Regioselective sulfation and desulfation of carbohydrates: Development of new methods and their applications

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CONTENTS

General introduction

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Sulfated polysaccharides are distributed in a variety of algal or animal tissues [1-3]. Among them, most of the polysaccharides of animal origin are sulfated glycosaminoglycan such as chondroitin sulfates, dermatan sulfate, heparin, and heparan sulfate, which are all important materials with various biological and pharmaceutical functions. Knowledge of the sulfated glycosaminoglycans has been accumulated. Their biological function, biosynthetic pathway, the behaviors in the biological processes and pharmaceutical utilization are now be able to be discussed.

The algal polysaccharide sulfates are widely used in various fields of industries. However, their biological functions are still under evaluation. The only some of them were proved as one of the components of hemicelluloses in the cell matrices [4]. These polysaccharides may be potential materials with biological and pharmaceutical activities; *e.g.*, sulfated fucose-containing polysaccharides from the brown algae such as *Ascophyllum nodosum, Fucus vesiculosus, Laminaria digitata, Hizikia fusiforme, etc.*, act as anticoagulant reagents [5,6]. Some red algal galactans from *Porphyra yezoyensis* and *Graciralia verrculosa* were also reported as potent activator for macrophages [7], and some carrageenans were considered as a potent anti-HIV active substance [8] or an antagonist for growth factors [9].

In addition to the naturally occurring sulfated polysaccharides, artificially modified sulfated glycans such as sulfated starch, dextran sulfate, lentinan sulfate, curdlan sulfate *etc.*, were demonstrated to be potential substances for various purposes in industrial and medical usages [10].

It is proved that the sulfate groups in these sulfated glycans may play important roles [11]. In blood coagulation systems, proteolyses catalyzed by the proteinases in the coagulation cascade

are controlled by such proteinous proteinase inhibitors as antithrombin III (AT-III) and heparin cofactor II (HC-II). The former is activated by associating with heparin. Structural investigations of heparin fragments with high affinity to AT-III indicated the presence of minimal binding site of heparin for AT-III consisting of a specific pentasaccharide sequence, where the sulfate groups were suitably constellated to interact to the protein [12]. Synthetic studies of this pentasaccharide and its analogues also supported this finding [13-15]. HC-II is activated by both heparin and dermatan sulfate. Heparin was reported to bind to HC-II by non-specific ionic interaction between sulfate groups and basic amino acid residues, while dermatan sulfate bound to HC-II specifically [16]. For the interaction of HC-II and dermatan sulfate, a minimal binding sequence in the polysaccharide chain was reported [17]. In this sequence, a strict arrangement of the acidic groups was required. The interaction between peptides and sulfated glycans was also observed in the process of cell adhesion or cell proliferation [18,19]. In this process, the sulfate groups which are located at ordinary positions in sulfated glycosaminoglycans were essential for the molecular recognition of various bioactive substances, such as firobrast growth factors [20-22], platelet factor 4 [23,24], and L-selectin [25]. Sulfate groups in appropriate positions may also play roles in functions of artificially sulfated polysaccharides.

Therefore, regioselective structural modification including both incorporation and removal of sulfate groups may provide strong tools for clearer understanding of sulfated polysaccharides, as well as confirmation of the location of sulfate moieties in the carbohydrate chain at the molecular level. For these purposes, the author developed new methods for regioselective sulfation and desulfation of carbohydrates. The method of desulfation was applied to structural analyses as well as to the study of the structure-activity relationships of sulfated polysaccharides.

This thesis consists of four sections. In **Part 1**, the author evaluates the regioselectivity in a

convenient method for sulfation using sulfuric acid and dicylhohexylcarbodiimide (DCC) and suggests that this method is useful for the regioselective sulfation of polysaccharides.

In **Part 2**, the author describes discovery of a unique elimination reaction of sulfate groups by silylating reagents. The reaction is then established as a novel desulfation method. Since the reaction is highly regioselective, it is applied to sulfated polysaccharides from algal origin in **Part 3** for the structural investigation of sulfated polysaccharides.

In **Part 4**, the author applies the desulfation methods to heparin and investigates changes in the structure and the function to evaluate the structure-function relationship, which is attributed to interaction of heparin with proteins and growth factors.

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Part 1

Regioselective sulfation of carbohydrates using sulfuric acid and dicyclohexylcarbodiimide

Part 1-I

Regioselectivity in sulfation of galactosides

Abstract

Methyl α- and β-D-galactopyranosides and 4-*O*-β-D-galactopyranosyl-3,6-anhydro-L-galactose dimethylacetal were sulfated with sulfuric acid and dicyclohexylcarbodiimide. The sulfated galactosides were purified by ion-exchange chromatography, and their structures were determined by methylation analyses. It was found that the sulfation at O-6 was predominant.

A variety of carbohydrate sulfates have been found in animals and plants. They are important substances playing physiological roles, and are also potential materials for various uses [1,2]. Sulfation of carbohydrates and related compounds is usually carried out by using sulfuryl chloride, chlorosulfonic acid, sulfur trioxide-pyridine complex, sulfur trioxide-DMF complex, or arylsulfuryl chloride [3,4]. Some of them are, however, very hygroscopic for handling. In addition, since those reagents possess very high reactivity, they may cause undesirable degradation or decrease the yield of desired products. Hoiberg and Mumma [5] reported a convenient method using sulfuric acid and dicyclohexylcarbodiimide (DCC) in DMF. This method was also applied to carbohydrate derivatives [6]. As this reaction proceeded under mild conditions, the method was expected to have both high selectivity and yield. In this chapter, the author performed the sulfation of methyl α/β-D-galactopyranosides and 4-*O*-β-D-galactopyranosyl-3,6-anhydro-L-galactose dimethylacetal [7] to examine the regioselectivity of the reaction using the combination of sulfuric acid and DCC.

Methyl α/β-D-galactopyranoside and 4-*O*-β-D-galactopyranosyl-3,6-anhydro-L-galactose

dimethylacetal (agarobiose dimethylacetal) were sulfated with sulfuric acid and DCC in a similar manner to that described by Mumma *et al.* [6]. The sulfated products produced in the reaction mixtures were adsorbed to a column of IR-45 (OH form) and eluted with aqueous ammonia. On the basis of quantitative analyses of the unchanged sugar passing through the ion exchange column, it was found that in both case *ca.* 85% of the starting materials were sulfated.

Mixtures of the sulfate isomers were fractionated by the method of Forrester *et al.* [8] with DEAE-cellulose (borate form) in a borate buffer. The fractions were purified by rechromatography. Characterization of the obtained products is summarized in Table I, and the details of the assignments are discussed later in this chapter. With the chromatography system using DEAE-cellulose, the compounds were separated based on the differences in ionic strength arising from their sulfate contents and from their potential to form borate complexes. Among the monosulfates of galactosides, 3-*O*-sulfates were eluted faster than 6-*O*-sulfates. The latter may form borate complex between O-2 and O-3 more easily than the former form the complex between O-4 and O-6. The separation of the disaccharide sulfates was better than that of the monosaccharide sulfates.

Methylation analyses of the isolated sugar sulfates were carried out by the method of Hakomori [9]. With this method, the methylation product is usually isolated from the reaction mixture by extraction. However, the extraction of the methylated product was unsuccessful due to the presence of the sulfate groups. The problem was overcome by use of gel filtration.

The permethylated products obtained were hydrolyzed, converted into alditol acetates, and then analyzed by GLC (Table I). In some cases, 3,6-anhydrogalactose derivatives were also identified. The 3,6-anhydro derivatives may be formed during the methylation reaction using a strong base. It was reported that a sugar residue carrying sulfated 6- and free 3-hydroxyl groups released the sulfate group and simultaneously formed a 3,6-anhydro ring [10]. The observed

Table I Methylation analyses of the fractions from sulfated methyl α- and β-galactosides, and from agarobiose dimethylacetal.

Fraction	Methylation product[a]	Location of sulfate[b]
A1	2,4,6-Me_3-Gal	3-
A2	mix. of 2,3,4-Me_3-Gal, 2,4-Me_2-AGal	6-
A3	mix. of 2,3,4-Me_3-, 2,3,6-Me_3-Gal	mix. of 6- and 4-
A4	2,3,6-Me_3-Gal	4-
A5	2,4-Me_2-Gal	3,6-
A6	mix. of 4,6-Me_2-, 2,3-Me_2-, 3,4-Me_2-Gal	mix. of 2,3-, 4,6- and 2,6-
A7	mix. of 3,4-Me_2-Gal, 4-Me-AGal	2,6-
B1	2,4,6-Me_3-Gal	3-
B2	2,3,4-Me_3-Gal	6-
B3	2,4-Me_2-Gal	3,6-
B4	mix. of 2,4-Me_2-, 2,3-Me_2-, 3,4-Me_2-Gal	mix. of 3,6-, 4,6- and 2,6-
B5	mix. of 2,3-Me_2-, 3,4-Me_2-Gal, 4-Me-AGal	mix. of 4,6- and 2,6-
B6	mix. of 2,3-Me_2-, 3,4-Me_2-Gal, 4-Me-AGal	mix. of 4,6- and 2,6-
B7	mix. of 3,4-Me_2-Gal, 4-Me-AGal	2,6-
C1	mix. of 2,4,6-Me_3-Gal, 2,5-Me_2-AGal	3-
C2	mix. of 2,3,4-Me_3-Gal, 2,5-Me_2-AGal	6-
C3	mix. of 2,3,4,6-Me_4-Gal, 2-Me-AGal	5'-
C4	mix. of 2,4-Me_2-Gal, 2,5-Me_2-AGal	3,6-
C5	mix. of 3,4-Me_2-Gal, 2,5-Me_2-AGal	2,6-
C6	mix. of 2,3,4-Me_3-Gal, 2-Me-AGal	5',6-

[a]2,4,6-Me_3-Gal = 1,4,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol; 2,4-Me_2-AGal = 1,5-di-*O*-acetyl-2,4-di-*O*-methyl-3,6-anhydrogalactitol, *etc.*

[b]Expressed as the sulfated position(s) in the galactose residue. O-5 of 3,6-anhydrogalactose dimethylacetal residue is expressed as 5'.

formation of 3,6-anhydro derivatives thus supports the presence of a 6-*O*-sulfate group.

The relative reactivity of the hydroxyl groups in methyl galactosides and agarobiose dimethylacetal by the present sulfation method was evaluated from the sum of the yields of the corresponding sugar sulfates obtained (Table II), *e.g.*, in the cases of the methyl galactosides, the reactivity of O-6 is expressed as total amounts of 6-sulfate, 2,6-disulfate, 3,6-disulfate, and 4,6-disulfate. In all the cases, 6-hydroxyl groups of the galactose residues exhibited the highest reactivity, the next highest being O-5 of the 3,6-anhydrogalactose residue in agarobiose dimethylacetal. The high reactivity may be explained from the lack of steric hindrance in the corresponding hydroxyl groups. The reactivity of O-2 and O-3 of α-galactoside were comparable, while O-2 of β-galactoside was much less reactive than O-3. In the case of agarobiose dimethylacetal possessing the anhydrogalactose dimethylacetal group in β-configuration as the aglycon, which is more bulky than a methyl group, the O-2 of the galactose residue was much less reactive. In the process of the sulfation, a DCC-sulfuric acid complex, the actual sulfating reagent, might approach to the hydroxyl group horizontally to the plane of the ring in the sugar residue, because the sugar residue itself sterically hinder a vertical approach of the bulky reagent. The aglycon in the *equatorial* β-configuration at the same plane may hinder the *equatorial* approach of sulfating reagent to cause low reactivity of the adjacent O-2, and the sulfation of the *axial* O-4 may not be favored due to the steric hindrance of the plane of the sugar ring. According to this scheme, the *equatorial* O-4 of glucoside or mannoside different from the *axial* O-4 of galactoside might be also presumably less sulfated due to the presence of C-6 at the same plane of the sugar ring. This is to be evaluated.

The order of the reactivity of the hydroxyl groups with sulfuric acid and DCC was similar to that described by Turvey and Williams [12] using a sulfur trioxide-pyridine complex. In the present case, the reactivity of the secondary hydroxyl groups was much lower than primary ones,

Table II Relative reactivity[a] of the hydroxyl groups in methyl galactosides and agarobiose dimethylacetal on sulfation by DCC-H_2SO_4 system

Hydroxyl group[b]	α-galactoside	β-galactoside	galactose (by SO_3-Pyr system[c])	agarobiose dimethylacetal
2	19	7	45	+
3	20	31	80	17
4	2	+	20	+
6[d]	100	100	100	100
5'				50

[a]Calculated from the yield of each fraction obtained by chromatography of the reaction mixtures. Minor fractions assigned as mixtures of sulfates are excluded from the calculation.

[b]Expressed as the sulfated position in the galactose residue. O-5 of the 3,6-anhydrogalactose dimethylacetal residue is expressed as 5'.

[c]Results by Turvey and Williams [11].

[d]Values for the 6-sulfates are taken as 100.

indicating higher regioselectivity. Since the sulfation at the O-6 occurs predominantly, the present method may be effective for the selective sulfation of carbohydrates without any protection of the hydroxyl groups in sugar residues.

Experimental

General Methods. Concentration of solutions was carried out *in vacuo* below 40°C with a rotary evaporator. Optical rotation in an aqueous solution was measured by Laurent's saccarimeter with a cell of 1 dm in length. Melting points were measured by a micro-melting point apparatus (Model MP-S2, Yanagimoto Co.). GLC was carried out by a gas chromatograph (Model 163, Hitachi Co.) equipped with a hydrogen flame ionization detector under the following conditions: injection temperature, 210°C; carrier gas (nitrogen) flow rate, 20 ml/min; hydrogen pressure, 0.6 kg/cm^2; air pressure, 2.0 kg/cm^2. Stainless steel columns (3 mm x 1 m) were packed with Chromosorb W NAW coated with following stationary phases: column *a*, 3% (w/w) Silar 10 C [13]; column *b*, 0.5 % PEG 20M. GLC-MS was carried out by a combined gas chromatograph-mass spectrometer (QP-1000, Shimadzu Corp.), using a fused silica capillary column (0.3 mm x 25 m) coated with SP-1000 operated at a temperature of 210°C. Helium was used as the carrier gas at 2 ml/min flow and 20:1 split ratio. Mass spectra were recorded at 70 eV ionization voltage. Infrared spectra were obtained with a spectrometer (Model 215, Hitachi Co.) by the KBr disk method.

Methyl α-D-galactopyranoside and β-D-galactopyranoside were obtained by treating commercial D-galactose with 1 M methanolic hydrogen chloride, chromatographically separating as described by Matsushima and Miyazaki [14], and purifying by recrystallization. Agarobiose dimethylacetal was obtained by partial methanolysis of commercial agar followed by charcoal chromatography and recrystallization.

Sulfation of methyl α-galactoside. DCC (5.15 g, 25 mmol) was dissolved in 60 ml of dry DMF, and 1.00 g (5 mmol) of methyl α-galactopyranoside in 40 ml of DMF was added. To the solution was added 0.55g (5.5 mmol) of sulfuric acid in 22 ml of DMF at 0°C under a nitrogen, and the solution was stirred for 15 min. The reaction mixture was poured into 200 g of crushed ice and neutralized with barium carbonate immediately. The resulting barium sulfate and the precipitate of *N,N'*-dicyclohexylurea were removed by filtration. The filtrate was applied to a column of Amberlite IR-120 cation exchange resin (H form, 1.26 x 23.5 cm) eluted with deionized water. The eluate was then applied to a column of Amberlite IR-45 anion exchange resin (OH form, 1.26 x 23.5 cm). The column was eluted with deionized water to remove the non-sulfated sugar until the eluate showed the absence of carbohydrates by the anthrone test. The amount of unreacted galactoside passing through in the eluate was estimated as 133 mg (13.3%). The IR-45 column was further eluted with 0.5 M ammonium hydroxide and then with 1 M ammonium hydroxide. Each eluate was evaporated to give a syrupy sulfate mixture (1.36 g from 0.5 M eluate, and 8 mg from 1 M eluate). The former eluate was further investigated.

Sulfation of methyl β-galactoside and agarobiose dimethylacetal. Methyl β-galactoside (1.00 g) was sulfated and fractionated with exactly the same procedure as described above for methyl α-galactoside. The amount of the unreacted galactoside passing through the columns was estimated as 160 mg (16.0%), and that of the sulfated mixture eluted from the IR-45 column with 0.5 M ammonium hydroxide was 1.52 g. No sulfated galactoside was eluted by 1 M ammonium hydroxide.

Agarobiose dimethylacetal (1.11 g, 3 mmol) was sulfated with 3.16 g (15 mmol) of DCC and 0.44 g (4.5 mmol) of sulfuric acid in 81 ml of DMF, and then fractionated similarly to the case of galactosides. The amount of the unreacted disaccharide was 0.14 g (13%). The IR-45 column was eluted with 0.1 M and 1 M ammonium hydroxide to give 1.21 g and 95 mg of syrupy sulfate

mixtures, respectively. The former 0.1 M eluate was further investigated.

Anion exchange chromatography of the sulfate mixture from methyl α-galactoside. A 288 mg portion of the sulfated mixture from the 0.5 M ammonium hydroxide eluate of IR-45 was dissolved in a borate buffer containing 200 mM boric acid and 10 mM ammonium hydroxide, then applied to a Whatman DE 52 DEAE-cellulose column (borate form, 2.2 x 50 cm) and eluted with the same buffer. The carbohydrate content in the eluate in each tube (containing 10 ml) was monitored by the orcinol test and the eluate was recombined into 3 fractions, A1, A2 and A3. From tube number 200, the column was eluted with a buffer containing 300 mM boric acid and 60 mM ammonium hydroxide to give 4 fractions, A4 to A7. This chromatography was repeated twice, and finally 1.07 g of the sulfate mixture was fractionated. Each recombined fraction was concentrated to dryness, then methanol (10 ml) was added and evaporated. This addition and evaporation of methanol were repeated several times to remove ammonium borate by co-distillation. The recombined fractions were rechromatographed under the same conditions, and the respective products were analyzed by methylation analyses.

Fraction A1. An amorphous solid (41 mg), eluted with the 200 mM borate buffer (tube numbers 100 to 140), was identified as methyl α-D-galactoside 3-sulfate, and yielded white crystals (22.8 mg) from ethanol. $[\alpha]_D^{15}$ +148.6° (*c* 1.2, H_2O); mp 192-193°C (dec.); IR spectrum: 1250-60, 840, 800 cm^{-1}.

Fraction A2. An amorphous solid (446 mg), eluted with the 200 mM borate buffer (tube numbers 141 to 177), was identified as methyl α-D-galactoside 6-sulfate, and yielded white crystals (323.4 mg) from ethanol. $[\alpha]_D^{15}$ +124.6° (*c* 1.2, H_2O); mp 127-129°C (dec.); IR spectrum: 1240-60, 800 cm^{-1}.

Fraction A3. Syrup (12 mg), eluted with the 200 mM borate buffer (tube numbers 178 to 199), was identified as a mixture of the 6-sulfate and 4-sulfate of methyl galactoside.

Fraction A4. Syrup (13 mg), eluted with the 300 mM borate buffer (tube numbers 200 to 212), was identified as methyl α-D-galactoside 4-sulfate. $[\alpha]_D^{15}$ +109.5° (*c* 1.1, H_2O); IR spectrum: 1240-60, 830, 810 cm^{-1}.

Fraction A5. Syrup (98 mg), eluted with the 300 mM borate buffer (tube numbers 271 to 304), was identified as methyl galactoside 3,6-disulfate. IR spectrum: 1240-60, 820-40 cm^{-1}.

Fraction A6. Syrup (17 mg), eluted with the 300 mM borate buffer (tube numbers 307 to 333), was identified as a mixture of the 2,3-disulfate, 4,6-disulfate, and 2,6-disulfate of methyl galactosides.

Fraction A7. Syrup (129 mg), eluted with the 300 mM borate buffer (tube numbers 334 to 376), was identified as methyl α-D-galactoside 2,6-disulfate and yielded white crystals (71.3 mg) from ethanol. $[\alpha]_D^{15}$ +102.2° (*c* 1.0, H_2O); mp 182-183°C (dec.); IR spectrum: 1240-60, 820 cm^{-1}.

Anion exchange chromatography of the sulfate mixture from methyl β-galactoside. A 1.00 g portion of the sulfate mixture from the 0.5 M ammonium hydroxide eluate of IR-45 was chromatographed under the same conditions as those for methyl α-galactoside, except that the elution buffer was changed to the 300 mM borate buffer after tube 270. The seven fractions obtained, B1 to B7, were rechromatographed, characterized and identified (Table I).

Fraction B1. Syrup (19 mg), eluted with the 200 mM borate buffer (tube numbers 111 to 142), was identified as methyl β-D-galactoside 3-sulfate. $[\alpha]_D^{15}$ +15.4° (*c* 1.1, H_2O); IR spectrum: 1240-50, 805 cm^{-1}.

Fraction B2. Syrup (408 mg), eluted with the 200 mM borate buffer (tube numbers 146 to 179), was identified as methyl β-D-galactoside 6-sulfate and yielded white crystals (139.2 mg) from ethanol. $[\alpha]_D^{15}$ -5.1° (*c* 1.0, H_2O); mp 142-143°C; IR spectrum: 1240-60, 820 cm^{-1}.

Fraction B3. An amorphous solid (177 mg), eluted with the 300 mM borate buffer (tube

numbers 270 to 295), was identified as methyl β-D-galactoside 3,6-disulfate and yielded white crystals (119.1 mg) from ethanol. $[\alpha]_D^{15}$ +22.0° (*c* 1.0, H_2O); mp 188-189°C (dec.); IR spectrum: 1240-60, 800-820 cm^{-1}.

Fraction B4. Syrup (7 mg), eluted with the 300 mM borate buffer (tube numbers 296 to 306), was identified as a mixture of the 3,6-disulfate, 4,6-disulfate, and 2,6-disulfate of methyl galactosides.

Fraction B5. Syrup (14 mg), eluted with the 300 mM borate buffer (tube numbers 307 to 317), was identified as a mixture of the 4,6-disulfate and 2,6-disulfate of methyl galactosides.

Fraction B6. Syrup (15 mg), eluted with the 300 mM borate buffer (tube numbers 318 to 327), was identified as a mixture of the 4,6-disulfate and 2,6-disulfate of methyl galactosides.

Fraction B7. An amorphous solid (48 mg), eluted with the 300 mM borate buffer (tube numbers 328 to 349), was identified as methyl β-D-galactoside 2,6-disulfate. $[\alpha]_D^{15}$ -3.66° (*c* 1.0, H_2O); IR spectrum: 1240-60, 820 cm^{-1}.

Anion exchange chromatography of the sulfate mixture from agarobiose dimethylacetal. A 401 mg portion of the sulfate mixture from IR-45 was applied to the same column that used for methyl galactosides. The column was eluted with a buffer solution containing 200 mM boric acid and 10 mM ammonium hydroxide from tubes 1 to 167 and thereafter eluted further with the buffer solution containing 200 mM boric acid and 40 mM ammonium hydroxide, to afford 6 fractions, C1 to C6. These fractions were rechromatographed and identified.

Fraction C1. Syrup (12 mg), eluted with the first buffer (tube numbers 74 to 112), was identified as agarobiose dimethylacetal 3'-sulfate. $[\alpha]_D^{15}$ -19.3° (*c* 0.8, H_2O); IR spectrum: 1240-50, 810 cm^{-1}.

Fraction C2. An amorphous solid (134 mg), eluted with the first buffer (tube numbers 113 to 143), was identified as agarobiose dimethylacetal 6'-sulfate. $[\alpha]_D^{15}$ -30.7° (*c* 0.8, H_2O); IR

spectrum: 1240-60, 810 cm^{-1}.

Fraction C3. Syrup (32 mg), eluted with the first buffer (tube numbers 144 to 166), was identified as agarobiose dimethylacetal 5-sulfate. $[\alpha]_D^{15}$ -28.5° (*c* 0.7, H_2O); IR spectrum: 1240-60, 790-810 cm^{-1}.

Fraction C4. Syrup (28 mg), eluted with the second buffer (tube numbers 256 to 289), was identified as agarobiose dimethylacetal 3',6'-disulfate. $[\alpha]_D^{15}$ -4.5° (*c* 1.2, H_2O); IR spectrum: 1240, 780-800 cm^{-1}.

Fraction C5. Syrup (23 mg), eluted with the second buffer (tube numbers 290 to 322), was identified as agarobiose dimethylacetal 2',6'-disulfate. $[\alpha]_D^{15}$ -15.7° (*c* 0.8, H_2O); IR spectrum: 1240-60, 810 cm^{-1}.

Fraction C6. Syrup (72 mg), eluted with the second buffer (tube numbers 323 to 414), was identified as agarobiose dimethylacetal 5,6'-disulfate. $[\alpha]_D^{15}$ -32.0° (*c* 0.8, H_2O); IR spectrum: 1240-60, 810 cm^{-1}.

Methylation analysis of the mono- and disaccharide sulfates. Each 5 mg portion of the fractions from sulfated methyl galactosides or agarobiose dimethylacetal was methylated by the method of Hakomori [9]. The reaction mixtures were chromatographed on Biogel P-2 (2.2 x 200 cm) with deionized water as the eluant. On the basis of the anthrone test, carbohydrates were eluted faster than DMSO and sodium iodide. The methylated products isolated were hydrolyzed with 1 M sulfuric acid at 100°C for 18 h and then neutralized with barium carbonate. For the compounds from agarobiose dimethylacetal and some of the galactosides liable to be converted into 3,6-anhydride during the methylation, hydrolysis with 0.02 M sulfuric acid at 100°C for 2 h and subsequent borohydride reduction were carried out before hydrolysis with 1 M sulfuric acid to protect the acid-labile 3,6-anhydrogalactose. The hydrolyzates were identified by GLC with columns *a* and *b* and by GLC-MS as alditol acetates [13,15].

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Part 1-II

Sulfation of polysaccharides

Abstract

Starch, agarose, κ-carrageenan and porphyran were sulfated using the combination of sulfuric and dicyclohexylcarbodiimide. In the all cases, sulfation at the position O-6 occurred predominantly. The difference in the reactivity of the hydroxyl groups may be due to difference in the steric hindrance. Sulfated agarose was further fractionated according to the difference in the solubility of cetylpyridinium salts of the components in aqueous potassium chloride. The major fraction was similar to the main polysaccharide of funoran (agarose 6-sulfate) in the solubility of its cetylpyridinium salt, NMR spectra, and the result of methylation analyses.

Sulfated polysaccharides are widely distributed among plants and animals, such as algal polysaccharides [1] and mucopolysaccharides [2]. They are known as materials with physiological and pharmaceutical functions and diverse applications. In addition to natural sulfated polysaccharides, artificially sulfated polysaccharides are also prepared. In Part I-1, the author investigated sulfation of monosaccharides and a disaccharide using sulfuric acid and dicyclohexylcarbodiimide (DCC) [3,4], where the sulfation at O-6 occurred predominantly. In the case of modification of polysaccharides, it may not be easy to protect particular hydroxyl groups. Therefore, an appropriate sulfation method with a suitable selectivity is necessary. Since the sulfation by the combination of sulfuric acid and DCC is regioselective as described in the previous chapter, this method is expected for regioselective sulfation of polysaccharides. In this chapter, the author describes an application of this method to sulfation of neutral and

sulfated polysaccharides.

The polysaccharides examined in the present chapter are starch, an α-(1→4) glucan, agarose consisting of a repeating unit of [→3)β-D-Gal(1→4)3,6-anhydro-α-L-Gal(1→], κ-carrageenan consisting of [→3)β-D-Gal-4-SO_3^-(1→4)3,6-anhydro-α–D-Gal(1→], and porphyran that predominantly contains [→3)β-D-Gal(1→4)α-L-Gal-6-SO_3^-(1→] and agarose moiety as minor components. The latter three polysaccharides are of red algal origin. The neutral polysaccharides, starch, and agarose, were successfully sulfated by the sulfuric acid and DCC. The natural sulfated polysaccharides were, however, insoluble in DMF which is the standard solvent for the method. To improve the solubility of sulfated polysaccharide in organic solvents, pyridinium and triethylammonium salts of the sulfated polysaccharides were employed for methylation analyses [5,6]. Tributylammonium salt was also used for sulfation of chondroitin sulfate using sulfur trioxide-pyridine complex [7]. Therefore, the sulfated natural polysaccharides tested in the present work, κ-carrageenan and porphyran, were converted into their pyridinium salts before sulfation. The sulfation using sulfuric acid and DCC appeared not to cleave glycosyl linkage of the polysaccharides, as judged from the results of gel-filtration, in which similar elution times and patterns were observed before and after the sulfation. With other sulfation methods using other reagents such as sulfur trioxide-amine complexes, pyperidine sulfonic acid, and chlorosulfonic acid, cleavages of glycosyl linkages may occur [8,9]. Thus, one of the advantages of the present method is that no cleavage of polysaccharide chains takes place even in the case of agarose and κ-carrageenan that contain considerably acid-labile 3,6-anhydrogalactosyl linkages.

