Glycobiology vol. 8 no. 9 pp. 869–877, 1998

Determination of the structure of oligosaccharides prepared from acharan sulfate

Yeong Shik Kim, Mi Young Ahn, Song Ji Wu, Dong-Hyun Kim[1], Toshihiko Toida[2], Lynn M.Teesch[3], Youmie Park[4], Guyong Yu[4], Jihon Lin[4] and Robert J.Linhardt[4,5]

Natural Products Research Institute, Seoul National University, Seoul 110–460 Korea, [1]College of Pharmacy, Kyunghee University, Seoul 130–701, Korea, [2]Faculty of Pharmaceutical Sciences, Chiba University, Chiba 263, Japan and [3]High Resolution Mass Spectrometry Facility and [4]Division of Medicinal and Natural Products Chemistry and Department of Chemical and Biochemical Engineering, University of Iowa, Iowa City, IA 52242, USA

Received on November 13, 1997; accepted on March 10, 1998

[5]To whom correspondence should be addressed at: University of Iowa, PHAR S328, Iowa City, IA 52242

The fine structure of acharan sulfate, a recently discovered glycosaminoglycan isolated from *Achatina fulica*, was examined. This glycosaminoglycan has a major disaccharide repeating unit of →4)-α-D-GlcNpAc(1→4)-α-L-IdoAp2S(1→ (where GlcNpAc is *N*-acetylglucosamine, IdoAp is iduronic acid, and S is sulfate) making it structurally related to both heparin and heparan sulfate. Using heparin lyases prepared from *Flavobacterium heparinum* and a newly isolated heparinase from *Bacteroides stercoris*, the controlled enzymatic depolymerization of acharan sulfate was undertaken to prepare a mixture of oligosaccharides. Fractionation of this mixture of oligosaccharides by strong-anion-exchange high performance liquid chromatography afforded oligosaccharides that capillary electrophoresis established were sufficiently pure for structural characterization. Electrospray ionization mass spectrometry identified two series of oligosaccharides, one derived from acharan sulfate's major repeating unit and a second minor group of undersulfated oligosaccharides. Proton nuclear magnetic resonance spectroscopy established the structure of these two classes of oligosaccharides to be ΔUAp2S(1→[4)-α-D-GlcNpAc(1→4)-α-L-IdoAp2S(1→]$_n$4)-D-GlcNpAcα,β (where n = 0,1,2,3 and ΔUAp is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid) and ΔUAp(1→[4)-α-D-GlcNpAc(1→4)-α-L-IdoAp2S(1→]$_m$-D-GlcNpAcα,β (where m = 1,2,3). These results suggest the presence of minor sequence variants in acharan sulfate containing unsulfated iduronic acid having the structure →4)-α-D-GlcNpAc(1→4)-α-L-IdoAp(1→.

Key words: acharan sulfate/oligosaccharide structure/heparin lyase/*Flavobacterium heparinum/Bacteroides stercoris*/electrospray ionization mass spectrometry

Introduction

Acharan sulfate is a glycosaminoglycan (GAG), having an average molecular weight of 29,000, which is isolated from the giant African snail, *Achatina fulica* (Kim *et al.*, 1996). This GAG has a major repeating disaccharide structure of →4)-α-D-GlcNpAc (1→4)-α-L-IdoAp2S(1→, where GlcNpAc is 2-acetamido 2-deoxy-glucopyranose, IdoAp is idopyranosyluronic acid, and S is sulfate. The structural determination of this GAG relied on high resolution NMR spectroscopy and degradative methods, including disaccharide analysis following its enzymatic breakdown with heparin lyase II (heparinase II) (Linhardt, 1994). NMR analysis shows that 90–95% of the GAG structure consists of its major disaccharide repeating unit. The remaining structure also contains unsulfated IdoAp. Treatment of acharan sulfate with heparin lyase II affords the expected disaccharide product, ΔUAp2S(1→4)-D-GlcNpAcα,β (where ΔUAp is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid) in 90% yield (Kim *et al.*, 1996). The remaining product consists of large oligosaccharides resistant to the action of this enzyme. From these data it was not possible to distinguish whether the IdoAp containing domains were an intimate part of the structure of acharan sulfate or simply a component of a second, minor contaminating polysaccharide (Kim *et al.*, 1996).

Acharan sulfate has a novel structure resembling both heparin and heparan sulfate, exhibiting interesting biological activity. Removal of the *N*-acetyl groups by treatment with hydrazine followed by *N*-sulfonation affords a polysaccharide with a repeating disaccharide unit of the structure →4)-α-D-GlcNpS (1→4)-α-L-IdoAp2S(1→. This sequence corresponds to the minimum required structure for binding to acidic and basic fibroblast growth factors (aFGF and bFGF) (Fahem *et al.*, 1996; Fromm *et al.*, 1997). Thus, *N*-sulfoacharan sulfate had a heparin-like effect on bFGF mitogenic activity (Wang *et al.*, 1997). Surprisingly, unmodified acharan sulfate, having the sequence →4)-α-D-GlcNpAc (1→4)-α-L-IdoAp2S(1→, also demonstrates an interesting mitogenic activity in the presence of heparin. Acharan sulfate interferes with heparin's effect on bFGF mitogenic activity, suggesting an important potential role for this polysaccharide as an inhibitor of angiogenesis (Wang *et al.*, 1997). Because of the importance of angiogenesis inhibitors in the treatment of diseases such as cancer (Folkman, 1995), studies on the fine structure of acharan sulfate were undertaken.

Results and discussion

Acharan sulfate was purified from *Achatina fulica* as previously described (Kim *et al.*, 1996). NMR spectroscopy of several acharan sulfate samples showed that in addition to its major repeating disaccharide unit, →4)-α-D-GlcNpAc(1→4)-α-L-IdoAp2S(1→, these preparations contained from approximately 5 to 15% unsulfated idopyranosyluronic acid (IdoAp). Complete depolymerization of a typical acharan sulfate sample with *Flavobacterial* heparin lyase II affords a single major product (~95%) (Kim *et al.*, 1996), as measured by strong-anion-exchange (SAX) high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) (not shown). Some of the larger oligosaccharides, the broad envelope of peaks eluting after **A8**, remain even following exhaustive treatment with *Flavobacterial* heparin lyase II (Kim *et al.*, 1996). Partial depolymerization of the same acharan sulfate sample affords a number of oligosaccharides as detected by SAX-HPLC (Figure 1A) and CE (not shown), consistent with the known endolytic

