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Note

Structural analysis and physico-chemical properties of charge-fractionated dermatan sulfate samples

Nicola Volpi

Chair of Biological Chemistry, Department of "Biologia Animale", University of Modena, via Berengario 14, I-4110 Modena, Italy

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The interest in studying the structure and biochemical properties of dermatan sulfate has grown following numerous studies. The efficacy of this naturally derived glycosaminoglycan drug as an anticoagulant [1] and antithrombotic agent [2,3] with possible applications in haemodialysis [3,4] and acute leukaemia [5] has stimulated pharmacological and clinical researches. Dermatan sulfate shows important biochemical (and possibly pharmacological) properties due to its effects on several molecular systems, such as interaction with histidine-rich glycoprotein and platelet factor 4 [6], lipoprotein, fibronectin, vitronectin, β-endorphin, and casein kinase [7]. Studies of the anticoagulant/antithrombotic activity of dermatan sulfate indicate that this glycosaminoglycan catalyzes the inhibition of thrombin by heparin cofactor II [8]; fragments of dermatan sulfate containing a minimum of 12–14 sugar residues are necessary to increase the inhibition of thrombin by heparin cofactor II [9]. Moreover, the presence of a specific hexasaccharide sequence composed of a unique disulfated disaccharide [(1 → 4)-*O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1 → 3)-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl 4-sulfate)] in intact dermatan sulfate is necessary to bind the polysaccharide chain to heparin cofactor II with high affinity [10]. On the contrary, Linhardt et al. [11] reported that the dermatan sulfate fraction with the highest heparin cofactor II-mediated activity is enriched in [(1 → 4)-*O*-(α-L-idopyranosyluronic acid)-(1 → 3)-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl 4,6-disulfate)]. Finally, Scully et al. [12] showed that 2-sulfatation increases and 6-sulfatation decreases the antithrombin activity of dermatan sulfate mediated by heparin cofactor II.

Dermatan sulfate extracted and purified from mammalian tissues is a complex glycosaminoglycan whose polysaccharide chains are composed mainly of disaccharide units of α-L-idopyranosyluronic acid β-(1 → 3) linked to 2-acetamido-2-deoxy-β-D-galactopyranosyl 4-sulfate [13]. Different nonsulfated and sulfated disaccharides are present inside the polysaccharide chains. The relative amount of the

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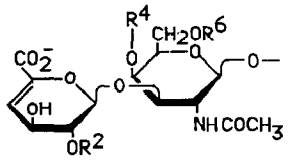
different constituent disaccharides is one of the factors responsible for the structural and biochemical heterogeneity of dermatan sulfate. Extraction and purification from different animal tissues produce heterogeneous dermatan sulfate preparations, variable in relative molecular mass (M_r), number of sulfate groups per disaccharide, constituent disaccharides, species of uronic acids, impurities (proteins and peptides, bivalent cations, pyrogen and dye substances, oxidising and reducing agents), or the presence of other glycosaminoglycans [14,15].

Preparative ion-exchange chromatography of pure heparin produces samples with different molecular mass, charge density, and biological and pharmacological properties, such as anticoagulant, antilipemic, and antithrombotic activities [16,17]. The aim of this research was to study the structures and physico-chemical properties of dermatan sulfate samples produced by charge fractionation. This preparative approach could be used to prepare dermatan sulfate fractions with different structural composition (and pharmacological activities).

Dermatan sulfate from beef mucosa was characterized with respect to structure and physico-chemical properties. A careful characterization is necessary since different species of dermatan sulfate having various structures and properties can be isolated depending on the source [15]. The peak M_r of this preparation is ca. 26000 and 90% of the heteropolysaccharide chains, evaluated from the chromatographic profile, range from M_r 40000 to 10000. The sulfate-to-carboxyl ratio is ca. 1.10 calculated by titrimetric determination and enzymatic cleavage: this value > 1 is due to disulfated disaccharides located inside the dermatan sulfate chains (Scheme 1).

The absence of chondroitin sulfates A and/or C and/or other species of chondroitin sulfates (D, E) as contaminants of the dermatan sulfate preparation was carefully tested by specific enzymatic degradation (by chondroitinase B) of the dermatan sulfate followed by agarose-gel electrophoresis, and by SAX-HPLC after treatment by chondroitinase ACII. These methods are very sensitive and can determine 0.2% (w/w) of chondroitin sulfates [18]: no appreciable amounts of these glycosaminoglycans were detected. On the other hand, small amounts of "fast moving" heparin species (ca. 2% w/w) were found in the dermatan sulfate preparation [18].

