Isolation, Fractionation, and Preliminary Characterization of a Novel Class of Sulfated Glycans from the Tunic of *Styela plicata* (Chordata Tunicata)*

Rodolpho M. Albano and Paulo A. S. Mourão

From the Departamento de Bioquímica, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Caixa Postal 68041, Rio de Janeiro 21910, RJ, Brasil

The sulfated glycans in the tunic of *Styela plicata* differ from the glycosaminoglycans of animal tissues and also from the sulfated polysaccharides isolated from marine algae. The ascidian glycans occur primarily as three fractions that differ markedly in molecular weight and chemical composition. The high molecular weight fraction encompasses a broad range of molecular weights but is chemically homogeneous and contains an unusual amount of galactose. The 20,000 molecular weight polysaccharide is rich in galactose and glucose while the 8,000 molecular weight fraction is rich in amino sugars and contains the neutral hexoses galactose, glucose, and mannose. All fractions contain large amounts of sulfate esters. The ascidians polysaccharides can be extracted from the tissue by proteolytic enzyme or by guanidine hydrochloride solutions. The high molecular weight fraction is preferentially extracted by papain while guanidine hydrochloride removes mainly the low molecular weight polysaccharides. We speculate that these sulfated glycans are essential for maintaining the structural integrity of the tunic, in analogy with the glycosaminoglycans of vertebrate connective tissues.

Together with fibrous proteins, sulfated glycosaminoglycans are essential for maintaining the structural integrity of connective tissues (1). Among these tissues, cartilages can be distinguished by their high concentrations of sulfated glycosaminoglycans, since about 10% of their dry weight corresponds to chondroitin 4- and 6-sulfates and keratan sulfate (2-6). These compounds occur in cartilage covalently linked to protein through O-sulphate or O-threonine linkages (7). They can be removed from the tissue as proteoglycans by concentrated salt solutions or as free polysaccharide chains by extraction with proteolytic enzyme or alkaline (8, 9).

The aim of our work is to investigate the presence of polyanionic glycans in invertebrate tissues that resemble the connective tissues of vertebrates. As will be shown, we have found sulfated glycans in the tunic of *Styela plicata* (Chordata Tunicata), in concentrations which resemble the great quantities of glycosaminoglycans that are characteristic of cartilages. Our results indicate that the sulfated glycans from the tunic of *S. plicata* are different from all the glycosaminoglycans described so far in animal tissues. They can be separated into three fractions: one of high molecular weight containing a high proportion of galactose and two other fractions of low molecular weight containing a higher proportion of amino sugars and glucose.

**EXPERIMENTAL PROCEDURES**

**Materials**

Tunicates were collected from Guanabara Bay (Urca) in Rio de Janeiro. Chondroitin 4-sulfate, chondroitin 6-sulfate, dextran sulfate (average molecular weight, 10,000), chondroitin sulfate (average molecular weight, 500,000), bovine liver β-galactosidase, *Aspergillus niger* α-β-galactosidase, Sephadex G-150 (superfine), Sepharose CL-4B, and guanidine hydrochloride were purchased from Sigma, dextran sulfate, chondroitin AC, and chondroitinase ABC from Miles Laboratories, Elkhart, IN, papain from E. Merck A. G. (Darmstadt, Germany), toluidine blue from Fisher, and agarose from Bio-Rad. Heparan sulfate was a gift from Dr. J. A. Cifonelli (Department of Pediatrics, University of Chicago, Chicago, IL). Keratan sulfate from ox cornea (10), crude extracts from heparan sulfate-induced *Flavobacterium heparinum* (11), and crude extracts from keratan sulfate-induced *Pseudomonas* sp. (IFO-13309) (12) were prepared by methods previously described.

**Isolation of the Sulfated Glycans from the Ascidian Tunic**

Isolation with Proteolytic Enzyme—The tunic of *S. plicata* was carefully separated from other tissues, immersed immediately in acetone, and kept for 24 h at 4 °C. The dried tissue (1 g) was cut in small pieces, suspended in 20 ml of 0.1 mM sodium acetate buffer, pH 5.5, containing 100 mg of papain, 5 mM EDTA, and 5 mM cysteine, and incubated at 60 °C during 24 h. The incubation mixture was then centrifuged (2000 × g for 10 min at room temperature), and 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 incubated for another 24 h. The clear supernatant of the two extractions was precipitated with 2 volumes of 95% ethanol and maintained at −10 °C for 24 h. The precipitate formed was collected by centrifugation (2000 × g for 15 min at room temperature), vacuum dried, dissolved in 20 ml of distilled water, exhaustively dialyzed against distilled water at 4 °C, and lyophilized. In some experiments, five consecutive extractions of the ascidian tunic polysaccharides were done, each extraction was for 24 h, and the supernatants obtained were precipitated with 2 volumes of 95% ethanol, collected as described above, dissolved in distilled water, and analyzed separately.

