

Molecular Profile and Mapping of Dermatan Sulfates from Different Origins

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Glycosaminoglycans (GAGs) are acidic polysaccharides that exhibit a variety of different biologic activities.¹ For example, heparin has been used clinically for over 50 years as an anticoagulant. In addition to heparin, GAGs that are structurally related, such as heparan sulfate, chondroitin sulfate, and dermatan sulfate, also have a variety of important biologic activities.^{2,3}

Dermatan sulfate is a complex, polydisperse, sulfated polysaccharide that may contain other GAG contaminants. Investigations of its anticoagulant/anti-thrombotic activities have indicated that dermatan sulfate primarily catalyzes the heparin cofactor II (HC II) inhibition of thrombin (Factor IIa). However, the possibility that this activity is the result of trace contamination of dermatan sulfate by heparin has not been conclusively ruled out (see other articles in this issue of *Seminars*).

The basic structural repeating unit of dermatan sulfate is a disaccharide sequence $\rightarrow 3)\text{-}\beta\text{-D-Gal-NAc4S-(1}\rightarrow 4)\text{-}\alpha\text{-L-IdoA(1}\rightarrow$. Structural variation in this disaccharide repeating unit is also observed and is responsible for the microheterogeneity of dermatan sulfate and much of its structural complexity.⁴⁻⁷ Specific structural features within this polymer may be responsible for many of its biologic activities.⁸ Therefore a method is needed for analyzing dermatan sulfate. This method should be useful for determining the presence of contaminating GAGs in a dermatan sulfate as well as giving information on its average molecular weight and polydispersity and,

finally, the type, content, and distribution of sequence variability within the polymer.

We have developed an oligosaccharide mapping method^{9,10} that uses gradient polyacrylamide gel electrophoresis (PAGE) and strong anion exchange (SAX)-high performance liquid chromatography (HPLC). Using this system we have studied depolymerized mixtures of dermatan sulfate sample obtained from different species and tissue origins. Depolymerization of dermatan sulfate by a bacterial lyase-type enzyme, chondroitinase ABC, yields oligosaccharide products containing unsaturated nonreducing terminal sugar residues¹¹ for analysis. Oligosaccharide mapping is then used to examine a variety of dermatan sulfates. High molecular weight and low molecular weight dermatan sulfates as well as charge fractionated dermatan sulfate, having substantially different HC II mediated anti-IIa activities were examined. Gradient PAGE, a method that was developed in our laboratory for general sequencing and analysis of glycosaminoglycans,^{9,10} was used to study the dermatan sulfate oligosaccharide mixtures prepared by chondroitinase ABC treatment. Commercially available disaccharide standards have been examined by SAX-HPLC and their retention times compared with the oligosaccharide mixture obtained from chondroitinase ABC-treated dermatan sulfate. On the basis of these studies, certain structural features associated with dermatan sulfates have been established. In addition, a beginning has been made to establish the dermatan structure-activity relationship as it pertains to its catalysis of HC II-mediated inhibition of Factor IIa.

MATERIALS AND METHODS

Materials

Dermatan sulfate from porcine mucosa, used for size and charge fractionation experiments, was a gift

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from Dr. Erwin Coyne of Loyola University Medical Center. Dermatan sulfate from porcine skin was obtained from Sigma (St. Louis, MO; C-4289) and Seikagaku Kogyo (Tokyo, Japan). Low molecular weight dermatan sulfate OP370, Oligo-DS OP518, and dermatan sulfate OP352 were from Opocrin (Corlo, Italy). Beef intestinal dermatan sulfate NF112(1-87) was from Rhone-Poulenc (Gennevilliers, France) and dermatan sulfate MF701 (485T) was from Mediolanum (Milan, Italy). Porcine mucosal heparin (161 U/mg) was from Hepar (Franklin, OH). Chondroitin and dermatan sulfate disaccharide standards and chondroitinase ABC (chondroitin lyase EC 4.2.2.4), 100 mIU, were from Seikagaku Kogyo. Spectra/Pore dialysis tubing molecular weight cutoff 1000 were from Spectrum Laboratories (Los Angeles, CA). Diethylaminoethanol (DEAE)-Sephacel and Sephacryl S-300 were from Sigma. Ultraviolet spectroscopy was performed with a Shimadzu model UV-160 spectrophotometer (Tokyo, Japan).