The presence of glycosaminoglycan contaminants in preparations of dermatan sulfate is related to the extraction and purification methods. In the present study, different fractionation techniques were chosen. Fractionation by anion-exchange chromatography is suitable for the separation of dermatan sulfate from other glycosaminoglycans by means of charge density. Under our conditions, large percentages of chondroitin sulfates, and other polysaccharides with low charge density, are eluted at NaCl ionic strength < 1.5–1.7 M whilst heparin is recovered with NaCl > 2 M. Dermatan sulfate is eluted at ca. 1.7–1.8 M NaCl. Precipitation with 0.3–0.5 vol of acetone is a further purification step, since heparin is precipitated whilst the dermatan sulfate is recovered with increasing volumes of organic solvent [19]. Finally, the precipitation of dermatan sulfate as a copper salt in alkaline medium produced a preparation with no detectable ($< 0.2\%$ w/w) amounts of chondroitin sulfates (A, C, and/or other highly sulfated species) and



	R^2	R^4	R^6
ΔDi-0S	H	H	H
ΔDi-6S	H	H	SO_3^-
ΔDi-4S	H	SO_3^-	H
ΔDi-4,6diS (E)	H	SO_3^-	SO_3^-
ΔDi-2,6diS (D)	SO_3^-	H	SO_3^-
ΔDi-2,4diS (B)	SO_3^-	SO_3^-	H
ΔDi-2,4,6triS	SO_3^-	SO_3^-	SO_3^-

Abbreviations. ΔDi-0S: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose. ΔDi-4S: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 4-sulfate. ΔDi-6S: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 6-sulfate. ΔDi-2,6diS: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2-sulfate)-D-galactose 6-sulfate. ΔDi-2,4diS: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2-sulfate)-D-galactose 4-sulfate. ΔDi-4,6-diS: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 4,6-disulfate. ΔDi-2,4,6triS: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2-sulfate)-D-galactose 4,6-disulfate. (B) = Disaccharide B. (D) = Disaccharide D (E) = Disaccharide E.

Scheme 1. Structural formulas of unsaturated, nonsulfated and sulfated disaccharides identified inside the polysaccharide chains of dermatan sulfate by specific enzymatic cleavage.

ca. 2% of the "fast moving" component of heparin. As a consequence of the absence of chondroitin sulfates in the preparation of dermatan sulfate, the disaccharide composition of the charge-fractionated fractions obtained by preparative strong-anion-exchange chromatography was performed by chondroitinase ABC due to the lower price of this enzyme than other lyases. On the other hand, the small amounts of heparin as contaminants are irrelevant to the purpose of this research.

Beef mucosa dermatan sulfate was fractionated on a strong anion-exchange resin by increasing the ionic concentration. The percentage recovery, calculated by weight of each fraction and referred to the total recovery of material (98%), for fractions at different ionic concentration is reported in Table 1. The dermatan sulfate fraction (470 mg) eluted by 0.10 M NaCl was recovered in 63% yield from the native glycosaminoglycan (750 mg). The physico-chemical properties and structure are comparable with those of native dermatan sulfate. This fraction shows a small increase in monosulfated disaccharides and a decrease in disulfated disaccharides (Table 2). The fractions eluted with 0.25 and 0.50 M NaCl (ca. 5%) show, in comparison to the native polysaccharide, the same M_r and a small decrease in 2,4-disulfated disaccharide and charge density (Table 1). Two fractions (ca. 4.5%) eluted at 0.75 and 1.00 M NaCl have low M_r and a low sulfate-to-carboxyl ratio due to an increase of nonsulfated disaccharide and to a decrease of disulfated

Table 1
Percentage recovery, M_r, and sulfate-to-carboxyl ratio of charge-fractionated dermatan sulfate.