Isolation with Guanidine Hydrochloride—About 2 g of the above-mentioned acetone powder of ascidian tunic were mixed with 160 ml of a 0.1 M guanidine hydrochloride solution in 0.05 M sodium acetate buffer (pH 7.0), containing 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, and 0.2 mg/ml soybean trypsin inhibitor and stirred for 8 h at 4 °C. The mixture was then centrifuged (2000 × g for 10 min at room temperature). The supernatant was dialyzed against distilled water during 24 h at 4 °C and lyophilized, while the precipitate was mixed with another guanidine hydrochloride solution of higher con-
Fractionation of the Sulfated Glycans

DEAE-cellulose Chromatography—About 50 mg of the sulfated glycans from the ascidian tunic were applied to a DEAE-cellulose column (3.5 cm × 2.0 cm) equilibrated with 0.1 M sodium acetate buffer (pH 6.0) and washed with 100 ml of the same buffer. The column was developed with a linear gradient of 100 ml of 0.1 M sodium acetate buffer (pH 6.0) in the flask and 100 ml of 0.1 M NaCl in the same buffer in the reservoir. The flow rate of the column was 10 ml/h, and fractions of 2.5 ml were collected and checked for hexose and for ultraviolet-absorbing material. The fractions containing the sulfated polysaccharides were pooled, precipitated with 2 volumes of 95% ethanol, and maintained at −10°C for 24 h. The precipitate formed was collected by centrifugation (2000 × g for 15 min at room temperature) and vacuum dried.

The presence of sulfated glycans eluted by dissociative chromatography was measured as follows: a 50-μl aliquot was mixed with 1 ml of a 0.01 mg/ml solution of toluidine blue, and after 10 min the absorbance was measured at 620 nm. The decrease in optical absorbance that occurs after addition of the sulfated glycans to the toluidine blue solutions is due to their metachromatic property and indicates the presence of glycans in each sample. The presence of sulfated glycans eluted by dissociative chromatography was measured as follows: a 50-μl aliquot of each fraction was applied to Whatman No. 1 paper for 48 h in ethyl acetate:pyridine:water (8:2:1, v/v) for 24 h (15) and by gas-liquid chromatography of the methanolic extract (13). Standard curves for hexose or hexose were constructed from glucosamine or galactosamine subjected to exactly the same hydrolytic procedures as the biological samples. The percentages of the different hexoses were estimated by chromatography on ethyl acetate:pyridine:water (8:2:1, v/v) for 24 h (15) and by gas-liquid chromatography of the acetylated hexoses (16). The percentages of amino sugars and amino acids were determined by means of a Beckman amino acid analyzer. The relative proportions of glucosamine and galactosamine were also determined by densitometry of chromatograms run on Whatman No. 1 paper for 48 h in ethyl acetate:pyridine:water (8:2:1, v/v). The hexuronic acid content was estimated by the carbazole reaction (17) and acetyl groups by a method previously described (18). Sulfate was measured both by densitometry on chromatograms of the acid hydrolysates (6.0 M HCl, 100°C for 6 h) stained with toluidine blue (19) and by the BaCl₂-gelatin method, as previously described (20). Protein was assayed according to Lowry et al. (21). All the densitometry was performed using a Quick Scan densitometer (Helena Laboratories, Beaumont, TX).

Agarose and Polyacrylamide Gel Electrophoresis—Sulfated polysaccharides were analyzed in sodium dodecyl sulfate gel electrophoresis, as previously described (22). About 100 μg of the sulfated glycans were applied to a 0.5% agarose gel in 0.05 M 1,2-diaminopropane:acetate buffer (pH 9.0) and after the electrophoretic run, the glycans in the gel were fixed with 0.1% N-acetyl-β-N,N,N-trimethylammonium bromide in water and stained with 0.1% toluidine blue in acetic acid:methanol (1:10, v/v). The molecular weights of the glycans were determined by polyacrylamide gel electrophoresis (23). About 50 μg of the sulfated glycans were applied to a 6% polyacrylamide gel slab 1 mm thick, and after the electrophoretic run the gel was stained with 0.1% toluidine blue in 1% acetic acid. After staining, the gel was washed for about 3 h in 1% acetic acid. In some experiments the relative proportions of the polysaccharides were determined by densitometry of the stained gel.

