Glycoconjugate Journal 17, 393–399, 2000

Effect of 6-*O*-sulfonate hexosamine residue on anticoagulant activity of fully *O*-sulfonated glycosaminoglycans

Toshihiko Toida, Atsushi Suzuki, Ken-ichi Nakajima, Amornrut Chaidedgumjorn and Toshio Imanari*

Faculty of Pharmaceutical Sciences, Chiba University, 1-33, Yayoi, Inage, Chiba, Japan 263-8522

Intact and fully *O*-sulfonated glycosaminoglycans (GAGs) including chondroitin sulfate, dermatan sulfate, hyaluronan, heparan sulfate and heparin were chemically de-*O*-sulfonated on their hexosamine C-6 position (6-*O*-desulfonation) using *N*,*O*-bis(trimethylsilyl) acetamide. ^{1}H NMR spectroscopy and chemical compositional analysis showed that the chemical de-*O*-sulfonation at C-6 position of hexosamine residues in both intact and fully *O*-sulfonated GAGs was completely achieved. Since GAGs and their derivatives are often used as anticoagulant agents, their anti-amidolytic activities were determined. While most of anticoagulant activity of fully *O*-sulfonated GAGs (FGAGs) and heparin disappeared following chemical 6-*O*-desulfonation, the activity of 6-*O*-desulfonated fully *O*-sulfonated dermatan sulfate (De6FDS) remained. This observation suggests the importance of the position of *O*-sulfonate groups for anti-coagulant activity.

Keywords: **chemical oversulfonation, glycosaminoglycans, anticoagulant activity, selective 6-*O*-desulfonation**

Abbreviations: **BTSA, *N*, *O*-Bis(trimethylsilyl) acetamide; GAG(s), glycosaminoglycan(s); FGAGs, fully *O*-sulfonated GAGs; De6FGAGs, 6-*O*-desulfonated FGAGs; Glcp, D-glucopyranoside; Galp, D-galactopyranoside; GlcpA, D-glucopyranosyluronic acid; IdopA, L-idopyranosyluronic acid; IdopA2S, 2-*O*-sulfo-L-idopyranosyluronic acid; GalpN, D-2-amino-2-deoxy galactopyranoside; GlcpN, D-2-amino-2-deoxyglucopyranoside; HexpN, D-2-amino-2-deoxy hexopyranoside; HexpA, hexuronic acid; Ac, acetyl; PAGE, polyacrylamide gel electrophoresis; ATIII, antithrombin III; HCII, heparin cofactor II; NHP, normal human plasma; 1D, one dimensional; 2D, two dimensional; NMR, nuclear magnetic resonance: CS, chondroitin sulfate; FCS, fully *O*-sulfonated CS; De6FCS, 6-*O*-desulfonated FCS; DS, dermatan sulfate; FDS, fully *O*-sulfonated DS; De6FDS, 6-*O*-desulfonated FDS; HA, hyaluronan; FHA, fully *O*-sulfonated HA; De6FHA, 6-*O*-desulfonated FHA; HP, heparin; De6HP, 6-*O*-desulfonated HP; FHP, fully *O*-sulfonated HP; De6FHP, 6-*O*-desulfonated FHP.**

Introduction

Glycosaminoglycans (GAGs) are structurally complex, *O*-sulfonated and linear polysaccharides comprised of alternating disaccharide units consisting of hexuronate (glucuronate (GlcpA) or iduronate (IdopA)) and hexosamine (glucosamine (GlcpN) or galactosamine (GalpN)) residue [1]. They are very heterogeneous polysaccharides in terms of relative molecular mass, charge density and physico-chemical properties [1] and are involved in a number of important biological processes and structurally altered in pathological conditions [2,3]. GAGs are divided into four categories based on their monosaccharide composition and glycosidic linkage: the chondroitin sulfate family including chondroitin sulfate and dermatan sulfate ($\rightarrow$3GalpNAcβ1$\rightarrow$4HexpA); the heparan sulfate family including heparan sulfate and heparin ($\rightarrow$4GlcpNXα1$\rightarrow$4HexpA, X = SO_3H or Ac); hyaluronan ($\rightarrow$3GlcpNAcβ1$\rightarrow$4GlcpA); and keratan sulfate ($\rightarrow$4GlcpNAcβ1$\rightarrow$3Galp); [1]. Chondroitin and dermatan sulfates are found attached to a core protein forming proteoglycans that are often localized on cell surfaces and in the extracellular matrix, where they are important in cell–cell communication [4]. While chondroitin sulfate and hyaluronan lack clinically relevant levels of anticoagulant activity [5], heparan sulfate and dermatan sulfate appear to be involved in