From the results of methylation analyses of the sulfated polysaccharide (Table Ia to IVa), relative reactivities of the respective hydroxyl groups towards the present sulfation reaction were evaluated (Table Ib to IVb). Relative reactivities of O-2 and O-6 in starch are calculated from

Table I

a Methylation analysis of starch sulfated with H_2SO_4-DCC system

Product*	Attributed to	mol%
2,3,6-Me_3-Gal	→4)Glc(1→	19
2,3-Me_2-Gal	→)Glc-6-SO_3^-(→	62
3-Me-Glc	→)Glc-2,6-di-SO_3^-(→	19

*2,3,6-Me_3-Gal = 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol,

2,3-Me_2-Gal = 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylgalactitol,

3-Me-Glc = 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methylgalactitol.

b Relative reactivities of OH groups in starch towards H_2SO_4-DCC system

Position	Relative reactivity*
2	23**
3	0
6	100

*The value for position 6 is taken as 100.

**Caluculated as follows; 100 x 19/(62 + 19) = 23

Table II

a Methylation analysis of agarose sulfated with H_2SO_4-DCC system

Product*	Attributed to	mol%
2-Me-AGal	→4)AGal(1→	48
AGal	→4)AGal-2-SO_3^-(1→	2
2,4,6-Me_3-Gal	→3)Gal(1→	8
2,4-Me_2-Gal	→3)Gal-6-SO_3^-(1→	31
4,6-Me_2-Gal	→3)Gal-2-SO_3^-(1→	2
4-Me-Gal	→3)Gal-2,6-di-SO_3^-(1→	10

*2-Me-AGal = 1,4,5-tri-*O*-acetyl-2-*O*-methyl-3,6-anhydrogalactitol,

2,4,6-Me_3-Gal = 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol *etc.*

b Relative reactivities of OH groups in agarose towards H_2SO_4-DCC system

Position	Relative reactivity*
2 at AGal	5
2 at Gal	29
4 at Gal	0
6 at Gal	100

*Reactivity of position 6 at Gal residue is taken as 100.

Table III

a Methylation analysis of κ-carrageenan sulfated with H_2SO_4-DCC system

Product*	Attributed to	mol%
2-Me-AGal	→4)AGal(1→	38
AGal	→4)AGal-2-SO_3^-(1→	12
2,6-Me_2-Gal	→3)Gal-4-SO_3^-(1→	5
4-Me-Gal	→3)Gal-4,6-di-SO_3^-(1→	32
Gal	→3)-Gal-2,4,6-tri-SO_3^-(1→	13

*2-Me-AGal = 1,4,5-tri-*O*-acetyl-2-*O*-methyl-3,6-anhydrogalactitol,

2,6-Me_3-Gal = 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylgalactitol *etc.*

b Relative reactivities of OH groups in κ-carrageenan towards H_2SO_4-DCC system

Position	Relative reactivity*
2 at AGal	27
2 at Gal	29
6 at Gal	100

*Reactivity of position 6 at Gal residue is taken as 100.

Table IV

a Methylation analysis of porphyran sulfated with H_2SO_4-DCC system

Product*	Attributed to	mol%
2-Me-AGal**	→4)AGal(1→	7
AGal**	→4)AGal-2-SO_3^-(1→	1
2,3-Me_2-Gal	→4)Gal-6-SO_3^-(1→	42
2,4,6-Me_3-Gal	→3)Gal(1→	25
2,4-Me_2-Gal	→3)Gal-6-SO_3^-(1→	17
4,6-Me_2-Gal	→3)Gal-2-SO_3^-(1→	2
4-Me-Glc	→3)Glc-2,6-di-SO_3^-(1→	6

*2,3,6-Me_3-Gal = 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol *etc.*

**Due to small amount of agarose moiety in porphyran chain.

b Relative reactivities of OH groups in porphyran towards H_2SO_4-DCC system

Position	Relative reactivity*
2 at 4-linked Gal	0
3 at 4-linked Gal	0
2 at 3-linked Gal	23
4 at 3-linked Gal	0
6 at 3-linked Gal	100

*Reactivity at position 6 of 3-linked Gal residue is taken as 100.

Contribution of small amount of agarose moiety was ignored.

the amount of 3-Me-Glc arising from the 2,6-disulfated glucose residues and the total amounts of 2,3-Me_2-Glc arising from the 6-sulfated glucose residues and 3-Me-Glc, respectively. The calculation suggested that sulfate groups were introduced predominantly to the 6-hydroxyl groups. Although the degree of sulfation of the other hydroxyl groups was far less than that of O-6, the relative reactivities of the respective hydroxyl groups varied depending on the position. The position O-2 of glucose residues was more reactive than O-3 in the case of starch. In the case of agarose, O-2 of galactose residues was more reactive than O-2 of 3,6-anhydrogalactose or O-4 of galactose residues. In the case of κ-carrageenan, O-2 at 3,6-anhydrogalactose residue was as reactive as O-2 at galactose residue. In the case of porphyran, hydroxyl groups at (1→4)-linked α-L-galactose 6-sulfate residues and that at O-4 of (1→3)-linked β-D-galactose residues were not sulfated, whereas O-2 of the latter residue was moderately reactive.

The reactivity of each hydroxyl group may be explained by steric effects, because the sulfuric acid-DCC complex, the active species in the sulfation reaction, is rather balky as described in the previous chaper. The hydroxyl group at O-6 is least sterically hindered to achieve the highest reactivity. The hydroxyl group that was not sulfated may be sterically hindered depending on conformation of the respective polysaccharide molecules in solution.

Funoran, a sulfated red algal galactan, is a heterogeneous polysaccharide. Its main polysaccharide is anticipated as agarose 6-sulfate consisting of a repeating unit [→3)-β-D-Gal-6-SO_3^-(1→4)-3,6-anhydro-α-L-Gal(1→] [10]. A conversion of agarose to funoran was reported by Guiseley [11] used DMF-sulfur trioxide complex as a sulfating reagent. The agarose sulfate thereby obtained was identical to funoran on the basis of IR spectra, but neither extensive structural analysis of the obtained polysaccharide nor the regioselectivity of his sulfation method was investigated. The reported structure of funoran is similar to that of the agarose sulfated by sulfuric acid and DCC. The sulfated agarose was fractionated according to the method applied

for funoran [12] including stepwise extractions of its cetyl pyridinium salt with 2M and hot 4M KCl solution to give two fractions. The polysaccharide containing more sulfate groups (PSI) was extracted at 2 M KCl, and the polysaccharide with less sulfate groups (PSII) was extracted at 4 M KCl. Methylation analysis (Table V) indicated that most of O-6 of galactose residue and some of O-2 of 3,6-anhydrogalactose and galactose residues were sulfated in PSI. However, in the case of PSII which was the major product, only O-6 was sulfated. ^{13}C-NMR spectra of PSI and PSII (Fig.1) are in good agreement with the results of the methylation analysis; the peak from the anomeric carbon atom adjucent to C-2 carrying O-sulfate group (109.5 ppm) was observed in the spectrum of PSI, while such peak was absent in the spectrum of PSII. The combination of regioselective sulfation and the subsequent fractionation described may be effective for the preparation of artificially sulfated polysaccharides.

Experimental

Materials and General Methods. Starch and agarose (Agarose LE) were purchased from Nacalai Tesque Co. κ-Carrageenan was a product of Sigma Co. Porphyran was extracted and purified from the red seaweed, *porphyra yezoyensis*. Colorimetric determination of hexoses and 3,6-anhydrogalactose was carried out by a modified method of Yaphe [13]. Sulfate content was determined colorimetrically using sodium rhodizonate method [14] after hydrolysis of sulfate group in the polysaccharide. GLC was carried out by a gas chromatograph GC-7A (Simadzu Corp.) equipped with a hydrogen flame ionization detector. Fused silica WCOT columns used were PEG-20M bonded (GL-Science Co.) and CP-Sil 88 (Chrompak Co.) operated at 200°C and 205°C, respectively. Nitrogen was used as a carrier gas; flow rate, 2 ml/min; split ratio, 20:1. GLC-MS was carried out using a gas chromatograph-mass spectrometer GCMS QP-1000 (Shimadzu Corp.) under identical conditions as GLC except for helium as a carrier gas. Mass

Table V Methylation analyses* of fractions from sulfated agarose

	2,4,6-Me_3-Gal**	2,4-Me_2-Gal	4,6-Me_2-Gal	2-Me-Gal	4-Me-Gal	2-Me-AGal***	AGal***
PSI	6	51	3	2	37	79	21
PSII	15	58	6	3	18	92	8

*Total of partially methylated Gal was taken as 100.

**2,4,6-Me_3-Gal = 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol; 2,4-Me_2-Gal = 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylgalactitol; 4,6-Me_2Gal = 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methylgalactitol; 2-Me-Gal = 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methylgalactitol *etc.*

***AGal = 3,6-anhydrogalactitol.

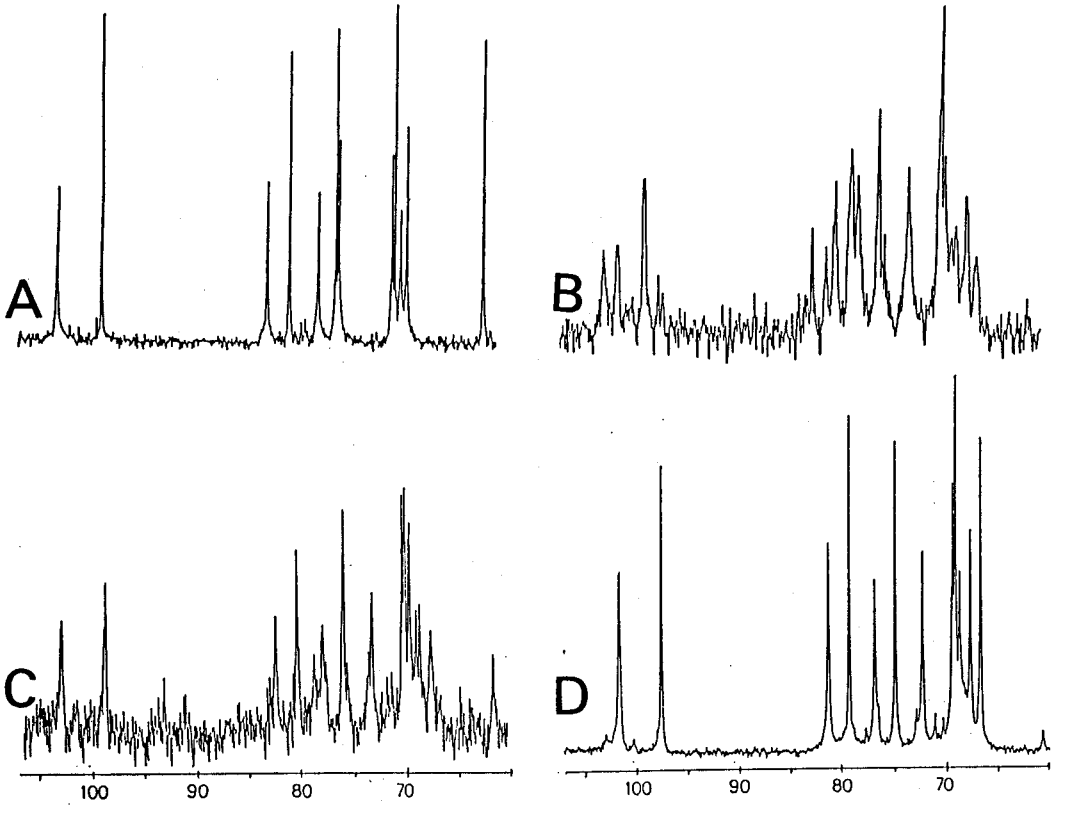

Fig.1 ^{13}C-NMR spectra of fractions from sulfated agarose and related polysaccharides. **A.** agarose; **B**, PSI; **C**, PSII; **D**, main polysaccharide from funoran from *Gloiopeltis complanata*.

spectra were recorded at the ionization voltage of 70 eV. Infrared spectroscopy was carried out by a spectrometer Model 215 (Hitachi) by KBr disk method. ^{13}C-NMR spectra were recorded by a Varian XL-200 spectrometer operated at 80°C and measured by 60° pulse width.

Sulfation of polysaccharides. Starch was dissolved in DMF (1.0 g/22 ml), and to the solution was added DCC dissolved in DMF (6.0 g/74 ml). The solution was cooled to 0°C, and then added sulfuric acid (630 mg) in DMF (32 ml). After stirring the solution at 0°C for 15 min under nitrogen, the resulting mixture was poured into crushed ice (100 g), neutralized with NaOH and dialyzed. The dialyzate was filtered with Celite 500 and centrifuged to remove dicyclohexylurea and then lyophilized to obtain sulfated polysaccharide (1.1 g). For 1.0 g of agarose, 3.0 g of DCC and 316 mg of sulfuric acid were used.

Sulfation of sulfated polysaccharides. A solution of the sulfated polysaccharide as Na salt was applied to a column of Amberlite IR-120 (H^+ form). The column was eluted with water, and the eluate was neutralized with pyridine and lyophilized. The resulting pyridinium salt of the sulfated polysaccharide was sulfated by a similar procedure to that described above. For 1.0 g of κ-carrageenan, 2.0 g of DCC and 224 mg of sulfuric acid were used. For 1.0 g of porphyran, 1.5 g of DCC and 168 mg of sulfuric acid were used.

Methylation analysis of sulfated polysaccharide. Methylation was carried out by the method of Isogai et al. [15] and Ciucanu and Kerek [16]. Before methylation, the sulfated polysaccharides were converted into triethylammonium salt according to Stevenson and Furneaux [7]. The methylation was repeated three times for each polysaccharide. The permethylated sample was hydrolyzed and the hydrolyzate was analyzed as mixtures of partially methylated alditol acetates by GLC and GLC-MS [17]. For the analysis of the sulfated polysaccharides containing acid-labile 3,6-anhydrogalactose residues, the double hydrolysis method described by Stevenson and Furneaux [7] was employed.

Fractionation of sulfated agarose [12]. The sulfated agarose (892 mg) was dissolved in water (30 ml), and an excess of 5% aqueous solution of cetylpyridinium chloride was added to the solution. The resulting precipitate of the cetylpyridinium salt of the sulfated agarose was washed twice with water and extracted three times with 50 ml of 2 M potassium chloride for 2 h. The combined extract was concentrated, and added four volumes of ethanol. The resulting precipitate was dissolved in water, dialyzed and then lyophilized to give a fraction, PSI (260 mg). The cetylpyridinium salt insoluble in 2 M potassium chloride was further extracted three times with 50 ml of 4 M potassium chloride for 2 h. The remaining cetylpyridinium salt was extracted with 50 ml of hot 4 M potassium chloride. The resulting solution was immediately poured into 200 ml of ethanol under vigorous stirring. The precipitate obtained was washed with ethanol, dissolved in water, dialyzed, and lyophilized to give PSII (442 mg).

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Part 2

Novel desulfation of sulfated carbohydrates by silylating reagents.

Part2-I

A novel regioselective desulfation method specific to carbohydrate 6-*O*-sulfates using silylating reagents

Abstract

Sulfated primary alcohols and methyl α-D-galactopyranoside 6-*O*-sulfate were converted into desulfated and trimethylsilylated alcohols and galactoside, respectively, by treating their pyridinium salts with *N,O*-bis(trimethylsilyl)acetamide (BTSA) or *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BTSTFA) in pyridine. Sulfated secondary alcohols and methyl galactoside 2-, 3-, and 4-*O*-sulfates did not lose their sulfate groups under similar conditions, indicating that the reaction was specific to primary hydroxyl groups. Methyl α-galactoside 2-*O*-sulfate was obtained by the BTSA treatment from methyl α-galactoside 2,6-di-*O*-sulfate in a preparative scale.

Sulfated carbohydrates are important substances which play a variety of roles in plants and animals. When considering the correlation between their structures and functions, one of important strategies is to compare the carbohydrate sulfates with their desulfated derivatives. The desulfation of sulfated carbohydrates was performed using dilute methanolic hydrogen chloride [1]. This method, however, caused considerable depolymerization owing to the concomitant cleavage of the glycosyl linkages. A milder and more convenient reaction generally used is based on the solvolysis of the pyridinium salts of sulfated carbohydrates in solvents such as DMSO, DMF, and pyridine [2], or DMSO containing a small amount of water or methanol [3]. One of the advantages of solvolytic desulfation is that careful control of the reaction

conditions enables selective *N*-desulfation. Although *O*-sulfates are usually resistant to alkaline hydrolysis, those linked to C-6 or C-3 of galactose residues with a free hydroxyl group on C-3 or C-6 are quantitatively removed by alkali to form simultaneously a 3,6-anhydro ring [4]. Similarly, O-sulfate groups adjacent to a free hydroxyl group in the *trans* configuration are also readily removed by alkali *via* epoxide ring formation [5]. A similar reaction was applied to heparin to remove the sulfate group at O-2 of the α-iduronic acid residues [6]. Since this reaction requires the appropriate location and configuration of free hydroxyl groups, they may not be applied to all sulfated carbohydrate. Enzymes with a specificity suitable for desulfation are rarely available. Thus, there is no universal desulfation method effective for *O*-sulfates linked to particular hydroxyl groups.

Recently a variety of reactions with silicon-containing reagents have been discovered and applied to syntheses of numerous substances including carbohydrates and related compounds. Alkyl phosphates, on the other hand, have been reported to release the phosphate group to yield dealkylated phosphates by a silylating reagent, bromotrimethylsilane [7]. A similar reaction might be expected for alkyl sulfates. Thus, pyridinium salts of sulfated primary and secondary alcohols and methyl α-D-galactoside 2-, 3-, 4-, and 6-*O*-sulfates dissolved in pyridine were heated with a large excess of a silylating reagent, *N,O*-bis(trimethylsilyl)acetamide (BTSA) or *N,O*-bis(trimethylsilyl)trifuluoroacetamide (BTSTFA). The reaction mixture was directly analyzed with GC and GLC-MS. The pyridinium salts of sulfated primary alcohols dodecanol and cyclohexylmethanol or methyl α-D-galactoside 6-*O*-sulfate were converted into the corresponding trimethylsilylated alcohols or pertrimethylsilylated methyl α-D-galactoside by the treatment with BTSA or BTSTFA in pyridine at 80°C for 2 h (Fig.1). Cyclohexyl sulfate, methyl α-D-galactoside 2-, 3-, or 4-*O*-sulfates, in contrast, did not yield desulfated and silylated cyclohexanol or galactosides under the same conditions (Fig. 1). Thus, conversion of a sulfate

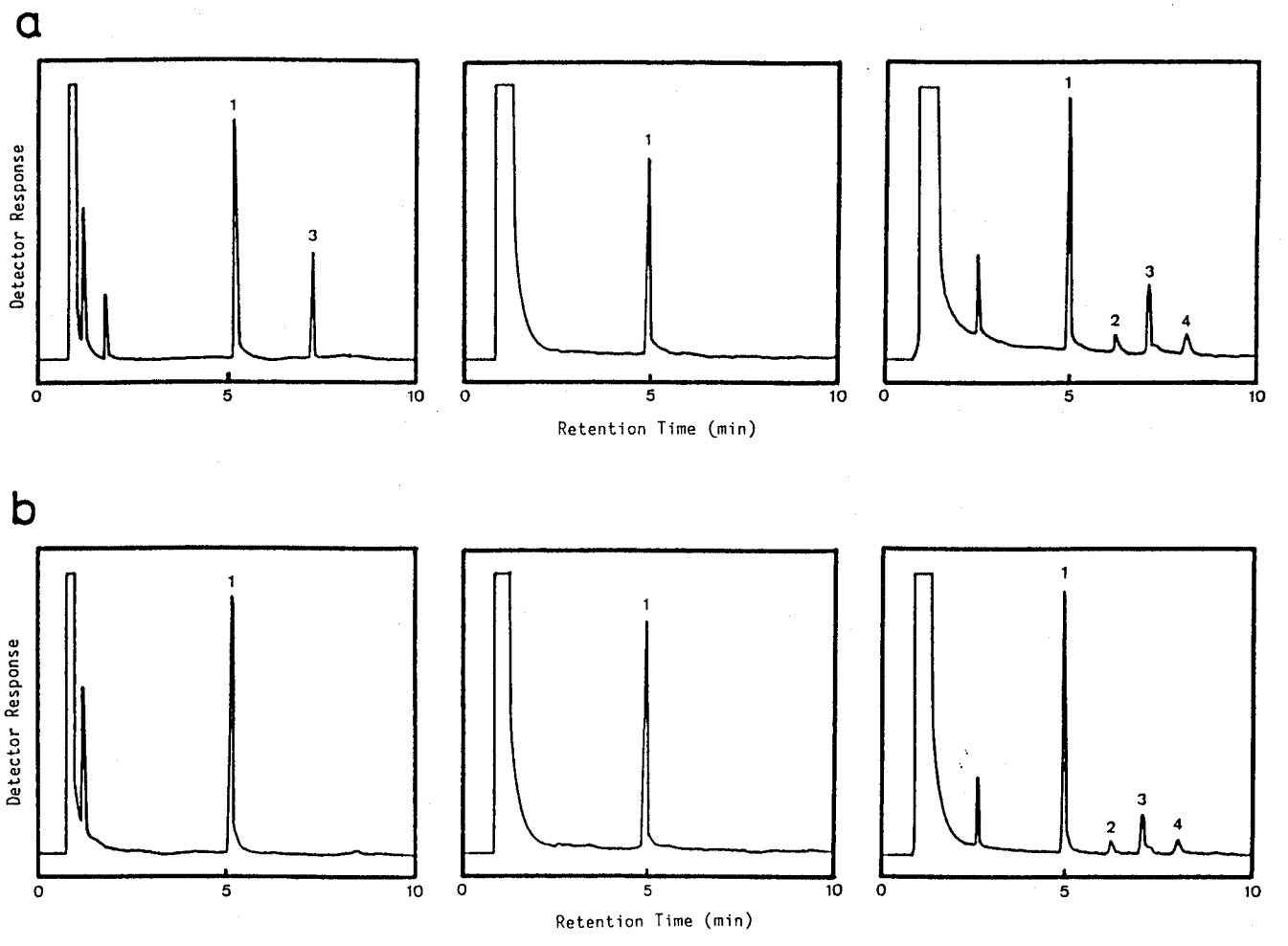

Fig. 1 GLC pattern of reaction mixtures from the BTSA treatment of methyl galactoside sulfates.

(a) Treatment of methyl α-D-galactoside 6-*O*-sulfate: left, a mixture of xylitol and the pyridinium salt of the galactoside 6-*O*-sulfate was treated with BTSA in pyridine at 80°C for 2 h; middle, the same mixture without BTSA was treated similarly and trimethylsilylated; right, the same mixture was methanolyzed and trimethylsilylated (see Experimental). (b) Treatment of methyl α-D-galactoside 3-*O*-sulfate: left, a mixture of xylitol and the pyridinium salt of the galactoside 3-*O*-sulfate was treated with BTSA; middle, the same mixture without BTSA was treated similarly and trimethylsilylated; right, the same mixture was methanolyzed and trimethylsilylated.

Peak assignment as TMS derivatives: 1, xylitol (internal standard); 2, methyl α-galactofuranoside; 3, methyl α-galactopyranoside; 4, methyl β-galactopyranoside.

into the corresponding trimethylsilyl ether appeared to be specific to sulfated primary alcohols.

The reaction of methyl α-D-galactoside 6-*O*-sulfate with BTSA was further investigated using GLC (Fig. 2). The silylating reagent was possibly consumed by silylation of free hydroxyl groups and hence no desulfation reaction took place with low BTSA concentrations. Under the reaction conditions of 80°C for 1 h, the minimum amount of BTSA required for complete desulfation was 15 equivalents to the sum of the sulfated and free hydroxyl groups. The time-course of the reactions with 20 equivalents of BTSA to the sum of the sulfated and free hydroxyl groups at 40°C, 60°C, and 80°C were plotted in Fig. 3. As the large excess of BTSA was used, the curves fit a kinetic equation for a pseudo-first-order reaction. The rate constants were estimated from a logarithmic plot of the galactoside sulfate concentration against the reaction period to be 5.5×10^{-4} sec^{-1}, 1.2×10^{-3} sec^{-1}, and 3.0×10^{-3} sec^{-1} at 40°C, 60°C, and 80°C, respectively. These rate constants was bigger than the reported value of 9.3×10^{-5} sec^{-1} for the hydrolysis of galactose 6-*O*-sulfate with 0.25 M hydrochloric acid at 100°C [8]. Using Arrhenius equation, an activation energy value of 9.4 kcal mol^{-1} for the BTSA desulfation was obtained. This activation energy may be comparable to that for the enzymatic hydrolysis of glycosides (ca. 12 kcal mol^{-1}) [9] and is lower than the value (*ca.* 30 kcal mol^{-1}) for the acidic hydrolysis of glycosides or disaccharides [10].

Other reagents such as trimethylsilylimidazole, iodotrimethylsilane, and *N,O*-bis(trimethylsilyl)carbamate were not effective for desulfation. BTSA treatment of methyl 6-*O*-*p*-tolylsulfonyl-α-D-galactoside did not afford deesterified and trimethylsilylated methyl galactoside.

In a preparative scale, the pyridinium salt of methyl α-D-galactoside 2,6-di-*O*-sulfate was treated with BTSA at 60 °C for 2 h. The product was subsequently desilylated and isolated by ion-exchange resins to afford a quantitative amount of methyl α-D-galactoside 2-*O*-sulfate.

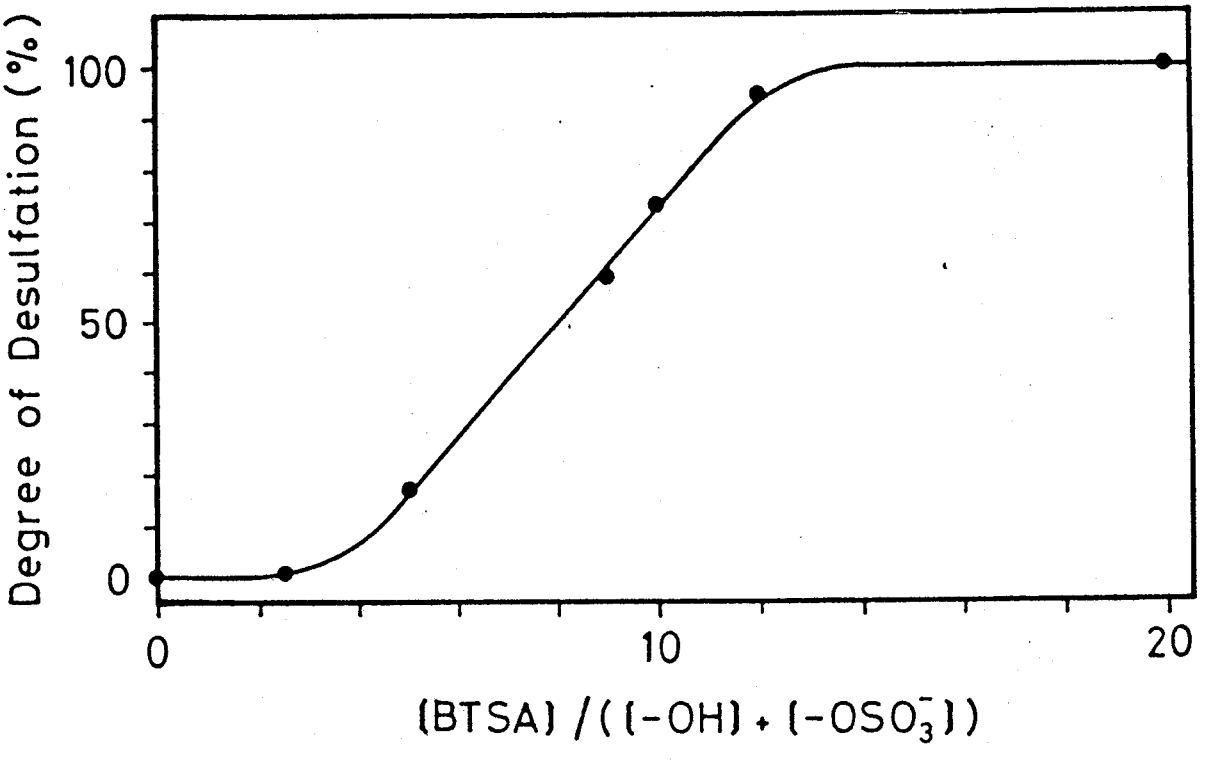

Fig. 2 Effect of the amount of BTSA on the desulfation of methyl α-D-galactopyranoside 6-*O*-sulfate.