**Chemical Modification of the Ascidian Glycans**

**Desulfation—**Desulfation of the sulfated glycans from the ascidian tunic was performed as described by Nagasawa et al. (24). About 200 mg of the sulfated glycans in 20 ml of water were mixed with 2 g (dry weight) of Dowex 50W × 8 (200-400 mesh). After Dowex Dowex 50W × 8 was removed by overnight descending chromatography in 1-M NaCl in the same buffer in the reservoir. The flow rate of the column was 10 ml/h, and fractions of 2.5 ml were collected and checked for hexose and for ultraviolet-absorbing material. The fractions containing the sulfated glycans to the toluidine blue solutions is due to their metachromatic property and indicates the presence of glycans in each sample. The presence of sulfated glycans eluted by dissociative chromatography was measured as follows: a 50-μl aliquot of each fraction was applied to Whatman No. 1 paper for 48 h in ethyl acetate:pyridine:water (8:2:1, v/v) for 24 h (15) and by gas-liquid chromatography of the methanolic extract (13). Standard curves for hexose or hexose were constructed from glucosamine or galactosamine subjected to exactly the same hydrolytic procedures as the biological samples. The percentages of the different hexoses were estimated by chromatography on ethyl acetate:pyridine:water (8:2:1, v/v) for 24 h (15) and by gas-liquid chromatography of the acetylated hexoses (16). The percentages of amino sugars and amino acids were determined by means of a Beckman amino acid analyzer. The relative proportions of glucosamine and galactosamine were also determined by densitometry of chromatograms run on Whatman No. 1 paper for 48 h in ethyl acetate:pyridine:water (8:2:1, v/v). The hexuronic acid content was estimated by the carbazole reaction (17) and acetyl groups by a method previously described (18). Sulfate was measured both by densitometry on chromatograms of the acid hydrolysates (6.0 M HCl, 100°C for 6 h) stained with toluidine blue (19) and by the BaCl₂-gelatin method, as previously described (20). Protein was assayed according to Lowry et al. (21). All the densitometry was performed using a Quick Scan densitometer (Helena Laboratories, Beaumont, TX).

**Enzymatic Degradations**

Incubation with α-D- and β-D-Galactosidase—About 300 μg of the sulfated polysaccharides from S. pictus were incubated with 0.5 unit of A. niger α-D-galactosidase in 1 ml of 0.05 M sodium acetate buffer (pH 4.5) or with 0.5 unit of bovine liver β-D-galactosidase in 1 ml of 0.05 M sodium acetate buffer (pH 7.0). After various incubation times, 100-μl samples were removed from each incubation mixture,
boiled for 2 min, and applied on Whatman No. 1 paper. After descending chromatography in ethyl acetate:pyridine:water (82:1, v/v) for 24 h followed by silver nitrate staining, the galactose was determined by densitometry of the chromatograms. To assess the α- or β-d-galactosidase activity, the p-nitrophenol liberated from p-nitrophenyl α-d-galactose or p-nitrophenyl β-d-galactose was quantified (29), after incubation under the same conditions described above.

Incubation with Mucopolysaccharides—About 100 μg of the sulfated glycans were incubated with 0.01 unit of chondroitinase AC or with 0.01 unit of chondroitinase ABC (30) in 0.5 M Tris-HCl buffer (pH 8.0) at 37 °C for 8 h; with 30 μg (as protein) of crude extract from heparan sulfate-induced F. heparinum in 0.05 M ethylene diamineacetate buffer, pH 8.0, at 30 °C for 8 h (11); or with 30 μg (as protein) of crude extracts from keratan sulfate-induced Pseudomonas sp. (IFO-13309) in 0.05 M Tris-HCl buffer, pH 7.2, at 37 °C for 8 h (12). Electrophoresis of control and enzyme-incubated glycans was used to assess the enzymatic activity.

RESULTS

Distinctive Features of the Ascidian-sulfated Glycans—The papain-extracted glycans from the tunic of S. plicata are compared with standard glycosaminoglycans after electrophoresis on agarose and polyacrylamide gels in Fig. 1. Two main metachromatic bands with electrophoretic mobilities which differ from those of standard glycosaminoglycans are observed on the agarose gel (Fig. 1A). Polyacrylamide gel electrophoresis of the ascidian polysaccharides (Fig. 1B) shows three fractions of different molecular weights: one fraction has a molecular weight of 100,000 or more, and thus stays at the origin, while the other two fractions migrate into the gel and have molecular weights of approximately 20,000 and 8,000.

