(SO_4)($6SO_4$)	23.7	39.3	31.1	25.5	58.5
4	ΔUA($2SO_4$)-1→4-GlcN(SO_4)	24.9	<1	<1	<1	<1
5	ΔUA($2SO_4$)-1→4-GlcNAc($6SO_4$)	27.5	<1	<1	<1	<1
6	ΔUA($2SO_4$)-1→4-GlcN(SO_4)($6SO_4$)	29.3	60.7	68.9	74.5	41.5

[a]Standard peak number in order of elution from a Spherisorb-SAX column.
[b]Retention time of the disaccharides on a Spherisorb-SAX column.
[c]The areas under the peaks corresponding to the disaccharides from the Spherisorb-SAX column, were integrated to obtain the disaccharide proportions.

disaccharide ΔUA($2SO_4$)-1→4-GlcN(SO_4)($6SO_4$), after degradation with the mixture of heparan sulfate and heparin lyases (Table III).

Antithrombin activity of the heparin-like polymers from the ascidian tissues

The most well known characteristic of heparin is its anticoagulant activity. This activity is related mainly to the ability of heparin to catalyze inhibition of thrombin by antithrombin (Bjork and Lindahl, 1982). In order to verify whether the heparin-like polymers from the ascidian tissues posses anticoagulant activity we assayed the thrombin inhibitory activity of the chondroitin ABC lyase-resistant glycans from the various tissues in the presence of antithrombin.

The dermatan sulfate-free glycans from all tissues studied were able to catalyze thrombin inhibition by antithrombin (Figure 3), however with a concentration at least 20 times higher, when compared to mammalian heparin. Among the glycans from the ascidian tissues, those from the pharynx possess the higher activity (IC_{50} = 0.03 μg/ml), whereas the glycans from the cloak are the less active (IC_{50} = 0.3 μg/ml). The antithrombin activity of the material obtained from the tissues disappears after degradation with heparin-lyase, excluding the presence of active contaminants in our preparations.

Histochemical analysis of the ascidian tissues

We used histochemical analysis of the intestine and pharynx with the cationic dye 1,9-dimethyl-methylene blue to determine the location of sulfated glycosaminoglycans in these tissues (Figure 4). Staining of intestine sections reveals a layer of cells along the intestinal lumen displaying a strong metachromasia associated with intracellular granules (arrow in Figure 4A). A diffuse extracellular metachromatic material is also observed (asterisk in Figure 4A). The metachromatic staining of the granules resists treatment with chondroitin ABC lyase (arrow in Figure 4B), while the metachromatic extracellular material completely disappears after treatment with the enzyme (asterisk in Figure 4B). The basal membrane is also metachromatic, but in this case, treatment with chondroitin ABC lyase has no effect on the staining (arrow in the inset of Figure 4B).

Similarly to the intestine, a diffuse extracellular metachromatic material is also present in the sections from the pharynx stained with 1,9-dimethyl-methylene (asterisk in Figure 4C). Treatment of the section with chondroitin ABC lyase abolishes the metachromatic staining (asterisk in Figure 4D). A layer of cells displaying a strong metachromasia associated with intracellular granules is also present in the sections from the pharynx treated with chondroitin ABC lyase (Figure 4D, inset).

Discussion

Sulfated glycosaminoglycans have been previously detected in the ascidian *Styela plicata* (Pavão *et al.*, 1994, 1998; Cavalcante *et al.*, 1999). A nitrous acid-sensitive glycan (Pavão *et al.*, 1994) and an oversulfated dermatan sulfate with high heparin cofactor II activity, composed mainly by disaccharide units of ΔUA($2SO_4$)-1→3GalNAc($4SO_4$) (Pavão *et al.*, 1998) occur in the visceras of this ascidian. In the present study, we investigated the distribution of these glycosaminoglycans in the various tissues of *S.plicata*.

