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Determination of the substitution pattern in the polymer chain of cellulose sulfates

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Abstract

The distribution of substituents in cellulose sulfates has been determined on different structural levels. Based on the determination of the monomer composition, a method has been developed to describe the sulfate pattern in the polymer chain. This approach comprises permethylation, desulfation, deuteromethylation, random cleavage, remethylation, fast-atom bombardment mass spectrum (FABMS) analysis and comparison of the experimental data with those calculated for a random sulfation pattern. Results are discussed with respect to the conditions of the sulfation reactions. In addition, the amount of glucose that could be hydrolyzed from the cellulose sulfates by enzymic digestion was determined. Results of both the random and the selective approach were in good agreement.

Keywords: Cellulose sulfates; Substituent distribution; FABMS; Enzymic degradation

1. Introduction

Polysaccharides such as starch or cellulose are often modified to prepare new materials, e.g. for oil recovery, waste water treatment, evaporation membranes, paper manufacturing, textile finishing, building material, cosmetic and food additives or pharmaceutical applications. Efforts have been made in the sulfation of several polysaccharides, e.g. of the β-(1→3)-glucans laminarin or curdlan or of the β-(1→)-4-glucan cellulose, to achieve synthetic materials with heparinoid properties [1], or to enhance the biological activity of glycosaminoglycans by additional introduction of sulfate groups [2]. Anti-tumor and anti-HIV activity of polysaccharide sulfates comprising dextran, pentosans, fucoidan and carrageenans has also been reported [3–8]. Location of sulfate groups in naturally occurring polysaccharide sulfates such as heparin and heparan sulfates is essential for antithrombin-binding [9]. In cellulose sulfates, esterification of the 2- and 3-OH of the anhydro glucose unit (AGU) induces biological activity, while sulfation in position 6 is inactive. Due to their ionic character, sulfate groups enhance water solubility and strongly influence gel formation and viscosity, as known for the carrageenans from red algae [10].

While reports on the synthesis of polysaccharide sulfates go back to 1844 [11], a more detailed analysis only became possible with modern instrumental techniques. Polysaccharide derivatives

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usually represent polydisperse mixtures with respect to DP and sequence of monomer units. Therefore, the substituent distribution has to be considered on different structural levels: in the AGU, along the polymer chain and between the polymer chains. The sulfate pattern in the monomer unit can be determined by modified methylation analysis [12,13]. To get some information on the substituent distribution on the polymer level, a defined partial degradation must be performed. For a selective approach, enzymes have been used [14–17]. Alternatively, a random cleavage establishes a defined relation of the degradation products with the original polymer. Based on the work of Arisz et al. [18] and our studies on methyl amyloses [19] we now report a new method for the determination of the sulfate pattern in the polymer chain.

2. Results and discussion

Monomer analysis.—Cellulose sulfates, prepared under different homogeneous and heterogeneous reaction conditions, were investigated (see Table 1). First, the distribution of sulfate groups in the AGU was determined by methylation analysis. Problems arose from loss of sulfate groups from position 2 by intramolecular nucleophilic displacement under the alkaline methylation conditions due to the 2,3-*trans*-diol structure in glucose (formation of the 2,3-anhydro derivative), and subsequent reactions. Further, sulfation of OH-2 turned out to enhance the acid lability of the glucosidic bond by some orders of magnitude [20,21], presumably by supporting the formation of the carboxonium ion via formation of a cyclic 1,2-sulfate. As a consequence, these early liberated moieties were partially destroyed during hydrolysis with 2 M trifluoroacetic acid. These drawbacks could be overcome by optimizing all parameters of the methylation reaction [13], and by the addition of *N*-methylmorpholine-borane complex during hydrolysis in agreement with the reductive hydrolysis procedure introduced by Garegg et al. [22] and Stevenson and Furneaux [12].

