Carbohydrate Research, 158 (1986) 183–190
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

CHEMICAL SULFATION OF PREPARATIONS OF CHONDROITIN 4- AND 6-SULFATE, AND DERMATAN SULFATE. PREPARATION OF CHONDROITIN SULFATE E-LIKE MATERIALS FROM CHONDROITIN 4-SULFATE

KINZO NAGASAWA*, HIDEKI UCHIYAMA, AND NORIE WAJIMA
School of Pharmaceutical Sciences, Kitasato University, 9-1, Shirokane 5 chome, Minato-ku, Tokyo 108 (Japan)

(Received February 10th, 1986; accepted for publication, April 1st, 1986)

ABSTRACT

A solution of the tributylammonium salts of chondroitin 4- or 6-sulfate, or dermatan sulfate in *N,N*-dimethylformamide was treated with 2.0–8.0 mol/hydroxyl group of pyridine–sulfur trioxide at 0° for 1 h. The progress of the sulfation was studied by chondroitinase ABC digestion and liquid chromatography. The results suggested that sulfation proceeded homogeneously according to the order of reactivity of the hydroxyl groups. Various chondroitin polysulfates, which resemble natural chondroitin sulfate E with respect to the disaccharide unit composition, were prepared from chondroitin 4-sulfate.

INTRODUCTION

The present study was initiated to establish the order of reactivity toward sulfation of the hydroxyl groups of chondroitin 4- and 6-sulfate (C-4-S and C-6-S), and of dermatan sulfate, and to develop a method for preparing polysulfated derivatives of these polysaccharides. To achieve this, (a) sulfation must be carried out in completely homogeneous medium[1,2], (b) the progress of the sulfation must be easily controllable, and (c) it must be free from side reactions, such as cleavage or transfer of the glycosidic linkages and decomposition of the sugar components. Recently, we devised a sulfation procedure that satisfies to some degree these requirements, and we report herein the preparation of chondroitin sulfate E-like materials.

RESULTS AND DISCUSSION

Commercial C-4-S and -6-S were successively purified by ion-exchange chromatography on AG 1-X2 anion-exchange resin and by gel filtration on

*To whom correspondence should be addressed.

0008-6215/86/$ 03.50

Sepharose 6B gel, respectively. The rooster-comb dermatan sulfate, RC-20 fraction[3], was fractionated by ion-exchange chromatography to obtain the fractions eluted with 2.0M sodium chloride on Dowex 1-X2 (Cl^-) anion-exchange resin. The K_{av} values obtained by analytical gel-filtration of these purified materials on Sepharose 6B were 0.41, 0.31, and 0.31 for C-4-S and -6-S, and dermatan sulfate, respectively. The composition of chondroitinase ABC digestion products and sulfur content are reported in Tables I–III.

As shown in Table I, the starting C-4-S used was a copolymer consisting mainly of chondroitin 4-sulfate (81%) and 6-sulfate units (17%). The tributylammonium salt was treated with 2.0–8.0 mol/mol equiv. of hydroxyl group of

TABLE I

ANALYTICAL DATA OF SULFATED PRODUCTS OF CHONDROITIN 4-SULFATE

Composition	*Starting chondroitin 4-sulfate*	*Sulfated chondroitin 4-sulfate*				*Chondroitin sulfate E*[b]
		Prep. I (2.0)[a]	*Prep. II (4.0)*	*Prep. III (6.0)*	*Prep. IV (8.0)*	
S content (mol/repeating unit)	1.02	1.26	1.60	1.72	2.04	1.55
Unsaturated disaccharide composition (%)						
ΔDi	1.3	0.8	0.4	0.3	0.2	0
ΔDi-6*S*	17.0	17.0	17.1	17.1	13.8	8.0
ΔDi-4*S*	81.0	70.1	33.2	17.9	10.6	30.0
ΔDi-diS_D	0.7	0.4	1.1	1.5	2.1	0
ΔDi-diS_E	0	11.7	48.2	58.4	66.0	62.0
ΔDi-tri*S*	0	0	0	4.8	7.3	

[a]Mol of pyridine–sulfur trioxide per mol equivalent of available hydroxyl group. [b]Ref. 7.