Initially we performed a biochemical analysis of the glycans extracted from different tissues: intestine, pharynx, heart, and cloak. We found that dermatan sulfate (identified by its sensitivity to chondroitin ABC lyase and resistance to chondroitin AC lyase) and a heparin-like polymer (identified by its sensitivity to heparin lyase and resistance to heparan sulfate lyase) are present at different concentrations in the tissues studied (see Figure 1 and Table I). The dermatan sulfate prevails in the pharynx, while the heparin-like polymer occurs in higher amounts in the intestine (Table I). The former polymer shows a retarded electrophoretic migration on agarose gel, when compared to the vertebrate standard (Figure 1A). This pattern is probably due to the higher charge density of the invertebrate glycan, allowing a stronger interaction between the sulfate groups on the polysaccharide and the diamine groups of the gel buffer.

The dermatan sulfate possesses the same chemical composition in all tissues: ΔUA($2SO_4$)-1→3GalNAc($4SO_4$) (~75%) and ΔUA-1→3GalNAc($4SO_4$) (~25%), as indicated by the analysis of the disaccharide units shown in Table II. In vertebrate dermatan sulfate only about 5% of the disaccharide units are O-sulfated at carbon 2 of iduronic acid moieties. This result is in accordance to our previous data on the chemical composition of

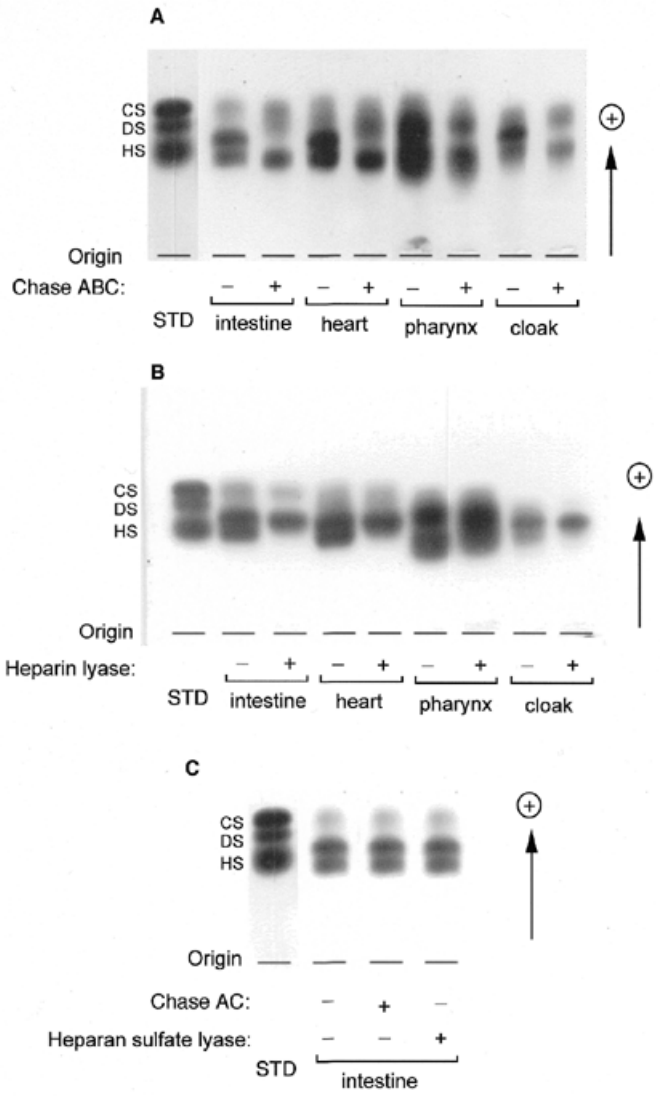

Fig. 1. Agarose gel electrophoresis of the sulfated glycans from the various tissues of *S.plicata* before and after incubation with chondroitin ABC lyase or heparin lyase. The glycans from intestine, heart, pharynx and cloak (15 µg as dry weight of each), before (–) and after (+) incubation with chondroitin ABC lyase (Chase ABC) (**A**), heparin lyase (**B**), and chondroitin AC lyase (Chase AC) and heparan sulfate lyase (**C**) (see *Materials and methods*), as well as a mixture of standard glycosaminoglycans, containing chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) (1.5 µg as uronic acid of each) were applied to a 0.5% agarose gel in 0.05 M 1,3-diaminopropane/acetate (pH 9.0), and run for 1 h at 110 mV. After electrophoresis the glycans were fixed with aqueous 0.1% cetylmethylammonium bromide solution and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v).