*To whom correspondence should be addressed: Tel.: +81-43-290-2894; Fax: +81-43-290-2895; E-mail: imanari@p.chiba-u.ac.jp

maintaining haemostasis [6,7]. The anticoagulant activity of heparin is ascribed to specific structural features as well as its high level of sulfation [8–10]. Heparin has been used for many years in clinical, pre-surgical and post-surgical prophylaxis of thrombotic events [11,12]. However, because of its side effects such as the decrease of platelets causing bleeding and other disadvantages, developing alternatives to heparin are an important research goal [9,13–16].

Recently, chemically modified GAGs and polysaccharides have been studied as potential heparin analogues in drug development [7,9,17,18]. Fully *O*-sulfonated GAGs, synthesized in our laboratory, have been shown to exhibit strong antithrombotic activity [17,18]. In those papers, we have shown that the conformation of glucuronate residues in the chemically modified glycosaminoglycans make them both structurally and conformationally similar to the 2-*O*-sulfo iduronic acid residues found in heparin and dermatan sulfate [19–21]. In this paper, we have investigated that the effect of 6-*O*-sulfonate group of HexpN residues for anticoagulant activity of intact and fully *O*-sulfonated GAGs by using the chemical selective desulfation procedure with *N*, *O*-bis(trimethylsilyl)acetamide (BTSA) [22]. The relationship of uronic acid conformation and 6-*O*-sulfonate to anticoagulant activity is also discussed.

Experimental

Materials

Chondroitin sulfate (average molecular weight (MWav, 16 000)) from bovine tracheal cartilage and dermatan sulfate (MWav, 30 000) from porcine skin were kindly gifted by Shin-Nippon Yakugyo Co. (Tokyo, Japan). Hyaluronan (MWav, 100 000) from *Streptococcus zooepidemicus* was purchased from Kibun Food Chemipha Co. (Tokyo, Japan). Heparin (MWav, 16 000) and heparan sulfate (MWav, 14 800) from porcine intestinal mucosa were kindly gifted from Professor Robert J. Linhardt (The University of Iowa).

Chemical sulfonation of glycosaminoglycans

Glycosaminoglycans were fully *O*-sulfonated as described previously [17]. In the cases of *O*-sulfonation of heparin and heparan sulfate, partial loss of *N*-sulfate group from GlcpNS residues was observed, requiring re-*N*-sulfation according to previously described method [23].

6-*O*-Desulfonation with BTSA

Selective desulfonation of intact GAGs and FGAGs were achieved according to the method described previously by Matsumoto *et al.* [22]. Briefly, an aqueous solution of the sodium salt of GAG (100 mg) was passed through a Dowex 50WX8 (H^+ form, 200–400 mesh) column (0.8 cm i.d. $\times$ 10 cm) at room temperature and the eluate was neutralized with pyridine, and lyophilized. The pyridinium salt of GAG (105 mg) was dissolved in 10 mL dry pyridine (Merck, Darmschtadt) and 2 mL BTSA was added. The mixture was kept for 2 h at 70 °C to give a clear solution and then, 10 mL water was added to the mixture to decompose the excess reagent and silyl ester. The mixture was dialyzed against distilled water and pH was adjusted to 7.0 with 0.1 M NaOH, and lyophilized to obtain the 6-*O*-sulfonated GAG sodium salt (75 mg).

Determination of molecular weight

Both the PAGE analysis [24] and GPC-HPLC [25] were used to estimate the average molecular weight (MWav) of each sample. A GPC-HPLC column (Asahipak GS-520 (7.6 mm i.d. $\times$ 50 cm) was eluted with 50 mM sodium acetate, pH 7.4 at a flow rate of 1 mL/min with detection at 206 nm [25]. The relative molecular weights of GAGs were determined from their elution position.