TABLE II

ANALYTICAL DATA OF SULFATED PRODUCTS OF CHONDROITIN 6-SULFATE

Composition	*Starting chondroitin 6-sulfate*	*Sulfated chondroitin 6-sulfate*		
		Prep. I (3.0)[a]	*Prep. II (5.0)*	*Prep. III (7.0)*
S content (mol/repeating unit)	1.08	1.17	1.29	1.48
Unsaturated disaccharide composition (%)				
ΔDi	1.3	0.9	0	0
ΔDi-6*S*	73.9	74.6	74.0	72.3
ΔDi-4*S*	17.7	14.1	2.6	0.8
ΔDi-diS_D	7.1	7.1	8.5	9.9
ΔDi-diS_E	0	3.3	13.8	14.9
ΔDi-tri*S*	0	0	1.1	2.1

[a]See footnote to Table I.

TABLE III

ANALYTICAL DATA OF SULFATED PRODUCTS OF DERMATAN SULFATE

Composition	*Starting dermatan sulfate*	*Sulfated dermatan sulfate*		
		Prep. I (2.0)[a]	*Prep. II (4.0)*	*Prep. III (6.0)*
S content (mol/repeating unit)	1.04	1.27	2.01	2.29
Unsaturated disaccharide composition (%)				
ΔDi	4.4	2.5	1.7	0
ΔDi-6*S*	3.0	4.4	1.4	1.5
ΔDi-4*S*	84.2	70.8	28.3	9.2
ΔDi-diS_D	0.6	1.6	4.3	6.0
ΔDi-diS_B	7.0	4.4	2.3	0
ΔDi-diS_E	0.8	14.6	56.4	76.9
ΔDi-tri*S*	0	1.7	5.6	6.4

[a]See footnote to Table I.

pyridine–sulfur trioxide in *N,N*-dimethylformamide at 0° for 1 h in homogeneous solution. The progress of sulfation was dependent on the amount of pyridine–sulfur trioxide used. The progress of the sulfation was monitored by digesting Preparations I–IV (Table I) with chondroitinase ABC and analyzing the digestion products by liquid chromatography under elevated pressure (l.c.)[4]. The data clearly showed the most important process to be the transformation of chondroitin 4-sulfate into chondroitin 4,6-disulfate units; for Preparation II, 60% of the chondroitin 4-sulfate units of the starting material were transformed into chondroitin 4,6-disulfate units without any change of the structure of other disaccharide units. On the contrary, transformation of chondroitin 6-sulfate units into chondroitin 4,6-disulfate units did not occur under the conditions used for Preparations I and II, although chondroitin 6-sulfate units were partially transformed into chondroitin disulfate (D type) units and chondroitin trisulfate units under the reaction conditions used for Preparation IV. These results suggest that the 6-sulfate group on the 2-acetamido-2-deoxy-D-galactose residue of the chondroitin 6-sulfate unit may prevent the 4-HO group on the same monosaccharide residue from undergoing sulfation.

As shown in Table I, the decrease of ΔDi-4*S** of Preparations I and II well correspond to the respective increase of ΔDi-diS_E. The decrease of ΔDi-4*S* of

*Abbreviations used: ΔDi, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose; ΔDi-4*S*, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 4-sulfate; ΔDi-6*S*, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 6-sulfate; ΔDi-diS_D, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2- or 3-sulfate)-D-galactose 6-sulfate; ΔDi-diS_B, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2- or 3-sulfate)-D-galactose 4-sulfate; ΔDi-DiS_E, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 4,6-bis(sulfate); ΔDi-tri*S*, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2- or 3-sulfate)-D-galactose 4,6-bis(sulfate); ΔDi-U*S*, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2- or 3-sulfate)-D-galactose.

