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Location of sulfate groups on sulfoacetate derivatives of cellulose☆

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Abstract

A water-soluble cellulose acetate sulfate (CAS) with a degree of acetylation (DS_{Ac}) 2.4 and a degree of sulfation (DS_{Sulf}) of 0.3 was obtained by direct acetylation of cellulose using sulfuric acid as catalyst. Using methylation analysis, IR and NMR spectroscopy, sulfate groups have been located on primary alcohol function of glucose residues. The distribution of the sulfate groups along the cellulose chain has been investigated using enzymatic hydrolysis. CAS was first de-acetylated under mild hydrolysis conditions (NaOH 0.25 mol/L at room temperature), and then cellulose sulfate was hydrolyzed by a cellulolytic complex (Celluclast 1.5L). Reaction products were separated by ion exchange chromatography on a DEAE Sepharose CL6B column into five fractions F_1, F_2, F_3, F_4 and F_5, which were analyzed for their chemical composition. F_1 was glucose and represented the main product of reaction ($\sim$50% of the initial glucose), F_2 was a dimer ($\sim$30%) with a ratio Sulfates–Glucose of 0.41 (about one sulfate group for two glucose units), F_3 a trimer ($\sim$10%) with a ratio Sulfates–Glucose of 0.62 (about two sulfate groups for three glucose units), and F_4 a tetramer ($\sim$5%) with a ratio Sulfates–Glucose of 0.69. The structure of the oligomers was established using ^1H and ^{13}C NMR. The observed proportion of the different blocks of sulfate groups was in good agreement with computed random distribution.

Keywords: Cellulose sulfate; Cellulose acetate; Acetylation; Sulfation; Enzymatic hydrolysis; Structures; NMR

1. Introduction

Cellulose acetates are the most important cellulose derivatives because of their broad application in plastic and fibers. Cellulose acetates are usually water-insoluble for substitution degree higher than 1.[3] Performing a sulfuric acid-catalyzed acetylation, a cellulose acetate with an acetylation degree of 2.4 was obtained, the latter being water-soluble.[1,4] Sulfate groups were shown to be present on the polymer and are responsible for its unusual water-solubility.

The competition between hydrophilic groups (sulfate) which confers a good solubility in water and hydrophobic groups (acetate) which probably mediate or modulate association between polymer chains led to very interesting properties as assessed by high reduced viscosity.[1] Gelling, thixotropic and shear thinning characters have been shown for this new cellulose derivative and the rheological properties of the cellulose acetate sulfate (CAS) solution appear comparable to the rheological behavior of associative polymers.[2]

The distribution of substituents in cellulose derivatives is considered to strongly influence the properties

Table 1
Composition of cellulose derivatives

	Cellulose	CAS	CS
Yield		168 [a]	57 [b]
Glc [c]	974	536	800
Ara	1	2	5
Man	0	8	14
Xyl	2	8	11
Acetates	0	328	5
Sulfates	0	96	136
Ashes	0	73	113
Total	977	1051	1084

[a] g/100 g of cellulose.
[b] g/100 g of CAS.
[c] In mg/g dry weight matter.

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of these derivatives. The precise determination and control of substituent distribution are thus important to understand structure–property relationships. Sulfate distribution on the anhydroglucose unit and on the polymer chain of CAS has been investigated using methylation, IR spectroscopy, enzymatic hydrolysis and ^{13}C NMR analysis.

2. Results

2.1. Characterization of starting cellulose, CAS and cellulose sulfate (CS)

2.1.1. Chemical composition. The chemical composition of starting cellulose and cellulose derivatives is reported in Table 1. Cellulose Avicel is almost pure cellulose but low amounts of arabinose (1 mg/g) and xylose (2 mg/g) are also detected. The yield of CAS was 168% (w/w), and 92.4% of glucose from initial cellulose is recovered. The main constituents of CAS are glucose (536 mg/g), acetate 328 mg/g ($DS_{Ac} = 2.4$) and sulfate 96 mg/g ($DS_{Sulf} = 0.30$). Traces of arabinose, xylose and mannose are also detected. Ash content is 73 mg/g (sulfate being quantified twice: in the sulfate content and in ashes). This composition is in agreement with previously published data.[1,4]

After de-acetylation, the recovery of glucose and sulfate in CS is 81 and 85%, respectively. CS is mainly composed of glucose (800 mg/g); sulfate 136 mg/g ($DS_{Sulf} = 0.29$) and traces of arabinose, xylose and mannose are also detected. Ash content is 113 mg/g, and no acetate is detected. The recovery obtained indicated that no degradation of the CAS occurred during de-acetylation.

Water solubility of CAS was clearly linked to the presence of sulfate groups since cellulose acetates are water-insoluble for an acetate content higher than 13–14% (w/w).[3]

2.1.2. Physicochemical characterization. An intrinsic viscosity of 120 mL/g in cupriethylene-diamine was measured for cellulose Avicel. A degree of polymerization ($\overline{DP_v}$) of 200 was determined using the Mark–Houwink–Sakurada relationship established for cellulose.[5,6] Reduced viscosity as function of concentration was determined for CAS and CS (Fig. 1). As previously reported,[1,2] a slight polyelectrolyte effect due to the presence of ionic sulfate groups was observed for cellulose derivatives and was more pronounced for CS than for CAS as shown on Fig. 1. Reduced viscosities determined in water at 1 mg/mL are reported for CAS and CS in Table 2. A large decrease of reduced viscosity occurred after de-acetylation (1027 and 227 mL/g for CAS and CS, respectively). NaCl (0.1 M) was used to screen the ionic effect of sulfate groups and an intrinsic viscosity of 110 mL/g was determined for CS. CAS was insoluble in 0.1 M NaCl.

