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Analysis of residue types in curdlan sulfate by nuclear magnetic resonance

Hiroshi Miyano [a], Ryusuke Nakagawa [a], Ei-ichiro Suzuki [a] and Toshiyuki Uryu [b]

[a] *Central Research Laboratories, Ajinomoto Co., Inc., Suzuki-cho, Kawasaki-ku, Kawasaki 210 (Japan)*
[b] *Institute of Industrial Science, University of Tokyo, Roppongi, Minato-ku, Tokyo 106 (Japan)*

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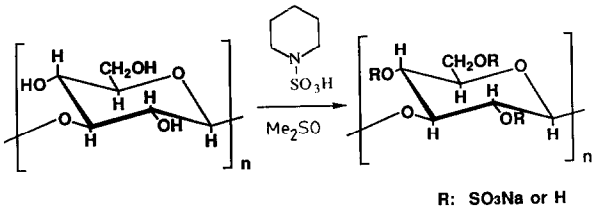
ABSTRACT

The degree of substitution at each position in curdlan sulfate (CRDS), a polysaccharide having a linear (1 → 3)-linked β-D-glucose backbone and activity against human immunodeficiency virus in vitro, has been determined by proton and carbon-13 NMR spectroscopy. Complete ^{1}H NMR assignment of CRDS was carried out by using a combination of two-dimensional double-quantum-filtered correlated spectroscopy and two-dimensional homonuclear Hartmann–Hahn spectroscopy. The latter was especially useful for identifying ^{1}H signals of the heavily substituted polysaccharide, which shows severely overlapped signals in its one-dimensional spectrum. Nine anomeric proton signals were identified, and four substitution patterns could be determined with reference to the proton chemical shifts of curdlan itself. It was revealed that the C-6 position of CRDS is completely sulfated and about one-third of the C-2 hydroxyls are also substituted, while the degree of substitution at the C-4 position is much smaller. Two-dimensional nuclear Overhauser enhancement spectroscopy of CRDS showed some sequential NOE connectivities between H-1 of one residue and H-3 of the neighboring residue. Carbon-13 NMR resonances were also completely assigned by using a two-dimensional ^{13}C–^{1}H heteronuclear correlation method. Crosschecks for these assignments were given by an isotope effect experiment; i.e., the chemical shift values of ^{13}C nuclei linked to free hydroxyl groups in the polysaccharide dissolved in H_2O were 6 to 8 Hz lower than those observed in D_2O solution, while the chemical shift difference was negligibly small for sulfated ^{13}C nuclei. The positions of sulfation in each glucose unit of CRDS derived from ^{13}C NMR analyses are consistent with those from ^{1}H NMR.

INTRODUCTION

Sulfated polysaccharides, such as carrageenan, heparin, dextran sulfate, fucoidan, pentosan polysulfate, polysulfated xylan, and mannan sulfate, have an anti-HIV (human immunodeficiency virus, AIDS virus) activity in vitro[1,2]. They inhibit the first step in the infection of human T lymphocytes by HIV type 1 by blocking the binding of *gp* 120 to the target cell receptor, CD4 antigen, and inhibit syncytia formation[3]. Recently, it has been reported that synthetic curdlan sulfate

Correspondence to: Dr. H. Miyano, Central Research Laboratories, Ajinomoto Co., Inc., Suzuki-cho, Kawasaki-ku, Kawasaki 210, Japan.



Scheme 1.

(CRDS) also has a strong inhibitory effect against HIV infection in vitro[1,2].

As curdlan is a linear (1 → 3)-β-D-glucan, each glucose unit has three hydroxyl groups, at the 2, 4, and 6 positions, that can be sulfated to some extent. The present work was performed in order to determine the positions and the degree of sulfation in each glucose unit of CRDS by using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) techniques. It is shown that two-dimensional NMR techniques such as double quantum-filtered correlated spectroscopy (DQF-COSY), two-dimensional homonuclear Hartmann–Hahn spectroscopy (2D-HOHAHA), and ^{13}C–^{1}H correlated spectroscopy (COSY) are useful for assigning the signals of CRDS. In addition, to investigate the possibility of sequence regularity of the sulfated residues in CRDS, we also utilized the results from two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY).

