Structure and Anticoagulant Activity of a Fucosylated Chondroitin Sulfate from Echinoderm

SULFATED FUCOSE BRANCHES ON THE POLYSACCHARIDE ACCOUNT FOR ITS HIGH ANTICOAGULANT ACTION

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A polysaccharide isolated from the body wall of the sea cucumber Ludwigia hurea grisea has a backbone like that of mammalian chondroitin sulfate: [4-β-D-GlcA–1–3-β-D-GalNAc–1], but substituted at the 3-position of the β-D-glucuronic acid residues with sulfated α-L-fucopyranosyl branches (Vieira, R. P., Mulloy, B., and Mourão, P. A. S. (1991) J. Biol. Chem. 266, 13530–13536). Mild acid hydrolysis removes the sulfated α-L-fucose branches, and cleaved residues have been characterized by 1H NMR spectroscopy; the most abundant species is fucose 4-O-monomosulfate, but 2,4- and 3,4-di-O-sulfated residues are also present. Degradation of the remaining polysaccharide with chondroitin ABC lyase shows that the sulfated α-L-fucose residues released by mild acid hydrolysis are concentrated toward the non-reducing end of the polysaccharide chains; enzyme-resistant polysaccharide material includes the reducing terminal and carries acid-resistant 1-fucose substitution. The sulfated α-L-fucose branches confer anticoagulant activity on the polysaccharide. The specific activity of fucosylated chondroitin sulfate in the activated partial thromboplastin time assay is greater than that of a linear homopolymeric α-L-fucan with about the same level of sulfation; this activity is lost on defucosylation or desulfation but not on carboxyl-reduction of the polymer. Assays with purified reagents show that the fucosylated chondroitin sulfate can potentiate the thrombin inhibition activity of both antithrombin and heparin cofactor II.

Sulfated polysaccharides constitute a complex group of macromolecules known to possess a wide range of important biological properties. These anionic polymers are widespread in nature, occurring in a great variety of organisms. In marine algae, for example, the carrageenans and fucoids are composed mainly of sulfated galactose and fucose, respectively (1). In the animal kingdom, sulfated glycosaminoglycans abound in vertebrate tissues (2). Invertebrate species are also a rich source of sulfated polysaccharides with novel structures (3–13).

Anticoagulant and antithrombotic activities are among the most widely studied properties of sulfated polysaccharides. The anticoagulant glycosaminoglycan heparin is an important therapeutic agent used in the prophylaxis and treatment of thrombosis (14); dermatan sulfate is also an anticoagulant, although of lower potency than heparin (15–17). A chemically sulfated xylan from beechwood, pentosan polysulfate, has been available for many years as an anticoagulant polysaccharide (18–20). Sulfated fucans from brown seaweed have anticoagulant activity due to the ability to potentiate inhibition of thrombin by antithrombin or heparin cofactor II (21, 22).

Recently, we isolated novel sulfated polysaccharides from the body wall of a sea cucumber (5, 7, 9–11). We found that the main fraction has a chondroitin sulfate-like structure, containing large numbers of sulfated α-L-fucopyranose branches linked to position 3 of the β-D-glucuronic acid residues (5, 7). We now present both revision and further refinement of our previous structure.

The analogy in structure between the fucosylated chondroitin sulfate from sea cucumber, heparin, or dermatan sulfate from mammalian tissues and sulfated fucans from brown algae led us to investigate the possible anticoagulant activity of the echinoderm polysaccharide. We observed a high anticoagulant activity in the fucosylated chondroitin sulfate due to its ability to potentiate inhibition of thrombin and factor Xa by antithrombin or heparin cofactor II. Measurements of anticoagulant activities of chemically modified polymers show that the high anticoagulant activity of the sea cucumber polysaccharide can be assigned mainly to sulfated fucose branches linked to the chondroitin sulfate core.
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EXPERIMENTAL PROCEDURES

Native and Chemically Modified Polysaccharides—Fucosylated chondroitin sulfate was extracted from the body wall of the sea cucumber _Stichopus japonicus_ (5) using papain digestion and purified by procedures previously described (5, 6). Desulfation of this polysaccharide by solvolysis in dimethyl sulfoxide/methanol (9.1, v/v) at 80 °C for 6 h (23) and reduction of the hexuronic acid carbonyl groups in the polysaccharide by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-NaBH₄ (24) were performed as described previously. Yield and integrity of the polysaccharides obtained after these two procedures were the same as in our previous study (7, 11). Partial removal of sulfated fucose branches from the fucosylated chondroitin sulfate was performed by mild acid hydrolysis. In these experiments, the fucosylated chondroitin sulfate (50 mg) was dissolved in 1.0 ml of 150 mM H₂SO₄, maintained at 100 °C for 30 min, and the pH of the solution was adjusted to 7.0 with 0.3 ml of ice-cold 1.0 M NaOH. Chondroitin 4-sulfate from whale cartilage, chondroitin 6-sulfate from shark cartilage, dermatan sulfate from bovine mucosa, and heparin from porcine intestinal mucosa were from Sigma. Dermatan sulfate was treated with nitric acid (25) to remove contaminating heparin. Heparan sulfate from human aorta was extracted and purified as described previously (26). The heparin for the APTT assay was the 4th International Standard (85/502), obtained from the National Institute for Biological Standards and Control, Potters Bar, UK.

3H Labeling of the Fucosylated Chondroitin Sulfate—Alkaline-catalyzed β-elimination of the linkage region of the fucosylated chondroitin sulfate (50 mg) was performed in 1.0 ml of 0.1 M NaOH in the presence of ~2 mCi of [3H]NaBH₄ at room temperature. After 8 h non-labeled NaBH₄ was added to a final concentration of 0.1 M and the solution was maintained at room temperature for an additional 8-h period. The solution was then neutralized with acetic acid, and the products were fractionated on a Bio-Gel P-4 column (see below). Fractions were collected, assayed by metachromasia using 1,9-dimethylmethylene blue (27), and the radioactivity was counted on a scintillation counter. The fractions containing the 3H-labeled fucosylated chondroitin sulfate were pooled and lyophilized.

