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Carbohydrate Research 337 (2002) 925–933

CARBOHYDRATE RESEARCH

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Effect of (1→3)- and (1→4)-linkages of fully sulfated polysaccharides on their anticoagulant activity

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Received 3 January 2002; accepted 12 March 2002

Abstract

Chemically fully sulfated polysaccharides including xylan (→4Xylβ-(1→4)Xylβ1→), amylose (→4Glcα-(1→4)Glcα1→), cellulose (→4Glcβ-(1→4)Glcβ1→), curdlan (→3Glcβ-(1→3)Glcβ1→) and galactan (→3Galβ-(1→3)Galβ1→), which have been isolated from Korean clam, were prepared, and their anticoagulant activity was investigated. The results strongly suggest that the activity might not be depending on anomeric configuration (α or β) or monosaccharide species but on the glycosidic linkage, either (1→3) or (1→4). ^{1}H NMR studies of these modified polysaccharides show that the neighboring sulfate groups at the C-2 and C-3 positions might have caused the conformational changes of each monosaccharide from $^4C_1$ to $^1C_4$. Furthermore, the effect of 6-sulfate residues on the anticoagulant activity was investigated using a specific desulfated reaction for the chemically fully sulfated polysaccharides. The 6-sulfate group is very important in determining anticoagulant activity of (1→3)-linked polysaccharides, whereas the activity is not affected by presence or absence of the 6-sulfate group in (1→4)-linked polysaccharides.

Keywords: Fully chemically sulfated polysaccharides; Anticoagulant activity; NMR spectroscopy

1. Introduction

Sulfated polysaccharides comprise a complex group of macromolecules with a wide range of important biological properties. These anionic polymers are widespread in nature, occurring in a great variety of organisms. In marine algae, the carrageenans and fucoidans are composed mainly of sulfated galactose and fucose, respectively.[1–3] Vertebrate tissues express abundant sulfated glycosaminoglycans.[4] Invertebrate species are also a rich source of sulfated polysaccharides with novel structures.[5–10] We have isolated and characterized the structure of a new sulfated β-galactan from clams.[9] In contrast to the algal polysaccharides, invertebrate galactans have simple structures, composed of a single repeating unit.[9] The specific pattern of sulfation and the position of the glycosidic linkage vary among different species.[6,8,11] One way to determine the relationship between structure and biological activity of sulfated polysaccharides is to compare their activity in various assays where the contributions of the polysaccharide backbone, and the extent and position of sulfation have been fully characterized.

On the other hand, we have reported the several biological activities of chemically fully sulfated poly- and oligosaccharides.[12–14] Anticoagulant and antithrombotic activities are among the most widely studied properties of sulfated polysaccharides.[11–16] The

Abbreviations: DQF-COSY, double quantum filtered chemical shift correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; HPLC, high-performance liquid chromatography; HPSEC, high-performance size-exclusion chromatography; NMR, nuclear magnetic resonance spectroscopy; PAGE, polyacrylamide gel electrophoresis; TOCSY, total chemical shift correlated spectroscopy.

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0008-6215/02/$ - see front matter
PII: S0008-6215(02)00078-2

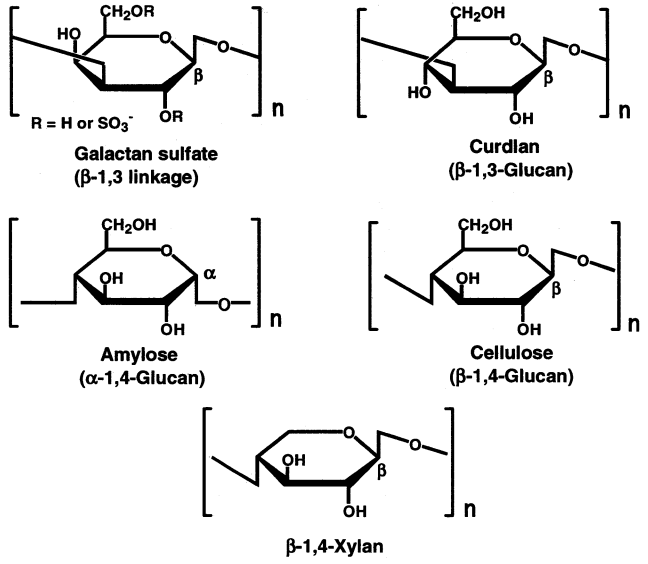

Fig. 1. Structure of polysaccharides.

anticoagulant glycosaminoglycan heparin is an important therapeutic agent for prophylaxis and treatment of thrombosis;[15] dermatan sulfate is also an anticoagulant agent, although of lower potency than heparin.[16–18] Other sulfated polysaccharides, either extracted from marine brown,[10,19–22] red,[23,24] and green alga,[25] or obtained by chemical sulfation of natural polysaccharides,[26–30] have been described as anticoagulants. In contrast to heparin and dermatan sulfate, the structural features of these chemically sulfated polysaccharides have not been correlated with anticoagulant activity. In the present study, we prepared and characterized the structure of chemically fully sulfated (1→3)-, or (1→4)-linked linear polysaccharides including xylan, amylose, cellulose, curdlan, which are commercially available, and sulfated D-galactan isolated from clams, and their anticoagulant activities were compared. Additionally, the contribution of the 6-sulfate group for anticoagulant activity of chemically fully sulfated polysaccharides was investigated by using the specific desulfation reaction.

