Carbohydrate Research, 255 (1994) 133–144
Elsevier Science B.V., Amsterdam

Dermatan sulfate from beef mucosa: structure, physicochemical and biological properties of fractions prepared by chemical depolymerization and anion-exchange chromatography

Nicola Volpi

Department of "Biologia Animale", Chair of Biological Chemistry, University of Modena, Via Berengario 14, I-41100 Modena (Italy)

(Received October 29th, 1992; accepted August 2nd, 1993)

ABSTRACT

Dermatan sulfate was extracted and purified from beef intestinal mucosa. The structure and physicochemical properties were evaluated by different techniques, such as, disaccharide pattern, relative molecular mass, sulfate-to-carboxyl ratio, and electrophoretic profile in agarose electrophoresis. The biological activity was evaluated as heparin cofactor II activity (HCII activity). The purity of dermatan sulfate was carefully evaluated by specific enzymatic cleavage, agarose electrophoresis, and HPLC. Different relative molecular masses of dermatan sulfate, from 25000 to 2000, were prepared by chemical degradation. The structures and physicochemical properties were checked to exclude a possible desulfation process. The HCII activities were evaluated for different relative molecular mass of dermatan sulfate. The capacity of chondroitinase ABC to cleave different relative molecular masses of dermatan sulfate was also studied. Native dermatan sulfate was fractionated according to charge density. Different fractions were obtained and analysed for disaccharide pattern, relative molecular mass, sulfate-to-carboxyl ratio, and HCII activities.

INTRODUCTION *

Interest in studying the structure and biological and pharmacological activities of nonheparin glycosaminoglycans has grown following many studies that have proved the efficacy of the naturally derived glycosaminoglycan (GAGs) drugs as antiinflammatory, antiatherosclerotic, cytoprotective, and immunomodulatory agents although they have none of the plasma anticoagulant effects typical for heparin (Hep)[1]. The nonheparin GAGs are mostly represented by natural or

* Abbreviations used: GAGs, glycosaminoglycans; DS, dermatan sulfate; LMW-DS, low molecular weight dermatan sulfate; CS, chondroitin sulfate; HS, heparan sulfate; Hep, heparin; HCII; heparin cofactor II; APTT, activated partial thromboplastin time; AXa, antifactor Xa; SAX, strong anion exchange; HPSEC, high-performance size-exclusion chromatography.

0008-6215/94/$07.00 © 1994 – Elsevier Science B.V. All rights reserved
SSDI 0008-6215(93)E0356-6

depolymerized heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfates (CS), and related substances. GAGs have been recently chemically modified and studies have been done on synthetic analogues of the natural substances[2].

DS extracted and purified from mammalian tissues is a complex GAG, the polysaccharide chains of which are constituted by the prevailing structural disaccharide unit sequences: [4)-*O*-(α-L-idopyranosyluronic acid)-(1 → 3)-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl 4-sulfate)-(1 →][3,4]. Different nonsulfated and sulfated disaccharides are present within the polysaccharide chains. The relative amount of different constituent disaccharides is a factor responsible for the structural and biological heterogeneity of DS (the percentage and structure formulas of variously nonsulfated and sulfated disaccharides, obtained by β-elimination by chondroitinase ABC lyase, identified in beef mucosa DS chains are reported in Table I)[5]. The extraction and purification processes of different animal tissues cause heterogeneous DS mixtures variable in relative molecular mass (M_r), quantities of sulfate groups per disaccharides, constituent disaccharides, and impurity or presence of possible other contaminating GAGs[6,7].

Studies on the anticoagulant/antithrombotic activity[8] of DS have showed that this GAG catalyzes the inhibition of the thrombin (factor IIa) by HCII. Fragments of DS containing a minimum of 12–14 sugar residues[9] are necessary to increase the inhibition of the thrombin by HCII. Also, the presence of a specific hexasaccharide sequence in intact DS is necessary to bind the polysaccharide chain to HCII with high affinity[10].

