NMR Spectroscopic Analysis of Sulfated β-1,3-Xylan and Sulfation Stereochimistry

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A novel sulfated β-1,3-xylan product was synthesized from algal cell wall microfibril homoxylan by the N,N-dimethylformamide (DMF)-SO4 complex sulfation method. Antithrombin activity appeared in this product was 6.5 times higher than that of standard heparin. From the results of 1H- and 13C-NMR spectroscopic analyses by DQF-COSY and HMOC and an infrared spectroscopic analysis, it was revealed that the ordered structure of β-1,3-xylan as a triple helix had decayed and the resulting conformational changes had been caused by the sulfation reaction. The sulfated positions on the C-4 hydroxyl groups of the xylene residues were determined from 13C-NMR chemical shifts, and it was found that regioselective sulfation had occurred predominantly with the C-4 secondary hydroxyl groups to produce a mono-substituent. Another type of sulfation of β-1,4-xylan that showed no regioselectivity is considered to have been due to the different conformation of both xylan chains such as the triple helix in β-1,3-xylan and the double straight chain like cellulose in β-1,4-xylan. Therefore, the different type of regioselective sulfation of β-1,3- and β-1,4-xylan was caused by the difference in steric hindrance due to these conformations. These different types of regioselective sulfation with different linkage positions are also discussed for the secondary hydroxyl groups in β-1,3- and β-1,4-glucan after chemoselective sulfation of the C-6 primary hydroxyl groups.

Key words: Caulerpa cell wall β-1,3-xylan; regioselective sulfation of the secondary hydroxyl groups; NMR spectroscopy; steric hindrance

Some types of sulfated polysaccharides from seaweed are known to show remarkable physiological activity such as antithrombinic9 and antiviral.10 These active sulfated polysaccharides were obtained as either natural or artificial products.35 In the latter case, when sulfated products from easily obtainable neutral polysaccharides with different types of linkage such as β-1,3- and β-1,4-glucan were synthesized, new properties like high heparinoid activity with different degrees were found in both products.13 This implies that the activity was deeply concerned with the structure and degree of substitution of the sulfate esters. The different types of reaction or sulfation mechanisms for polysaccharides are considered due to different types of linkage such as β-1,3- and β-1,4-, as well as to the kinds of constituent sugar residues in the polysaccharides. In general, chemoselective ether formation with the primary hydroxyl groups is predominant compared with the secondary hydroxyl groups. Therefore, the effect of regioselective sulfation on the secondary hydroxyl groups in polysaccharides is influenced by the varying degree of steric hindrance due to the polysaccharide structure. Although the properties of a sulfated β-1,4-xylan product obtained from a land plant cell wall have been reported,5 sulfated β-1,3-xylan products remain to be determined.

In previous studies, the fine chemical structure of the cell wall microfibril polysaccharides from Caulerpa brachypus belonging to Siphonales have been demonstrated to be non-branched straight chains of β-1,3-xylan.6 Clarification of this structure by NMR spectroscopy, including the total signal assignments of 1H- and 13C-NMR spectra by various 2D techniques, have been achieved,7 and all of the obtained results agree well with those of a chemical analysis.8 The obtained β-1,3-xylan was chemically sulfated in the present study, and the sites of sulfate ester substitution in the products were determined by 13C-NMR spectroscopy. Regioselective sulfation of the secondary hydroxyl groups in β-1,3- and β-1,4-xylan is also discussed in detail, as well as the different types of linkage in β-glucan.

Experimental

Materials. Cell wall β-1,3-xylan was obtained from algal fronds of Caulerpa brachypus as described elsewhere.9 Spectroscopic measurements. IR spectral and optical rotation measurements were similar to those described elsewhere.6 NMR spectra were measured in a D2O solution at 25°C with a JEOL α-500 spectrometer at 499.65 MHz for 1H and at 125.50 MHz for 13C, using sodium-2,2-dimethyl-2-silapentane-5-sulfonate-d4 (DSSMP) as the internal reference.

Sulfation of β-1,3-xylan.8 β-1,3-Xylan (0.5 g) from Caulerpa brachypus was allowed to swell well in N,N-dimethylformamide (DMF, 5 ml) and was then stirred for 12 h at room temperature under nitrogen gas. A DMF-SO3-complex reagent (50 ml) was added to the solution, and the mixture was stirred at 0°C for 30 min under nitrogen gas. The resulting inorganic sulfuric acid was neutralized by an aqueous solution of NaOH. Sulfated xylan was precipitated by adding 4 volumes of ethanol to the reaction mixture. After centrifugation, the precipitate was dissolved in water (100 ml), and the supernatant obtained after the second centrifugation from the insoluble part was dialyzed and lyophilized (yield, 0.8 g).