2. Results

Preparation and characterization of fully sulfated polysaccharides.—The polysaccharides were subjected to persulfation with sulfur trioxide–pyridine to both increase their negative charge and to decrease (or eliminate in the case of D-galactan from clam) their sequence heterogeneity (Fig. 1). Sulfation of each polysaccharide was performed at different conditions and resulted in oversulfated polysaccharides having different levels of sulfation. The different types of reaction or full sulfation mechanisms for polysaccharides are considered due to different types of glycosidic linkages, such as β-(1→3) and β-(1→4), as well as to the kinds of constituent sugar residues in the polysaccharides. The degree of sulfate substitution of preparations is shown in Table 1, presented in molar ratio to a monosaccharide unit. The degree of sulfation of each fully sulfated polysaccharide was determined to be three, except for xylan that has two hydroxyl groups/monosaccharide. Table 1 shows that the sulfation reaction for each polysaccharide might be almost completely achieved. The molecular weight of each sample was determined using both gradient polyacrylamide gel electrophoresis (PAGE) and high-performance size-exclusion chromatography (HPSEC) analyses.[31] Fig. 2 shows the PAGE profile of chemically modified polysaccharides.

Changes in the optical rotation of polysaccharides shown in Table 1 accompany full sulfation. The difference of the optical rotation from the intact polysaccharide was significant in the case of fully sulfated samples. The magnitude and direction of this change are consistent with either a significant change in the molecular conformation of these derivatives or the dilution effect of newly substituted sulfate groups.[32] IR spectroscopy of intact and fully sulfated cellulose (Fig. 3(A and B)) strongly suggested the conversion of hydroxyl groups to axial–equatorial sulfate groups. The intensity of the absorbances at 1240 cm^{-1} and 800–820 cm^{-1} attributed to the stretching of S=O bond and C–O–S bonds on the sulfate ester of the hexose, respectively, is dramatically changed by sulfation of cellulose. Simi-

Table 1
Chemical composition, average molecular mass and specific optical rotation of sulfated polysaccharides

Sample	Linkage	Monosaccharide constituents	Sulfation degree	Mw_{av} (Da)	$[\alpha]_D^{25}$	
					Intact	Sulfated
Amylose	α-(1→4)	Glc	2.9	22,000	+190	+87.9
Cellulose	β-(1→4)	Glc	2.9	24,000	ND [a]	−9.0
Curdlan	β-(1→3)	Glc	2.8	39,000	ND	−21.7
Galactan	β-(1→3)	Gal	2.9	8300	+42.2	ND
Xylan	β-(1→4)	Xyl	2.1	16,000	−71.1	−57.1

[a] Not determined.

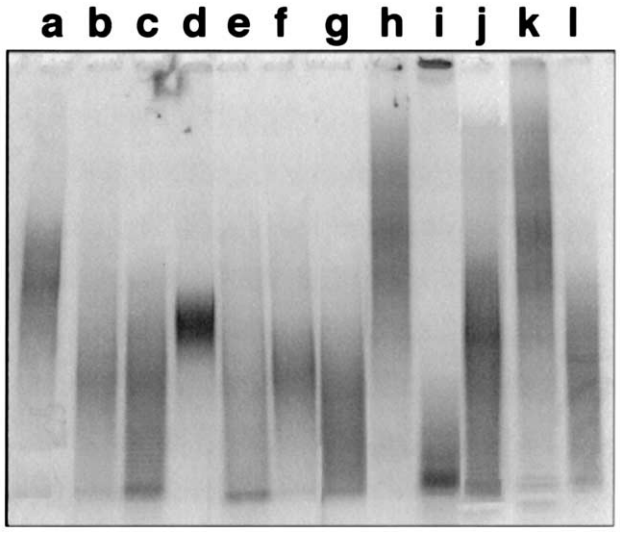

Fig. 2. Gradient PAGE analysis of chemically modified polysaccharides. Lanes: (a) fully sulfated chondroitin sulfate from whale cartilage (Mw_{av} 46.4 kDa); (b) fully sulfated chondroitin sulfate from bovine tracheal cartilage (Mw_{av} 22.5 kDa); (c) fully sulfated chondroitin sulfate from shark cartilage (Mw_{av} 18.0 kDa); (d) fully sulfated dermatan sulfate from porcine intestinal mucosa (Mw_{av} 24.2 kDa); (e) fully sulfated heparan sulfate from porcine intestinal mucosa (Mw_{av} 22.0 kDa); (f) fully sulfated heparin from porcine intestinal mucosa (Mw_{av} 21.0 kDa); (g) dextran sulfate (Mw_{av} 10.0 kDa); (h) fully sulfated curdlan; (i) fully sulfated galactan; (j) fully sulfated cellulose; (k) fully sulfated amylose; (l) fully sulfated xylose.