Recently, great interest has been stimulated by the low molecular weight derivatives of DS (LMW-DSs) for their possible use as prophylactic antithrombotic drugs[11,12]. In addition, the processes producing LMW-derivatives give different structures and distributions of M_r, responsible for the heterogeneity of products and therefore the biological activity and pharmacological properties.

Native DS was depolymerized by a controlled free radical process. The structure of the depolymerized DS has been studied by various techniques; DS with different M_r have been evaluated on HCII and on bacterial lyase (chondroitinase ABC)[5] activity. Besides, high M_r fractions with high charge density have been isolated from natural DS through SAX-HPLC. The purity of products has been determined by agarose electrophoresis or specified enzymatic depolymerization by bacterial lyases.

EXPERIMENTAL

Extraction and purification of dermatan sulfate.—DS extraction and purification was performed as reported by Volpi et al.[5]. 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 product was filtered, and the solution containing the polysaccharides was percolated through a SAX resin column (Amberlite IRA-900, Rohm & Haas Co.). DS was eluted with 1.7–1.8 M NaCl, and the recovered solution was treated with

0.3–0.5 vol of acetone. The precipitate was discarded and 1.0–1.5 vol of acetone were added to the filtrate. The DS was purified by selective precipitation with copper acetate and acetone. The DS copper salt was transformed into the DS sodium salt by cation-exchange resin. The crude DS sodium salt was collected by precipitation with 1.0–1.5 vol of acetone and dried.

Preparation of different relative molecular mass fragments from dermatan sulfate.—Different M_r fragments were obtained by controlled chemical depolymerization of DS induced by free radicals. Five g of DS and 0.2 g of copper acetate monohydrate (0.02 M) were dissolved in 50 mL of water in a reaction vessel. The temperature was kept at 60°C and the pH adjusted to 7.5 by adding N NaOH solution. A 9% hydrogen peroxide solution was added at a rate of 10 mL/h. The reaction was stopped at different times, and after the reaction, Chelex 100 chelating resin (Bio-Rad, 142-2832) was utilized to remove contaminating copper from the product, and a SAX resin in the OH^- form was used to remove acidic contaminants. The pH of the percolate was adjusted to 6.0 by the addition of excess acetic acid, and then 2 vol of acetone were added. The precipitate was collected by filtration, washed with acetone and dissolved again in 100 mL of water. Five g of sodium acetate was added to this solution, and then the LMW-DS sodium salt was precipitated by 2 vol of acetone. The precipitate was collected and dried. DS with different M_r were prepared by stopping the chemical depolymerization process at different times.

Charge fractionation of native dermatan sulfate.—HPLC Jasco equipment was used for semi-preparative SAX separation; 750 mg of native DS (peak M_r, 25800) was fractionated on a 30 cm × 7.8 mm column packed with 10 g of 10-μm Spherisorb-S10-SAX resin ([trimethylammoniopropyl groups $Si-CH_2-CH_2-CH_2-N^+(CH_3)_3$ in the Cl^- form] from Phase Separations Limited, Deeside Industrial Park, Deeside, Clwyd, UK). 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 for each fraction were collected.

The different NaCl mole fractions were concentrated by use of a Rotavapor; dialyzed with molecularporous dialysis membranes (Spectrapore dialysis tubing from Spectrum; M_r cut-off, 1000) against a decreasing molarity NaCl solution and then against bidistilled water; finally, they were concentrated and lyophilized. The fractions were analysed and characterized.

Determination of relative molecular mass.—HPLC equipment was from Jasco: pump model 880 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 N NaOH. Flow rate was 0.9 mL/min with a back pressure of 25 Kg/cm^2. Two columns of Protein Pak 125 and 300 assembled in a series (Waters, 84601 and T72711) were used. Different M_r GAGs were solubilized in the mobile phase at a concentration of 5 mg/mL; an aliquot (10 μL, 50 μg) was injected into the HPLC.

The M_r was determined by a calibration curve plotted with GAG standards whose M_r were evaluated by analytical ultracentrifuge, according to Nieduszynski [13]. A third-grade polynomial regression was performed with a Macintosh computer program.

