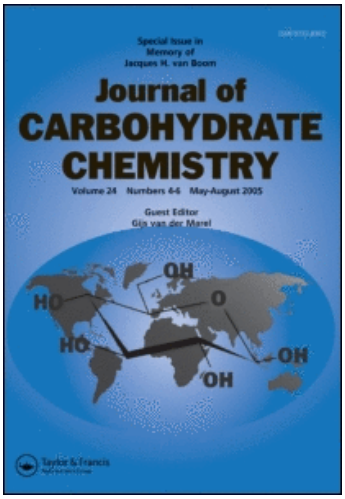

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Ryo Takano[a]; Shuichi Yoshikawa[a]; Takashi Ueda[a]; Kaeko Hayashi[a]; Susumu Hirase[b]; Saburo Hara[a]
[a] Department of Chemistry and Materials Technology, Faculty of Engineering and Design, Kyoto Institute of Technology, Kyoto, Japan [b] Taito Co. Ltd., Research Laboratory, Kobe, Japan

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SULFATION OF POLYSACCHARIDES WITH SULFURIC ACID MEDIATED BY DICYCLOHEXYLCARBODIIMIDE

Ryo Takano, Shuichi Yoshikawa, Takashi Ueda, Kaeko Hayashi, Susumu Hirase[a] and Saburo Hara*

Department of Chemistry and Materials Technology, Faculty of Engineering and Design, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606, Japan
[a]Present address: Research Laboratory, Taito Co. Ltd., 1-26 Higashi-shiriikeshinmachi, Nagata-ku, Kobe 653, Japan

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ABSTRACT

Starch, agarose, κ-carrageenan and porphyran were sulfated by sulfuric acid with dicyclohexylcarbodiimide as a condensation reagent. In all the cases, the sulfation at O-6 appeared to be predominant on the basis of results from methylation analyses. Although the reactivities of the other hydroxyl groups towards the present sulfation reaction were less than that at C-6, they varied depending on the position in the sugar residue of the polysaccharides. The sulfated agarose was further fractionated in terms of the difference in the solubility of its cetylpyridinium salt to KCl solution. The major fraction resembled the main polysaccharide of funoran (agarose 6-sulfate) on the basis of the behavior of its cetylpyridinium salt, ^{13}C NMR measurements and methylation analyses.

INTRODUCTION

Sulfated polysaccharides are widely distributed in nature, e.g., as algal polysaccharides[1] and sulfated glycosaminoglycans of animal origin.[2] They are known as materials with diverse applications and physiological functions, e.g., heparin, dermatan sulfate and fucoidan etc. as anti-blood coagulation substances, and heparan sulfate as a

regulator of a variety of growth factors, etc. Besides such naturally occurring polysaccharide sulfates, artificially sulfated polysaccharides are also synthesized for similar interests. When introducing the sulfate groups, regioselectivity of the reaction is an important issue because the activities of the sulfated polysaccharides depend on the position of the sulfate groups in the polymers.

As a model experiment,[3] we investigated sulfation of methyl α- and β-galactopyranosides and 4-*O*-β-D-galactopyranosyl-3,6-anhydro-L-galactose dimethylacetal, a derivative of the repeating disaccharide of agarose,[4] using sulfuric acid as a sulfating reagent as activated by dicyclohexylcarbodiimide (DCC).[5,6] It has been indicated that the sulfation at O-6 is predominant. When modifying polysaccharides, the selective protection and deprotection of the hydroxyl groups is often difficult and inconvenient. However, such protection/deprotection procedures are unnecessary if an appropriate sulfation method with a suitable selectivity is chosen. As demonstrated by the experiment using the model compounds mentioned above, the sulfation by the sulfuric acid-DCC system is regioselective, and thus expected to be applicable to the regioselective sulfation of polysaccharides. In the present paper, we describe the application of this method to sulfation of neutral polysaccharides and introduction of extra sulfate groups into sulfated polysaccharide (over-sulfation).

RESULTS AND DISCUSSION

The polysaccharides examined in the present work are: an amylose-rich starch, i.e., an α-(1→4) glucan; agarose, consisting of a repeat 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 mainly consists of →3)β-D-Gal(1→4)α-L-Gal-6-SO_3^-(1→ with a smaller amount of the agarose moiety as a part of the polysaccharide.[1] The latter three polysaccharides are of red algal origin. The neutral polysaccharides, starch and agarose, could be successfully sulfated by the sulfuric acid-DCC system. However, the naturally sulfated polysaccharides are insoluble in DMF, a solvent that is required for the present method. In order to improve the solubility of sulfated polysaccharides in organic solvents, the pyridinium and triethylamine salts of the sulfated polysaccharides have been employed for methylation.[7,8] The tributylamine salt has been used for over-sulfation of chondroitin sulfate using the sulfur trioxide-pyridine complex.[9] The sulfated polysaccharides examined in the present work, κ-carrageenan and porphyran, were therefore converted into their pyridinium salts before sulfation. The present method did not affect the molecular weights of the polysaccharides on the basis of the results from gel-filtration analysis, as the elution patterns of the polysaccharides

