



REGULAR ARTICLE

Chemical Sulfonation and Anticoagulant Activity of Acharan Sulfate

Song Ji Wu^{1,2}, Moon Woo Chun², Kuk Hyun Shin¹, Toshihiko Toida³, Youmie Park⁴, Robert J. Linhardt⁴ and Yeong Shik Kim¹
¹Natural Products Research Institute, Seoul National University, Seoul 110-460, Korea; ²College of Pharmacy, Seoul National University, Seoul 151-742, Korea; ³Faculty of Pharmaceutical Sciences, Chiba University, Chiba 263, Japan; ⁴Division of Medicinal and Natural Products Chemistry and Department of Chemical and Biochemical Engineering, PHAR S328, University of Iowa, Iowa City, Iowa 52240, USA.

(Received 25 May 1998 by Editor N. Sakuragawa; revised/accepted 11 August 1998)

Abstract

Acharan sulfate is a glycosaminoglycan prepared from the giant African snail, *Achatina fulica*. This polysaccharide has a repeating disaccharide structure of $\rightarrow 4$ -2-deoxy-2-acetamido- α -D-glucopyranose (1 \rightarrow 4)-2-sulfo- α -L-idopyranosyluronic acid (1 \rightarrow). Its structure is related to heparin and heparan sulfate but is distinctly different from all known members of these classes of glycosaminoglycans. Because of its structural similarities to heparin, chemically modified acharan sulfate was studied to understand the chemical structure effected its anticoagulant activity. After de-*N*-acetylation, acharan sulfate was *N*-sulfonated using either chlorosulfonic acid-pyridine or sulfur trioxide-trimethylamine complex. The sulfate level in these products ranged from 22 to 24% (w/w), significantly less than that of heparin at 36%. The molecular weight

of both *N*-sulfoacharan sulfates were comparable with that of heparin. In vitro anticoagulant activity assays showed that *N*-sulfoacharan sulfate derivatives were moderately active for the inhibition of thrombin and neither product showed any measurable anti-factor Xa activity. The differences in the activities of *N*-sulfoacharan sulfates produced by these two methods are probably ascribable to a small level of concomitant *O*-sulfonation obtained when using chlorosulfonic acid-pyridine. © 1998 Elsevier Science Ltd.

Key Words: Acharan sulfate; Chemical *N*-sulfonation; Anticoagulant activity

Glycosaminoglycans (GAGs) are linear anionic polysaccharides having characteristic disaccharide repeating sequences of uronic acid and hexosamine residues. These biopolymers are biosynthesized as macromolecular proteoglycans linked to protein core but are typically isolated as GAGs [1–3]. The biological functions of GAGs result from their interaction with proteins, such as clotting factors found in plasma, growth factors, lipoproteins, and extracellular matrix components [4–6]. In a previous study, we reported a new GAG, acharan sulfate, isolated from the giant African snail, *Achatina fulica* [7]. This GAG has a molecular weight of 29000 and a uniform repeating disaccharide structure of $\rightarrow 4$ - β -D-

Abbreviations: GAGs, glycosaminoglycans; MW, molecular weight; NMR, nuclear magnetic resonance; SAX, strong-anion exchange chromatography; HPLC, high-performance liquid chromatography; APTT, activated partial thromboplastin time; TBE, tris-borate-EDTA buffer; GPC, gel-permeation chromatography; GlcN, glucosamine; S, sulfate; Ac, acetate; IdoA, iduronic acid; Δ UA, 4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid; GlcA, glucuronic acid; ATIII, antithrombin III.

Corresponding author: Yeong Shik Kim, Natural Products Research Institute, Seoul National University, 28 Yeonkun-Dong, Jongno-Ku, Seoul 110-460, South Korea. Tel: +82 (2) 740 8929; Fax: +82 (2) 765 4768; E-mail: <kims@plaza.snu.ac.kr>.

GlcNAc (where GlcNAc is 2-acetamido-2-deoxy- α -D-glucopyranose (1 \rightarrow 4)- α -L-IdoA2S (1 \rightarrow and (where IdoA2S is α -L-idopyranosyluronic acid and S is sulfate). The unusual structure of this GAG, being similar but structurally different from both heparin or heparan sulfate, suggests that it might be a good precursor for the preparation of semi-synthetic heparin or heparan sulfate analogs having important biological activities. A recent study on *N*-sulfoacharan sulfate, for example, showed a heparin-like effect on basic fibroblast growth factor mitogenicity [8].

In an effort to prepare heparin-like anticoagulant agents having simple structures, acharan sulfate was chemically *N*-sulfonated using two methods. The anticoagulant activity of these derivatives was measured using amidolytic assays as well as a clotting based assay. The structural features of these derivatives were characterized by ^1H -nuclear magnetic resonance (NMR) spectroscopy and by using strong-anion exchange (SAX) high-performance liquid chromatography (HPLC) after treatment with heparinases.