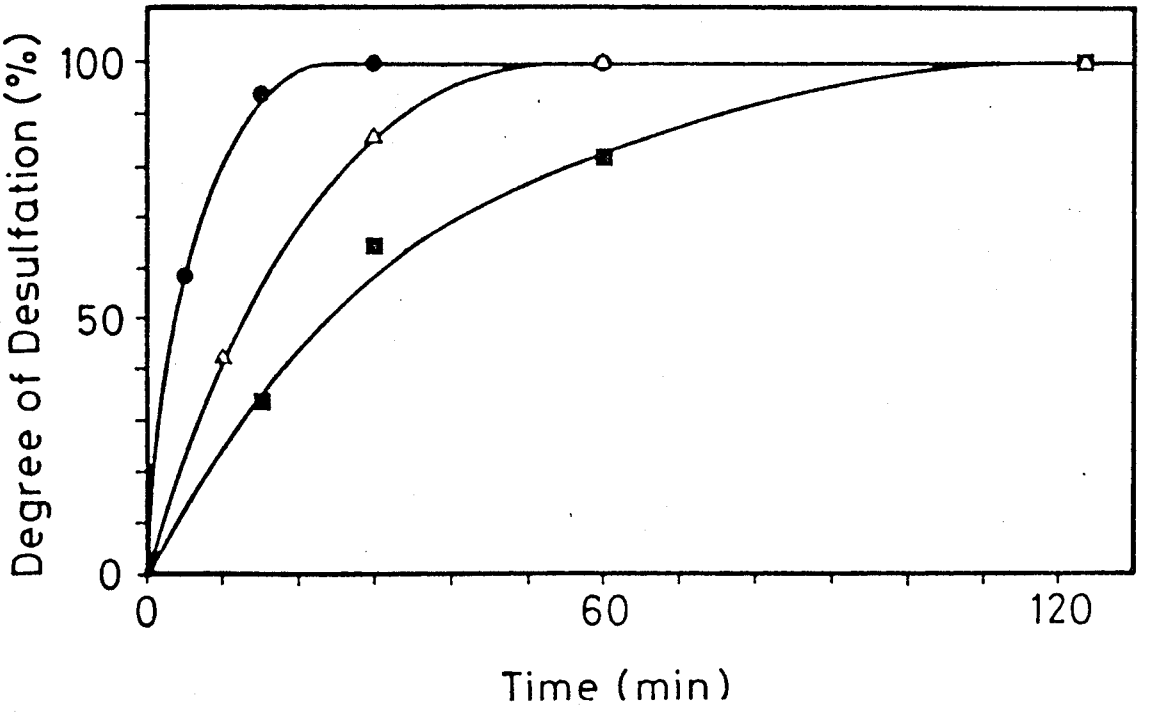

Fig. 3 Time-course for the BTSA desulfation of methyl α-D-galactoside 6-*O*-sulfate at 40°C, 60°C and 80°C. -■-, 40°C; -△-, 60°C; -●- 80°C.

From the ^{13}C-NMR analysis using the DEPT sequence [13], signal from the methylene carbon at C-6 shifted upfield by 6.43 ppm and a C-5 signal downfield by 2.18 ppm after the BTSA treatment. Thus, the BTSA treatment proved to be an efficient method for regioselective 6-*O*-desulfation of sulfated carbohydrates.

Experimental

General methods. GLC was carried out with a chromatograph (Model GC-8, Shimadzu Corp.) equipped with a flame ionization detector and a fused silica capillary column (OV-1 bonded, 0.3 mm x 25 m, GL-Science Co. Ltd.). Nitrogen was used as a carrier gas at a flow rate of 20 ml/min, with a split ratio of 20 : 1 and operating temperature of 180°C (for galactosides) or 100°C (for alcohols). GLC-MS was carried out with a mass spectrometer (Model GCMS QP-1000, Shimadzu Corp.). The chromatographic conditions were same as employed for GLC described above, except for the use of helium as a carrier gas. The ^{13}C-NMR spectrum at 75.4 MHz was recorded on a spectrometer (QE-300, General Electric Co.) operated at 21.0°C. Chemical shifts were measured relative to internal methanol (51.60 ppm), and converted into values relative to internal sodium 3-trimethylsilyl-1-propanesulfonate.

Silylating reagents. Iodotrimethylsilane was prepared as an acetonitrile solution by the method of Olah et al [12]. *N,O*-bis(trimethylsilyl)acetamide (BTSA), *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BTSTFA) and *N*-(trimethylsilyl)imidazole were purchased from Wako Pure Chemicals Ind., while *N,O*-bis(trimethylsilyl)carbamate was a product of Petrarch Systems.

Sulfonated sugar, sulfated alcohols, and sulfated sugars. Methyl 6-*O*-tolylsulfonyl-α-D-galactopyranoside was synthesized according to the literature [13]. Sodium dodecyl sulfate was purchased from Wako Pure Chemicals Ind. Barium cyclohexylmethyl sulfate and cyclohexyl sulfate were synthesized by sulfating the respective alcohols with sulfur trioxide-pyridine

complex in DMF [14]. The sulfated methyl galactosides were prepared from methyl galactoside with sulfuric acid and dicyclohexylcarbodiimide and purified by ion-exchange chromatographies as described in Part 1-I, except that methyl α-D-galactoside 2-*O*-sulfate was prepared from methyl α-D-galactoside 2,6-di-*O*-sulfate as described later.

Reactions of sulfonated sugar, sulfated alcohols and sulfated sugars with silylating reagents. The silylating reagents tested were iodotrimethylsilane, BTSA, BTSTFA, trimethylsilyl-imidazole, and *N,O*-bis(trimethylsilyl)carbamate. The esters tested were 6-*O*-tolylsulfonyl-α-D-galactopyranoside, dodecyl sulfate, cyclohexylmethyl sulfate, cyclohexyl sulfate, and 2-, 3-, 4-, 6- and 2,6-di-*O*-sulfates of methyl-α-D-galactosides. Each ester (0.2 mg) was dissolved in water and converted into its acid form by ion-exchange resin (Amberlite IR-120, H^+ form, 0.5 ml) before neutralization with pyridine and lyophilization. The resulting dry pyridinium salt was dissolved in dry pyridine (40 μl) containing dibutyl phthalate (for simple alcohols) or xylitol (for sugars) as the internal standard. To the solution, a silylating reagent (80 equiv. to the sulfate and sulfonate) was added, and the mixture kept stand at 80°C for 2 h. The resulting reaction mixture was directly analyzed by GLC and GC-MS. As a control, another portion of each ester was methanolyzed with 0.2 ml of 1 M methanolic hydrogen chloride in a sealed tube at 70°C for 16 h, and then neutralized with silver carbonate. After the resulting silver chloride and excess silver carbonate had been filtered off, the methanolyzate containing dibutyl phthalate (for alcohols) or xylitol (for sugars) was trimethylsilylated with BTSTFA (for alcohols) or trimethylsilylimidazole (for sugars), and then analyzed by GLC. In the case of the BTSA and BTSTFA treatments of dodecyl sulfate, cyclohexylmethyl sulfate, and methyl α-D-galactopyranoside 6-*O*-sulfate, the respective trimethylsilylated, desulfated alcohols and sugar were identified on the basis of their retention times in GLC and their mass spectra. In the case of the other sulfates and the sulfonate, no peak corresponding to the respective deesterified, trimethylsilylated alcohol or galactoside

appeared. The other silylating reagents also did not afford any peaks corresponding to desulfated, trimethylsilylated materials.

Effect of BTSA amount on degree of desulfation. Each portion from a mixture of the pyridinium salt of methyl α-D-galactopyranoside 6-*O*-sulfate and dibutyl phthalate as an internal standard was treated with BTSA in pyridine at 80°C for 1 h. The recovery of desulfated, trimethylsilylated methyl galactoside in each reaction mixture was determined by a GLC analysis.

Time-course of desulfation at various temperatures. The pyridinium salt of methyl α-D-galactoside 6-*O*-sulfate and dibutyl phthalate was treated with BTSA (20 equiv. to the total amount of -OH and $-OSO_3^-$) in pyridine at 40°C, 60°C, and 80°C. Aliquots of the reaction mixtures were periodically analyzed by GLC.

Methyl α-galactopyranoside 2-O-sulfate. The ammonium salt of methyl α-D-galactoside 2,6-di-*O*-sulfate (12.5 mg, 0.032 mmol) was converted into the pyridinium salt by neutralizing its acid form by pyridine as already mentioned. To the resulting salt in 2.0 ml of dry pyridine was added 1.2 ml of BTSA. The solution was heated at 60°C for 2 h, 10 ml of 20% methanol was added, and the mixture kept stand at 20°C for 18 h. The reaction mixture was evaporated *in vacuo* to dryness at 40°C, dissolved in 2 ml of water, and applied to serial columns of IR-120 (H^+ form) and IR-45 (OH^- form) of analytical grade (1 x 3 cm each). After columns had been washed with 100 ml of water, the acidic substance adsorbed to the IR-45 column was eluted with 50 ml of 1 M ammonium hydroxide. The eluate was evaporated to dryness to give a syrup (9.5 mg, 0.033 mmol as an anhydrous ammonium salt), which was assigned to methyl α-galactopyranoside 2-*O*-sulfate on the basis of ^{13}C- NMR data using the DEPT sequence; δ (D_2O): 57.79 ($-OCH_3$), 63.66 (C-6, negative peak), 70.03 (C-3), 72.07 (C-4), 73.21 (C-5), 78.06 (C-2) and 100.06 (C-1). Chemical shifts for the starting methyl α-galactoside 2,6-di-*O*-sulfate,

δ: 57.78 (-OCH_3), 69.81 (C-3), 70.09 (C-6, negative peak), 71.03 (C-5), 71.73 (C-4), 77.89 (C-2), 100.06 (C-1).

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Part2-II

Desulfation of sulfated carbohydrates by silylating reagents

Abstract

Potential of silylating reagents for desulfation of carbohydrate sulfates was investigated. Pyridinium salt of methyl α-D-galactoside 3- or 6-*O*-sulfate was heated in pyridine in the presence of a variety of silylating reagents. As compared with a reaction without silylating reagent (equivalent to solvolytic desulfation), the silylating reagents investigated were classified into three categories A, B, and C. The reagents of the category A suppressed solvolytic desulfation. Ones of the category B regioselectively removed sulfate groups at O-6 but suppressed desulfation of 3-sulfates. Ones of the category C, by contrast, removed both 3- and 6-*O*-sulfate groups.

Efficient methods for desulfation are often required in carbohydrate chemistry and biochemistry. In addition to conventional desulfation methods [1,2], the author described, in the preceding chapter, a novel desulfation method employing a silylating reagent, *N*,*O*-bis(trimethylsilyl)acetamide (BTSA). With BTSA, the 6-*O*-sulfate groups of sugar moieties are regioselectively removed and converted into trimethylsilyloxyl groups. The desulfated carbohydrates are easily recovered after desilylation with water or aqueous methanol. Therefore, silylating reagents may be considered as potential reagents for desulfation reaction. In the present chapter, the author examined various silylating reagents to find the most effective one for desulfation of carbohydrate sulfates.

Pyridinium salt of methyl α-D-galactoside 3- or 6-*O*-sulfate was treated with a large excess of silylating reagents (listed in table) in pyridine at 40 °C. Since the sulfate group is substituted by a trimethylsilyloxyl group when desulfation occurs with the silylating reagent, the degree of desulfation was estimated by the amount of pertrimethylsilylated galactoside using GLC. But the reactivity of several reagents of complete silylation of hydroxyl groups was found to be insufficient, so that the degree of desulfation with each reagent was estimated after further treatment with *N*-(trimethylsilyl)imidazole that can completely silylate hydroxyl groups but not catalyze desulfation as described in the preceding chapter. Since a large excess of the reagent was present in the system, the reaction was a pseudo first-order reaction, the rate constant for each reagent thus being estimated from the degree of desulfation. By comparison with the rate constant for the solvolytic reaction that proceeds under similar conditions but in the absence of silylating reagent [3], the silylating reagents used were classified into three categories (Table 1). The reagents of category A are incapable of desulfation, those of the category B accelerate 6-*O*-desulfation but are ineffective for 3-*O*-desulfation, and those of the category C non-specifically remove sulfate groups.

Pyridinium salt of a carbohydrate sulfate was known to be solvolyzed when treated in pyridine to release the sulfate group [3]. A small amount of water facilitated the solvolytic desulfation in DMSO [4,5]. Accordingly, the silylating reagents were expected to prevent the solvolytic reaction since they can remove residual moisture from the system. The reagents of the category A thus caused a decrease in the rate of desulfation, whereas those of the categories B and C showed different effects.

In the reaction with the reagents of the category B preferential desulfation at O-6 occurred, which may involve a mechanism other than solvolytic desulfation. The reagents of the category C are also likely to induce the desulfation in a mechanism different from solvolysis.

Table I Ratio of rate constants for the reaction of methyl galactoside 6- and 3-*O*-sulfates in pyridine with (k_{sil}) and without (k_{sol}) silylating reagent. The k_{sol} values at 40°C for 6- and 3-*O*-sulfates were estimated at 9.0×10^{-6} sec^{-1} and 1.8×10^{-6} sec^{-1}, respectively.

cate-gory	silylating reagent	k_{sil}/k_{sol} 6-sulfate	3-sulfate
A	*N*-(trimethylsilyl)imidazole	0	0
	1-(trimethylsilyl)-1,2,4-triazole	0.6	0.2
	N-(trimethylsilyl)acetamide	0.2	0.5
	3-(trimethylsilyl)-2-oxazolidinone	0.9	0.2
	nonamethyltrisilazane	0	0.2
	N,N-dimethylaminotrimethylsilane	0.5	0
	hexamethyldisiloxane	0.5	0.1
	isopropenoxytrimethylsilane	0	0
	1-methoxy-3-(trimethylsilyloxy)-1,3-butadiene	0.1	0.2
	1-(trimethylsilyl)propyne	0.7	0.1
	bis(trimethylsilyl)acetylene	0.5	0
B	*N,O*-bis(trimethylsilyl)acetamide	21.6	0
	N-methyl-*N*-(trimethylsilyl)acetamide	14.9	0
	N-methyl-*N*-(trimethylsilyl)trifluoroacetamide	36.4	0
	hexamethyldisilazane	1.2	0.4
	heptamethyldisilazane	4.5	0
	N,N-diethylaminotrimethylsilane	1.4	0
	1-methoxy-1-(trimethylsilyloxy)-2-methyl-1-propene	1.1	0.2
C	trimethylsilyl azide	1.7	3.1
	trimethylsilyl isocyanate	2.4	3.8
	bis(trimethylsilyl)carbodiimide	2.1	7.8
	1-methoxy-2-(trimethylsilyloxy)propane	2.1	3.1
	trimethylethoxysilane	2.1	4.2
	4-(trimethylsilyloxy)-3-pentene-2-one	4.5	3.8
	trimethylsilyl acetate	1.5	3.8
	trimethylsilyl trifluoroacetate	2.3	2.0
	bis(trimethylsilyl) adipate	1.9	3.4
	trimethylchlorosilane	3.9	3.4

Among the reagents of the category B, *N*-methyl-*N*-(trimethylsilyl)acetamide (MTSA) and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MTSTFA) proved to be new reagents for effective 6-*O*-specific desulfation. Although the reagents of the category C was less effective than the category B, the former would be potential desulfation reagents for sulfate groups at both primary and secondary hydroxyl groups; a high degree (minimum 80%) of desulfation was achieved by a reaction at 80 °C with the reagents of the category C. BTSA belonging to the category B was successfully employed for desulfation of sulfated monosaccharide (see preceding section) and polysaccharides as will be discussed in the following section. Treatment with the silylating reagents described here may be a convenient method for the preparation of desulfated carbohydrates and constitutes an alternative method to solvolytic desulfation, since the procedure is simple and the introduced trimethylsilyl groups are easily removed. An appropriate choice of a silylating reagent may be helpful for preparing variously desulfated carbohydrates, as the position and the degree of the desulfation vary depending on the reagents employed.

Experimental

General methods. Ammonium salts of methyl α-D-galactoside 3- and 6-*O*-sulfates were prepared as described in Part 1 of the present thesis. Pyridinium salts of these compounds were obtained by neutralization of the acidic form of sugar sulfate with pyridine after passing through a column of Amberlite IR 120 (H^+ form) resin. The silylating reagents employed were products of Petrarch systems, Tokyo Chemical Ind. Co., Aldrich Chemical Co. Inc., or Wako Pure Chemicals Ind. Other reagents were purchased from Wako Pure Chemicals Ind. GLC was carried out using a gas chromatograph (GC-8A, Shimadzu Corp.) equipped with FID and a WCOT column (OV-1 bonded, 0.3 mm x 25 m, GL-Science Co.) at 180 °C. Nitrogen was used as a carrier gas; flow rate, 20 ml/min; split ratio, 20:1.

Reaction with silylating reagents. The pyridinium salt of methyl α-D-galactoside 3- or 6-*O*-sulfate (100 μg) was dissolved in pyridine (100 μl) containing methyl α-D-glucoside (50 μg) as an internal standard. To the solution was added the silylating reagent (40 μl), and the mixture was heated to 40 °C. To the reaction mixture was added *N*-(trimethylsilyl)imidazole (TSIM, 40 μl), the solution was heated at 80 °C for 5 min and then analyzed by GLC. The degree of desulfation was estimated based on the peak area ratio of desulfated and silylated galactosides and the internal standard. As a control, the same mixture without the silylating reagent was heated in pyridine (100 μl) at 40 °C, the solution silylated with TSIM (40 μl), and then analyzed with GLC.

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Part 2-III

Regioselective 6-*O*-desulfation of sulfated polysaccharide using *N*,*O*-bis(trimethylsilyl)acetamide

Abstract

Treatment of pyridinium salt of glycosaminoglycans and galactan sulfates with *N*,*O*-bis(trimethylsilyl)acetamide (BTSA) in pyridine at 60°C for 2 h effected specific 6-*O*-desulfation without depolymerization or other side reactions.

Treatment of pyridinium salt of sulfated monosaccharide with *N*,*O*-bis(trimethylsilyl)acetamide (BTSA) or *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BTSTFA) in pyridine causes regioselective removal of 6-*O*-sulfate group. Methyl α-D-galactoside 2,6-di-*O*-sulfate is converted into the 2-*O*-sulfate in quantitative yield. In this section, the author reports that BTSA in pyridine is also applicable to sulfated polysaccharides for the specific 6-*O*-desulfation without depolymerization.

Funoran, porphyran, dermatan sulfate, or chondroitin sulfate was treated with BTSA. The structure of the polysaccharides are summarized in Fig. 1. The sulfur contents of the polysaccharides before and after the treatment are summarized in Table I. Funoran, which has the repeating unit [→3)β-D-Gal*p*-6SO_3^-(1→4)3,6-anhydro-α-L-Gal*p*(1→], was completely desulfated by the treatment with BTSA and pyridine. About 75% of the sulfate groups in porphyran, which has the repeating unit [→3)β-D-Gal*p*(1→4)α-L-Gal*p*-6SO_3^-(1→], were removed. In contrast, dermatan sulfate, which consists mainly of the repeating unit [→4)β-D-GlcA*p*(1→3)β-D-GalNAc*p*-4SO_3^-(1→], was less affected. In the case of chondroitin sulfate,

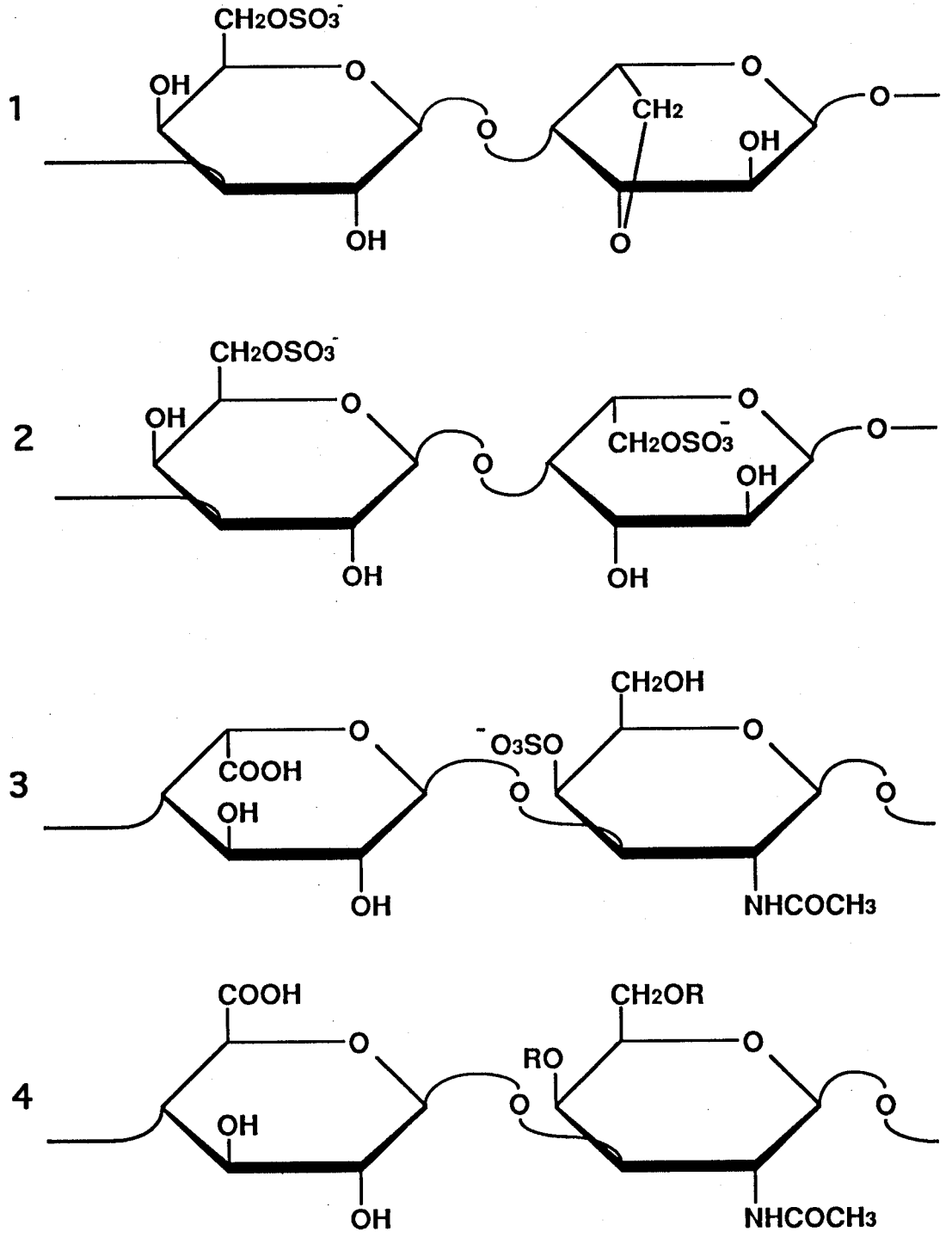

Fig. 1 Predominant chemical structures of the polysaccharides tested for the treatment with BTSA. **1**, Funoran; **2**, porphyran; **3**, dermatan sulfate; **4**, chondroitin sulfate.
R = H, SO_3^-

Table I Sulfur contents (wt %) of polysaccharides before and after treatments with silylating reagents.

Reagent		BTSA	-	TSIM	BTSA
Solvent		pyridine	pyridine	pyridine	DMSO
Conditions	(Native)	60°C, 2 h	60°C, 2 h	60°C, 2 h	80°C, 5 h
Funoran	6.2	0	6.0	6.1	4.0
Porphyran	4.4	1.1	4.3	4.2	-
Dermatan Sulfate	5.1	4.7	5.0	5.1	4.6
Chondroitin Sulfate	6.6	2.2	6.5	6.4	-

which contains the units [→4)β-D-GlcA*p*(1→3)β-D-GalNAc*p*-6SO_3^-(1→] and [→4)β-D-GlcA*p*(1→3)β-D-GalNAc*p*4SO_3^-(1→] (see below), *ca.* 30% of the sulfate groups survived. These results suggest that the treatment with BTSA and pyridine occurred at O-6.

The locations of the sulfate groups in the polysaccharides before and after treatment with BTSA and pyridine were determined by ^{13}C-NMR spectroscopy (Fig. 2). The spectrum of the BTSA-treated funoran was identical to that of agarose [1], which has the same sugar chain but contains no sulfate groups. The spectrum of the BTSA-treated porphyran was also identical to that of desulfated porphyran reported previously [2]. The spectra of dermatan sulfates before and after the treatment with BTSA were identical, indicating that the sulfate groups, mainly at the position 4, were not removed. In the case of ^{13}C-NMR of chondroitin sulfate using DEPT pulse sequence [3], a negative signal (70.0 ppm) which is associated with 6-*O*-sulfate group disappeared after the BTSA-treatment. In addition, the signal corresponding to C-5 (75.2 ppm) disappeared after the treatment. Those results indicated that the desulfation by the treatment of BTSA and pyridine occurred at O-6 of sulfated sugar moieties.

The changes in structure of dermatan sulfate and chondroitin sulfate were further evaluated by the disaccharide analysis using chondroitinase ABC (Fig. 3,4). As shown in Fig. 3 of dermatan sulfate, a disaccharide containing the [→3)GalNAc*p*-4SO_3^-(1→] residue and a small amount of that with [→3)GalNAc*p*-6SO_3^-(1→] residue were detected, whereas the latter was absent after digestion of the BTSA-treated polysaccharide. This indicates that only the removal of 6-*O*-sulfate group occurred.

The almost all 6-*O*-sulfate groups of chondroitin sulfate were removed by the treatment with BTSA because disaccharides containing [→3)GalNAc*p*-6SO_3^-(1→] disappeared and that with [→3)GalNAc*p*(1→] appeared (Fig. 4). It was found that 4-*O*- and 2-*O*- sulfate groups at [→3)GalNAc*p*-4SO_3^-(1→] and [→3)GlcA*p*-2SO_3^-(1→] residues were not affected by the

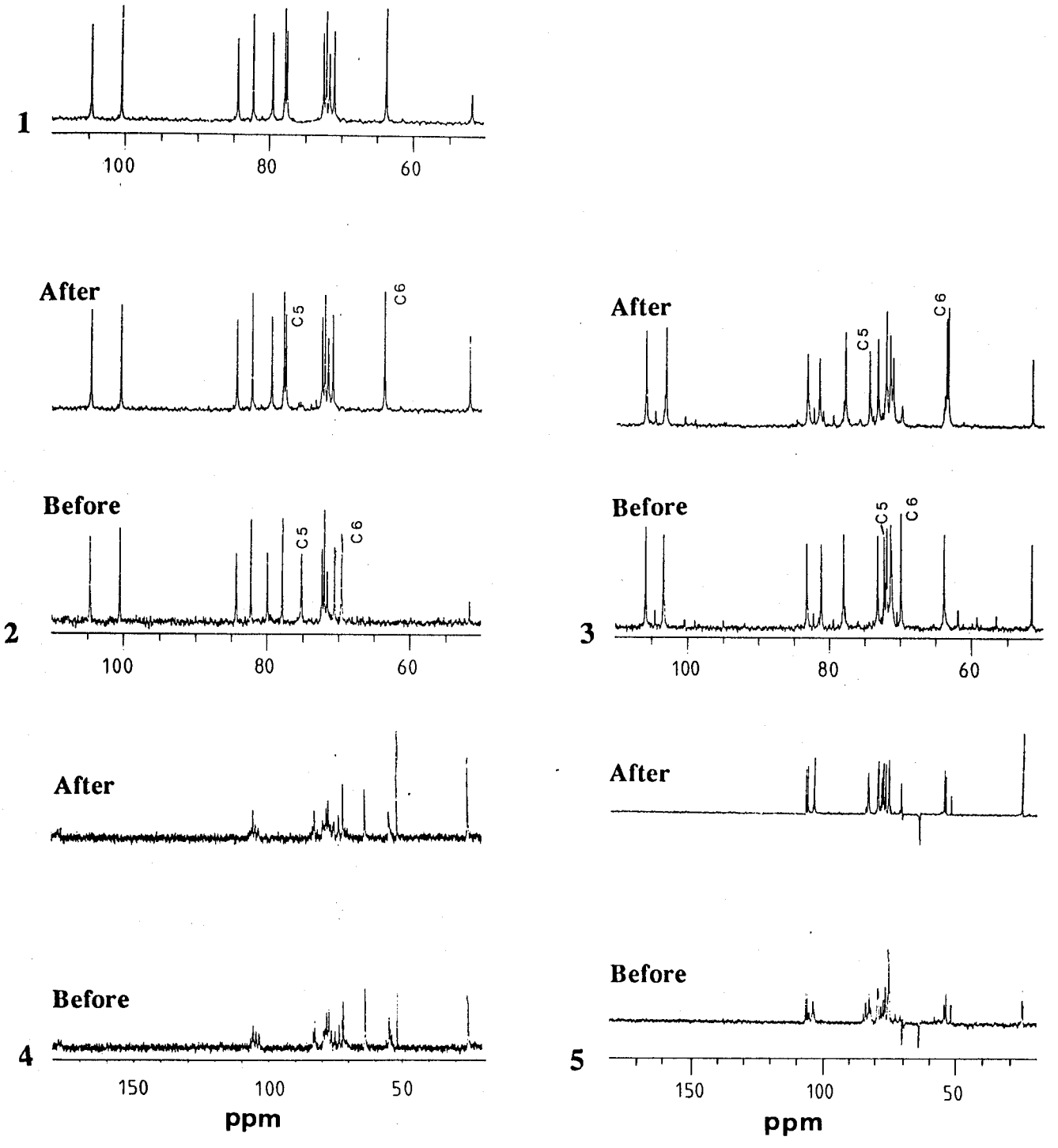

Fig. 2 ^{13}C-NMR spectra (75.4 MHz) of polysaccharides before and after the treatment with BTSA. **1**, agarose; **2**, funoran; **3**, porphyran; **4** dermatan sulfate; **5**, chondroitin sulfate. Spectra of **4** were recorded using DEPT pulse sequence.