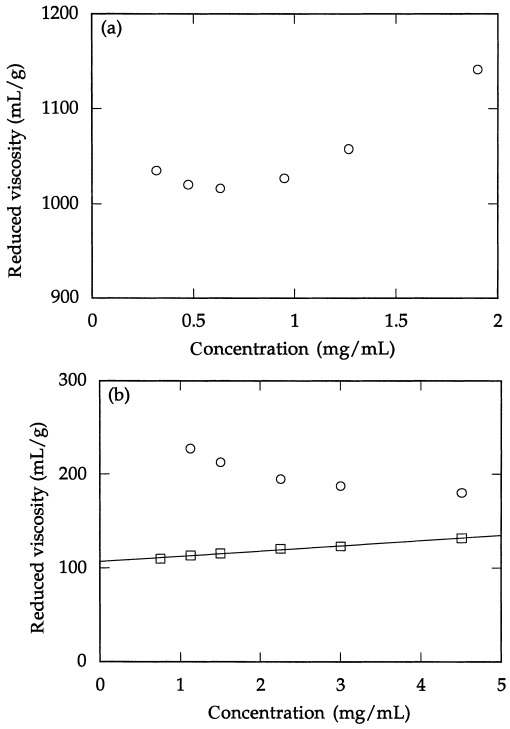


Fig. 1. Reduced viscosity as a function of concentration for: (a) CAS in water at 25 °C; (b) CS in water at 25 °C (circles) and in 0.1 mol/L NaCl at 30 °C (squares).

Table 2
Physico-chemical properties of Avicel cellulose and cellulose derivatives

	Cellulose	CAS	CS
$[\eta]$ (mL/g)	120 [a]		110 [b]
η_{red} (mL/g)		1027 [c]	227 [c]
$\overline{M_v}$	32,400 [a]		
$\overline{M_w}$			26,950 [d]

[a] Determined in cupriethylenediamine at 25 °C.
[b] Determined in NaCl 0.1 mol/L at 25 °C.
[c] Determined in water at 25 °C for a concentration of 0.5 mg/mL.
[d] Determined by HPSEC-MALLS in $NaNO_3$ 5×10^{-3} mol/L at 25 °C.

A weight average molar mass ($\overline{M_w}$) of 27,000 ($\overline{DP_w} = 170$) was measured for CS using HPSEC-MALLS, and the polymer exhibited a moderate polydispersity ($\overline{M_w}/\overline{M_n} = 1.6$). According to the degree of polymerization determined by viscometry for original cellulose, it is likely that the different chemical treatments (acetylation and de-acetylation) had little affected the length of the chains in CS.

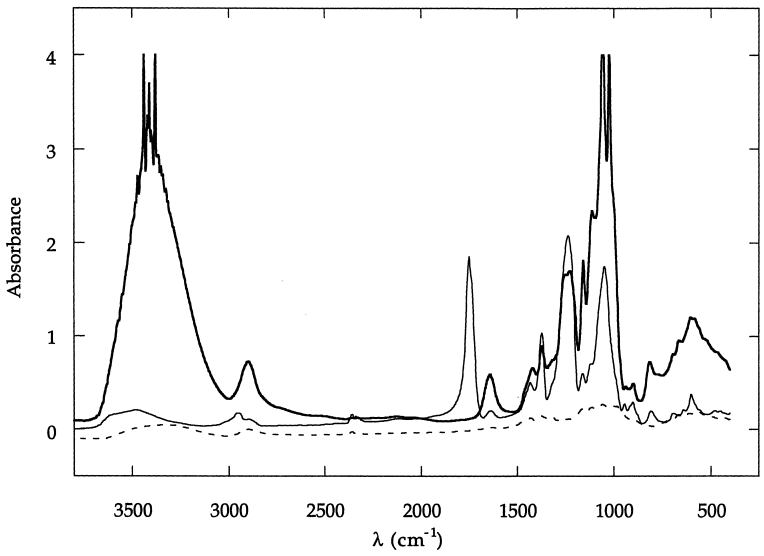


Fig. 2. IR spectra of cellulose (dotted line), CAS (continuous line) and CS (bold line).

2.1.3. Structural characterization. Infrared spectroscopy was performed on original cellulose and on cellulose derivatives (Fig. 2). Typical IR spectra of polysaccharides were observed with specific peaks for C–O–C linkage of glucose rings (between 950 and 1160 cm^{-1}), CH groups (between 2700 and 3000 cm^{-1}) and OH groups (between 3000 and 3700 cm^{-1}).[7] Acetylation of CAS was clearly evidenced by the disappearance of the hydroxyl absorption at 3300 cm^{-1} and concomitant appearance of ester carbonyl absorption at 1750 cm^{-1}.[8] CS exhibited no peak at 1750 cm^{-1}, confirming that de-acetylation was complete. Peaks at 1238 cm^{-1} confirmed the presence of sulfate ester and the peak at 813 cm^{-1} indicated that sulfate groups were located on position 6 of glucose ring.[9]

Methylation according to the Hakomori method[10] was performed on CAS and CS. Results are reported in Table 3. Three methyl ethers (2,3,4,6-tetramethyl-1-acetyl-glucose; 2,3,6-trimethyl-1,4-diacetyl-glucose and 2,3-dimethyl-1,4,6-triacetyl-glucose) are observed, each in similar proportion for CAS and CS. Acetate groups are lost in the strong alkaline conditions of the methylation, whereas sulfate groups are resistant, thus only OH groups that are not sulfated nor involved in a glycosidic linkage are methylated. The presence of 2,3-methyl glucose indicated that sulfate groups are esterified on the primary alcohol functions. DS_{Sulf} determined from methylation analysis (0.25 for CAS and 0.23 for CS) were in fairly good agreement with composition analysis, but suggested that some sulfate groups might have been lost during methylation analysis. As a matter of fact, previous authors have shown that sulfate groups substituted at positions C-2 or C-3 of glucose ring can be lost under the conditions of methylation.[11]

2.2. Enzymatic hydrolysis of CS

Two commercial enzymes were tested (Celluclast 1.5L and an endoglucanase) for the degradation of CAS and CS. Viscometric measurements showed that CAS was not hydrolyzed by enzymes. The high substitution degree of CAS (DS_{Ac} 2.4 and DS_{Sulf} 0.3) prevents the action of cellulolytic enzymes and CAS was thus de-acetylated.