1. Materials and Methods

1.1. Materials

Acharan sulfate (molecular weight [MW] 29000) was prepared as described previously [7]. Heparin from porcine mucosa (MW 12000) and low molecular weight heparin (MW 3000–5000) were purchased from Sigma (St. Louis, MO). The reagents for the measurement of activated partial thromboplastin time (APTT) and thrombin time were also from Sigma. Coatest kits for the assay of factor Xa and factor IIa inhibition were from Chromogenix AB (Mölnådal, Sweden). Heparin lyase I (heparinase, EC 4.2.2.7) and heparin lyase II (heparitinase II) were from Seikagaku (Tokyo, Japan).

1.2. General Assays

Uronic acid was assayed by the modified carbazole method [9]. Sulfate content of samples were measured according to a previously reported method [10]. Briefly, samples or standards containing 0–40 nmol sulfate containing sample was added into pyrex tubes together with 5 μl of 0.02 M NaOH and freeze-

dried. The residues were then pyrolyzed at 600°C for 10–15 minutes. Barium buffer (0.6 ml) and rhodizonate reagent (0.3 ml) were added. After a 10-minute incubation at room temperature, the absorbance was determined at 520 nm.

1.3. Deacetylation of Acharan Sulfate

Deacetylation was performed according to the published procedure [11,12]. Briefly, acharan sulfate (50 mg) was dissolved in 3 ml of anhydrous hydrazine containing 1% hydrazine sulfate and heated in a sealed glass tube at 98°C for 10 hours. After cooling, toluene was added and the sample was subjected to repeated evaporation under N_2 . The residue was dissolved in 1.5 ml of 0.25 M iodic acid. The small amount of I_2 that was formed was extracted with ether and aqueous layer was dialyzed exhaustively and freeze-dried.

1.4. Sulfonation of Deacetylated Acharan Sulfate

The de-*N*-acetylated acharan sulfate (12 mg) was suspended in anhydrous pyridine (5 ml). To this suspension 5 ml of a mixture of chlorosulfonic acid and pyridine (1:6) was added and the reaction was refluxed for 2 hours [13]. The solution was cooled, 10 ml of ice-water was added, and its pH adjusted to 7.0 with 2 M sodium hydroxide. Three volumes of ethanol was then added and the mixture left overnight at 4°C. The precipitate formed was recovered by centrifugation, dissolved in water, dialyzed exhaustively against water, and freeze-dried. Alternatively, de-*N*-acetylated acharan sulfate (10 mg) was dissolved in water (1 ml). Sodium carbonate (36 mg) was added, and the solution was heated to 55°C. Sulfur trioxide-trimethylamine complex (3 \times 15 mg) was added to the solution at 0-, 5-, and 10-hour intervals and the reaction was maintained at 55°C for 15 hours [12]. The sample was then cooled and diluted with 1 ml of 16% sodium chloride, and 9 ml of methanol was added to precipitate the product. The precipitate was recovered by centrifugation, dissolved in water, exhaustively dialyzed against water and freeze-dried.

1.5. Measurement of Clotting Time

APTT was measured according to the manufacturer's procedure by using normal human plasma. The

activity was calculated by comparison with the heparin standard curve (clotting time vs. units/mg).

1.6. Inhibition of Factor IIa and Xa

Anti-IIa activity was determined by incubating 100 μ l of 0.1 U of AT III and 50 μ l of human thrombin (10 NIH units/ml) at 37°C for 30 seconds. Then, 50 μ l (2.5 μ mole/ml) of S2238 (ethylmalonyl-Pro-Arg-*p*-nitroanilide hydrochloride) chromogenic substrate was added and the amidolytic thrombin activity was measured at 405 nm [14]. Measurements were performed on a JASCO V550 spectrophotometer (Tokyo, Japan). Anti-Xa activity was also determined using Coatest® Heparin kit (Möln dal, Sweden) by the “End Point” method. In brief, 200 μ l of human plasma containing heparin (0.1–0.7 USP units/ml) or acharan sulfate derivatives (0.005–1 mg/ml) was incubated at 37°C for 3 minutes. Then, 100 μ l (0.125 nkat) of bovine factor Xa was added into test solutions preincubated and mixed briefly. The reactions were initiated by adding 200 μ l (2.9 μ mole/ml) of benzoyl-Ile-Glu-(γ -Piperidyl)-Gly-Arg-*p*-nitroanilide hydrochloride substrate (S-2222) to each mixture. After a 3-minute incubation, the reaction was stopped by adding 200 μ l of 20% acetic acid and the absorbance was measured at 405 nm. Anti-Xa activity was calculated by comparison with the heparin standard curve (absorbance at 405 nm vs. units/mg).