Acrylamide (ultrapure), Tris, Alcian blue, bromophenol blue, and ammonium persulfate were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Glycine hydrochloride, disodium ethylene diamine-tetraacetic acid (EDTA), azure A, boric acid, sucrose, N,N-methylene bisacrylamide and N,N,N,N-tetramethylenediamine (TEMED) were from Fisher Chemical Company (Fair Lawn, NJ). All other chemicals and reagents were of reagent grade. A 32 by 16 cm vertical slab gel unit (SE 620) and SG500 linear gradient marker apparatus were from Hoefer Scientific Instruments (San Francisco, CA). Strong anion exchange HPLC was performed on a Spherisorb (5 μ m particle size) column of dimensions 0.46 by 25 cm from Phase Separation (Norwalk, CT). A dual pump LC-7A HPLC system (Shimadzu, Tokyo) coupled to an LKB 2141 variable wavelength monitor (Stockholm, Sweden) was used for SAX-HPLC.

Charge Fractionation of Dermatan Sulfate

DEAE-Sephacel anion exchange (70 ml) suspended in 0.02 M Tris-hydrochloride containing 0.25 M sodium chloride packed into a column (2.5 \times 19 cm) was used to fractionate dermatan sulfate. Dermatan sulfate (100 mg in 100 ml water) was applied to the column. Elution of the sample from the column was performed using a linear gradient (800 ml) between 0.25 and 1.5 M of sodium chloride, in Tris-hydrochloride pH 7.4 at a flow rate of 60 ml/hour, and 3 ml was collected in each tube. The amount of dermatan sulfate was determined using an azure-A assay.¹² The collected samples were pooled into five fractions and were dialyzed against distilled water and freeze dried.

Size Fractionation of Dermatan Sulfate

Sephacryl S-300 was packed into a column (1.25 \times 100 cm) and equilibrated with a solution containing 0.02 M Tris-hydrochloride (pH 7.4) and 0.25 M sodium chloride. Dermatan sulfate (71.4 mg in 3 ml water) was applied to the top of the column. Elution was carried out using the equilibrating buffer as already described. Two liters of buffer was used and the sample was collected at a flow rate of 20 ml/hour. The collected samples were pooled into five fractions, which were dialyzed against distilled water and freeze dried.

Depolymerization of Dermatan Sulfates

Each dermatan sulfate sample (500 μ g in 100 μ l) was made up in sodium phosphate buffer (5 mM, pH 7.0). Chondroitinase ABC (10 U/ml, 1 μ l) was added and the reaction mixture was incubated at 37°C for over a period of 2 hours. The reaction was monitored by removing 5 μ l aliquots, adding these to 995 μ l of 30 mM hydrochloric acid, and absorbance was determined at 232 nm. The reaction was complete within 2 hours as demonstrated by constant absorbance at 232 nm. The sample was frozen and stored at -70°C.

Preparation of Polyacrylamide Gels

The resolving gel buffer and lower buffer chamber contained 0.1 M boric acid, 0.1 M Tris, and 0.1 M disodium EDTA pH 8.3, and the upper gel buffer was 0.2 M Tris and 1.25 M glycine at pH 8.3, as previously described.^{9,13} Gradient polyacrylamide resolving gel was prepared from two resolving gel buffer solutions; one containing 11.52% (w/v) acrylamide with 0.48% (w/v) N,N-bisacrylamide and 1% sucrose and the second containing 20.02% (w/v) N,N-bisacrylamide, 2% (w/v) N,N-bisacrylamide, and 15% (w/v) sucrose. Gels were poured vertically between glass plates (16 \times 32 cm), which were separated by 1.5 mm spacers.¹³ Gradient gels were poured by adding 35 ml of 12% solution to the reservoir and 35 ml of 22% solution to the mixing chamber of the linear gradient marker. Ammonium persulfate, 200 μ l of 10% in water, was added to the reservoir and 100 μ l to the mixing chamber, followed by addition of 30 μ l TEMED to both reservoir and mixing chamber. Solution in the mixing chamber was continuously mixed using a magnetic stirrer. The valve between the reservoir and the mixing chamber was open. Polyacrylamide solution from the mixing chamber, passed by gravity into two channels leading to the top of the glass plates, forms a linear gradient from the bottom to the top. The unpolymerized gel was overlaid with water (1 ml) and polymerization occurred from top to bottom. After

polymerization was completed, the water layer was removed and 10 ml solution of stacking gel made of 4.75% (w/v) acrylamide and 0.25% (w/v) N,N-bisacrylamide in stacking gel buffer (same as the resolving buffer but at pH 6.3) containing 10 μ l of TEMED and 150 μ l of 10% (w/v) ammonium persulfate was added to the top of the resolving gel. A comb (well former) was inserted. After polymerization, the comb was removed and each well was rinsed with stacking buffer and then with upper chamber buffer.