Chromatographic purification of sulfated xylan. The sulfated xylan (100 mg) was subjected to ion-exchange chromatography by DEAE-Toyopearl 650C (50 cm x 22 mm i.d.), being eluted by an aqueous KCl solution (1.5 liters) with a linear concentration gradient from 0 to 1.0 M. Gel filtration chromatography by Toyopearl HW-55F (100 cm x 9 mm i.d.) was eluted by distilled water for 1 mg of the sample. The elution profile

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Abbreviations: DQF-COSY, double quantum filtered-COSY; DS, degree of substitution; HSSQC, heteronuclear single quantum coherence.
was colorimetrically monitored by the phenol-sulfuric acid method. The final recovery of the purified materials, \([\alpha]_{D}^{20} = -76.2 (c = 0.5, \text{H}_2\text{O}),\) was estimated to be about 68%.

Cellulose-acetate membrane electrophoresis. Electrophoresis on a cellulose acetate strip (Fuji Film Co., 1 x 11 cm) was conducted with a 0.5 M phosphate buffer (pH 6.0) at a constant current of 0.5 mA/cm within 30 to 90 min. The strip was developed by dipping it into a toluidine blue reagent.

Microdetermination of sulfur by the combustion flask method. A dried sample (5 mg) was placed in a platinum bucket in the combustion flask (300 ml, Hamada Rika Co.) with an aliquot of hydrogen peroxide (0.5 ml). After the flask had been filled with oxygen to replace the air, the sample was ignited in the flask. The generated SO\(_2\) gas was absorbed by the hydrogen peroxide solution. The resulting solution was neutralized by 2 N NaOH to pH 6-7 and then titrated against a standard solution of 0.005 M BaCl\(_2\), using a 0.1% dimethylsulfoxonazo III reagent as the indicator. Since the standard solution (1 ml) corresponds to 0.1603 mg of sulfur, the percentage by weight of sulfur was calculated. The degree of substitution (DS) was evaluated as the molar ratio of sulfur to the anhydroxylpyranosyl residue.

Assay for antithrombin activity. All biological activities are expressed on the basis of the weight of material vacuum-dried over P\(_2\)O\(_5\) and are compared to those of the same weight of standard heparin (140 IU mg\(^{-1}\)), supplied by National Institute of Hygienic Science (Japan). Human serum and commercial bovine thrombin were purchased from Sigma. The thrombin was dissolved in a physiological saline solution to produce a concentration of 50 NIH unit ml\(^{-1}\). Fibrinogen (purchased from Oriental Yeast) was dissolved to 0.5% in physiological saline solution.

The antithrombin activity of each sulfated polysaccharide was assayed with human serum as the heparin cofactor. After a mixed solution of thrombin, serum and a 0.1 M barbital buffer solution (pH 7.2) had been preincubated at 37 C for 30 min, an aliquot of the fibrinogen solution was added and the clotting time (s) of fibrin was measured as a blank (T\(_0\)). The clotting time for the sample (T\(_x\)) was evaluated with a mixture of heparin or the sulfated polysaccharide solution with thrombin and serum. Antithrombin activity is expressed by the difference in clotting times as T\(_x\) - T\(_0\).

Results and Discussion

After successive column chromatography, the sulfated \(\beta\)-1,3-xylan had a homogeneous charge and molecular size distribution. Cellulose acetate membrane electrophoresis of the sulfated xylan from this reaction revealed a single band that migrated toward the cathode. The sulfur content was determined to be 14.63%. Therefore, it was concluded that the sulfated product under the reaction conditions employed contained 1 mol of sulfate ester residue per xylpyranosyl residue. When the antithrombin activity (ATA) was compared for the synthetic sulfated xylan, it was about 6.5 times higher than that of standard heparin. This value is similar to or much higher than that of rhamnan sulfate from Monostromace, or arabinan sulfate from Codiaeaceae.