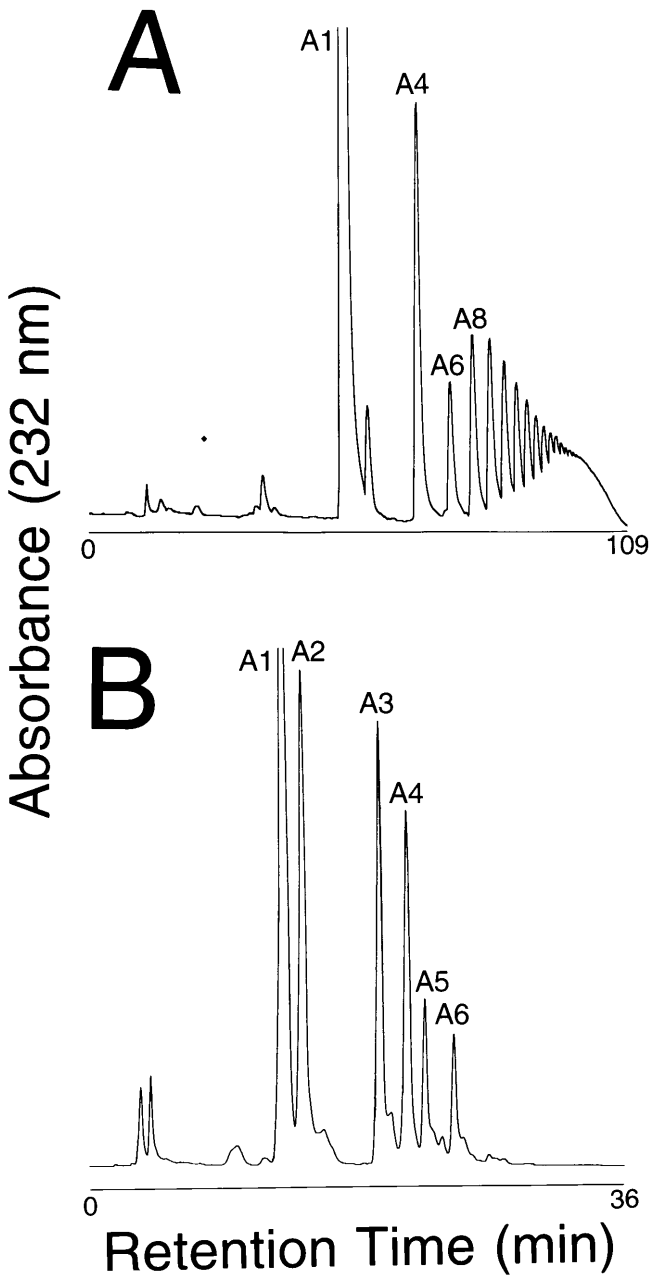

Fig. 1. SAX-HPLC analysis of acharan sulfate derived oligosaccharides. (**A**) Semipreparative SAX-HPLC of acharan sulfate partially (55% reaction completion) depolymerized by heparin lyase II; (**B**) analytical SAX-HPLC of acharan sulfate partially (47% reaction completion) depolymerized by *Bacteroides* heparin lyase.

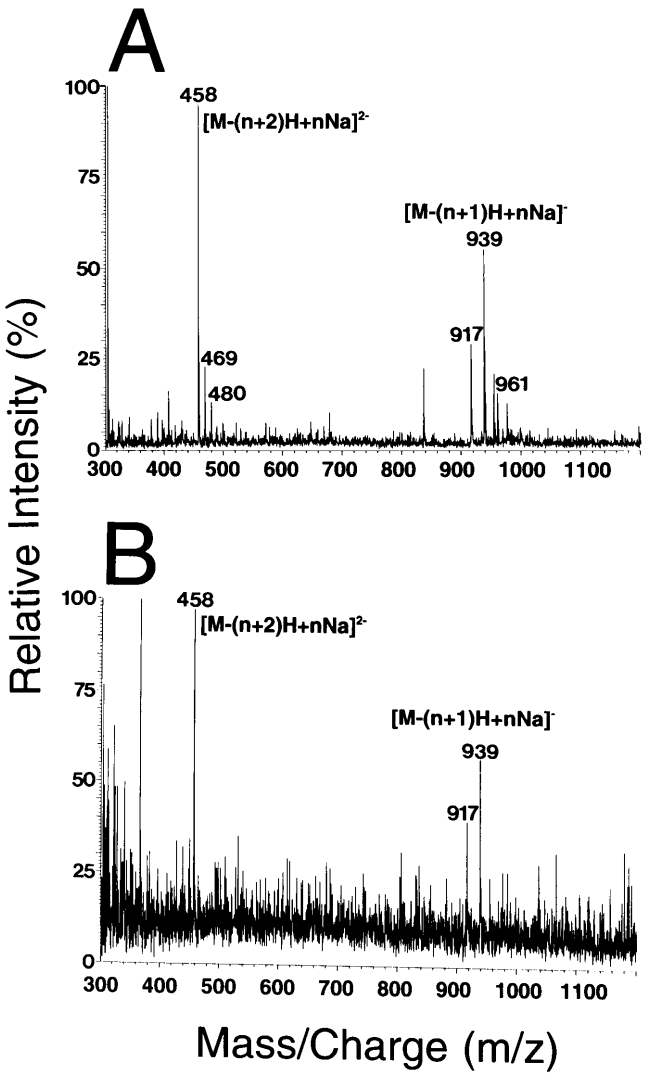

Fig. 2. ESI-MS analysis of fully sulfated tetrasaccharide **A4**. (A) ESI-MS of 375 ng (400 pmol) of **A4**, where n = 0, 1, 2 (singly charged) and n = 0, 1, 2 (doubly charged). (**B**) ESI-MS of 47 ng (50 pmol) of **A4**, where n = 0, 1 (singly charged) and n = 0 (doubly charged). Raw (unprocessed) data are shown.

action pattern of this enzyme (Jandik *et al.*, 1994). The major peaks, **A1**, **A4**, **A6**, **A8** observed on SAX-HPLC, were collected and desalted, and their purity assessed using CE (Table I). The purity of each oligosaccharide and the amount obtained by analytical SAX-HPLC was sufficient to undertake analysis using electrospray ionization (ESI)–mass spectrometry (MS).

ESI-MS has recently been applied to the analysis of charged oligosaccharides available only in small quantities (Silvesto *et al.*, 1996; Siegel *et al.*, 1997). ESI-MS analysis of each acharan sulfate-derived oligosaccharide afforded molecular ion information (Table II). This extremely sensitive method was used to determine the mass of oligosaccharides **A1–A6** and **A8**. The spectra of the fully sulfated (one sulfate group per disaccharide repeating unit) tetrasaccharide **A4** is shown in Figure 2A. The conditions were optimized with respect to needle voltage, carrier solution, nebulizing and bath gas flows. Using the optimal conditions a range of sample amounts 9 ng to 1.9 µg (10 pmol to 2 nmol) was examined. At a signal/noise of 3:1 the peaks at 939 and 458 corresponding to singly and doubly charged ions $[M\text{-}2H+Na]^-$, $[M\text{-}2H]^{2-}$, respectively, could be detected with as little as 19 ng (20 pmol) of sample. The ESI-MS spectrum of 47 ng (50 pmol) of **A4** is shown in Figure 2B. No fragmentation was evident at any of the concentrations. As the sample concentration was decreased, the intensity of $[M\text{-}H]^-$ (at 917) increased and the multisodiated species corresponding to peaks at 939, 961, 469, and 480 decreased. No change in the ratio of doubly to singly charged species resulted from a change in the amount of sample analyzed.