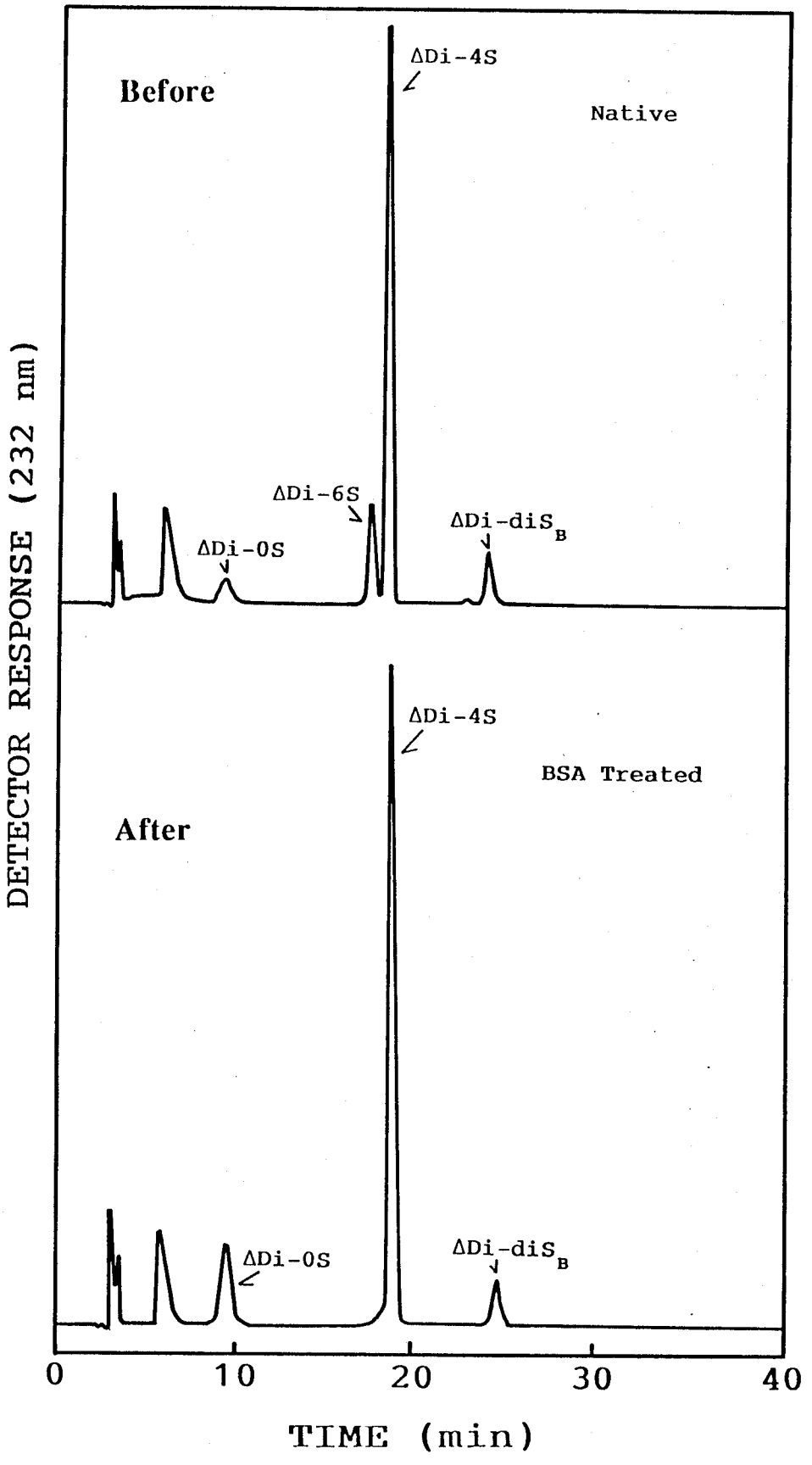

Fig. 3 HPLC profiles on a Shimadzu PNH_2 column (see Experimental) after digestion with chondroitinase ABC of dermatan sulfate, before and after treatment with BTSA; ΔDi-0S, ΔUA(1→3)GalNAc; ΔDi-4S, ΔUA(1→3)GalNAc4SO_3^-; ΔDi-6S, ΔUA(1→3)GalNAc-6SO_3^-; ΔDi-diS$_B$, ΔUA2SO_3^-(1→3)GalNAc4SO_3^-, where ΔUA is referred to 4,5-unsaturated hexuronate residue.

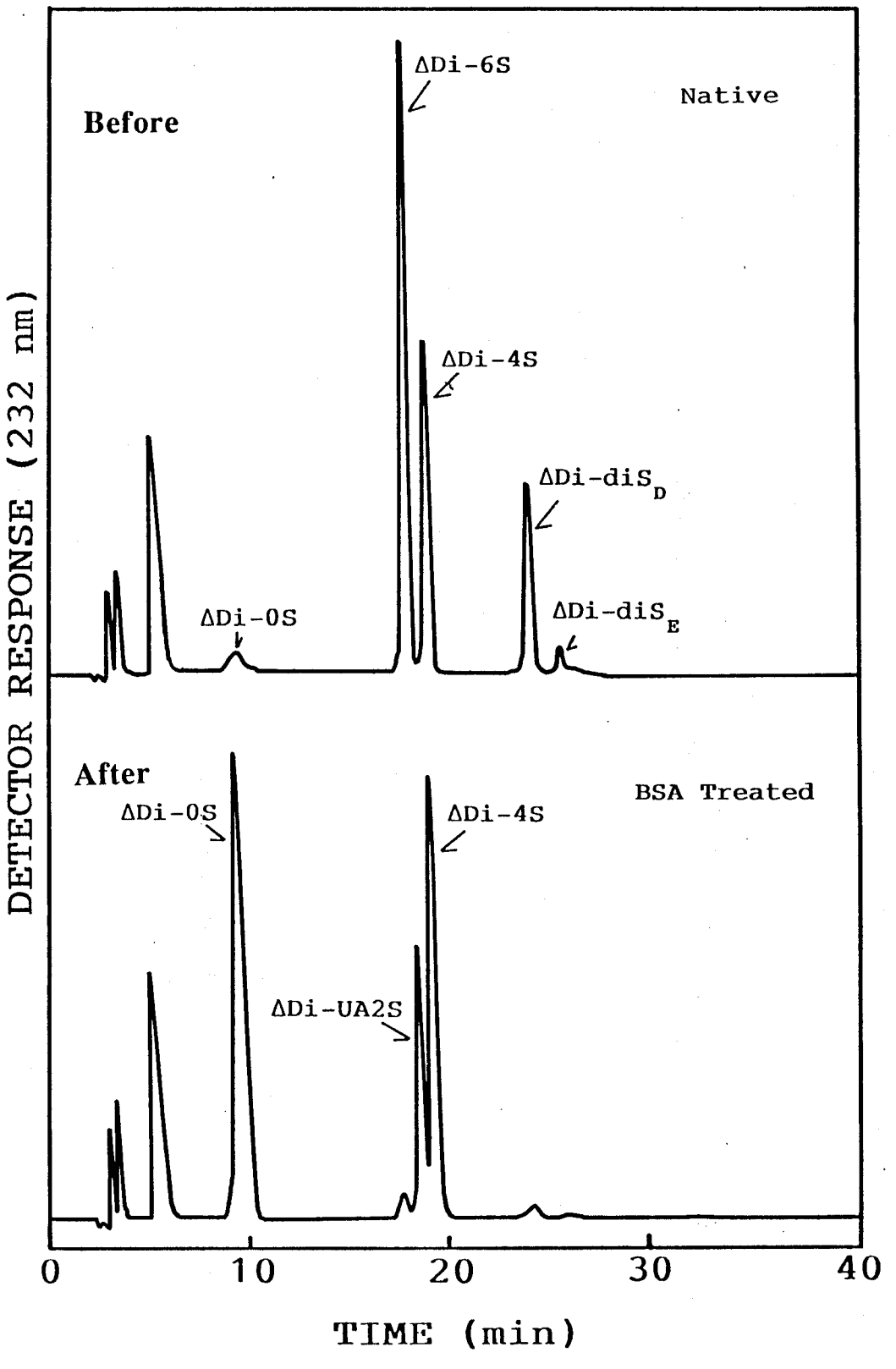

Fig. 4 HPLC profiles on a Shimadzu PNH_2 column (see Experimental) after digestion with chondroitinase ABC of chondroitin sulfate, before and after treatment with BTSA; ΔDi-0S, ΔUA(1→3)GalNAc; ΔDi-4S, ΔUA(1→3)GalNAc4SO_3^-; ΔDi-6S, ΔUA(1→3)GalNAc-6SO_3^-; ΔDi-UA2S, ΔUA2SO_3^-(1→3)GalNAc; ΔDi-diS$_B$, ΔUA2SO_3^-(1→3)GalNAc4SO_3^-; ΔDi-diS$_D$, ΔUA2SO_3^-(1→3)GalNAc6SO_3^-; ΔDi-diS$_E$, ΔUA(1→3)GalNAc4,6-di-SO_3^-, where ΔUA is referred to 4,5-unsaturated hexuronate residue.

treatment with BTSA.

Gel filtration profiles of dermatan sulfate and chondroitin sulfate showed no remarkable change by the BTSA-treatment (Fig. 5), indicating no cleavage of the polysaccharide chain.

The treatment of sulfated polysaccharide with TSIM in pyridine did not cause desulfation (Table 1). This suggests again that TSIM suppressed solvolytic desulfation [5] which is usually carried out under similar conditions in the absence of silylating reagent. It was reported that dermatan sulfate was desulfated at 80°C for 5 h in DMSO containing small amount of water [6-8]. In contrast, by the treatment of dermatan sulfate with BTSA at 80°C for 5 h in DMSO, the extent of desulfation was lower than that by the same treatment in pyridine (Table 1). This result suggests that the silylating reagent does not assist solvolytic desulfation, but prevents desulfation presumably by removal of the remaining water in the solvent. Therefore, it is suggested that the mechanism of BTSA/pyridine dependent desulfation is different from that of solvolysis. Since the extent of desulfation of funoran by the treatment with BTSA-DMSO was far less than that by the treatment with BTSA-pyridine, pyridine may play an important factor.

Experimental

Materials. BTSA and *N*-(trimethylsilyl)imidazole (TSIM) were obtained from Wako Pure Chemicals Ind. Funoran [9] and porphyran [2] were prepared from the red algae, *Gloiopeltis complanata* and *Porphyra yezoyensis*, respectively. Dermatan sulfate, chondroitin sulfate, and chondroitinase ABC were obtained from Seikagaku Corp., and pullulan from Showa Denko Co.

Desulfation with BTSA. An aqueous solution of the sodium salt of a sulfated polysaccharide (200 mg) was passed through a column (1 x 10 cm) of IR-120 (H^+) resin, the eluate was neutralized with pyridine, then lyophilized to give the pyridinium salt (*ca.* 220 mg). The pyridinium salt (220 mg) was soaked in dry pyridine (20 ml), BTSA (4 ml) was added, and the

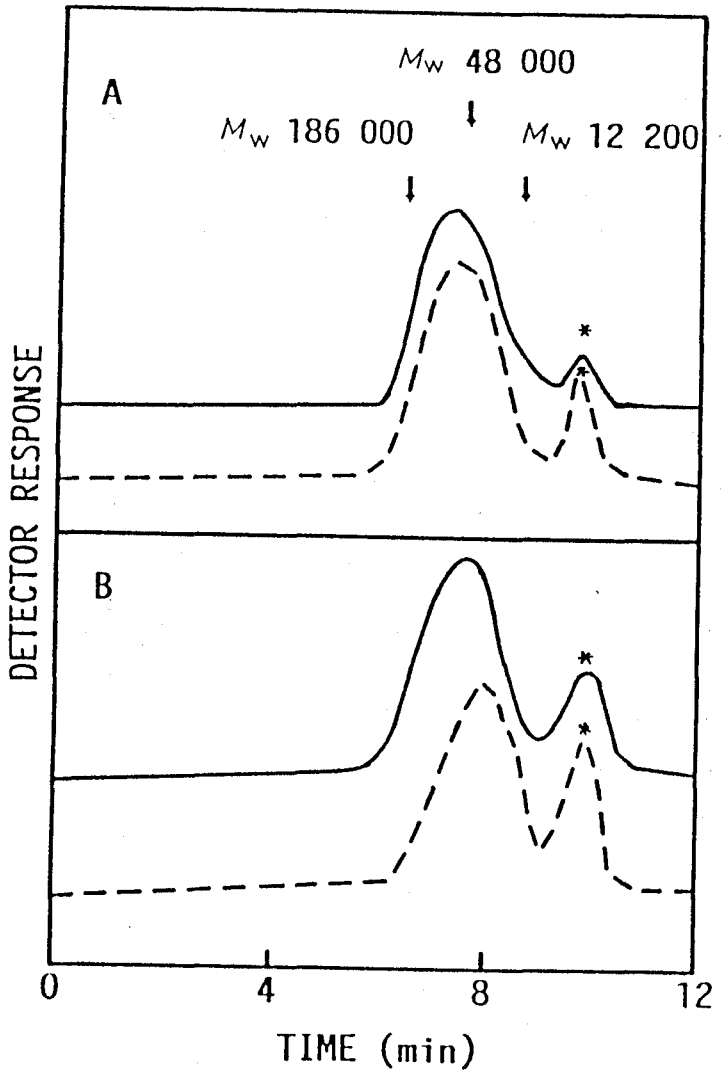

Fig. 5 Gel filtration on TSKgel-G4000PWXL of **A** dermatan sulfate, **B** chondroitin sulfate before (——) and after (----) treatment with BTSA. Arrows indicate the elution positions of pullulan molecular-weight markers and * indicates inorganic ions.

mixture was kept at 60°C for 2 h to give a homegenenous solution. After decomposing the excess reagent and silyl ethers by the addition of water (20 ml), the mixture was dialyzed. Then the pH of the retentate was adjusted to pH >7 with sodium hydroxide. The solution was dialyzed, and the retentate was lyophilized to give the BTSA-treated product (140 mg).

The pyridinium salt of a sulfated polysaccharide (100 mg) was treated similarly in pyridine (10 ml) without BTSA, and also in pyridine with TSIM (2.8 ml). The latter treatment gave a homogeneous solution. In addition, a solution of the pyridinium salt of the sulfated polysaccharide (100 mg) in DMSO (10 ml) was treated with BTSA (4 ml) at 80°C for 5 h.

Analysis of sulfates. A sulfated polysaccharide (300 μg) was hydrolyzed by 3 M HCl (1 ml) at 100°C for 18 h. The hydrolyzate was filtered with a membrane filter (0.45 μm) and the filtrate was analyzed by HPLC (Waters, ILC-1) using an ion-exchange column (Waters, IC Pak Anion, 4.6 mm x 5 cm) eluted with borate/gluconate buffer (1.3 mM sodium tetraborate, 1.5 mM sodium gluconate, 6 mM boric acid, 1.25% of glycerol, and 12% of acetonitrile) at a flow rate 0.8 ml/min. The sulfate ion in the eluate was monitored by a conductivity detector (Waters, 430).

NMR Spectroscopy. The ^{13}C-NMR spectra (75.4 MHz) of 10% solutions of polysaccharide sulfates in D_2O were recorded at 80°C with a spectrometer (General Electric Co., QE-300). Spectral widths of 10 kHz and relaxation delays of 1.0 s were used. Chemical shifts were measured in ppm from internal methanol and converted into values related to sodium 3-trimethylsilyl-1-propanesulfonate (conversion factor, 51.6). For chondroitin sulfate, distortionless enhancement by the polarization transfer (DEPT) pulse [3] was used at 80°C.

Digestion with chondroitinase. 0.4 M Tris-HCl buffer (20 μl, pH 8.0) containing 0.4 M sodium acetate, 0.1% of bovine serum albumin, and water (120 μl) was added to an aqueous 1% solution (20 μl) of dermatan sulfate or chondroitin sulfate. Chondroitinase ABC (5 U/ml, 20 μl) was added to each mixture, which was incubated at 37°C for 2 h.

Chromatography of enzyme digests. The digest of chondroitinase ABC was analyzed by HPLC (Irica 852) using an amine-bound silica column [10] (Shimadzu-PNH_2, 4.0 x 250 mm). The elution was performed with 16 mM sodium dihydrogen phosphate in a 60-min linear gradient from 16 mM to 0.5 M sodium dihydrogen phosphate at 1 ml/min. The elution profiles were monitored at 232 nm.

Gel filtration of polysaccharide sulfates. An aqueous 3% solution (10 µl) of sulfated polysaccharide was analyzed by HPLC (Shimadzu Corp., LC-5A) using a gel-filtration column (Tosoh Co., TSKgel G4000PWXL, 7.8 mm x 30 cm) eluted with 0.2 M potassium sulfate. The elution profiles were monitored by a refractive index detector (Shimadzu, RID-2A).

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Part 2-IV

Reaction process of desulfation of sulfated carbohydrates by silylating reagents

Abstract

Comparative studies on the desulfation reaction by various trimethylsilylating reagents classified into three categories (Part 2-II, Table I) are discussed to elucidate the mode of reactions. In the case of reaction with silylating reagents of category C, sulfate groups are supposed to be eliminated by attack of a nucleophile generated by release of trimethylsilyl group from trimethylsilylating reagent. In the case of reagents of category B, removal of 6-*O*-sulfate groups was supposed to be accelerated by sterically favored configuration of the intermediate or the transition state.

The author described the desulfation reactions of carbohydrates using a variety of silylating reagents. These reagents have been classified into three categories (Part 2-II, Table I); A, reagents inhibiting desulfation; B, accelerating removal of 6-*O*-sulfate; and C, capable of non-specific desulfation.

The conditions for the desulfation reactions using the silylating reagents are similar to those for the solvolytic desulfation [1,2] as already described: in both cases, pyridinium salt of sulfated carbohydrate is heated in pyridine. The two reactions are distinguished only by the presence or absence of the silylating reagents. A possible reason for the different reactions could be thus removal of water from the medium by the silylating reagents, since the presence of water is known to accelerate solvolytic desulfation [2]. In fact, several silylating reagents, namely

those of the category A, suppressed desulfation. If only this effect is operative, however, all the silylating reagents should have exerted suppression of desulfation. Accordingly, other factors have to be included in the case of reagents of the categories B and C.

For the desulfation with silylating reagents, conversion of the sulfate group into a salt with amine (such as pyridinium salt) was crucial. Sodium salts of funoran and chondroitin sulfate (insoluble in pyridine) or tetrabutylammonium salt of funoran and chondroitin sulfate (completely soluble to pyridine) was not desulfated with the silylating reagents. In contrast, pyridinium salt of these sulfated polysaccharides were readily desulfated with efficient silylating reagents. In the first step of the desulfation reaction with the reagents of both category B and C, trimethylsilylation of sulfate hemi-ester may occur as follows:

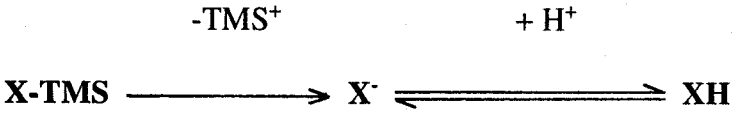
$$\underset{\mathbf{1}}{\mathbf{R\text{-}OSO_3^-}} + (\mathbf{PyrH^+}) \rightleftharpoons \underset{\mathbf{2}}{\mathbf{R\text{-}OSO_3^{(-)}H^{(+)}}} \rightleftharpoons \underset{\mathbf{3}}{\mathbf{R\text{-}O\text{-}SO_3\text{-}TMS}}$$

where $PyrH^+$ and TMS represents pyridinium cation and trimethylsilyl group, respectively. Such trimethylsilylated sulfate ester **3** may be reasonably expected, since it was reported that inorganic sulfate was trimethylsilylated to yield bis(trimethylsilyl)sulfate [3] and glucose 6-phosphate was also silylated to yield pertrimethylsilylated phosphate [4].

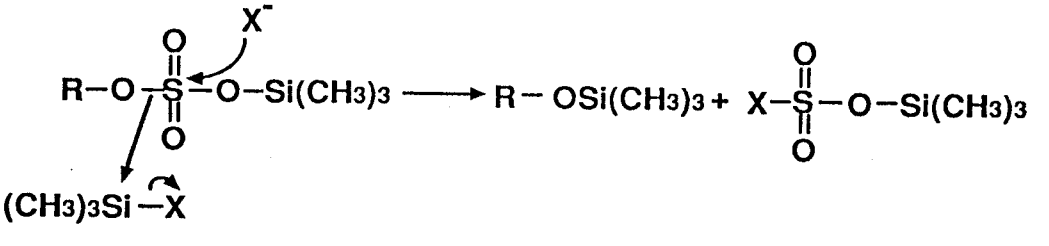
Different factors should be considered for the reactions with reagents of the category B (regiospecific 6-*O*-desulfation) and the category C (non-regiospecific desulfation). 6-*O*-Sulfate groups in tributylammonium or guanidium salts of funoran and chondroitin sulfate were not removed by 4-trimethylsilyloxyl-3-penten-2-one (TPENON), one of the reagents of the category C, but completely removed by *N,O*-bis(trimethylsilyl)acetamide (BTSA) or *N*-methyl-*N*-

(trimethylsilyl)trifluoroacetamide (MTSTFA), which are classified in the category B. The reagents in the category B and C are distinguished by their silylating activity; the category B involves strong silylating reagents, whereas the category C involves weak or moderately active silylating reagents. With the reagents in the category C with stronger base than pyridine, trimethylsilylated sulfate ester **3** can be hardly formed, because protonated sulfate hemiester **2** would much less contribute to the equilibrium than in the case of the pyridinium salt.

In silylation reaction, a trimethylsilyl group donor, **X-TMS**, releases trimethylsilyl group to result an acid (**XH**) or its conjugated base ($\mathbf{X}^-$)as follows.

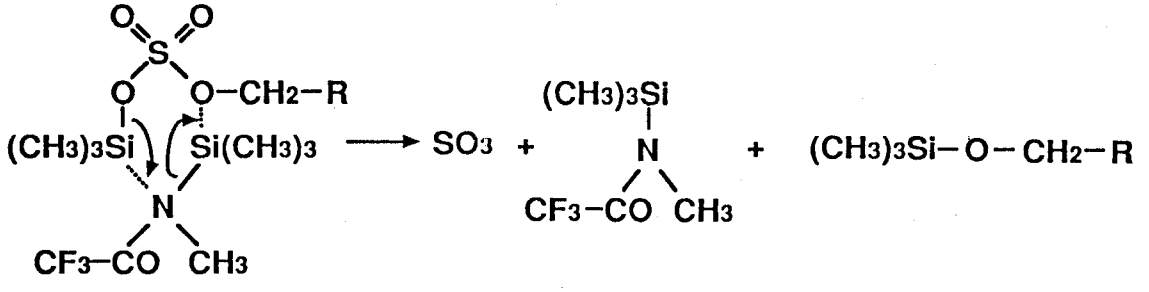


In the case of the stronger silylating reagents, such as BTSA and MTSTFA, the reagents release trimethylsilyl group to form acetamide and *N*-methyltrifluoroacetamide that may predominantly exist as **XH**. In contrast, the weaker silylating reagent such as TPENON may produce enol anion of 2,4-pentanedione, because pKa value of 2,4-pentanedione is close to that of pyridinium cation to make the contribution of the conjugate base considerable. Then the conjugate base may act as a nucleophile to attack the trimethylsilylated sulfate ester **3** and eliminate sulfate group as the following scheme.



This may explain the results that sulfate group was not removed by TPENON when the methyl α-galactoside 6-sulfate was pretreated with a strong silylating reagent, *N*-(trimethylsilyl)-imidazole (TSIM). Because pretreatment with TSIM silylates all hydroxyl and sulfate hemiester groups, TPENON added thereafter cannot release trimethylsilyl group and cannot yield the conjugate base of 2,4-pentanedione as the nucleophile.

Although the above scheme may explain the reaction with TPENON belonging to the category C, it may not explain the mechanism for the stronger silylating reagents of the category B such as BTSA and MTSTFA, which afford very stable acetamide derivatives after release of trimethylsilyl group. These reaction products do not act as nucleophiles due to negligible contribution of their conjugate bases in the equilibrium. As already discussed, formation of the trimethylsilylated sulfate ester **3** is expected to precede the desulfation. The desulfation reaction of methyl α-galactoside 6-*O*-sulfate occurred by the treatment of BTSA or MTSTFA even after treating with TSIM. This suggests that another additional transition state is formed by a reaction between BTSA or MTSTFA and intermediate **3**. The specificity to 6-*O*-sulfate group may be explained by a steric allowance of C-6 in a transition state favorable for a concerted cleavage of the ester linkage and a formation of silyl ether linkage. The formation of the transition state may also explain the low activation energy of the reaction mentioned in Part 2-I. A possible example of six-membered transition state is suggested as follows.



The desulfation mechanism might also be explained by another scheme involving a nucleophilic substitution initiated by an anion attack on the carbon atom adjacent to the sulfate group, the process being analogous to the alkaline desulfation of carbohydrates [6,7]. However, a nucleophile such as silyloxyl anion may not produced. This may be consistent with that methyl 6-*O*-tosylgalactoside carrying a good leaving group did not deesterified by the treatment with BTSA in pyridine.

Experimental

Sulfated polysaccharide used were funoran extracted and purified from the red seaweed, *Gloiopeltis complanata*, and chondroitin sulfate (mixture of 6-sulfate and 4-sulfate). Tributylammonium salt of the sulfated polysaccharide was prepared as described by Nagasawa *et al.* [8]. Tetrabutylammonium salt of the sulfated polysaccharides were prepared by neutralization of acid form of the polysaccharides obtained by passing through cation exchange resin IR-120 (H^+ form) with tetrabutylammonium hydroxide, followed by dialysis and lyophilization. Treatments of the polysaccharides with silylating reagents were carried out as described in Part 2-II. Structural changes of the polysaccharides by the treatment were monitored by ^{13}C-NMR. As another model experiment, pyridinium salt of methyl α-galactoside 6-*O*-sulfate was heated in pyridine at 60°C for 2 h with TSIM, to the resulting solution was added BTSA or TPENON, the mixture heated at 60°C for 2 h, and then the reaction mixture was analyzed using GLC.

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Part 3

Application of the regioselective desulfation method to structural analysis of sulfated polysaccharides

Part 3-I

Structure of a water-soluble sulfated polysaccharide from the red seaweed, *Joculator maximus* Manza

Abstract

Main acidic polysaccharide, designated PS1, was extracted from the red seaweed, *Joculator maximus* Manza. It consists of D-Gal, L-Gal, 2-*O*-methyl-L-Gal, D-Xyl and sulfate (100 : 69 : 32 : 49 : 73 in molar ratio). By partial hydrolysis study of the polysaccharide, oligosaccharides indicating the repeating backbone of [→3)-β-D-Gal-(1→4)-α-L-Gal-(1→] were identified. In this structure, (1→4)-linked L-Gal residue was found to be partially 2-*O*-methylated in terms of trideuteriomethylation study. On the basis of the results from ^{13}C-NMR spectroscopy, controlled Smith degradation, specific 6-*O*-desulfation, and methylation analysis, it was demonstrated that all the Xyl residues were linked to O-6 of a D-Gal residue in β-configuration, and sulfate groups were located at O-6 of D-Gal and O-2 or O-3 of L-Gal residues.

Water-soluble galactans are widely distributed among red seaweeds, and their common backbone consists of an alternating repeat of (1→4)-linked α-Gal (and/or 3,6-anhydrogalactose) and (1→3)-linked β-Gal residues [1]. The latter residue is always of D-form, while the former is either of D- or L-form depending on the origin. The algal galactans are thus classified into the two types: carrageenan (containing only the D-enantiomer of Gal) and agaroid (containing both D- and L-enantiomers). This backbone unit is modified by substituents such as 4,6-pyruvate ketal, *O*-methyl, *O*-sulfate, or *O*-glycosyl groups in various extent, thus giving rise to structural heterogeneities. Biological functions of the algal polysaccharides have not been fully

understood. However, some of them were found in the cell matrices as components of hemicellulose [2]. This fact suggests that the acidic groups such as sulfate or carboxylate influence some properties and functions of the matrices.

A calcareous red seaweed, *Joculator maximus*, an alga commonly found in Japan, is classified into the family Corallinaceae, which is the largest family in the order Cryptonemiales [3]. However, polysaccharide from calcareous algae of this family was scarcely reported except for the studies [4,5] for *Corallina officinalis*, owing to the low production of such polysaccharides.

The seaweed, *Joculator maximus*, is covered with thick layer of calcium carbonate that may occlude sulfated polysaccharides. Although it is possible to solubilize the calcified layer in an acidic medium to improve the yield of extraction, the use of the acid may cause hydrolysis of the polysaccharide. To avoid such undesirable structural alteration, finely milled alga was directly extracted with neutral hot water in this study. From the extract obtained, sulfated polysaccharide was isolated as water-insoluble cetylpyridinium (CP) salt. The CP salt was extracted with 1 M potassium chloride to obtain potassium salt of sulfated polysaccharide, termed PS1. As shown in Table I, the constituents of PS1 were D-Gal, L-Gal, 2-*O*-methyl-L-Gal, D-Xyl, and sulfate. However, 3,6-anhydrogalactose found in many algal polysaccharides was not detected. The molar ratio of D-Gal was almost equivalent to the sum of L-Gal and 2-*O*-methyl-L-Gal, in agreement with a composition of an agarose type polysaccharide.