larly, the intensity of the bands at 2900 cm^{-1}, attributed to the stretching and/or deformation vibration of C–O–H bonds, was decreased in the spectrum of the fully sulfated polysaccharides. Assignment of IR absorption bands at 1240 cm^{-1} in the spectrum of fully sulfated cellulose was based on the report of Cabassi and co-workers.[33] The absorptions at about 800–820 cm^{-1} were tentatively ascribed to sulfate half-esters based on the report of Grant and coworkers,[32] and the band at 800 cm^{-1} was ascribed to C–O–S stretching within predominantly axial–equatorial 2-, and 3-*O*-sulfo groups of glucose residues based on the work of Sanderson et al.[34]

^1H NMR spectra of fully sulfated polysaccharides prepared at 40 °C are shown in Fig. 4. Because of the insolubility of cellulose in DMF for the sulfation reaction, the sample was treated with the reagents repeatedly to obtain fully sulfated cellulose. It should be mentioned that the solubility of the sulfated derivatives in water is influenced by the number of sulfate ester groups. Several mg of fully sulfated cellulose could, for example, be dissolved in 1 mL of a water–DMSO mixture at ambient temperatures. Partial sulfation prepared under mild conditions resulted in an expected appearance in the structural heterogeneity (data not shown). This increased heterogeneity is caused by the introduction of sulfate groups at the 2- and/or 3- and/or 6-positions of the 4-linked monosaccharide residues, or at the 2- and/or 4- and/or 6-positions of the

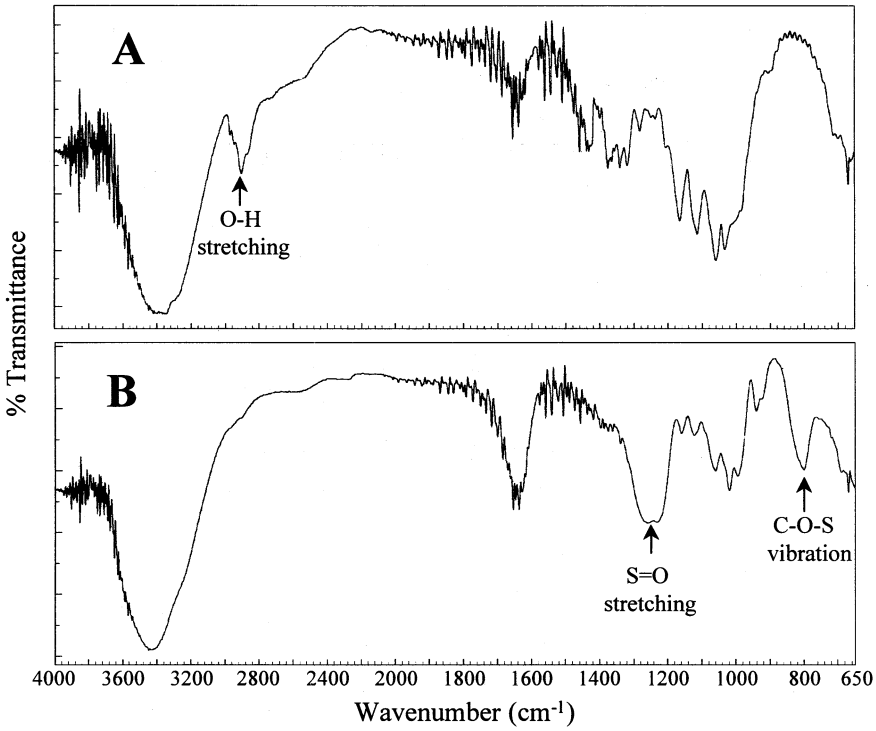

Fig. 3. IR spectra of intact and chemically sulfated cellulose. (A) Unmodified cellulose; (B) fully sulfated cellulose. Each arrow indicates the absorbance band attributed to each functional group.