Both enzymes cause a rapid and large loss of viscosity of CS solution attesting a decrease of the degree of polymerization of the chain (Fig. 3). The plot of $1/\eta_{spe}$ as a function of time is linear, indicating the action of endoglucanase acting in a random mode of action.[12] Furthermore, the similar slopes indicate similar hydrolysis rate in the first hour of reaction for both enzymes. However, a higher percentage of hydrolysis was observed for Celluclast (8%) than for Cellulase (4%) over longer incubation time (40 h), indicating a more extensive degradation of CS. As a matter of fact, beside endoglucanase activity Celluclast exhibits exoglucanase and β-glucosidase activities.

Degradation products of Celluclast were analyzed by HPSEC. In the early steps of the degradation (15 min,

Table 3
Glycosyl analysis [a] of methylated cellulosic derivatives

Sugar	CAS	CS
2,3,4,6-Glc [b]	2	2
2,3,6-Glc	73	75
2,3-Glc	25	23

[a] mol%.
[b] 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, etc…

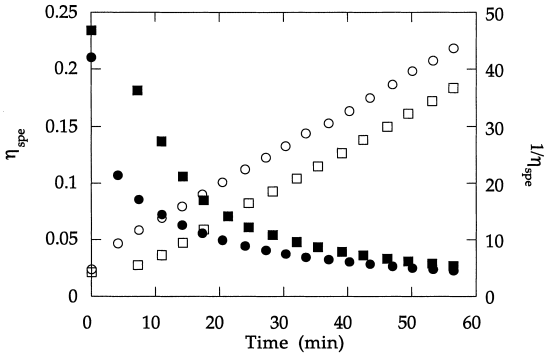


Fig. 3. Specific viscosity (closed symbols) and inverse ratio of specific viscosity (open symbols) during kinetics of hydrolysis in water at 30 °C of CS by celluclast (circles) and cellulase (square).

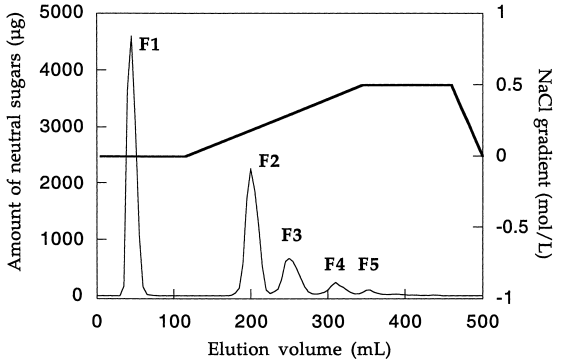


Fig. 5. DEAE sepharose CL6B fractionation (continuous line) of hydrolyzed CS by celluclast (40 °C, 72 h) and NaCl gradient (bold line).

Fig. 4(a)) glucose, cellobiose, cellotriose and cellotetraose were detected together with oligomers of higher degree of polymerization probably bearing sulfate groups. After 72 h of reaction, the chromatographic profile exhibited 5 major peaks (Fig. 4(b)) f_1, f_2, f_3, f_4 and f_5 representing, respectively 50, 32, 11 and 4% of the total quantity eluted. These peaks are final degradation products of CS by endoglucanase and glucosidase enzymes of Celluclast complex. Peak f_1 has similar retention time to glucose and other fractions are sulfated oligomers of higher DP.

Products of CS degradation by Celluclast were isolated on a preparative scale using anion exchange chromatography (Fig. 5). A neutral fraction F_1 was eluted by water whereas 4 sulfated oligomers (F_2–F_5) were eluted by a linear gradient of NaCl according to their charge. Eighty and 98% (w/w) of the neutral sugars and sulfate, respectively, were recovered after chromatography. Each fraction (F_1–F_5) was isolated and injected on HPSEC giving a peak with a similar retention time to the corresponding (f_1–f_5) fractions. The chemical composition of the five fractions is reported in Table 4. F_1 is glucose, whereas fractions (F_2–F_5) are constituted of sulfate and glucose, with a molar ratio sulfate–glucose increasing from F_2 (0.41) to F_5 (0.81).

2.3. ^{13}C NMR spectroscopy

The ^{13}C NMR spectra of oligosaccharide fractions (F_2–F_5) were measured (Fig. 6) and peaks were assigned by comparison with literature data for cellobiose, cellotriose[13] and a sulfated glucan isolated from red

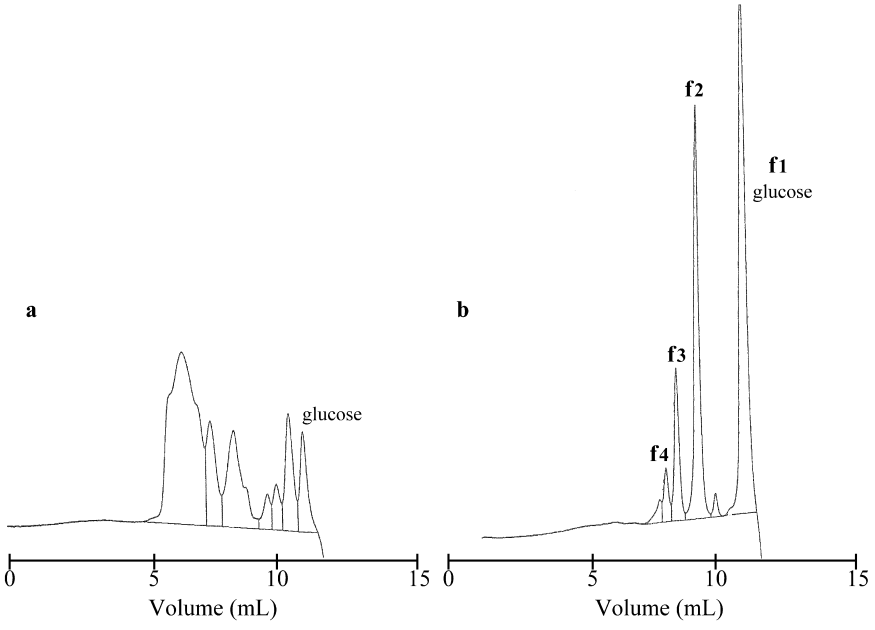


Fig. 4. Chromatography on Shodex OHpak-802.5 of the celluclast degraded CS after 15 min (a) and 72 h (b) of hydrolysis at 40 °C.