Oligomer analysis.—Due to the polydispersity of polysaccharide derivatives, no sequence analysis could be performed. If each AGU of a cellulose molecule reacted with equal probabilty, a random distribution of the eight different monomer units (un-, 2-, 3-, 6-mono-, 2,3-, 2,6-, 3,6-di- and trisubstituted AGU) would be obtained. If some of the β-(1→4)-linked glucosyl residues were less accessible than others, e.g. due to strong hydrogen bonds in crystalline regions, areas of higher and lower degree of substitution compared to the random pattern would result and be called a heterogeneous distribution. On the other side, a negative intermonomeric effect of a substituent that effects a decreased reactivity in the vicinity of a substituted AGU, e.g. due to steric hindrance or electrostatic repulsion of an ionic reagent [23], would yield a more regular distribution along the polymer chain with respect to the random reference model. Competing reactions will be indicated by overlapping of different distribution patterns [19]. To get this information, the polymer molecules must be randomly cleaved to smaller oligosaccharides, that could be quantitatively analyzed by mass spectrometry.

Table 1
Derivatisation conditions and monomer composition of cellulose sulfates CS1-7

Sample	Educt	Derivatisation conditions[a]	Sulfate group in position								
			—	2	3	6	23	26	36	236	DS
CS1	Cellulose	N_2O_4/DMF/SO_3 (homogeneous)	51,30	2,17	1,35	41,95	0,24	1,84	0,74	0,40	0,53
CS2	Cellulose	Propanol/H_2SO_4 (heterogeneous)	27,88	2,29	0,98	61,66	0,13	5,06	1,59	0,41	0,80
CS3	Cellulose	DMF/Ac_2O/$ClSO_3H$ (subsequent acetyl cleavage)	19,92	5,98	1,17	50,84	0,27	20,44	0,92	0,45	1,03
CS4	Cellulose acetate DS(Ac) 1,48	DMF/NH_2SO_3H	28,92	13,45	6,92	6,14	25,52	4,95	1,93	12,10	1,28
CS5	Cellulose acetate DS(Ac) 2,5	DMF/$ClSO_3H$	69,10	5,33	4,37	17,25	0,40	1,89	1,37	0,27	0,35
CS6	Cellulose acetate DS(Ac) 2,0	DMF/$ClSO_3H$	41,97	7,35	6,12	29,53	0,51	7,57	6,16	0,79	0,74
CS7	Cellulose	N_2O_4/DMF/SO_2 (homogeneous)	45,42	11,14	1,99	32,18	0,5	7,29	0,82	0,65	0,65

[a]For more detailed information see [25–28].

Direct hydrolysis of the permethylated cellulose sulfates does not fulfill these requirements, since 2-sulfation strongly increases the acid-lability of the glucosidic bond as outlined above. Several desulfation methods have been reported in the literature, but these are usually not quantitative and are accompanied by partial cleavage of glycosidic bonds and loss of material. After detailed studies, the acetylative desulfation method of Hyatt [24] turned out to be the best compromise. In a subsequent reaction step, the acetyl groups were exchanged by deuteromethyl groups with sodium hydroxide/methyl iodide-d_3. The resulting cellulose methyl ether could now be randomly cleaved by methanolysis. After remethylation with MeI-d_3, quantitative FABMS analysis was performed [18,19]. Originally sulfated positions are isotope-labeled (Me-d_3).