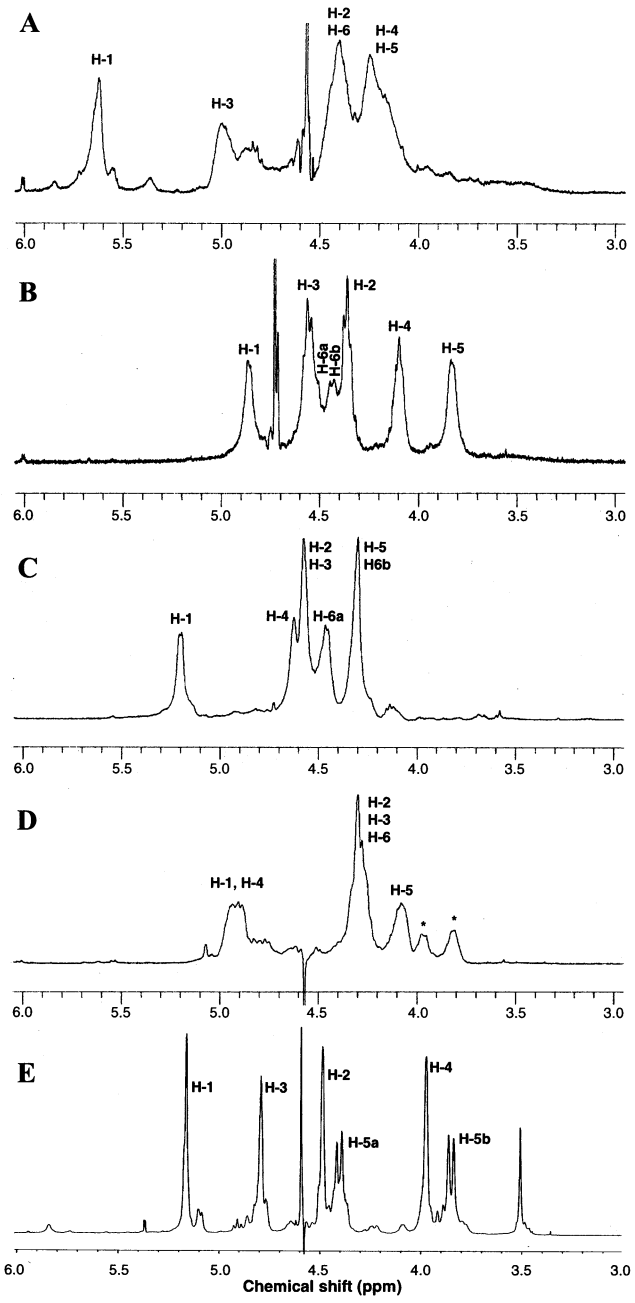

Fig. 4. 1D ^{1}H NMR spectra of chemically fully sulfated polysaccharides. (A) Fully sulfated amylose; (B) fully sulfated cellulose; (C) fully sulfated curdlan; (D) fully sulfated galactan; (E) fully sulfated xylan; *, signals from contaminant(s).

3-linked monosaccharide residues. Sulfation at 40 °C resulted in a simple 1D ^{1}H NMR spectrum consistent with full sulfation. Two-dimensional (2D) ^{1}H NMR experiments involving DQF and NOESY spectroscopy of fully sulfated cellulose (Fig. 5) clearly show the downfield shifts of ring protons attached to the sulfated carbons, and also afford sequence confirmation. NOESY spectra of the fully sulfated polysaccharides showed cross-peaks that strongly suggest that the sequence and linkage positions of the polysaccharides are maintained.

Table 2 summarizes the chemical shifts and coupling constants of ring protons of fully sulfated amylose, cellulose, galactan, xylan, and curdlan samples. The coupling constant of each ring proton of glucose of fully sulfated amylose at 30 °C was around 1–2 Hz, and this value did not correspond to the dihedral angle by 180°, which is typically observed for a glucose residue in amylose. These data strongly suggest that the glucose residue had undergone a conformational change from $^{4}C_{1}$ to $^{1}C_{4}$, resulting from the full sulfation of this residue in solution. The coupling constant between H-1 and H-2 of a glucose residue of cellulose, curdlan, and galactan at 30 °C dramatically changed from <1.5 to ~ 4–6 Hz (Table 2). This observation strongly suggests that the conformation of the glucose residue, in the fully sulfated cellulose, curdlan, and galactan at 30 °C, might have changed from $^{1}C_{4}$ to $^{2}S_{0}$. On the other hand, an unusual coupling constant ($<$ 1.5 Hz) between H-2 and H-3, H-3 and H-4, and H-4 and H-5 of the xylose residues of xylan were observed. The coupling constants among ring protons of xylose residues of intact xylan are typically in the range of 8–9 Hz at 30 °C (data not shown). This observation may suggest that full sulfation might have caused distortion of xylose residues in xylan.