Table 4
Yields and composition of oligomers isolated on DEAE Sepharose CL6B after enzymatic digestion of CS by Celluclast

	Glucose (mg)	Sulfate (mg)	Sulf/Glc (molar ratio)
F_1	129.0	0.0	0.00
F_2	803	19.5	0.41
F_3	23.9	7.9	0.62
F_4	11.0	4.5	0.69
F_5	4.6	2.2	0.81
Yield [a]	80	98	

[a] Recovery in g/100 g of glucose (sulfate) injected on the column.

seaweed.[14] This particular glucan is composed of a majority of 4-linked glucose 6-sulfate with typical ^{13}C NMR signals for C-6, C-5 and C-4 at 67.7, 73.7 and 79.9 ppm, respectively. Attributions of the signal for methylene carbons were further checked by their inversion in a DEPT experiment (Fig. 7). The chemical shift of the different carbon signals in these spectra are reported in Table 5. The chemical shift of signals of the F_2 fraction agreed with those of cellobiose except that for C-6 and C-5 of the non-reducing end unit. The C-6nr was attributed by comparison with the C-6 signal of the algal sulfated glucan and its inversion on the DEPT spectrum. The chemical shift for C-5nr was attributed by deduction to the signal at 74.8 ppm. The signals on the spectrum of the F_3 fraction were attributed by comparison with the data of the F_2 fraction and those of cellotriose. The data from the latter oligosaccharide helped in the attribution of the resonance of C-1, C-2 and C-3 of the internal glucose unit. The chemical shifts of C-4, C-5 and C-6 of this unit (67.6, 73.7, 79.9 ppm) were in good agreement with those of the algal sulfated glucan. The signals on the spectrum from fraction F_4 were identified based on the attributions of those of F_3. The additional signal at 103.5 ppm (C-1) and the signals splitting at 73.9 and 73.7 ppm, for C-2 and C-5 of the internal glucose residues, respectively, indicate some differences in the environment of the additional internal unit. The degree of polymerization of the different oligosaccharide fractions was calculated on the basis of the C-1 intensity from the reducing (α and β, 92.9 and 96.9 ppm) end, non-reducing end (103.8 ppm) and internal units (103.6, 103.5 ppm). F_2, F_3 and F_4 are thus identified as di-, tri- and tetrasaccharide of the cellodextrin family sulfated at C-6 of all the 4-linked glucose residues except the reducing end unit (Scheme 1).

3. Discussion and conclusion

CAS exhibits interesting rheological properties, which are due to the balance between hydrophobic acetate and hydrophilic sulfate groups. The water solubility of CAS is clearly due to the presence of sulfate groups on the polysaccharide chain. Using IR and NMR spectroscopy as well as methylation analysis, we have shown that sulfate groups were mainly located on primary alcohol function of glucose residues. Degradation by cellulolytic enzymes has provided information concerning the distribution of sulfate groups along the polymer chain. Final products of degradation of CAS by the mixture of endoglucanase and β-glucosidase from Celluclast are glucose and a series of sulfated oligomers. Sulfated oligomers have been identified by NMR as di-, tri- and tetrasaccharide of the cellodextrin family sulfated at the primary hydroxyl group C-6 of all the glucose residues except the reducing end unit. Fraction F_5, which represents 2% (w GLC fraction/w total GLC) of the polymer, was not isolated but has a degree of polymerization higher than 4 and a ratio sulfate–glucose of to 0.81 and is likely to be a pentasaccharide of the same family (Table 4). Furthermore, oligosaccharides sulfated on position 2 or 3, were not observed attesting that 6 position was the unique site of sulfation. We have compared the distribution of blocks of sulfate groups obtained from enzymatic degradation with a theoretical random distribution of these groups along the polymer chain (Table 6). Although blocks of sulfate groups over a DP 4 were not experimentally observed, probably due to their low amount, a pretty good agreement was obtained between experimental and simulated distributions.

4. Experimental

4.1. Materials

Cellulose Avicel was purchased from Fluka (France). Cellulase, an *endo*-(1→4)-β-glucanase from *Trichoderma sp.* was purchased from Megazyme (Ireland). Celluclast 1.5L (Novo Nordisk A/S, Denmark) is an enzymatic complex from *Trichoderma reesei*.