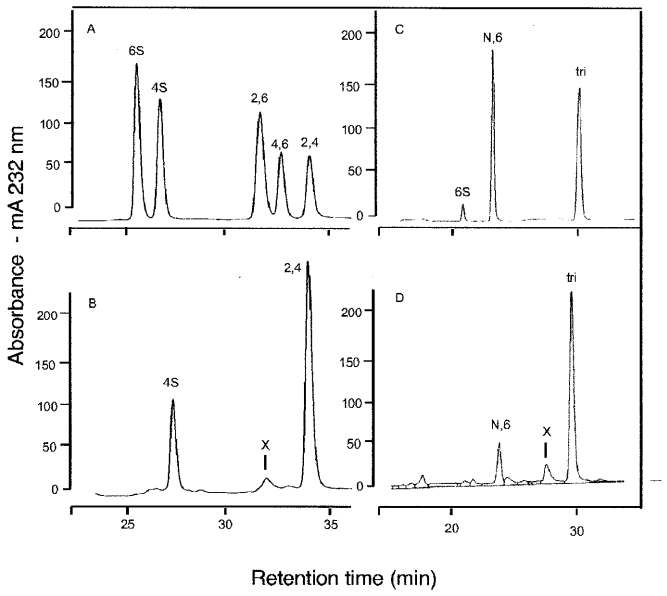

Fig. 2. Strong anion-exchange HPLC analysis of the disaccharides formed by specific lyases on the ascidian glycosaminoglycans. A mixture of chondroitin (**A**) and heparin (**C**) standard disaccharides and the disaccharides formed by exhaustive action of chondroitinase ABC (**B**) and heparin- plus heparan sulfate-lyases (**D**) on the glycans from intestine (see *Materials and methods*) were applied to a 25 cm × 4.6 mm Spherisorb-SAX column, linked to an HPLC system. The column was eluted with a linear gradient of NaCl, as described in *Materials and methods*. The eluent was monitored for UV absorbance at 232 nm. The chondroitin sulfate disaccharide standards used were: 6S, ΔUA-1→3-GalNAc(6SO_4); 4S, ΔUA-1→3-GalNAc(4SO_4); 2,6, ΔUA(2SO_4)-1→3-GalNAc(6SO_4); 4,6, ΔUA-1→3-GalNAc(4SO_4)(6SO_4); 2,4, ΔUA(2SO_4)-1→3-GalNAc(4SO_4). The heparin disaccharide standards used were: 6S, ΔUA-1→4-GlcNAc(6SO_4); N,6, ΔUA-1→4-GlcN(SO_4)(6SO_4); tri, ΔUA(2SO_4)-1→4-GlcN(SO_4)(6SO_4). X, Unidentified peak.

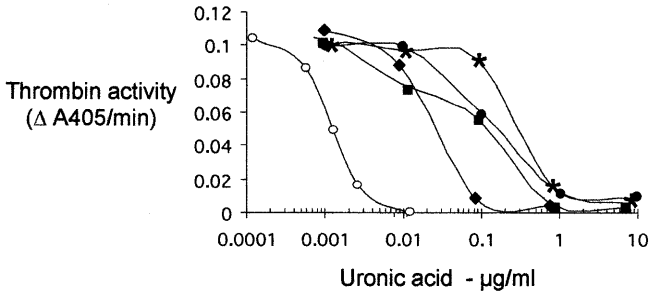

Fig. 3. Antithrombin activity of the glycans from the tissues of *S.plicata*. Antithrombin (100 nM) was incubated with thrombin (20 nM) in the presence of various concentrations of the chondroitin ABC lyase-treated glycans (see *Materials and methods*) from intestine (squares), heart (solid circles), pharynx (diamonds), and cloak (asterisks) of *S.plicata*, and mammalian heparin (open circles). After 60 s, the remaining thrombin activity was determined with a chromogenic substrate (A_{405nm}/min).

the *S.plicata* oversulfated dermatan (Pavão *et al.*, 1998) and suggests the presence of only one type of dermatan sulfate in this ascidian.

The ascidian dermatan sulfate occurs in the extracellular matrix surrounding the cells (asterisk in Figure 4A,C), as indicated by the sensitivity of a metachromatic extracellular material to chondroitin ABC lyase in the sections from intestine and pharynx (asterisk in Figures 4B,D).