Determination of sulfate-to-carboxyl ratio and uronic acid percentage.—Sulfate and carboxyl groups were determined by potentiometric titration[14] with 0.1 N NaOH in water–DMF of the DS acid obtained by removal of the metal cations and possible contaminating anions by ion-exchange resins (Amberlite IRA-400, strongly basic polystyrene gel-type resin and Amberlite IR-120, strongly acidic polystyrene gel-type resin; Rohm & Haas Co.). The sulfate-to-carboxyl ratio was also determined by enzymatic degradation after HPLC separation of constituent disaccharides (see structural analysis of LMW-DS). The ratio was calculated considering the percentage and the presence of carboxyl and sulfate groups for each disaccharide.

Specific optical rotation.—The specific optical rotation was determined at 25°C on 10-mL samples of 5% (w/v) of DS in water.

Determination of the purity of dermatan sulfate preparations.—Possible contaminant GAGs i.e., CS A and C, "slow-moving" components of Hep (constituted by the most highly sulfated and higher-M_r species) and "fast-moving" components of Hep (less sulfated and lower-M_r species[15]) in the preparations of DS were determined by gel electrophoresis on agarose in barium trimethylenediamine. An agarose solution (0.50%) in barium acetate buffer (0.04 M, pH 5.8) was prepared by heating at 100°C. After cooling to 60°C, the solution was layered (2-mm thickness) on glass plates (5 × 7.5 cm) and cooled to 4°C; 5-μL samples (1 mg/mL of GAGs in water) were deposited by micropipet.

The first run was performed in barium acetate buffer 0.04 M pH 5.8 for 30 min at 60 mA (~ 120 V) per plate and the second in propanediamine buffer 0.05 M, pH 9.0, for 60 min at 50 mA (~ 120 V) per plate. After migration, the plate was soaked into cetavlon (1% solution) for ~3 h, dried and then stained with Toluidine Blue (0.2% in 50:49:1 EtOH–water–acetic acid) for 30 min. After decoloration with 50:49:1 EtOH–water–acetic acid, quantitative analysis of GAGs was performed by a photodensitometer at 583 nm. Calibration curves were obtained by measuring the absorbance of increasing concentration (1 to 6 μg) of CS A and C (Sigma, C-4134, Sigma C-4384), "fast moving" and "slow moving" components of Hep (prepared as described[15]) and DS (Fig. 1).

Small amounts of Hep (and/or HS) as contaminants in DS preparation were detected by enzymatic degradation by chondroitinase ABC (EC 4.2.2.4) and agarose electrophoresis. This procedure is necessary to determine small amounts of Hep that could tightly bind the polysaccharidic chains of DS; 500 μg of DS (500 μg/10 μL of distilled water) were treated with 0.5 units of chondroitinase ABC in 40 μL of 50 mM Tris · HCl buffer, pH 8.0. After incubation at 37°C for 3 h and boiling for 1 min, 5 μL of the solution was deposited on the agarose plate as reported, and quantified by calibration curves.

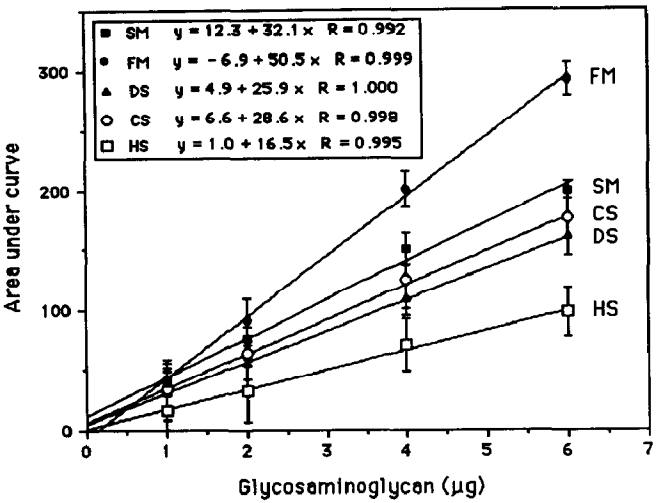


Fig. 1. Calibration curves for "fast moving" Hep (FM), "slow moving" Hep (SM), heparan sulfate (HS), Dermatan sulfate (DS), and chondroitin sulfate (CS) measured in agarose-gel electrophoresis.