To analyze the backbone structure, PS1 was partially hydrolyzed. Among the resulting mixture of the hydrolysis products, neutral hydrolyzate was gel-filtrated using Bio-Gel P-2. Since methylated sugars behave as saccharides with higher degree of polymerization when chromatographed on Bio-Gel [6], the fractions corresponding to di- and tri-saccharides (DP2 and DP3) were further purified by semipreparative HPLC to obtain true disaccharide (DP2-1) and trisaccharide (DP3-1) fractions. From DP2, 2-*O*-methyl-L-Gal was isolated. The obtained DP2-

Table I Composition* of main polysaccharide, PS1, from *Joculator maximus*

component	molar ratio
D-Xyl	49
D-Gal	100**
L-Gal	69
2-*O*-Me-L-Gal	32
-SO_3^-	72

* Values are based on color reactions of pentose and hexoses, and sulfate analysis. Ratio of Gal and 2-*O*-Me-Gal was determined by GLC. D/L assignment and its ratio was determined as described in ref. [23].

** The value for D-Gal was taken as 100.

1 and DP3-1 were independently analyzed by ion-exchange chromatography using borate buffer to identify the disaccharides **1** and **2** and the trisaccharides **3** and **4** (Table II). These di- and tri-sachharide are reasonably assumed to be derived from an agarose type repeating backbone, [→3)β-D-Gal(1→4)α-L-Gal(1→]. Oligosaccharide containing 2-*O*-methyl-L-Gal was not isolated in the present study. However, as discussed later in trideuteriomethylation study, 2-*O*-methyl-L-Gal residue is likely (1→4)-linked, thus making an agarose type unit, →3)β-D-Gal(1→4)2-*O*-methyl-α-L-Gal(1→. Although xylose-containing oligosaccharide was not isolated possibly due to acid-lability of xylosyl linkage, Xyl is likely to occur as a single unit branching, because essentially all Xyl was detected as 2,3,4-tri-*O*-methylxylose attributable to non reducing end in methylation analysis (Table III). This was supported from loss of the Xyl after successive periodate oxidation and borohydride reduction.

The location of sulfate group and Xyl residue was evaluated by methylation analyses of PS1 and chemically modified PS1s (Table III). Since the D- and L-Gal residue appeared to be 3- and 4-linked, respectively, 2,4,6-tri- and 2,4-di-*O*-methyl-D-Gal from the native PS1 are concluded to be derived from free and 6-substituted (1→3)-linked D-Gal units, respectively. The other products, 2,3,6-tri-, 2,6-di-, and 3,6-di-*O*-methyl-L-Gal are likewise assumed to be derived from free (and/or naturally 2-methylated), 3-substituted, and 2-substituted (1→4)-linked L-Gal units, respectively. When compared with the results from native PS1, the methylation analysis after the successive periodate oxidation, borohydride reduction, and partial hydrolysis (controlled Smith degradation), caused increase in 2,4,6-tri-*O*-methyl-D-Gal and decrease in 2,4-di-*O*-methyl-D-Gal, while there was no essential change in the ratio of di-*O*-methylated L-Gal residues (Table III). Accordingly, the Xyl branches are to be located at O-6 of (1→3)-linked D-Gal residue in the native PS1, and sulfate groups at O-2 and O-3 of (1→4)-linked L-Gal residue.

Table II Oligosaccharide obtained from partial hydrolyzate from PS1

product	structure
1	β-D-Gal(1→4)L-Gal
2	α-L-Gal(1→3)D-Gal
3	β-D-Gal(1→4)α-L-Gal(1→3)D-Gal
4	α-L-Gal(1→3)β-D-Gal(1→4)L-Gal

Table III Methylation analyses* of polysaccharides from *Joculator maximus*.

Methylation product**	Attributed to	Native PS1	Controlled Smith Degraded PS1	BTSA-treated PS1
2,3,4-Me_3-D-Xyl	D-Xyl(1→	24	0	25
2,4,6-Me_3-D-Gal	→3)D-Gal(1→	10	29	21
2,4-Me_2-D-Gal	→3)D-Gal-6-Xyl(1→ and →3)D-Gal-6-SO_3^-(1→	40	21	29
2,3,6-Me_3-L-Gal	→4)L-Gal(1→ and →4)L-Gal-2-Me(1→	33	25	32
2,6-Me_2-L-Gal	→4)L-Gal-3-SO_3^-(1→	7	9	7
3,6-Me_2-L-Gal	→4)L-Gal-2-SO_3^-(1→	10	13	11

*Expressed as the molar ratio of partially methylated alditol acetate after hydrolysis of permethylated polysaccharides and subsequent derivatization (Total of partially methylated galactitols is taken as 100.). D/L assignment is determined by the method in ref [23].

As the amount of 2,4-di-*O*-methyl-D-Gal in the methylation analysis of the native PS1 is larger than its Xyl content, the excess 2,4-di-*O*-methyl-D-Gal suggests 6-sulfation at (1→3)-linked D-Gal. This is consistent with that 2,4-dimethyl-D-Gal was also detected in the methylation analysis after controlled Smith degradation (Table III). Sulfate linked to O-6 of carbohydrates are regioselectively removed by a treatment with a silylating reagent, bis(trimethylsilyl)acetamide (BTSA) as described in Part 2. In the methylation analysis after the treatment with BTSA (Table III), the content of 2,4-di-*O*-methyl-D-Gal decreased to essentially the same amount of methylated Xyl, when compared with the results from native PS1. The decrease of the 2,4-di-*O*-methyl derivative accompanied an almost equal increase in the amount of 2,4,6-tri-*O*-methyl-D-Gal. The results indicate the presence of 6-sulfate.

Linkage and substitution of 2-*O*-methyl-L-Gal residue were evaluated by a trideuteriomethylation study. With GLC-MS analysis of the alkylated (*i.e.*, methylated and/or trideuteriomethylated) alditol acetates, the peak of the 2,3,6-tri-*O*-alkylgalactose derivative was identified as a mixture of 2,3,6-tris-*O*-trideuteriomethyl- and 2-*O*-methyl-3,6-bis-*O*-trideuteriomethylgalactitol, as judged from the peaks at m/z 120 and 117 (Fig. 1A) indicating fragment ions from 2-*O*-trideuteriomethylated and 2-*O*-methylated residues, respectively [8]. The mass chromatogram monitoring m/z 117 afforded only one peak attributable to 2-*O*-methyl-3,6-bis-*O*-trideuteriomethylgalactitol. In contrast, all the 2-*O*-alkylated component afforded peaks in the mass chromatogram monitoring m/z 120, which indicated free hydroxyl groups at C-2 of the Gal residues (Fig. 1B). This denotes that all 2-*O*-methyl-L-Gal residue in PS1 is (1→4)-linked and contains no other substituent.

Although ^{13}C-NMR spectrum of native PS1 was complicated due to various substituents in the polysaccharide chain, there were five conspicuous peaks (asterisked in Fig. 2A), which disappeared after controlled Smith degradation (Fig. 2B). They were assigned for β-xylosyl

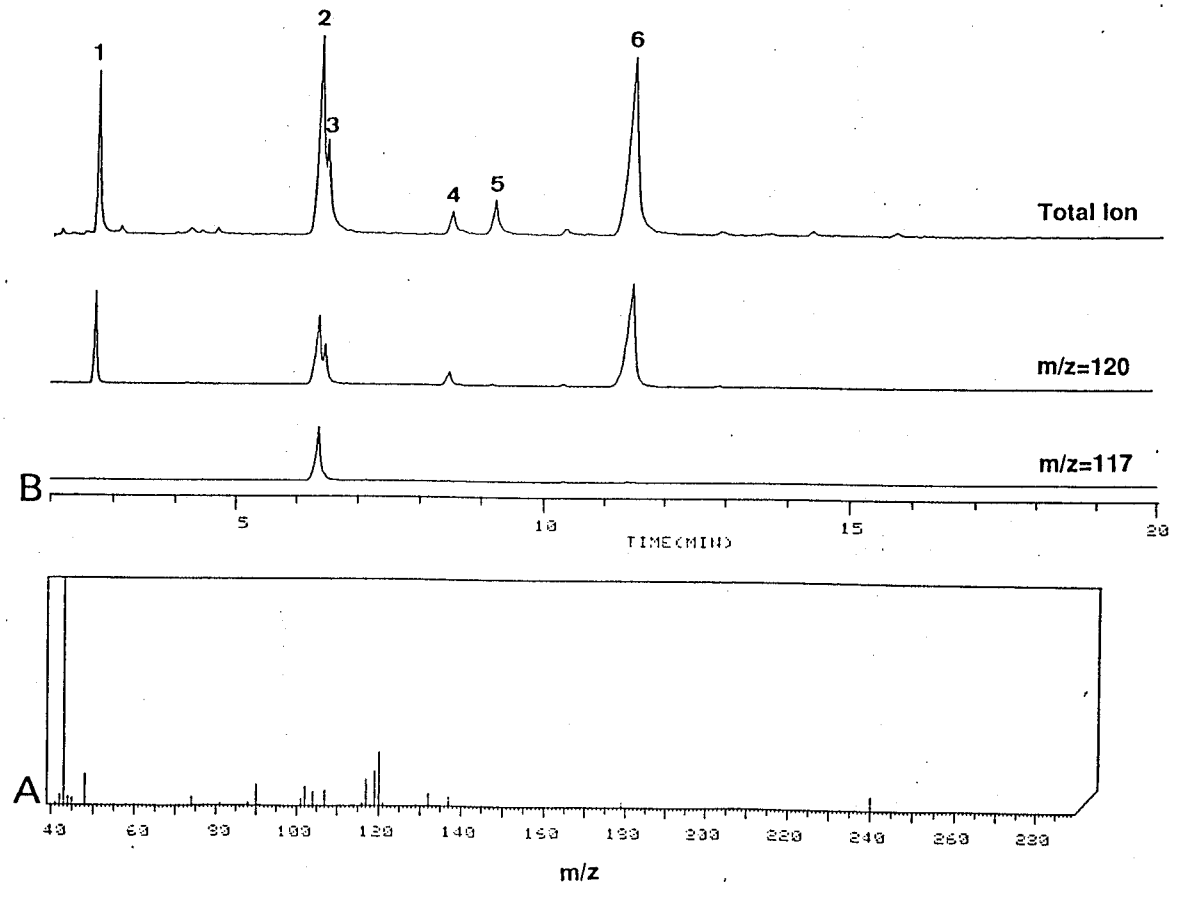


Fig. 1 GC-MS of alditol acetates from trideuteriomethylated PS1.

A. Mass spectrum of 2,3,6-tri-*O*-alkylated galactose derivative (peak 2 in **B**) from pertrideuteriomethyalted PS1.

B. Mass chromatograms and total ion chromatogram of alditol acetates derived from pertrideuteriomethylated PS1. The fragment peak at m/z 120 is characteristic of derivatives carrying 2-0-trideuteriomethyl group, and m/z 117, 2-*O*-methyl group. Assignment; **1**, 2,3,4-Md_3-Xyl; **2**, mixture of 2,3,6-Md_3-Gal and 2-Me-3,6-Md_2-Gal; **3**, 2,6-Md_2-Gal; **4**, 3,6-Md_2-Gal; **5**, 2,4-Md_2-Gal. Abbreviations; 2,3,4-Md_3-Xyl, 2,3,4-tris-*O*-trideuteriomethylxylose; 2-Me-3,6-Md_2-Gal, 2-*O*-methyl-3,6,-bis-*O*-trideuteriomethylgalactose, *etc*.

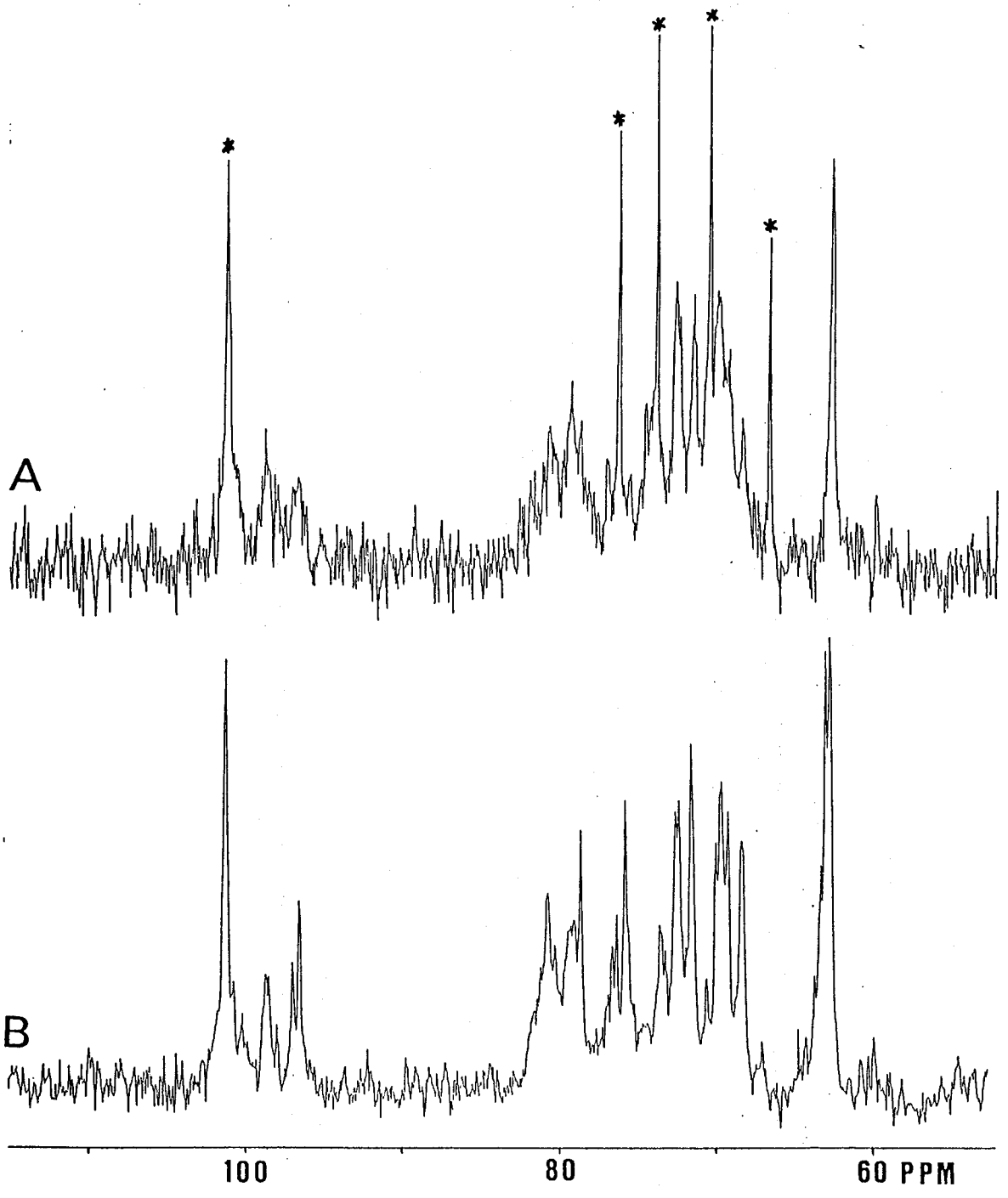


Fig. 2 ^{13}C-NMR spectra

A. Native PS-1. Asterisked peaks were attributed to a branching Xyl residue (see text).

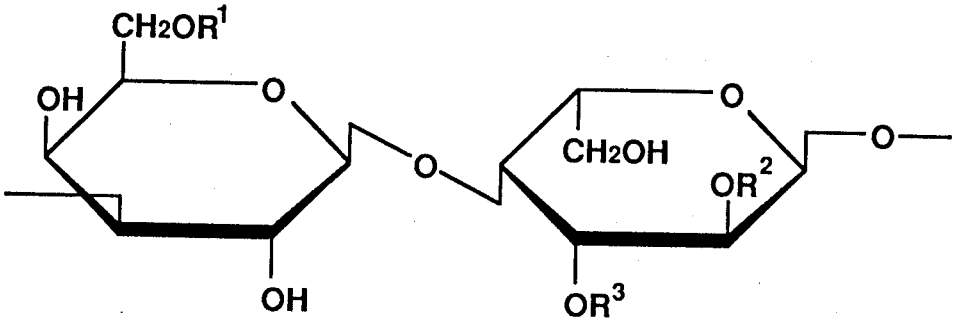
B. Controlled Smith degraded PS-1.

residue with no substituent; 105.9 ppm (C-1), 75.5 ppm (C-2), 78.1 ppm (C-3), 72.9 ppm (C-4) and 67.6 ppm (C-5). As for methyl β-xyloside, Gorin and Mazurek [9] reported following chemical shift data; 105.1 ppm (C-1), 74.0 ppm (C-2), 76.9 ppm (C-3), 70.4 ppm (C-4) and 66.3 ppm (C-5).

From the evidence above, the structure of PS1 is proposed as shown in Fig. 3. Regarding several carrageenans that consist of two types of basic repeating units, [→3)β-D-Gal(1→4)3,6-anhydro-α-D-Gal(1→] and [→3)β-D-Gal(1→4)α-D-Gal(1→], Lawson *et al.* [10] proposed that each unit exists consecutively in the polysaccharide chain. The proposal was derived from the fact that they obtained disaccharide derivative of 4-*O*-β-D-galactopyranosyl-3,6-anhydro-D-galactose by partial methanolysis in a fairly good yield. In regard to PS1 from *J. maximus*, the repeating unit containing unmodified (1→4)-linked L-Gal residue is likely to present consecutively in the polysaccharide chain, since non-dialysable polysaccharide was recovered in an almost quantitative yield after the controlled Smith degradation. A serious depolymerization of the polysaccharide chain may occur if the oxidation-labile unmodified (1→4)-linked L-Gal residue was distributed randomly or homogeneously in the chain. This periodate-labile moiety may be less substituted at (1→3)-linked unit, because larger amount of 2,4,6-tri-*O*-methylgalactose was obtained in methylation analysis of controlled Smith degraded PS1 (Table III).

The high rates of substitution with single Xyl residue at O-6 of D-Gal residue and substitution with methyl group at O-2 of L-Gal residue are the characteristic structural features of PS1. From a closely related alga *Corallina officinals*, a highly methylated, sulfated xylogalactan was also isolated [4,5]. Single Xyl residue was also discovered in a *Laurencia intermedia* polysaccharide [11]. With regard to the glycosylation of the main polysaccharide chain, in *Gracilaria tekvahiae* agar [12], single 4-*O*-methyl-L-Gal residue at O-6 of D-Gal residue was also found.

Backbone



Modification

$R^1 = H$	20%	$R^2 = R^3 = H$	35%
$R^1 = Xyl$	50%	$R^2 = CH_3$, $R^3 = H$	30%
$R^1 = SO_3^-$	30%	$R^2 = SO_3^-$, $R^3 = H$	20%
		$R^2 = H$, $R^3 = SO_3^-$	15%

Fig. 3 Proposed covalent structure of PS1 from *Joculator maximus*.

Among the red seaweed belonging to the order Cryptonemiales, several galactan sulfate have been isolated from the families Grateloupiaceae [13,14] and Endocladiaceae [15]. These polysaccharides are classified into the carrageenan type and/or agarose-carrageenan hybrid type. It may be informative that the agarose type polysaccharide was isolated from the red seaweed of the taxonomically confused family Corallinaceae belonging to this order. It is interesting that the sulfated polysaccharide, even in quite lower yield than those from other red seaweeds, was isolated from the firmly calcified seaweed, which may not require any structural polysaccharide, such as cellulose or hemicellulose, to maintain the shape of the algal body.

Experimental

Materials and general methods. The red seaweed *Joculator maximus* Manza was collected at a sublittoral of Shirahama coast in Wakayama Prefecture, Japan. Amount of hexoses was determined colorimetrically by anthrone-sulfuric acid method [16]. Xyl amount was estimated by the Bial method [17]. Analysis of 3,6-anhydrogalactose was carried out as described by Yaphe and Arsenault [18], as well as by methanolysis of polysaccharide as described later. Sulfate content was determined by titration with poly(vinylsulfate) using toluidine blue as indicator after addition of aliquot of glycolchitosan. GLC was carried out by a gas chromatograph (GC-8A, Shimadzu Corp.) equipped with flame ionization detector using fused silica capillary columns, OV-1 Bonded (column *a*, 0.3 x 25 m, GL-Science Co.), PEG-20M Bonded (column *b*, 0.3 x 25 m, GL-Science Co.), and CP-Sil 88 (column c, 0.25 x 25 m, ChromPack Co.) operated at 170°C, 200°C, and 215°C, respectively. Nitrogen was used as the carrier gas in a flow rate 20 ml/min and split ratio 20 : 1. GLC-MS was carried out by a mass spectrometer (GC/MS QP-1000, Shimadzu Corp.) in the conditions similar to GLC except for the use of helium as a carrier gas. Ion-exchange liquid chromatography as sugar borate complex

and gel filtration were carried out as described [7] using #2630 (column *d*, borate form, 0.8 x 23 cm, Hitachi Co.) and Bio-Gel P-2 (column *e* 2.2 mm x 2 m), respectively. HPLC was carried out by a liquid chromatograph LC-5A (Shimadzu Corp.) equipped with a refractive index detector RID-2A (Shimadzu Corp.). An SCR-101N column (7.5 mm x 25 cm, Shimadzu Corp.) eluted with water at a flow rate of 1 ml/min was used as column *f* for HPLC.

Extraction and fractionation of sulfated polysaccharide. Dried whole body of the seaweed *Joculator maximus* (702 g) milled with a ball mill was extracted twice with water (5000 ml) at 100°C, the combined extract was centrifuged, dialyzed, evaporated *in vacuo* to 2000 ml, and then 5% cetylpyridinium (CP) chloride solution was added to give an insoluble CP salt of sulfated polysaccharides. The resulting precipitate was separated by centrifugation. The CP salt was washed successively with ethanol and acetone, and then dried. The salt (1.5 g) was extracted twice with 1 M potassium chloride at 20°C for 2 hr. The combined extract was concentrated, poured into 4 vol. of ethanol. The resulting precipitate was dissolved in water, dialyzed. The dialyzate was concentrated, poured into 4 vol. of ethanol, and the resulting precipitate was then washed with ethanol and acetone to give the potassium salt of sulfated polysaccharide, PS1 (1.02 g). The remaining precipitate from 1 M potassium chloride extraction was successively extracted with 2 M and 4 M potassium chloride at 20°C and the respective extracts were treated similarly to give PS2 (0.2 g) and PS3 (0.2 g). The precipitate insoluble to 4 M potassium chloride at 20°C was further extracted with 4 M potassium chloride at 90°C. The solution obtained was immediately poured into 4 vol. of ethanol, the resulting precipitate was treated similarly, dissolved in water, dialyzed, concentrated, and poured into ethanol. The resulting precipitate was washed with ethanol and acetone to give PS4 (0.1 g). The main acidic polysaccharide fraction, PS1, was further investigated.

Composition analysis of PS1. PS1 was methanolyzed with 1 M methanolic hydrogen chloride

at 70°C for 18 h, and the mixture was neutralized with silver carbonate. After the resulting silver chloride and excess silver carbonate were filtered off, the methanolyzate was trimethylsilylated as described by Sweeley *et al.* [19], and then analyzed by GLC and GC/MS (column *a*). In addition, PS1 was hydrolyzed with 1 M sulfuric acid at 100°C for 18 h and the mixture was neutralized with barium carbonate. After the resulting barium sulfate and excess barium carbonate were filtered off, the hydrolyzate was trimethylsilylated, and then analyzed by GLC (column *a*). Another portion of the sulfuric acid hydrolyzate was derivatized into alditol acetates [20] for GLC and GC/MS analyses GLC (column *b*), and the other was used for assignment of absolute configuration of the component sugars by diastereomeric derivatization with a chiral alcohol [21-23]. The D/L ratio of Gal was determined from the peak area ratio of each derivative in GLC. Quantitative analysis of each component sugar was based on the results from colorimetric determinations of hexoses and xylose. Then ratio of Gal to 2-*O*-methylgalactose was determined by GLC as alditol acetates from the hydrolyzate of PS1.

Partial hydrolysis of PS1. PS1 (400 mg) was hydrolyzed with 0.1 M sulfuric acid (25 ml) at 100°C for 2 h and neutralized with barium carbonate. After the resulting barium sulfate and excess barium carbonate were filtered off, the hydrolyzate was evaporated to dryness and then applied to serially connected ion-exchanger columns of Amberlite IR-120 (H^+ form, 2 x 20 cm), Dowex 1 x 4 (sulfate form, 2 x 20 cm), and IR-45 (OH^- form, 2 x 20 cm). The neutral hydrolyzate fraction (flowing through fraction) was further investigated. The neutral hydrolyzate (100 mg) was gel-filtrated using column *e*. The resulting fractions corresponding to disaccharide (designated as DP2) and trisaccharide (DP3) were further investigated. DP2 was semipreparatively chromatographed with column *f* to afford two fractions DP2-1 (10.3 min, 2.3 mg) and DP2-2 (11.5 min, 2.1 mg). DP2-2 was trimethylsilylated, analyzed with GLC and GC/MS (column *a*). DP2-1 was analyzed as borate complex with column *d*. DP3 was applied to

column *f* to afford two fractions DP3-1 (9.6 min, 2.9 mg) and DP3-2 (10.5 min, 1.4 mg). The latter fraction was not further investigated. DP3-1 was analyzed similarly to DP2-1.

Methylation and trideuteriomethylation analyses of PS1. PS1 was methylated two times in DMSO by using powdered sodium hydroxide and methyl iodide as described by Isogai *et al.* [24] and Ciucanu and Kerek [25]. The methylated PS1 was hydrolyzed, and analyzed by GLC and GLC-MS as the partially methylated alditol acetates as described by Jansson *et al.* [8]. The absolute configuration of the partially methylated monosaccharide in the hydrolyzate was assigned as described elsewhere [23]. Another portion of PS1 was trideuteriomethylated and analyzed by GLC and mass chromatography using GLC-MS as partially alkylated (*i.e.*, methylated and/or trideuteriomethylated) alditol acetates under exactly the same conditions as the above methylation except for the use of trideuteriomethyl iodide.

Controlled Smith degradation of PS1. PS1 (102 mg) was dissolved in water (20 ml), sodium metaperiodate was added, and then the solution was kept in dark at 4°C for 48 h. After addition of ethylene glycol, the reaction mixture was dialyzed, potassium borohydride was added, the solution kept stand for 24 h, dialyzed, and then lyophilized. The resulting "polyalcohol" was dissolved in 0.1 M sulfuric acid, kept at 15°C for 24 h, neutralized with potassium hydroxide, dialyzed, and then lyophilyzed to afford the controlled Smith degraded PS1 (64 mg).

Treatment of PS1 with BTSA. PS1 (100 mg) was converted into pyridinium salt and treated with BTSA (2 ml) in pyridine (10 ml) at 60°C for 2 h as described in Part 2. The BTSA-treated PS1 (85 mg) was methylated and analyzed as already described.

^{13}C-NMR spectra. ^{13}C-NMR spectra of 10 % D_2O solution at 50.3 MHz were recorded on a spectrometer (XL-200, Varian Instruments) operated at 80°C. Chemical shift values were measured relative to internal methanol, and converted into values relative to sodium 3-trimethylsilyl-1-propanesulfonate (conversion factor, 51.60).

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Part 3-II

Fractionation and structural analyses of funoran, a sulfated polysaccharide from the red seaweed, *Gloiopeltis complanata*

Abstract

Funoran extracted from the red seaweed, *Gloiopeltis complanata*, was fractionated in terms of the differences in the solubility of cetylpyridinium salt in KCl solution to afford fractions designated CP1 and CP2. CP2 was further fractionated into CP2G that formed a gel in 0.5 M KCl, and CP2S that did not form gel in saturated KCl. CP1, CP2G and CP2S were different in their composition and property to suggest a heterogeneity of the funoran. Structures of CP2G and CP2S were further evaluated. On the basis of the results of composition analysis, partial methanolysis, methylation, ^{13}C-NMR measurements, and regioselective desulfation with a silylating reagent *N,O*-bis(trimethylsilyl)acetamide (BTSA), it was found that CP2G consisted mainly of 6-*O*-sulfated agarose with a repeating unit of [→3)6-SO_3^--β-D-Gal(1→4)3,6-anhydro-α-L-Gal(1→]. Also CP2S was found to contain a repeating unit of [→3)6-SO_3^--β-D-Gal(1→4)6-SO_3^-- α-L-Gal(1→], as well as smaller amounts of [→3)2,6-di-SO_3^--β-D-Gal(1→] and [→3)2-SO_3^--β-D-Gal(1→] residues.

Funoran is a sulfated galactan from the red seaweeds, *Gloiopeltis* spp. [1]. The funoran from *G. furcata* consists of several components fractionated in terms of solubility of their quaternary ammonium salts in aqueous potassium chloride [2]. The main polysaccharide fraction is an agarose type polysaccharide, which consists of a repeating unit, →3)6-SO_3^--β-D-Gal(1→4)3,6-anhydro-α-L-Gal(1→. In addition, the presence of a precursor moiety which is converted to the

agarose sulfate under alkaline conditions was proposed in an unfractionated funoran from a closely related species, *G. cervicornis*, [3,4].