Analysis of the Products Formed by Digestion of the Partial Defucosylated Chondroitin Sulfate—H-Labeled fucosylated chondroitin sulfate (50 mg) was submitted to mild acid hydrolysis with acid (see above). After neutralization with NaOH, the solution was applied to a Bio-Gel P-4 column (88 × 1.5 cm) and eluted with 50 mM pyridine/acetate buffer (pH 6.0) at a flow rate of 6 ml/h. Fractions of 1.5 ml were collected and assayed by metachromasia and the absence of carbazole and metachromatic reactions. The released “sulfated fucose” was identified by positive Dubois and the absence of carbazole and metachromatic reactions. Both fractions were pooled and lyophilized.

In order to remove small amounts of unsulfated fucose (and also of inorganic sulfate) from the sulfated fucose sample, the fraction was re-applied to a Bio-Gel P-2 column (90 × 0.8 cm) and eluted with distilled water at a flow rate of 6 ml/h. Fractions of 1.0 ml were collected and assayed by the Dubois reaction (28). Fractions containing 3H-labeled fucosylated chondroitin sulfate were identified by the absence of carbazole and metachromatic reactions. Both fractions were pooled and lyophilized.

Analysis of the Products Formed by Mild Acid Hydrolysis of the Fucosylated Chondroitin Sulfate—H-Labeled fucosylated chondroitin sulfate (50 mg) was submitted to mild acid hydrolysis with acid (see above). After neutralization with NaOH, the solution was applied to a Bio-Gel P-4 column (88 × 1.5 cm) and eluted with 50 mM pyridine/acetate buffer (pH 6.0) at a flow rate of 6 ml/h. Fractions of 1.5 ml were collected and assayed by metachromasia and the absence of carbazole and metachromatic reactions. The released “sulfated fucose” was identified by positive Dubois and the absence of carbazole and metachromatic reactions. Both fractions were pooled and lyophilized.

To determine the percentage of 3H-labeled fucosylated chondroitin sulfate, the acid-resistant fragments (50 mg) was applied to a Mono Q column-FPLC (HR 5/5) from Pharmacia Biotech Inc., equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The column was developed by a linear gradient of 0–2 M NaCl in the same buffer. The flow rate of the column was 0.45 ml/min, and

![Figure 1](image.png)

**FIG. 1. Analysis of the products formed by partial acid hydrolysis of the fucosylated chondroitin sulfate.** In Panel A, the 3H-labeled fucosylated chondroitin sulfate (50 mg) was submitted to mild acid hydrolysis (see “Experimental Procedures”). After neutralization with NaOH, the solution was applied to a Bio-Gel P-4 column (88 × 15 cm) and eluted with 50 mM pyridine/acetate buffer (pH 6.0) at a flow rate of 6 ml/h (inset in Panel A). Fractions of 1.0 ml were collected and assayed by the Dubois reaction (O) and Dubois (○) reactions, for metachromasia (Δ), and counted in a liquid scintillation counter (▲). The fractions containing the acid-resistant fragments and sulfated fucose (horizontal bars) were pooled and lyophilized. The sulfated fucose was re-purified on a Bio-Gel P-2 column (90 × 0.8 cm), eluted with distilled water at a flow rate of 6 ml/h (inset in Panel A). Fractions of 1.0 ml were collected and assayed by the Dubois reaction (○). Fractions 72, 73, and 74 (horizontal bar) were pooled and lyophilized. In Panel B, the purified sulfated fucose (~15 µg), before (−) and after (+) strong acid hydrolysis and a mixture of standard sugars containing 10 µg each of galactose, mannose, fucose, and galactosamine were spotted on Whatman No. 1 paper and subjected to chromatography in isobutyric acid, 1.0 M NH₄OH (5.3, v/v), for 24 h. The products were located on the chromatogram by silver nitrate staining. In Panel C, the sulfated fucose (~15 µg) before (−) and after (+) strong acid hydrolysis and a mixture of standard sugars containing 10 µg each of glucuronic acid, fucose, and galactosamine were spotted on Whatman 3MM chromatographic paper and submitted to electrophoresis in 0.3 M pyridine/acetate buffer (pH 5.0), run for 4 h at 500 V. The electrophoresis was stained with silver nitrate.

applied to Whatman No. 1 paper, separated by descending chromatography in isobutyric acid, 1.0 M NH₄OH for 48 h and stained with silver nitrate.

Analysis of the Fucosylated Chondroitin Sulfate by Mono Q-FPLC before and after Mild Acid Hydrolysis and Incubation with Chondroitin ABC lyase—Native fucosylated chondroitin sulfate, the acid-resistant and chondroitin lyase-resistant fragments (~2.0 mg of each) or a solution containing standard hyaluronic acid (50 µg), heparan sulfate (200 µg), chondroitin sulfate (200 µg), dermatan sulfate (200 µg), and heparin (200 µg) were applied to a Mono Q column-FPLC (HR 5/5) from Pharmacia Biotech Inc., equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The column was developed by a linear gradient of 0–2 M NaCl in the same buffer. The flow rate of the column was 0.45 ml/min, and

1 The abbreviation used is: APTT, activated partial thromboplastin time.
fractions of 0.5 ml were collected and assayed by metachromasia using 1,9-dimethylmethylene blue (27) and by the carbazole (28) and Dubois (29) reactions.