Table I. Structure and purity of acharan sulfate-derived oligosaccharides

Sample	Structure	Purity by CE (%)	*Flavobacterial* heparin lyase II (mole%)	*Bacteroides* heparin lyase (mole%)
A1	ΔUAp2S(1→4)GlcNpAcα,β	>99	51.3	34.0
A2	ΔUAp(1→[4)GlcNpAc(1→4)IdoAp2S(1→]4)GlcNpAcα,β	92.2	0	19.0
A3	ΔUAp(1→[4)GlcNpAc(1→4)IdoAp2S(1→]$_2$4)GlcNpAcα,β	>99	0	17.6
A4	ΔUAp2S(1→[4)GlcNpAc(1→4)IdoAp2S(1→]4)GlcNpAcα,β	96.1	8.57	14.3
A5	ΔUAp(1→[4)GlcNpAc(1→4)IdoAp2S(1→]$_3$4)GlcNpAcα,β	80.5	0	5.85
A6	ΔUAp2S(1→[4)GlcNpAc(1→4)IdoAp2S(1→]$_2$4)GlcNpAcα,β	96.7	2.91	3.08
A8	ΔUAp2S(1→[4)GlcNpAc(1→4)IdoAp2S(1→]$_3$4)GlcNpAcα,β	>99	4.53	—

Table II. ESI-MS analysis of acharan sulfate-derived oligosaccharides

	A1	A2	A3	A4	A5	A6	A8
$[M-H]^-$	458.0	837.1		917.0			
$[M-2H+Na]^-$		859.1	1318.1	939.0			
$[M-3H+2Na]^-$		881.1	1340.1	961.0			
$[M-4H+3Na]^-$			1362.1	983.0			
$[M-5H+4Na]^-$			1384.1				
$[M-2H]^{2-}$		418.0	647.6	458.0			
$[M-3H+Na]^{2-}$		429.0	658.6	469.0	888.1	698.5	
$[M-4H+2Na]^{2-}$			669.6	480.0	899.1	709.5	939.0
$[M-5H+3Na]^{2-}$			680.6		910.1	720.5	950.0
$[M-6H+4Na]^{2-}$					921.1	731.5	961.0
$[M-7H+5Na]^{2-}$					932.1		972.0
$[M-8H+6Na]^{2-}$							983.0
$[M-3H]^{3-}$			431.4	305.0	584.4	458.0	
$[M-4H+Na]^{3-}$			438.7	312.3	591.7	465.4	618.4
$[M-5H+2Na]^{3-}$			446.1		599.1	472.7	625.7
$[M-6H+3Na]^{3-}$					606.4	480.0	633.0
$[M-7H+4Na]^{3-}$					613.7		640.3
$[M-8H+5Na]^{3-}$							647.6
$[M-4H]^{4-}$					343.3		458.0
$[M-5H+Na]^{4-}$					348.8		463.5
$[M-6H+2Na]^{4-}$					354.3		469.0
$[M-7H+3Na]^{4-}$							474.5
$[M-8H+4Na]^{4-}$							480.0

M, molecular weight of fully protonated each oligosaccharide; **A1**, fully sulfated disaccharide (M = 459); **A2**, undersulfated tetrasaccharide (M = 838); **A3**, undersulfated hexasaccharide (M = 1297); **A4**, fully sulfated tetrasaccharide (M = 918); **A5**, undersulfated octasaccharide (M = 1756); **A6**, fully sulfated hexasaccharide (M = 1377); **A8,** fully sulfated octasaccharide (M = 1836).

The full structural assignment of the acharan sulfate-derived oligosaccharides relied on high field ^{1}H-NMR spectroscopy. Additional oligosaccharide sample was prepared and purified using semi-preparative SAX-HPLC. One dimensional NMR of **A1**, **A4**, **A6**, and **A8** confirmed that these oligosaccharides corresponded to a di-, tetra-, hexa-, and octasaccharide, respectively. The structure of **A1** had previously been established using ^{1}H-NMR spectroscopy (Kim *et al.*, 1996). The assignments presented in Table III for **A1** are consistent with the known disaccharide structure ΔUAp2S(1→4)-D-GlcNpAcα,β. The spectra of **A4** showed the appropriate number of signals and integration corresponding to the presence of one unsaturated uronate, two glucosamine and one iduronate residue, confirming it to be a tetrasaccharide (Table IV). Two singlets at 2.04 and 2.05 ppm correspond to the *N*-acetyl methyl signals of two unsulfated GlcNpAc residues. The 2-*O*-sulfo group of the unsaturated uronate residue was confirmed by the downfield shift of the H-1 proton from 5.08 (for ΔUAp) to 5.51 ppm (for ΔUAp2S). Signals at 4.26 and 4.81 for H-2 and H-5 confirm the presence of the internal IdoAp2S residue. **A6** and **A8** gave similar spectra (Table V and VI), suggesting these to be a hexasaccharide and octasaccharide of the structure ΔUAp2S(1→[4)-α-D-GlcNpAc(1→4)-α-L-IdoAp2S (1→]$_n$4) GlcNpAcα,β where n = 2 and 3, respectively. All of these oligosaccharides contained an α and β anomeric mixture of the reducing terminal GlcNpAc. Two-dimensional (2D) double quantum filtered chemical shift correlation spectroscopy (DQF-COSY), performed on fully sulfated octasaccharide **A8**, is presented in Figure 3A. Assignment can be conveniently made based on the chemical shifts of the structure reporter signals, such as anomeric protons and H-2 of IdoAp2S observed at 5.2 and 4.3 ppm, respectively. A typical splitting ratio (7:3) of α and β anomeric signals of GlcNpAc at the reducing terminal was observed at 5.2 and 4.7 ppm, respectively. Since the β anomeric signal was overlapped with irradiated HOD signal in Figure 3A, the chemical shift of this signal was confirmed by a second experiment at 333°K. Because of the consecutive sequence of multiple →4)-α-D-GlcNpAc(1→4)-α-L-IdoAp2S (1→ disaccharide units in **A8**, the spectrum of this octasaccharide is considerably simpler than an octasaccharide previously prepared from heparin (Toida *et al.*, 1996).

Table III. Chemical shifts and coupling constants of tetrasaccharide **A1**

	ΔUAp2S-α GlcNpAc	ΔUAp2S-β GlcNpAc	αGlcNpAc	βGlcNpAc
H-1	5.50	5.49	5.22	4.69
$^3J_{1,2}$	3.0	2.9	2.4	8.0
H-2	4.55	4.53	3.87	3.70
$^3J_{2,3}$	3.2	2.4	6.5	6.9
H-3	4.32	4.31	3.87	3.69
$^3J_{3,4}$	4.7	4.7	8.6	8.4
H-4	5.97	5.96	3.79	3.78
$^3J_{4,5}$	1.20 ($^4J_{2,4}$)	1.20 ($^4J_{2,4}$)	6.7	7.9
H-5	—	—	3.97	3.59
$^3J_{5,6a}$	—	—	5.2	5.5
H-6a	—	—	3.85	3.91
$^2J_{6a,6b}$	—	—	9.9	10.0
H-6b	—	—	3.84	3.81
$^3J_{5,6b}$	—	—	3.4	2.4
N-Acetyl methyl	—	—	2.04	2.04