Compositional analysis of GAG derivatives

GAG samples were prepared for the determination of sulfate and hexosamines by exhaustive dialysis (MWCO 3500) against distilled water, lyophilization and drying for 2 days in a desiccator over P_2O_5. Determination of sulfate group was performed by anion-exchange HPLC after acid hydrolysis of the sample in 6 M HCl at 100 °C for 2.5 h using conductivity detection (Tosoh model CM-8, Tokyo, Japan). Hexosamine was analyzed by the postcolumn HPLC derivatization method [26] after acid hydrolysis under identical conditions as described for sulfate analysis.

IR spectroscopy

The IR spectra were obtained with a Jasco model FTIR 230 (Tokyo, Japan). Glycosaminoglycan (100 μg) was mixed with 500 μg of dried KBr and compressed to prepare a salt disk (3 mm diameter).

^{1}H NMR spectroscopy

^{1}H NMR spectroscopy was performed under the conditions described previously [18]. All samples for NMR experiments were subjected to ion-exchange chromatography to remove paramagnetic impurities such as iron and manganese ions. A column (1 cm $\times$ 10 cm) of AG 50W-X8 (Bio-Rad Japan, Tokyo) was converted into sodium form by treatment with 5 mL of 0.1 M NaOH and washed with water for 12 h before use. Samples for NMR experiment were applied to the column, eluted with 20 mL of water and freeze dried. Two to five mg of each sample was freeze dried three times from 99.8% D_2O (Merck, Germany), then dissolved in 0.5 mL of 100% D_2O (Aldrich Japan, Tokyo) for NMR spectroscopy in a

5 mm tube. 3-(Trimethylsililpropionic-2,2,3,3-d_4 acid was used as an internal stand for chemical shifts.

Magnitude-mode 2D spectra were obtained from 512 experiments resulting 1024 data points for a spectral width of 2000 Hz and the time domain data were multiplied after zero filling (data matrix size, 1 K × 1 K) with a shifted sine-bell window functions for 2D double and triple quantum filtered (DQF and TQF)-COSY, NOESY and TOCSY experiments. The instrument manufacturer's standard pulse sequences were used in all cases. An MLEV-17 mixing sequence of 100 ms was used for 2D TOCSY experiments.

Figure 1. Gradient PAGE of fully *O*-sulfonated and 6-*O*-desulfonated glycosaminoglycans. *Lanes:* a. fully *O*-sulfonated chondroitin sulfate; b. a sample as same as lane a after 6-*O*-desulfonation; c. fully *O*-sulfonated hyaluronan; d. a sample as same as lane c after 6-*O*-desulfonation; e. fully *O*-sulfonated dermatan sulfate; f. a sample as same as lane e after 6-*O*-desulfonation; g. heparin; h. a sample as same as lane g after 6-*O*-desulfonation.

Table 1. Compositional analysis of intact and chemically modified glycosaminoglycans.

GAG	*Sulfate/ disaccharide**	*MWav (kDa)***	*HexN*	*GlcAP****	*IdoA*
CS	0.93	16	GAlNAc	100	0
FCS	3.94	24			
De6FCS	2.96	21			
DS	1.04	30	GalNAc	11.8	88.2
FDS	3.95	48			
De6FDS	3.06	41			
HA	0	100	GlcNAc	100	0
FHA	3.97	195			
De6FHA	2.88	170			
HP	2.68	16	GlcNS/NAc	8.9	91.1
De6HP	1.54	13			
FHP	4.88	19			
De6FHP	3.76	16			

CS, chondoritin sulfate; FCS, fully *O*-sulfonated CS; De6FCS, 6-*O*-desulfonated FCS; DS, dermatan sulfate; FDS, fully *O*-sulfonated DS; De6FDS, 6-*O*-desulfonated FDS; HA, Hyaluronan; FHA, fully *O*-sulfonated HA; De6FHA, 6-*O*-desulfonated FHA; HP, heparin; De6HP, 6-*O*-desulfonated HP; FHP, fully *O*-sulfonated HP; De6FHP, 6-*O*-desulfonated FHP.
*Sulfate group and aminosugar were analyzed independently by Ion Chromatography and HPLC after hydrolysis.
**The values were estimated by both PAGE analysis and the gel filtration HPLC method.
***Uronate residues were analyzed by proton NMR.