Since the sulfated product obtained in this way showed a typical IR absorbance at 1240 cm\(^{-1}\) due to the \(\text{S=O}\) stretching vibration of the sulfate ester, it was confirmed that effective sulfation had proceeded under the experimental conditions with the xylopyranosyl residues in \(\beta\)-1,3-xylan. The product also showed distinct IR spectral features at 920 cm\(^{-1}\) due to ring-breathing vibration (type 1) and at 760 cm\(^{-1}\) due to ring-bending vibration (type 3), both of which are characteristic of a pyranose ring. In the original \(\beta\)-1,3-xylan, no types 1 and 3 absorption bands were detected in the IR spectrum, and only a type 2b absorption band was found at 890 cm\(^{-1}\), representing a \(\beta\)-configuration due to axial-axial interaction between the anomic and C-5 protons. These results are similar to those of the cellulose IR spectrum, only a type 2b absorption at 890 cm\(^{-1}\) being detectable for crystalline cellulose, and not types 1 and 3. Types 1 and 3 absorption bands, however, have appeared distinctly in amorphous cellulose and deuterated derivatives. Due to intra- and intermolecular hydrogen bonding in a triplet helical structure, the original \(\beta\)-1,3-xylan formed a crystalline structure like that of cellulose, and ring breathing or bending vibrations in the IR spectrum were restricted. Such a structure was, however, broken by sulfation, and sulfated xylan was obtained in a noncrystalline form.

The \(^1\)H- and \(^13\)C-NMR spectra for sulfated xylan (Figs. 1 and 2) show major peaks due to regularly produced monosubstituted sulfated xylan, and several minor peaks in the sulfated product. These minor peaks may have originated from minor amounts of irregularly monosubstituted and/or dissubstituted sulfated xylan. In this present study, the discussions will focus on only the major peaks of monosubstituted sulfated xylan.

Since one anomic signal was observed in both the \(^1\)H- and \(^13\)C-NMR spectra, it appears that complete sulfation had occurred throughout the xylan chain. Total assignment of the \(^1\)H-NMR spectrum signals was completed with the DQF-COSY spectrum (Fig. 3 and Table A). Comparing the sulfated and original \(\beta\)-1,3-xylan in their \(^1\)H-NMR spectra, differences in chemical shifts at H-1 to H-5 were 0.5 (H-1), 1.2 (H-2), 0.7 (H-3), 1.0 (H-4), 0.5 ppm.

![Fig. 1. \(^1\)H-NMR Spectrum of Sulfated \(\beta\)-1,3-Xylan.](image1)

![Fig. 2. \(^13\)C-NMR Spectrum of Sulfated \(\beta\)-1,3-Xylan.](image2)
Table Chemical Shifts (δ ppm from DMSO) in the 1H-NMR (A) and 13C-NMR (B) Spectra of Sulfated and Original β-1,3-Xylan

<table>
<thead>
<tr>
<th></th>
<th>H-1</th>
<th>H-2</th>
<th>H-3</th>
<th>H-4</th>
<th>H-5ax</th>
<th>H-5eq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfated β-1,3-xylan a</td>
<td>5.16</td>
<td>4.61</td>
<td>4.28</td>
<td>4.61</td>
<td>3.80</td>
<td>4.41</td>
</tr>
<tr>
<td>Original β-1,3-xylan a</td>
<td>4.63</td>
<td>3.44</td>
<td>3.55</td>
<td>3.62</td>
<td>3.28</td>
<td>3.93</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th></th>
<th>C-1</th>
<th>C-2</th>
<th>C-3</th>
<th>C-4</th>
<th>C-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfated β-1,3-xylan</td>
<td>101.2</td>
<td>76.7 or 77.5</td>
<td>77.5</td>
<td>76.7 or 77.5</td>
<td>63.3</td>
</tr>
<tr>
<td>Original β-1,3-xylan a</td>
<td>107.1</td>
<td>76.5</td>
<td>88.9</td>
<td>70.7</td>
<td>68.3</td>
</tr>
</tbody>
</table>

* Spin-coupling constants could not be obtained because of signal broadening.