RESULTS AND DISCUSSION

Assignments of ^{1}H NMR signals of CRDS.—The one-dimensional (1D) proton NMR spectrum of CRDS is shown in Fig. 1. All the proton signals of CRDS appear in the region between 3.5 and 5.0 ppm and so are heavily overlapped. Since two-dimensional NMR spectroscopy has been found useful for the separation of the complicated signals given by proteins and nucleic acids[4], we turned to 2D NMR for the assignment of the ^{1}H signals of the substituted polysaccharide CRDS. The DQF-COSY spectrum of CRDS is shown in Fig. 2. Cross-peak

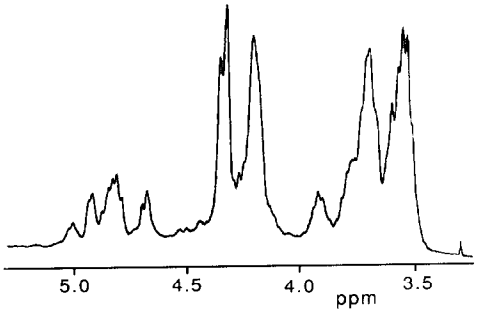


Fig. 1. The 400 MHz ^{1}H NMR spectrum of CRDS in D_2O at 25°.

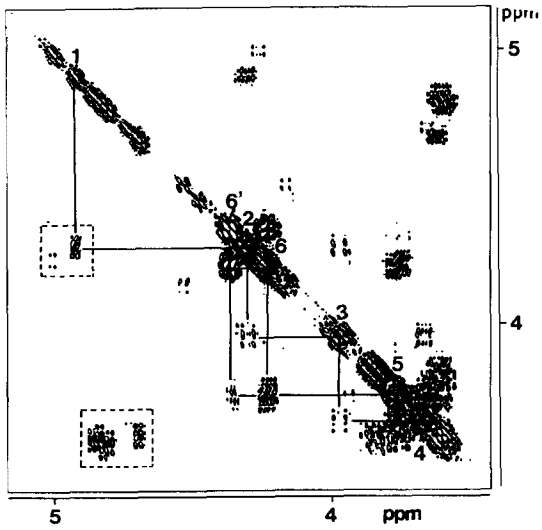


Fig. 2. DQF-COSY spectrum of CRDS at 25°. The dashed squares are the cross-peak regions between H-1 (horizontal) and H-2 (vertical). Scalar connectivities from H-1 to H-6 and H-6′ of residue *II* are illustrated by the solid lines. The numbers near the diagonal peaks are the assignments of the proton resonances of residue *II*.

connectivities were observed from the H-1 resonance at 5.00 ppm (glucose unit designated as residue *II*) to H-6 and H-6′ via H-2, H-3, H-4, and H-5 as shown in Fig. 2. As for the identification of the signals from the other residues, however, we could only recognize the cross-peaks between H-1 (4.70 to 5.02 ppm) and H-2, while the other resonances in the DQF-COSY spectrum are heavily overlapped and obscure especially in the range of 3.5–3.8 ppm.

Fig. 3 shows the cross-peaks between the anomeric proton (H-1) and other proton (H-2, H-3, H-4, and H-5) resonances in a 2D-HOHAHA spectrum taken with a mixing time of 75 ms. In general, a HOHAHA spectrum shows three or more bond connectivities between coupled protons. Actually, the anomeric proton region in Fig. 3 apparently consists of seven signals and each has four cross-peaks. As for the cross-peaks of the H-1 resonance at 5.00 ppm (residue *II*) assigned by DQF-COSY (vide supra), the four cross-peaks at 4.30, 3.95, 3.77, and 3.66 ppm corresponded to the chemical shifts of H-2, H-3, H-5, and H-4, respectively. It is very interesting that the cross-peaks between H-1 and other protons have unique and characteristic shapes. In the cross-peak region for H-1 of the other residues, we also found patterns similar to those of the H-1 in residue *II*. The shape of a cross-peak is uniquely related to the number of chemical bonds connecting the protons involved, so cross-peaks of the same shape are observed when the number of chemical bonds between H-1 and another proton is the same. For instance, the cross-peaks between H-1 and H-2 in the DQF-COSY spectrum could be observed in exactly the same region as in the 2D-HOHAHA spectrum, in which all