Table 1a. Methylation analysis of starch sulfated by the H_2SO_4-DCC method

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

b. Relative reactivities of OH groups in starch using the H_2SO_4-DCC method

Position	Relative reactivity
2	23
3	0
6	100

before and after the sulfation were essentially identical. With methods using the sulfur trioxide-amine complex or other sulfate donors carried out under drastic conditions, some cleavages of the glycosidic linkages may occur. One of the advantages of the present method is that no cleavage of the polysaccharide chains appeared to take place even in the cases of agarose and κ-carrageenan that contain acid-labile 3,6-anhydrogalactosyl linkages. From methylation analyses of the obtained sulfated or over-sulfated polysaccharides (Table 1a to 4a), the relative reactivities of the respective hydroxyl groups towards the present sulfation reaction were estimated, e.g., the reactivity of O-2 of starch was calculated from the amount of 3-*O*-methylglucose and the reactivity of O-6 from the total amount of 3-*O*-methylglucose and 2,3-di-*O*-methylglucose, etc., (Table 1b to 4b). In all the cases, sulfate groups were apparently introduced predominantly to O-6. Although the degree of the sulfation of the other hydroxyl groups is far less than that of O-6, the relative reactivities of the respective hydroxyl groups varied depending on the position, e.g., O-2 in the Glc residue of amylose rich starch was more reactive than O-3, which was not sulfated (contribution of the amylopectin moiety to the estimation was ignored). The O-2 in the Gal residue of agarose was more reactive than the O-2 in 3,6-anhydrogalactose residue and the O-4 in the Gal residue. In the case of κ-carrageenan, the O-2 at the 3,6-anhydrogalactose residue and the O-2 at the Gal residue were similar in their reactivities. In the case of porphyran, O-2 and O-3 at (1→4)-linked α-L-Gal 6-sulfate residue and O-4 of (1→3)-linked β-D-Gal residue were not sulfated, whereas O-2 of the latter residue was moderately reactive.

The difference in the reactivity of each hydroxyl group is likely due to the fact that the sulfuric acid-DCC complex, the efficient donor of the sulfate group, is rather bulky.[5]

Table 2a. Methylation analysis of agarose sulfated by the H_2SO_4-DCC method

Product	Assignment	mol %
2-Me-AGal[a]	→4)AGal(1→	48
AGal[a]	→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

a. AGal=3,6-anhydrogalactose

b. Relative reactivities of OH groups in agarose using the H_2SO_4-DCC method

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

Table 3a. Methylation analysis of κ-carrageenan sulfated by the H_2SO_4-DCC method

Product	Assignment	mol %
2-Me-AGal[a]	→4)AGal(1→	38
AGal[a]	→4)AGal-2-SO_3^-(1→	12
2,6-Me_2-Gal	→3)Gal-4-SO_3^-(1→	5
2-Me-Gal	→3)Gal-4,6-di-SO_3^-(1→	32
Gal	→3)Gal-2,4,6-tri-SO_3^-(1→	13

a. AGal=3,6-anhydrogalactose

b. Relative reactivities of OH groups in κ-carrageenan using the H_2SO_4-DCC method

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

Table 4a. Methylation analysis of porphyran sulfated by the H_2SO_4-DCC method

Product	Assignment	mol %
(2-Me-AGal[a]	→4)AGal(1→	7)
(AGal[a]	→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-Gal	→3)Gal-2,6-di-SO_3^-(1→	6

a. AGal=3,6-anhydrogalactose; arising from small amount of agarose moiety

b. Relative reactivities of OH groups in porpyran using the H_2SO_4-DCC method

Position	Relative reactivity
(2 at AGal	5)
2 at 4-linked Gal	0
3 at 4-linked Gal	0
2 at 3-linked Gal	35
4 at 3-linked Gal	0
6 at 3-linked Gal	100