1.7. Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed in a 1% gel poured in TBE buffer (0.045 M Tris-borate, 0.001 M EDTA). One hundred micrograms of acharan sulfate and chemically modified acharan sulfate derivatives were loaded on the gel and constant voltage (80 V) was applied for 1 hour at room temperature. The gel was visualized with 0.5% Azure A in 1% acetic acid.

1.8. ¹H-NMR Spectroscopic Analysis

Approximately 5 mg of each sample was exchanged three times with 1 ml portions of ²H₂O (99.9%; Sigma), followed by in vacuo desiccation over P₂O₅. The thoroughly dried sample was redissolved in 0.7 ml of ²H₂O (99.96%), and spectra were obtained using a UNITY-Varian 500 spectrometer at the

operating frequency of 500 MHz equipped with a VXR 5000 computer system or a JEOL 500 MHz instrument equipped with a VAX computer. The operating conditions for one-dimensional (1D) spectra were as follows: frequency, 500 MHz; sweep width, 6kHz; flip angle, 90° (12.8 μ s); sampling point, 48 K; accumulation, 256 pulses; temperature, 298 K. The water resonance was suppressed by selective irradiation during the relaxation delay.

1.9. Determination of Molecular Weight

The molecular weight of chemically modified acharan sulfates was determined by HPLC on GPC columns of TSK G3000SW coupled with 2000SW (Uppsala, Sweden) equilibrated with 100 mM NaCl at a flow rate of 1.0 ml/minute. The standards for the calibration of the column were heparin (12000), low molecular weight heparin (5000), and chondroitin sulfate A (40000) of known molecular weight.

1.10. Enzymatic Digestion

N-sulfoacharan sulfate derivatives (100 μ g) were depolymerized by 1 mU of both heparin lyase I and II in 0.5 ml of 20 mM phosphate buffer (pH 7.0) for 2 hours at 37°C, after which the samples were heated in a boiling water bath for 3 minutes. The products were analyzed by SAX-HPLC described previously [14]. Standard disaccharides from Sigma were coinjected to tentatively identify the structure of the major peak. The major peak in the chromatogram was collected and its structure was confirmed by NMR as described above.

2. Results

2.1. Preparation and Structural Characterization of *N*-Sulfoacharan Sulfate Derivatives

The structure of de-*N*-acetylated acharan sulfate and *N*-sulfoacharan sulfate was assigned based on 500 MHz ¹H NMR spectroscopy (Figure 1 and Table 1). On de-*N*-acetylation, the signal corresponding to *N*-acetyl CH₃ group at 2.07 ppm completely disappeared. This was accompanied by the appearance of a new signal at 3.49 ppm, corresponding to the H-2 of the newly formed D-GlcN residue [12]. The

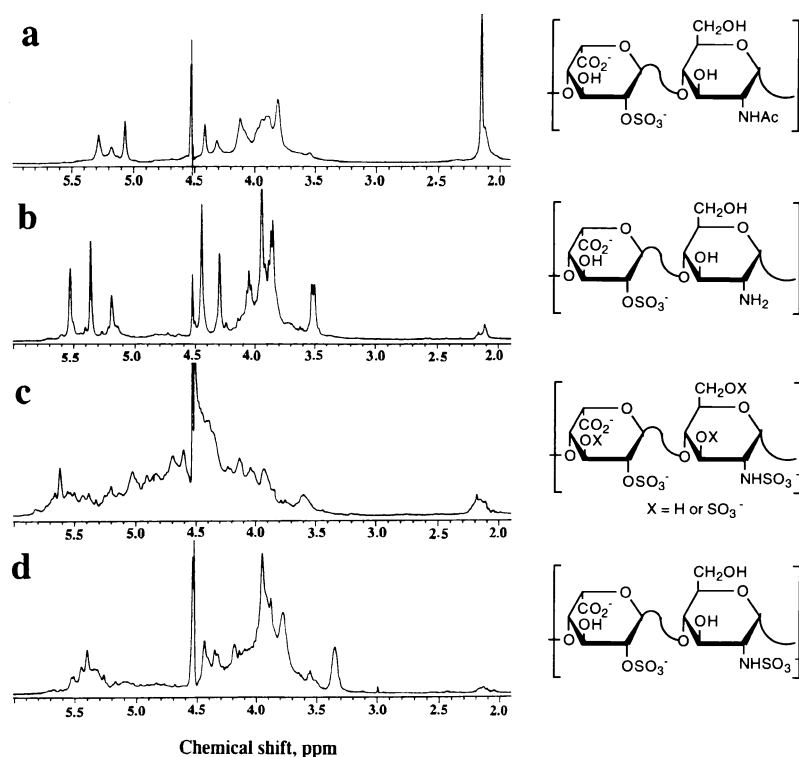


Fig. 1. $^1\text{H-NMR}$ spectra of (a) acharan sulfate, (b) de-*N*-acetylated acharan sulfate (c) *N*-sulfoacharan sulfate prepared by chlorosulfonic acid-pyridine, (d) *N*-sulfoacharan sulfate prepared by sulfur trioxide-trimethylamine.