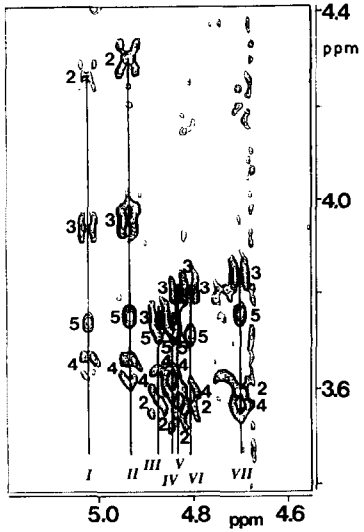


Fig. 3. 2D HOHAHA spectrum of CRDS in the cross-peak region of the anomeric proton and other sulfated-glucose proton resonances at 25°. Mixing time was 75 ms. The vertical lines indicate the chemical shifts of H-1. The numbers near the cross-peaks refer to the assignments of proton resonances of the scalar coupling network belonging to H-1.

cross-peaks between H-1 and the neighboring H-2 have the same shapes (Fig. 3), like a St. Andrew's cross. The resonances of the other six groups of the residues (*I*, *III*, *IV*, *V*, *VI*, and *VII*) in CRDS could be unambiguously assigned by using this type of pattern analysis of the characteristic cross-peak shapes (Fig. 3 and Table I).

Some weaker signals, especially in the region between 4.46 and 4.55 ppm, could also be assigned from the DQF-COSY and 2D-HOHAHA spectra of CRDS which was synthesized so as to contain a little higher content of these residues (residues *VIII* and *IX*, data not shown).

Magnetization transfer in the HOHAHA experiment is especially efficient when all coupling constants are of a similar order of magnitude, so it is recognized as an excellent technique for identifying the proton resonances of the sugar components of oligosaccharides and glycolipids[5–8]. However, this is the first study to apply the HOHAHA technique to signal assignment for a substituted polysaccharide with a molecular mass greater than 10000 daltons. In the case of CRDS, which is a linear β-glucan derivative, a sulfated glucose unit has vicinal coupling constants of 6 to 9 Hz all around the ring, so the cross-peaks can be obtained very efficiently. It is then useful to observe the cross-peak patterns in the HOHAHA spectrum, which depend on the number of chemical bonds, even if signals are heavily overlapped in the one-dimensional spectrum. The two-dimensional HOHAHA technique is one of the most important methods for signal assignment and structure analysis of polysaccharides.

Of course we should be careful in using the above "cross-peak shape rule" for

TABLE I

Proton chemical-shift assignments of CRDS

Residue type	Chemical shifts (ppm) [a]						
	H-1	H-2	H-3	H-4	H-5	H-6	H-6′
I	5.02	4.27	3.94	3.66	3.77	4.23	4.36
	(0.32)	(0.80)	(0.31)	(0.24)	(0.35)	(0.57)	(0.46)
II	4.92	4.30	3.95	3.66	3.77	4.23	4.36
	(0.22)	(0.83)	(0.32)	(0.24)	(0.35)	(0.57)	(0.46)
III	4.87	3.57	3.74	3.61	3.73	4.23	4.36
	(0.17)	(0.10)	(0.11)	(0.19)	(0.31)	(0.57)	(0.46)
IV	4.84	3.59	3.74	3.61	3.73	4.23	4.36
	(0.14)	(0.12)	(0.11)	(0.19)	(0.31)	(0.57)	(0.46)
V	4.82	3.54	3.80	3.55	3.73	4.23	4.36
	(0.12)	(0.07)	(0.17)	(0.13)	(0.31)	(0.57)	(0.46)
VI	4.81	3.57	3.80	3.58	3.73	4.23	4.36
	(0.11)	(0.10)	(0.17)	(0.16)	(0.31)	(0.57)	(0.46)
VII	4.70	3.58	3.83	3.55	3.77	4.23	4.36
	(0.00)	(0.11)	(0.20)	(0.13)	(0.35)	(0.57)	(0.46)
VIII	5.01	4.45	4.46	4.44	4.09	4.17	4.46
	(0.31)	(0.98)	(0.83)	(1.02)	(0.67)	(0.51)	(0.56)
IX	4.70	3.71	4.17	4.23	3.94	4.14	4.53
	(0.00)	(0.24)	(0.54)	(0.79)	(0.52)	(0.48)	(0.63)