Sample (M NaCl)	Recovery (%)	M_r ($\times 10^{-3}$)	SO_3^-/COO^-
0.10	63.1	24.9	1.08
0.25	2.6	25.6	1.07
0.50	2.2	26.4	1.07
0.75	2.3	4.2	0.92
1.00	2.5	6.9	1.04
1.25	16.6	20.0	1.13
1.50	8.8	23.5	1.19
1.75	1.3	33.3	1.16
2.00	0.5	39.2	1.24

disaccharides. The elution of charge-fractionated dermatan sulfate fractions from 1.25 to 2.00 M NaCl was dependent on their M_r and charge density. The fractions at 1.25 and 1.50 M NaCl represent a significant percentage of native dermatan sulfate: 16.6 and 8.8%, respectively. These two fractions have about the same percentage of 2,4-disulfated disaccharide (10.0 and 10.9%) but different amounts of 4,6-disulfated disaccharide (2.1% for 1.25 M and 6.3% for 1.50 M) inside their polysaccharide chains. The fraction eluted at 1.75 M shows an increased M_r and 4,6-disulfated disaccharide percentage, and a decreased amount of 2,4-disulfated disaccharide with respect to native dermatan sulfate. The last fraction (2.00 M) shows a very high M_r and charge density with a significant increase of 4,6-disulfated disaccharide and the same amount of 2,4-disulfated disaccharide as native polysaccharide.

Samples of dermatan sulfate fractionated by anion-exchange chomatography show heterogeneity in their physico-chemical properties. This preparative ap-

Table 2
Disaccharide composition of beef mucosa dermatan sulfate (B.M.) and charge-fractionated samples. For structural formulas, see Scheme 1

Sample B.M.	Composition (%)						
	ΔDi-OS 1.3	ΔDi-6S 3.0	ΔDi-4S 84.7	ΔDi-2,6diS 0.3	ΔDi-4,6diS 1.4	ΔDi-2,4diS 9.2	ΔDi-2,4,6triS 0.0
M NaCl							
0.10	1.3	2.7	87.2	0.3	1.0	7.5	0.0
0.25	1.2	2.3	87.9	0.6	1.2	6.7	0.0
0.50	1.4	2.3	87.8	0.6	1.3	6.7	0.0
0.75	8.8	2.6	87.6	0.0	0.0	0.9	0.0
1.00	0.8	2.2	93.0	0.2	0.7	3.2	0.0
1.25	0.5	2.2	84.1	0.3	2.1	10.0	0.8
1.50	0.9	3.8	76.3	0.5	6.3	10.9	1.3
1.75	1.9	3.6	77.4	0.1	11.2	5.8	0.3
2.00	1.2	3.0	71.0	0.2	16.3	8.4	0.0

proach represents a useful technique for producing dermatan sulfate species with different structural (and possibly biological and pharmacological) properties. In particular, it is possible to produce fractions with very low M_r and charge density. These fractions can be considered as the "fast moving" component of dermatan sulfate and, although present in an appreciable amount (ca. 5%) in the native polysaccharide, it is not possible to separate these species by conventional agarose-gel electrophoresis, as for the "fast moving" heparin [18]. Chains with very high M_r and charge density have also been produced by anion-exchange chromatography. These dermatan sulfate species are composed of highly sulfated disaccharides, in particular disaccharides with sulfate groups at positions 4 and 6 of 2-acetamido-2-deoxy-β-D-galactopyranosyl residues and/or position 2 of α-L-iduronic acid and position 4 of 2-acetamido-2-deoxy-β-D-galactopyranosyl residues. These disulfated disaccharides can be associated to form oligosaccharide sequences with high charge density and are present in polysaccharide chains with high M_r chains. Nevertheless, one would expect that highly sulfated dermatan sulfate chains purified by strong anion-exchange chromatography should contain higher amounts of disulfated disaccharide, similar to those inside the chains of native polysaccharide (the 2,4-disulfated disaccharide). On the contrary, only 4,6-disulfated disaccharide, and not 2,4-disulfated disaccharide, 2,6-disulfated disaccharide, or trisulfated disaccharide, is enriched. However, the same results have been obtained by Linhardt et al. [14], who studied a dermatan sulfate from porcine mucosa.

From this study, it is evident that purified dermatan sulfate contains chains with different M_r, structural composition, and charge density. In particular, chains with low M_r are composed of undersulfated disaccharides whilst oversulfated disaccharides (and possibly oligosaccharides) are present in high M_r polysaccharide chains. Finally, dermatan sulfate chains with very high M_r are enriched with a specific disulfated disaccharide, such as the 4,6-disulfated disaccharide E, which is not detectable in the native polysaccharide.