6-Desulfation of each fully sulfated polysaccharide was performed at different reaction times because of different stabilities for each sulfated polysaccharide un-

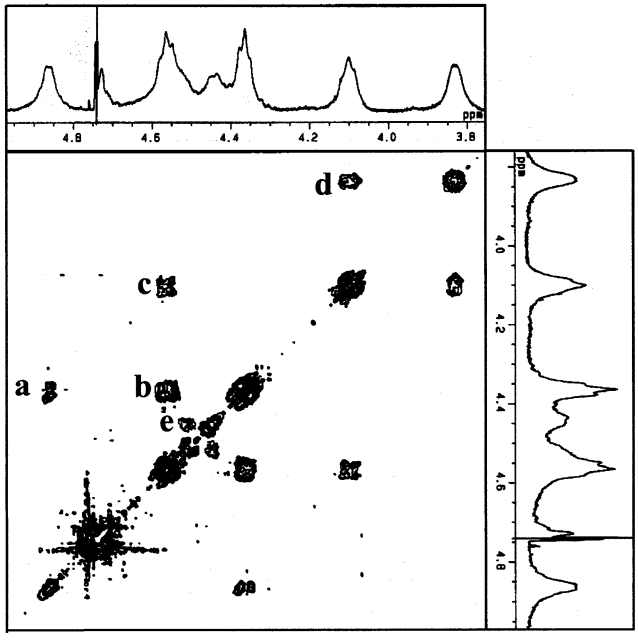

Fig. 5. 2D DQFCOSY ^{1}H NMR spectrum of chemically fully sulfated cellulose. Cross peaks: (a) H-1/H-2; (b) H-2/H-3; (c) H-3/H-4; (d) H-4/H-5; (e) H-6a/H-6b. Cross peaks between H-5 and H-6 protons were observed at the lower threshold (data not shown).

Table 2
Chemical shifts and coupling constants of fully sulfated polysaccharides

Sample	Chemical shift (ppm) Coupling constant (Hz)								
	H-1 $J_{1,2}$	H-2 $J_{2,3}$	H-3 $J_{3,4}$	H-4 $J_{4,5}$	H-5 $J_{5,6}$	H-6a $J_{6a,6b}$	H-6b	H-5a $J_{5a,5b}$	H-5b
Xylan	5.19 <1.5	4.49 <1.5	4.82 <1.5	3.99 <1.5	[b]			4.33 −11.2	3.78
Curdlan	5.20 5.2	4.57 n.d. [a]	4.57 n.d.	4.62 <1.5	4.30 n.d.	4.45 −6.4	4.30		
Amylose	5.67 <1.5	4.42 n.d.	5.00 n.d.	4.20 n.d.	4.28 n.d.	4.40 n.d.	4.40		
Cellulose	4.86 4.1	4.36 7.2	4.57 5.1	4.10 <1.5	3.84 n.d.	4.54 −9.8	4.43		
Galactan	4.92 5.9	4.32 n.d.	4.35 n.d.	4.94 <1.5	4.10 n.d.	4.32 n.d.	4.32		

[a] Not detected.
[b] Not present.

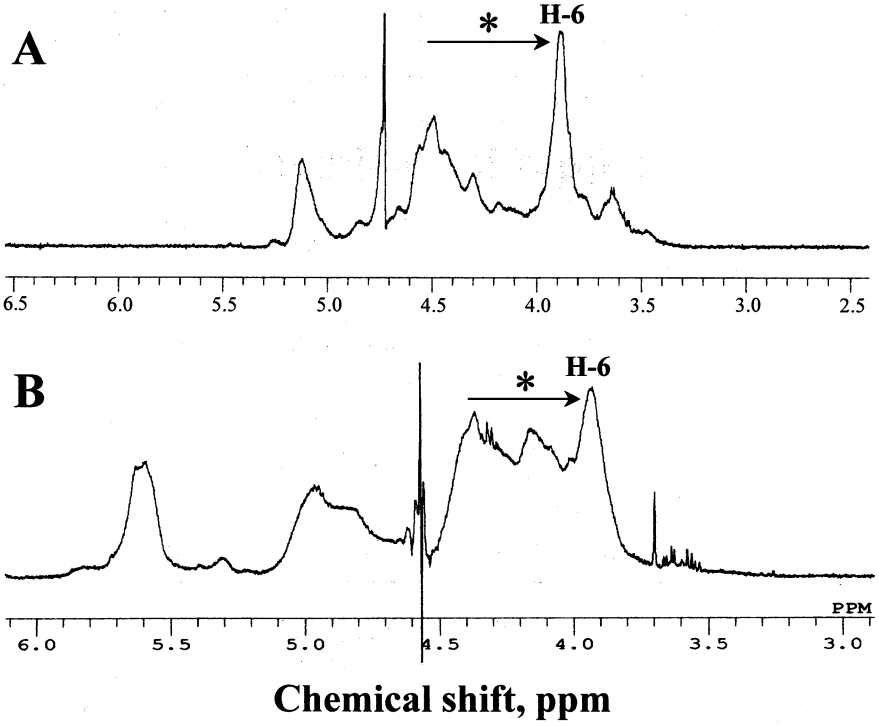

Fig. 6. 1D ^{1}H NMR spectra of 6-desulfated fully sulfated curdlan and amylose. * and arrows indicate the upfield shifts of H-6 protons by desulfation (see Fig. 2).

der the reaction conditions, and resulted in 6-desulfated fully sulfated sugars with a degree of sulfation ~2.0. Harsh conditions for 6-desulfation, such as the reaction at 70 °C, have caused overdesulfation at not only the C-6 primary sulfate group, but also at the C-2 and/or C-3, or C-4 positions as suggested by their ^{1}H NMR spectra (data not shown). Fig. 6 shows the ^{1}H NMR spectra of 6-desulfated, fully sulfated curdlan and amylose, and clearly shows an upfield shift of the H-6 protons upon desulfation (see also Fig. 4).