[a] Chemical shifts are referenced to internal DSS at 32°. The numbers in parentheses are chemical-shift differences with respect to curdlan.

signal assignment as these shapes are sensitive to the experimental procedures such as the isotropic mixing sequence used for spin locking. In other words, the shapes are reproducible only when the experimental conditions applied are the same for similar sample systems.

Determination of substitution positions in the glucose units of CRDS.—The substitution pattern in each glucose unit could be determined with reference to the proton chemical shifts of curdlan (Table I). The chemical shifts of curdlan were determined from the 2D HOHAHA spectrum taken in 0.5 N NaOD–D_2O (10 mg/mL) at ambient temperature (data not shown). As curdlan can be dissolved only at higher pH because of its hydrogen-bonding tendencies, the chemical shifts of curdlan in this condition were used for reference.

On comparison of the values for curdlan and CRDS large downfield shifts (0.46 to 0.63 ppm) were observed for H-6 in all the residues of CRDS, obviously influenced by a sulfate group at the primary position[5]. Larger downfield shifts of the proton resonances for H-2 in residues *I*, *II*, and *VIII* (0.80, 0.83, and 0.98 ppm, respectively) and H-4 of residues *VIII* and *IX* (1.02 and 0.79 ppm, respectively), indicated that sulfate groups are linked at the C-2 and C-4 positions, respectively. On the other hand, the high-field signals for H-2 in residues *III*, *IV*, *V*, *VI*, *VII*, and *IX* (range 3.54–3.71 ppm) and H-4 in residues *I* to *VII* (around 3.6 ppm) show that no sulfate groups exist at these positions. Therefore, we could assign the following substitution patterns in the NMR spectra of CRDS: residues *I* and *II*

have two sulfate groups at the C-2 and C-6 positions; residues *III*, *IV*, *V*, *VI*, and *VII* have a single sulfate group at the C-6 position; residue *IX* has two sulfate groups at the C-4 and C-6 positions; and residue *VIII* has three sulfate groups at the C-2, C-4, and C-6 positions.

A deshielding effect of the sulfate group was observed for all protons, especially at positions vicinal to those carrying sulfate groups. Protons H-3 and H-5 of residues *VIII* and *IX*, in which the vicinal C-4 hydroxyl group is sulfated, show especially large downfield shifts. These protons are presumably influenced not only by the deshielding effect of neighboring sulfate groups but also by some other factors.

Degree of sulfate substitution on each hydroxyl group.—As revealed by ^{1}H NMR, the C-6 position in each residue is substituted by sulfate, while the C-2 and C-4 positions are partially sulfated. It is difficult to state exactly the degree of the sulfate substitution at the C-2 and C-4 positions because of the severe overlapping of the proton signals.

However, as mentioned above a smaller number of sulfate groups were introduced at the C-4 position (residues *VIII* and *IX*) than at C-2. So we may assume that the degree of sulfate substitution at the C-2 position of CRDS is approximately the same as the proportion of residues *I* and *II*. The peak intensity in the anomeric region suggested that the value of this fraction was about one third. Thus, the degree of substitution of CRDS by sulfate groups is estimated to be in the range of 1.3 and 1.4 groups per glucose unit. This value is in good agreement with the results from elemental analysis for sulfur.