The presence of Hep (and/or HS) in DS preparations has also been evaluated by specific degradation with heparinase I (EC 4.2.2.7), heparinase II (no assigned EC number), and heparinase III (EC 4.2.2.8), performed as reported by Volpi et al.[16] and SAX-HPLC analysis. The presence of Hep was evaluated by measuring the unsaturated trisulfated disaccharide [*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-(1 → 4)-2-deoxy-6-*O*-sulfo-2-sulfoamino-D-glucose] and the presence of HS by measuring the unsaturated nonsulfated disaccharide [*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-(1 → 4)-2-acetamido-2-deoxy-D-glucose] developed by enzymatic degradation. This procedure revealed 0.5% Hep (w/w) contaminating the DS preparations.

Quantitation of constituent disaccharides by cleavage with chondroitinase ABC.— A sample (100 μg, 10 mg/mL in H_2O) of native DS or LMW-DS was incubated with 625 mU of chondroitinase ABC, (EC 4.2.2.7) (Seikagaku Kogyo, 100320) 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 SAX-HPLC[17]. HPLC equipment was from Jasco as reported. The column used was Spherisorb 5 SAX, 250 × 4.6 mm. Isocratic separation was performed from 0 to 5 min with 0.20 M NaCl pH 4.00; linear gradient separation was from 5 to 60 min with 100% 0.20 NaCl, pH 4.00, to 100% 1.20 M NaCl, pH 4.00. The flow rate was 1.4 mL/min. The UV wavelength was set at 232 nm. Thirty μg of enzymatically degraded DS was injected (20 μL).

Separation of unsaturated disaccharides of DS was performed according to the standards and retention times of Seikagaku Kogyo[18,19].

Quantitative determination of the different sulfated disaccharides and tetrasaccharides by HPSEC.—HPLC Jasco equipment connected with Columns Protein Pak 60 and 125 assembled in series was used.

Determination of M_r and the relative concentration of the two main species of oligosaccharides (disaccharides and tetrasaccharides), obtained by enzymatic cleavage of native DS and different M_r DS, were performed by the use of purified disaccharides (Seikagaku Kogyo) and oligosaccharides having known M_r.

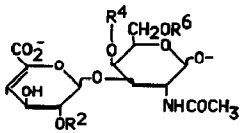
Biological activity in vitro.—The biological activities of native DS, different M_r DS, and DS fractions obtained by SAX-HPLC were measured on HCII (Kit Stachrom by Stago)[20]. DS were incubated with a known amount of thrombin in the presence of purified HCII at 37°C. After 10 s, the chromogenic substrate CBS 34.47 was added. Three min later, the reaction was stopped by the addition of acetic acid and the thrombin amidolytic activity was measured at 405 nm. The DS activity on HCII was calculated in comparison with WHO IV Hep international standard and expressed as units/mg.

RESULTS AND DISCUSSION

The beef mucosa DS has been characterized by different analytical techniques. M_r was calculated by a third grade polynomial curve that fits the experimental retention time vs. the log of M_r of 12 different M_r GAG standards[21]. The sulfate-to-carboxyl ratio was calculated by considering the disaccharide pattern of DS after cleavage by chondroitinase ABC and the potentiometric titration in water–DMF at different ratios. Table II reports the physicochemical properties and biological activity on HCII of native DS.