The hydroxyl groups at C-6 that project from the polysaccharide chains are least sterically hindered and achieve the highest reactivities. The hydroxyl groups that were not sulfated are likely to have been sterically hindered, presumably due to the conformations of polysaccharide molecule. In the case of starch, the moderately reactive O-2 is close to O-1 that is axially linked to the adjacent Glc residue, whereas the less reactive O-3 is close to equatorially linked O-4. In contrast, in the case of porphyran, O-2 and O-3 at the 4-linked L-Gal residue that are close to the *axial* O-1 and O-4, respectively, exhibited no reactivity, whereas O-2 of the 3-linked D-Gal residue close to *equatorial* O-1 exhibited the moderate reactivity. In general, *equatorial* hydroxyl groups appeared to be more reactive than *axial* hydroxyl groups. In the cases of porphyran and agarose, *axial* hydroxyl groups such as O-2 of 4-linked 3,6-anhydro-L-galactose residue and O-4 of 3-linked D-Gal residues were not reactive. This is consistent with the results from the model experiments using monosaccharides.[3] However, the *axial* O-2 of 4-linked 3,6-anhydro-D-galactose residue of κ-carrageenan was as reactive as the *equatorial* O-2 of 3-linked D-Gal residue.

Table 5. Methylation analyses[a] 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[b]	AGal[b]
PS-I	6	51	3	2	37	79	21
PS-II	15	58	6	3	18	92	8

a. Total of partially methylated Gal was taken as 100.

b. AGal=3,6-anhydrogalactose

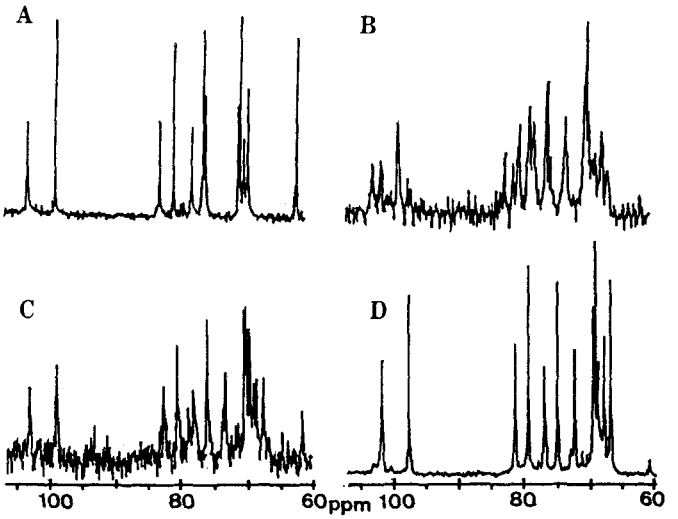

Figure. ^{13}C NMR spectra of fractions from sulfated agarose and related polysaccharides. **A**, agarose; **B**, PS-I; **C**, PS-II; **D**, the main polysaccharide from funoran (ref. 11).

Although funoran, a sulfated galactan of red algal origin, is a heterogeneous compound, its main polysaccharide is ideally illustrated as agarose-6 sulfate, which has the alternating repeat of →3)β-D-Gal-6-SO_3^-(1→4)3,6-anhydro-α-L-Gal(1→.[10,11] A conversion experiment of agarose into funoran has been already reported by Guiseley,[12] who used sulfur trioxide-DMF complex as the sulfating reagent. The agarose sulfate thereby obtained appeared to be identical to funoran on the basis of IR spectral comparisons, but a detailed structural analysis and examination of the regiospecificity of his sulfation method have not been investigated. Since the indicated structure of the

sulfated agarose made by the present method was similar to that of funoran, this polysaccharide was fractionated by the method applied to funoran[11,13] including stepwise extraction of its cetylpyridinium salt with 2M and hot 4M KCl to afford the two polysaccharide fractions termed PS-I and PS-II, respectively. In agreement with the results from the fractionation studies of funoran from a natural source,[11,13] the polysaccharide with the most abundant sulfate (PS-I) was eluted in the relatively low concentration of KCl, while PS-II with a smaller amount of the sulfate was eluted in the higher KCl concentration. Methylation analyses (Table 5) indicate that most of O-6 in Gal residue and considerable amounts of O-2 in 3,6-anhydrogalactose and O-2 in Gal residue are sulfated in PS-I, while PS-II, the major product of the sulfation, can be regarded as a homologue of agarose 6-sulfate. Their ^{13}C NMR spectra (Figure) also supported these structures. The combination of the regioselective sulfation method and the subsequent suitable fractionation was thus effective for the preparation of the sulfated polysaccharides with the fairly regular structure.