The reactivity of the reagent (piperidine-*N*-sulfonic acid) toward hydroxyl groups is thought to depend mainly on the steric environment in each individual case. The hydroxyl group at the C-6 position is a primary alcoholic OH located outside the glucose ring, so the reagent can attack it easily. On the other hand, the hydroxyl groups at the C-2 and C-4 positions are secondary alcoholic OH groups, having a relatively higher steric hindrance than the OH at C-6. In a (1 → 3)-β-D-glucan, especially, the hydroxyl group at C-4 is close to both the glycosidic linkage and the sulfate group at C-6, and these would be expected to interrupt the access of the reagent. Such steric hindrance may explain why such a low level of sulfation at the C-4 position was observed.

Sequential connectivities of sulfated residues in CRDS.—We could extract some NOE cross-peaks between H-1 of a particular residue and H-3 of the adjacent unit toward the reducing end, as well between H-1 and H-3 of the same residue (Fig. 4). It was revealed in this way that the residue *II* is adjacent to residue *VII* in the non-reducing direction, and that the next neighboring unit in that direction is residue *III*. This result suggests that one-dimensional sequential regularity in a substituted polysaccharide can be recognized by the NOESY technique. Thus, this technique could become important for structure determination of polysaccharides. The NOESY spectrum shows that a proton resonance shift can be affected by the sulfate groups introduced not only in the same glucose unit but also in the nearest

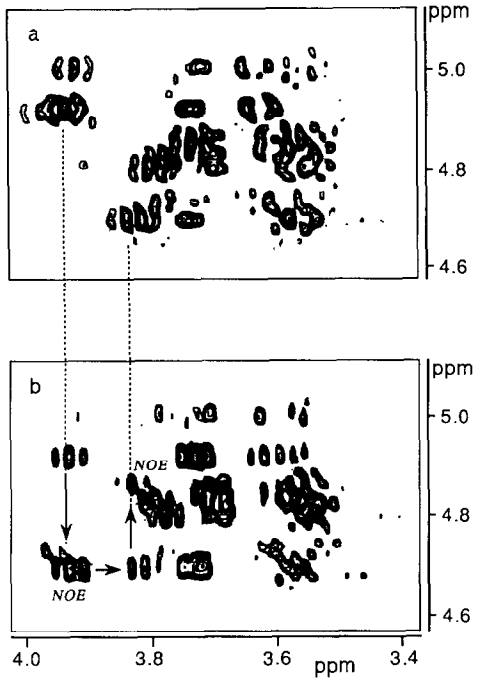


Fig. 4. 2D HOHAHA(a) and NOESY(b) spectra of CRDS in the cross peak region of the anomeric proton and other sulfated-glucose proton resonances. The mixing times were 75 ms for HOHAHA and 100 ms for NOESY. The NOESY spectrum contains extra cross-peaks (labelled NOE) corresponding to the short interproton distances (spatial proximity) between H-1 and H-3 of the neighboring unit toward the reducing end. The arrows in the NOESY spectrum show "sequential assignments" of sulfated glucose residues. The horizontal arrow points to intraresidue correlation cross-peaks and the vertical arrows lead to interresidue correlation cross-peaks.

neighbor. This is suggested as the explanation for the chemical shift difference that was observed between glucose residues having the same sulfate substitution (C-6), such as *III* and *VII*.

However, as for the other residues of CRDS the sequential connectivities could not be determined because of severe overlapping of the signals in the relevant region.

Assignment and characterization of ^{13}C NMR signals of CRDS.—The ^{13}C signals of CRDS could be completely assigned by using ^{13}C–^{1}H COSY (Fig. 5). The chemical shifts and the differences from the ^{13}C chemical shifts of unsubstituted curdlan are listed in Table II.