TABLE I

Proportions of unsaturated nonsulfated and sulfated disaccharides derived from the polysaccharide chains of beef mucosa dermatan sulfate by specific enzymatic cleavage



Disaccharide	Proportion (% of total)	Structure (see formula above)		
		R^2	R^4	R^6
ΔDi-0S	≤ 1%	H	H	H
ΔDi-6S	≤ 3%	H	H	SO_3^-
ΔDi-4S	≤ 86%	H	SO_3^-	H
ΔDi-4, 6diS	≤ 1%	H	SO_3^-	SO_3^-
ΔDi-2, 6diS	≤ 0.5%	SO_3^-	H	SO_3^-
ΔDi-2, 4diS	≈ 8%	SO_3^-	SO_3^-	H
ΔDi-2, 4, 6triS	≤ 0.5%	SO_3^-	SO_3^-	SO_3^-

TABLE II

Physicochemical characteristics and biological activity of purified beef mucosa dermatan sulfate

Peak M_r	25800
Sulfate-to-carboxyl ratio (titrimetric)	1.12
Sulfate-to-carboxyl ratio (enzymatic cleavage)	1.09
Optical rotation	−55°
Agarose electrophoresis	
mobility %	62
percentage of glycosaminoglycan	100%
HCII activity (units/mg)	176

The different values of sulfate-to-carboxyl ratio of DS by titrimetric determination in the presence of different percentages of DMF in water is shown in Fig. 2. Considering the sulfate-to-carboxyl ratio determined by enzymatic lyase (see Table I) to be 1.09, the more reliable percentage of DMF in water, calculated from the experimental curve reported in Fig. 2, is 42. The determination of the sulfate-to-carboxyl ratio of DS in water in presence of 35–45% of DMF was in good agreement with that determined by enzymatic cleavage.

The absence of other GAGs as contaminants in the preparations of DS is important for biological and pharmacological studies. In fact, small amounts of Hep can modify the biological activity with regard to HCII. Then, small amounts of Hep and other GAGs could aggregate as supramolecular complexes with DS chains in vitro and in tissues[22]. To date, the biological and pharmacological effects of these complexes have been poorly understood[23]. DS is known to cause either self-association or association with other GAGs[24]. The current analytical techniques, such as agarose electrophoresis, HPSEC, specific optical rotation, and NMR[25], are not sensitive enough to quantify small amounts of Hep as contaminants in preparations of DS. The DS extracted and purified from beef mucosa was controlled for possible Hep (and/or HS) contamination. The GAGs formed by enzymolysis with chondroitinase ABC were quantified at 2% (w/w) and were found to be similar to HS or "fast moving" Hep. The DS was reprocessed by the

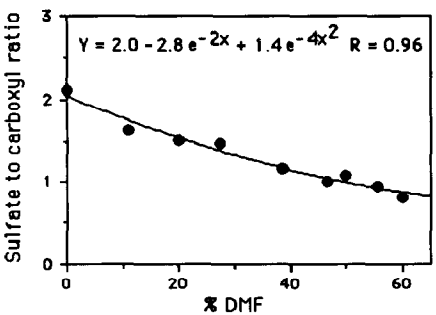


Fig. 2. Values of the sulfate-to-carboxyl ratio of dermatan sulfate given by titrimetric determination in the presence of varying percentages of DMF in water.

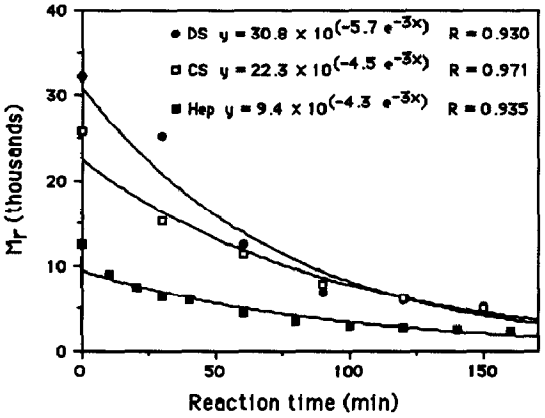


Fig. 3. Chemical process of glycosaminoglycan depolymerization. Coefficients of exponential regression and correlation coefficients are shown for heparin (Hep), dermatan sulfate (DS), and chondroitin sulfate (CS).

main steps of purification until no appreciable GAG contaminants (< 0.2%) were detected.