Electrophoresis of Oligosaccharide Samples

Samples (in distilled water at desired concentration) were combined with equal volume of 50% (w/v) sucrose in distilled water containing 5 μ l phenol red (10 μ g/ml) and bromophenol blue (10 μ g/ml) and loaded into the bottom of the well using a microsyringe. Electrophoresis was performed at 400 V under circulating tap water (10° to 15°C) for 16 hours. The gel was removed from the glass plates and stained with Alcian blue 0.5% (w/v) and 2% (v/v) acetic acid solution in water for 30 minutes. Destaining was carried out with several 200 ml volumes of 5% (v/v) aqueous acetic acid. If increased staining sensitivity was required, the Alcian blue-stained gel could be silver stained. First, all the acetic acid used in destaining was removed by soaking the gel in 30% (v/v) aqueous methanol for 12 hours followed by a 1-hour washing with distilled water. The gel was placed in silver-staining solution prepared from 200 ml of distilled water, 2 ml of 7.6 M sodium hydroxide, 3 ml of aqueous ammonium hydroxide (29%), and 2 ml of 1 gm/ml silver nitrate solution. The gel was agitated gently in the above solution for 1 hour. The gel was then washed three times (each time for 10 minutes) with distilled water and placed in developing buffer, which was made of 500 ml of distilled water, 1 ml of 2.5% citric acid, and 0.25 ml of (37% w/v) aqueous formaldehyde solution. The reaction was terminated within 15 minutes by soaking the gel in 10% solution of acetic acid.

SAX-HPLC of Dermatan Sulfates and Disaccharide Standards

Chondroitinase ABC depolymerized dermatan sulfate samples prepared at 2 mg/ml in phosphate buffer were analyzed directly by SAX-HPLC. Disaccharide standards were dissolved in distilled water at 1 mg/ml for analysis. A 3 μ g sample in 3 μ l was injected onto the analytical SAX-HPLC column equilibrated with 0.2 M sodium chloride at pH 3.5. Elution was performed using a linear gradient (0 to 2 M of sodium chloride) at a flow

rate of 1.5 ml/min. Detection was at 232 nm at 0.02 absorbance units full scale (AUFS).

Gel Permeation Chromatography-HPLC of Dermatan Sulfates

The average molecular weight of each dermatan sulfate fraction was determined using gel permeation chromatography (GPC)-HPLC. Sample (100 μ L, 10 mg/ml) was injected onto a GPC-HPLC column equilibrated with 0.3 M sodium chloride at a flow rate of 0.4 ml/minute. The sample elution was monitored at A_{206} at 0.02 AUFS. The void volume and total volume of the column were measured using blue dextran and sodium azide and K_{av} was calculated for each sample. A porcine mucosal heparin (M_r 13,000), low molecular weight heparin (RD heparin, Hepar, M_r 5000), and a sized fraction of heparin (M_r 43,000) previously standardized by GPC-HPLC were used for the calibration curve of $\log M_r$ versus K_{av} . The correlation coefficient (r^2) was 0.998.

Measurement of Dermatan Sulfate IC_{50}

Antithrombin III (AT III) and HC II mediated anti-IIa activity assays were performed on dermatan sulfate and fractionated dermatan sulfate using an end point chromogenic assay.¹⁴ HC II and AT III were prepared and purified as previously described.¹⁴ Thrombin (10 NIH U/vial) and Chromozym TH were from Sigma. Percent inhibition values were calculated using equation 1, where A and B are the change in absorbance over a fixed time interval in the absence and presence of dermatan sulfate, respectively.

$$\text{Percent inhibition} = \frac{(A - B)}{A} \times 100$$

A plot of percent inhibition versus the log of inhibitor concentration was used to obtain a value for the concentration of dermatan sulfate resulting in 50% inhibition of Factor IIa (IC_{50}).