Results are presented for four selected cellulose sulfates, CS1–CS4. Conditions of derivatisation and the distribution in the AGU are listed in Table 1. The random distribution pattern for the dimer-, trimer- and tetramer fraction (DP 2–4) was calculated from the monomer composition and compared with the experimental data (Fig. 1a–d). The average degree of substitution (DS) for each oligomeric fraction should agree with the DS of the whole sample, if the results are representative for the original polymer. Sample CS1 was sulfated via the nitrite ester in the system dinitrogen tetroxide/dimethylformamide with sulfur trioxide as described and discussed by Wagenknecht et al. [25]. The DS values showed a slight drift from DP 2 to DP4 (0,56–0,59–0,61), but within experimental error a random distribution of sulfate groups in the cellulose molecules was found, as expected for this homogeneous reaction (Fig. 1a). For CS2, the agreement of experimental and calculated data was even better (Fig. 1b), although this derivative was prepared in the heterogeneous system H_2SO_4/propanol [26]. Sulfuric acid is known to break the intermolecular hydrogen bonds of cellulose (swelling) and therefore may enable an equal accessibility of the AGUs. In addition, the reversibility of the sulfation process can be responsible for a random pattern by thermodynamic control in contrast to a kinetically controlled etherification [19]. The same explanation can be applied in the discussion of the results for CS3, which also showed a random distribution of sulfate groups within experimental error (Fig. 1c). This sample was treated with acetic anhydride and chlorosulfonic acid in *N,N*-dimethylformamide at elevated temperature, changing from a heterogeneous to a homogeneous system during the reaction, and deacetylated [27]. In contrast, a strong deviation from a statistical substitution is evident for CS4 (Fig. 1d), which was prepared from a cellulose acetate with a DS of 1,48. The latter was obtained from cellulose triacetate by regioselective deacetylation, to achieve subsequently a high degree of sulfation in positions 2 and 3 due to the above mentioned correlation with the anticoagulant activity [28]. Since 84% of the OH-groups available were sulfated, the distribution is mainly determined by the acetate pattern of the cellulose acetate. Acetate cleavage was carried out with 3 equiv of 1,6-diaminohexane in dimethylsulfoxide at 80 °C for 14 h. Comparison of the experimental and the calculated data reflects a very marked heterogeneity with an enhanced ratio of low- and high-substituted regions. This can be interpreted as a strong DS gradient within the material, i.e. a heterogeneity of first order, as well as a heterogeneous sulfation pattern in each polymer molecule (heterogeneity of second order). Presumably, both types of heterogeneity (first and second order) contribute.

Enzymic degradation.—Cellulose degrading enzymes ("cellulase", containing exo- and endoglucanases and cellobiohydrolase) are hindered by substituents in the cellulose molecule. For a given DS, the amount of glucose that can be liberated by cellulase under defined conditions should increase with increasing heterogeneity of substitution in the polymer chain, since the average length of unsubstituted sequences is larger when the substituents are more clustered [14–16]. First, the optimal conditions for a maximum yield of glucose were established. The sample was dissolved in acetic acid at pH 4.6 and treated with cellulase from *Trichoderma reesei* (0.61 U mg^{-1}) at 40 °C. After deactivation of the enzymes, the amount of glucose was determined by an enzymatic/UV-test and referred to the unsubstituted fraction. Fig. 2 shows the time course for three cellulose sulfates. For CS5 (Table 1) with a DS of 0,35, nearly 60% of the unsubstituted glucose moities were hydrolyzed, while for CS3 with a DS of 1,03 only 7% of this fraction could be liberated by the enzymes. The results obtained for seven cellulose sulfates after 50 h of incubation are shown in Fig. 3. The data for CS1, CS2, CS3, and CS7 and one further comparable sample can be connected to a curve, that represents the randomly substituted cellulose