The chemical analysis of these ascidian polysaccharides indicates the presence of amino sugars, hexoses, and sulfate (Table I). The polysaccharides obtained from S. plicata were incubated with chondroitinase AC, chondroitinase ABC, crude extracts from heparan sulfate-induced F. heparinum, or crude extracts from keratan sulfate-induced Pseudomonas sp. (IFO-13309). In addition, these glycans were submitted to deaminative cleavage by nitrous acid at pH 4.0 and 2.0. The galactose and polyacrylamide gel electrophoresis of the control and the enzyme- or nitrous acid-incubated glycans (not shown) indicated that these compounds are resistant to all the mucopolysaccharidases and to the deaminative cleavage by nitrous acid, thus differing from all previously described glycosaminoglycans.

Fractionation and Chemical Analysis of the Ascidian Polysaccharides—The glycans extracted from the ascidian tunic by papain were purified by DEAE-cellulose chromatography (Fig. 2). The ultraviolet-absorbing materials were eluted before or at the beginning of the salt gradient while 98% of the heparin was eluted with higher NaCl concentrations. As shown in the inset of Fig. 2, the sulfated glycans from the ascidian tunic have the same electrophoretic pattern on an agarose gel before and after purification on the DEAE-cellulose column.

The DEAE-cellulose column-purified glycans were fractionated by gel chromatography on Sepharose CL-4B (Fig. 3A). The glycans were separated into two peaks: a broad peak designated as F-1 that accounts for approximately 75% of the extracted polysaccharides and a sharp peak eluted near the total volume, designated as F-2. The elution of F-1 near the void volume of the Sepharose CL-4B (nominal exclusion of neutral polysaccharides on this gel is approximately 5 × 10^5 daltons) could indicate that this fraction is either a large molecular weight polysaccharide, an aggregate of complex carbohydrate molecules as demonstrated for cartilaginous proteoglycans (8, 9), or even a product of partial proteolysis. The results of the experiments of Fig. 3B indicate that the elution profile of the ascidian glycans after β-elimination, after incubation of the extracted glycans with proteolytic enzyme, and after hydrazinolysis (same result as in Fig. 3B, but not shown) remained essentially unchanged. The same result was obtained when the ascidian polysaccharides were chromatographed directly in 4.0 M guanidine hydrochloride (Fig. 3C). These results exclude the above-mentioned hypothesis of partial proteolysis or macromolecular aggregates contributing to fraction F-1.

The chemical analysis of the fractions of polysaccharides obtained from the Sepharose CL-4B column is shown in Table I. Hexosamines, hexoses, and sulfate are detected in both fractions; however, the proportions of these components vary from one fraction to another. F-1 has a high molar proportion of hexose (mainly galactose), and the predominant amino sugar is galactosamine. F-2 has a lower proportion of hexose to hexosamine; the predominant amino sugar is glucosamine and among the neutral hexoses, glucose and galactose are present in approximately the same proportion while mannose is detected in a small proportion. Both fractions have a strong negative optical rotation and high amounts of sulfate. Besides those sugars listed in Table I, no other sugars were detected in these ascidian polysaccharides, the level of detection of the methods used being approximately 0.02 mg/mg of polysaccharide. Also, amino acids were not detected in these polysaccharides, the level of detection for amino acids being approximately 0.01 mg/mg of polysaccharide.

F-1 forms broad peaks on the Sepharose CL-4B columns (Fig. 3, A–C). In order to determine whether this fraction has a homogeneous chemical composition, F-1 was subfractionated into F-1-A and F-1-B (Fig. 3A). F-1-A shows a slightly lower electrophoretic mobility on agarose gel when compared to F-1-B (Fig. 3D), and it does not enter into the polyacrylamide gel due to its very high molecular weight, while F-1-B shows a slight migration on the polyacrylamide gel (Fig. 3E).
Sulfated Glycans from Ascidians