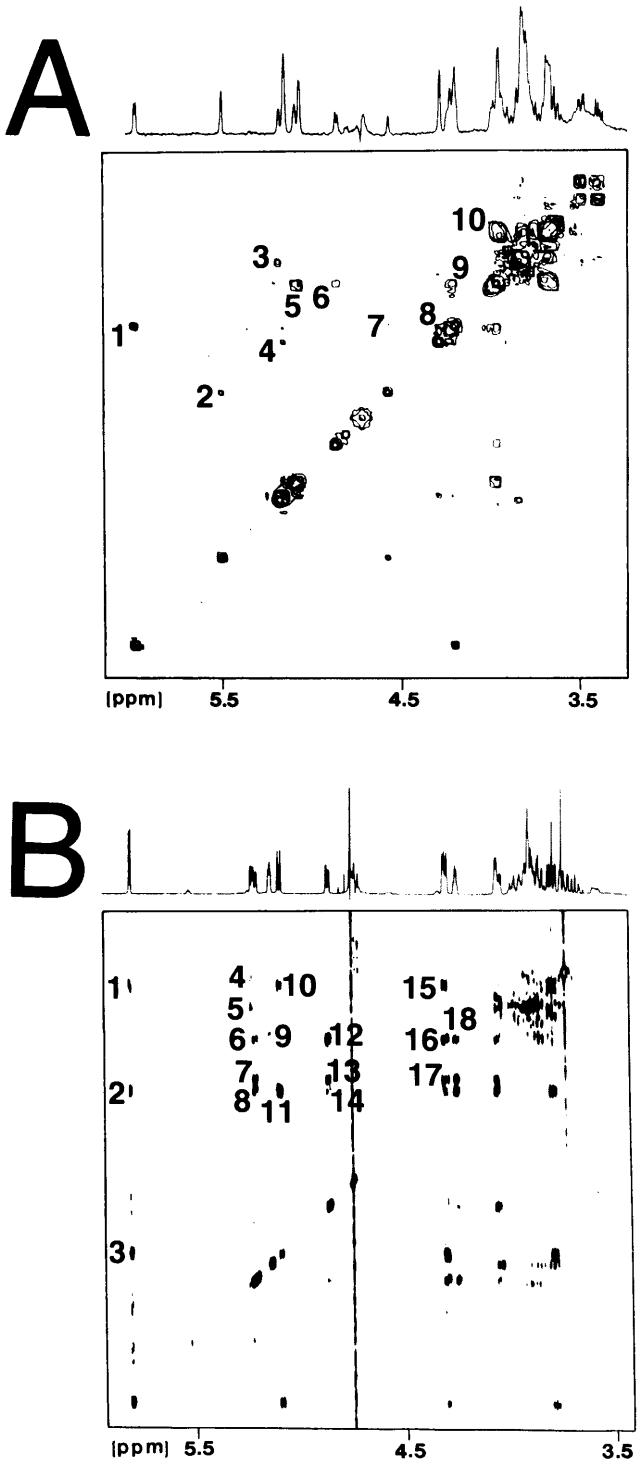

Fig. 3. 2D DQF COSY (**A**) of fully sulfated octasaccharide **A8** and 2D TOCSY (**B**) of undersulfated tetrasaccharide **A2**. Cross peaks in (**A**): 1, H-3/H-4 of ΔUAp2S; 2, H-1/H-2 of ΔUAp2S; 3, H-1/H-2 of GlcNpAcα (reducing end); 4,H-1/H-2 of IdoAp2S; 5, H-1/H-2 of GlcNpAc (internal); 6, H-4/H-5 of IdoAp2S; 7, H-2/H-3 of ΔUAp2S; 8, H-2/H-3 of IdoAp2S; 9, H-3/H-4 of IdoAp2S; 10, H-2/H-3 of GlcNpAc (internal). Cross peaks in (**B**): 1, H-4/H-2 of ΔUAp; 2, H-4/H-3 of ΔUAp; 3, H-4/H-1 of ΔUAp; 4, H-1/H-4 of GlcNpAcα (reducing end); 5, H-1/H-3 of GlcNpAcα (reducing end); 6, H-1/H-4 of IdoAp2S; 7, H-1/H-3 of IdoAp2S; 8, H-1/H-2 of IdoAp2S; 9, H-1/H-2 of GlcNpAc (internal); 10, H-1/H-2 of ΔUAp; 11, H-1/H-3 of ΔUAp; 12, H-5/H-4 of IdoAp2S; 13, H-5/H-3 of IdoAp2S; 14, H-5/H-2 of IdoAp2S; 15, H-3/H-2 of ΔUAp; 16, H-2/H-4 of IdoAp2S; 17, H-2/H-3 of IdoAp2S; 18, H-3/H-4 of IdoAp2S

Table IV. Chemical shifts and coupling constants of tetrasaccharide **A4**

	ΔUAp2S	GlcNpAc	IdoAp2S ($^2S_0$)	GlcNpAc	
				α	β
H-1	5.51	5.11	5.17	5.20	4.69
$^3J_{1,2}$	<1.0	2.4	6.0	3.0	8.2
H-2	4.60	4.02	4.26	3.85	3.70
$^3J_{2,3}$	<1.0	7.8	4.2	6.4	7.0
H-3	4.23	3.72	4.21	3.85	3.69
$^3J_{3,4}$	4.9	9.2	4.0	8.0	8.2
H-4	6.00	3.70	3.99	3.77	3.78
$^3J_{4,5}$	—	9.2	4.5	6.9	8.0
H-5	—	3.88	4.81	3.94	3.59
$^3J_{5,6a}$	—	5.4	—	5.4	5.5
H-6a	—	3.87	—	3.83	3.91
$^2J_{6a,6b}$	—	n.d.	—	9.8	9.8
H-6b	—	3.85	—	3.82	3.81
$^3J_{5,6b}$	—	n.d.	—	3.6	2.4
N-acetyl methyl	—	2.05	—	2.04	2.04

The $^2S_0$ conformation of IdoAp2S residue next to the reducing terminal was confirmed by the dihedral angles calculated by the coupling constants (4–7 Hz) under the Karplus equation. This characteristic conformation, of each IdopA2S residue next to the reducing terminal GlcNpAc, was observed in all oligosaccharides except **A8.** This observed difference in conformation is particularly important since oligosaccharides **A2–A6** and **A8** all have identical saccharide residues surrounding this IdoAp2S residue. Indeed, it is interesting to note that **A5** and **A8** are both octasaccharides and have identical structures except for the ΔUAp and ΔUAp2S residues at the nonreducing terminus of **A5** and **A8**, respectively. This means that the presence of a sulfate group, in the ΔUAp residue at the nonreducing end, affects the conformation of the IdoAp2S six residues away. The alteration of the IdoAp2S conformation by such a remote change in primary structure suggests a change in the global conformation of the molecule. A similar observation of an unusual IdoAp2S conformation was previously reported for a heparan sulfate-derived tetrasaccharide having the structure ΔUAp(1→4)-α-D-GlcNpS(1→4)-α-L-IdoAp2S(1→4)-D-GlcNpAcα,β (Hileman *et al.*, 1997). Studies are underway to better understand the affect of remote changes in the primary structure of oligosaccharides on local and global conformation.