Chemical Analysis—After strong acid hydrolysis (4.0 M HCl, 100 °C for 6 h) of the polysaccharide total hexosamine and sulfate were estimated by a modified Elson-Morgan reaction (30) and by the BaCl2-gelatin method (31), respectively. Standard curves for hexosamine and sulfate were constructed from glucosamine and Na2SO4. The hexuronic acid content was estimated by the carbazole reaction (28). The percentages of fucose and galactosamine in the acid hydrolysates were estimated by paper chromatography in 1-butanol/pyridine/water (3:2:1, v/v) for 48 h or in isobutyric acid, 1.0 M NH4OH (5:3, v/v) for 24 h and by gas-liquid chromatography of the corresponding alditol acetates (32).

NMR Methods—1H spectra were recorded at 500 MHz and 13C spectra at 125 MHz using a Varian Unity 500 spectrometer in the FT mode. Polysaccharide samples were converted to their sodium salts by passage through a column 1 × 10 cm of Dowex 50 × 8 Na+ form. About 15 mg of each polysaccharide sample was dissolved in approximately 0.7 ml of 99.8% D2O (Goss Scientific, Ingatestone, United Kingdom) for NMR spectroscopy. The polysaccharide spectra were recorded at 60 °C, with suppression of the HOD signal by presaturation. 13C spectra were recorded with full proton decoupling using the WALTZ sequence (33). Two-dimensional double-quantum filtered COSY (34), TOCSY (35), and NOESY (36) spectra were recorded in the phase-sensitive mode using the pulse programs supplied by the manufacturer. TOCSY spectra were run with a spin-lock field of about 10 kHz and a mixing time of 120 ms; the NOESY spectrum was run with a mixing time of 100 ms. All chemical shifts are relative to internal or external trimethylsilylpropionic acid.

Anticoagulant Action Measured by APTT (Activated Partial Thromboplastin Time)—APTT clotting assays were carried out as described previously (37, 38). Normal human plasma (90 μl) was incubated with 10 μl of a solution of polysaccharide (0–100 μg) and 100 μl of kaolin + bovine phospholipid reagent (National Institute for Biological Stand-
Anticoagulant Action of a Fucosylated Chondroitin Sulfate

TABLE I

| Sugar                        | Anomeric form | H-1  | H-2  | H-3  | H-4  | H-5  | H-6  | Chemical shifts ppm |
|------------------------------|---------------|------|------|------|------|------|------|---------------------|
| Standard unsulfated fucose   | α             | 5.20 | 3.76 | 3.86 | 3.81 | 4.20 | 1.21 |
| Fucose 4-sulfate (major)     | α             | 5.22 | 3.81 | 3.97 | 4.61 | 4.31 | 1.26 |
| Fucose 2,4-disulfate         | α             | 5.49 | 4.41 | 4.13 | 4.67 | 4.34 | 1.29 |
| Fucose 3,4-disulfate         | α             | 5.28 | 3.94 | 4.60 | 1.89 |
| Fucose 4-sulfate (minor)     | α             | 5.26 | 3.92 | 4.13 | 4.54 |
| Standard unsulfated fucose   | β             | 4.55 | 3.46 | 3.63 | 3.75 | 3.80 | 1.25 |
| Fucose 4-sulfate             | β             | 4.60 | 3.48 | 3.75 | 4.54 | 3.95 | 1.31 |
| Fucose 2,4-disulfate         | β             | 4.73 | 3.92 | 4.21 | 4.61 | 3.93 | 1.28 |
| Fucose 3,4-disulfate         | β             | 4.68 | 3.60 | 4.37 | 4.82 | 3.92 | 1.28 |
| Fucose 3-sulfate             | β             | 4.65 | 3.64 | 4.31 | 4.09 | 3.84 | 1.26 |

- The 500 MHz 1H spectrum was recorded at 55°C in D2O. Chemical shifts are referenced to internal trimethylsilylpropionic acid at 0 ppm. Values in boldface indicate positions bearing sulfate esters.

TABLE II

| Poly saccharide          | GlcUA | GalNH | Fuc | Sulfate/total sugar |
|--------------------------|-------|-------|-----|--------------------|
| Native fucosylated chondroitin sulfate | 1.00 | 0.92 | 1.23 | 0.70 |
| Acid-resistant fragments | 1.00 | 0.87 | 0.27 | 0.62 |
| Chondroitin lyase-resistant fragments | 1.00 | 0.93 | 0.41 | ND |
| Fucosylated chondroitin sulfate after desulfation | 1.00 | 0.97 | 0.64 | 0.05 |

- ND, not determined.
- Desulfation of the polysaccharide was by solvolysis in dimethyl sulfoxidemethyl (9:1, ν/ν) at 80°C for 6h.

RESULTS AND DISCUSSION

Fucose 4-O-Monosulfate, Fucose 2,4-O-Disulfate, and Fucose 3,4-O-Disulfate Are the Major Species Released by Partial Acid Hydrolysis of the Fucosylated Chondroitin Sulfate—The fucosylated chondroitin sulfate from the body wall of sea cucumber has a chondroitin sulfate-like core, containing side chains of sulfated α-L-fucose linked at the C-3 position of the β-D-glucuronic acid. The intact polysaccharide is totally resistant to chondroitin lyase digestion (5, 7).

Since fucose forms a glycosidic linkage that is more sensitive to acid than that formed by glucuronic acid or by hexosamine (41), we attempted to defucosylate the polysaccharide using partial hydrolysis with acid. Indeed, this chemical treatment releases sulfated fucose and leaves “acid resistant-fragments,” eluted at Vv and Vc of Bio-Gel P-4 column, respectively (Fig. 1A). The 1H radioactivity (closed triangles in Fig. 1A) introduced to the labeling terminal of the polysaccharide still eluted at the Vv of the column.