Preparation III correspond to the sum of the increase of ΔDi-diS_E and ΔDi-tri*S* (%). These results strongly suggest that the chondroitin trisulfate unit was preferentially formed by sulfation of the chondroitin 4,6-disulfate (E type) unit, and not by that of the chondroitin disulfate (D type) unit.

As shown in Table II, C-6-S was more difficult to sulfate than C-4-S. This seems to be exclusively due to the marked resistance of the chondroitin 6-sulfate unit against further sulfation, as the data suggest that the proportion of chondroitin 6-sulfate units remains unchanged in Preparations I–III, and the increase in sulfur content depends mostly on the transformation of the chondroitin 4-sulfate units present in chondroitin 4,6-disulfate units. These data also indicate that a limited proportion of the chondroitin 6-sulfate units underwent sulfation to give chondroitin disulfate (D type) units. Accordingly, more drastic reaction conditions would result in the formation of additional chondroitin disulfate (D type) units and chondroitin trisulfate units as suggested by the results obtained previously[5].

Subsequently, a preparation of rooster-comb dermatan sulfate was subjected to sulfation under the same conditions as just described. The preparation of dermatan sulfate investigated contained more disaccharide 4-sulfate and less disaccharide 6-sulfate units than the preparation of C-4-S. As shown in Table III, the sulfation of the polysaccharide was very similar to that of C-4-S, indicated by the preponderant transformation of disaccharide 4-sulfate into the disaccharide 4,6-disulfate units. The data also show a slow but steady transformation of the disaccharide disulfate (B type) into disaccharide trisulfate units for Preparations I–III, suggesting that HO-6 of the 2-acetamido-2-deoxy-D-galactose residue of the disaccharide disulfate (B type) units is highly sensitive to sulfation under the reaction conditions used. Comparison between Tables I and III revealed that the sulfated products of dermatan sulfate were more sulfated than those of C-4-S, owing in part to the preponderance of unsubstituted HO-6 groups in the disaccharide units of dermatan sulfate (Table III), as compared with C-4-S (Table I), and in part to some difference in the three-dimensional structures of both starting materials.

A sulfated C-4-S obtained by the present method (Preparation IV in Table I) was digested with chondroitinase ABC alone or chondroitinase ABC plus chondrosulfatases, and each digestion product was analyzed for unsaturated disaccharide composition by l.c. (Table IV). The data suggest that ΔDi-tri*S* was produced by chondroitinase ABC digestion of a disaccharide trisulfate unit, in which one sulfate group is located either at O-2 or -3 of the 4,5-unsaturated uronic acid residue and the other two sulfate groups at O-4 and -6 of the acetamido-2-deoxy-D-galactose residue.

The data of Tables I–III indicate that the sulfation progressed homogeneously according to the order of reactivity of the hydroxyl groups within the polysaccharide molecules. A gel-filtration analysis of Preparation III (Table I) did not show any scission of the polysaccharide chain (Fig. 1).

A chondroitin polysulfate (ChS-E), isolated from squid cranial cartilage[6,7], has been shown to have physiological roles in mammals[8–11]. The data in Table I

TABLE IV

UNSATURATED DISACCHARIDE COMPOSITION OF DIGESTION PRODUCTS GENERATED FROM SULFATED CHONDROITIN 4-SULFATE WITH CHONDROITINASE ABC ALONE, OR CHONDROITINASE ABC PLUS CHONDROSULFATASES

Unsaturated disaccharide composition (%)	*Digestion product of sulfated chondroitin 4-sulfate[a] with enzyme(s)*		
	Chondroitinase ABC alone[b]	*Chondroitinase ABC plus chondro-6-sulfatase*	*Chondroitinase ABC plus chondro-6- and -4-sulfatases*
ΔDi	0.2	14.4	90.7
ΔDi-U*S*	0	2.1	9.3
ΔDi-6*S*	13.8	0	0
ΔDi-4*S*	10.6	75.5	0
ΔDi-diS_D	2.1	0	0
ΔDi-diS_B	0	8.0	0
ΔDi-diS_E	66.0	0	0
ΔDi-tri*S*	7.3	0	0

[a]Preparation IV in Table I. [b]Data from Table I.