Large downfield shifts were observed for all C-6 signals (8.1 to 8.3 ppm), the C-2 signals of the residues *I*, *II*, and *VIII* (7.1 to 7.8 ppm), and the C-4 signals of the residues *VIII* and *IX* (7.7 and 7.9 ppm, respectively). These results also indicate that the hydroxyl groups at the C-6 position in all the residues, at C-2 in residues *I*, *II*, and *VIII*, and at C-4 in residues *VIII* and *IX* are substituted by sulfate

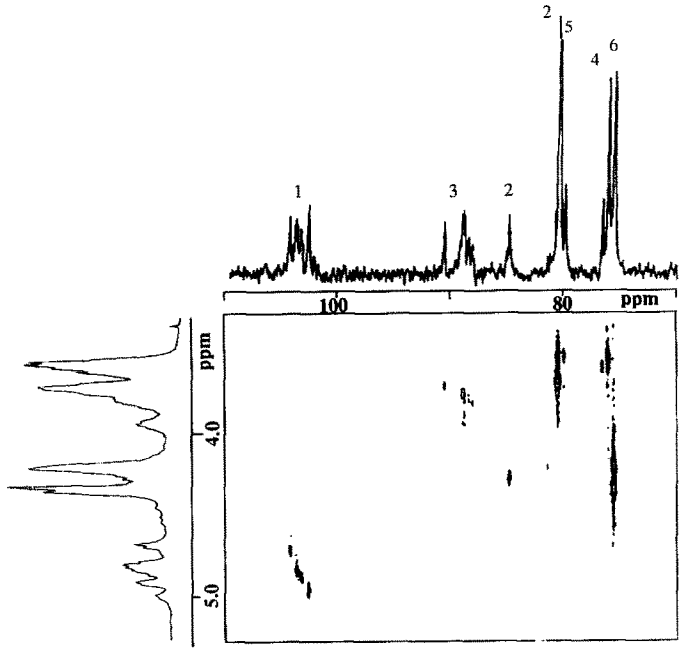


Fig. 5. Two-dimensional $^{13}C-^{1}H$ COSY spectrum of CRDS at 25°.

groups[9]. Small upfield shifts were observed for the signals of carbons adjacent to the substituted positions, such as C-5, owing to the β-effect of the sulfate group.

We compared the ^{13}C chemical shifts of CRDS in D_2O solution and in H_2O (containing 10% of D_2O for signal locking). Some C-2 and C-4 signals in H_2O were shifted 6–8 Hz lower than those in D_2O solution, while the others were not

TABLE II

Carbon chemical-shift assignments of CRDS

Residue types	Chemical shifts (ppm) [a]					
	C-1	C-2	C-3	C-4	C-5	C-6
I, II	103.7	81.3	86.8	70.4	75.9–76.1	69.8
	(−0.6)	(7.1)	(0.1)	(1.3)	(−0.9–−0.7)	(8.1)
III, IV, V, VI	104.5–105.2	75.2/75.8	85.8–87.0	71.0	75.9–76.1	69.8
	(0.2–0.9)	(1.0/1.6)	(−0.9–0.3)	(1.9)	(−0.9–−0.7)	(8.1)
VII	105.8	75.2/75.8	85.8–87.0	71.0	75.9–76.1	69.8
	(1.5)	(1.0/1.6)	(−0.9–0.3)	(1.9)	(−0.9–−0.7)	(8.1)
VIII	103.7	82.0	85.9	76.8	75.9–76.1	69.9
	(−0.6)	(7.8)	(−0.8)	(7.7)	(−0.9–−0.7)	(8.2)
IX	105.8	76.3	85.9	77.7	75.9–76.1	70.0
	(1.5)	(2.1)	(−0.8)	(7.9)	(−0.9–−0.7)	(8.3)

[a] Chemical shifts are referenced to internal DSS at 32°. The numbers in parentheses are chemical-shift differences with respect to curdlan.