Different M_r DS were prepared by controlled chemical degradation. The depolymerization of DS was controlled through reaction kinetics calculated by withdrawing the samples at different times and evaluating their M_r by HPSEC. M_r decreases with time according to an exponential-like function. The same reaction kinetics has been observed for chemical depolymerization of CS (from bovine trachea) and Hep (from beef mucosa) (Fig. 3). No significant differences were detected for the sulfate-to-carboxyl ratio, agarose electrophoresis, constituent disaccharides, or ^{13}C NMR spectra of LMW-DS in comparison with unfractionated DS. This confirms the retention of the primary structure of DS after chemical depolymerization, as previously reported for Hep[16].

By enzymatic cleavage with chondroitinase ABC of LMW fractions of DS we can obtain different sulfated disaccharides and tetrasaccharides. Fig. 4 reports the

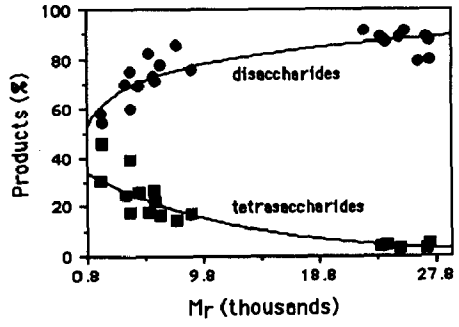


Fig. 4. Relative percentages of disaccharides and tetrasaccharides obtained from dermatan sulfate by enzymatic cleavage, as a function of polymer molecular weight. Values determined by HPSEC.

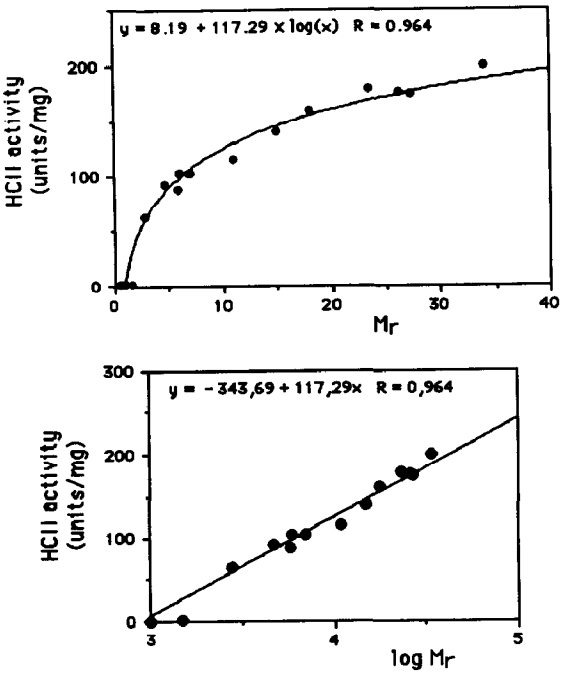


Fig. 5. Heparin cofactor II activity of dermatan sulfates obtained from the chemical process as a function of M_r. The equations of the experimental curves and their correlation coefficients are shown.

relative percentage of disaccharides and tetrasaccharides, obtained by enzymatic cleavage, of DS having different M_r as estimated by HPSEC. The percentages of tetrasaccharides (fragments not depolymerized by enzyme) increase in inverse proportion to the M_r of DS. In particular, the percentage of tetrasaccharides derived from DS of M_r 2000–3000 approaches 50%. Similar results[16] were obtained with different M_r Hep by cleavage with heparinases I, II, and III. Bacterial lyases with different substrate specificity (DS or Hep) show the same behaviour in cleaving different M_r fractions. In particular, they do not yield 100% disaccharides when catalyzing the cleavage by β-elimination of LMW fractions. Different percentages of tetrasaccharides remain uncleaved.