anomeric proton of D-GlcN is also downfield shifted from 5.2 ppm to 5.5 ppm by the absence of the anisotropic effect of *N*-acetyl group. The resulting de-*N*-acetylated acharan sulfate was next *N*-sulfoated using either chlorosulfonic acid-pyridine (Figure 1C) or sulfur trioxide-trimethylamine complexes (Figure 1D). Because sulfonation by chlorosulfonic acid-pyridine occurred not only on the amino groups of D-GlcN but also on some of the hydroxy groups of IdoA and GlcN, the spectrum shows a complex splitting pattern of the signals of the sugar ring protons (Figure 1C). In contrast, the simple splitting pattern in the sulfate derivative prepared by sulfur trioxide-trimethylamine com-

plex reagent shows well resolved signals for the H-2 of IdoA2S and GlcNS at 4.4 and 3.3 ppm, respectively. This suggests that only re-*N*-sulfonation resulted under these milder reaction conditions. Both 1% agarose electrophoresis and HPLC-gel permeation chromatography (GPC) was next used to determine the molecular weight and polydispersity of the derivatives (Figure 2A and B). The average molecular weight of acharan sulfate was 29000, while the molecular weights of de-*N*-acetylated acharan sulfate and *N*-sulfoacharan sulfate were reduced to 20000 and 8000, respectively. Lane 3 in Figure 2A showed that average molecular weights of two *N*-sulfoacharan sulfates were similar

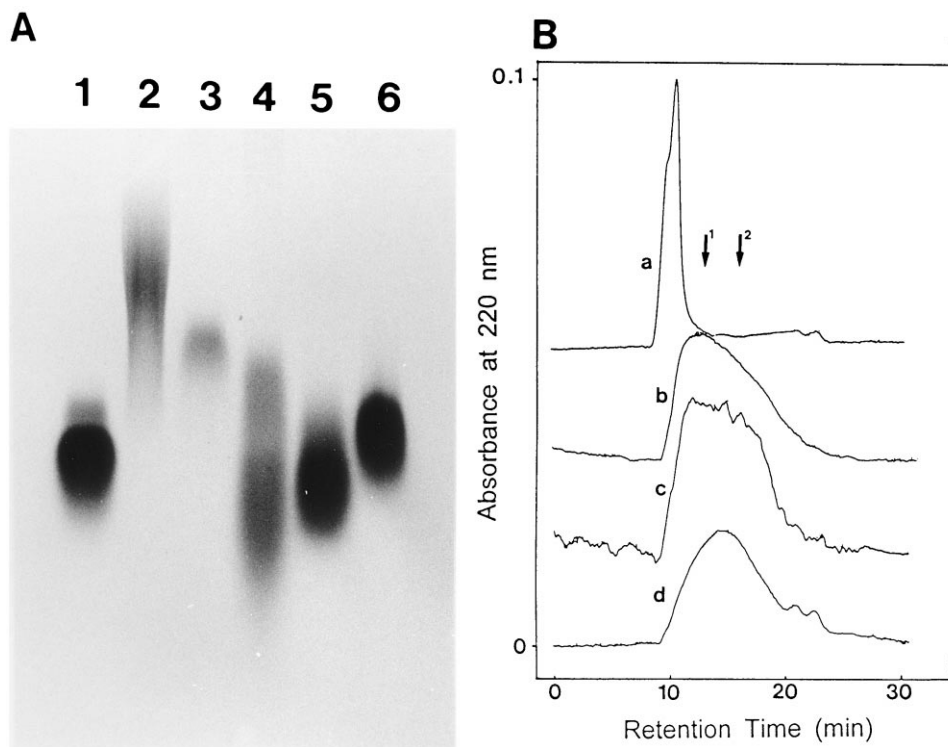
Table 1. Chemical shifts of structural reporter groups in acharan sulfate and its derivatives

	L-iduronic acid 2- <i>O</i> -sulfate residue (chemical shift in ppm)		D-glucosamine residue (chemical shift in ppm)	
	H-1	H-2	H-1	H-2
Acharan sulfate	5.19	4.35	5.11	4.02
De- <i>N</i> -acetylated acharan sulfate	5.53	5.20	5.37	3.49
<i>N</i> -sulfoacharan sulfate ^a	5.28	4.35	5.35	3.60
<i>N</i> -sulfoacharan sulfate ^b	5.22	4.33	5.45	3.33

^a *N*-sulfoacharan prepared by chlorosulfonic acid-pyridine.

^b *N*-sulfoacharan prepared by sulfur trioxide-trimethylamine.