Sulfated fucose was further purified on a Bio-Gel P-2 column (inset to Fig. 1A), and on paper chromatography shows a major component (Fig. 1B) which migrated as glucuronic acid standard on paper electrophoresis at pH 5.0 (Fig. 1C). Upon strong acid hydrolysis it releases exclusively fucose (Fig. 1, B and C). Another component, which migrated as disulfated fucose, was also observed on paper electrophoresis (Fig. 1C). Overall, these experiments indicate that the major products released from the fucosylated chondroitin sulfate by partial acid hydrolysis are fucose monosulfate and fucose disulfate.

Characterization of Released Sulfated Fucose by 1H NMR—The 1H spectrum of fucose released from the fucosylated chondroitin sulfate by mild acid treatment is shown in Fig. 2.

Eight spin systems consistent with assignment to fucose residues could be identified using the TOCSY and DQ COSY 1H spectra of the released fucose, and a further five minor anomeric doublets were visible which may also come from α-fucose. The DQ COSY and TOCSY spectra gave connectivities for H1 through to H4, and H5 to H6. The coupling constant between H4 and H5 is small for fucose residues, so H4-H5 cross-peaks cannot be seen, but the connectivity can be established by cross-peaks in the NOESY spectrum resulting from the close spatial proximity of H4 and H5. Comparison of the chemical
be seen that the disaccharide mixture obtained from partially defucosylated chondroitin sulfate contains a higher proportion of saturated disaccharides, originating from units at the non-reducing terminal, than disaccharides obtained from standard glycosaminoglycans. The relative proportion of saturated to unsaturated disaccharides is 15:85, as measured by quantitative densitometry of a silver nitrate-stained chromatogram using a Quick Scan densitometer (Helena Laboratories, Beaumont, TX). See also Ref. 7 for the characterization of the disaccharides obtained from defucosylated chondroitin sulfate.

Fig. 3. Analysis of the products formed by digestion of the partially defucosylated chondroitin sulfate with chondroitin ABC lyase. Panel A, the acid resistant-fragments obtained in the experiment of Fig. 1A (~20 mg) was incubated with 1 unit of chondroitin ABC lyase (see "Experimental Procedures"). After incubation at 37 °C for 12 h, the reaction mixture was applied to a Bio-Gel P-4 column and chromatographed as described in the legend of Fig. 1. The fractions containing the chondroitin lyase-resistant fragments and the "disaccharides" (horizontal bars) were pooled and lyophilized. In Panel B, the native fucosylated chondroitin sulfate, the acid-resistant fragments and the chondroitin lyase-resistant fragments (~20 μg of each) were submitted to 6% polyacrylamide gel electrophoresis in 0.02 M sodium barbitral buffer (pH 8.6). The gels were run on a Bio-Rad vertical mini-gel electrophoresis apparatus at 100 V for about 30 min. After electrophoresis, the gels were stained with 0.1% toluidine blue in 1% acetic acid solution. The molecular mass markers were: high molecular weight dextran sulfate (DexSO4) (70–100 kDa), chondroitin 6-sulfate from shark cartilage (C-6-S) (average molecular mass = 60 kDa), chondroitin 4-sulfate from whale cartilage (C-4-S) (average molecular mass = 20 kDa), and low molecular weight dextran sulfate (average molecular mass = 8 kDa). In Panel C, the disaccharide fraction from Panel A and standard disaccharides obtained by incubation of chondroitin 4/6-sulfate (CS) or chemically desulfated chondroitin sulfate (COS) with chondroitin ABC lyase (~100 μg of each) were applied to Whatman No. 1 paper, separated by descending chromatography in isobutyric acid, 1.0 M NH4OH for 48 h and stained with silver nitrate. The abbreviations for the disaccharides are the same as in our previous publication (7). It can shifts for each of these fucose residues with shifts for standard, unsulfated fucose (Table I) showed strong downfield shifts of some signals consistent with sulfation at those positions, and less strong downfield shifts attributable to sulfation at the adjoining position. These signals are marked in bold type in Table I.

Three α- and β-fucose systems correspond: those for fucose 4-O-mono­sulfate, fucose 2,4-O-disulfate, and fucose 3,4-di­sulfate. Approximate integration of the anomeric signals gives the proportions of these three as 49, 20, and 17% of the sample, respectively. A further α-fucose 4-O-mono­sulfate makes up at least 4% of the sample, and a β-fucose 3-O-mono­sulfate at least 6%. It was not possible to identify the partner anomeric forms of these last two species; either they are the non-reducing residues of disaccharides or the anomeric equilibrium strongly favors the form seen over the form not seen.

Fig. 4. Analysis of standard glycosaminoglycans and of defucosylated chondroitin sulfate before and after partial acid hydrolysis and incubation with chondroitin ABC lyase by Mono Q-FPLC. In Panel A a solution (1 ml) containing standard hyaluronic acid (50 μg, HA), heparan sulfate (200 μg, HS), chondroitin sulfate (200 μg, CS), dermatan sulfate (200 μg, DS), and heparin (200 μg) was applied to a Mono Q-FPLC column (HR 515) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The column was developed by a linear gradient of 0–2.0 M NaCl in the same buffer. The flow rate of the column was 0.45 ml/min, and fractions of 0.5 ml were collected and assayed by metachromasia (△), by the carbazole (○) and Dubois (●) reactions, and for NaCl concentration (—–). In Panels B–D, the native fucosylated chondroitin sulfate, the acid-resistant fragments (see Fig. 1A) and the chondroitin lyase-resistant fragments (see Fig. 3A) (~2 mg of each) were applied to a Mono Q-FPLC, and the column was developed as described under A. In Panel B, after the linear gradient with NaCl, the column was washed with 10 ml of 3 M NaCl solution (see arrow). The numbers in the panels indicate the average concentration of NaCl necessary to elute the various sulfated polysaccharides from the Mono Q-FPLC column.
The Non-reducing Portion of the Acid-resistant Fragments Are Cleaved by Chondroitin Lyase—The acid-resistant fragments obtained after partial acid hydrolysis of the fucosylated chondroitin sulfate still contain fucose (open circles in Fig. 1A and C). Thus, this chemical treatment produces only a partially defucosylated polysaccharide.