Anti-factor IIa activity

Normal human plasma (NHP) was collected from healthy volunteers. Anti-factor IIa activity was measured by incubating 50 µL of intact and chemically modified GAG in 30 µL of NHP or AT III from human plasma (Wako Pure Chemicals, Japan) with 20 µL of human thrombin (1.2 NIH units/mL) at 37 °C for 30 s. Chromogenic TH (ethylmalonyl-Pro-Arg-p-nitroanilide hydrochloride) substrate 50 µL (1.9 µmol/mL) was added and the amidolytic activity of thrombin was determined at 405 nm. An ACL 300 plus instrument (Lexington, MA) was used and activity was calculated in comparison with USP Heparin Reference Standard (K-3) supplied by U.S. Pharmacopeial Convention (Rockville, MD) [27].

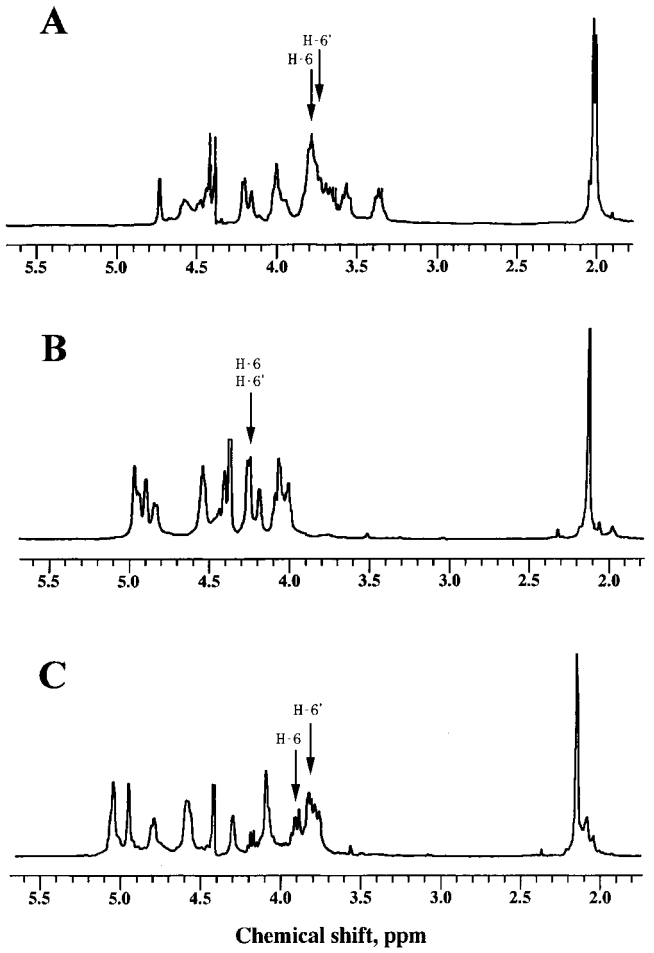

Figure 2. One-dimensional ^{1}H NMR spectra of intact, fully *O*-sulfonated and 6-*O*-desulfonated fully *O*-sulfonated dermatan sulfate measured at 60 °C. A. Intact dermatan sulfate; B. fully *O*-sulfonated dermatan sulfate; C. 6-*O*-desulfonated fully *O*-sulfonated dermatan sulfate. Arrows indicate the signals of H-6 protons of GalpNAc.

Results

Preparation and characterization of 6-*O*-desulfonated intact and fully *O*-sulfonated GAGs

6-*O*-Desulfonation of each GAG derivative was performed at different temperatures and resulted in 6-*O*-desulfonated fully *O*-sulfonated GAGs (De6FGAGs). Harsh conditions for 6-*O*-desulfonation, such as the reaction at 70 °C for 5 h, cause desulfonation not only on C-6 position of hexosamine residues but also on C-2 and/or C-3 position of hexuronate residues as suggested by ^{1}H NMR spectra (data not shown). Molecular weight of each sample was determined using gradient polyacrylamide gel electrophoresis (PAGE) analysis [26] (Figure 1). A slight decrease in molecular weight was observed on 6-*O*-desulfonated GAG derivatives compared to those of FGAGs. The results obtained were consistent with the decreased mass of the *O*-sulfonate groups and the expected stability of the glycosidic linkages in the polysaccharides under the reaction conditions. Table 1 shows the chemical compositional analyses of the samples.