In the present study, a funoran extracted from *Gloiopeltis complanata* was fractionated in a similar manner used for the polysaccharide from *G. furcata* [2] based on a method for mucopolysaccharides [5]. A sulfated polysaccharide was precipitated from the algal extract as its insoluble cetylpyridinium (CP) salt, which was then dissolved at 18°C with 4 M KCl, to obtain potassium salt of polysaccharide designated as CP1. The insoluble part of CP salt was extracted at 100°C with 4 M KCl to give potassium salt of a sulfated polysaccharide, CP2. Fujiki [6] reported that a funoran from a closely related *G. tenax*, was fractionated into a gel-forming and non-gel-forming fractions by addition of KCl to the solution. Therefore, CP2 was further fractionated using 0.5 M KCl to afford a gel-forming subfraction designated CP2G. The supernatant was obtained as a subfraction CP2S, which did not form gel even in saturated potassium chloride solution.

As summarized in Table I, sum of D-Gal and its methyl ether in CP2G and CP2S was almost equal to that of L-Gal, 3,6-anhydro-L-Gal (L-AGal), and the respective methyl ethers. In contrast, CP1 consists of a high content of D-Gal but contains only low amounts of L-AGal and L-Gal. CP1 was also distinguished from CP2G and CP2S in the positive optical rotations. CP2G and CP2S were different in their AGal contents. The funoran thus appeared to be a mixture containing at least three fractions corresponding to CP1, CP2G, and CP2S. CP2G and CP2S were further investigated.

By the partial methanolysis, CP2G and CP2S afforded dimethyl acetals of disaccharides, 4-*O*-β-D-galactopyranosyl-3,6-anhydro- L-galactose (agarobiose) and a small amount of 4-*O*-(6'-*O*-methyl-β-D-galactopyranosyl)-3,6-anhydro-L-galactose (6'-*O*-methylagarobiose). These suggest backbone structures consisting of agarose and its 6-O-methyl ether, respectively (Fig. 1A). Since

Table I Yields, compositions, and specific optical rotation values of CP1, CP2G, CP2S from the red alga, *Gloiopeltis complanata*, and their derivatives.

	yield(%)[b]	composition[a]						$[\alpha]_D$
		D-Gal	6M-D-Gal[c]	L-Gal	2M-L-Gal[c]	L-AGal[d]	$-SO_3^-$	
CP1	0.9	87.7	12.3	4.3	1.7	3.6	158.8	+64°
CP2G	4.8	97.4	2.6	8.9	1.3	76.0	109.0	-20°
CP2GA	-	98.0	2.0	0.1	0	85.9	89.0	-27°
CP2S	1.4	95.8	4.2	54.5	2.0	33.4	139.7	-19°
CP2SA	-	96.5	3.5	25.5	0	52.4	77.5	-14°

[a]Molar ratio. Sum of D-series galactoses is taken as 100.

[b]Based on the dry seaweed.

[c]6M-D-Gal = 6-*O*-methyl-D-galactose; 2M-L-Gal = 2-*O*-methyl-L-galactose.

[d]3,6-anhydro-L-galactose

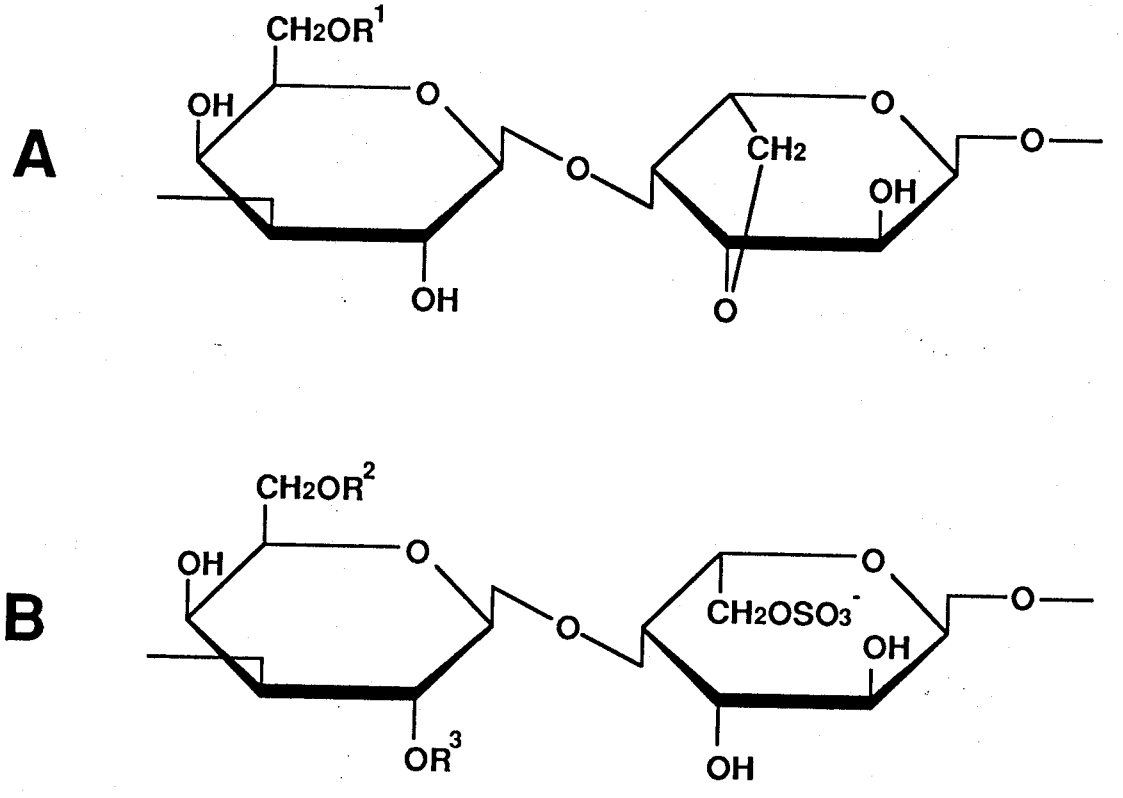

Fig. 1 Idealized structural moieties in the funorans from *Gloiopeltis complanata*.

A Agarose sulfate moiety: $\mathbf{R^1}$=SO_3^-, CH_3

B "Precursor" moiety: $\mathbf{R^2}$=SO_3^-, CH_3, H; $\mathbf{R^3}$=H, SO_3^-

2,4-di-*O*-methyl-D-Gal and 2-*O*-methyl-3,6-anhydro-Gal were the principal products in the methylation analysis of CP2G (Table II), the most part of the (1→3)-linked Gal residues are expected to carry 6-sulfate, and the (1→4)-linked AGal residues have no sulfate. This was supported from the ^{13}C-NMR spectra (Fig. 2), in which the 12 signals had chemical shift values (Table III) almost identical to those from agarose 6-sulfate moiety of the polysaccharide from a red alga, *Gracilaria dominguensis* [7]. The treatment of CP2G with 6-*O*-specific desulfating reagent, *N,O*-bis(trimethylsilyl)acetamide (BTSA), yielded a non-sulfated polysaccharide whose ^{13}C-NMR was indistinguishable from that of agarose. This result also confirms the agarose 6-sulfate structure in CP2G. In the ^{13}C-NMR spectrum of CP2S, the identical set of the above mentioned 12 signals attributable to the structure of 6-*O*-sulfated agarose was also observed (Fig. 3A). In addition, the peaks indicated with arrows suggested another structural moiety in CP2S. This was further evaluated.

In algal polysaccharides such as porphyran or carrageenans, it was reported that (1→4)-linked L- or D-Gal 6-sulfate residue was converted into 3,6-anhydride under alkaline conditions [8]. The 6-*O*-sulfated residue was called as "precursor residue", because the 3,6-anhydride formation also enzymatically took place in the biosynthetic process [9,10]. In addition to the agarose 6-sulfate structure in CP2G and CP2S, another moiety containing L-Gal residue with sulfate at O-6 was suggested, because decrease of L-Gal content and increase of AGal content were found after the alkaline treatment (CP2GA and CP2SA in Table I). The precursor may be enriched in CP2S, because increase in AGal content and decrease in L-Gal content by the alkaline treatment were more significant in the case of CP2S than CP2G. The main components in the methylation analysis of CP2S (Table II) were 2,3-di-*O*-methyl-L- and 2,4-di-*O*-methyl-D-Gal derivatives attributable to 6-*O*-sulfated (1→4)-linked L- and 6-*O*-sulfated (1→3)-linked D-Gal residues, respectively. In addition, 4-*O*-methyl- and 4,6-di-*O*-methyl-D-Gal derivatives may arise from

Table II Methylation analyses[a] of polysaccharides from *Gloiopeltis complanata.*

product[e]	CP2G	CP2GA[b]	CP2S	CP2SA[b]	CP2SB[c]	CP2SR[d]
2,3,4,6-Me_4-D-and L-Gal	0	0	0	0	0	10
2,4,6-Me_3-D-Gal	7	8	21	10	63	91
2,3,6-Me_3-L-Gal	1	1	15	10	100[f]	100[f]
4,6-Me_2-D-Gal	0	0	12	9	37	1
2,6-Me_2-D- and L-Gal	0	0	0	0	0	3
2,4-Me_2-D-Gal	100[f]	100[f]	100[f]	100[f]	41	21
2,3-Me_2-L-Gal	8	0	98	18	17	12
4-Me-D-Gal	0	0	33	27	2	0
2-Me-L-AGal	95	106	44	95	40	0

[a]Based on peak area of GLC of partially methylated alditol acetates from hydrolyzed methylated polysaccharides.

[b]Alkaline treated CP2G and CP2S.

[c]BTSA-treated CP2S.

[d]Partial methanolysis residue of CP2S.

[e]2,3,4,6-Me_4-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol; 2,4,6-Me_3-Gal = 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol; 2-Me-L-AGal = 1,4,5-tri-*O*-acetyl-2-*O*-methyl-3,6-anhydro-L-galactitol, etc.

[f]The value are taken as 100

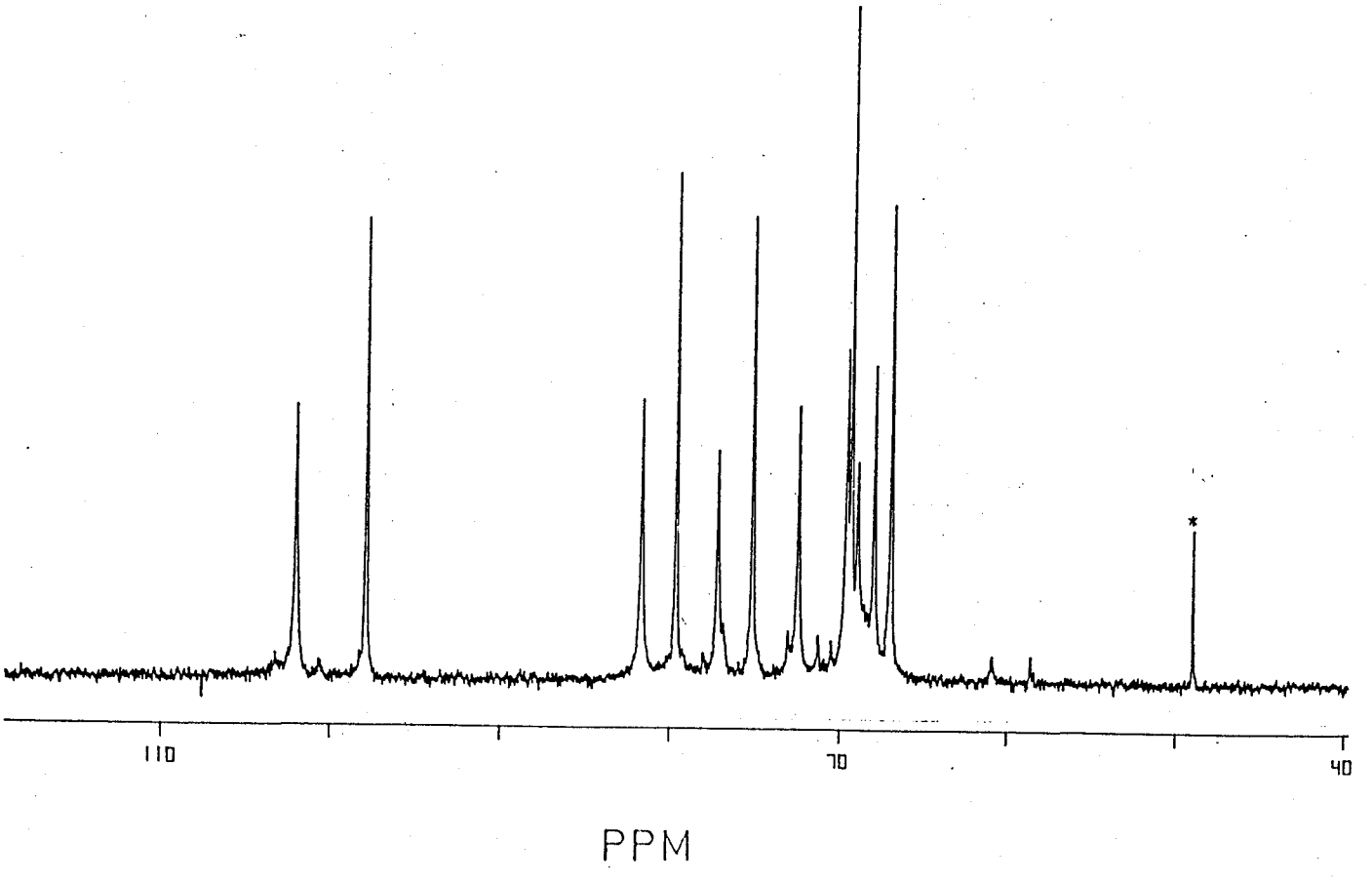

Fig.2 ^{13}C-NMR spectrum of CP2G. The asterisked signal arises from internal methanol.

Table III ^{13}C-NMR chemical shift values and peak assignments of PS2G, PS2S, and structurally related other polysaccharides

	C1	C2	C3	C4	C5	C6
→3)β-D-Gal-6-SO_3^-(1→ (agarose sulfate moiety in CP2G and CP2S)	102.35	69.91	81.99	68.21	72.80	67.22
→3)β-D-Gal-6-SO_3^-(1→ (*Gracilaria dominguensis* agar)[a]	102.5	70.1	82.1	68.4	72.9	67.4
→3)β-D-Gal(1→ (agarose)[b]	102.26	70.07	82.04	68.59	75.29	61.24
→3)β-D-Gal(1→ (BTSA-treated CP2G)	102.28	70.08	82.05	68.59	75.30	61.24
→3)β-D-Gal-6-SO_3^-(1→ (precursor moiety in CP2S)	103.59	69.92	80.55	68.71	73.23	67.21
→3)β-D-Gal(1→ (porphyran)[c]	103.48	69.96	80.90	69.02	75.65	61.46
→4)3,6-anhydro-α-L-Gal(1→ (agarose sulfate moiety in CP2G and CP2S)	98.16	69.64	79.96	77.58	75.51	69.23
→4)3,6-anhydro-α-L-Gal(1→ (agarose)[b]	98.14	69.69	79.95	77.21	75.46	69.26
→4)3,6-anhydro-α-L-Gal(1→ (BTSA-treated CP2G)	98.16	69.69	79.97	77.22	75.48	69.26
→4)α-L-Gal-6-SO_3^-(1→ (precursor moiety in CP2S)	100.94	68.96	70.85	79.55	69.64	67.36
→4)α-L-Gal-6-SO_3^-(1→ (porphyran)[c]	101.09	69.60	70.87	78.91	69.96	67.55

[a]Chemical shift values and assignment based on Fernandez *et al.* (1989).
[b]Assignment based on Nicolaisen *et al.* (1980).
[c]Assignment based on Lahaye and Yaphe (1985).

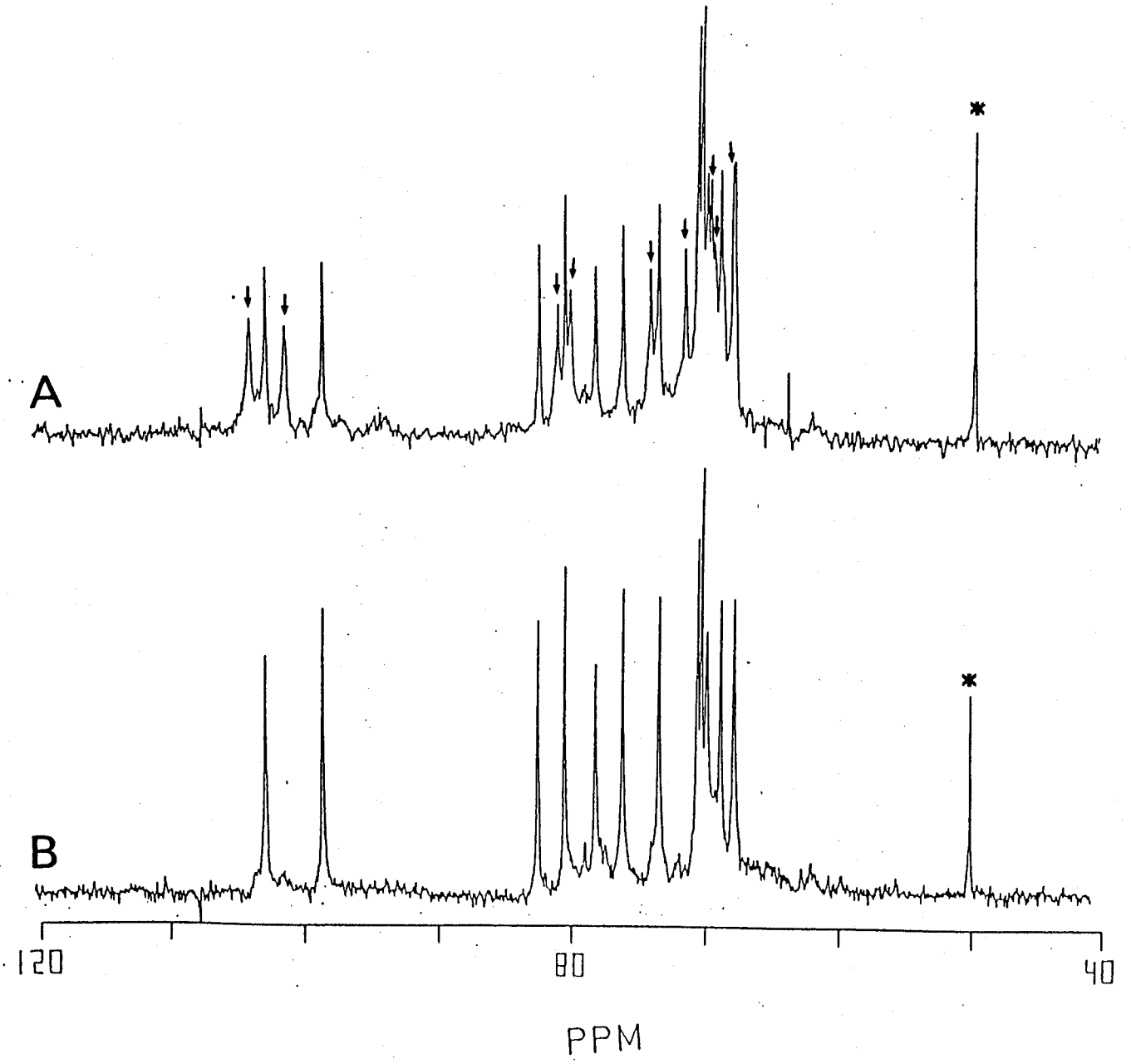

Fig.3 ^{13}C-NMR spectra of CP2S. The asterisked signals arise from internal methanol.

A Native CP2S. The arrows indicate the signals attributable to "precursor" moiety.

B Alkali-treated CP2S (CP2SA)

(1→3)-linked 2,6-disulfated and 2-sulfated (or 2-sulfated 6-*O*-methyl-) D-Gal residues, respectively. This was supported by the fact that the remarkable increase in 2-*O*-methyl-AGal accompanied the decrease in 2,3-di-*O*-methylgalactose derivative in the methylation analysis by the alkaline treatment of CP2S (CP2SA in Table II). This was further confirmed by a treatment of CP2S with BTSA, which regioselectively removes the 6-sulfate group. In the methylation analysis of the BTSA-treated CP2S (CP2SB in Table II), decreases in 2,3-di-*O*-, 2,4-di-*O*-, and 4-*O*-methylgalactose derivatives and increases in 2,3,6-tri-*O*-, 2,4,6-tri-*O*-, and 4,6-di-*O*-methylgalactose derivatives were observed. The structure of the "precursor" moiety, thus, is illustrated in Fig. 1B. The peaks indicated with arrows in the NMR spectrum of CP2S (Fig. 3A) may arise from the major "precursor" moiety, [→3)β-D-Gal-6-SO_3^-(1→4)α-L-Gal-6-SO_3^-(1→], by referring the reported spectral data for porphyran (Table III) consisting of a repeating unit, [→3)β-D-Gal(1→4)α-L-Gal-6-SO_3^-(1→] [11,12]. The spectrum obtained after the alkaline treatment (Fig. 3B) was almost identical to that of CP2G, a virtually idealized agarose 6-sulfate. However, signals attributable to the minor repeating structure carrying 2-sulfate at the (1→3)-linked residues were not clearly observed because of the low intensity in the spectra.

Although CP2S yielded agarobiose derivatives by partial methanolysis, this polysaccharide was not completely degraded but gave a remarkable amount of methanol-insoluble material termed CP2SR. This material may arise from a moiety that consists of consecutive repeating unit containing the precursor residues, because almost the same amount of (1→3)-linked D- and (1→4)-linked L-Gal residues were obtained in the methylation analysis of CP2SR (Table II).

The treatment of CP2S with BTSA removed 6-*O*-sulfate groups. However, the removal was incomplete, while CP2G, porphyran, chondroitin sulfate, and dermatan sulfate were almost completely 6-*O*-desulfated as described in the previous chapter. The incomplete removal of 6-*O*-sulfate group was also found in BTSA-treatment of heparin (see Part 4), suggesting the influence

of conformation of sulfated polysaccharide to the degree of desulfation by BTSA.

Experimental

General methods. Colorimetric determination of galactose (Gal) and 3,6-anhydrogalactose (AGal) contents in polysaccharides was carried out by the method of Yaphe [14] and the result was expressed as wt. % of their anhydrohexose units. Sulfate contents expressed as wt. % of SO_3K were estimated by titration; a mixture of sulfated polysaccharides and an aliquot of glycolchitosan was titrated with potassium poly(vinylsulfate) using toluidine blue as an indicator. Optical rotation was measured by a Laurent's saccharimeter with a 1 dm cell. GLC was carried out with a chromatograph (GC-7A, Shimadzu Corp.) equipped with an FID using following WCOT columns at the respective operating temperatures unless otherwise mentioned; column *a*, PEG-20M bonded (GL-Science Co.) at 200°C; column *b*, CP-Sil 88 (Chrompak Co.) at 205°C; column *c*, OV-1 bonded (GL-Science Co.) at 170°C. Nitrogen was used as the carrier gas at a flow rate of 20 ml/min and a split ratio of 20 : 1. GLC-MS was carried out by a mass spectrometer (GC/MS QP-1000, Shimadzu Corp.) at 70 eV ionization potential. The chromatographic conditions were identical to those mentioned above except for the use of helium as a carrier gas. ^{13}C-NMR spectra in D_2O were recorded by spectrometers (XL-200. Varian Instruments and GE-300, General Electric Co.) at 80°C. In calculation for Fourier transformation, apodization using 4 Hz line broadening followed by zero filling was executed. Chemical shifts were measured relative to internal methanol, the chemical shift value of which was taken as 49.30 ppm.

Extraction and fractionation of polysaccharides. The red alga, *Gloiopeltis complanata*, was harvested at Shirahama coast in Shizuoka Prefecture. Dried seaweed (50 g) was macerated with water, homogenized with a blender and extracted twice with water (2500 ml) at 100°C. To the

combined extract, 5% cetylpyridinium (CP) chloride was added until no more CP salt of sulfated polysaccharide was precipitated. The CP salt of polysaccharide was washed successively with water, methanol and acetone, and then extracted twice with 4 M KCl at 18°C. The combined extract was concentrated and poured into 4 vol. of ethanol to afford a precipitate, which was dissolved in water, dialyzed, concentrated, and then poured into 4 vol. of ethanol to obtain potassium salt of a polysaccharide termed CP1 (425 mg, $[\alpha]_D^{27}$ +64.0° (*c* 0.4, H_2O), Gal 33.6%, AGal 1.1%, sulfate 36.1%). The residue insoluble in 4 M KCl at 18°C was extracted with 4 M KCl at 100°C, and was immediately poured into 4 vol. of ethanol. The resulting precipitate was dissolved in water, dialyzed, and precipitated by 4 vol. of ethanol to afford a polysaccharide termed CP2 (3.78 g, $[\alpha]_D^{18}$ -26.3° (*c* 1.2, H_2O), Gal 40.2%, AGal 19.9%, sulfate 31.1%).

Sub-fractionation of CP2. To an aqueous solution of CP2 (3 g in 150 ml) was added 1 M KCl (150 ml) and the solution was kept overnight at 4°C. The resulting gel was collected by centrifugation at 4°C. The gel was dialyzed, concentrated and poured into 4 vol. of ethanol to give CP2G (1.92 g, $[\alpha]_D^{20}$ -20.0° (*c* 1.5, H_2O), Gal 36.8%, AGal 22.8 %, sulfate 26.7%). KCl concentration of the supernatant was adjusted to 1.6 M, to afford weak gel, which was not further investigated because of its small quantity (0.17 g). The supernatant, which did not afford gel even in saturated KCl at 4°C, was dialyzed, concentrated and precipitated with ethanol to give CP2S (0.57 g, $[\alpha]_D^{18}$ -18.5° (*c* 1.5, H_2O), Gal 46.1%, AGal 8.8%, sulfate 30.2%).

Composition analyses of Polysaccharides. Polysaccharide (10 mg) was methanolyzed with 0.5 ml of 3% methanolic hydrogen chloride at 70°C for 18 h. The methanolyzate was trimethylsilylated [15] and analyzed by GLC and GLC-MS (column *c*). Absolute configuration of component sugars except for AGal was determined by GLC-MS (column *c*, 210°C) of trimethylsilylated derivatives of L-2-octyl glycosides [16]. Ratio of D- to L-enantiomer of Gal was calculated from the peak area corresponding to each enantiomer. The configuration of AGal

was assigned to L-form, because agarobiose derivatives were detected whereas no carrabiose derivative was detected after the partial methanolysis as mentioned later. For quantitative analysis, component sugars were analyzed as their alditol acetates [17] by the double hydrolysis method [13]. Briefly, the polysaccharides were hydrolyzed with 0.02 M sulfuric acid at 100°C for 1 h, and treated with $NaBH_4$ at room temperature for 2 h. The resulting hydrolyzate was further hydrolyzed with 1 M H_2SO_4, neutralized, reduced with borohydride. The hydrolyzate obtained finally was acetylated with acetic anhydride and pyridine, and analyzed by GLC and GC/MS (column *a*, 200°C, and column *b*, 205°C). Respective total areas of peaks arising from Gal series and that arising from AGal series were converted to the analytical values based on colorimetric determination.

Partial methanolysis of CP2G. CP2G (400 mg) in 0.1 M methanolic hydrogen chloride was heated at 70°C for 2 h, and then the solution was neutralized with Ag_2CO_3. A portion of the dried methanolyzate was trimethylsilylated and analyzed by GLC and GLC-MS (column *c* at 250°C).

Alkaline treatment of Polysaccharides. To an aqueous solution of CP2G (100 mg/70 ml) was added 20 mg of KBH_4 and the solution was kept at 15°C for 18 h. Then KOH (2.8 g) and KBH_4 (50 mg) were added and heated to 80°C for 2 h. The solution was neutralized with acetic acid, dialyzed, concentrated, and precipitated with 4 vol. of ethanol to give alkali-treated CP2G, (designated CP2GA, 80 mg, $[\alpha]_D$ -26.8°(*c* 0.9, H_2O), Gal 35.3%, AGal 26.6%, sulfate 23.2%). CP2S was treated similarly to give alkali-treated CP2S (CP2SA, $[\alpha]_D$ -14.4° (*c* 1.0, H_2O), Gal 43.8%, AGal 20.1%, sulfate 19.9%).

Methylation analyses of Polysaccharides. A polysaccharide sample was methylated by powdered NaOH and iodomethane based on the methods of Isogai et al. [18] and Ciucanu and Kerek [19] except for the use of triethylammonium salt of polysaccharide according to Stevenson

and Furneaux [13]. The methylated polysaccharide was hydrolyzed with 0.1 M H_2SO_4, treated with $NaBH_4$ and further hydrolyzed with 1 M H_2SO_4. The hydrolysis products were analyzed by GLC and GLC-MS (column *a*, 200°C, and column *b*, 205°C) as partially methylated alditol acetates [20]. Absolute configuration of the partially methylated sugar obtained after total hydrolysis of methylated polysaccharide was assigned as described in ref. [16].

Partial methanolysis of CP2S. CP2S (80 mg) was partially methanolyzed as above. The remaining sediment was methanolyzed again, and the final remaining sediment was recovered as CP2SR (33 mg). The combined methanolyzate was analyzed in a similar way to that for CP2G by GLC. CP2SR was methylated and analysed as above.