If the sulfated \( \mathbf{\alpha} \)-fucose branches released by partial acid hydrolysis were distributed randomly through the chondroitin sulfate core, we would expect that chondroitin ABC lyase digestion of the acid-resistant fragments would produce a wide variety of oligosaccharides with different molecular weights. Instead, mainly disaccharides and small amounts of tetrasaccharides were released, as well as a proportion of polymeric chondroitin lyase-resistant material (Fig. 3).\(^2\) Of the disaccharides produced, 15% are saturated and therefore originated from the non-reducing ends of polysaccharide molecules (Fig. 3, A and C). The average molecular mass of the fucosylated chondroitin sulfate decreases from \( \approx 30 \) kDa in the native polysaccharide to \( \approx 15 \) kDa after partial acid hydrolysis and to \( \approx 10 \) kDa after subsequent incubation with chondroitin ABC lyase (Fig. 3B). In addition, the \( ^{3\text{H}} \) label at the reducing terminal of the polysaccharide (closed triangles in Fig. 3A) is found in the 10-kDa chondroitin lyase-resistant fragments.

Our interpretation of these experiments is that sulfated \( \mathbf{\alpha} \)-fucose branches susceptible to release by mild acid treatment are located as a cluster at the non-reducing terminal of the polysaccharide.\(^3\)

Overall, the combination of partial acid hydrolysis and incubation with chondroitin lyase allows a sequential degradation of the fucosylated chondroitin sulfate. The fucose:glucuronic acid molar ratio decreases in the polysaccharide after partial acid hydrolysis and increases again after digestion with chondroitin ABC lyase (Table II).

Analysis of the fucosylated chondroitin sulfate by anion exchange chromatography on a Mono Q-FPLC column (Fig. 4) shows a homogeneous compound and confirms the high negative charge density of this polysaccharide. Thus, the sea cucumber chondroitin sulfate (Fig. 4B) was eluted from the column at a higher NaCl concentration than mammalian glycosaminoglycans (Fig. 4A). After sequential degradation by partial acid hydrolysis (Fig. 4C) and incubation with chondroitin lyase (Fig. 4D), the resistant fragments showed increasingly wider chromatographic fractions and eluted at lower NaCl concentrations than the native polysaccharide.



\section*{Study of the Sequential Degradation of the Fucosylated Chondroitin Sulfate Using $^1H$ NMR Spectra—The $^1H$ NMR spectra of the fucosylated chondroitin sulfate, before and after partial acid hydrolysis and degradation with chondroitin ABC lyase, are shown in Fig. 5.}

The two signals at 3.39 and 3.59 ppm, attributable to H-2 and H-3, respectively, of non-substituted glucuronic acid residues (42, 43) are almost absent in the \( ^{1\text{H}} \) NMR spectrum of the fucosylated chondroitin sulfate (Fig. 5A). After mild acid hydrolysis, which partially releases the sulfated fucose branches, the intensity of these two signals increases markedly (Fig. 5B),

\begin{table}[h!]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
& H-1 & H-2 & H-3 & H-4 & H-5 & H-6 \\
\hline
Major & 5.22 & 3.96 & 4.13 & ND\(^a\) & 4.73 & 1.30 \\
Minor & 5.44 & 3.90 & 4.13 & ND & 4.88 & 1.26 \\
\hline
\end{tabular}
\caption{$^1H$ chemical shifts of fucose residues in the chondroitin lyase-resistant fragments obtained from the sea cucumber chondroitin sulfate. $^1H$ spectrum was recorded at 500 MHz, 60 °C in D$_2$O. The chemical shifts are referenced to internal TSP at 0 ppm.}
\end{table}
and decreases after incubation with chondroitin lyase (Fig. 5C).

Some L-fucose residues remain after partial acid hydrolysis of the sea cucumber chondroitin sulfate (Fig. 5B), and are concentrated in the polymeric remainder after chondroitin lyase degradation (Fig. 5C). A signal at 5.22 ppm in the spectrum of these preparations is particularly noticeable, but there are several possible fucose anomeric signals between 5.1 and 5.7 ppm. The TOCSY spectrum (not shown) displays cross-peaks for two of these signals, the signal at 5.22 ppm and a further less intense signal at 5.44 ppm. Two H5-H6 cross-peaks can also be identified in the TOCSY spectrum, one more intense than the other. The chemical shifts for fucose residues which can be inferred from these cross-peaks may be tabulated as in Table III. There is no evidence other than relative intensity to connect the spin system from H1 with the H5,H6 pairs.

Comparison of these chemical shifts with those for the acid-liberated fucose residues and the standard fucose listed in Table I indicates that the remaining fucose residues after partial acid hydrolysis and chondroitin lyase treatment are unlikely to be sulfated at the 2 or 3 positions, but we have no evidence as to the state of sulfation at the 4-position.

Comparison of the various 1H NMR spectra in Fig. 5 shows clearly that the fucose residues removed by partial acid hydrolysis are distinguished from those in the resistant polysaccharide. Thus, the signal at 5.69 ppm consistent with the anomic proton of sulfated α-fucose is intense in the fucosylated chondroitin sulfate but almost absent in the acid-resistant and chondroitin lyase-resistant fragments. In contrast, the signal at 5.22 ppm remains in the polymer after both treatments.