Fig. 2. (A) Agarose gel electrophoresis of acharan sulfate and derivatives. Lane 1, heparin; lane 2, acharan sulfate; lane 3, de-*N*-acetylated acharan sulfate; lane 4, *N*-sulfoacharan sulfate prepared by chlorosulfonic acid-pyridine; lane 5, *N*-sulfoacharan sulfate prepared by sulfur trioxide-trimethylamine; lane 6, chondroitin sulfate. (B) Determination of average molecular weight by GPC-HPLC. a, acharan sulfate; b, de-*N*-acetylated acharan sulfate; c, *N*-sulfoacharan sulfate prepared by chlorosulfonic acid-pyridine; d, *N*-sulfoacharan sulfate prepared by sulfur trioxide-trimethylamine. The arrows indicate the retention time of heparin (1) and low molecular weight heparin (2), respectively.



to that of heparin. The molecular weight of the *N*-sulfoacharan sulfate is probably reduced owing to a low level of hydrolysis of the polymer's glycosidic linkages by acid produced in the sulfonation reactions. Sulfonation under anhydrous conditions with chlorosulfonic acid-pyridine afforded a considerably more polydisperse product than that formed using sulfur trioxide-trimethylamine complex in aqueous sodium carbonate solution. The *N*-sulfoacharan sulfates were next enzymatically depolymerized and analyzed by SAX-HPLC (Figure 3). Two enzymes, heparin lyase I (heparinase, E.C. 4.2.2.7), and heparin lyase II (heparitinase II) were used to ensure complete depolymerization [15]. The *N*-sulfoacharan sulfate prepared with sulfur trioxide-trimethylamine complex was sensitive to these enzymes, but the derivative prepared using chlorosulfonic acid-pyridine was resistant. These data suggest that other structural modifications limiting the action of these enzymes, such as *O*-sulfonation, might also have taken place under the anhydrous sulfonation conditions. Additionally, *O*-sulfonation is consistent with a slightly but not significantly, higher sulfate content observed when using chlorosulfonic acid-pyridine. After enzymatic depolymerization, the *N*-sulfoacharan sul-

fate prepared using sulfur trioxide-trimethylamine was analyzed by SAX-HPLC. A single peak corresponding to the production of one oligosaccharide was observed. Coinjection with a disaccharide standard suggested that this major oligosaccharide product was $\Delta\text{UA}2\text{S}-(1\rightarrow4)-\alpha\text{-D-GlcNS}$ (where ΔUA is 4-dexoy- $\alpha\text{-L-threo-hex-4-enopyranosyluronic acid}$). This major peak was collected and its structure was confirmed using $^1\text{H-NMR}$ (data not shown). These results establish that one of the structure of the polysaccharide after *N*-sulfonation is primarily the expected $\rightarrow4\text{-}\alpha\text{-D-GlcNS}(1\rightarrow4)\text{-}\alpha\text{-L-IdoA}2\text{S}(1\rightarrow$.

2.2. Determination of Anticoagulant Activity

Anticoagulant activity was evaluated using heparin as a standard and measuring clotting time (Figure 4) and the conversion of chromogenic substrates using anti-factor IIa and anti-factor Xa amidolytic assays (Table 2). Neither the parent acharan sulfate nor the de-*N*-acetylated acharan sulfate showed anticoagulant activity. Both *N*-sulfoacharan sulfate derivatives, however, showed elongation of clotting time and substantial anti-factor IIa activity. Neither *N*-sulfoacharan sulfate derivative showed