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*. Other reagents used were obtained from Wako Pure Chemical Co. Colorimetric determination of hexoses and 3,6-anhydrogalactose was carried out by a modified method of Yaphe.[14] Sulfate content of the polysaccharide was estimated by the sodium rhodizonate method[15] after hydrolysis of the polysaccharide. GLC was carried out using a gas chromatograph, GC-7A (Shimadzu Corp.), equipped with FID. 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. N_2 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, GC/MS QP-1000 (Shimadzu Corp.), under the identical conditions to GLC except for the use of He as the carrier gas. Gel-filtration was carried out using a high-performance liquid chromatograph, LC5A (Shimadzu corp.), equipped with a refractive index detector, RID-2A (Shimadzu Corp.), and a column, TSK-Gel G-3000 PWXL (7.6 mm x 30 cm), eluted with 0.2 M NaCl at 40 °C. IR spectra were recorded using a spectrometer, Model 215, (Hitachi Co.) by the KBr disk method. ^{13}C NMR spectra were recorded by a Varian XL-200 spectrometer operated at 80 °C using 60° pulse width. The chemical shift values were expressed relative to the signal from internal methanol (49.3 ppm).

Sulfation of neutral polysaccharides. Starch was dissolved in DMF (1 g/54 mL), and to the solution was added DCC dissolved in DMF (6.4 g/82 mL). The solution was cooled to 0 °C, and after addition of H_2SO_4 dissolved in DMF (700 mg/35 mL), the solution was stirred for 15 min at 0 °C under N_2 atmosphere. The resulting mixture was poured into crushed ice (500 g), neutralized with NaOH and dialyzed. The dialysate was filtered with Celite 500 and centrifuged to remove dicyclohexylurea derived from DCC and then lyophilized to obtain the sulfated polysaccharide (1.45 g; Glc, 50.1%; sulfate as -SO_3Na, 32.3%). For 1 g of agarose (Gal, 46.2%; 3,6-anhydroGal, 42.6%), 3 g of DCC and 316 mg of H_2SO_4 was used (yield, 0.93 g; Gal, 28.3%; 3,6-anhydroGal, 25.2%; sulfate as -SO_3Na, 23.5%).

Over-sulfation of sulfated polysaccharides. Solution of the sulfated polysaccharide as Na and/or K salt was applied to a column of Amberlite IR-120 (H^+ form), and the eluate was neutralized with pyridine and lyophilized. The resulting pyridinium salt of the sulfated polysaccharide was over-sulfated similarly as described in the preceding paragraph. For 1 g of κ-carrageenan (Gal, 32.1%; 3,6-anhydroGal, 27.9%; sulfate as SO_4, 19.2%), 2 g of DCC and 224 mg of H_2SO_4 were used (yield, 1.22 g; Gal, 31.5%; 3,6-anhydroGal, 27.1%; sulfate as -SO_3Na, 24.4%), and for 1 g of porphyran (Gal, 58.9%; 3,6-anhydroGal, 4.8%; sulfate as -SO_3K, 18.6%), 1.5 g of DCC and 168 mg of H_2SO_4 was used (yield, 1.05 g; Gal 52.7%; 3,6-anhydroGal, 4.2%; sulfate as -SO_3Na, 26.3%).

Methylation analyses of polysaccharides. The methylation was carried out by the method of Isogai et al.[16] and Ciucanu and Kerek[17] using powdered NaOH and CH_3I in DMSO. Before the methylation, the sulfated polysaccharides were converted into their triethylamine salt according to the recommendation by Stevenson and Furneaux.[8] After the polysaccharides were methylated three times, the permethylated sample was hydrolyzed with 1 N H_2SO_4 for 16 h at 100 °C and the hydrolyzates were analyzed as mixtures of partially methylated alditol acetates[18] using GLC and GLC-MS. For the analyses of the sulfated polysaccharides containing acid-labile 3,6-anhydrogalactose residue, the double hydrolysis method described by Stevenson and Furneaux[8] was adopted.

Fractionation of sulfated agarose. The fractionation was carried out as described elsewhere.[11,13] The sulfated agarose (892 mg) was dissolved in water (30 mL), to the solution was added excess of 5% solution of cetylpyridinium chloride (100 mL), the resulting precipitate of the cetylpyridinium salt of the sulfated agarose was collected by centrifugation, and then washed three times with water (50 mL). The cetylpyridinium salt was extracted twice with 2 M KCl (50 mL) at room temperature, the combined extract was concentrated and poured into x 4 volumes of EtOH. The resulting

precipitate was dissolved in water, dialyzed and then lyophilized to afford a polysaccharide fraction termed PS-I (recovery, 260 mg; Gal, 26.4%; 3,6-anhydroGal, 23.5%; sulfate as -SO_3K, 33.2%). The cetylpyridinium salt insoluble during the extraction with 2 M KCl was further extracted with 4 M KCl (50 mL) at 100 °C, and the extract was treated similarly to afford a polysaccharide fraction termed PS-II (recovery, 442 mg; Gal, 30.7%; 3,6-anhydroGal, 27.3%, sulfate as -SO_3K, 21.8%).

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