Effect of fully sulfated polysaccharides on the inactivation of factor IIa by human plasma.—The correlation between the sulfation level of chemically *O*-sulfated GAGs and their inactivation of factor IIa activity has

been previously demonstrated.[12] In the current study, the anticoagulant activities of the chemically fully sulfated and specifically 6-desulfated (1→3)-, or (1→4)-linked linear compounds were examined using the amidolytic method, and the results were compared. Heparin (172 units/mg) was used as a reference substance to estimate the relative anticoagulant action of these sulfated polymers. The effect of the fully sulfated and 6-desulfated, fully sulfated polysaccharides on inactivation of chromogenic amidolytic activity of thrombin via human plasma is illustrated in Fig. 7. The concentration-dependent inhibitions of liberated chromogenic substance can be seen with all of the sulfated polysaccharides and with heparin. Because of the concern that both antithrombin III and heparin cofactor II are present in plasma, thrombin inhibition was independently examined in the systems that were plasma-free, as well as with purified antithrombin. These studies showed that all of the sulfated polysaccharides failed to inhibit thrombin activity, both in the absence of plasma and in the presence of the purified antithrombin III (data not shown). These results conclusively demonstrate that sulfated polysaccharides show no direct thrombin inhibition, but accelerated thrombin inhibition via heparin cofactor II, not through antithrombin III. The antithrombin activity via human plasma of the fully sulfated and 6-desulfated, fully sulfated polysaccharides is measured as relative activity compared with a standard heparin (172 unit/mg) sample. It was apparent that each of the fully sulfated (1→4)- or (1→3)-linked polysaccharides exhibited plasma-mediated antithrombin activity, which was 1/4 less active than that of heparin. The fully sulfated galactan showed the lowest anti-IIa activity among the samples tested, presumably attributable to its having the lowest molecular weight among the compounds. (The correlation between molecular mass and anticoagulant activity of persulfated polysaccharides will be described in the near future.)

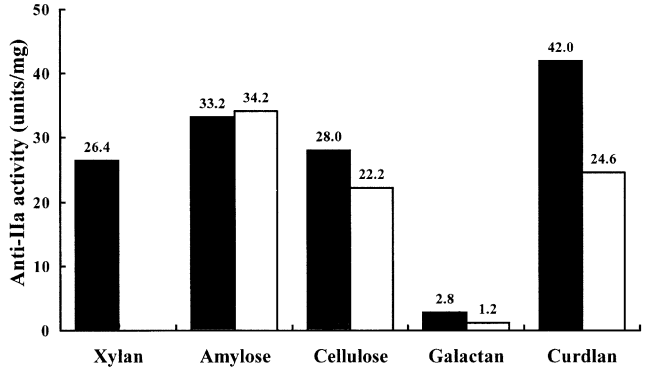

Fig. 7. Anti-IIa activities of chemically fully sulfated polysaccharides and 6-desulfated derivatives. Data were obtained by heparin standard. ■, fully sulfated; □, 6-desulfated, fully sulfated.

Unexpectedly, 6-desulfated, fully sulfated (1→3)-linked polysaccharides, such as curdlan and galactan, lost ~41 and ~57%, respectively, of their anti-factor IIa activity compared to those of their fully sulfated polysaccharides, whereas the activity of 6-desulfated, fully sulfated (1→4)-linked polysaccharides, such as amylose and cellulose, are significantly unchanged. It should be noted that the data obtained here may not be sufficient to generalize the necessity of having a 6-sulfate group for the anticoagulant activity of fully sulfated polysaccharides; however, these data strongly suggest that the presence of two adjacent sulfate groups at the C-2 and C-3 positions are required to inhibit factor IIa activity, and the contribution of 6-sulfate group might be significant for activity in (1→3)-linked polysaccharides.

Additionally, a clear and dramatic increase in anti-factor IIa activity was observed on the complete sulfation of polysaccharides. Since this dramatic activity increase was only observed with full sulfation, the increased anti-factor IIa activity probably does not merely result from an increase in overall charge. It is, instead, likely that some other structural change, such as a shift in conformation, is responsible for the increased anti-factor IIa activity of the fully sulfated polysaccharide derivatives. The absence of anti-factor Xa activity (data not shown) may simply result from a non-specific effect associated with an increase in the overall molecular charge.