TABLE I

"Total" is the main hexose peak from the DEAE-cellulose chromatography (Fig. 2). The fractions F-1, F-1-A, F-1-B, and F-2 were obtained on Sepharose CL-4B column under associative conditions (Fig. 3A), and F-2-A and F-2-B were obtained by chromatography on a Sephadex G-150 column (Fig. 3F). The total sulfated glycans were analyzed for acetyl groups, and the results indicated the presence of about 0.84 mol of acetyl groups/mol of total hexosamines, ND, not determined.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hexose/Hexosamine</th>
<th>Hexose</th>
<th>Hexosamine</th>
<th>Sulfate/Total sugar</th>
<th>[o]β°</th>
<th>Hexose</th>
<th>Hexosamine</th>
<th>Sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>5.48</td>
<td>57</td>
<td>38</td>
<td>5</td>
<td>74</td>
<td>26</td>
<td>0.95</td>
<td>-79</td>
</tr>
<tr>
<td>F-1</td>
<td>24.77</td>
<td>85</td>
<td>15</td>
<td>0</td>
<td>29</td>
<td>71</td>
<td>0.66</td>
<td>-126</td>
</tr>
<tr>
<td>F-1-A</td>
<td>23.87</td>
<td>84</td>
<td>16</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>0.93</td>
<td>ND</td>
</tr>
<tr>
<td>F-1-B</td>
<td>28.17</td>
<td>89</td>
<td>11</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>0.78</td>
<td>ND</td>
</tr>
<tr>
<td>F-2</td>
<td>3.54</td>
<td>43</td>
<td>47</td>
<td>10</td>
<td>82</td>
<td>18</td>
<td>0.69</td>
<td>-32</td>
</tr>
<tr>
<td>F-2-A</td>
<td>3.45</td>
<td>26</td>
<td>74</td>
<td>0</td>
<td>90</td>
<td>10</td>
<td>0.73</td>
<td>ND</td>
</tr>
<tr>
<td>F-2-B</td>
<td>1.49</td>
<td>34</td>
<td>46</td>
<td>20</td>
<td>80</td>
<td>20</td>
<td>0.67</td>
<td>ND</td>
</tr>
</tbody>
</table>

The fraction F-2 is more resistant than F-1 to papain extraction, as suggested by the experiment of Fig. 4. The polysaccharides were extracted from the ascidian tunic by incubation of the acetone powder of this tissue with papain for different periods of time. F-1 is the main fraction present in the extracts of the first two to three days, while F-2 is the predominant fraction in the last two extracts. Polyacrylamide gel electrophoresis of the F-2 extracts in Fig. 4 (not shown) revealed that the ratio of F-2-A to F-2-B was about 6.7 to 1 in all 5 extracts.

Preliminary Structural Studies of the Ascidian Glycans—In order to obtain information about the structure of the saccharide chains, the ascidian glycans were analyzed by their infrared spectra, by periodate oxidation, and by degradation with α- and β-D-galactosidase.

The infrared spectra of the total sulfated glycans and of the fractions F-1 and F-2 showed absorption bands at 820 cm⁻¹, at 1240 cm⁻¹, and bands between 1550 and 1680 cm⁻¹ which are attributed to the primary equatorial ester sulfate (C—S—O), to S=O linkage, and to C—O and/or C—N— groups, respectively (31, 32). The 1550-1680 cm⁻¹ bands are more intense for fraction F-2, which correlates with the chemical analysis of Table I, showing higher amino sugar content in F-2 than in F-1. Desulfation of the polysaccharide resulted in the disappearance of the C—O—S band at 820 cm⁻¹ as well of the S=O band at 1240 cm⁻¹, as expected.

The periodate consumption of the total sulfated polysaccharides was 0.94, 0.53, and 0.15 mol of periodate reduced/mol of total sugar in the first, second, and third rounds of consecutive oxidation of the polysaccharide with a periodate solution. About 0.27 mol of formic acid was produced after one round of oxidation of the ascidian polysaccharide with periodate. The products obtained by acid hydrolysis of the periodate-oxidized ascidian polysaccharides after borohydride reduction are shown in Table II. Glycerol, erythritol, and threitol are formed from the crude polysaccharide and from fraction F-1, while the formation of glycerol and the total disappearance of glucose are observed for fractions F-2-A and F-2-B. However, a considerable proportion of the hexosamines and hexoses are resistant to the periodate oxidation.

The desulfated polysaccharide produces a significantly higher proportion of glycerol, erythritol, and threitol in the first round of periodate oxidation when compared with the sulfated glycans. This difference could be attributed to the increased resistance to periodate oxidation of those polysaccharides that contain sulfate esters (33). The amounts of glycerol formed from the desulfated glycans and from the
sulfated glycans after 3 rounds of periodate oxidation are similar (Table II), although more threitol is formed from the desulfated glycan. From these results it is possible to speculate that small amounts of sulfate ester are blocking the access of periodate to galactose residues.

Incubation of the desulfated polysaccharides from *S. plicata* with *A. niger* α-D-galactosidase or with bovine liver β-D-galactosidase results in the liberation of about 2% of the total galactose by β-D-galactosidase (Fig. 5A), while no detectable galactose was formed after incubation with α-D-galactosidase.

The velocity of liberation of galactose by β-D-galactosidase from polysaccharides whose molecular weights were reduced by mild acid hydrolysis was markedly increased (Fig. 5B). About 5% of the total galactose was released from the polysaccharides by incubation with β-D-galactosidase, while the glycan continued to resist hydrolysis by α-D-galactosidase.