RESULTS

Dermatan sulfate, low molecular weight dermatan sulfate, and fractionated dermatan sulfates were examined in this study. Porcine mucosal dermatan sulfate was fractionated on the basis of size (Fig. 1) and on the basis of charge (Fig. 2) and separated into five major fractions. Each fraction was desalted by dialysis, freeze dried, and weighed. Stock solutions of each fraction (10 mg/ml)

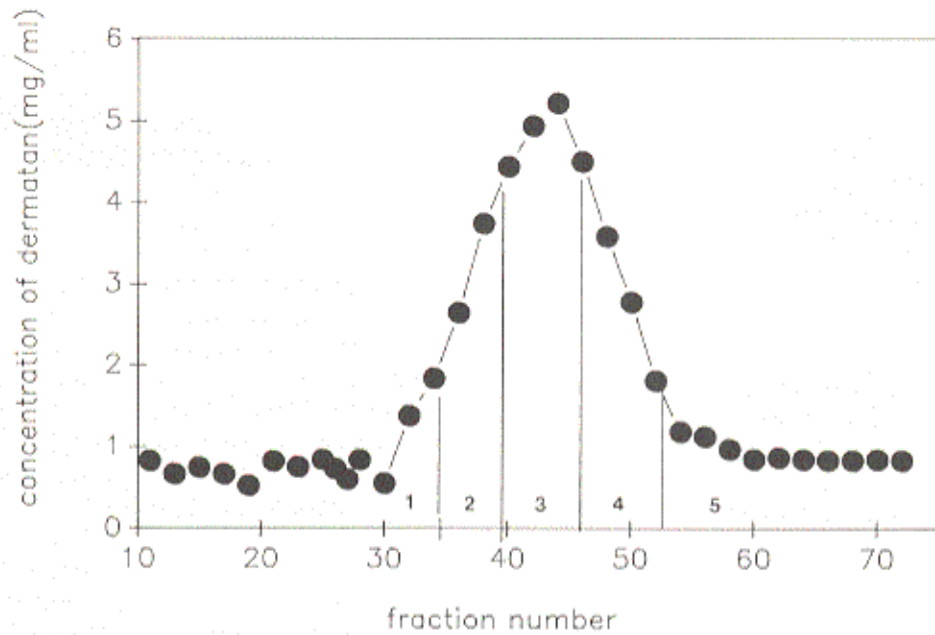


FIG. 1. Size fractionation of dermatan sulfate.

were prepared in distilled water to carry out further analysis. The molecular weight of the charge fractionated dermatan sulfate was determined using GPC-HPLC and is shown in Table 1. The AT III and HC II mediated anti-IIa activities of these dermatan sulfate fractions were also determined (Table 1). These dermatan sulfate samples were analyzed by gradient PAGE together with a

number of low molecular weight dermatan sulfates. The results of these fractionations are given in Figure 3.

Oligosaccharide mapping^{9,10} was performed on the dermatan sulfate samples following their chondroitinase ABC catalyzed depolymerization. The depolymerization reaction was monitored by removing aliquots and measuring their absorbance at 232 nm in 30 mM hydrochloric

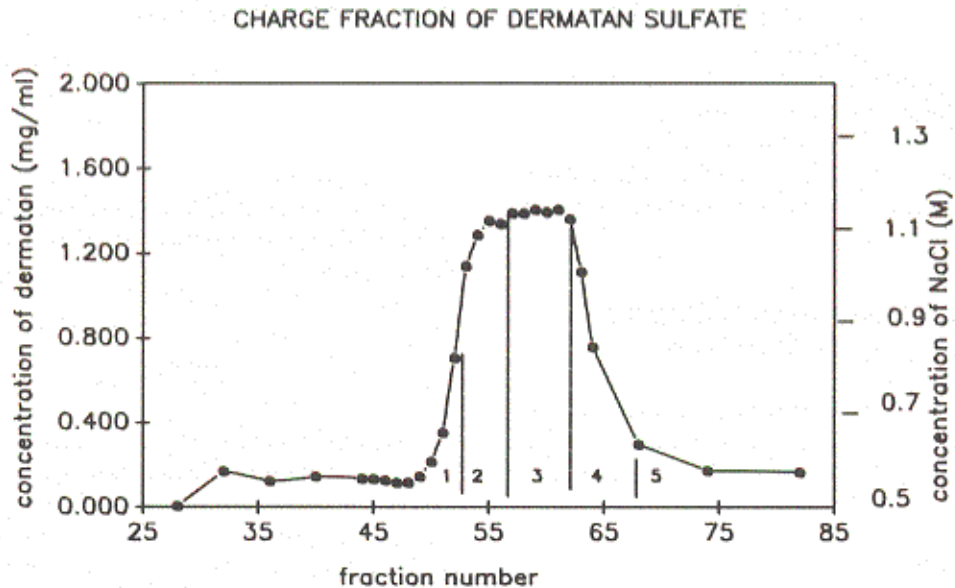


FIG. 2. Charge fractionation of dermatan sulfate.