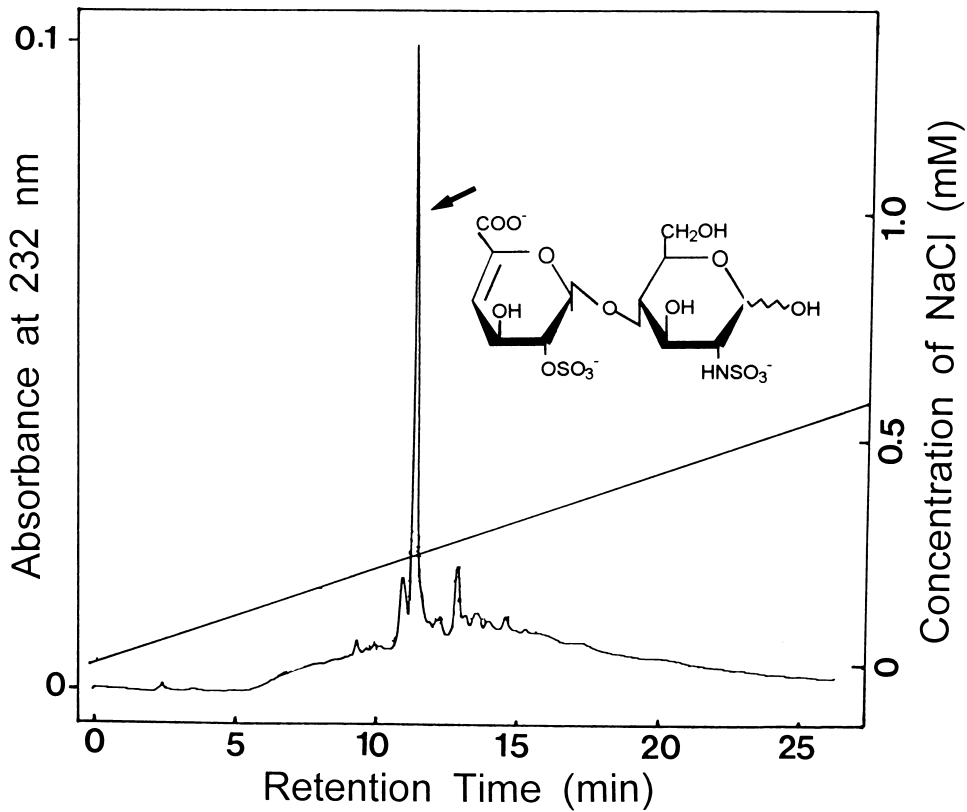


Fig. 3. SAX-HPLC analysis of *N*-sulfoacharan sulfate prepared by sulfur trioxide-trimethylamine on treatment with heparin lyases. The peak indicated by arrow was collected and its structure was determined.

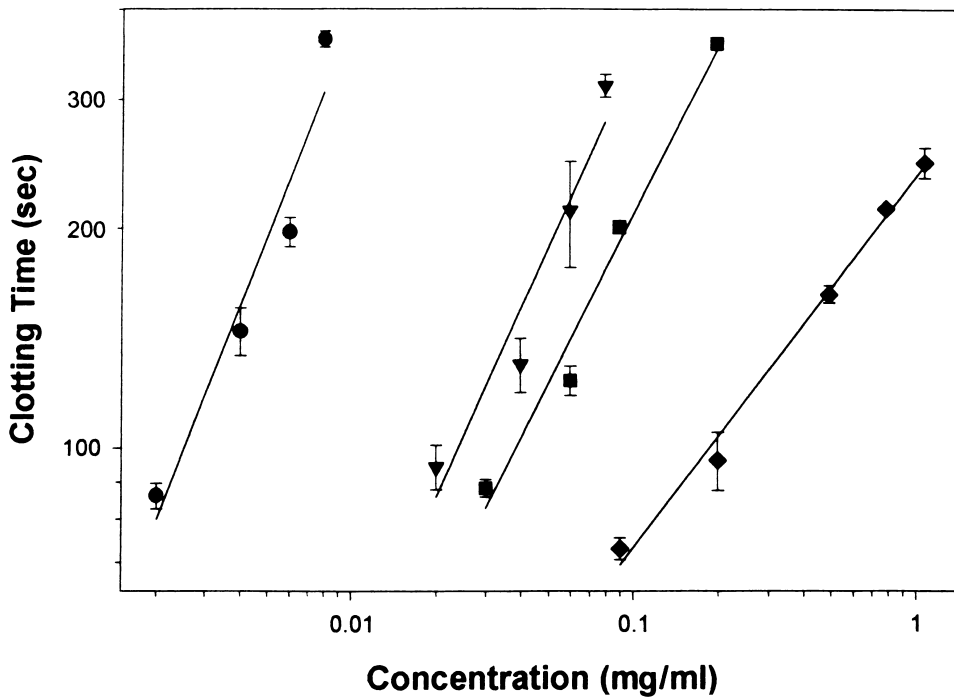


Fig. 4. Measurement of clotting time by APTT in the presence of heparin (circles), dermatan sulfate (triangles), *N*-sulfoacharan sulfate prepared by chlorosulfonic acid-pyridine (squares), and *N*-sulfoacharan sulfate prepared by sulfur trioxide-trimethylamine (diamonds).

Table 2. Anticoagulant (amidolytic) activities and sulfate content of modified acharan sulfate

Samples	Anti-factor Xa activity (U/mg)	Anti-factor IIa activity (U/mg)	Sulfate content (%)
Acharan sulfate	<1	<1	nd ^a
de- <i>N</i> -acetylated acharan sulfate	<1	<1	nd ^a
<i>N</i> -sulfoacharan sulfate ^b	2.5	69	24.1±0.1
<i>N</i> -sulfoacharan sulfate ^c	2.1	21	22.1±2.1
Heparin	150	150	35.9±2.4

^a nd, not determined.

^b *N*-sulfoacharan prepared by chlorosulfonic acid and pyridine.