On the contrary, the heparin-like polymer possesses a more heterogeneous sulfation pattern in the tissues of this ascidian. A more sulfated polymer, composed mainly by the tri-sulfated disaccharide ΔUA(2SO_4)-1→4-GlcN(SO_4)(6SO_4) is present in the intestine, heart and pharynx, whereas a less sulfated molecule, composed mainly of the disaccharide ΔUA-1→4-GlcN(SO_4)(6SO_4) occurs in the cloak (Table III).

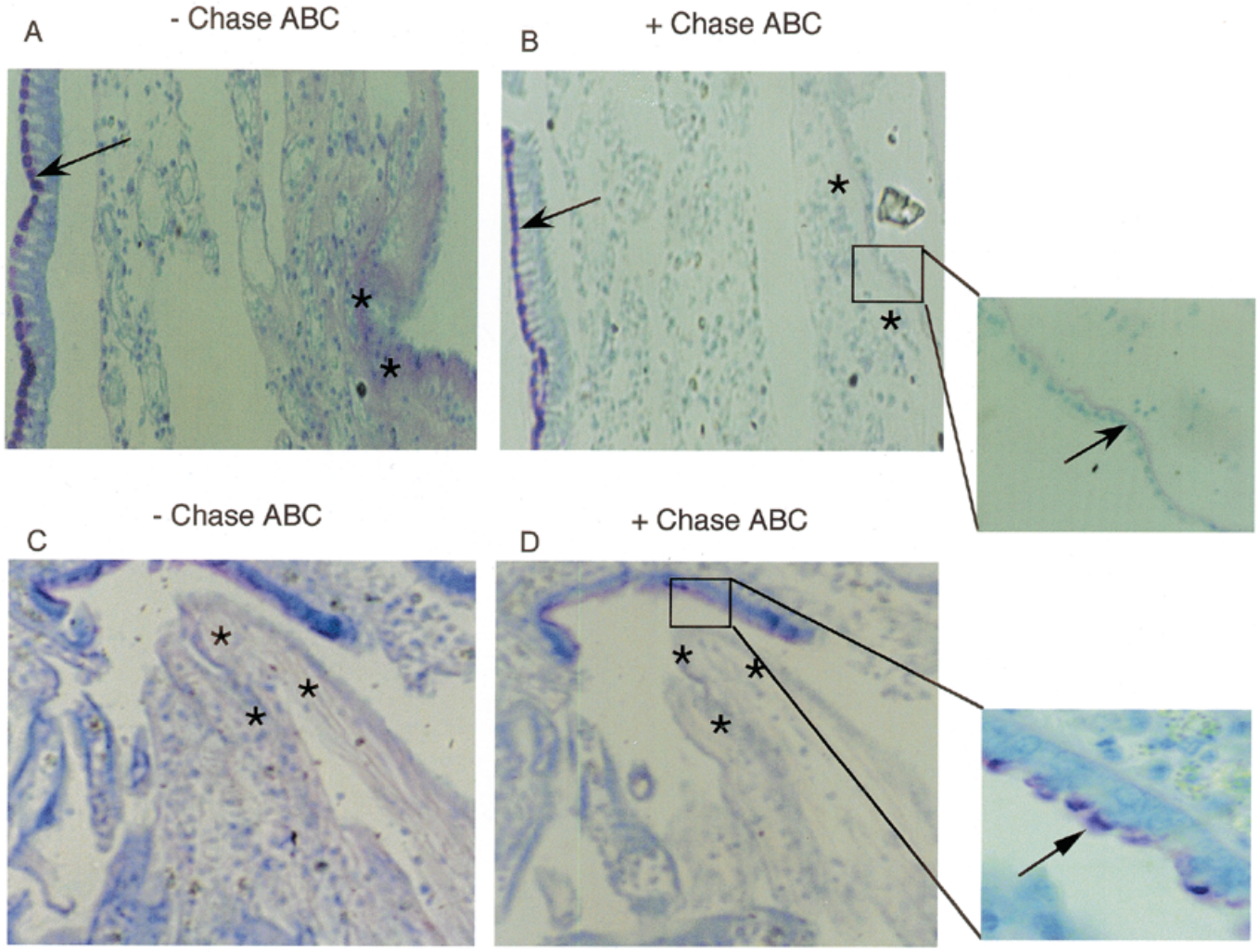

Fig. 4. Histological sections from the intestine and pharynx of *S.plicata* stained with 1,9-dimethylmethylene blue before and after incubation with chondroitin ABC lyase. Sections from intestine (**A** and **B**) and pharynx (**C** and **D**) were stained with the cationic dye 1,9-dimethylmethylene blue before (**A** and **C**) and after (**B** and **D**) incubation with chondroitin ABC lyase (see *Materials and methods*). Arrows indicate the metachromatic granules inside cells, and basal membrane (inset in **B**). Asterisks indicate the extracellular metachromatic material.

The occurrence of high percentage (about 25%) of the disulfated disaccharide ΔUA-1→4-GlcN(SO_4)($6SO_4$) in the ascidian heparin is noteworthy (Table III). In the biosynthetic pathway of heparin, described by Lindahl and co-workers for mammalian systems (Lindahl, 1989; Lindahl *et al.*, 1989; Salmivirta *et al.*, 1996), the 6-O-sulfation of $GlcNSO_3$ occurs preferentially after iduronic acid formation and 2-O-sulfation. During the biosynthesis of the ascidian heparin, this mechanism seems to be less relevant.

In addition to the susceptibility to heparin lyase and the occurrence of high amounts of trisulfated disaccharide units in the ascidian heparin-like polymers, two other features of these glycans are present in mammalian heparin, namely, antithrombin activity and localization in cytoplasmic granules. Although less potent than heparin, the heparin-like polymers from all ascidian tissues are able to catalyze thrombin inhibition by antithrombin (Figure 3). Preliminary data of our laboratory indicated that the ascidian heparin is eluted from an antithrombin affinity column with approximately the same salt concentration (not shown), suggesting the presence of the antithrombin-high-affinity pentasaccharide in the ascidian heparin. However, at the moment, we do not have any chemical evidence that confirm this evidence. More detailed experiments are required to properly address this question.