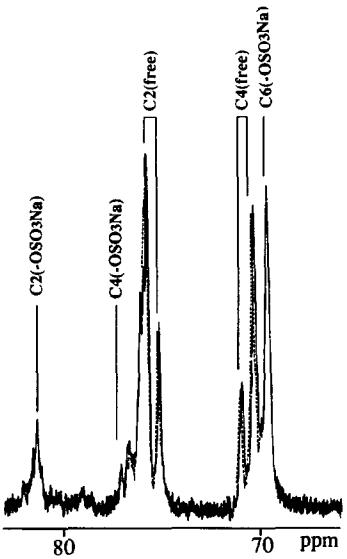


Fig. 6. Comparison of the ^{13}C NMR spectra of CRDS dissolved in D_2O (solid line) and in 90% H_2O–10% D_2O (dashed line).

changed (Fig. 6). It is known that ^{13}C resonances show an upfield shift when an atom connected to the ^{13}C directly or through two bonds is replaced by a heavier isotope[10]. The effective distance between the ^{13}C atom and the heavier isotope (deuterium in this study) becomes shorter than that between the ^{13}C atom and the lighter ligand, due to vibration anharmonicity, which results in shielding of the ^{13}C atom. Thus, in the case of CRDS, it can be concluded that a ^{13}C nucleus downshifted in H_2O is bound to a free hydroxyl group, while a ^{13}C the chemical shift of which is not changed is substituted. Replacement of OD by OH changes the magnetic environment of ^{13}C, while that of SO_3D by SO_3H has no influence because there are four bonds between the carbon and the hydrogen of the sulfate group. This signal characterization is consistent with the results derived from the assignments described above. It is useful in determining which ^{13}C is esterified by sulfate and which is not.

In the ^{13}C NMR spectrum of CRDS, fewer signals could be observed than in the ^{1}H NMR. Thus, ^{1}H NMR has more information than ^{13}C NMR for structure analysis for the purpose of determining the degree of substitution on each hydroxyl group.

EXPERIMENTAL

Curdlan was purchased from Wako Pure Chemicals (Osaka). It was sulfated with piperidine–*N*-sulfonic acid in dimethyl sulfoxide[11]. The average molecular mass of the CRDS sample using in this study was found by gel permeation

chromatography to be 4.8×10^4 daltons. The sulfur content was 14.8% by elemental analysis.

All NMR spectra were recorded on a JEOL GX 400 spectrometer operating at 400 MHz. A sample for NMR spectroscopy contained about 100 mg of CRDS dissolved in 0.5 mL of D_2O (99.95%, Merck). DQF-COSY spectra and 2D-HOHAHA spectra were used for the proton signal assignments of CRDS. HOHAHA spectra were recorded at various mixing times, with the most useful one being 75 ms. The NOESY spectrum of CRDS was also observed at a mixing time of 100 ms for investigation of the sulfated residue sequence regularity. For all proton–proton 2D NMR, 256 spectra of 2048 data points were recorded with 48 scans. The sweep width employed was 2500 Hz, and the total measurement time was ~ 14 h. The HDO signal was suppressed by presaturation at all times except during acquisition. Apodization was carried out in each domain; in the t_2 domain using a Gaussian function and in the t_1 domain using a phase-shifted sine-bell function for the spectra. Phase sensitive Fourier transformation was performed after zero-filling to a 4096×1024 data matrix size. Two-dimensional shifted correlated spectra (^{13}C–^1H COSY) were collected into 4096 points for 256 t_1 values with a recycle delay of 1.0 s. Spectra were acquired with sweep width of 6000 Hz for ^{13}C and 2000 Hz for ^1H. A pulse delay of 1.8 ms was used before and after the data acquisition. Apodizations were carried out in both domains using a Lorentzian function for the spectrum and transformation was performed after zero-filling to 4096×512 data matrix size.

The temperature of all 2D NMR experiments was 25°. However, chemical shifts (Tables I and II) are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) as the internal standard at 32°. At this temperature, no peaks are overlapped with that of HOD, so it is possible to recognize all signals in the 1D proton spectra, especially those needed for the estimation of the degree of sulfation. Chemical shift differences over this temperature range are negligibly small, except for the HOD signal.

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