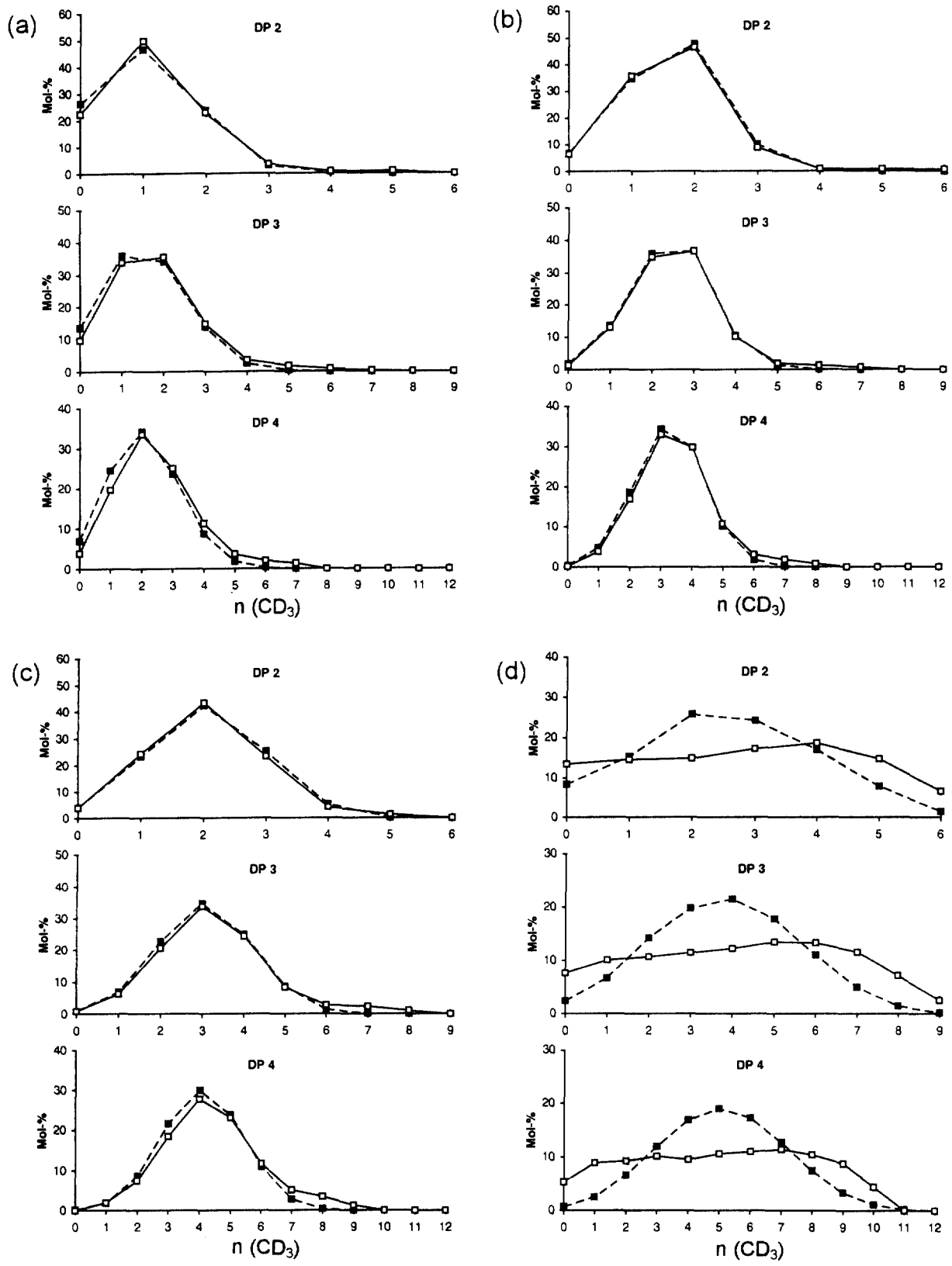

Fig. 1. Distribution of Me-d_3-groups in the dimeric (DP2), trimeric (DP3), and in the tetrameric fraction (DP4) obtained from cellulose sulfates CS1-4 in comparison with the pattern calculated for a random distribution of the monomer units in the polymer chain. The Me-d_3-groups represent original sulfate groups. (a): CS1, (b): CS2, (c) CS3, and (d): CS4. (Preparation and monomer composition are listed in Table 1.) ■ = calculated, □ = experimental.

sulfates. As expected, a higher ratio of glucose is accessible in CS5 and CS6, which showed a significant heterogeneity in the oligomer analysis by FABMS (data not shown). These sulfates were prepared from commerical cellulose acetates (DS 2,5 and 2,0) with chlorosulfonic acid in *N,N*-dimethylformamide and reflect the heterogeneity of the acetyl pattern of the cellulose acetate used. This approach is limited to a DS of about 1. It allows a relative classification of water-soluble cellulose derivatives degraded under defined conditions.