Different M_r DS have been evaluated for their anticoagulant activity by inhibition of HCII. The HCII activity decreases in proportion to the M_r of DS following a logarithmic-like function (Fig. 5). A linear plot can be calculated for the log of M_r of DS vs. HCII activity. The biological activity of different M_r DS on HCII is similar to that of different M_r Hep expressed as APTT units and AXa units[16]. Biological activity in vitro decreases with different M_r Hep and DS: AXa activity by Hep and HCII activity by DS decreases according to a logarithmic-like function and, to the contrary, APTT activity by Heps decreases according to a linear function[16]. The potentiation of thrombin inhibition (and prolongation of APTT activity) by Hep requires at least an oligosaccharide chain of about 18–20

TABLE III

Percentage recovery, M_r, sulfate-to-carboxyl ratio, and HCII activities of charge fractionated dermatan sulfate fractions

M NaCl	Recovery (%)	$10^{-3} \times M_r$	SO_3^-/COO^-	HCII activity (units/mg)
0.10	63.1	24.9	1.08	172
0.25	2.6	25.6	1.07	158
0.50	2.2	26.4	1.07	167
0.75	2.3	4.2	0.92	31
1.00	2.5	6.9	1.04	40
1.25	16.6	20.0	1.13	208
1.50	8.8	23.5	1.19	288
1.75	1.3	33.3	1.16	334
2.00	0.5	39.2	1.24	350

units ($M_r = 5000–6000$)[26]. On the other hand, the antifactor Xa activity of Hep fractions having different M_r decreases more slowly and remains high even for $M_r = 2000–3000$ fractions. This is consistent with the model of interaction between ATIII, factor Xa, and Hep described by Lane et al.[27]. Fragments of DS containing a minimum of 12–14 sugar residues[9] are necessary to increase the inhibition of thrombin by HCII. The mechanism of interaction between DS, thrombin, and HCII could be similar to that described for Hep, thrombin, and ATIII, and different in comparison with the interaction between Hep, factor Xa, and ATIII (ref 27).

Beef mucosa DS was submitted to charge fractionation by SAX-HPLC. DS fractions with different M_r, disaccharide patterns, and biological properties were obtained at different NaCl ionic strength (from 0.1 to 2.0 M). Table III reports the percentage recovery, M_r, sulfate-to-carboxyl ratio, and activity on HCII of differ-

TABLE IV

Composition of charge fractionated dermatan sulfate fractions

DS fraction (M NaCl)	Disaccharides in enzymatic digest (%)[a]						
	ΔDi-0S	ΔDi-6S	ΔDi-4S	ΔDi-2,6diS	ΔDi-4,6diS	ΔDi-2,4diS	ΔDi-2,4,6triS
Native	1.3	3.0	84.7	0.3	1.4	9.2	0.0
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

[a] For structures of the disaccharides see Table I and formula.

ent charge fractionated DS, whereas the disaccharide pattern is shown in Table IV. The first fractions, from 0.1 to 0.5 M, are similar in their physicochemical characteristics to native DS and they constitute ~68% of its weight. Two DS fractions, at 0.75 and 1.00 M, have low M_r and sulfate-to-carboxyl ratio. These fractions, that represent about 4.5% of the native DS, can be considered the "fast moving" component of DS. The charge fractionated DS fractions from 1.25 to 2.00 M are eluted depending on their M_r and charge density. The fractions eluted at 1.25 and 1.50 M NaCl represent a significant percentage of native DS: 16.6 and 8.8%. The HCII activity of fractions eluted with high NaCl molar strength (from 1.25 to 2.00 M) increases depending on higher M_r and charge density. The increase of HCII activity of these fractions could depend on the presence of large amounts of (4,6)-disulfated disaccharide in comparison to native DS (Table IV) according to Linhardt et al.[6].

The aim of this work was to gain a finer knowledge of the structure and biological activity of beef DS. Different analytical approaches and techniques are required for more reliable information on the structure and physicochemical properties of natural and LMW derived fraction DS. Another key point is the knowledge of the possible presence of GAG contaminants the preparations used for biological and, expecially, pharmacological purposes: the presence of small amounts of contaminating GAGs can change the properties of DS. Several questions remain to be explained and further investigations will be required to establish the relationship between the structure and physicochemical properties of DS, M_r, charge density, position of sulfate groups, and in vitro and in vivo activity.

ACKNOWLEDGMENT

The research was supported by a 60% grant from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (M.U.R.S.T.).