This proposition is also supported by the time course experiment of partial acid hydrolysis of the fucosylated chondroitin sulfate at 60 °C, followed by 1H NMR spectroscopy (Fig. 6). The integrals of the signal at ~5.60 ppm decrease (Fig. 6C) concomitantly with an increase in the integrals of the signal at ~3.39 ppm (Fig. 6D). These two signals are ascribed to anomic protons of α-fucose residues and to H-2 of unsubstituted β-D-glucuronic acid in the chondroitin sulfate (42, 43), respectively. However, the integral of the peak at ~5.22 ppm (Fig. 6E) also attributed to anomic protons of α-fucose is only slightly affected during the time course experiment of acid hydrolysis. This peak is slightly modified during the partial acid hydrolysis (Fig. 6A), possibly as a consequence of the other chemical modifications that occur within the polysaccharide. Therefore, we believe that not all L-fucose residues in the fucosylated chondroitin sulfate are equally susceptible to removal by mild acid hydrolysis; the resistant fucose residues are in some way structurally distinct from those removed.

13C NMR Spectra—The 13C-spectrum of the partially defucosylated chondroitin sulfate from sea cucumber (Fig. 7B) resembles for the most part that of standard chondroitin 4- or 6-sulfate (42, 43). Signals attributable to fucose residues in the spectrum of the original fucosylated chondroitin sulfate (Fig. 7A) are much reduced after mild acid treatment (for example, the fucose CH2 at 18.6 ppm). The spectrum of chondroitin backbone residues is also simplified (for example, the C2 signal from N-acetyl-β-D-galactosamine, split into two separate reso-
Summary of the Structural Features of the Fucosylated Chondroitin Sulfate—Fig. 8 shows our proposition for the structure of the major components found in the fucosylated chondroitin sulfate from sea cucumber. The results presented in this study and in our previous publications (5, 7) suggest a highly heterogeneous polysaccharide. But, some structural features are now very clear.

Most of the \( \beta \)-d-glucuronic acid units from the chondroitin sulfate core are substituted at the O-3 position. This conclusion is based on the near absence of signals at 3.59 and 3.39 ppm in the \( ^1H \) NMR spectrum (Fig. 5A), which correspond to H-2 and H-3, respectively of non-substituted glucuronic acid residues (42, 43) and in the formation of 2,6-di-O-methylglucitol after methylation of the carboxyl-reduced chondroitin sulfate from sea cucumber (7).

Our earlier conclusion that only half of the \( \beta \)-d-glucuronic acid residues would be fucosylated (5, 7) was based on the formation of approximately equimolar proportions of 2,3,6-tri-O-methyl- and 2,6-di-O-methylglucitol after methylation of the desulfated and carboxyl-reduced polysaccharide. However, sulfated fucose is highly sensitive to chemical treatment. The desulfation reaction, which requires heating of the polysaccharide solution in dimethyl sulfoxide/methanol (9:1, v/v) at 80°C for several hours, partially defucosylates the molecule (Table II). In addition, we observed that it is difficult to obtain reliable proportions of methylated derivatives from polysaccharides rich in sulfated fucose residues (9, 10). The current study uses less severe conditions to approach the structure of the fucosylated chondroitin sulfate.

We have also previously suggested the presence of a high proportion of sulfate esterification at position O-3 of the \( \beta \)-d-glucuronic acid residues (7). This suggestion was based on a mistaken assumption that fucose was not removed during the desulfation reaction by solvolysis in dimethyl sulfoxide. We have now shown that fucose branches are in fact partially removed from the polysaccharide during this reaction (Table II). Therefore, high amounts of 3-sulfo-\( \beta \)-d-glucuronosyl residues do not occur in the sea cucumber chondroitin sulfate. However, the immunoreaction of this polysaccharide with anti-Leu-7 monoclonal antibody (7), which specifically recognizes 3-sulfoglucuronic acid residues, suggests that these residues do occur in the sea cucumber chondroitin sulfate, although in a smaller proportion of disaccharide units than was previously proposed (7). It may be that these residues are located in the chondroitin lyase-resistant region, which is apparently a more heterogeneous portion of the molecule.

Mild acid hydrolysis produces a partially defucosylated chondroitin sulfate, releasing a mixture of mono- and disulfated fucose. The non-reducing portion of the partially defucosylated polysaccharide is totally degraded by chondroitin AC or ABC lyase (Fig. 3). These experiments suggest a cluster of sulfated \( \alpha \)-L-fucose branches susceptible to release by mild acid treatment at the non-reducing portion of the polysaccharide (Fig. 8).

The fucose branches which resist mild acid hydrolysis are in some way structurally distinct, as suggested by the \( ^1H \) NMR spectra (Figs. 5 and 6). These residues are not sulfated at positions 2 and 3 (Table III), but 4-O-sulfation and the presence of disaccharides formed by fucosyl residues cannot be excluded. They are clustered toward the reducing end of the polysaccharide. The precise distinction between acid-releasable and non-releasable fucose branches was not determined by the methods used.

\(^4\) Apparently L-fucose residues removed during the desulfation reaction are evenly distributed along the polysaccharide since no disaccharide is produced by chondroitin lyase digestion of the desulfated polymer (7), in contrast with results from digestion of the partially defucosylated material produced by mild acid hydrolysis (Fig. 3).
Anticoagulant Action of a Fucosylated Chondroitin Sulfate

The APTT assays indicate a high degree of anticoagulant activity in the fucosylated chondroitin sulfate (a in Table IV). Comparison between native and chemically modified (desulfated, carboxyl-reduced, and partial defucosylated) polysaccharides suggests that sulfated fucose branches, which are released in the course of mild acid hydrolysis, are responsible for the high anticoagulant activity of the fucosylated chondroitin sulfate. This higher anticoagulant activity is not a specific property of polymers composed of sulfated fucose units, since a sulfated \( \alpha-L \)-fucan from sea urchin has a low anticoagulant activity (b in Table IV) in spite of its high sulfate/fucose molar ratio (9). This higher activity also cannot be attributed exclusively to the higher anionic nature of the fucosylated chondroitin sulfate, since the partially defucosylated polysaccharide has no discernible anticoagulant activity (a in Table IV) in spite of its elution from a Mono Q-FPLC column at a higher NaCl concentration than heparin (Fig. 4, A and C). Carboxyl reduction of the sea cucumber chondroitin sulfate does not affect its anticoagulant action.5

Fucosylated Chondroitin Sulfate Accelerates Thrombin Inhibition by Antithrombin and Heparin Cofactor II—Native and carboxyl-reduced fucosylated chondroitin sulfates have inhibitory effect on thrombin amidolytic activity of normal human plasma (Fig. 9A), whereas the desulfated and partially defucosylated polysaccharides have no effect. These results and the APTT assays (a in Table IV) indicate the requirement of sulfated fucose branches for the anticoagulant activity of the fucosylated chondroitin sulfate. Fucosylated chondroitin sulfate is more effective than mammalian dermatan sulfate on inhibition of thrombin activity of normal human plasma (Fig. 9B).