TABLE 1. Properties of Fractionated Dermatan Sulfate

Fractions*	Average Molecular Weight	IC ₅₀ (μg/ml)	
		Anti-IIa/AT III	Anti-IIa/HC II
DSC1,2	11,400	663.8	69.4
DSC3	39,000	695.6	3.1
DSC4	45,500	10.3	0.67
DSC5	60,000	6.2	0.70
UF-DS	43,300	6.5	1.45
UF-heparin	14,000	0.032	0.30

*Dermatan sulfate charge fractionated samples (DSC1 to DSC5), unfractionated porcine mucosal dermatan sulfate (UF-DS), and unfractionated porcine mucosal heparin (UF-heparin).

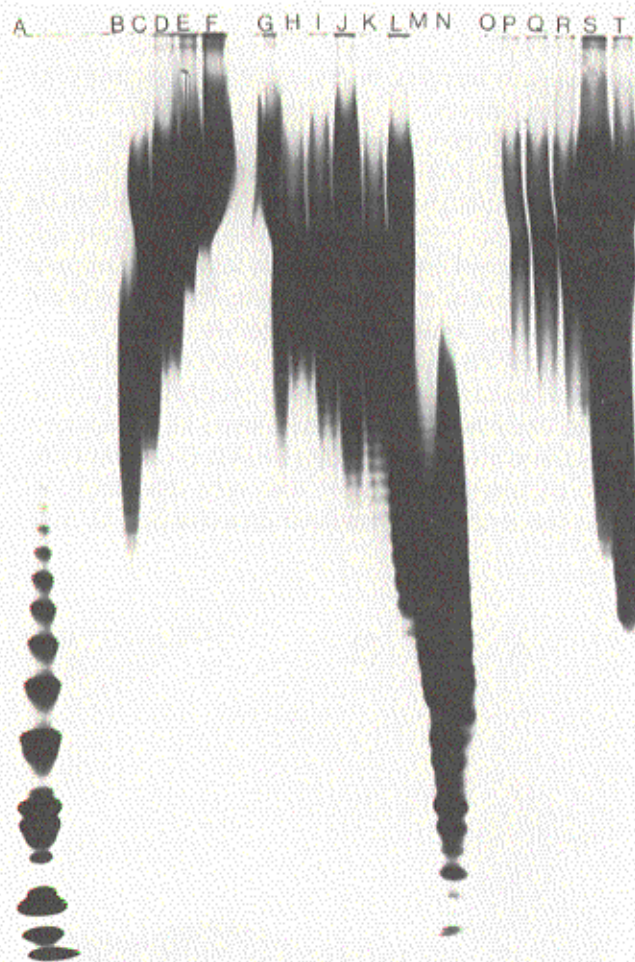


FIG. 3. Gradient-PAGE analysis of intact dermatan sulfates. Lane A contains partially depolymerized bovine heparin. Lanes B, C, D, E, and F are size fractions (1 to 5, Fig. 2). Lane G is unfractionated dermatan sulfate (E. Coyne). Lanes H-N are dermatan sulfates (Sigma, Seikagaku, OP352, NF112, MF701, OP518, and OP370, respectively). Lane O is empty and lanes P, Q, R, S, and T are size fractions (1 to 5, Fig. 1).

acid. When the reaction was completed, the oligosaccharide products were analyzed using SAX-HPLC (Fig. 4) and the retention times of the peaks observed were compared to the values obtained using disaccharide standards of known structure (Table 2). The oligosaccharide mixtures were also examined using gradient PAGE (Fig. 5). Because of the low level of sulfation in the dermatan sulfate oligosaccharide products, Alcian blue-stained bands were only observable in the gradient PAGE gel when heavy sample loadings were applied. The intensity of the bands could be enhanced by silver staining the gel (Fig. 6).