It is important to note the relationship between the sulfate content of the ascidian glycans with antithrombin activity. The heparin-like polymer from pharynx, which has the higher content of trisulfated disaccharide (see Table III), possesses the higher antithrombin activity (0.03 µg/ml), as measured by the IC_{50} for thrombin inhibition (see Table IV). The less sulfated glycans from the cloak, enriched in disulfated disaccharides (see Table III) is 10 times less potent (0.3 µg/ml) (Table IV).

It is known that heparin occurs exclusively within cytoplasmic granules of mast cells (Straus *et al.*, 1982; Stevens, 1987). In fact, some authors (Kjellén and Lindahl, 1991) suggest that the term heparin should be reserved for those glycosaminoglycans that are synthesized by mast cells and stored in cytoplasmic granules. In Figure 4, we provide

evidence that suggests the occurrence of the heparin-like polymer in cytoplasmic granules of cells that are located at the lumen of the intestine (arrow in Figure 4A) and pharynx (inset in Figure 4C). The evidence comes from the fact that the metachromatic materials in the cytoplasmic granules are resistant to treatment with chondroitin ABC lyase (arrows in Figure 4B and inset in Figure 4D). Recent data from our laboratory reinforces this evidence. We detected the presence of an oversulfated heparan sulfate, enriched in trisulfated disaccharide units, in cytoplasmic granules of the test cells that surround the oocytes of *S.plicata* (Cavalcante *et al.*, 1999).

This is the first report on the distribution and localization of sulfated glycosaminoglycans in the tissues of ascidians. The results described here may bring new insights about the function of this class of polysaccharides in animals. It is likely, however, that the physiological function of the sulfated glycosaminoglycans described here has no relation to their anticoagulant activity, since the prevention of body fluid loss in this invertebrate does not involve coagulation of the hemolymph.