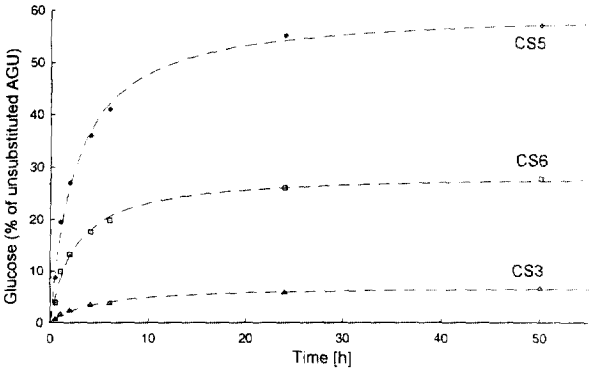

Fig. 2. Time course study for the enzymic digestion of cellulose sulfates CS3, 5 and 6 (for DS and monomer composition see Table 1) with cellulase from *Trichoderma reesei*. The amount of glucose liberated by the enzyme was determined and referred to the unsubstituted AGUs present in the CS.

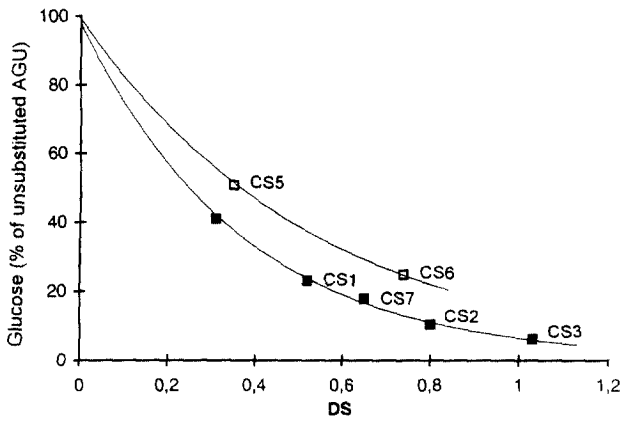

Fig. 3. Relative amount of glucose referred to the unsubstituted AGUs in the CS, which was liberated by cellulase from *Trichoderma reesei* from homogeneously and heterogeneously substituted cellulose sulfates (data see Table 1).

Enzyme selectivity.—After enzymic degradation, one sample (CS7, DS 0,65, Table 1) was reduced with sodium borohydride and permethylated. After total hydrolysis, a second reduction was performed with sodium borohydride-d_4 to label the alditols formed *after* hydrolysis. After acetylation, the product mixture was analyzed by GLC–MS. By this derivatization sequence, terminal glucosyl groups, inner (1→4-linked) glucosyl residues, and glucose residues could be differentiated. The relative composition of all types of glucosyl and glucose moieties is listed in Table 2. Glucose liberated by the enzyme is always unsubstituted (DS 0,0). Further, it turned out that no significant amount of substituted glucose residues could be detected for the reducing end (DS 0,05), while the composition of the terminal glucosyl residues (DS 0,39) indicates the principal acceptance of 2-, 6- and also 2,6-disubstituted residues as the aglycon part of the hydrolyzed glycosidic bond by the cellulase from *Trichoderma reesei*. The DS for the fraction of the inner glucosyl moieties was 0,85 and therefore increased for 30% compared to the original sample. It still comprises 33% unsubstituted glucosyl units in an arrangement that obviously did not allow the attack of the enzymes.

3. Conclusion

It has been demonstrated that cellulose sulfate can be transformed to mixed *O*-methyl/*O*-methyl-d_3

Table 2
Relative composition (in mol%) of CS7 and its enzymic degradation products. Each fraction—glucose monomers, terminal non-reducing glucosyl residues, inner (1→4)-linked glucosyl residues, and glucose residues (reducing end groups)—are normalized to 100%. The DS of each fraction is calculated. Average DP of the oligomeric fraction = 7.6

		Degradation products			
		Monomers	Oligomers		
Substitution	CS7	Glucose	*t*-glucosyl groups	inner (1→4)-linked	glucose residues
un-	45.4	100	63.8	32.9	95.3
mono-	45.3	—	33.3[a]	51.7	4.7
di-	8.6	—	2.9[b]	14.0	—
tri-	0.7	—	—	1.4	—
DS	0.65	0.00	0.39	0.85	0.05

[a]2- and 6-sulfate.
[b]2.6-sulfate.

ethers, where methyl-d_3 represent the positions of the original sulfate groups. After random degradation and FABMS-analysis the composition of the oligomeric mixture was compared with a calculated pattern for a random distribution of sulfate groups. It turned out that all cellulose sulfates prepared from cellulose showed a statistical distribution in the polymer chain, independently from homogeneous or heterogeneous reaction conditions. This was attributed to the reversibility of the sulfation reaction, allowing thermodynamic control. In contrast, cellulose sulfates prepared from cellulose acetates reflect the heterogeneity of the intermediate derivative.