4.2. Preparation of CAS and CS

CAS was prepared as previously described.[1,4] Cellulose (cellulose Avicel PH-101, Fluka, 1 g) was activated in glacial AcOH and then dispersed in a mixture of glacial AcOH and H_2SO_4 (20 mL and 0.24 g, respectively) and Ac_2O was finally added (3.2 mol/mol of anhydroglucose). The mixture was stirred for 30 min at 40 °C. The reaction was stopped by addition of 3:7 water–AcOH and the soln was slowly added to de-ionized water to

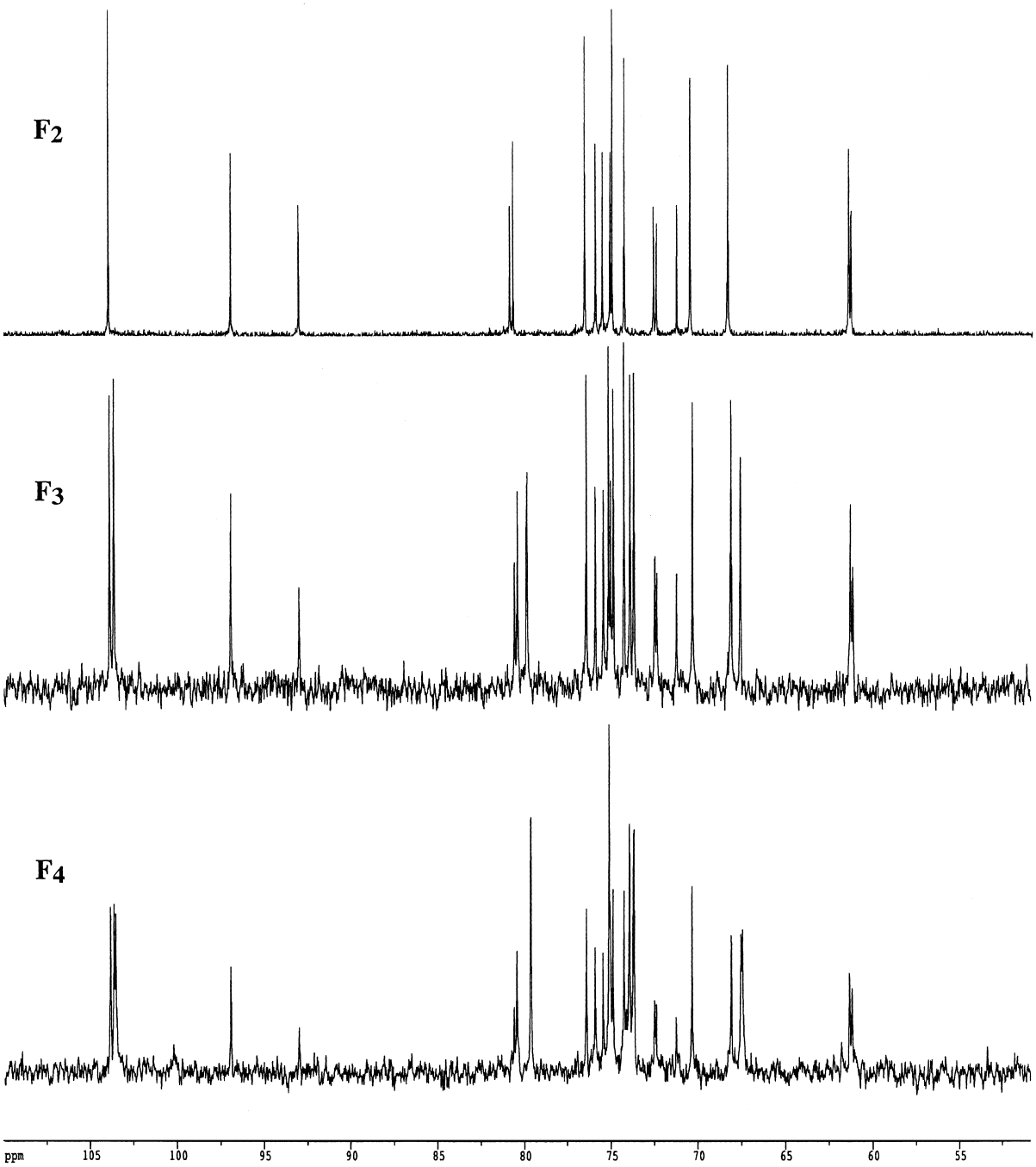


Fig. 6. ^{13}C NMR spectra of oligosaccharide fraction F_2, F_3 and F_4. The number of scans recorded were, respectively 512, 1024 and 4096.

precipitate cellulose triacetate (CTA). After 16 h at 4 °C, CTA was removed by centrifugation (17,500*g*, 20 min, and 4 °C). The supernatant was neutralized at pH 7.5 by addition of 4 M NaOH, dialyzed and the water-soluble ester was finally freeze-dried.

CAS (1 g) was de-acetylated in 0.25 M NaOH (200 mL) for 1 night at room temperature (rt) under constant stirring. The solution was then dialyzed and freeze-dried to get CS (0.57 g).

4.3. Enzymatic hydrolysis

4.3.1. Kinetics. CAS and CS were dissolved in distilled water (5 mg/mL), and incubated at 40 °C either with Cellulase or Celluclast 1.5L (1.22 nkat of endoglucanase/mg cellulose) for various periods of times. The kinetic was followed either by viscometric measurements with an Ostwald capillary viscometer (diameter: 0.46 mm), or by HPSEC using a Shodex OHpak SB-

802.5 HQ (30 × 0.8 cm) column eluted at 0.5 mL/min by 50 mM $NaNO_3$. A differential refractometer (ERC 7517 A) was used for detection of peaks. Increase in reducing ends in the hydrolysis mixture was also followed.[15]

4.3.2. Preparation of sulfated oligomers. A solution of CS (9.4 mL, 50 mg/mL) in water was incubated at 40 °C with Celluclast 1.5L (49 μL), further enzyme (24 μL) was added every 24 h, up to 72 h. Then, boiling for 5 min stopped the reaction, and the hydrolysis mixture was filtered over 0.45 μm.

Degradation products were isolated by anion exchange chromatography using a DEAE Sepharose CL6B (Pharmacia) column (45 × 1.6 cm). Two runs (3.5 mL) were injected on the column, which was eluted at a flow rate of 1.7 mL/min at rt. The column was eluted with water for 1 h, then a linear gradient of NaCl (0– > 0.5 M) was applied over 2 h and finally the column was eluted with NaCl 0.5 M for 1 h. Fractions (5 mL) were collected and analyzed for their sugar content by the automated orcinol method[16] using glucose as the standard. Fractions were pooled and desalted on a Sephadex G10 column (85 × 2 cm) and eluted at rt with water at 0.9 mL/min.