Materials and methods

Materials

Heparan sulfate from human aorta was extracted and purified as described previously (Cardoso and Mourão, 1994). Chondroitin 4-sulfate from whale cartilage, dermatan sulfate from bovine intestinal mucosa, twice-crystallized papain (15 units/mg protein) and the standard disaccharides α-ΔUA-1→3-GalNAc(6SO_4), α-ΔUA-1→3-GalNAc(4SO_4), α-ΔUA(2SO_4)-1→3-GalNAc(6SO_4), α-ΔUA-1→3-GalNAc(4SO_4)(6SO_4), α-ΔUA(2SO_4)-1→3-GalNAc(4SO_4), α-ΔUA-1→4-GlcN(SO_4), α-ΔUA-1→4-GlcNAc(6SO_4), α-ΔUA(2SO_4)-1→4-GlcNAc, α-ΔUA(2SO_4)-1→4-GlcN(SO_4), α-ΔUA(2SO_4)-1→4-GlcNAc(6SO_4), α-ΔUA-1→4-GlcN(SO_4)-(6SO_4) and α-ΔUA-(2SO_4)-1→4-GlcN(SO_4)-(6SO_4) were purchased from Sigma (St. Louis, MO); chondroitin AC lyase (EC 4.2.2.5) from *Arthrobacter aurenses*, chondroitin ABC lyase (EC 4.2.2.4) from *Proteus vulgaris*, heparan sulfate lyase (EC 4.2.2.8) and heparin lyase (EC4.2.2.7) from *Flavobacterium heparinum* were from Seikagaku America Inc. (Rockville, MD); agarose (standard low M_r) was from Bio-Rad (Richmond, CA); toluidine blue was from Fisher Scientific (USA) and 1,9-dimethylmethylene blue from Serva Feinbiochimica (Heidelberg, Germany); cetyltrimethylammonium bromide from Merck (Darmstadt, Germany) and DEAE-cellulose (DE-52) from Whatman International (Maidstone UK); human antithrombin and thrombin were from Hematologica Technologies Inc. (USA); thrombin chromogenic substrate tosyl-Gly-Pro-Arg-p-nitroanilide acetate (Chromozyn TH) was from Boehringer Mannheim (Germany).

Isolation of the glycans

Adult specimens of *S.plicata* were collected at Guanabara Bay, Rio de Janeiro. Animals were maintained in an aerated aquarium until use. Tissues from different organs (intestine, heart, pharynx, and cloak) of several ascidians were carefully isolated from other tissues, under magnifying lenses, cut in small pieces and dried. The dried tissues (~1 g) were individually suspended in 20 ml of 0.1 M sodium acetate buffer (pH 5.5), containing 100 mg papain, 5 mM EDTA, and 5 mM cysteine and incubated at 60°C for 24 h. The mixtures were centrifuged (2000 × *g* for 10 min at room temperature), another 100 mg of papain in 20 ml of the same buffer, containing 5 mM EDTA and 5 mM cysteine was added to the precipitate, and the mixture was incubated for another 24 h. The clear supernatants from the two extractions were combined and the polysaccharides precipitated with 2 vol. of 95% ethanol and maintained at 4°C for 24 h. The precipitates formed were collected by centrifugation (2000 × *g* for 10 min at room temperature), freeze-dried, and dissolved in 2 ml of distilled water.

Chemical analysis

The hexuronic acid content of the glycans from the various tissues was estimated by the carbazole reaction (Bitter and Muir, 1962).

Agarose gel electrophoresis

The intact or enzyme-degraded glycans from the ascidian tissues were analyzed by agarose gel electrophoresis, as described previously (Dietrich and Dietrich, 1976). About 1.5 μg (as uronic acid) of the glycans, and a mixture of standard glycosaminoglycans, containing chondroitin sulfate, dermatan sulfate, and heparan sulfate (1.5 μg as uronic acid of each) were applied to a 0.5% agarose gel in 0.05 M 1,3-diaminopropane/acetate (pH 9.0), and run for 1 h at 110 mV. After electrophoresis the glycans were fixed with aqueous 0.1% cetylmethylammonium bromide solution and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v).

Enzymatic treatments

Chondroitin lyases. the glycans extracted from the ascidian tissues (~100 μg) were incubated with 0.01 U of chondroitin AC or ABC lyase in 0.1 ml 50 mM Tris–HCl buffer (pH 8.0), containing 5 mM EDTA and 15 mM sodium acetate. After incubation at 37°C for 12 h, another 0.01 U of enzyme was added to the mixture, and the reaction continued for an additional 12 h period. The percentage of the sulfated glycans degraded by the enzymes was estimated by densitometry of the metachromatic bands on a Bio-Rad densitometer, following agarose gel electrophoresis of intact and enzyme-degraded glycans.