Both the ESI-MS and ^{1}H-NMR analyses (Table II–VI) showed that all the major oligosaccharides obtained from acharan sulfate upon partial depolymerization with *Flavobacterial* heparin lyase II were fully sulfated, corresponding to the major repeating disaccharide unit of acharan sulfate. Thus, the question of the presence of the unsulfated IdoAp residues, observed by NMR analysis of the intact acharan sulfate polymer, remained unanswered. The two other lyases obtained from *Flavobacterium heparinum* heparin lyase I and III fail to act on acharan sulfate providing little additional information on the fine structure of this polymer (Kim *et al.*, 1996). Recently, *Bacteroides stercoris* (strain HJ-15) has also been found to produce a heparin lyase capable of acting on acharan sulfate (Ahn *et al.*, 1998). When a heparin lyase, partially purified from this organism, was used to completely depolymerize acharan sulfate, a major disaccharide product was again obtained. The structure of this disaccharide was tentatively identified as **A1** by CE analysis, and its structure was confirmed by ^{1}H-NMR spectroscopy (not shown). These results suggested that the *Bacteroides* heparin lyase enzyme was similar in specificity to *Flavobacterial* heparin lyase II.

Table V. Chemical shifts and coupling constants of hexasaccharide **A6**

	ΔUAp2S	GlcNpAc	IdoAp2S ($^1C_4$)	GlcNpAc	IdoAp2S ($^2S_0$)	GlcNpAc	
						α	β
H-1	5.51	5.08	5.17	5.11	5.17	5.20	4.74
$^3J_{1,2}$	<1.0	3.0	<1.0	3.0	6.2	3.0	8.0
H-2	4.60	4.0	4.31	4.0	4.27	3.85	3.69
$^3J_{2,3}$	<1.0	9.2	<1.0	9.2	4.4	6.8	7.8
H-3	4.23	3.72	4.24	3.72	4.23	3.85	3.67
$^3J_{3,4}$	4.2	n.d.	<1.0	n.d.	4.2	7.8	8.2
H-4	6.00	3.70	4.00	3.70	4.02	3.78	3.78
$^3J_{4,5}$	—	8.6	<1.0	8.6	4.5	6.9	8.0
H-5	—	3.88	4.88	3.88	4.83	3.94	3.55
$^3J_{5,6a}$	—	5.0	—	5.0	—	5.4	n.d.
H-6a	—	3.87	—	3.87	—	3.83	n.d.
$^2J_{6a,6b}$	—	n.d.	—	n.d.	—	9.9	n.d.
H-6b	—	3.84	—	3.84	—	3.82	n.d.
$^3J_{5,6b}$	—	n.d.	—	n.d.	—	3.4	n.d.
N-acetyl methyl	—	2.05	—	2.05	—	2.04	2.04

Table VI. Chemical shifts and coupling constants of octasaccharide **A8**

	ΔUAp2S	GlcNpAc		GlcNpAc	IdoAp2S	GlcNpAc	
						α	β
H-1	5.51	5.09		5.11	5.17	5.20	4.74
$^3J_{1,2}$	<1.0	3.5		3.0	<1.0	2.8	8.2
H-2	4.60		4.01		4.32	3.85	3.69
$^3J_{2,3}$	<1.0		9.4		<1.0	7.0	7.8
H-3	4.23		3.72		4.24	3.85	3.66
$^3J_{3,4}$	4.9		8.8		<1.0	7.8	n.d.
H-4	5.99		3.70		4.00	3.77	3.78
$^3J_{4,5}$	—		5.4		7.4	7.0	n.d.
H-5	—		3.80		4.88	3.94	3.55
$^3J_{5,6a}$	—		n.d.		—	5.4	n.d.
H-6a	—		3.88		—	3.83	3.90
$^2J_{6a,6b}$	—		n.d.		—	n.d.	n.d.
H-6b	—		3.85		—	3.82	3.81
$^3J_{5,6b}$	—		n.d.		—	n.d.	n.d.
N-acetyl methyl	—		2.05		—	2.04	2.04

Partial depolymerization of acharan sulfate by the *Bacteroides* heparin lyase was next performed under conditions identical to those used for *Flavobacterial* heparin lyase II. Oligosaccharide analysis by SAX-HPLC (Figure 1B) showed many of the same peaks were obtained using the *Flavobacterial* heparin lyase II. While a broad envelope of very large resistant oligosaccharides (similar to that observed in Figure 1A) were detected, the kinetics of depolymerization monitored by CE suggested this was an endolytic enzyme. In addition to **A1**, **A4** and **A6**, the *Bacteroides* enzyme also afforded additional peaks, **A2**, **A3**, and **A5**, not obtained using *Flavobacterial* heparin lyase II (Table I). ESI-MS analysis of these products indicated that they were undersulfated (<1 sulfate/disaccharide) oligosaccharides (Table II). ^{1}H-NMR analysis confirmed that these oligosaccharides (Table VII, VIII, IX) all contained an unsulfated ΔUAp at their nonreducing terminus (Table I). The unsaturated uronate residue in these oligosaccharides was unsulfated as demonstrated by the 5.06–5.09 ppm shift of the H-1 signals in these spectra. The one

dimensional ^{1}H-NMR spectra of **A2**, **A3**, and **A5** clearly showed these to be a tetra-, hexa-, and octasaccharide. The number of *N*-acetyl methyl signals at 2.0–2.1 ppm shows the number of GlcNpAc residues in each oligosaccharides. Two dimensional total spin correlation spectroscopy (TOCSY) (Figure 3B) was used to confirm the structure of tetrasaccharide **A2** and to assist in peak assignments (Table VII). The signal of H-2 of IdoAp2S was also downfield shifted by *O*-sulfonation. As with those spectra obtained for **A4** and **A6**, the conformation of each IdoAp2S residue next to the reducing terminal GlcNpAc in **A2**, **A3**, and **A5** was confirmed to reside in the $^2S_0$ conformation based on the observed dihedral angles.

The presence of nonreducing terminal ΔUAp residues suggest that the *Bacteroides* heparin lyase is distinctly different from *Flavobacterial* heparin lyase II, which does not act at unsulfated IdoAp residues. Since the *Bacteroides* enzyme is not homogeneous, it is possible that the undersulfated products might arise from the action of a second minor enzymatic activity present in this preparation, such as a 2-*O*-sulfoesterase (2-*O*-sulfatase). A 2-*O*-sulfoesterase has been reported in *Flavobacterium heparinum* (Dietrich *et al.*, 1973). While it was initially believed that this enzyme acted only on the ΔUAp2S residues present in disaccharides (Dietrich *et al.*, 1973), this enzyme has also been demonstrated to act on heparin-derived tetrasaccharide substrates containing nonreducing terminal ΔUAp2S residues (McClean *et al.*, 1984). Treatment of **A1**, **A4**, **A6**, and **A8** with *Flavobacterial* 2-*O*-sulfoesterase, followed by CE (Pervin *et al.*, 1994b) and ESI-MS analysis, showed that the acharan sulfate-derived disaccharide, tetrasaccharide hexasaccharide, and octasaccharide were all converted to a product having one less sulfate group. Comigration on CE analysis confirmed that these products were undersulfated oligosaccharides ΔUAp(1→[4)-α-D-GlcNpAc(1→4)-α-L-IdoAp2S(1→]$_m$-D-GlcNpAcα,β; ΔUAp(1→4)-α-D-GlcNpAcα,β (m = 0), **A2** (m = 1), **A3** (m = 2), and **A5** (m = 3), respectively. The loss of a single sulfate group from **A1** and **A6** was also confirmed by ESI-MS analysis. Careful examination of the heparin lyase preparation from *Bacteroides stercoris* failed to detect the presence of 2-*O*-sulfoesterase using colorimetric assay based on the hydrolysis of *p*-nitrophenyl sulfate (Waheed and Etlen, 1980). In addition, exhaustive treatment of **A1** with the *Bacteroides* enzyme failed to form any ΔUAp(1→4)-D-GlcNpAcα,β product.