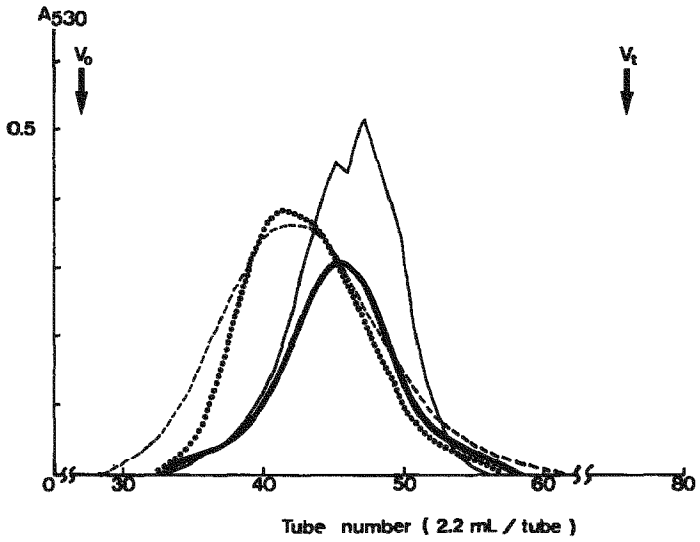

Fig. 1. Elution diagrams of starting chondroitin 4- and 6-sulfate and dermatan sulfate, and of sulfated chondroitin 4-sulfate on Sepharose 6B: (——) chondroitin 4-sulfate, (----) chondroitin 6-sulfate, (●●●●●●●) dermatan sulfate, and (——) sulfated chondroitin 4-sulfate (Preparation III, Table I).

suggest that several chondroitin polysulfates, which possess a similar degree of disaccharide unit composition as those of natural ChS-E preparations[8–11], may be obtained by sulfation of C-4-S. Thus, a sufficient supply of semi-synthetic ChS-E preparations will contribute to the study of the physiological functions of ChS-E.

EXPERIMENTAL

Materials. — Chondroitin 4-sulfate (whale cartilage, reagent grade) and chondroitin sulfate (shark cartilage, reagent grade), chondroitinase ABC from *Proteus vulgaris* (chondroitin sulfate lyase, EC 4.2.99.6), chondro-4- and -6-sulfatases from *Proteus vulgaris,* and 4,5-unsaturated disaccharide standards (ΔDi, ΔDi-4*S*, and ΔDi-6*S*) were obtained from Seikagaku Kogyo Co. Ltd. (Tokyo). The chondroitin 4-sulfate was further purified by ion-exchange chromatography on AG 1-X2 (Cl^-) resin with a linear gradient elution of 1.5–2.5M NaCl. The fraction eluted with 1.52–1.66M NaCl was used for the present experiments. A preparation of rooster-comb dermatan sulfate[3] (RC-20) was fractionated into 1.5 and 2.0M NaCl fractions by ion-exchange chromatography on Dowex 1-X2 (Cl^-) resin, and the 2.0M NaCl fraction, a dermatan sulfate–chondroitin sulfate copolymer having an IdoA/GlcA ratio of 41:9, was used for the present experiments. The elution diagrams of these starting materials on Sepharose 6B gel are shown in Fig. 1. Pyridine–sulfur trioxide obtained from Aldrich Chem. Co. Inc. (Milwaukee, Wis 53201) was purified according to the procedure described[12].