DISCUSSION

The porcine mucosal heparin examined had an average molecular weight of 43,000 and an IC₅₀ (HC II-mediated anti-IIa) of 1.45 μg/mL. This dermatan sulfate was fivefold less potent as an HC II-mediated inhibitor of Factor IIa than porcine mucosal heparin. Fractionation of dermatan sulfate on the basis of charge, however, resulted in fractions having both considerably higher and lower IC₅₀ values than unfractionated dermatan sulfate (Table 1). Dermatan sulfate charge fractions 4 and 5 (DSC4 and DSC5) had IC₅₀ values comparable with heparin. DSC4 and DSC5 had increased sulfation and required 1.1 to 1.2 M sodium chloride to elute from DEAE-Sephacel. Analysis by GPC-HPLC and by gradient PAGE also showed these samples to have a higher average molecular weight than the unfractionated dermatan sulfate from which they were obtained. We have reported a similar trend for DEAE-Sephacel fractionated heparins.¹⁵ Dermatan sulfate fractions having lower levels of sulfation (DSC1, DSC2, and DSC3) had reduced molecular weights and increased IC₅₀ values.

From these studies, it appeared that dermatan sulfate's HC II-mediated (and also its AT III-mediated) inhibition of Factor IIa was dependent on either its molecular size or charge (degree of sulfation). Dermatan sulfate fractions were prepared using Sephacryl S-300. The resulting fractions, ranging from 10,000 to 60,000, showed no significant differences in IC₅₀ values. All of these size fractions were well above the minimum chain length required for dermatan sulfate's HC II-mediated anti-Factor IIa activity.¹⁵ The molecular weight and polydispersity of the size fractionated and charge fractionated dermatan sulfates were comparable as determined by GPC-HPLC and by gradient PAGE (Fig. 5). Unfractionated dermatan sulfates, however, showed greater polydispersity (Fig. 3).

In an effort to understand the tenfold difference (IC₅₀ of 0.7 to 69.4 μg/mL) between charge fractionated dermatan sulfates (DSC5 and DSC1), we used an oli-



FIG. 4. SAX-HPLC analysis of chondroitinase ABC depolymerized dermatan sulfates. A is dermatan sulfate (E. Coyne). B is size fraction 5 (Fig. 1). C is size fraction 1 (Fig. 1). D is charge fraction 1 (Fig. 2). E is charge fraction 5 (Fig. 2). The tick-marks on the X-axis are at 10-minute intervals.

TABLE 2. SAX-HPLC Analysis of Dermatan Sulfate Disaccharide Standards

Disaccharide Standards*	Elution Time (Min)	Salt Required to Elute (M)
1 Δ UA(1 \rightarrow 3)- β -D-GalNAc	9.633	0.054
2 Δ UA(1 \rightarrow 3)- β -D-GalNAc6S	15.332	0.085
3 Δ UA(1 \rightarrow 3)- β -D-GalNAc4S	16.082	0.090
4 Δ UA2S(1 \rightarrow 3)- β -D-GalNAc	16.397	0.091
5 Δ UA2S(1 \rightarrow 3)- β -D-GalNAc6S	25.200	0.140
6 Δ UA(1 \rightarrow 3)- β -D-GalNAc4S6S	27.115	0.151
7 Δ UA2S(1 \rightarrow 3)- β -D-GalNAc4S	28.733	0.160
8 Δ UA2S(1 \rightarrow 3)- β -D-GalNAc4S6S	42.373	0.236

* Δ UA is 4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid; S is sulfate.

gosaccharide mapping developed in our laboratory for analyzing heparin.^{9,10} The dermatan sulfate fractions were first depolymerized using chondroitinase ABC. Analysis by SAX-HPLC demonstrated that all the fractions had a major peak at 16 minutes corresponding to the expected Δ UA(1 \rightarrow 3)- β -D-GalNAc-4-S. The major difference observed between dermatan sulfate fractions was the enrichment of a single peak from DSC5 observed at 27.5 minutes, which was assigned to Δ UA(1 \rightarrow 3)- β -D-GalNAc4S6S (Fig. 2). It is expected that highly sulfated dermatan sulfate chains enriched by ion-exchange chromatography should contain higher amounts of disulfated disaccharide, Δ UA(1 \rightarrow 3)- β -D-GalNAc4S6S. It is unexpected, however, that only this disulfated disaccharide and not disulfated disaccharides Δ UA2S-