3. Discussion

We previously reported the preparation of fully sulfated GAGs that showed strong anticoagulant activity.[12,35] The activity of these derivatives was ascribed to a conformational change in the glucuronate residue. Oversulfated disaccharide sequences account for a minor but important part of the structure of GAGs derived from mammalian tissues.[31] Amylose, cellulose, curdlan, xylan, and galactan were chemically sulfated to investigate their physical, chemical, and biological properties. Optimum conditions for complete sulfation of these polysaccharides were determined to be 40 °C for 6 h with 15 equiv of sulfation reagent/mol of hydroxyl group. ^{1}H NMR experiments (Figs. 3–5) and compositional analysis data (Table 1), demonstrated that they were fully sulfated, and the conformational flipping of core sugar residues caused by negative charges on sulfate groups were observed as well as in the cases of fully sulfated GAGs.[12]

This study demonstrates that both chemically sulfated (1→3)- and (1→4)-linked polysaccharides show anti-factor IIa activities (Fig. 7) comparable with the activities displayed by previously described heparin analogues[36] and low-molecular-weight heparins.[37] Opti-

cal rotation measurements suggest a change in conformation, and bands at 820 cm^{-1} in the IR spectra suggest sulfate ester groups in the glucose residues of amylose and cellulose. Unexpectedly, the repulsion of core sugar structures of (1→3)-linked polysaccharides was also observed. NMR spectroscopy at 30 °C (Figs. 4–6) demonstrates an altered conformation of monosaccharide residues from $^4C_1$ to $^1C_4$ in the fully sulfated polysaccharide derivatives. These conformational changes afford a substantial increase in anti-factor IIa activity (Table 2).

It is also interesting that desulfation at the specific position, C-6, has caused a loss of the activity in only (1→3)-linked fully sulfated polysaccharides, and this reaction does not affect on the activity of (1→4)-linked fully sulfated polysaccharides. Consequently, a couple of neighboring sulfate groups such as at C-2/C-3 or C-4/C-6 might be required for the anticoagulant activity. The anticoagulant activity of (1→4)-linked, 6-desulfated, fully sulfated polysaccharides might be rendered further useful by removing nonspecific binding to proteins of sulfated polysaccharides compared to (1→3)-linked polysaccharides.

4. Materials and methods

Polysaccharides.—Xylan from birchwood, curdlan from *Alcaligenes faecalis*, cellulose (fribrous, medium), and amylose (type III) from potato, were purchased from Sigma–Aldrich Chemical Co. (Tokyo). All other chemicals used were of analytical grade unless otherwise stated.

Preparation of chemically fully sulfated polysaccharides.—Chemical sulfation to obtain fully sulfated polysaccharides was carried out under mild conditions with adducts of sulfur trioxide (SO_3) in aprotic solvents.[38] Fully sulfated polysaccharides were prepared according to the method described previously,[35,39] which was slightly improved for neutral polysaccharides in this paper. Briefly, 20 mg of each polysaccharide was suspended in 3.2 mL of *N*,*N*-dimethylformamide (DMF) and was then stirred for 14 h at rt under N_2. A required excess (15 mol/equiv of available hydroxyl group in amylose) of pyridine–sulfur trioxide complex was added to the sample suspension, and the mixture was stirred at 40 °C for 6 h under N_2. The reaction was interrupted by addition of 3.2 mL of water, and the raw product was precipitated with 3 vol of cold EtOH saturated with anhyd AcONa, and then collected by centrifugation. Each of the resulting fully sulfated polysaccharides was dissolved in water, dialyzed to remove salts, and lyophilized. In the case of cellulose, because of the very high molecular weight of this polysaccharide, a sample was partially decomposed under acid hydrolysis, resulting in depolymerization to give a polymer of molecular weight 10,000–20,000 before sulfation. The sulfation reaction was repeatedly conducted according to the method described previously[35] to obtain fully sulfated cellulose.

Compositional analysis of modified polysaccharides.—Chemically modified polysaccharide samples were prepared for the determination of sulfate and monosaccharide residues by exhaustive dialysis (MWCO 3,500) against distilled water, lyophilization and drying for 2 days in a desiccator over P_2O_5. Determination of the sulfate group was performed by ion chromatography using conductivity detection (Tosoh model CM-8, Tokyo, Japan) after acid hydrolysis of the sample in 2.5 M trifluoroacetic acid at 100 °C for 3–5 h, depending on the type of sulfated polysaccharide. Monosaccharide residues were analyzed by a post-column HPLC derivatization method[40] using a TSKgel sugar AXI column (4.0 mm i.d. × 30 mm, Tosoh Co., Japan) after acid hydrolysis under identical conditions as described for sulfate analysis.

IR analysis.—IR spectroscopy of solid samples was carried out on a JASCO model FT-IR 230 (Nihonbunko Co., Japan). The dried sample (100 μg) was mixed with 500 μg of dried KBr and compressed to prepare a salt disc (3 mm diameter).