is, the substituted carbon signal in the 13C-NMR spectrum was shifted toward a lower magnetic field due to the electron negativity of the substituent, and both vicinal carbon signals were slightly shifted toward a higher magnetic field. To determine the substitution site of sulfation on xylopyranosyl residues by the 13C-NMR spectrum, the chemical shifts of the sulfated and original xylan were compared. Individual carbon signals in the 13C-NMR spectrum of sulfated xylan were assigned by using HSQC. In the 13C-NMR spectrum (Fig. 2), the C-2 and C-4 signals were closely related at 76.7 or 77.5 ppm. When the chemical shifts were compared with the original β-1,3-xylan, however, a marked shift to a lower magnetic field was observed at C-4 with different values of 6.0 or 6.8 ppm. In contrast, the signals for both vicinal carbon atoms at C-3 and C-5 were shifted to a higher magnetic field. These results show that regioselective sulfation occurred regularly at C-4 for each xylopyranose residue in β-1,3-xylan, and that random sulfation did not occur. Consequently, the majority of sulfation products were esterified at the C-4 hydroxyl group site. 13C-NMR signals for the C-2 carbons of sulfated xylan, another candidate site for sulfation, were shifted to a slightly higher magnetic field at 0.2 or 1.0 ppm. This slight shifts was not marked, and no substitution effects at the xylopyranosyl residue of the C-2 hydroxyl groups were recognized. From these results, it is concluded that, in the major β-1,3-xylan sulfation products, sulfation scarcely occurred at C-2, or randomly at C-2 and C-4 simultaneously, but predominately occurred at the C-4 hydroxyl group in each xylopyranose residue of the original xylan. DS values calculated as 1 from a sulfur analysis of all xylopyranosyl residues support these conclusions.

The possibility of disubstitution by two sulfate esters at both the C-2 and C-4 sites was not the main sulfation effect,
although the presence of major peaks in the $^1$H- and $^{13}$C-NMR spectra may suggest such substitution. In the $^1$H-NMR spectrum, the differences in chemical shifts at H-2, H-3, and H-4 were large, as already described. The difference in H-4 (1.0 ppm) was directly caused by the bulky anionic substituents of sulfate esters in the C-4 hydroxyl groups. The conformation change by substitution of the sulfate esters in the molecule influenced the chemical shift of the H-3 proton atom that was at the glycosidic linkage position, such a change being in the strain and/or stretch of the helical conformation in the original $\beta$-1,3-xylan. The largest difference in H-2 (1.2 ppm) was also caused by conformation changes with the substitution of sulfate esters and the decay of hydrogen-bonding networks in the helical conformation. Thus, the substitution of sulfate esters in the original $\beta$-1,3-xylan caused the change in conformation of the xylpyranose ring and/or macromolecule of $\beta$-1,3-xylan.

Since the type of sulfation of a polysaccharide depends on the sugar residues and linkage positions, the sulfation of xylan was quite different from that of glucan. In $\beta$-1,4- and $\beta$-1,3-xylan, there are two equivalent secondary hydroxyl groups. Regioselective substitution specifically and predominantly proceeded in the C-4 hydroxyl groups for $\beta$-1,3-xylan sulfation. With $\beta$-1,4-xylan, substitution reportedly occurred equally in the C-2 and C-3 hydroxyl groups to produce a mixture of equal amounts of monosubstituted xylan in the 2- or 3-sulfated xylose residues. No disubstituted reaction products were seen in either the 2- or 3-hydroxyl groups of the xylose residues. The type of regioselective sulfation in the C-4 hydroxyl group of the xylose residues in $\beta$-1,3-xylan differed specifically from that in the secondary hydroxyl group of $\beta$-1,4-xylan.

With hexosan, chemoselective sulfation of a more reactive primary hydroxyl group at C-6 proceeded before regioselective substitution of a secondary one. Sulfation, therefore, progressed in the order of C-6, C-2, and C-3 in a ratio of about 10:5:1 in $\beta$-1,4-glucan (cellulose). A similar type of substitution has also been seen in $\beta$-1,3-glucan (curdlan), in which substitution occurred primarily at C-6, and less but equally at C-2 and C-4. Different regioselective sulfation of secondary hydroxyl groups should arise from the different types of linkage in cellulose and curdlan. These results suggest similar considerations for the sulfation products of $\beta$-1,3- and $\beta$-1,4-xylan due to their different types of linkage.