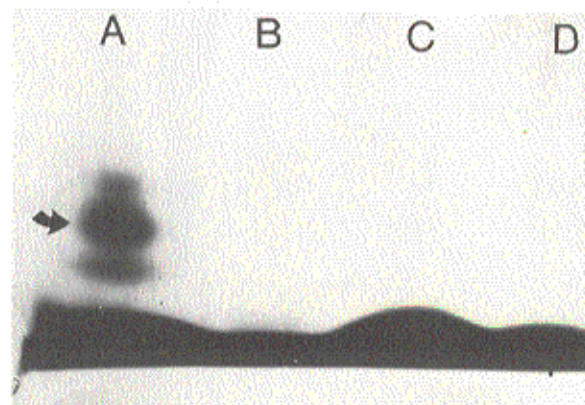


FIG. 5. Gradient-PAGE analysis of chondroitinase ABC depolymerized charge and size fractionated dermatan sulfates. Lanes A and B are charge fractions 1 and 5 (Fig. 2) and lanes C and D are size fractions 1 and 5 (Fig. 1). The arrow in lane A points to tetrasaccharide components.

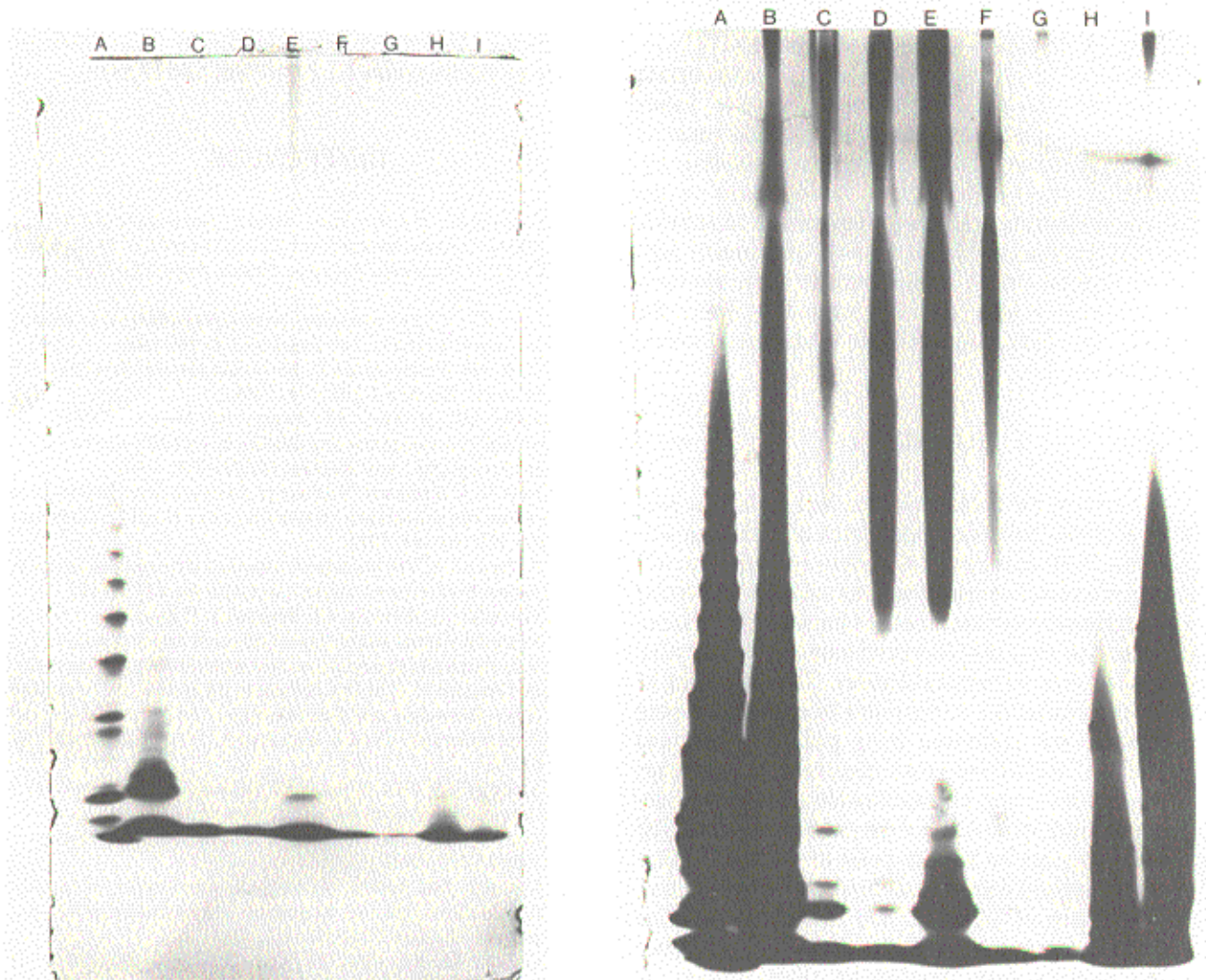


FIG. 6. Gradient-PAGE analysis of chondroitinase ABC depolymerized dermatan sulfates. Lane A is partially depolymerized bovine heparin. Lanes B to I are dermatan sulfate (E. Coyne) (Sigma, Seikagaku, OP352, NF112, MF701, OP518 and OP370, respectively). Gel A (on left) is stained with Alcian blue and gel B is the same gel after being silver stained (the bottom quarter of gel B is not shown).

(1 \rightarrow 3)- β -D-GalNAc6S and Δ UA2S(1 \rightarrow 3)- β -D-GalNAc4S or trisulfated disaccharide Δ UA2S(1 \rightarrow 3)- β -D-GalNAc4S6S are enriched. This suggests that the most highly charged chains having the greatest HC II-mediated anti-Factor IIa activity are preferentially enriched with Δ UA(1 \rightarrow 3)- β -D-GalNAc4S6S. This is contrary to a report that 2-sulfation increases and 6-sulfation decreases dermatan sulfate's HC II-mediated anti-Factor IIa activity.⁸ Further investigation, including the oligosaccharide mapping of dermatan sulfates fractionated by HC II affinity chromatography¹⁴ will be required to establish clearly the relationship between the position of sulfation and activity.

In addition to SAX-HPLC, gradient PAGE analysis of DSC5 clearly demonstrated an increase in the band

intensity assignable to tetrasaccharide-sized oligosaccharides. The structures of these tetrasaccharides were not established, but their presence in unfractionated porcine skin dermatan sulfate (92 mol% disaccharide, 5 mol% tetrasaccharide, and 3 mol% higher oligosaccharide) was observed by low-pressure GPC analysis following chondroitinase catalyzed depolymerization. All ten disaccharide standards comigrated on gradient PAGE. These results suggest that future studies may require the isolation and characterization of tetrasaccharides, which probably contain linkages that are resistant to cleavage by chondroitinase ABC.