BTSA-treatment of Polysaccharide. CP2S (50 mg) was converted into pyridinium salt, and treated with BTSA (4 ml) in dry pyridine (20 ml) as described in Part 2-III to afford 6-*O*-desulfated polysaccharide (CP2SB, 37 mg).

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Part 4

Applications of the regioselective desulfation to structural modification of heparin: Relations between structures and protein-binding properties

Part 4-I

Selective 6-*O*-desulfation of heparin by *N,O*-bis(trimethylsilyl)-acetamide and its effect on anti-coagulant activity.

Abstract

Treatment of heparin with *N,O*-bis(trimethylsilyl)acetamide (BTSA) caused regiospecific partial 6-*O*-desulfation, where no elimination of sulfate groups at other positions occurred. From disaccharide analysis after enzymatic degradation and repeated BTSA-treatment, it was suggested that the removal of 6-*O*-sulfate groups was regulated by the conformation of heparin chain. From the results of antithrombin III (AT-III) affinity chromatography, and factor Xa- and thrombin-inhibitory activity of AT-III enhanced by heparin, it was suggested that the BTSA-treatment of heparin caused partial removal of 6-*O*-sulfate groups required for the enhancement of AT-III activity.

Heparin is one of glycosaminoglycans of mammalian origin, and consists of a repeating disaccharide backbone modified with sulfate and/or acetamide groups [1]. Heparin is composed of both regular repeat of [→4)IdoA-2-SO_3^-(1→4)GlcN-6,*N*-di-SO_3^-(1→] and irregular parts of the molecule due to incomplete actions of sulfotransferases and C-5 epimerase in the biosynthetic pathway. The heterogeneity in the distribution of the sulfate groups along the heparin chain gives rise to, in part, specific sequences to invest the molecule with affinities for proteins participating to a variety of biological process including blood coagulation. In the blood coagulation system, heparin interacts with a serine proteinase inhibitor, antithrombin III (AT-III), to enhance its inhibitory activity towards such enzymes as factor Xa and thrombin *etc*. Minimal

sequence for binding to AT-III in heparin was established as a pentasaccharide unit, where the sulfate groups and carboxyl groups of uronic acid residues forms a cluster of anionic charges making the chain accessible to AT-III [2]. It was also reported that the inhibition by AT-III towards proteinases such as thrombin, factor IXa, and factor XIa involved ternary complex formation, for which a chain sequenced with more than 18 sugar residue of heparin is required for fixing the proteinase to make them accessible to AT-III [3]. In contrast, only the specific pentasaccharide unit was enough for the inhibition of the proteinases such as factor Xa, factor VIIa, and kallikrein. In the cases of both type of proteinases, heparin was released from the complex after the activation of AT-III, thus acting like a catalyst.

As described in Part 2, sulfates linked to O-6 of carbohydrates are regiospecifically removed by the treatment of their pyridinium salt in pyridine with a silylating reagent, *N,O*-bis(trimethylsilyl)acetamide (BTSA). The results thus imply potential usefulness of the reaction as a method in investigating molecular basis for functions of sulfate groups of glycosaminoglycans. In this section, the author applied the desulfation method to heparin in order to know the applicability of this method and to evaluate the influence of the 6-*O*-sulfate group of heparin on its anti-coagulant activity.

Sulfate content in heparin decreased by the treatment with BTSA (Table I). The position of the removed sulfate groups were evaluated by ^{13}C-NMR using DEPT [4] pulse sequence, with which a signal from a secondary carbon atom such as C-6 appears as a negative peak. In the spectrum of the native heparin (Fig 1A), there were negative peaks attributable to C-6. After BTSA-treatment (Fig 1B), one of these peaks arising from sulfated C-6 (69.1 ppm) decreased in its intensity accompanying increase in the intensity of the other peak (62.8 ppm) arising from C-6 carrying no sulfate, although sulfated C-6 still remained even after the treatment. This indicates that the BTSA-treatment caused partial removal of 6-*O*-sulfate group in heparin. In

Table I Composition of native and BTSA-treated heparins.

	Uronic acid[a]	Sulfate[b]
Native	37.1	33.8
BTSA-treated	36.7	27.6
BTSA-treated[c]	38.2	28.2

[a]Calculated as wt% of $C_6H_8O_6$.

[b]Calculated as wt% of SO_3Na.

[c]Treatment was repeated three times.

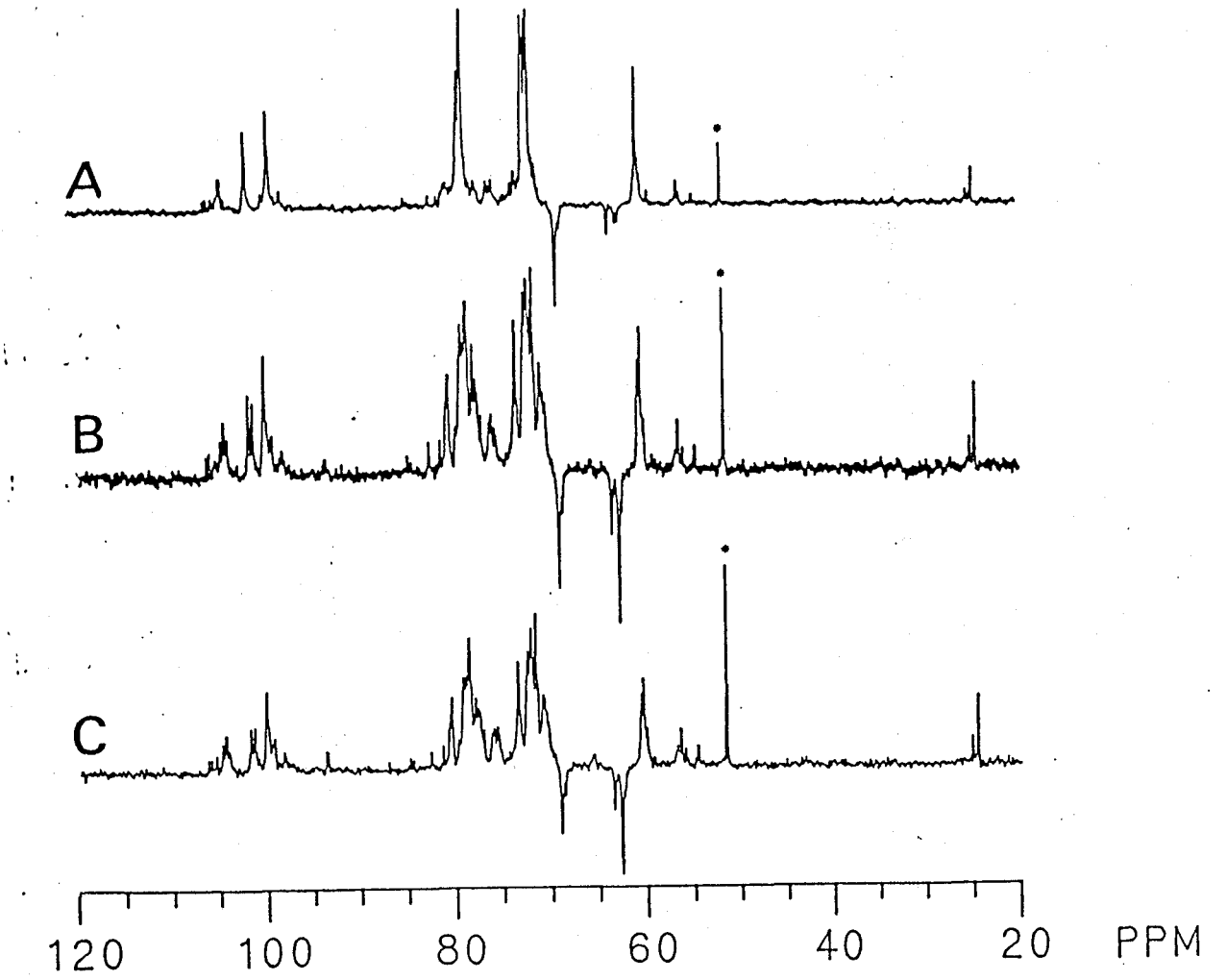

Fig. 1 ^{13}C-NMR spectra of native and BTSA-treated heparins using DEPT pulse sequence.

A, Native heparin; B, heparin after single BTSA-treatment; C, three times BTSA-treated heparin.

Asterisked peaks are due to methanol as internal standard.

addition, there was no loss of the sulfate groups as the other main heparin constituents, *i.e.*, *N*-sulfates of GlcN residues and 2-sulfate of IdoA residues, as judged from the ^{13}C-NMR spectrum. There was negligible change in intensity and chemical shift values of the signals from C-1, C-2, and C-3 of GlcNS and IdoA2S residues compared with the native heparin. The presence of sulfate groups on the positions other than O-6 was also confirmed from the results of the analyses of disaccharide fraction obtained after heparinase/heparitinase digestion (Table II) of the native and the BTSA-treated heparins. By the BTSA-treatment, trisulfated disaccharide, ΔUA2S-GlcNS6S decreased and disulfated disaccharide, ΔUA2S-GlcNS increased.

The results obtained here are consistent with the previous investigation and again demonstrate that the BTSA-treatment is capable of regioselective 6-*O*-desulfation. The treatment did not affect *N*-sulfate groups that were labile under conditions of other desulfation methods [6,7]. In the case of heparin, however, some of 6-*O*-sulfate groups still remained even after the treatment with BTSA as indicated by the NMR measurement and the disaccharide analysis. By contrast, all 6-*O*-sulfate groups were completely removed in the case of less sulfated polysaccharides such as funoran (agarose 6-sulfate), porphyran, or chondroitin sulfate as described in Part 2-III. Such incomplete removal of 6-*O*-sulfate groups in heparin may reflect the occurrence of site-specific desulfation. Repeated (3 times) BTSA-treatment practically did not result any further desulfation (Table I and Fig. 1C) as compared with those after the single treatment.

In Table III, the effect of BTSA-treatment on the AT-III-binding affinity of heparin was evaluated by affinity chromatography. Under the identical chromatographic conditions, BTSA-treated heparin showed decrease in the high affinity fraction and increases in low affinity fractions with respect to the native heparin. This shift to the lower affinity suggests the loss of the 6-*O*-sulfate groups required for AT-III binding sequence in the heparin. Next, the effect of BTSA-treatment of heparin on the inhibitory activity of AT-III towards thrombin or factor Xa

Table II Disaccharide composition of native and BTSA-treated heparins after heparin lyase/heparan sulfate lyases digestion.

product	relative ratio	
	native heparin	BTSA-treated heparin
ΔUA-GlcNAc	8.2	12.6
ΔUA-GlcNS	3.2	4.9
ΔUA-GlcNAc6S	6.0	2.8
ΔUA2S-GlcNAc	1.2	1.1
ΔUA-GlcNS6S	13.2	12.6
ΔUA2S-GlcNAc6S	1.6	3.1
ΔUA2S-GlcNS	7.2	31.7
ΔUA2S-GlcNS6S	59.4	31.2

Abbreviations; ΔUA, 4,5-unsaturated hexuronate; ΔUA2S, 4,5-unsaturated hexuronate 2-sulfate; GlcNAc, *N*-acetyl-glucosamine; GlcNS, *N*-sulfoamino-glucosamine; GlcNAc6S, *N*-acetyl-glucosamine 6-sulfate; GlcNS6S, *N*-sulfoamino-glucosamine 6-sulfate.

Table III Recovery (%) of fractions of heparin and BTSA-treated heparin from antithrombin-agarose affinity column eluted with NaCl

	NaCl concentration			
	0.01	0.05	0.1	2.0
Native heparin	4.7	70.5	13.5	11.3
BTSA-treated heparin	15.2	65.5	11.3	8.0

was evaluated (Fig. 2, and 3). In agreement with the results of the affinity chromatography, the ability of heparin to enhance the inhibitory activity of AT-III was reduced by the BTSA-treatment. In the case of thrombin, IC_{50} value was ca. 7.3 times increased by the treatment (3.3 ng/ml for native heparin and 24.0 ng/ml for BTSA-treated heparin), and in the case of factor Xa that was increased ca. 6.4 times (36.0 ng/ml for native and 230.0 ng/ml for BTSA-treated). Since it was reported that there were two 6-*O*-sulfate groups in the AT-III-binding pentasaccharide unit in heparin [2], the results may indicate removal of the 6-*O*-sulfate group(s) in the pentasaccharide sequence.

The BTSA-treatment of heparin may preferentially remove the 6-*O*-sulfate groups located in a particular circumstance. BTSA-treatment caused decrease in both affinity to AT-III and activation of AT-III, but did not abolish completely these properties. Therefore, the treatment may not distinguish the AT-III binding sequence from the other part. The site-specific 6-*O*-desulfation might be due to the conformation of heparin chain, a property of BTSA, or both.

Heparin is extensively used for a clinic treatment owing to its anti-coagulant activity, it often causes several harmful side effects, *i.e.*, heparin interacts to platelets to induce platelet aggregation and intravascular thrombosis. Recently, the regular repeating trisulfated disaccharide unit including 6-*O*-sulfate group in the heparin has been suggested to be a crucial element for the interaction with platelets [8,9]. BTSA-treatment might be a potential method for preparing a safer heparin with reduced side effect, since BTSA-treated heparin still possessed anti-coagulant activity.

Experimental

Materials and general methods. Porcine mucosal heparin investigated was a product of Shanghai Biochemical and Pharmaceutical Laboratory, China. Heparinase, heparitinase I, and

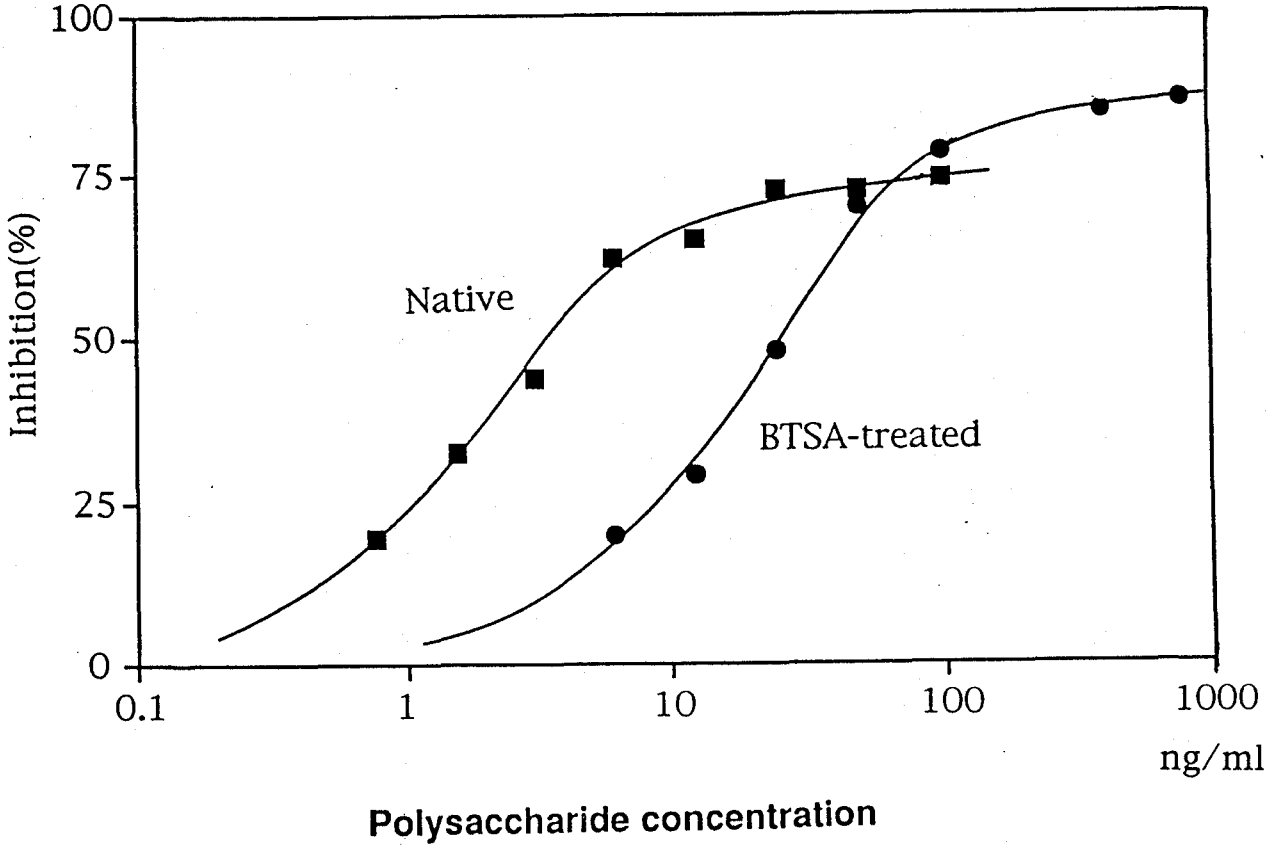

Fig. 2 Thrombin inhibitory activity in the presences of AT-III and varying amounts of native or BTSA-treated heparin.

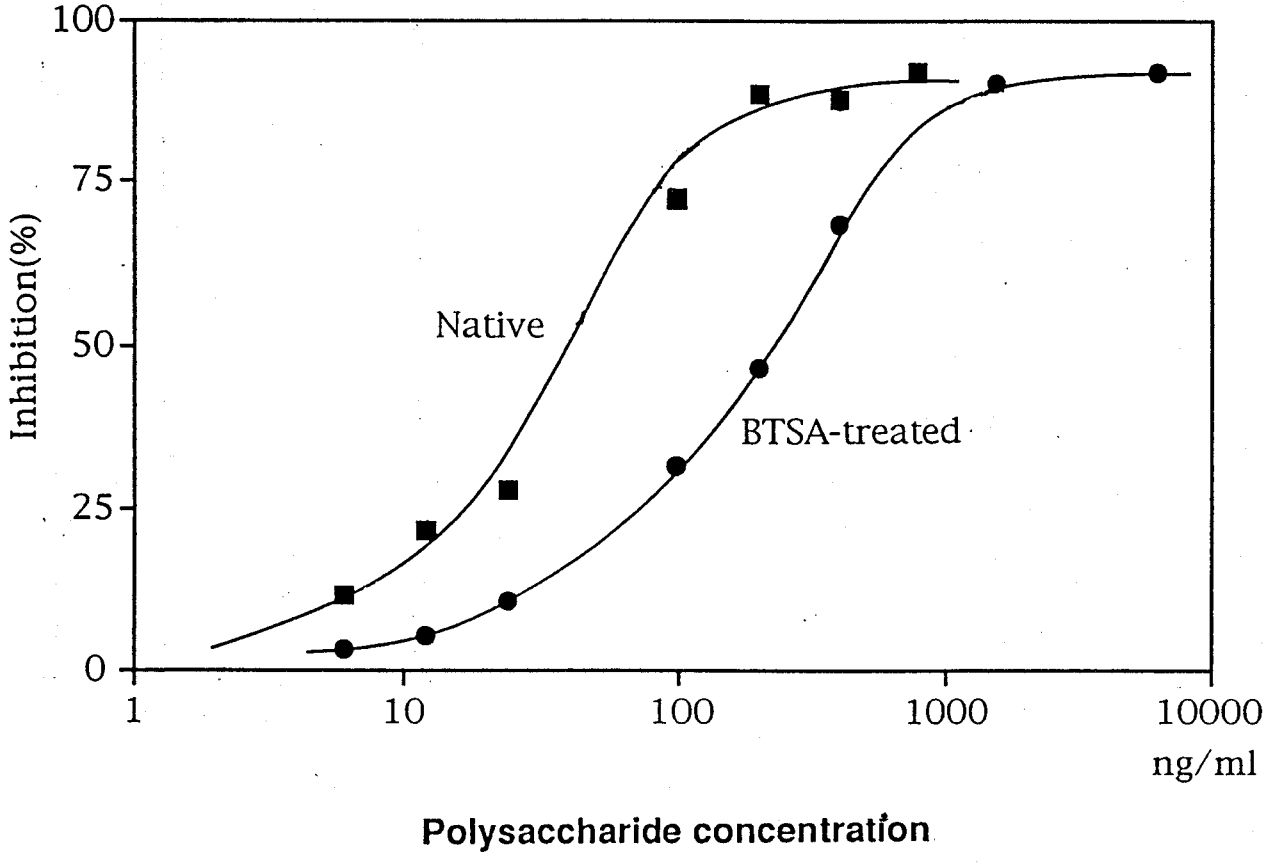

Fig. 3 Factor Xa inhibitory activity in the presences of AT-III and varying amounts of native or BTSA-treated heparin.

heparitinase II were from Seikagaku Corp. AT-III bound agarose matrix, thrombin, and factor Xa were purchased from Sigma Chem. Co. Substrates S-2238 (H-D-Phe-Pip-Arg-*p*NA) and S-2222 (Bz-Ile-Glu-Gly-Arg-*p*NA) were obtained Kabi Diagnostica, Sweden. Human AT-III was a product of Green Cross Corp. BTSA and other reagents were products of Wako Pure Chemicals Ind. Uronic acid content was determined by carbazole-sulfuric acid method [10], and sulfate content by the sodium rhodizonate method [11] after hydrolysis of the glycosaminoglycan.

Treatment with BTSA. Pyridinium salt of heparin, prepared from sodium salt (200 mg) by the method of Nagasawa and Inoue [6], was heated in pyridine (20 ml) with BTSA (4 ml) at 60°C for 2 h and then cooled to room temperature. After addition of 20% methanol (20 ml), the reaction mixture was dialyzed, 1 M NaOH was added to adjust pH 9-10, and the solution was dialyzed again. Then the dialyzate was lyophilized to recover sodium salt of BTSA-treated heparin (150 mg).

^{13}C-NMR measurement. The spectra of 10 % D_2O solution of heparin samples at 75 MHz were recorded using a QE-300 spectrometer (General Electric) at 80°C. Chemical shift values were expressed relative to TMS using internal methanol (51.60 ppm). DEPT [4] sequence was supplied by the manufacturer.

Disaccharide analysis after enzymatic degradation. A sample (0.1 mg) was incubated with mixture of heparinase (50 mU), heparitinase I (20 mU), and heparitinase II (20 mU) at 37°C for 2 h in 0.22 ml of 20 mM sodium acetate buffer containing 2 mM calcium acetate (pH 7.0). The reaction mixture was gel-filtrated with serial columns of TSK-Gel PW4000, PW3000, and PW2500 (Tosoh Co.) eluted with 0.2 M NaCl and monitoring absorbance at 230 nm. The digest was analyzed by ion-exchange chromatography with CarboPac PA-1 according to the literature [13].

AT-III-binding experiment. Affinity column of AT-III-agarose (10 x 0.9 cm) was equilibrated with 0.02 M Tris buffer (pH 7.4) for at 4°C for 72 h. A solution of a sample (ca. 3 mg) in the starting buffer (50 ml) was applied to the column, and eluted stepwise with the buffer solutions (50 ml each) containing NaCl at concentrations of 0.01, 0.05, 0.1, and 2 M, respectively. Each effluent was monitored by carbazole-H_2SO_4 method, the absorbance at 530 nm being used for calculation of the recovery of each fraction with respect to the unfractionated heparin.

Anticoagulant activity. The inactivation of thrombin or factor Xa with AT-III enhanced by heparin were assayed using chromogenic substrate, H-D-Phe-Pip-Arg-*p*NA or Bz-Ile-Glu-Gly-Arg-*p*NA, respectively, according to the literature [14]. The activity was expressed in terms of ratio of inhibition.

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Part 4-II

Effect of 6-*O*-sulfate group of glucosamine residues in heparin on the activation of FGF-1 and FGF-2

Abstract

Treatment of the pyridinium salt of heparin with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MTSTFA) in pyridine was effective for the specific 6-*O*-desulfations without detectable depolymerization or other chemical modifications. The 6-*O*-desulfated heparins were tested as enhancers or inhibitors of specific FGF-1- and FGF-2-induced proliferation of BALB/c3T3 clone A31 cells and the chlorate-treated cells. It was found that a high content of 6-*O*-sulfate groups in GlcNS residues was required for the activation of FGF-1, but not for FGF-2. However, a complete 6-*O*-desulfation of tri-sulfated disaccharide units in heparin resulted in a loss of the ability to activate FGF-2, even it bound to FGF-2.

Heparin or heparan sulfate (HS) has been implicated in the regulation of cell growth [1], although their action mechanisms are not fully understood. There may be a consensus that these polymers exert their effects by modulating the activity of various growth factors. Fibroblast growth factor (FGF)-1 (acidic FGF) and FGF-2 (basic FGF) bind to heparin or HS with high affinity. Mitogenic activity, and biochemical stability of these growth factors are modulated by heparin or HS. It was reported that heparin enhanced the mitogenic activity of FGF-1, but not FGF-2 [2]. Heparin or HS also protected FGF-1 and FGF-2 from inactivation caused by acid and heat, and from enzymatic degradation by proteases [3]. Furthermore, there was an evidence that heparin or HS served as a cofactor for the binding of FGF-1 and FGF-2 to their high affinity

receptor, enhancing their mitogenic activities [4-6].

HS possesses a heterogeneous polymeric structure in which *N*- and/or *O*-sulfated residues are clustered in a series of short domains separated by relatively long sulfate-poor oligosaccharide domains [7,8]. The highly *N*-sulfated domains may be related to the biological activities, it participating in the binding of HS to FGF-2 [7,8]. *N*-sulfated fragments derived from various animal tissues or cells having high affinity to FGF-2 were enriched with 2-*O*-sulfated iduronate [9-11]. It was also reported that high content of 2-*O*-sulfated iduronate in decasaccharides or larger oligosaccharide were required for specific binding to FGF-2 and for the activation. However, 6-*O*-sulfate groups of *N*-sulfated glucosamine residue did not affect the interaction with FGF-2 [12]. In contrast to FGF-2, a high content of 6-*O*-sulfate groups in GlcNS residues was necessary for the specific binding to FGF-1 and FGF-4 and for their activations [2].

As described in the preceding chapter, treatment of heparin with *N,O*-bis(trimethylsilyl)-acetamide (BTSA) resulted in an incomplete 6-*O*-desulfation. Since the silylating reagent, *N*-methyl-*N*-trimethysilyltrifluoroacetamide (MTSTFA) was more effective for 6-*O*-desulfation than BTSA, heparin was desulfated in various extents using MTSTFA in the present section. And the effect of the 6-*O*-sulfate groups in heparin on the activation of FGF-1 or FGF-2 was investigated.

Regioselective 6-*O*-desulfations of heparin by the treatment with MTSTFA at various temperatures were confirmed from analysis of disaccharide fractions obtained by heparinase/heparitinases digestion of the MTSTFA-treated or untreated heparins. As shown in Table I, the MTSTFA-treatment caused decrease of the tri-sulfated disaccharide (ΔUA2S-GlcNS6S) and corresponding increase of di-sulfated disaccharide (ΔUA2S-GlcNS). However, decrease of di-sulfated disaccharide (ΔUA-GlcNS6S) and increase of mono-sulfated disaccharide (ΔUA-GlcNS) were not distinct. Furthermore, these treatments did not affect both *N*-sulfate

Table I Disaccharide composition of heparin treated with MTSTFA at various temperatures

product	relative ratio					
	native	65°C	75°C	85°C	90°C	95°C
ΔUA-GlcNAc	4.5	5.9	6.4	7.4	6.5	6.8
ΔUA-GlcNS	4.0	3.1	4.1	4.1	5.6	10.2
ΔUA-GlcNAc6S	0.7	1.2	0.5	1.0	0.3	0.0
ΔUA2S-GlcNAc	1.4	3.0	1.3	8.5	4.4	2.6
ΔUA-GlcNS6S	8.5	8.5	6.3	6.5	7.1	5.7
ΔUA2S-GlcNAc6S	0.0	1.8	0.1	1.4	0.6	0.6
ΔUA2S-GlcNS	25.9	37.2	51.4	55.2	62.2	73.0
ΔUA2S-GlcNS6S	55.0	39.3	29.8	15.9	13.3	1.7
Degree of 6-O-desulfation	0	24.4	44.8	62.2	66.8	88.8

Abbreviations; ΔUA, 4,5-unsaturated hexuronate; ΔUA2S, 4,5-unsaturated hexuronate 2-sulfate; GlcNAc, *N*-acetyl-glucosamine; GlcNS, *N*-sulfoamino-glucosamine; GlcNAc6S, *N*-acetyl-glucosamine 6-sulfate; GlcNS6S, *N*-sulfoamino-glucosamine 6-sulfate.

and 2-*O*-sulfate groups as judged from the results of disaccharide analysis. The MTSTFA-treatments in the increasing temperature caused the increasing degree of 6-*O*-desulfations, and the treatment at 95°C almost completely removed 6-*O*-sulfate groups.

A cell-based assay for the evaluation of the interactions of heparin-like compounds with FGF-2 was reported [13]. Here a similar method was applied to measure the ability of selectively 6-*O*-desulfated heparin to inhibit the binding of COS-1 cells to FGF-2-coated wells. The binding was inhibited in a dose dependent manner by heparin or HS (Fig. 1), whereas CS-A (4-*O*-sulfate-rich chondroitin sulfate), CS-B (dermatan sulfate), and CS-C (6-*O*-sulfate-rich chondroitin sulfate) had almost no effect on the binding at similar concentration range. When COS-1 cells was treated by either heparinase, heparitinase, or trypsin at 37°C for 10 min, the cells lost the binding activity. These results indicated that COS-1 cells bind to FGF-2-coated plates through a specific interaction between cell surface heparan sulfate proteoglycan and immobilized FGF-2, and that the exogenous heparin or HS competitively inhibit the interactions.