The De6FGAGs afforded no unsaturated disaccharide products by treatment with chondroitin/heparin lyases as expected based on the known resistance of highly *O*-sulfonated domains to these enzymes [28]. IR spectroscopy of FDS and De6FDS strongly suggested the loss of 6-*O*-sulfonate group from GalpNAc residues (data not shown). The intensity of the bands at 1440, 1380 and 1100 cm^{-1}, attributed to the stretching and/or deformation vibration of C−O−H bonds, was increased in the spectrum of the De6FDS.

^{1}H NMR spectra of intact, fully *O*-sulfonated and 6-*O*-desulfonated dermatan and chondroitin sulfates prepared at 70 °C for 2 h are shown in Figure 2 and 3, respectively. The signals correspond to H-6 protons of GalpNAc residues in the derivatized GAGs were significantly shifted upfield by 6-*O*-desulfonation. Two dimensional (2D) ^{1}H NMR experiments measured at both 30 and 60 °C, involving DQF- and TQF-COSY, and NOESY spectroscopy, of the 6-*O*-desulfonated FGAGs, clearly show the upfield shifts of protons attached to

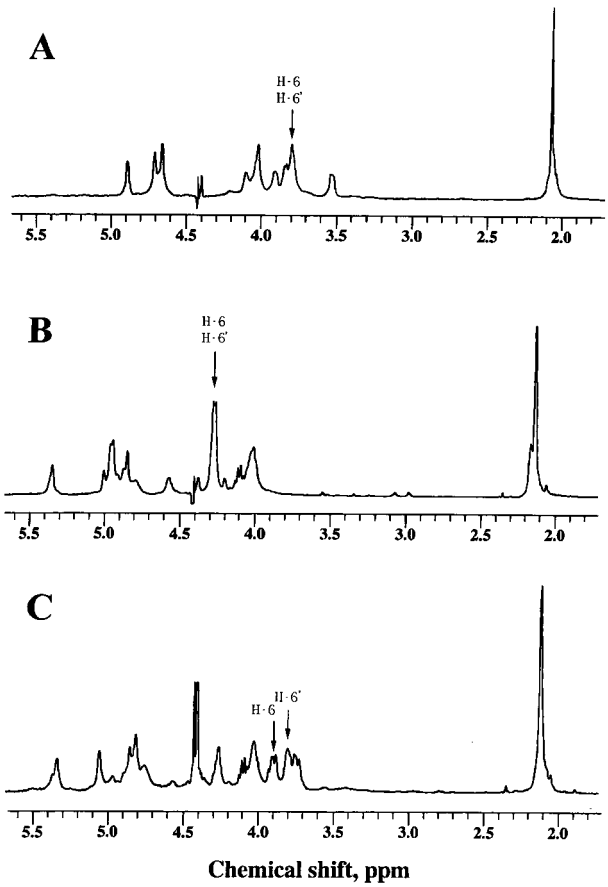

Figure 3. One-dimensional ^{1}H NMR spectra of intact, fully *O*-sulfonated and 6-*O*-desulfonated fully *O*-sulfonated chondroitin sulfate measured at 60 °C. A. Intact chondroitin sulfate; B. fully *O*-sulfonated chondroitin sulfate; C. 6-*O*-desulfonated fully *O*-sulfonated chondroitin sulfate. Arrows indicate the signals of H-6 protons of GalpNAc.