4.4. General methods

All results are expressed relative to the dry matter content determined by drying at 120 °C for 3 h. Ash was measured after incineration overnight at 500 °C then for 1 h at 900 °C.

Individual neutral sugars were analyzed by gas–liquid-chromatography (GLC) after total hydrolysis of cellulose derivatives in 1 M H_2SO_4 at 100 °C for 6 h and derivation of the sugars into alditol acetates[17]

Sulfate was quantified, after hydrolysis by 2 M TFA at 120 °C for 2 h, on an IonPac II column (4 mm × 25 cm) with a conductimetric detection (ASII DIONEX, Sunnyvale, U.S.), as previously described.[1,18]

Acetate was quantified after hydrolysis by 0.4 M NaOH in a 1:1 water–EtOH for 2 h at rt. Acetic acid was then analyzed on an Aminex HPX-87H column as previously described.[1,19]

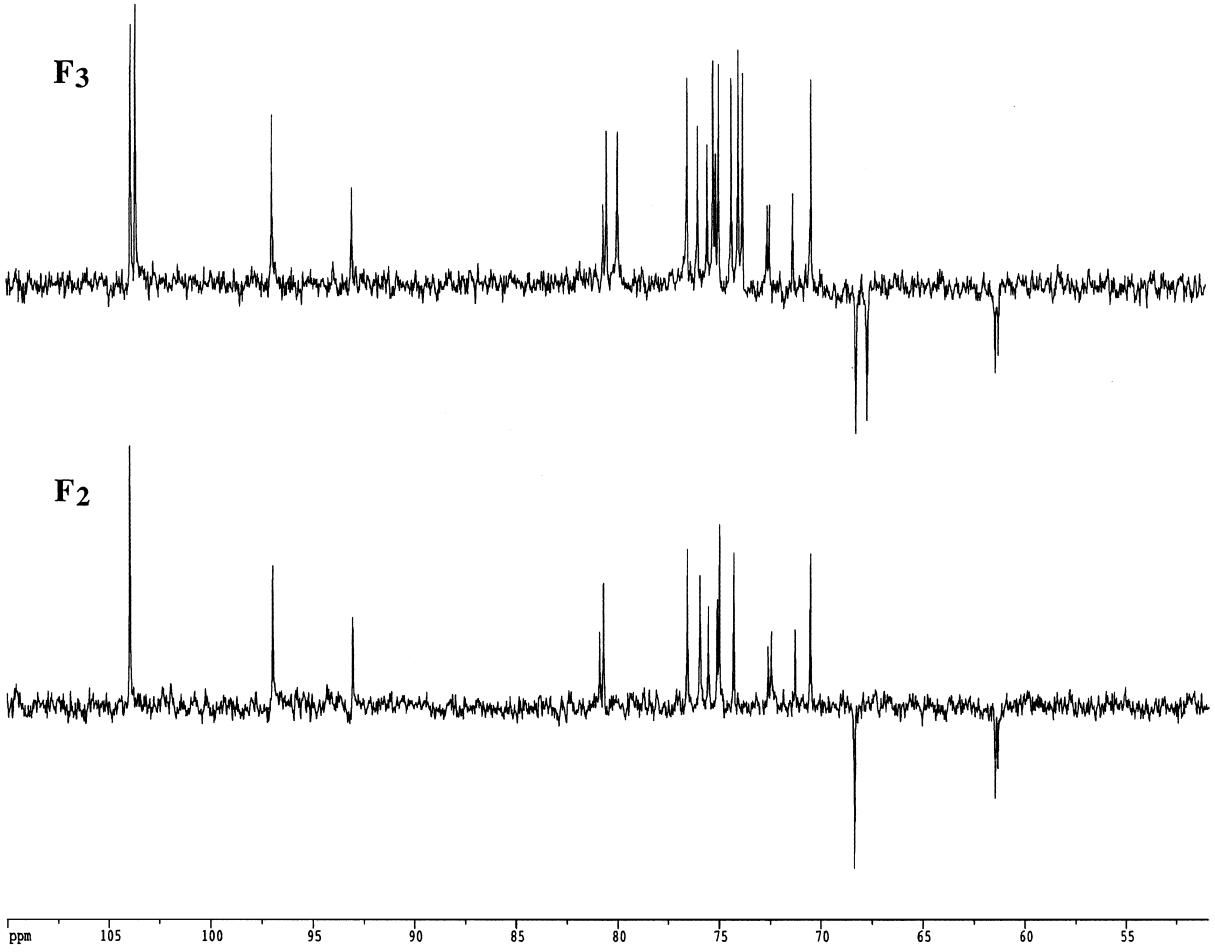
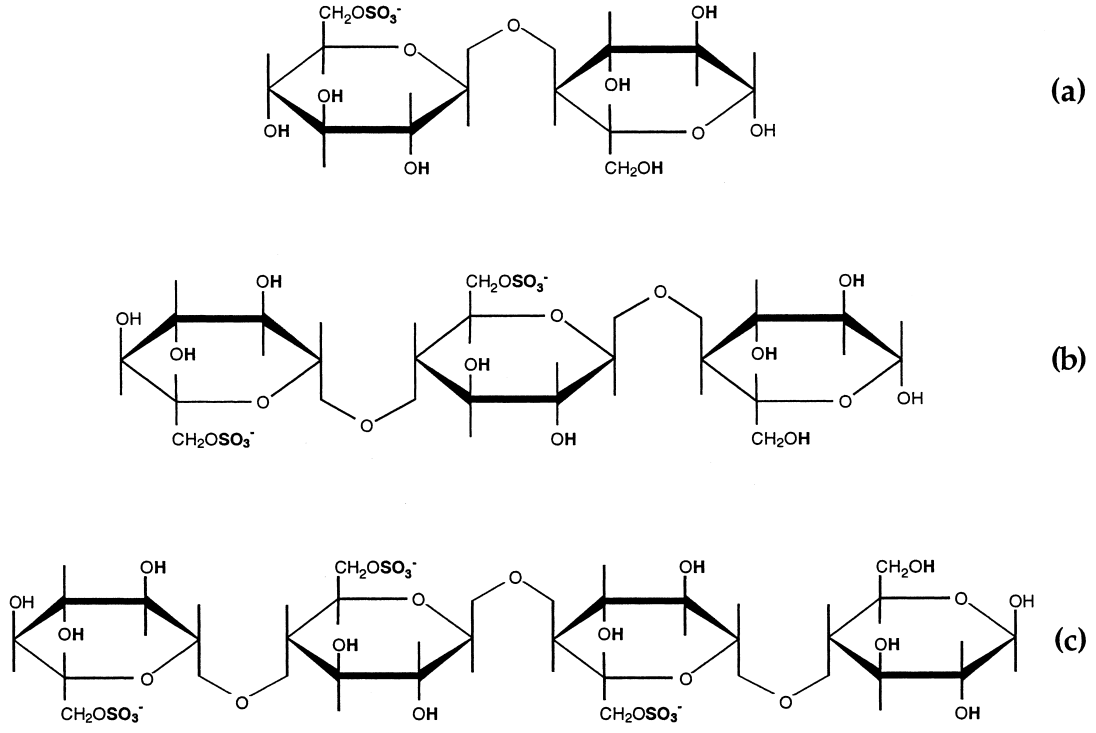