As shown in Fig. 1, 6-*O*-desulfated heparins strongly inhibited the binding of COS-1 cells to FGF-2-coated wells in a dose dependent manner, which was similar to native heparin or HS, although highly 6-*O*-desulfated heparins (88.8% and 66.8% 6-*O*-desulfated heparin) exhibited slightly lower inhibitory activities. These results suggests that high content of 6-*O*-sulfate groups in heparin may not be required for the specific binding of heparin or HS to FGF-2.

It was reported that sodium chlorate, a potent inhibitor of sulfate adenyltransferase, reduced sulfations of various carbohydrates and proteins. Chlorate blocked the stimulation of FGF-1 and FGF-2 for proliferation of Swiss 3T3 fibroblasts [5] as well as adrenocortical endothelial cells [6]. The chlorate blockage of FGF-mediated growth was overcome by exogenous heparin. The effect of chlorate on FGF-induced A31 cell growth was investigated. By the addition of 20 mM chlorate into sulfate-free medium (Fig. 2), both FGF-1 and FGF-2 induced proliferations of A31

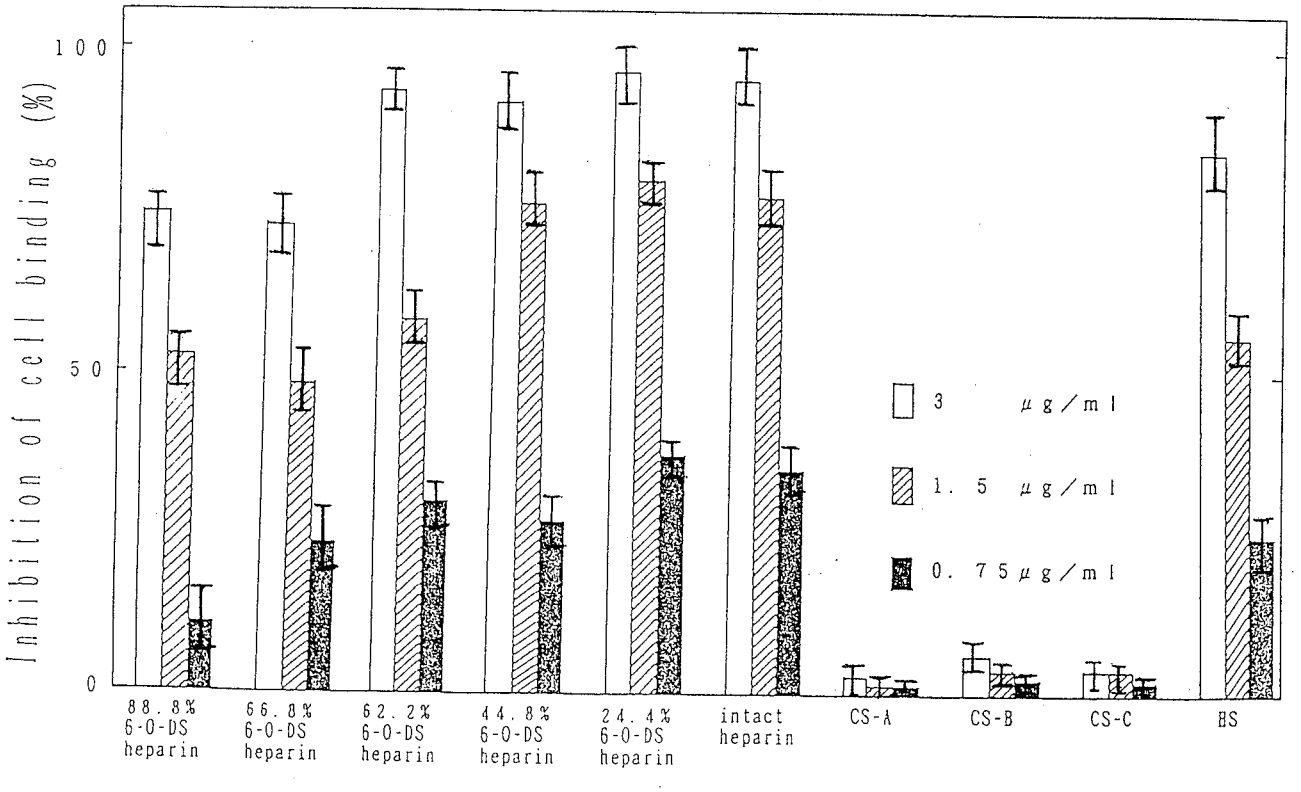

Fig. 1 Inhibitory activity of 6-*O*-desulfated heparins to binding of COS-1 cell to FGF-2-coated plates. The COS cells were collected and resuspended in PBS containing 5% fetal bovine serum including various 6-*O*-desulfated heparins at the indicated concentrations. The cells were applied to FGF-2-coated plates and incubated for 10 min, and protein of bound cells was measured as described in the text. The results represent the means of triplicate determinations. 6-O-DS = 6-*O*-desulfated.

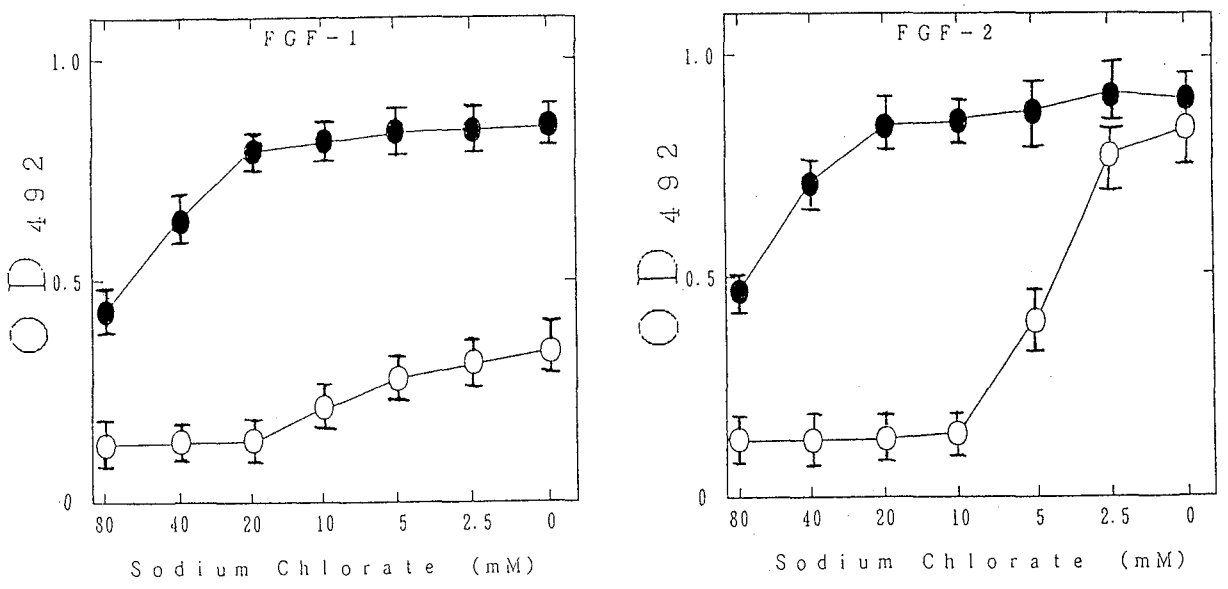

Fig. 2 Restoration of FGF-1- and FGF-2-induced proliferation of chlorate-treated A31 cells by heparin. A31 cells were incubated for 3 days in sulfate-free DMEM containing either 5 ng/ml human recombinant FGF-1 or 2 ng/ml human recombinant FGF-2, ITS+, 100 units/ml penicillin G, and sodium chlorate at the indicated concentrations, with (O) and without (O) 2 μg heparin. Cell proliferation was assessed as described in the text. The results represent the means of triplicate determinations.

cells were significantly inhibited. This chlorate inhibition of the FGF-induced A31 cell growth was overcome by the addition of 2 μg/ml exogenous heparin (Fig. 2). In addition, 2μg/ml heparin potentiated mitogenic activity of FGF-1 even in the absence of chlorate.

Then the effect of various 6-*O*-desulfated heparins was evaluated on the mitogenic activity of FGFs for chlorate-treated A31 cells. A31 cells were grown in sulfate-free medium containing 5 ng/ml FGF-1 and 20 mM sodium chlorate for three days at OD_{492}=0.15. The addition of heparin (2 μg/ml) increased cell proliferation at OD_{492}=0.9 (Fig. 3A). But the extent of FGF-1-induced proliferation was decreased with increasing degree of 6-*O*-desulfation (Fig. 3A).

Regarding FGF-2, when A31 cells were incubated in sulfate-free medium containing 2 ng/ml FGF-2 and 20 mM sodium chlorate, the cells were not proliferated (Fig. 4A). However, the addition of intact heparin (more than 0.25 μg/ml) stimulated the FGF-2-induced A31 cell growth. The similar potentiating of 6-*O*-desulfated heparins on the chlorate-inhibited cell growth was observed, except for 88.8% 6-*O*-desulfated heparin. These facts suggest that FGF-1 requires more 6-*O*-sulfate group in heparin than FGF-2 for the activation.

It was reported that FGF-2-induced adrenocortical endothelial (ACE) cell growth was inhibited by high concentrations of heparin. However, the similar concentrations of heparin potentiated FGF-1-induced ACE cell growth [2]. Various 6-*O*-desulfated heparins were tested for their abilities to modulate the mitogenic activities of FGF-1 and FGF-2 on A31 cells (Fig.3B, Fig. 4B). Native heparin was effective in potentiating the mitogenic activity of FGF-1 over a wide range of concentrations (5-80 μg/ml). However, 6-*O*-desulfated heparins showed lower activities, which was dependent on degree of 6-*O*-desulfations (Fig. 3B). Thus, it is again suggested that heparin-like structure including high content of tri-sulfated disaccharide units are required for the mitogenic activity of FGF-1. In contrast, the exogenous heparins did not affect remarkably on the FGF-2-mediated cell proliferation, suggesting that endogenous HS is enough

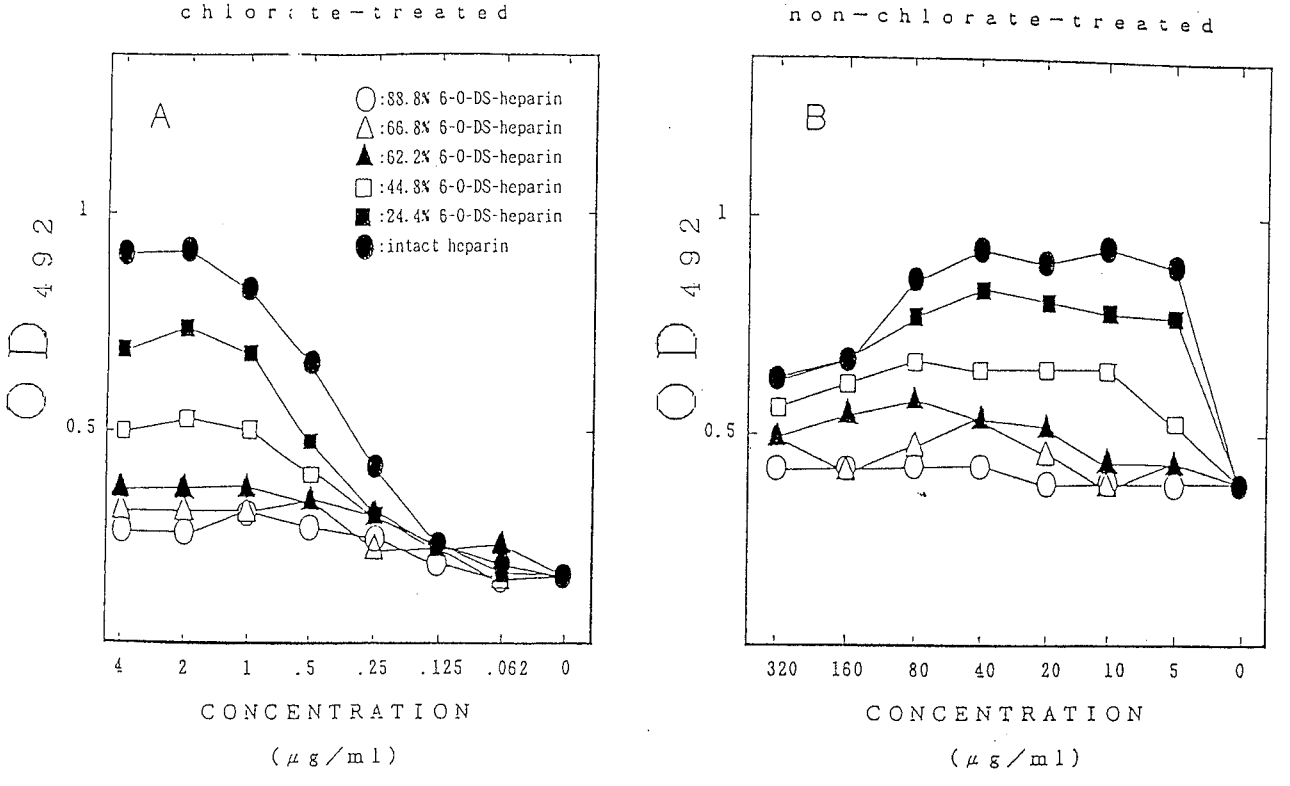

Fig. 3 Ability of 6-*O*-desulfated heparins to potentiate FGF-1 activity on chlorate-treated and non-chlorate-treated A31 cells. The chlorate-treated cells were grown for 3 days in sulfate-free DMEM containing 5 ng/ml human recombinant FGF-1, ITS+, 100 units/ml penicillin G, 20 mM sodium chlorate, and various 6-*O*-desulfated (6-O-DS-) heparins at the indicated concentrations. The non-chlorate-treated cells were also grown for 3 days in DMEM containing 5 ng/ml human recombinant FGF-1, ITS+, 100 units/ml penicillin G and the various 6-O-DS-heparins. Cell proliferation was assessed as described in the text. The results represent the means of triplicate determinations; $p<0.05$.

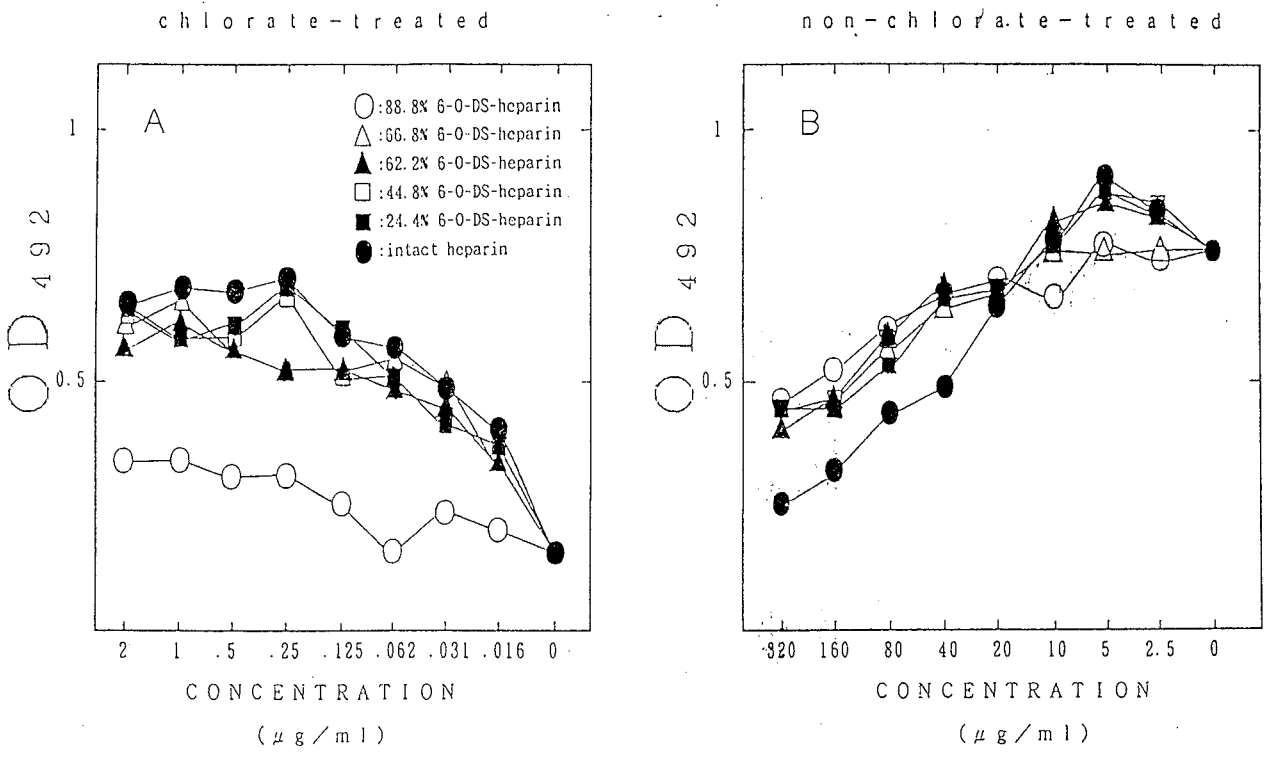

Fig.4 Ability of 6-*O*-desulfated heparins to modulate FGF-2 activity on chlorate-treated and non-chlorate-treated A31 cells. The chlorate-treated cells were grown for 3 days in sulfate-free DMEM containing 2 ng/ml human recombinant FGF-2, ITS+, 100 units/ml penicillin G, 20 mM sodium chlorate, and various 6-*O*-desulfated (6-O-DS-) heparins at the indicated concentrations. the non-chlorate-treated cells were also grown for 3 days in DMEM containing 2 ng/ml human recombinant FGF-2, ITS+, 100 units/ml penicillin G, and the various 6-O-DS-heparins. Cell proliferation was assessed as described in the text. The results represent the means of triplicate determinations; $p<0.05$.

to potentiate the mitogenic activity of FGF-2. Intact heparin inhibited FGF-2-induced A31 cell growth with high concentrations (>20 μg/ml), while 6-*O*-desulfated heparins showed a slightly lower inhibitory effect on FGF-2-induced A31 cell growth (Fig. 4B).

Many studies demonstrated that FGF-1 and FGF-2 played an important role in regulating a variety of physiological functions. The biological activities of FGF-1 and FGF-2 are currently believed to derive from their interactions with a "dual receptor system" [14], that is, a low affinity-association with heparan sulfate proteoglycan and high affinity-association with integral membrane receptors containing intracellular tyrosine kinase domains. A number of studies have provided evidence that heparin or HS works as a cofactor to promote the binding of FGFs to its high affinity receptor, enhancing the mitogenic activities of FGFs [4,5]. In addition, recent studies demonstrated that signalings of FGF-1, FGF-2, and FGF-4 required specific oligosaccharide sequences in heparin or HS [2,15].

Previous studies showed that structural requirements in heparin for binding and activation of FGF-1 were different from that for FGF-2 [2]. Deca- or larger saccharides enriched with IdoA2S-GlcNS6S disaccharide sequences were required for the binding to both FGF-1 and FGF-4 and to promote mitogenic activities. In contrast, a high content of 6-*O*-sulfate groups in GlcNS residues was not required to bind to FGF-2 and to promote FGF-2-induced cell proliferation [2,12]. The 6-*O*-sulfate in heparin or HS may not be essential for the FGF-2 binding.

Intact heparin promoted the mitogenic activity of FGF-1 on both A31 (non-chlorate-treated) and chlorate-treated A31 cells. Partial 6-*O*-desulfation from heparin resulted in a decrease of ability to promote the mitogenic activity of FGF-1 and a complete loss of the ability was obtained by 62.2% or higher 6-*O*-desulfation. On the other hand, FGF-2 did not require exogenous heparin for the mitogenic activity and heparin inhibited the FGF-2 activity at high concentrations (>20 μg/ml), suggesting that endogenous heparan sulfate proteoglycan levels are

sufficient for activation of FGF-2. Intact heparin efficiently recovered the mitogenic activity of FGF-2 on chlorate-treated A31 cells. In contrast to the results for FGF-1, the recovery of FGF-2-induced proliferation of chlorate-treated A31 cells was not significantly influenced by the partial 6-*O*-desulfation. These results were in agreement with the reported observation [2].

Almost complete 6-*O*-desulfation in heparin resulted in a loss of ability to restore FGF-2 activity on chlorate-treated A31 cells, whereas it did not significantly influence the binding affinity for FGF-2 (Fig. 1). The results suggest that an essential tri-sulfated disaccharides in heparin and HS is only required for the activation of FGF-2, but not for high affinity to FGF-2. This is in agreement with the result of Guimond *et al.* [16], suggesting that the binding of FGF-2 to its receptor may involve the formation of a ternary complex, where HS chain binds both to the growth factor and to the receptor.

Heparin or HS interacts with numerous growth factors and control their biological activities. There may be some differences in their structural requirements for binding to the growth factors and their activation. From a pharmaceutical perspective, studies to understand the molecular basis of specific interaction between each growth factor and heparin or HS may lead to the design of analogues that activate or suppress the action of each growth factor.

Experimental

Preparation of 6-O-desulfated heparins and analyses of disaccharide compositions. Bovine intestinal heparin (Seikagaku Corp.) was treated in a similar manner to that described in Part 2 except for the reagent, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MTSTFA), used. Briefly, pyridinium salt of the heparin (200 mg) was heated in pyridine (20 ml) with MTSTFA (4 ml) for 2 h at 65°C, 75°C, 85°C, 90°C or 95°C. After addition of 20 ml of 20% methanol, the reaction mixture was dialyzed, and 1 M NaOH was added to adjust to pH 9-10. The dialyzate was

lyophlized to obtain sodium salt of various 6-*O*-desulfated heparins. Any depolymerization in each 6-*O*-desulfated heparins was not detected in their gel-filtration profile.

Composition analysis of 6-*O*-desulfated heparins was performed as follows [17,18]. The polysaccharides (0.1 mg) were treated with a mixture of heparinase (50 mU), heparitinase I (20 mU), and heparitinase II (20 mU) (Seikagaku Corp.) in 0.22 ml of 2 mM calcium acetate and 20 mM sodium acetate (pH 7.0) at 37°C for 2 h. The digestion was checked by gel-filtration with serial columns of TSK-Gel PW4000, PW3000, and PW2500 (Tosoh Co.) using 0.2 M NaCl as eluant and by monitoring both absorbance at 230 nm and refractive index for detection. The disaccharide composition was analyzed by ion-exchange chromatography of the reaction mixture with Dionex CarboPac PA-1 (4 x 250 mm).

Inhibition of COS-1 cell binding to FGF-2-coated well. Ability of the various 6-*O*-desulfated heparins to inhibit the binding of COS-1 cells to immobilized FGF-2 was examined by a modification of the procedure reported [13,19,20]. Briefly, wells of a 96-well tissue culture plate were coated with 50 µl of 10 µl/ml human recombinant FGF-2 (Promega) at 4°C overnight. The solution was aspirated, wells were rinsed twice with PBS, and blocked by incubation with 5% (v/v) fetal bovine serum in PBS at room temperature for 1 h. A pellet of COS-1 cells was prepared by incubation with 2 mM EDTA in PBS at 37°C for 20 min, trituration, and collection by centrifugation. The cells were then resuspended at a cell density of 5×10^5 cells/ml with 5% fetal bovine serum in PBS. The 6-*O*-desulfated heparins were then added to the suspension. The cell suspension (0.1 ml) was immediately applied to the coated wells and incubated for 10 min at room temperature. Each well was washed twice with PBS, and then 20 µl of 5% SDS was added to lyse the bound cells. Two hundred microliter of Micro BCA protein assay reagent (Pierce) was added to each well and the protein concentration of the lysate was determined. Bovine serum albumin was used as a standard by measuring absorbance at 520 nm of each well.

Cell proliferation assay. A31 cells (BALB/c 3T3, clone A 31) were obtained from Japanese Cancer Research Bank and were maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin in 5% CO_2 and 100% relative humidity. For the assay of non-chlorate-treated A31 cell growth, the cells were seeded at an initial density of 4,000 cells/well in 96-well tissue culture plates and were grown for 3 days in 100 μl of DMEM supplemented with ITS+ (Collaborative Research; containing insulin [6.25 μg/ml], transferrin [6.25 μg/ml], selenium [6.25 ng/ml], bovine serum albumin [1.25 mg/ml], and linoleic acid [5.35 μg/ml]), 2 ng/ml human recombinant FGF-2 or 5 ng/ml human recombinant FGF-1, 50 μg/ml gentamicin, and 6-*O*-desulfated heparins. For testing the ability of the 6-*O*-desulfated heparins to restore the FGF-induced proliferation of chlorate-treated A31 cells, A31 cells were grown for 3 days in DMEM lacking inorganic sulfate but supplemented with ITS+, 2 ng/ml FGF-2 or 5 ng/ml FGF-1, 20 mM sodium chlorate, 100 units/ml penicillin G, and the 6-*O*-desulfated heparins. After incubation, 20 μl of MTS/PMS solution (Cell titer 96 aqueous non-radioactive cell proliferation assay kit, Seikagaku Corp.) was added into each well. After 3 h incubation at 37°C, the absorbance of each well at 490 nm was directly measured from the 96 well tissue culture plate.

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Summary

Summary

Sulfate groups play central roles for physical and biological functions of sulfated polysaccharides. Especially, the linkage position of sulfate groups and degree of sulfation affect the functions of these compounds. For modification of these properties and investigation of the structure-function relationship of the sulfated polysaccharides, development of controlled conversion methods are required. In this point of view, the works presented in the this thesis were designed to develop new methods for regioselective introduction and removal of the sulfate groups in polysaccharides.

In **Part 1** of this thesis, the author examined a sulfation method using sulfuric acid and dicyclohexylcarbodiimide (DCC). It is not easy to introduce various protecting groups into particular positions of complex carbohydrates. Regioselective reactions are, therefore, required. The author thus examined regioselectivity in the sulfation reaction using sulfuric acid and DCC, since the method was expected to have stereoselective reactivity owing to the formation of a balky intermediate. Consequently, for mono- or di-saccharide, the sulfuric acid-DCC system showed 6-*O*-specificity better than the traditional sulfation method using sulfur trioxide-pyridine complex. Then, the method was applied to polysaccharides to achieve regioselective sulfation. The results suggested that the method is useful for functional modification of various polysaccharides.

In **Part 2**, the author found novel 6-*O*-regiospecific desulfation reactions of sulfated carbohydrates using trimethylsilylating reagents. From an extensive evaluation using various kinds of trimethylsilylating reagents and monosaccharides as model compounds, the silylating reagents was classified into three categories; the reagents suppressing solvolytic desulfation, those capable of 6-*O*-specific desulfation, and those removing sulfate groups non-specifically.

The author then discussed about the nature of the reactions of the trimethylsilylating reagents with sulfated carbohydrates, and proposed three different mechanisms respective for the three different categories. The author also demonstrated that these desulfation reactions were applicable to sulfated polysaccharides. Among the desulfation reactions, the 6-*O*-specific desulfation is expected to be a strong tool for the functional study on sulfated polysaccharides.

Structural analysis of polysaccharides is a fundamental work for elucidating functions and properties of the polysaccharides. In regard to sulfated polysaccharides, the structural analyses should be carried out by specifying the glycosyl linkage, their sequence, and the location of sulfate groups. By methylation analysis alone, the positions of substituents on the component sugars and the positions of glycosyl linkages can not be unquivocally determined. Combined with the desulfation method described in this thesis, the locations of sulfate groups can be determined more precisely. In **Part 3**, the author was successful to assign the linkage position of sulfate groups in the sulfated polysaccharides from red algae. From methylation analyses before and after the 6-*O*-specific desulfation, the presence of 6-*O*-sulfate groups in the polysaccharide was quantitatively identified.

It would be rational to assume that the interaction of sulfated glycosaminoglycans with proteins are influenced by their sulfate groups which might be precisely arranged to form anionic cluster to make the glycan chain specifically susceptible to their target proteins. Therefore, the positions and degree of substitution of the sulfate groups in the bioactive sulfated polysaccharide may be crucial for the expression of their activities. In **Part 4**, the regiospecific desulfation methods was applied to heparin. By the treatment with *N,O*-bis(trimethylsilyl)acetamide, partial 6-*O*-desulfation occurred. The sulfate groups required for binding to antithrombin-III (AT-III) proved to be partially removed as concluded from the experiment of binding and activation. On the other hand, treatments of heparin with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide gave

heparins with various degrees of further 6-*O*-desulfation. Using the heparins having various content of 6-*O*-sulfate, the author evaluated the effects of the 6-*O*-sulfate groups in heparin on the binding abilities and activations of fibroblast growth factors, FGF-1 and FGF-2. It was found that FGF-1 required 6-*O*-sulfate groups for both binding and activation, while FGF-2 requires 6-*O*-sulfate groups for activation but not for binding to heparin. The latter results may support the earlier assumption reported.

In conclusion, the author has developed regiospecific sulfation and desulfation methods. The results demonstrate potential applicability of the methods for the modification of bioactive carbohydrates for their chemical and biochemical investigation as well as for pharmaceutical uses.

List of publications

Pubilications concerning to the present thesis

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