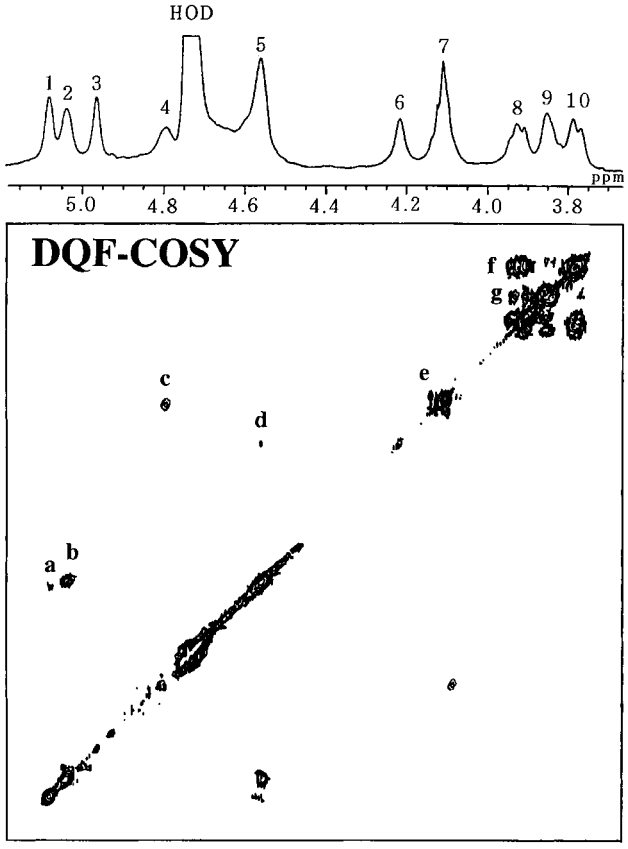

Figure 4. Two-dimensional DQF-COSY spectrum of 6-*O*-desulfonated fully *O*-sulfonated chondroitin sulfate measured at 30 °C. *Peaks:* 1. GlcpA H-3; 2. GlcpA H-1; 3. GalpNAc H-4; 4. GalpNAc H-1; 5. GlcpA H-2 and H-4; 6. GlcpA H-5; 7. GalpNAc H-2 and H-3; 8. GalpNAc H-6; 9. GalpNAc H-5; 10. GalpNAc H-6′. HOD signal was presaturated during pulse delay time (3 s). *Cross peaks:* a. GlcpA H-2/H-3; b. GlcpA H-1/H-2; c. GalpNAc H-1/H-2; d. GlcpA H-4/H-5; e. GalpNAc H-2/H-3; f. GalpNAc H-6/H-6′; g. GalpNAc H-5/H-6.

the 6-*O*-desulfonated carbon (Figure 4 and Table 2). NOESY spectra of the De6FGAGs showed cross-peaks that strongly suggest that the sequence and linkage positions of the GAGs are maintained (data not shown).

Table 2 summarizes the chemical shifts and coupling constants of ring protons of 6-*O*-desulfonated fully *O*-sulfonated chondroitin sulfate, dermatan sulfate, hyaluronan and heparin samples. The coupling constant of each ring proton of glucuronate of hyaluronan at 30 °C was used to calculate the dihedral angles of vicinal protons of glucuronate. As we described in previous paper [17], this dihedral angle was not the 180°, typical observed for a glucuronate residue after 6-*O*-desulfonation. These data strongly suggest that a conformational change from $^4C_1$ to $^1C_4$ of the glucuronate residue was still maintained on De6FGAGs (Figure 5). Unusual coupling constants ($\sim$4 Hz) between H-1 and H-2 of GlcpA residues of 6-*O*-desulfonation of the FCS and FHA was observed. These observations strongly suggest that at 60 °C the conformation of the glucuronate residue in the FCS, De6CS, FHA and De6FHA has changed from $^1C_4$ to $^2S_0$ (Figure 5) [17].

Effect of 6-*O*-desulfonated GAGs on the inactivation of factor IIa and factor Xa by human plasma

The anti-factor IIa or Xa of the FGAGs are summarized in Figure 6. The anticoagulant activities of GAG derivatives and heparin with the exception of dermatan sulfate were significantly decreased by 6-*O*-desulfonation. Surprisingly, the activity of De6FDS was almost same value with that of FDS. It is well established that the consecutive sequence of [→4IdopA2Sα1→3GalNAc4Sβ1→] in dermatan sulfate can bind to heparin cofactor II and is required to inhibit blood coagulation. Consequently, in the case of dermatan sulfate, both 2- and 4-*O*-sulfate groups of IdopA and GalpNAc, respectively, might be sufficient for the strong activity shown in Figure 6.

In the previous papers [17,18], we have shown the correlation between the sulfonation level of chemically *O*-sulfonated GAGs and their inactivation of factor IIa activity. Since this dramatic increase of activity was only observed with full *O*-sulfonation, the increased anti-factor IIa activity probably does not merely the result from an increase in overall charge. Consequently, we have concluded that some other structural change, such as a shift in conformation, is responsible for the increased anti-factor IIa activity of the FCS derivative.