^1H NMR spectroscopy.—^1H NMR spectroscopy was performed using conditions described previously.[41] Briefly, a sample (approx 1–2 mg) was dissolved in 0.5 mL of D_2O (99.9%) and freeze-dried repeatedly to remove exchangeable protons. The sample was kept in a desiccator over phosphorus pentoxide in vacuo overnight at rt. The thoroughly dried sample was then dissolved in 0.5 mL of D_2O (99.96%) and passed through a 0.45-μm syringe filter and transferred to an NMR tube (5.0 mm o.d. × 25 cm; Wilmad Glass Co. (Buena, NJ)). 1D and 2D NMR experiments were performed on a JEOL ECP600 spectrometer equipped with a 5-mm field-gradient tunable probe with standard JEOL software at 45 °C for all experiments on 500 μL samples. The HOD signal was suppressed by presaturation during 3 or 1.5 s for 1D or 2D spectra, respectively. To obtain 2D spectra, 512 experiments resulting 1024 data points for a spectral width of 2000 Hz were measured, and the time domain data were multiplied after zerofilling (data matrix size, 1 × 1 K) with a shifted sine-bell window functions for 2D double quantum filtered (DQF) and triple quantam filtered (TQF)-COSY, NOESY or TOCSY experiments. An MLEV-17 mixing sequence of 100 ms was used for 2D TOCSY experiments, and NOESY experiments were carried out using 150, 250, and 500 ms as the mixing times.

Gradient PAGE and HPSEC analyses of sulfated polysaccharides.—Gradient PAGE was used to monitor the distribution of the molecular weight of sulfated polysaccharides. Polyacrylamide linear gradient resolving gels (90 × 73 mm, 10–20% acrylamide gel, NPG

1020L, Atto Co., Japan) were purchased and run as previously described.[42] Sulfated polysaccharides were visualized by using Alcian Blue staining.

The molecular weight of each intact and chemically modified polysaccharide was evaluated by high-performance size-exclusion chromatography (HPSEC). The HPSEC system consisted of a JASCO 980-PU pump (Nihonbunko, Co., Japan) and Rheodyne 7725i loop injector (USA), and a conductivity detector (CM-8, TOSOH Co., Japan). A TSKgel G3000SWXL (6 μm, 7.8 mm i.d. × 300 mm, TOSOH Co., Japan) column connected with a Dowex 50W-X8 suppressor column (200–400 mesh, H^+ form, 5.0 mm i.d. × 200 mm) was used at 30 °C. The mobile phase, comprised of 5 mM borate (pH 7.0 adjusted by 10 mM NaOH), was delivered at a flow rate of 0.5 mL/min. A Hitachi D-2500 integrator was used to acquire and analyze the data.

6-Desulfation of fully sulfated polysaccharides with BTSA.—Selective desulfation of fully sulfated polysaccharides was achieved according to the method described previously by Matsuo et al.[43] Briefly, an aqueous solution of the sodium salt of fully sulfated polysaccharide (100 mg) was passed through a Dowex 50W-X8 (H^+ form, 200–400 mesh) column (0.8 cm i.d. × 10 cm) at rt, and the eluate was neutralized with pyridine, and then lyophilized. The pyridinium salt of fully sulfated polysaccharide (105 mg) was dissolved in 10 mL of dry pyridine (E. Merck, Darmstadt), and 2 mL of BTSA was added. The mixture was kept for 1 h at 60 °C to give a clear solution and then, 10 mL of water was added to the mixture to decompose the excess reagent and silyl ester. The mixture was dialyzed against distilled water, the pH was adjusted to 7.0 with 0.1 M NaOH. The mixtures was then lyophilized to obtain the 6-desulfated, fully sulfated polysaccharide sodium salt (75 mg).

Anti-factor IIa activity.—Normal human plasma (NHP) was collected from healthy volunteers. Anti-factor IIa activity was measured by incubating 50 μL of chemically sulfated polysaccharides in 50 mM Tris–HCl buffer containing 227 mM NaCl, pH 8.3, with 30 μL of NHP and 20 μL of human thrombin (1.2 NIH units/mL) at 25 °C for 30 s. Chromozym TH (tosyl-glycyl-prolyl-arginine-4-nitanilide acetate) 50 μL (1.9 μmol/mL) was added, and the amidolytic activity of thrombin was determined at 405 nm using a Shimadzu model 1200 UV–Vis spectrophotometer (Shimadzu Co., Japan). Activity was calculated in comparison with heparin (172 units/mg) purchased from Sigma.[44]

Acknowledgements

This work was supported in part by Grants-in-Aid from the Ministry of Culture and Education of Japan (11672136, TT), the Sankyo Life Science Foundation (TT) and the San-Ei Gen Foundation for Food Chemical Research (IT).

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