^c *N*-sulfoacharan prepared by sulfur trioxide and trimethylamine.

any anti-factor Xa activity (Table 2). The *N*-sulfoacharan sulfate prepared using chlorosulfonic acid-pyridine showed approximately 40% activity of heparin's anti-factor IIa activity. Kinetic analysis indicated that at the concentration of 75 µg/ml, this *N*-sulfoacharan sulfate showed the same anti-factor IIa activity as 5 U/ml of heparin (150 U/mg), corresponding to a specific activity of approximately 70 U/mg. By clotting assay this sample was somewhat less active, requiring 10-fold higher concentration than heparin for the same extension of clotting time. The *N*-sulfoacharan sulfate prepared by sulfur trioxide-trimethylamine showed a slightly lower anti-factor IIa activity of 21 U/mg and more than 50-fold higher concentration was required to obtain the same clotting time as heparin.

3. Discussion

The structure of acharan sulfate, $\rightarrow 4$ - α -D-GlcNAc(1 \rightarrow 4)- α -L-IdoA2S (\rightarrow 1 is closely related to heparin, which is mainly comprised of a repeating disaccharide unit $\rightarrow 4$ - α -D-GlcNS6S(1 \rightarrow 4)- α -L-IdoA2S. Spectral data suggest that while both the chemically modified acharan sulfates contain *N*-sulfo groups, the *N*-sulfoacharan sulfate prepared in non-aqueous solvent by using chlorosulfonic acid-pyridine has also undergone some *O*-sulfonation. The resistance of the *N*-sulfoacharan sulfate prepared using chlorosulfonic acid-pyridine to heparin lyase I and II suggests that some of the secondary hydroxyl groups were also sulfonated. In contrast, the *N*-sulfoacharan sulfate prepared using sulfur trioxide-trimethylamine complex appears to be only sulfonated at the 2-*N*-position. While the molecular weights of the *N*-sulfoacharan sulfate derivatives are reduced

under the acidic conditions on sulfonation, they are similar to that of heparin, suggesting it is of sufficient size to exhibit full anticoagulant activity. The sulfate content of the products after sulfonation was approximately 24%, corresponding to approximately 60% of heparin's level of sulfation. The observed anticoagulant activities of the two *N*-sulfoacharan sulfates, corresponding from 20 to 40% of heparin's activity based on amidolytic factor IIa inhibition assay, are surprisingly high based on such a simple structural modification. There are many reports that increased sulfation of GAGs results in their anticoagulant activity [16–18]. While sulfation is indispensable for anticoagulant activity, it is certainly not sufficient. A unique pentasaccharide sequence, $\rightarrow 4$ - α -D-GlcNS-6S(1 \rightarrow 4)- α -D-GlcA(1 \rightarrow 4)- α -D-GlcN2S3S6S(1 \rightarrow 4)- α -L-Ido2S(1 \rightarrow 4)- α -D-GlcNS6S(1 \rightarrow (where GlcA is glucopyranosyluronic acid), found within heparin binds antithrombin III (ATIII) affording heparin its full anticoagulant activity [4,6,19]. This heparin pentasaccharide is sufficiently large to bind to ATIII and inhibit factor Xa. However, the minimum sized heparin chain required for ATIII mediated inhibition of factor IIa (thrombin) is a hexadecasaccharide [20]. The structure of acharan sulfate contains no GlcA residue nor any GlcN2S3S6S residues, both critically important for binding ATIII [21]. Thus, sulfonation of acharan sulfate alone cannot generate a complete ATIII pentasaccharide binding site. It appears therefore that factor IIa activity is inhibited by *N*-sulfoacharan sulfate through an ATIII independent pathway [22,23]. There are several examples of neutral and acidic polysaccharides that have been *O*-sulfonated resulting in derivatives that exhibit AT III independent anti-factor IIa activity [24–26]. The current study suggests that enhanced levels of both *N*- and *O*-sulfo groups

increase AT III independent anti-factor IIa activity. Further studies are required to examine the anticoagulant activity of more fully *N*- and *O*-sulfonated acharan sulfates. The modified *N*-sulfoacharan sulfate derivatives prepared in this study are currently being evaluated for new biological activities including antiviral and growth factor regulating activities.

This work was supported from Korea Science Foundation Grants 961-0720-114-2 and Ministry of Health and Welfare HMP-96-D-1-1002 (YSK) and NIH Grants GM38060 and HL52622 (RJL).