Analytical methods. — The uronic acid content was determined by the

modified method of Bitter and Muir[13,14]; the uronic acid composition of the dermatan sulfate by the procedure previously described[3]; and the sulfur content by the method of Dodgson and Price[15]. Analytical gel-chromatography on Sepharose 6B was carried out by the same procedure described previously[16], modified by increasing the NaCl concentration to 0.2M.

Determination of constitutional disaccharide composition in chondroitin 4- and 6-sulfate, and dermatan sulfate, and their sulfated products. — The determination was carried out according to the procedure of Seldin *et al.*[4]. To a solution of the sample (200 μg) in water (40 μL) were added 50mM Tris·HCl buffer (pH 8.0, 20 μL) containing 10mM NaF, and chondroitinase ABC (0.4 unit) in water (40 μL), and the mixture was incubated for 2 h at 37°. It was diluted with ethanol (4 vol.), cooled to 0° for 2 h, and centrifuged at 5500 *g* for 15 min. The supernatant (0.45 mL) was evaporated under a stream of nitrogen, and the residue dissolved in l.c. solvent *A* (3:1, v/v, 70% acetonitrile–methanol and 30% 0.2M ammonium acetate–acetic acid, pH 5.3) (36 μL). L.c. was performed with a chromatographic apparatus equipped with a liquid delivery pump (NP-D, 5SK25GK-A, Oriental motor Co., Tokyo), a variable-wavelength u.v. detector (S-310A model-II, Nihonseimitsu Co., Tokyo), a Whatman Partisil-10 PAC column (4.6 × 250 mm), and a precolumn (4.6 × 25 mm) containing the same packing. The u.v. absorbance of the column eluate was monitored with a detector at 232 nm. The unsaturated disaccharide (ΔDi) and unsaturated disaccharide monosulfates (ΔDi-4*S*, ΔDi-6*S*, and ΔDi-U*S*) were eluted with solvent *A*. The unsaturated disaccharide disulfates (ΔDi-diS_B, ΔDi-diS_D, and ΔDi-diS_E) and unsaturated disaccharide trisulfate (ΔDi-tri*S*) were eluted with solvent *B* (3:1, v/v, 70% acetonitrile–methanol and 30% 0.4M ammonium acetate–acetic acid, pH 5.3) and solvent *C* (3:1, v/v, 65% acetonitrile–methanol and 35% 0.55M sodium acetate–acetic acid, pH 5.3), respectively. The peaks of ΔDi, ΔDi-4*S*, and ΔDi-6*S* were assigned by comparison with the retention times of commercial standards. Each peak of the unsaturated disaccharide di- and tri-sulfates was assigned by comparison of the chromatographic data of each digestion product of the same sample with chondro-4- and chondro-6-sulfatase with those of reference samples (ΔDi, ΔDi-4*S*, and ΔDi-6*S*). The conditions of sulfatase digestion were as follows. To a solution of the sample (200 μg) in enriched Tris buffer[17] [0.3M sodium acetate, 0.25M NaCl, 0.25M Tris·HCl (pH 8.0), and bovine serum albumin (0.5 mg/mL)] (40 μL) was added chondroitinase ABC (0.4 unit) in water (20 μL). The mixture was incubated for 2 h at 37°, and then chondro-6-sulfatase (0.8 unit) in water (40 μL) alone, or chondro-4- and -6-sulfatases (each 0.8 unit) in water (40 μL) were added, and the incubation was continued for additional 16 h at 37°. The mixture was diluted with ethanol (4 vol.), cooled to 0° for 2 h, and centrifuged at 5500 *g* for 20 min. The precipitate was resuspended in water (0.1 mL) and the mixture centrifuged at 5500 *g* for 20 min. The combined supernatants were evaporated under a stream of N_2. The residue was dissolved in solvent *A* (40 μL) and analyzed by l.c. with the same solvent and the 4,5-unsaturated disaccharide standards (ΔDi, ΔDi-4*S*, and ΔDi-6*S*) as reference.