Fig. 7. DEPT ^{13}C NMR spectra of oligosaccharide fraction F_2 and F_3. The number of scans recorded were, respectively 512 and 2048.

Table 5
^{13}C NMR chemical shifts (ppm) of cellobiose and cellotriose[13] and oligosaccharide fractions F_2, F_3 and F_4 recovered from DEAE sepharose CL6B

Sample	Residue [a]	Carbon					
		1	2	3	4	5	6
Cellobiose	α	92.9	72.3	72.4	79.9	71.2	61.0
	β	96.8	75.0	75.4	79.6	75.9	60.9
	nr	103.5	74.3	76.7	70.6	77.0	61.8
F_2	α	92.9	72.3	72.4	80.7	71.1	61.2
	β	96.8	74.9	75.4	80.5	75.8	61.3
	nr	103.8	74.1	76.4	70.3	74.8	68.2
Cellotriose	α	92.9	72.3	72.4	79.9	71.2	61.0
	β	96.8	75.0	75.4	79.6	75.9	60.9
	int	103.4	74.0	75.1	79.5	75.9	61.0
	nr	103.5	74.3	76.7	70.6	77.0	61.8
F_3	α	92.9	72.3	72.4	80.6	71.2	61.1
	β	96.9	75.0	75.4	80.4	75.9	61.3
	int	103.6	73.9	75.1	79.9	73.7	67.6
	nr	103.8	74.2	76.4	70.3	74.8	68.1
F_4	α	92.9	72.3	72.4	80.6	71.2	61.1
	β	96.9	75.0	75.4	80.4	75.9	61.3
	int	103.6	73.9	75.1	79.6	73.7	67.5
	int	103.5	73.9	75.1	79.6	73.7	67.4
	nr	103.8	74.2	76.4	70.3	74.8	68.1

[a] α, α anomer of the reducing residue; β, β anomer of the reducing residue; nr, non-reducing residue; int, internal residue.



Scheme 1. Structural characterization of the DEAE-sepharose purified F_2 (a), F_3 (b) and F_4 (c) fractions obtained by celluclast hydrolysis (40 °C, 72 h) of CS.

Table 6
Distributions of sulfated blocks of glucose residues in CS

	Fractions isolated with Celluclast		DP of sulfated blocks	Sulfated glucose [b]	
	% of CS [a]	Structure		Experimental	Simulated
F_1	51.8	G			
F_2	32.3	1S/2G	DP1	16.1	14.7
F_3	9.7	2S/3G	DP2	6.5	8.1
F_4	4.4	3S/4G	DP3	3.3	3.4
F_5	1.8	4S/5G	DP4	1.4	1.2
			DP>4	n.d. [c]	0.5

[a] g of Glc in the fraction/100 g of glucose in CS.
[b] g of sulfated Glc in the fraction/100 g of Glc in CS.
[c] Not detected.

4.5. Physico-chemical characterization

CS was dissolved (5 mg/mL) for 2 h at 40 °C under magnetic stirring, filtered over 0.2 μm membrane and injected at 25 °C on a high performance size exclusion chromatography (HPSEC) system constituted of two Shodex OH-pack SB HQ 804 and 805 columns eluted at 0.7 mL/min with 50 mM $NaNO_3$, containing 0.02% NaN_3. An on-line molar mass determination was performed at rt using a multi-angle laser light scattering (MALLS) detector (mini-Dawn®, Wyatt, USA; operating at three angles: 41, 90 and 138°), a differential refractometer (ERC 7517 A) ($dn/dc = 0.146$ mL/g). $\overline{M_w}$ and $\overline{M_n}$ were determined using Astra 1.4 software (Wyatt, USA).

Avicel cellulose was dissolved in 0.5 M cupriethylenediamine at rt. Viscosity was measured in an Ostwald capillary viscometer (diameter: 0.46 mm) and intrinsic viscosity was estimated from specific viscosity as described.[20] Viscometric molar mass and polymerization degree were calculated using the Mark–Houwink–Sakurada relationship[5,6] established for cellulose in cupriethylenediamine:

$$[n] = (1.01 \times 10^{-4})\bar{M}_v^{0.9}$$

Reduced viscosities of CAS and CS were determined with an Ubbelhode viscometer (diameter 0.52 mm, Viscologic TI.1 SEMATech, Nice—France). Six determinations were carried out at 25 °C for each dilution and intrinsic viscosity was deduced from the extrapolation to zero concentration of the reduced viscosity. Solutions were prepared in water or in 0.1 M NaCl and were filtered (pore diameter 15 μm) before measurement.