Analysis of the Sulfated Glycans Extracted from the Ascidian Tunic by Guanidine Hydrochloride Solutions—In order to compare the ascidian-sulfated glycans with proteoglycans extracted from cartilage, the ascidian tunic was submitted to successive extractions with guanidine hydrochloride solutions of increasing concentrations. The results of Table III indicate that about 50% of the glycans can be extracted with guanidine hydrochloride, while the remaining polysaccharides are obtained after papain treatment. The molar ratio of hexose to hexosamine is lower in the guanidine hydrochloride extracts than in the glycans extracted by papain.

In the experiments of Fig. 6, the relative proportions of F-1 and F-2 fractions in the guanidine hydrochloride and papain extracts were analyzed. Both agarose gel electrophoresis (Fig. 6A) and chromatography on Sepharose CL-4B columns (Fig. 6, B–E) indicate that the F-2 is removed mainly by guanidine hydrochloride extractions while F-1 is present in higher proportion in the papain extracts. Because of the leftward shift in the position of the F-2 peak in Fig. 6C, we analyzed the F-2 fractions from all the 4 extracts (B–E) on polyacrylamide gel; all 4 had the same two bands of 20,000 and 8,000 daltons.

The high molecular weight fraction (F-1) obtained by guanidine hydrochloride extraction (see Fig. 6, B and C) could be attributed to glycans covalently linked to protein or to molecular aggregates. However, F-1 still eluted near the void volume of the column after β-elimination (Fig. 6F), after incubation with papain (same result as in Fig. 6F, but not shown), or when the column was done under dissociative conditions (Fig. 6G), as already observed for the fraction F-1 obtained by papain extraction (see Fig. 3).

From the experiments of Fig. 6, it is difficult to decide whether the fraction F-2 extracted by guanidine hydrochloride is resistant or susceptible to hydrazinolysis, β-elimination, or incubation with papain. However, Fig. 7 shows the polyacryl-
Sulfated Glycans from Ascidians

TABLE II

Analysis of the products formed from the periodate-oxidized ascidian polysaccharides after acid hydrolysis

The results in parenthesis show the percentage amounts of hexoses and hexosamines that disappeared after periodate oxidation.

<table>
<thead>
<tr>
<th>Product</th>
<th>Total polysaccharides</th>
<th>Total polysaccharides</th>
<th>Desulfated polysaccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hexosamines</td>
<td>Glucose</td>
<td>F-1</td>
</tr>
<tr>
<td></td>
<td>8.20 (47)</td>
<td>10.17 (68)</td>
<td>3.26 (60)*</td>
</tr>
<tr>
<td></td>
<td>8.20 (47)*</td>
<td>2.36 (93)</td>
<td>2.21 (45)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.47 (89)</td>
<td>2.33 (89)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.11 (42)</td>
<td>5.65 (86)*</td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td>42.99 (11)</td>
<td>Trace</td>
</tr>
<tr>
<td></td>
<td>21.40 (56)</td>
<td>16.61 (65)</td>
<td>Trace</td>
</tr>
<tr>
<td></td>
<td>Mannose</td>
<td>1.31 (68)</td>
<td>Trace</td>
</tr>
<tr>
<td></td>
<td>0.01 (100)</td>
<td>1.52 (64)</td>
<td>Trace</td>
</tr>
<tr>
<td></td>
<td>Erythritol</td>
<td>5.98</td>
<td>Trace</td>
</tr>
<tr>
<td></td>
<td>9.8</td>
<td>7.16</td>
<td>Trace</td>
</tr>
<tr>
<td></td>
<td>Threitol</td>
<td>11.73</td>
<td>Trace</td>
</tr>
<tr>
<td></td>
<td>8.6</td>
<td>27.57</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>19.61</td>
<td>40.41</td>
</tr>
<tr>
<td></td>
<td>49.7</td>
<td>26.62</td>
<td>89.62</td>
</tr>
</tbody>
</table>

ND, not detected.

FIG. 5. Degradation of the chemically desulfated glycans from Styela plicata with α- and β-D-galactosidase.

The incubation conditions are described under "Experimental Procedures." A shows the amounts of galactose formed after incubation of the chemically desulfated polysaccharides from S. plicata with α- and β-D-galactosidase while B shows the amounts of galactose formed by the action of these galactosidases on the desulfated glycans whose molecular weights were reduced by mild acid hydrolysis. The straight arrows in B indicate the time at which another 0.1 unit of α- or β-D-galactosidase was added to the incubation mixture.