Heparan sulfate and heparin lyases. About 50 μg (as dry weight of each) of the glycans extracted from the ascidian tissues were incubated with 0.005 U of either heparan sulfate lyase or heparin lyase in 100 μl of 100 mM sodium acetate buffer (pH 7.0), containing 10 mM calcium acetate for 17 h at 37°C. At the end of the incubation period the mixtures were analyzed by agarose gel electrophoresis, as described earlier. The percent of the sulfated glycans degraded by the enzymes was estimated by densitometry of the metachromatic bands, as described in the previous paragraph.

In order to determine the disaccharide composition of the glycosaminoglycans present in the various ascidian tissues, the glycans obtained from each tissue were incubated with chondroitin ABC lyase, or with a mixture of heparin and heparan sulfate lyase, as described above. After the incubation period, the reaction mixture was applied to a Superdex-Peptide 10/30 column (Amersham Pharmacia Biotech), connected to

an HPLC system. The column was equilibrated with an aqueous solution of 20% acetonitrile (pH 3.5) and developed at a flow rate of 0.25 ml/min. Fractions of 0.5 ml were collected and checked for absorbance at 232 nm. The fractions containing the disaccharides, identified by their position of elution from the column, were pooled, freeze-dried, and analyzed by HPLC.

Analysis of the disaccharides formed by digestion of the glycans with specific glycosidases

The disaccharides formed by digestion of the glycans from different tissues with chondroitin ABC lyase, and/or with heparin and heparan sulfate lyases were analyzed by HPLC on a Supelco 4.5 mm × 25 cm Spherisorb SAX column, using a linear gradient of 0–1.0 M aqueous NaCl (pH 3.5) at a flow rate of 0.5 ml/min. The elution of the disaccharides was followed by absorbance at 232 nm, and they were identified by comparison with elution positions of known disaccharide standards.

Inhibition of thrombin by antithrombin in the presence of the glycans from the various ascidian tissues

The glycans extracted from intestine, heart, pharynx, and cloak were incubated separately with chondroitin ABC lyase, as described above. After the incubation period, 2 volumes of 95% ethanol was added. The precipitate formed after incubating the sample at –10°C for 12 h, containing the enzyme-resistant glycans, was separated by centrifugation (2000 × *g* for 15 min at room temperature) and used to estimate the antithrombin activity of the chondroitin ABC lyase-resistant glycans from different tissues. Incubations were performed in disposable polystyrene cuvettes. The reactants included 100 nM antithrombin, 20 nM thrombin and 0–10 μg/ml (as uronic acid) chondroitin lyase ABC-resistant glycans from different tissues in 100 μl 0.02 M Tris–HCl, 0.15 M NaCl, and 1.0 mg/ml polyethylene glycol, pH 7.4 (TS/PEG buffer). Thrombin was added last to initiate the reaction. After a 60 s incubation at room temperature, 500 μl of 100 μl Chromosym TH in TS/PEG buffer was added, and the absorbance at 405 nm was recorded continuously for 100 s. The rate of change of absorbance was proportional to the thrombin activity remaining in the incubation. No inhibition occurred in control experiments in which thrombin was incubated with antithrombin in the absence of glycan, nor did inhibition occur when thrombin was incubated with glycan alone over the range of concentrations tested.

Histochemistry of the ascidian tissues

For histochemical preparations the intestine and pharynx were carefully isolated from other tissues and fixed in 5% formaldehyde in seawater for 2 h at room temperature. After fixation, the organs were washed with water, dehydrated in graded ethanol, cleared in xylol, and embedded in Para-plast (m.p. 55.6°C). Approximately 7 μm sections from intestine and pharynx were cut longitudinally on a Spencer microtome. Sections were stained with 0.05 M 1,9-dimethyl-methylene blue in 0.1 M HCl, before or after incubation with chondroitin ABC lyase in 50mM Tris–HCl buffer (pH 8.0), containing 5 mM EDTA and 15 mM sodium acetate, for 12 h at room temperature.

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Abbreviations

α-ΔUA(2SO_4), α-$\Delta^{4,5}$ unsaturated hexuronic acid 2-sulfate; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography.

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