Results from enzymic degradation are in agreement with those of mass spectrometric analysis.

4. Experimental

General.—The cellulose sulfates (sodium form) were obtained from the Fraunhofer Institute für Angewandte Polymerforschung, Teltow (Germany). Their preparation, and the regioselectivity of the sulfation in the AGU has been described and discussed elsewhere [25–28]. For all reactions in Me_2SO, they were transformed to the triethylammonium salt by ion exchange as described [19]. All reagents were of highest purity available and purchased from Fluka, Aldrich or from E. Merck.

Standard methylation analysis was carried out as described [13]. Hydrolysis was carried out according to Stevenson and Furneaux [12].

Desulfation.—The permethylated cellulose sulfate (20 mg) was dissolved in dry acid-free acetic anhydride (2,5 mL) in a 3 mL-V-vial and heated to 120 °C for 14 h. After cooling the solution was concentrated in a stream of nitrogen. Loss of sulfate (ca. 80%, >90% for the critical 2-sulfate) was examined by IR and by methylation analysis.

Alkylation.—Exchange of acetyl groups against Me-d_3 and remethylation of the oligomeric mixture from partial degradation was carried out according to Ciucanu and Kerek [29] with MeI-d_3 and NaOH in Me_2SO.

Partial random cleavage of the cellulose sulfate.—Permethylated cellulose (*O*Me/*O*Me-d_3, 15 mg) was dissolved in 0,1 M MeOH/HCl (1.5 ml) and heated to 90 °C for 1.5 h. The methanolic HCl was evaporated in a stream of nitrogen at 30 °C. The oligomeric mixture was remethylated as described.

GLC.—GLC separations were carried out on a Carlo Erba GC 6000 Vega Series 2 instrument equipped with an on-column injector, a flame ionization detector, a 25 m capillary column CPSil 8CB (Chrompack) connected with a retention gap (2 m), and a Merck Hitachi D-2500 integrator. Hydrogen was used as carrier gas (80 kPa).

GLC–MS.—EI (70 eV)-Mass spectra were recorded on a VG Analytical VG/70-250S instrument. For CI, ammonia was used as the reactant gas.

FABMS.—FAB mass spectra were recorded in the range *m/z* 200–1500 on a VG Analytical VG/70-250S instrument with a xenon-gun and *m*-nitrobenzylalcohol as matrix, saturated with NaI. The data were accumulated over 10 scans. (Acceleration voltage: 8 kV in the positive ion mode. Scan time: 20 s, Resolution: 2500.) The relative signal intensities (R.I.) at *m/z* [M + Na] were corrected for their signal/noise ratio with respect to the corresponding isotopic peak at *m/z* [M + 1 + Na]. Th.I. = theoretical intensity of the M + 1 signal, calculated from the molecular formula. Noise = R.I.[M + 1 + Na]–(R.I. [M + Na] * 0.01 * Th.I. [M + 1 + Na])/(1–0.01 * Th.I[M + 1 + Na]).

IR.—IR specta were recorded with a FTIR-spectrometer of ATI Mattson (Genesis Series FTIR). KBr tablet was pressed.

Enzymic degradation.—CS (100–200 mg depending on the DS) was dissolved in distilled water and adjusted to pH 4.6 with acetic acid. Cellulase from *Trichoderma reesei* (Serva TE, 0.61 U mg^{-1}, 25 mg) was added and the soln incubated in a shaking water bath at 40 °C for 50 h. Glucose was determined by the UV-test of Boehringer. The soln was heated to 100 °C for 10 min to denature the enzyme, filtered and freeze dried. Further investigation of the reaction products was carried out by a special order of reaction steps according to methylation analysis.