Discussion

We previously reported the preparation of FGAGs that showed strong anticoagulant activity [17,18]. The activity of these derivatives was ascribed to a conformational change of glucuronate residue, and because of the well-known molecular-size dependence of the anticoagulant activity, the difference of the activity among the modified GAGs might also be ascribed to their average molecular weight [29]. In this present study, 6-*O*-desulfonation of FGAGs and intact heparin have dramatically caused decrease of the anticoagulant activity, with exception of dermatan sulfate. ^{1}H NMR experiments (Figures 2, 3 and 4), and compositional analysis data (Table 1), demonstrated that FGAGs and intact heparin were completely 6-*O*-desulfonated by the reaction with BTSA. Although the reaction mechanism has not been elucidated, the results indicate that the desulfation performed in BTSA in

Table 2. Chemical shifts (ppm) and coupling constants (Hz) of 6-*O*-desulfonated fully *O*-sulfonated GAGs.

*GAG**	*Residue*	*H-1* *J1,2*	*H-2* *J2,3*	*H-3* *J3,4*	*H-4* *J4,5*	*H-5* *J5,6*	*H-6a*	*H-6b*	*N-Ac*
CS	GalpNAc	4.59 n.d.**	3.76 n.d.	4.09 n.d.	4.96 <1.5	4.08 n.d.	3.91	3.82	2.15
	GlcpA	5.03 3.8	4.78 n.d.	5.03 n.d.	4.59 n.d.	4.30	–	–	–
DS	GalpNAc	4.82 n.d.	3.72 n.d.	4.02 n.d.	4.82 n.d.	4.02 n.d.	3.89	3.80	2.11
	IdopA	5.35 <1.5	4.82 n.d.	4.80 <1.5	4.28 <1.5	4.58	–	–	–
HA	GlcpNAc	4.91 n.d.	3.96 n.d.	4.18 8.7	3.96 n.d.	4.22 <1.5	3.90	3.81	2.14
	GlcpA	5.02 4.1	4.56 n.d.	4.91 n.d.	4.56 <1.5	4.29	–	–	–
HP	GlcpNS	5.47 2.4	3.81 10.0	4.80 <1.5	4.12 n.d.	4.12 n.d.	3.88	3.84	–
	IdopA	5.35 <1.5	4.90 <1.5	4.61 <1.5	4.49 <1.5	5.14	–	–	–

*CS, chondroitin sulfate; DS, dermatan sulfate; HA, hyaluronan; and HP, heparin.
n.d.** not determined.

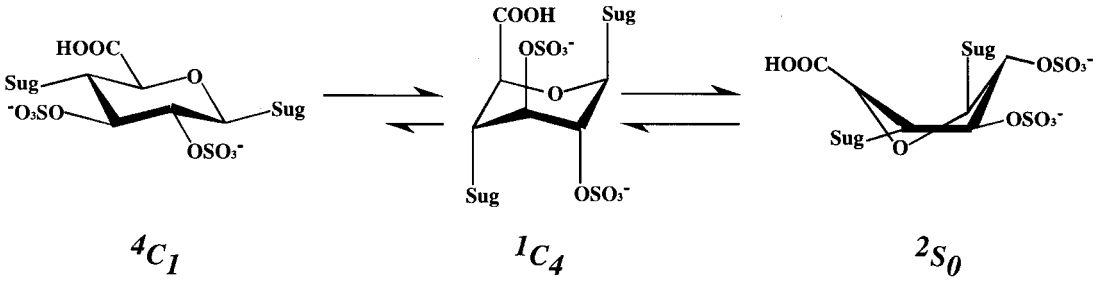

Figure 5. Effect of *O*-sulfonation on the conformation of glucuronate residue in chondroitin sulfate. The glucuronate residue of fully *O*-sulfonated chondroitin sulfate residues primarily in the $^1C_4$ conformer at 30 °C and in the $^2S_0$ conformer at 60 °C.