4.6. Structural characterization

Cellulose derivatives were methylated according to the method of Hakomori[10] using lithium-methylsulfinyl-methanide carbanion. Dried samples (4 mg) were dissolved in Me_2SO (0.5 mL) and 2 M lithium dimsyl carbanion (0.5 mL) was added and kept for 1 h at rt under Ar. Methylation was then performed as described[21] using MeI (0.5 mL). Methylated polysaccharides were dialyzed against water and freeze-dried and the permethylation step was repeated once. After dialysis and freeze-drying, methylated polysaccharides were hydrolyzed with 2 M trifluoroacetic acid (100 °C, 2 h) and converted into alditol acetates.[17] The partially methylated alditol acetates were analyzed by GLC on DB-225 and DB-1 fused-silica capillary columns (J&W, USA; 30 m × 0.32 mm i.d.).[22] Peak areas were corrected by response factors.[23] Identification was based on relative retention times.

IR spectra of initial cellulose and cellulose derivatives were carried out as follows. Avicel cellulose was dried (1 week, 40 °C) then milled with KBr (2 mg/100 mg KBr) and pressed (200 kg/cm^2) to form pellets. Aqueous solutions of CAS and CS (1 mL, 11 mg/mL) were evenly spread on a Teflon support and maintained at rt in order to form thin films. The spectra were recorded on a Brücker IFS 2S spectrometer (wavelengths: 400–4000 cm^{-1}, by steps of 4/cm).

Proton decoupled ^{13}C NMR spectra of oligosaccharide fractions dissolved in D_2O (100% D_2O) were recorded at 300 K on a Bruker ARX 400 instrument with a total recycling time of 0.56 s. The distortionless enhancement by polarization transfer experiment (DEPT) was set to flip negative methylene carbons following the pulse sequence provided by Bruker. Carbon chemical shifts were calculated from internal acetone attributed at 31.4 ppm.

4.7. Simulation of sulfate distribution

Simulation of sulfate distribution along the cellulose backbone was performed using a program written in Fortran, running on Silicon Graphics computer. Chain

lengths of 170 glucose units were used for statistics sake, the percentage of substituents was set equal to the sulfate content (28.7%) and one sulfate group could substitute each glucose residue. Using these imposed parameters, the program simulated a random distribution of sulfate groups on the cellulose chain.

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References

1. Chauvelon, G.; Buléon, A.; Thibault, J.-F.; Saulnier, L. *Carbohydr. Res.* **2003**, *338*, this issue, doi:10.1016/S0008-6215(03)00008-9.
2. Chauvelon, G.; Doublier, J.-L.; Buléon, A.; Thibault, J.-F.; Saulnier, L. *Carbohydr. Res.* **2003**, *338*, this issue, doi:10.1016/S0008-6215(03)00009-0.
3. Engelhardt, J. *Carbohydr. Eur.* **1995**, *12*, 5–14.
4. Chauvelon, G.; Saulnier, L.; Buléon, A.; Thibault, J.-F. FR. Patent 2789080, 2000; Chem Abstr.
5. Marx, M.; Schultz, G. V. *Papier* **1955**, *9*, 13–16.
6. Marx-Figini, M.; Schultz, G. V. In *Polymer Handbook*; Brandrup, J.; Immergut, E. H., Eds.; Wiley: New York, 1975; p V-107.
7. Mitchell, A. J. *Carbohydr. Res.* **1993**, *241*, 47–54.
8. Tezuka, Y.; Kiyokazu, I. *Carbohydr. Res.* **1990**, *196*, 1–10.
9. Craigie, J. S.; Leigh, C. Carrageenans and agars. In *Handbook of Phycological Methods, Physiological and Biochemical Methods*; Hellebust, S. A.; Craigie, J. S., Eds.; University Press: Cambridge, 1978; Vol. 12, pp 109–131.
10. Hakomori, S. I. *J. Biochem.* **1964**, *55*, 205–208.
11. Gohdes, M.; Mischnick, P.; Wagenknecht, W. *Carbohydr. Polym.* **1997**, *33*, 163–168.
12. Vink, H. *Makromolekulare Chemie* **1963**, *67*, 105–123.
13. Heyraud, A.; Rinaudo, M.; Vincendon, M. *Biopolymers* **1979**, *18*, 167–185.
14. Lechat, H.; Amat, M.; Mazoyer, J.; Buléon, A.; Lahaye, M. *J. Phycol.* **2000**, *36*, 891–902.
15. Nelson, N. A. *J. Biol. Chem.* **1944**, *153*, 375–380.
16. Tollier, M. T.; Robin, J.-P. *Ann. Technol. Agric.* **1979**, *28*, 1–15.
17. Englyst, H. N.; Cummings, J. H. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 808–814.
18. Quemener, B.; Lahaye, M.; Bobin-Dubigeon, C. *J. Appl. Phycol.* **1997**, *9*, 179–188.
19. Voragen, A. G. J.; Schols, H. A.; Pilnik, W. *Foods Hydrocoll.* **1986**, *1*, 65–70.
20. Norme Française NF T 12-005, mars 1953, *J. O.*, 20-6-1953.
21. Harris, P. J.; Henry, R. J.; Blakeney, A. B.; Stone, B. A. *Carbohydr. Res.* **1984**, *127*, 59–73.
22. Saulnier, L.; Brillouet, J.-M.; Joseleau, J.-P. *Carbohydr. Res.* **1988**, *181*, 63–78.
23. Sweet, D. P.; Shapiro, R. H.; Albersheim, P. *Carbohydr. Res.* **1975**, *40*, 217–225.