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References

[1] (a) G. Franz, S. Alban, and J. Kraus, *Macromol. Symp.*, 99 (1995) 187–200; (b) D.L. Williams, H.A. Pretus, R.B. McNamee, E.L. Jones, H.E. Ensley, and I.W. Browder, *Carbohydr. Res.*, 235 (1992) 247–257.

[2] K.G. Ludwig-Baxter, Z. Liu, and AS. Perlin, *Carbohydr. Res.*, 214 (1991) 245–256.

[3] M. Baba, R. Snoeck, R. Pauwels, and E. de Clercq, *Antimicrob. Agents Chemother.*, 32 (1988) 1742–1745.

[4] R.S. Chang, H.D. Tabba, Y. He, and K.M. Smith, *Proc. Soc. Exp. Biol. Med.*, 189 (1988) 304–309.

[5] S. Lederman, R. Gulick, and L. Chess, *J. Immunol.*, 143(4) (1989) 1149–1154.

[6] I. Yamamoto, K. Takayama, K. Honma, T. Gonda, and K. Matsuzaki, *Carbohydr. Pol.*, 14 (1991) 53–63.

[7] T. Uryu, K. Katsuraya, and T. Yoshida, *Macromol. Sci., Pure Appl. Chem.*, A33(12) (1996) 1863–1874.

[8] D.H. Paper, H. Vogl, and G. Franz, *Macromol. Symp.*, 99 (1995) 219–225.

[9] (a) C.A.A. van Boeckel, and M. Petitou, *Angew. Chem.*, 105 (1993) 1741–1894, *Angew. Chem., Int. Ed. Engl.*, 32 (1993) 1641; (b) M. Maccarana, Y. Sakura, A. Tawada, K. Yoshida, and U. Lindahl, *J. Biol. Chem.*, 271 (1996) 17804–17810.

[10] H. Scherz, *Hydrokolloide*. Behr, Hamburg, 1996, pp 52–55.

[11] B. De Carolles, *Ann.*, 52 (1844) 412–416.

[12] T.T. Stevenson and R.H. Furneaux, *Carbohydr. Res.*, 210 (1991) 277–298.

[13] M. Gohdes, P. Mischnick, and W. Wagenknecht, *Carbohydr. Pol.*, 33 (1997) 163–168.

[14] M.G. Wirick, *J. Polym. Sci.*, 6, Part A-1 (1968) 1705–1718.

[15] M.G. Wirick, *J. Polym. Sci.*, 6, Part A-1 (1968) 1965–1974.

[16] R.A. Gelman, *J. Appl. Polym. Sci.*, 27 (1982) 2957–2964.

[17] P.A.M. Steeneken and A.J.J. Woortman, *Carbohydr. Res.*, 258 (1994) 207–221.

[18] P.W.F. Arisz, H.J.J. Kauw, and J.J. Boon, *Carbohydr. Res.*, 271 (1995) 1–14.

[19] P. Mischnick and G. Kühn, *Carbohydr. Res.*, 290 (1996) 199–207.

[20] P.F. Lloyd and P.F. Forrester, *Carbohydr. Res.*, 19 (1971) 430–431.

[21] M. Gohdes, Thesis, University of Hamburg 1997.

[22] P.J. Garegg, B. Lindberg, P. Konradsson, and I. Kvarnström, *Carbohydr. Res.*, 176 (1988) 145–148.

[23] O. Wilke and P. Mischnick, *Starch/Stärke*, 49 (1997) 453–458.

[24] J.A. Hyatt, *Carbohydr. Res.*, 239 (1993) 291–296.

[25] W. Wagenknecht, I. Nehls, and B. Philipp, *Carbohydr. Res.*, 240 (1993) 245–252.

[26] B. Lukanoff, H. Dautzenberg, and G. Holzapfel, DE Patent 4 021 049 (1992); *Chem. Abstr.*, 116 (1992) 86110j.

[27] B. Philipp and D. Klemm, *Das Papier*, 49 (1995) 3–7; B. Philipp, D. Klemm, and W. Wagenknecht, *ibid.* 58–64.

[28] B. Philipp, D. Klemm, and A. Stein, *Das Papier*, 49 (1995) 102–108.

[29] I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.