Analysis of a variety of commercially available dermatan sulfates using oligosaccharide mapping techniques suggests that most afford primarily disaccharide

products on treatment with chondroitinase ABC, although some show tetrasaccharide-sized oligosaccharide products (Fig. 5). On silver staining, however, bands corresponding to tetrasaccharide-sized products could be seen in all dermatan sulfates that were examined (Fig. 6). In addition, silver staining also showed many of the commercial dermatan preparations to contain chondroitinase ABC-resistant polymeric material (presumably heparin or heparan sulfate contamination). This approach may represent a very sensitive method to detect the presence of contaminating GAGs in dermatan sulfate preparations.

SUMMARY

A method for characterization and molecular profiling of acidic polysaccharides (such as dermatan sulfates) has been developed. A variety of dermatan sulfates, fractionated dermatan sulfates and low molecular weight dermatan sulfates, were examined. First, bacterial lyase-type enzymes (chondroitinase ABC) were used to depolymerize the polysaccharides. Then, mapping of these oligosaccharides (comparable to peptide mapping of proteins) was performed using gradient PAGE and SAX-HPLC. Bands and peaks observed in these maps were identified using oligosaccharide standards of defined chemical structures and physical properties. The resulting map can be used to point to structural differences among these dermatan sulfates regarding their size, charge, degree of sulfation, and contamination. Fine details of fragmentation patterns and absence or presence of contaminants were detected by silver staining of gels. These differences, particularly the content of $\rightarrow 4$ - α -IdoA(1 \rightarrow 3)- β -D-GalNAc4S6S(1 \rightarrow) sequences (detected using SAX-HPLC as Δ UA(1 \rightarrow 3)- β -D-GalNAc4S6S) may play an important role influencing the activity of dermatan sulfates to potentiate HC II inhibition of Factor IIa.

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