Regioselective sulfation of the secondary hydroxyl groups were compared for $\beta$-1,3-xylan and $\beta$-1,3-glucan. The $\beta$-1,3-xylan chains are reported to twist to form a triple helix, so that C-4 hydroxyl group would be oriented toward the outside of the chains to react more easily in the surrounding environment. The C-2 hydroxyl group is less reactive than the C-4 one, since it is oriented toward the inside and is obscured by the chain. $\beta$-1,3-glucan seems to be similar. In the twisted conformational structure of $\beta$-1,3-glucan, the primary hydroxyl group is oriented toward the outside as a bulky substituent on the polysaccharide chain, but the secondary hydroxyl groups at C-2 and C-4 are oriented toward the inside and obscured by the chain. Therefore, chemoselective sulfation progressed predominantly with the primary hydroxyl groups at C-6 and a lesser regioselective reaction with the C-2 or C-4 hydroxyl group following.

When the regioselective substitution in the secondary hydroxyl groups of $\beta$-1,3- and $\beta$-1,4-xylan are compared, each type of reaction seems to have been different due to steric hindrance of these hydroxyl groups. Since equatorial hydroxyl groups at C-2 of $\beta$-1,3-xylan (Fig. 5A) exist between two adjacent xylopyranosyl residues (R$_1$ and R$_2$ in Fig. 5) through the glycosidic bond at C-3 and C-1, both xylopyranosyl residues are bulky substituents, sterically hindering the C-2 hydroxyl groups during sulfation. The secondary hydroxyl group at C-4 is in a spatially open area and has smaller neighboring methylene groups at C-5. It is thus more difficult for sulfite groups to approach the secondary hydroxyl group at C-2 than the secondary hydroxyl group at C-4. With $\beta$-1,4-xylan (Fig. 5B), the correlation of the C-2 and C-3 hydroxyl groups to the neighboring xylopyranosyl residues is in almost the same spatially open area, allowing sulfation to progress equally without any marked steric hindrance. A type of steric hindrance quite different from that with xylan due to the C-6 primary hydroxyl group was observed for $\beta$-1,3-glucan. First, chemoselective sulfation progressed in the primary hydroxyl groups, which was followed by regioselective sulfation of the less-reactive C-4 or C-2 secondary hydroxyl group. The resulting chemoselective sulfation products

![Fig. 5. Steric Hindrance to the Secondary Hydroxyl Groups of $\beta$-1,3-Xylan (A) and $\beta$-1,4-Xylan (B).](image)

![Fig. 6. Steric Hindrance to the Secondary Hydroxyl Groups of $\beta$-1,3-Glucan after Chemoselective Sulfation.](image)
Fig. 7. Steric Hindrance to the Secondary Hydroxyl Groups of β-1,4-Glucan after Chemoselective Sulfation. Circles indicate the steric hindrance effects of bulky substituents to the secondary hydroxyl groups. (●●● indicates a hydrogen bond.)

of the sulfate ester at C-6 (Fig. 6) behaved as bulky substituents, and successive regioselective sulfation at C-2 and C-4 of the secondary hydroxyl groups of β-1,3-glucan progressed almost equally. These proposals are reasonable when the experimental results of sulfation are considered. As shown in the structural diagram for β-1,4-glucan (cellulose) in Fig. 7, the distance between the C-6 and C-2' hydroxyl groups in adjacent glucopyranose residues in the 4C1 conformation is so little that intermolecular hydrogen bonding is formed along the polysaccharide chain. After chemoselective sulfation had progressed at C-6 of the primary hydroxyl groups, the substituted sulfate group at C-6 behaved as a bulky substituent. To avoid steric hindrance of the C-2' hydroxyl groups, this bulky substituent was forced to turn in a direction different from the neighboring C-3 hydroxyl groups of the glucopyranose residues. Products with bulky substituents in the primary hydroxyl groups (C-6) seem almost covered around the C-3 hydroxyl groups of neighboring glucose residues. With cellulose sulfation, the resulting steric hindrance from the first sulfation due to the bulky sulfate substituent at C-6 causes the second regioselective sulfation of the C-2 hydroxyl groups to be slightly more dominant than of the C-3 hydroxyl groups. These considerations explain the sulfation order of C-6, C-2, and C-3 in a ratio of about 10:5:1 for β-1,4-glucan as described, and equivalent substitution in the C-2 and C-3 hydroxyl groups of β-1,4-xylan. It is thus noteworthy that sulfation of the secondary hydroxyl groups differed for β-1,3-xylan, β-1,4-xylan and glucans due to steric hindrance resulting from different glycosidic linkages and sugar residues. The physiological activity of sulfated β-1,3-xylan will be reported elsewhere.

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References