Table VII. Chemical shifts and coupling constants of undersulfated tetrasaccharide **A2**

	ΔUAp	GlcNpAc	IdoAp2S ($^2S_0$)	GlcNpAc	
				α	β
H-1	5.08	5.12	5.19	5.21	4.71
$^3J_{1,2}$	7.0	3.5	7.0	3.0	7.8
H-2	3.78	4.04	4.30	3.90	3.70
$^3J_{2,3}$	n.d.	6.5	3.5	7.8	8.0
H-3	4.30	3.79	4.24	3.87	3.73
$^3J_{3,4}$	3.5	7.4	3.0	7.0	7.0
H-4	5.79	3.80	4.05	3.74	3.74
$^3J_{4,5}$	—	8.0	3.0	7.8	7.8
H-5	—	3.83	4.85	3.97	3.57
$^3J_{5,6a}$	—	5.4	—	5.0	5.4
H-6a	—	3.89	—	3.88	3.88
$^2J_{6a,6b}$	—	9.8	—	10.2	9.8
H-6b	—	3.86	—	3.86	3.86
$^3J_{5,6b}$	—	4.8	—	4.4	2.8
N-Acetyl methyl	—	2.09	—	2.04	2.04

Table VIII. Chemical shifts and coupling constants of undersulfated hexasaccharide **A3**

	ΔUAp	GlcNpAc	IdoAp2S	GlcNpAc	IdoAp2S ($^2S_0$)	GlcNpAc	
						α	β
H-1	5.09	5.10	5.20	5.10	5.20	5.22	4.70
$^3J_{1,2}$	4.5	3.0	3.0	3.0	4.0	3.0	8.0
H-2	3.78	4.02	4.33	4.02	4.28	3.89	3.71
$^3J_{2,3}$	5.4	n.d.	<1.0	n.d.	4.2	n.d.	9.4
H-3	4.27	3.79	4.24	3.79	4.24	3.87	3.73
$^3J_{3,4}$	3.5	n.d.	<1.0	n.d.	n.d.	n.d.	n.d.
H-4	5.80	3.82	4.02	3.82	4.04	3.74	3.74
$^3J_{4,5}$	—	n.d.	<1.0	n.d.	5.5	n.d.	n.d.
H-5	—	3.84	4.91	3.84	4.87	3.91	3.57
$^3J_{5,6a}$	—	n.d.	—	n.d.	—	n.d.	5.4
H-6a	—	3.88	—	3.88	—	3.88	3.88
$^2J_{6a,6b}$	—	n.d.	—	n.d.	—	n.d.	10
H-6b	—	3.87	—	3.87	—	3.87	3.87
$^3J_{5,6b}$	—	n.d.	—	n.d.	—	n.d.	4.4
N-Acetyl methyl	—	2.08	—	2.06	—	2.04	2.04

Table IX. Chemical shifts and coupling constants of undersulfated octasaccharide **A5**

	ΔUAp	GlcNpAc	IdoAp2S ($^1C_4$)	IdoAp2S ($^2S_0$)	GlcNpAc	
					α	β
H-1	5.06	5.10	5.20	5.21	5.21	4.74
$^3J_{1,2}$	6.8	n.d.	<1.0	4.0	3.0	n.d.
H-2	3.78	4.04	4.32	4.28	3.89	3.71
$^3J_{2,3}$	n.d.	n.d.	<1.0	3.8	n.d.	n.d.
H-3	4.27	3.78	4.23	4.24	3.87	3.73
$^3J_{3,4}$	3.2	n.d.	<1.0	n.d.	n.d.	n.d.
H-4	5.79	3.81	4.01	4.04	3.74	3.74
$^3J_{4,5}$	—	n.d.	<1.0	<1.0	n.d.	n.d.
H-5	—	3.83	4.92	4.87	3.92	3.54
$^3J_{5,6a}$	—	n.d.	—	—	n.d.	5.2
H-6a	—	3.88	—	—	3.88	3.88
$^2J_{6a,6b}$	—	n.d.	—	—	n.d.	10.4
H-6b	—	3.87	—	—	3.87	3.87
$^3J_{5,6b}$	—	n.d.	—	—	n.d.	4.2
N-Acetyl methyl	—	2.08, 2.06, 2.06	—	—	2.04	2.04

The tetra-, hexa-, and octasaccharides isolated contain both the primary disaccharide repeating structure, →4)-α-D-GlcNpAc(1→4)-α-L-IdoAp2S (1→ making up acharan sulfate, and unsulfated ΔUAp at the nonreducing terminus arising from →4)-α-D-GlcNpAc(1→4)-α-L-IdoAp(1→. This strongly suggest that these unsulfated IdoAp residues are a rare but intimate part of the structure of acharan sulfate. Thus, the structure of acharan sulfate may best be described as $[\rightarrow 4)\text{-}\alpha\text{-D-GlcNpAc}(1\rightarrow 4)\text{-}\alpha\text{-L-IdoAp2S}(1]_n$ $[\rightarrow 4)\text{-}\alpha\text{-D-GlcNpAc}(1\rightarrow 4)\text{-}\alpha\text{-L-IdoAp}(1]_m\rightarrow$, where the undersulfated domains are distributed and represent only about 10% of the polymer ($n \sim 9 \times m$).

Further studies are underway to establish the distribution of these undersulfated domains within the acharan sulfate polymer and to further purify the new heparin lyase prepared from *Bacteroides stercoris.* In addition, the role of these undersulfated domains in the biological activity of acharan sulfate (Wang *et al.*, 1997) also requires further consideration.

Materials and methods

Materials

The sodium salt form of acharan sulfate was prepared according to the previous procedure (Kim *et al.*, 1996). Heparin lyase II and 2-*O*-sulfatase from *Flavobacterium heparinum* were provided as gifts from IBEX (Montreal, Canada) and Dr. Yoshida of Seikagaku (Tokyo, Japan), respectively. Gel permeation chromatography was performed on Bio-Gel P-2 (fine) from Bio-Rad (Richmond, CA) and Sephadex G-50 (superfine) from Pharmacia (Piscataway, NJ). The ΔUAp(1→4)-D-GlcNpAcα,β disaccharide standard was from Grampian Enzymes (Aberdeen, Scotland). BSA (biological grade) was from Sigma Chemical Co. (St. Louis, MO). Imidazole (enzyme grade) was from Fisher Scientific (Fair Lawn, NJ). Tetraethylammonium iodide was from Aldrich (Milwaukee, WI). All other reagents used were analytical grade.