Sulfation of chondroitin 4- and 6-sulfates, and dermatan sulfate. — A solution of the Na salt of each polysaccharide (~50 mg) in water (5 mL) was passed through a column of Dowex 50W-X8 (H^+, 50–100 mesh) at 0°, and the pH of the effluent was adjusted to 5.0 by the addition of 10% tributylamine in ethanol. The solution was extracted three times with diethyl ether (each 50 mL) and lyophilized to give the tributylammonium salt (~50 mg) as a white powder.

To a solution of the tributylammonium salt of each polysaccharide (~6 mg) in *N,N*-dimethylformamide (0.5 mL) was added a solution of pyridine–sulfur trioxide (2.0–8.0 mol/equiv. of available hydroxyl group) in *N,N*-dimethylformamide (0.5 mL), and the mixture was stirred for 1 h at 0°. The reaction was terminated by the addition of cold water (1 mL) and the pH of the solution was adjusted to 9.0 with M NaOH. The solution was diluted with ethanol (3 vol.) saturated with anhydrous sodium acetate and kept for 1 h at 0° to give a white precipitate. The precipitate was collected by centrifugation at 1500 *g* for 15 min and dissolved in a small volume of water. The solution was dialyzed against distilled water (3 × 20 L) for 20 h and the dialyzate lyophilized to give the sodium salt of the sulfated product (4–5 mg) as a white powder.

REFERENCES

1 E. E. GILBERT, *Sulfonation and Related Reactions, Interscience,* New York, 1965, pp. 354–360.
2 J. F. KENNEDY, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 305–405.
3 K. NAGASAWA, A. OGAMO, H. ICHIHARA, AND K. YOSHIDA, *Carbohydr. Res.*, 131 (1984) 301–314.
4 D. C. SELDIN, N. SENO, K. F. AUSTEN, AND R. L. STEVENS, *Anal. Biochem.*, 141 (1984) 291–300.
5 A. OGAMO, A. NAGAI, AND K. NAGASAWA, *Biochim. Biophys. Acta,* 841 (1985) 30–41.
6 Y. KAWAI, N. SENO, AND K. ANNO, *J. Biochem. (Tokyo),* 60 (1966) 317–321.
7 S. SUZUKI, H. SAITO, T. YAMAGATA, K. ANNO, N. SENO, Y. KAWAI, AND T. FURUHASHI, *J. Biol. Chem.*, 243 (1968) 1543–1550.
8 E. RAZIN, R. L. STEVENS, F. AKIYAMA, K. SCHMID, AND K. F. AUSTEN, *J. Biol. Chem.*, 257 (1982) 7229–7236.
9 Y. HOJIMA, C. G. COCHRANE, R. C. WIGGINS, K. F. AUSTEN, AND R. L. STEVENS, *Blood,* 63 (1984) 1453–1459.
10 L. ENERBÄCK, S. O. KOLSET, M. KUSCHE, A. HJERPE, AND U. LINDAHL, *Biochem. J.*, 227 (1985) 661–668.
11 S. KOBAYASHI, K. OGURI, E. YAOITA, K. KOBAYASHI, AND M. OKAYAMA, *Biochim. Biophys. Acta,* 841 (1985) 71–80.
12 P. BAUMGARTEN, *Ber. Dtsch. Chem. Ges.*, 59 (1926) 1166–1171.
13 T. BITTER AND H. M. MUIR, *Anal. Biochem.*, 4 (1962) 330–334.
14 M. KOSAKAI AND Z. YOSIZAWA, *Anal. Biochem.*, 93 (1979) 295–298.
15 K. S. DODGSON AND R. G. PRICE, *Biochem. J.*, 84 (1962) 106–110.
16 H. UCHIYAMA, A. OGAMO, AND K. NAGASAWA, *Carbohydr. Res.*, 99 (1982) 87–92.
17 H. SAITO, T. YAMAGATA, AND S. SUZUKI, *J. Biol. Chem.*, 243 (1968) 1536–1542.