1. Conclusions

The results of this study confirm that dermatan sulfate purified from tissues is a heteropolysaccharide, heterogeneous both in structure and physico-chemical properties. This heterogeneity was found for dermatan sulfate from different sources [15], and even in the same preparation in which a variable percentage of the chains shows different M_r, charge density, and highly sulfated disaccharide (and possibly oligosaccharide) sequences.

Dermatan sulfate fractions with a specific structural characteristic can be isolated by anion-exchange chromatography. In particular, dermatan sulfate can be obtained with high charge density and M_r, and also enriched in a particular kind of disulfated disaccharide. Dermatan sulfates with different and known structure and physico-chemical properties show various biological and pharmacological activities, as also reported elsewhere [2,12,14], and the preparation of species with specific molecular characteristics can help to elucidate the structure–function

relationship of this polysaccharide, as also reported for chondroitin sulfates with different structures [20].

2. Experimental

Extraction and purification of dermatan sulfate.—The extraction and purification of dermatan sulfate was performed as reported [18]. Beef intestinal mucosa was ground and treated with proteolytic enzymes at 65°C for 12 h. After heating at 100°C for 30 min, the mixture was brought to pH 9.0 by adding 2 M NaOH. After 24 h at 40°C, the product (brought to pH 6.0 with 2 M AcOH) was filtered on a Diatomite filter (High Performance Filter Aids from Dicalite, Los Angeles, CA, USA). The solution containing polysaccharides was percolated through a column of strong anion-exchange resin (Purolite A860, batch 1/88 from Purolite International) in HO^- form. Dermatan sulfate was eluted with 1.7–1.8 M NaCl, and acetone (0.3–0.5 vol) was added to the recovered solution. The precipitate was discarded and acetone (1.0–1.5 vol) was added to the filtrate. The new precipitate was recovered and dried. Dermatan sulfate was purified by selective precipitation with $Cu(OAc)_2$ [15] in alkaline medium and acetone. To the powder (10 g) dissolved in 100 mL of bidistilled water were added $Cu(OAc)_2$ (500 mg) and 10 M NaOH (10 mL). The precipitate formed after 24 h at 4°C was collected by centrifugation and dried.

Dermatan sulfate Cu^{II} salt was transformed into dermatan sulfate Na salt by cation-exchange resin. Dermatan sulfate Cu^{II} salt (5 g) was percolated through a column (150 × 25 mm) packed with Chelex 100 resin (Bio-Rad, Richmond, USA) in Na^+ form (washed with two bed volumes of 2 M HCl, two volumes of M NaOH, and five volumes of bidistilled water) and eluted with bidistilled water. Sodium acetate (1%) was added to the recovered solution (100 mL) and the crude dermatan sulfate Na salt was collected after precipitation with acetone (1.0–1.5 vol) and dried.

Determination of the purity of dermatan sulfate preparations.—Possible contaminating glycosaminoglycans (chondroitin sulfate A and C, heparan sulfate, "slow-moving" and "fast-moving" components of heparin) in the preparation of dermatan sulfate were determined by agarose-gel electrophoresis in $Ba(OAc)_2$/1,2-diaminopropane [18] and electrophoresis on cellulose polyacetate (Titan III) [21].

Small amounts of chondroitin sulfates as contaminants in dermatan sulfate preparations were detected by enzymatic degradation by chondroitinase B (EC 4.2.2.) and agarose-gel electrophoresis. Dermatan sulfate (500 μg in 10 μL of distilled water) was treated with 0.01 unit of chondroitinase B (Seikagaku Co., Tokyo) in 50 mM Tris-HCl buffer, pH 8.0 (40 μL). After incubation at 30°C for 24 h and boiling for 1 min, the solution (5 μL) was deposited on the agarose plate as reported elsewhere [18] and quantified by specific calibration curves. This procedure revealed 0.2% chondroitin sulfates (w/w) contaminating the dermatan sulfate preparations.

The presence of chondroitin sulfates in dermatan sulfate preparations has also been evaluated by specific degradation with chondroitinase ACII (EC 4.2.2.5; Seikagaku Co., Tokyo), and SAX-HPLC analysis [18]. The presence of chondroitin sulfate A and/or C was evaluated by measuring the unsaturated monosulfate disaccharides [2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 4-sulfate and 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 6-sulfate] produced by enzymatic degradation. This procedure detects 0.5% chondroitin sulfates (w/w) contaminating the dermatan sulfate preparations.