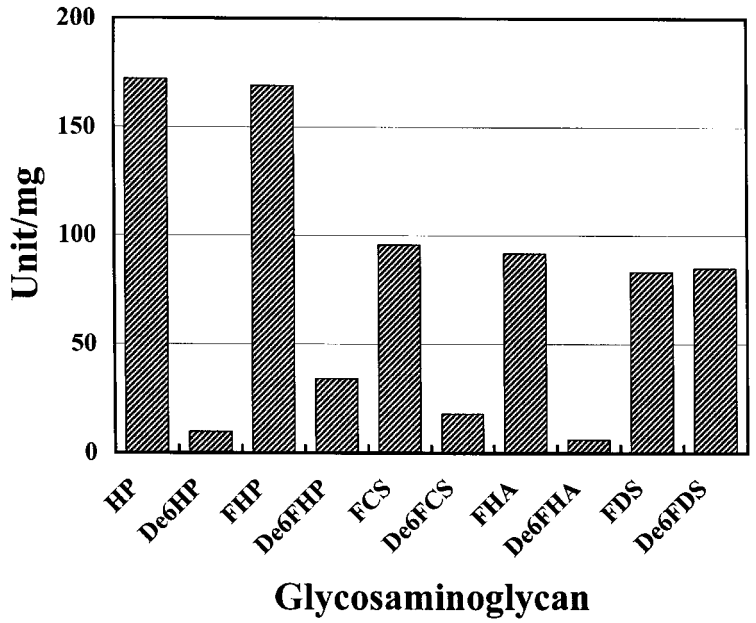

Figure 6. Anti-factor IIa activity of *O*-sulfonated and 6-*O*-desulfonated fully *O*-sulfonated GAGs and intact heparin. HP, heparin; De6HP, 6-*O*-desulfonated HP; FHP, fully *O*-sulfonated HP; De6FHP, 6-*O*-desulfonated FHP; FCS, fully *O*-sulfonated chondroitin sulfate; De6FCS, 6-*O*-desulfonated FCS; FHA, fully *O*-sulfonated hyaluronan; De6FHA, 6-*O*-desulfonated FHA; FDS, fully *O*-sulfonated dermatan sulfate; De6FDS, 6-*O*-desulfonated FDS.

pyridine is specific for 6-*O*-sulfonates and that glycosidic linkages are not cleaved (see Figure 1).

This study demonstrates that chemically 6-*O*-desulfonation of FGAGs and intact heparin affords products that show decrease of anti-factor IIa activities (Figure 6) comparable with the activities displayed by previously described FGAGs [17,18]. ^{1}H NMR spectroscopy demonstrates that an altered conformational change of glucuronate residues from $^4C_1$ to $^1C_4$ in the De6FCS and De6FHA derivatives [17,18]. These two GAGs both still showed $^1C_4$ glucuronate, closely resembling the 2-*O*-sulfo-iduronate residue commonly found in heparin and dermatan sulfate. However, the anti-factor IIa activity of these two GAGs was dramatically decreased. Consequently, it might be necessary not only conformational change of glucuronate residues but also 6-*O*-sulfonate group of hexosamine residues for anticoagulant activity.

Interestingly, while the anticoagulant activity of heparin was significantly decreased by 6-*O*-desulfonation, the activity of dermatan sulfate derivatives was unchanged by 6-*O*-desulfonation. These observations confirm that while ATIII is known to bind to a specific pentasaccharide sequence found within heparin's structure, HCII binds with considerably less specificity to oversulfated domains of heparin [30,31], dermatan and chondroitin sulfates [7,17,18,32]. Therefore, it is likely that the large enhancement of anti-factor IIa activity, observed for De6FDS, is an HCII mediated activity. None of intact and chemically modified GAGs used in this paper, except unfractionated intact heparin, showed anti-thrombin activity under the assay system using commercially available AT III instead of normal human plasma.

The De6FDS might be useful for anticoagulant reagent, because of less nonspecific binding of chemically sulfonated

polysaccharides to plasma proteins. Such a chemical derivatization of GAGs might represent a new route for the generation of new compounds with a variety of pharmacologically relevant biological activities.

Acknowledgement

This work was supported in part by Grants-in-Aid from the Ministry of Culture, Sports and Education of Japan (09672185 and 11672136 (TT) and 09470490 (TI)).

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Received 7 July 2000; revised 28 August 2000; accepted 5 September 2000