TABLE IV
Anticoagulant properties of fucosylated chondroitin sulfate, sulfated fucan, and vertebrate glycosaminoglycans

| Source  | Polysaccharide                          | Chemical modification             | APTT\(^{a}\) |
|---------|-----------------------------------------|-----------------------------------|-------------|
| Invertebrates | a, Fucosylated chondroitin sulfate from sea cucumber | Native                           | 40          |
|         |                                          | Partially defucosylated              | <1          |
|         |                                          | Desulfated                          | <1          |
|         |                                          | Carboxyl-reduced                     | 39          |
|         | b, Sulfated fucan from sea urchin (see Ref. 9) |                                  | 8           |
|         | c, Dermatan sulfate from porcine mucosa |                                  | 4           |
| Vertebrates | d, Chondroitin 6-sulfate from shark    |                                  | <1          |
|         | e, Unfractionated heparin\(^{b}\)      |                                  | 193         |
|         | f, Low molecular weight heparin\(^{b}\) |                                  | 30          |

\(^{a}\) The clotting times were recorded as described under “Experimental Procedures.” The activity is expressed as international units/mg using a parallel standard curve based on the International Heparin Standard (193 units/mg).

\(^{b}\) International standards.

used in this study.

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5 Preliminary experiments suggest that the fucosylated chondroitin sulfate has no effect on the bleeding time when the scarified rat tail was placed in a polysaccharide solution up to concentration of 1.0 mg/ml.
Heparin and some other sulfated polysaccharides have anticoagulant action mediated mainly by plasma protease inhibitors (14, 39, 40, 44, 45). Thus, heparin inhibits thrombin, factor Xa, and other coagulation enzymes in the presence of antithrombin. Dermatan sulfate and heparin have an additional inhibitory effect on coagulation through heparin cofactor II (46).

In order to trace a parallel between the anticoagulant actions of mammalian glycosaminoglycans and that of the fucosylated chondroitin sulfate we compared the influence of these sulfated polysaccharides on thrombin and factor Xa inactivation by antithrombin and heparin cofactor II.

Native and carboxyl-reduced fucosylated chondroitin sulfate inhibits the thrombin amidolytic activity in the presence of antithrombin (Fig. 10A), but higher concentrations are required to obtain the same effect as with heparin (Fig. 10B). Mammalian dermatan sulfate has no effect on this assay, as expected.

The effect of the sea cucumber chondroitin sulfate is essentially the same if factor Xa instead of thrombin is the target protein for antithrombin inactivation (Fig. 11A) although slight differences are observed between the native and the carboxyl-reduced polysaccharide (Fig. 11B). In addition, a marked difference is observed in the concentration of fucosylated chondroitin sulfate for thrombin and factor Xa inhibition in the presence of antithrombin. The IC_{50} for fucosylated chondroitin sulfate inhibition of thrombin and factor Xa are ~10 and ~500 times greater when compared with IC_{50} for heparin inhibition, respectively (see Figs. 10B and 11B).

Finally, the sea cucumber chondroitin sulfate also inactivates thrombin in the presence of heparin cofactor II, and again, carboxyl reduction of the polysaccharide does not abolish the inhibitory effect (Fig. 12A). In this case, the inhibitory effect occurs in approximately the same range of concentrations required for mammalian dermatan sulfate or heparin and slight differences were observed between the IC_{50} for native and the carboxyl-reduced polysaccharide (Fig. 12B).

The sulfated fucose branches are apparently essential for the anticoagulant action of fucosylated chondroitin sulfate (α in Table IV, Figs. 9A and 12B) and thus these branches could constitute the structural requirement for the binding of the polysaccharide to heparin cofactor II and antithrombin. Nevertheless the IC_{50} for fucosylated chondroitin sulfate inactivation of heparin cofactor II was unchanged in the presence of 100 μg/ml sulfated fucose released by partial acid hydrolysis of the sea cucumber chondroitin sulfate (Fig. 13). In addition, a sulfated α-L-fucan from sea urchin, composed of fucose units sulfated at O-2 and/or O-4 positions (9) has a low anticoagulant activity (β in Table IV). Therefore we believe that the specific spatial array of the sulfated fucose branches in the fucosylated chondroitin sulfate is essential for its anticoagulant action. There are few compounds which provide a suitable comparison for the anticoagulant activity of the fucosylated chondroitin sulfate from the sea cucumber L. grisea. An anticoagu-
A fucosylated chondroitin sulfate extracted from the sea cucumber body wall exhibits a potent anticoagulant action due to its ability to potentiate inhibition of thrombin by both heparin cofactor II and antithrombin. Comparison between native and chemically modified (desulfated, partially defucosylated, or carboxyl reduced) polysaccharides suggests that the sulfated fucose branches are responsible for the high anticoagulant activity of the fucosylated chondroitin sulfate. This activity is mediated mainly through heparin cofactor II, but the fucosylated chondroitin sulfate can also potentiate antithrombin; this pattern of anticoagulant activity is similar to that described for some algal fucoidans (22, 50), but is in contrast to that of other fucoidan preparations (21, 51) and of dermatan sulfate (46) which act only through heparin cofactor II. The potent anticoagulant action of the fucosylated chondroitin sulfate and the possible absence of bleeding side effect make this polysaccharide a promising molecule for testing in experimental thrombosis.