SAX-HPLC was performed on 5 μm Spherisorb columns from Phase Separation (Norwalk, CT) of dimensions 2.5 × 25 cm and 0.46 × 25 cm using dual-face programmable LC-7A titanium-based pumps from Shimadzu (Kyoto, Japan). This system was equipped with a Rheodyne (Cotati, CA) titanium injector and a Pharmacia LKB variable-wavelength UV detector (Piscataway, NJ) and with a Shimadzu Chromatopac C-R2A integrating recorder. CE was performed using a Dionex Capillary Electrophoresis system with advanced computer interface, model I, equipped with high-voltage power supply capable of constant or gradient voltage control using a fused silica capillary, 50 μm i.d., 375 μm o.d., 75 cm in long, 70 cm effective length, from Dionex Corporation (Sunnyvale, CA). UV spectrometry was performed on a Shimadzu model UV 160 spectrometer equipped with a thermostated cell or a JASCO model V550 (Tokyo, Japan). A Varian 500 MHz NMR spectrometer controlled by a SUN SPARC station 2 workstation was used for all 1D and 2D NMR experiments.

Methods

Preparation of Bacteroides *heparin lyase. Bacteroides stercoris* (strain HJ-15, Korean Culture Collection #KCCM-10096) was cultured in general anaerobic media (Kim *et al.*, 1986), the cell pellet was isolated by centrifugation, resuspended in 20 mM pH 7.2 sodium phosphate buffer, disrupted by sonication, and the supernatant recovered following centrifugation. To this supernatant, $(NH_4)_2SO_4$ was added to 30% saturation, the precipitate was removed by centrifugation and discarded. Next, $(NH_4)_2SO_4$ was added to 60% of saturation, and the precipitate was recovered by centrifugation and dissolved in 20 mM pH 7.2 sodium phosphate and dialyzed exhaustively against the same buffer. This partially purified enzyme preparation contained a heparin lyase activity but no sulfatase activity as demonstrated by the failure of this enzyme preparation to act on *p*-nitrophenyl sulfate (Waheed and Etlen, 1980).

Depolymerization of acharan sulfate with heparin lyases. Acharan sulfate (200 μl at 1 mg/ml) in 50 mM sodium phosphate buffer, pH 7.6 was treated with heparin lyase II (12 mU) at 30°C.

At various time points, the absorbance at 232 nm was measured and digestion was continued until the absorbance was constant (complete digestion). The percent reaction completion at each time point was calculated by dividing the absorbance at 232 by the absorbance measured at reaction completion. Acharan sulfate (3 ml of 1 mg/ml) in the same buffer was again digested with heparin lyase II. The digestion mixture heated at 100 °C for 3 min when the absorbance at 232 nm indicated the digestion was 55% complete. The partial digestion mixture was freeze-dried and reconstituted with 1.5 ml of distilled water and stored frozen for the next SAX-HPLC analysis.

Acharan sulfate (5 ml, 10 mg/ml) in 20 mM sodium phosphate buffer, pH 7.0, was depolymerized with 50 µl *Bacteroides* heparin lyase (2.75 mg/ml of a specific activity 7.5 mU/mg) for 6 and 48 h at 37 °C. Aliquots were withdrawn at 2 h intervals and immediately frozen and stored at -70 °C.

Enzymatic desulfation of oligosaccharides. Acharan sulfate oligosaccharides (disaccharide **A1**: 30 µg, tetrasaccharide **A4**: 20 µg, hexasaccharide **A6**: 200 µg, octasaccharide **A8**: 200 µg) were dissolved in 10 µl of 25 mM imidazole (HCl), 25 mM NaCl containing 0.05% BSA, pH 6.5 buffer (McClean *et al.*, 1984). *Flavobacterial* 2-*O*-sulfatase (0.1 nU to 1 nU, where 1 nU liberates 1 nmol inorganic sulfate/min from **A1**) was added to each oligosaccharide, and the reaction mixture was incubated at 25 °C. The progress of digestion was monitored for 24–48 h by CE.

Each product was analyzed by CE and identified by comigration with standards. The structure of the desulfated products prepared from **A1** and **A6** were desalted on Sephadex G-50 (1.8 cm × 48 cm) and Bio-Gel P-2 (1 cm × 48 cm), respectively, and their structures were confirmed by ESI-MS analysis.

CE analysis of acharan sulfate oligosaccharides. The CE system was operated in the reverse polarity mode by applying the sample at the cathode and run with 20 mM phosphoric acid adjusted to pH 3.5 with 1 M dibasic sodium phosphate as described previously (Pervin *et al.*, 1994a). The capillary (50 µm inner diameter, 375 µm outer diameter, 75 cm long, 70 cm effective length) was manually washed before use with 0.5 ml of 0.5 M sodium hydroxide followed by 0.5 ml of distilled water and then 0.5 ml running buffer. Samples were applied using pressure injection (5 psi, 2 or 3 sec) resulting in a sample volume of 70 or 105 nl. Each experiment was conducted at a constant 18,000 V. Data collection was at 232 nm.

SAX-HPLC fractionation of acharan sulfate oligosaccharides. The *Bacteroides* heparin lyase digested samples were injected on an analytical SAX-HPLC column to monitor the reaction. A linear NaC1 gradient of 0.1–1.6 M, pH 3.5, at a flow rate of 1.0 ml/min was used and detection was at 232 nm. The *Flavobacterial* heparin lyase II digested samples were injected on a semipreparative SAX-HPLC column equilibrated with water at pH 3.5 and eluted from the column using a 120 min gradient from 0.0 to 1.8 M of NaC1 pH 3.5 at a flow rate of 4.0 ml/min. The elution profile was monitored by absorbance at 232 nm at 0.5–1.5 absorbance unit full scale (AUFS). Each peak was pooled, lyophilized, and desalted on a Bio-Gel P-2 column (1.8 cm × 60 cm). Elution from the P-2 column was monitored by absorbance at 232 nm. The pooled fractions were lyophilized. Each oligosaccharide was analyzed by CE, ^{1}H-NMR, and ESI-MS.

ESI-MS analysis. The negative ion mass spectra were obtained by using a Micromass, Inc. (England) Autospec equipped with an electrospray interface. Samples were initially dissolved in 1:1 water/acetonitrile with 0.05% NH_4OH which was also used for the mobile phase. A Harvard syringe pump was used to deliver the mobile phase at a flow rate of 20 µl/min. Samples were introduced into the system through 20 µl injection loop on a Rheodyne (Cotati, CA) injection port. Nitrogen gas was used both as bath and nebulizer gas at flow rates of 250 l/h and 12 l/h, respectively. The electrospray ion source was held at 80 °C and the spray needle was held at 7.7 kV. Tetraethylammonium iodide in acetonitrile was used as the calibrant (Hop, 1996). In measuring detection limits a stock solution of **A4** at 100 pmol/µl was used to make dilutions down to 0.5 pmol/µl. Spectra were obtained by injecting 20 µl of each solution. Multiple injections were performed. The spectra shown are 30–40 scans of one representative injection. The manufacturer's software (OPUS) was used to acquire and process data.