REFERENCES

1 E.F. Mammen (Ed.), *Seminars in Thrombosis and Hemostasis*, Vol. 17, suppl. 1-2, Thieme Medical, New York, 1991.
2 H. Engelberg, in E.F. Mammen (Ed.), *Seminars in Thrombosis and Hemostasis*, Vol. 17, suppl. 1-2, Thieme Medical, New York, 1991, pp 5–8.
3 F.A. Ofosu, I. Danishefsky, and J. Hirsh (Eds.), *Ann. N.Y. Acad. Sci.*, 556 (1989).
4 M.O. Longas, *Anal. Biochem.*, 187 (1990) 355–358.
5 N. Volpi, G. Mascellani, P. Bianchini, and L. Liverani, *Farmaco*, 47 (suppl. 5) (1992) 841–853.
6 R.J. Linhardt, A. Al-Hakim, S.V. Liu, Y.S. Kim, and J. Fareed, in E.F. Mammen (Ed.), *Seminars in Thrombosis and Hemostasis*, Vol. 17, suppl. 1-2, Thieme Medical, New York, 1991, pp 15–22.
7 L. Fransson, B. Havsmark, and I. Silverberg, *Biochem. J.*, 269 (1990) 381–388.
8 D.M. Tollefsen, C.A. Pestka, and W.J. Monafo, *J. Biol. Chem.*, 258 (1983) 6713–6717.
9 D.M. Tollefsen, M.E. Peacock, and W.J. Monafo, *J. Biol. Chem.*, 261 (1986) 8854–8858.
10 M.M. Maimone and D.M. Tollefsen, *J. Biol. Chem.*, 265 (1990) 18263–18271.
11 J. Van Ryn-McKenna, F.A. Ofosu, E. Gray, J. Hirsh, and M.R. Buchanan, *Ann. N.Y. Acad. Sci.*, 556 (1989) 305–312.

12 D.P. Thomas, E. Merton, and T.W. Barrowcliffe, *Ann. N.Y. Acad. Sci.*, 556 (1989) 313–322.
13 I. Nieduszynski, in D.A. Lane and U. Lindahl (Eds.), *Heparin, Chemical and Biological Properties, Clinical Applications*, Edward Arnold, London, 1989, pp 51–63.
14 K.E. Kuettner and A. Lindenbaum, *Biochim. Biophys. Acta*, 101 (1965) 223–229.
15 P. Bianchini, H.B. Nader, H.K. Takahashi, B. Osima, A.H. Straus, and C.P. Dietrich, *J. Chromatogr.*, 196 (1980) 455–462.
16 N. Volpi, G. Mascellani, and P. Bianchini, *Anal. Biochem.*, 200 (1992) 100–107.
17 R.J. Linhardt, K.G. Rice, Y.S. Kim, D.L. Lohse, H.M. Wang, and D. Loganathan, *Biochem. J.*, 254 (1988) 781–795.
18 K. Murata, Y. Yokoyama, and K. Yoshida, *J. Chromatogr.*, 496 (1989) 27–33.
19 K. Yoshida, S. Miyauchi, H. Kikuchi, A. Tawada, and K. Tokuyasu, *Anal. Biochem.*, 177 (1989) 327–334.
20 D. Dupouy, P. Sie, and B. Boneu, *Thromb. Haemostasis*, 60 (1988) 236–239.
21 N. Volpi and L. Bolognani, *J. Chromatogr.*, 630 (1993) 390–396.
22 J.E. Scott, *FASEB J.*, 6 (1992) 2639–2645.
23 T.J. Racey, P. Rochon, F. Mori, and G.A. Neville, *J. Pharm. Sci.*, 78 (1989) 214–218.
24 L.A. Fransson and L. Cöster, *Biochim. Biophys. Acta*, 582 (1979) 132–144.
25 G.A. Neville, F. Mori, K.R. Holme, and A.S. Perlin, *J. Pharm. Sci.*, 78 (1989) 101–104.
26 E. Holmer, K. Söderberg, D. Bergqvist, and U. Lindahl, *Haemostasis*, 16 (suppl. 2) (1986) 1–13.
27 D.A. Lane, J. Denton, A.M. Flynn, L. Thunberg, and U. Lindahl, *Biochem. J.*, 218 (1984) 725–734.