References

1. Hardingham TE, Fosang AJ. Proteoglycans: Many forms and many functions. *FASEB J* 1992;6:861–70.
2. Kjellen L, Lindahl U. Proteoglycans: Structures and interactions. *Ann Rev Biochem* 1991;60:443–75.
3. Jolles P. Proteoglycans. Basel: Birkhauser; 1994.
4. Lane DA, Lindahl U. Heparin. Chemical and Biological Properties, Clinical Applications, Boca Raton: CRC Press; 1989.
5. Harenberg J, Casu B. Nonanticoagulant Actions of Glycosaminoglycans. New York: Plenum Press; 1996.
6. Linhardt RJ, Toida T. Heparin analogues—development and applications. In: Witzak ZB, Nieforth KA, editors. Carbohydrates as Drugs. New York: Marcel Dekker; 1997. p. 277–341.
7. Kim YS, Jo YY, Chang IM, Toida T, Park YM, Linhardt RJ. A new glycosaminoglycan from African giant snail *Achatina fulica*. *J Biol Chem* 1996;27:11750–5.
8. Wang H, Toida T, Kim YS, Capila I, Hileman RE, Bernfield M, Linhardt RJ. Glycosaminoglycans can influence growth factor–2 mitogenicity without significant growth factor binding. *Biochem Biophys Res Commun* 1997;235:369–73.
9. Taylor KA, Bucanan-Smith JG. A colorimetric method for the quantitation of uronic acid and a specific assay for galacturonic acid. *Anal Biochem* 1992;201:190–6.
10. Silvestri LJ, Hurst RE, Simpson L, Settine J. Analysis of sulfate in complex carbohydrate. *Anal Biochem* 1982;123:303–9.
11. Shaklee PN, Conrad HE. Hydrazinolysis of heparin and other glycosaminoglycans. *Biochem J* 1984;217:187–97.
12. Nadkarni VD, Toida T, Van Gorp CL, Schubert RL, Weiler JM, Hansen KP, Caldwell EEO, Linhardt RJ. Preparation and biological activity of *N*-sulfonated chondroitin and dermatan sulfate derivatives. *Carbohydr Res* 1996;290:87–96.
13. Doctor VM, Sauls V. Isolation and anticoagulant properties of a new sulfated xylan: Comparison with heparin and a sodium pentosan polysulfate (SP-54). *Thromb Res* 1983;30:573–8.
14. Linhardt RJ, Rice KG, Kim YS, Lohse DL, Wang HM, Loganathan L. Mapping and quantification of the major oligosaccharide components of heparin. *Biochem J* 1988;254:781–7.
15. Linhardt RJ. Analysis of glycosaminoglycans with polysaccharide lyases. Varki A, editor. *Current Protocols in Molecular Biology*. Boston: Wiley Interscience; 1994. p. 17.13.17–32.
16. Scully MF, Ellis V, Seno N, Kakar VV. Effect of oversulfated chondroitin and dermatan sulfate upon thrombin and factor inactivation by antithrombin III or heparin cofactor II. *Biochem J* 1988;254:547–51.
17. Maaroufi RM, Tapon-Bretondiere J, Mardiguian J, Sternberg C, Dautzenberg MD, Fischer AM. Influence of the oversulfation method and the degree of sulfation on the anticoagulant properties of dermatan sulfate derivatives. *Thromb Res* 1990;59:749–58.
18. Casu B, Grazioli G, Razi N, Guerrini M, Naggi A, Torri G, Oreste P, Tursi F, Zoppetti G, Lindahl U. Heparin-like compounds prepared by chemical modification of capsular polysaccharide from *E. coli* K5. *Carbohydr Res* 1994;263:271–84.
19. Casu B. Structure and biological activity of heparin. *Adv Carbohydr Chem Biochem* 1985;43:51–134.
20. Lane AD, Denton J, Flynn AD, Thunburg L, Lindahl U. Anticoagulant activities of heparin oligosaccharides and their neutralization by platelet factor 4. *Biochem J* 1984;218:725–32.
21. Lindahl U, Backström B, Thunberg L, Leder IG. Evidence for a 3-*O*-sulfated D-glucosamine residue in the antithrombin-binding sequence of heparin. *Proc Natl Acad Sci USA* 1980;77:6551–5.

22. Tollefsen DM, Pestka CA, Monafó WJ. Activation of heparin cofactor II by dermatan sulfate. *J Biol Chem* 1983;258:6713–6.
23. Linhardt RJ, Hileman RE. Dermatan sulfate as a potential therapeutic agent. *Gen Pharmacol* 1995;26:1609–91.
24. Nagase H, Eniyoji K, Minamiguchi K, Kitazato K, Saito H, Kato H. Depolymerized halothrium glycosaminoglycan with novel anticoagulant actions: Antithrombin III- and heparin cofactor II-independent inhibition of factor X activation by factor IXa-Factor VIIIa complex and heparin cofactor II-dependent inhibition of thrombin. *Blood* 1995;85:1527–34.
25. Maaroufi RM, Jozefowicz M, T-Brethaudiere J, Fisher AM. Mechanism of thrombin inhibition by antithrombin and heparin cofactor II in the presence of heparin. *Biomaterials* 1997;4:359–66.
26. Dace R, McBride E, Brooks K, Gander J, Buszko M, Doctor VM. Comparison of the anticoagulant action of sulfated and phosphorylated polysaccharides. *Thromb Res* 1997;87:113–21.