TABLE III

Analysis of the glycans extracted from the tunic of Styela plicata with guanidine hydrochloride and papain

<table>
<thead>
<tr>
<th>Extracted with</th>
<th>Protein</th>
<th>Hexosamine</th>
<th>Hexose</th>
<th>Hexose/Hexosamine</th>
<th>Extracted glycans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% dry weight of tissue</td>
<td>mol/mol</td>
<td>% of total</td>
<td></td>
</tr>
<tr>
<td>0.1 M Guanidine</td>
<td>0.954</td>
<td>0.106</td>
<td>0.336</td>
<td>3.30</td>
<td>10.1</td>
</tr>
<tr>
<td>HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 M Guanidine</td>
<td>0.950</td>
<td>0.070</td>
<td>0.267</td>
<td>4.57</td>
<td>7.7</td>
</tr>
<tr>
<td>HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 M Guanidine</td>
<td>1.960</td>
<td>0.088</td>
<td>0.457</td>
<td>6.22</td>
<td>12.4</td>
</tr>
<tr>
<td>HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0 M Guanidine</td>
<td>1.421</td>
<td>0.290</td>
<td>0.337</td>
<td>1.52</td>
<td>15.0</td>
</tr>
<tr>
<td>HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papain</td>
<td>0.225</td>
<td>2.175</td>
<td>11.58</td>
<td>54.8</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5.285</td>
<td>0.779</td>
<td>3.602</td>
<td>5.64</td>
<td>190.8</td>
</tr>
</tbody>
</table>

The sulfated polysaccharides from the tunic of S. plicata occur as three main fractions, which are markedly different in their molecular weight and chemical composition. F-1 has a very high molecular weight (Fig. 3), a high proportion of hexose (mainly galactose), and galactosamine as the predominant amino sugar, whereas F-2-A and F-2-B have molecular weights of 20,000 and 8,000 daltons, respectively, and the predominant amino sugar of these two fractions is glucosamine. Among the hexoses, F-2-A has a high proportion of glucose, while F-2-B has glucose, galactose, and mannose. The chemical analysis in Table I and the infrared spectra indicate the presence of high amounts of sulfate ester in all fractions. The experiment shown in Table II suggests the presence of a small proportion of galactose and glucose 1→4 linkages in fraction F-1, since galactose and glucose without substitution at positions 2 and 3 would produce threitol and erythritol, respectively, after periodate oxidation, borohydride reduction, and acid hydrolysis. The presence of 1→6 linkages and/or the presence of nonreducing end sugars is suggested by the formation of glycerol together with production of formic acid in the experiments of Table II. The fractions F-2-A and F-2-B produce glycerol together with the total disappearance of glucose after periodate oxidation, borohydride reduction, and amidic gel electrophoresis of F-2 before and after β-elimination or incubation with proteolytic enzyme. The results indicate that F-2 extracted by guanidine hydrochloride has two components with molecular weights of 20,000 (F-2-A) and 8,000 (F-2-B), and neither is affected by β-elimination, incubation with proteolytic enzyme, or hydrazinolysis (not shown in Fig. 7). All these results show the similarity of F-2 obtained by guanidine hydrochloride with F-2 that is extracted by papain.

DISCUSSION

The present study reports the presence of a novel class of sulfated glycans in the tunic of the ascidian S. plicata. The chemical analysis, the resistance to all the mucopolysaccharidases, and the resistance to deaminative cleavage by nitrous acid indicate that these compounds differ from the glycosaminoglycans. They are also distinct from the sulfated polysaccharides isolated from marine algae (34). They differ from the carrageenan-type polysaccharides by the absence of anhydrogalactose and from other algal-sulfated polysaccharides containing neutral sugars by the absence of fucose, xylose, and arabinose. The presence of amino sugars in the ascidian glycans also distinguishes them from the marine algal polysaccharides.

Recently, various sulfated glycoproteins have been reported in bacterial and animal tissues. Wieland et al. (35) reported a sulfated glycoprotein in the Holobacterium cell wall that contained glucose and glucuronic acid, while Slomiany and Meyer (36) isolated various sulfated glycoproteins from hog gastric mucosa that differed from one another in their proportions of fucose, galactose, and N-acetyl glucosamine. The presence in embryonic chicken liver and lung of a sulfated carbohydrate chain composed of fucose, sialic acid, and N-acetyl glucosamine and joined to protein by N-glycosidic linkages was reported by Heifetz et al. (37). However, the chemical compositions of all these sulfated glycoproteins are markedly different from the ascidian polysaccharides described in this paper.
Sulfated Glycans from Ascidians