NMR spectroscopy. For ^{1}H-NMR spectroscopy, each sample was exchanged three times with 0.5 ml portions of ^2H_2O (99.90%, Sigma), followed by *in vacuo* desiccation over P_2O_5. The thoroughly dried sample was redissolved in 0.7 ml of ^2H_2O (99.90%), and spectra were obtained using a UNITY-Varian 500 spectrometer at the operating frequency of 500 MHz equipped with a VXR 5000 computer system (Varian Instruments) or a JEOL 500 MHz instrument equipped with VAX 32 computer. The operation conditions for one-dimensional spectra were as follows: frequency, 500 MHz; sweep width, 6 kHz; flip angle, 90° (11.1 µs or 12.8 µs); sampling point, 48 K; accumulation, 256 pulses; temperature, 298 K. The water resonance was suppressed by selective irradiation during the relaxation delay.

Two-dimensional double quantum-filtered COSY and TOCSY spectra were recorded using the phase-sensitive mode. All two-dimensional spectra were recorded using the phase-sensitive mode. All two-dimensional spectra were recorded with 512 × 2048 data points and a spectral width of 3200 Hz. The water resonance was suppressed by selective irradiation during the relaxation delay. A total of 128–256 scans were accumulated for each t_1, with a relaxation delay of 2 s. The digital resolution was 1.6 Hz/point in both dimensions with zero-filling in the t_1 and t_2 dimensions in the case of double quantum-filtered COSY, and a Lorentz-Gauss function was applied in all other cases. An MLEV-17 mixing sequence of 120 ms was used for 2D TOCSY.

Acknowledgments

We are grateful for support from the National Institutes of Health (GM38060 and HL52622) (R.J.L.), the University of Iowa, Center for Biocatalysis and Bioprocessing (as a fellowship to Y.M.P.), KOSEF 961–071–114–2 and HMP-96–1–1002 (Y.S.K.), Grants-in-Aid from the Ministry of Culture and Education of Japan (09672185) (T.T.), and US National Science Foundation Grant CHE-9510004 (L.T.).

Abbreviations

GAG, glycosaminoglycan; GlcNpAc, *N*-acetylglucosamine; IdoAp, iduronic acid; ΔUAp, 4-deoxy-α-L-*threo*-hex-enopyranosyluronic acid; aFGF, bFGF, acidic and basic fibroblast growth factors; SAX-HPLC, strong-anion-exchange high performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; 2D, two dimensional; COSY, correlation spectroscopy; 1D, one dimensional.

References

Ahn,M.Y., Shin,K.H., Kim,D.-H., Jung,E.-A., Toida,T., Linhardt,R.J. and Kim,Y.S. (1998) Characterization of a *Bacteroides* species from human intestine that degrades glycosaminoglycans. *Can. J. Microbiol.*, in press.

Dietrich,C.P., Silva,M.E. and Michelacci,Y.M. (1973) Sequential degradation of heparin in *Flavobacterium heparinum*. *J. Biol. Chem.*, **248,** 6408–6415.

Faham,S., Hileman,R.E., Fromm,J.R., Linhardt,R.J. and Rees, D.C. (1996) Crystallographic analyses of heparin oligosaccharides binding to basic fibroblast growth factor. *Science*, **271,** 1116–1120.

Folkman,.J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature Med.*, **1,** 27–31.

Fromm,J.R., Hileman,R.E., Weiler,J.M. and Linhardt,R.J. (1997) Interaction of fibroblast growth factor-1 and related peptides with heparan sulfate and its oligosaccharides. *Arch. Biochem. Biophys.*, **346,** 252–262.

Hileman,R.E., Smith, A.E., Toida,T. and Linhardt,R.J. (1997) Preparation and structure of heparin-lyase-derived heparan sulfate oligosaccharides. *Glycobiology*, **7,** 231–239.

Hop,C.E.C.A. (1996) Generation of high molecular weight cluster ions by electrospray ionization: implications for mass calibration. *J. Mass Spectrom.*, **31,** 1314–1316.

Jandik,K.A., Gu,K. and Linhardt,R.J. (1994) Action pattern of polysaccharide lyases on glycosaminoglycans. *Glycobiology*, **4,** 289–296.

Kim,D.H., Konishi,L. and Kobayashi,K. (1986) Purification, characterization and reaction mechanism of novel sulfotransferase from an anaerobic bacterium in human intestine. *Biochem. Biophys. Acta*, **872,** 33–41.

Kim,Y.S., Jo,Y.T., Chang,I.M., Toida,T.E., Park,Y. and Linhardt,R.J. (1996) A new glycosaminoglycan from the giant African snail *Achatina fulica*. *J. Biol. Chem.*, **271,** 11750–11755.

Linhardt,R.J. (1994) Polysaccharide lyases for glycosaminoglycan analysis. In Varki,A. (ed.), *Current Protocols in Molecular Biology, Analysis of Glycoconjugates.* Vol. 2, John Wiley & Sons, Boston, pp. 17.13.17–17.13.32.

McClean,M.W., Bruce,J.S., Long,W.F. and Williamson,F.B. (1984) *Flavobacterium heparinum* 2-*O*-sulphatase for 2-*O*-sulphato-$\Delta_{4,5}$-glycuronate-terminated oligosaccharides from heparin. *Eur. J. Biochem.*, **145,** 607–615.

Pervin,A., Al-Hakim,A. and Linhardt,R.J. (1994a) Separation of glycosaminoglycan-derived oligosaccharides by capillary electrophoresis using reversed polarity. *Anal. Biochem.*, **221,** 182–188.

Pervin,A., Gu,K. and Linhardt,R.J. (1994b) Capillary electrophoresis to measure sulfoesterase activity on chondroitin sulfate and heparin derived disaccharides. *Appl. Theoret. Electrophor.*, **3,** 297–303.

Siegel,M.M., Tabei,K., Kagan,M.Z., Vlahov,I.R., Hileman,R.E. and Linhardt,R.J. (1997) Polysulfated carbohydrates analyzed as ion-paired complexes with basic peptides and proteins using electrospray negative ionization mass spectrometry. *J. Mass Spectrom.*, **32,** 760–772.

Silvesto,L., Savu,S.R., van Veelen,P.A. and Jacobs,P.L. (1996) Application of mass spectrometry to the analysis of natural and synthetic sulfated oligosaccharides. In Harenberg,J. and Casu,B. (eds.), *Nonanticoagulant Actions of Glycosaminoglycans*. Plenum Press, New York, pp. 27–46.

Toida,T., Hileman,R.E., Smith,A.E., Vlahova,P.I. and Linhardt,R.J. (1996) Enzymatic preparation of heparin oligosaccharides containing antithrombin III binding sites. *J. Biol. Chem.*, **271,** 32040–32047.

Waheed,A. and Etlen,R.L. (1980) Chemical characterization and substrate specificity of rabbit liver aryl sulfatase A, *Biochem. Biophys. Acta*, **614,** 92–101.

Wang,H.M., Toida,T., Kim,Y.S., Capila,I., Hileman,R.E., Bernfield,M. and Linhardt,R.J. (1997) Glycosaminoglycans can influence fibroblast growth factor-2 mitogenicity without significant growth factor binding, *Biochem. Biophys. Res. Commun.*, **235,** 369–373.