Small amounts of heparin (and/or heparan sulfate) as contaminants in dermatan sulfate preparations were detected by enzymatic degradation by chondroitinase ABC (EC 4.2.2.4) and agarose-gel electrophoresis. Dermatan sulfate (500 μg in 10 μL of distilled water) was treated with 0.5 unit of chondroitinase ABC (Sigma, 0.34 unit/mg of protein) in 50 mM Tris-HCl buffer pH 8.0 (40 μL). After incubation at 37°C for 3 h and boiling for 1 min, the solution (5 μL) was deposited on the agarose plate as reported elsewhere [18].

Charge fractionation of native dermatan sulfate.—Jasco HPLC equipment was used for semi-preparative strong anion-exchange separation; native beef mucosa dermatan sulfate (peak M_r 25 800) (750 mg) was fractionated on a column (30 cm × 7.8 mm) packed with 10 g of 10-μm Spherisorb SAX (trimethylammoniumpropyl groups, $Si-CH_2-CH_2-CH_2-N^+(CH_3)_3$ in Cl^- form; from Phase Separations Limited, Deeside Industrial Park, Deeside Clwyd, UK). Native polysaccharide (750 mg) in 0.02 M NaCl (10 mL) was charged on the column and elution was performed at 1.5 mL/min with solutions of increasing NaCl molarity (0.10, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, and 2.00 M); 40-mL fractions were collected, concentrated by Rotavapor, and dialyzed with molecularporous dialysis membranes (Spectrapore dialysis tubing from Spectrum; molecular mass cut-off of 2000) against decreasing molarity NaCl solution and then against bidistilled water; finally, they were concentrated and lyophilized. The relative amounts of samples were determined (mg of powder for each fraction) and expressed as a percentage of the total recovery (98%).

Determination of relative molecular mass.—HPLC equipment was from Jasco: pump, model 800 PU; system controller, model 801 SC; ternary gradient unit, model 880-02; injector, Rheodyne equipped with a 100-μL loop; UV detector, model 875 UV. The mobile phase was composed of 125 mM Na_2SO_4 and 2 mM NaH_2PO_4 adjusted to pH 6.0 with 0.1 M NaOH. The flow rate was 0.9 mL/min with a back pressure of 25 kg/cm^2. Protein Pak 125 and 300 columns assembled in series (Waters, 84601 and T72711) were used. Different dermatan sulfate fractions were solubilized in the mobile phase at a concentration of 5 mg/mL; 10 μL (50 μg) were injected in HPLC [22].

Determination of sulfate-to-carboxyl ratio.—Sulfate and carboxyl groups were determined by potentiometric titration, with 0.1 M NaOH in water DMF, of dermatan sulfate fractions in the acid form obtained by removal of the cations by a strong cation-exchange resin (Amberlite IR-120). The sulfate-to-carboxyl ratio was also determined by enzymatic degradation after HPLC separation of constituent

disaccharides. The ratio was calculated by considering the percentage and the presence of carboxyl and sulfate groups for each disaccharide.

Determination of constituent disaccharides by cleavage with chondroitinase ABC. —Samples (100 μg; 10 mg/mL in water) of charge-fractionated dermatan sulfates were incubated with 625 munit of chondroitinase ABC (EC 4.2.2.7) (Sigma, Code C-2905; specific activity of 0.34 unit/mg of protein) in 50 mM Tris-HCl buffer, pH 8.0. The reactions were stopped, after 3 h incubation at 37°C, by boiling for 1 min. The constituent disaccharides were determined by strong-anion-exchange(SAX)–HPLC as reported elsewhere [18]. Jasco HPLC equipment as described and a Spherisorb 5 SAX column (250 × 4.6 mm) were used. Isocratic separation was from 0 to 10 min with 0.10 M NaCl, pH 4.00; linear gradient separation was from 10 to 90 min with 0.10 M NaCl, pH 4.00, to 1.2 M-NaCl, pH 4.00. The flow rate was 1.4 mL/min.

The complete degradation of samples to disaccharides was tested by high-performance size-exclusion chromatography as reported [22] (see "Determination of relative molecular mass" section).

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