FIG. 6. Analysis of the sulfated glycans extracted from the ascidian tunic by increasing concentration of guanidine hydrochloride. A, about 100 µg of the sulfated glycans extracted from the ascidian tunic by 0.1 M (1), 0.5 M (2), 1.0 M (3), 4.0 M (4) guanidine hydrochloride solutions and the residue of these four extractions treated with papain (5) were submitted to agarose gel electrophoresis as described in the legend to Fig. 1. B–E, the sulfated glycans extracted from the ascidian tunic by 0.1 M (B), 0.5 M (C), and 4.0 M (D) guanidine hydrochloride solutions and the sulfated glycans resistant to these extractions but removed by papain extraction (E) were applied to a Sepharose CL-4B column and eluted with 0.5 M pyridineacetate buffer, pH 6.0 (associative conditions). F, the sulfated glycans extracted by 0.1 M guanidine hydrochloride were submitted to β-elimination before the fractionation on Sepharose CL-4B. The numbers under the peaks indicate the relative amounts of fractions F-1 and F-2 in each column.

FIG. 7. Polyacrylamide gel electrophoresis of the fraction F-2 extracted from the ascidian tunic by guanidine hydrochloride solutions. A, about 50 µg of each of the following polysaccharides were submitted to polyacrylamide gel electrophoresis as described in the legend to Fig. 1: fraction F-2 extracted from the ascidian tunic by 0.1 M (1) or 4.0 M (2) guanidine hydrochloride and purified on Sepharose CL-4B (see Fig. 6); Fraction F-2 extracted by 0.1 M guanidine hydrochloride and submitted previously to β-elimination (3) or incubation with trypsin for 12 h (4). The molecular weight markers used in this experiment are the same as those described in Fig. 1, and the gel was stained with toluidine blue. B, densitometry of lanes 1, 3, and 4 of the polyacrylamide gel in A shows fractions F-2-A and F-2-B before (top) and after β-elimination (middle) or after incubation with trypsin (bottom).

Acid hydrolysis, while about 40% of the galactose is resistant to periodate oxidation. This result suggests that all the glucose and some of the galactose forms 1→6 linkages and/or that they occur at the nonreducing end, while part of the galactose is substituted at position 3.

The presence of β-linked galactose in these polysaccharides is indicated by the liberation of galactose after incubation of the desulfated polysaccharide with β-D-galactosidase while these compounds are resistant to α-D-galactosidase degradation (Fig. 5). The limited action of β-D-galactosidase may reflect the presence of amino sugars and glucose near the nonreducing end of the molecule, rather than indicating that only a small amount of the linkages are β; the resistance to α-D-galactosidase after mild acid hydrolysis of the glycan suggests that newly exposed internal linkages may not be α. The strong negative optical rotation (Table I) is another indication of β-linkages (38).

The sulfated polysaccharides can be extracted from the ascidian tunic by proteolytic enzyme or by guanidine hydrochloride solutions. F-1 is preferably extracted by proteolytic enzyme (Fig. 4) while F-2-A and F-2-B are better removed from the tunic by guanidine hydrochloride solutions (Fig. 6). From the results of these experiments and from those of Fig. 3, it could be speculated that F-1 is a high molecular weight compound that would not be extracted by guanidine hydrochloride solutions or this polysaccharide interacts strongly with other proteins of the tissue through noncovalent bonds. The experiments of Fig. 7 indicate that F-2-A and F-2-B are probably either free polysaccharide chains or linked to very small peptides.

The function of these polysaccharides in the tunic of the ascidian is a matter of speculation. Since the tunic of ascidians is an external supportive and protective skeleton (39) and
these polysaccharides occur in concentrations which resemble the great quantities of the glycosaminoglycans that are characteristic of cartilages (1–6); it may be that the ascidian polysaccharides are essential for maintaining the structural integrity of the tunic, resembling the structural function of the glycosaminoglycans in the connective tissues. Further support for this hypothesis is our recent finding that the glycosaminoglycans in the connective tissues. Further details of the collections of sulfated glycans in the tunics of other ascidian species and in the body wall of a sea cucumber (40). Although these polysaccharides were not investigated in as great detail, they resemble the polysaccharides reported in this study in chemical composition, in the concentration as per cent of dry tissue, and in the electrophoretic mobilities on agarose and polyacrylamide gels.

Acknowledgments—We would like to thank Dr. Celuta S. Alviano, Dr. Lucia Mendonça-Previato, and Dr. José O. Previato for the valuable discussions and for the facilities of the Instituto de Microbiologia, Universidade Federal do Rio de Janeiro; Dr. Lewis J. Greene (Centro Interdepartamental de Química de Proteínas, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo) for the amino acid determination; and Dr. Martha M. Sorenson for the help in the preparation of this manuscript. Thanks are also due to Inass G. Bastos for the excellent technical assistance.

REFERENCES