**CONCLUSION**

A fucosylated chondroitin sulfate extracted from the sea cucumber body wall exhibits a potent anticoagulant action due to its ability to potentiate inhibition of thrombin by both heparin cofactor II and antithrombin. Comparison between native and chemically modified (desulfated, partially defucosylated, or carboxyl reduced) polysaccharides suggests that the sulfated fucose branches are responsible for the high anticoagulant activity of the fucosylated chondroitin sulfate. This activity is mediated mainly through heparin cofactor II, but the fucosylated chondroitin sulfate can also potentiate antithrombin; this pattern of anticoagulant activity is similar to that described for some algal fucoidans (22, 50), but is in contrast to that of other fucoidan preparations (21, 51) and of dermatan sulfate (46) which act only through heparin cofactor II. The potent anticoagulant action of the fucosylated chondroitin sulfate and the possible absence of bleeding side effect make this polysaccharide a promising molecule for testing in experimental thrombosis.

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REFERENCES

1. Painter, T. J. (1983) in *The Polysaccharides* (Aspinall, G. O., ed) Vol. 2, pp. 195–265, Academic Press, New York
2. Mathews, M. B. (1975) *Connective Tissue, Macromolecular Structure and Evolution*, pp. 93–206, Springer-Verlag, Berlin
3. Albano, R. M., and Mourão, P. A. S. (1986) *J. Biol. Chem.* 261, 758–765
4. Mourão, P. A. S., and Perlin, A. R. (1987) *Eur. J. Biochem.* 166, 431–436
5. Vieira, R. P., and Mourão, P. A. S. (1988) *J. Biol. Chem.* 263, 18176–18183
6. Pavão, M. S. G., Albano, R. M., Lawson, A. M., and Mourão, P. A. S. (1989) *J. Biol. Chem.* 264, 9972–9979
7. Vieira, R. P., Mulloy, B., and Mourão, P. A. S. (1991) *J. Biol. Chem.* 266, 15530–15536
8. Santos, J. A., Mulloy, B., and Mourão, P. A. S. (1992) *Thromb. Res.* 66, 669–677
9. Mulloy, B., Ribeiro, A.-C., Alves, A.-P., Vieira, R. P., and Mourão, P. A. S. (1994) *J. Biol. Chem.* 269, 22113–22123
10. Ribeiro, A. C., Vieira, R. P., Mourão, P. A. S., and Mulloy, B. (1994) *Carbohydr. Res.* 325, 225–240
11. Ruggiero, J., Vieira, R. P., and Mourão, P. A. S. (1994) *Carbohydr. Res.* 256, 275–287
12. Pavão, M. S. G., Rodrigues, M. A., and Mourão, P. A. S. (1994) *Biochim. Biophys. Acta* 1199, 229–237
13. Pavão, M. S. G., Mourão, P. A. S., Mulloy, B., and Tollesøen, D. M. (1995) *J. Biol. Chem.* 270, 31027–31036
14. Kakkar, V. V., and Hedges, A. R. (1989) in *Heparin*, pp. 13530–13536, Academic Press, New York
15. Ribeiro, A. C., Vieira, R. P., Mourão, P. A. S., and Mulloy, B. (1994) *Arterioscler. Thromb.* 14, 115–124
16. Farndale, R. W., Buttle, D. J., and Barret, A. J. (1986) *Biochim. Biophys. Acta* 883, 173–177
17. Bitter, T., and Muir, H. M. (1962) *Anal. Biochem.* 4, 330–334
18. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) *Anal. Chem.* 28, 350–356
19. Ruggiero, J., Vieira, R. P., and Mourão, P. A. S. (1994) *J. Biol. Chem.* 269, 22113–22123
20. Colliec, S., Fisher, A. M., Tapon-Bretaudiere, J., Boisson, C., Durant, P., and Dupuy, C. (1991) *Thromb. Res.* 64, 143–154
21. Nagasawa, K., Inoue, Y., and Kamata, T. (1977) *Carbohydr. Res.* 58, 47–55
22. Taylor, R. L., Shively, J. E., and Conrad, H. E. (1976) *Methods Carbohydr. Chem.* 7, 149–151
23. Shively, J. E., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
24. Scully, L. E. M., and Mourão, P. A. S. (1994) *Arterioscler. Thromb.* 14, 115–124
25. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
26. Donati, M. B. (1987) *J. Biol. Chem.* 262, 18176–18183
27. Ruggiero, J., Vieira, R. P., and Mourão, P. A. S. (1994) *Arterioscler. Thromb.* 14, 115–124
28. Pavao, M. S. G., Mourao, P. A. S., and Tollefsen, D. M. (1995) *Biochim. Biophys. Acta* 1280–1285
29. Ruggiero, J., Vieira, R. P., and Mourão, P. A. S. (1994) *J. Biol. Chem.* 269, 22113–22123
30. Colliec, S., Fisher, A. M., Tapon-Bretaudiere, J., Boisson, C., Durant, P., and Dupuy, C. (1991) *Thromb. Res.* 64, 143–154
31. Nagasawa, K., Inoue, Y., and Kamata, T. (1977) *Carbohydr. Res.* 58, 47–55
32. Taylor, R. L., Shively, J. E., and Conrad, H. E. (1976) *Methods Carbohydr. Chem.* 7, 149–151
33. Shively, J. E., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
34. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
35. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
36. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
37. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
38. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
39. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
40. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
41. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
42. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
43. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
44. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
45. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
46. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
47. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
48. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
49. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942
50. Carbohydr., V. V., and Conrad, H. E. (1976) *Biochemistry* 15, 3932–3942