New Approaches to the Analysis of Enzymatically Hydrolyzed Methyl Cellulose. Part 1. Investigation of the Influence of Structural Parameters on the Extent of Degradation

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Six methyl celluloses (MCs), one with a degree of substitution (DS) of 1.32 and five with DS between 1.83 and 1.88, were thoroughly investigated. Monomer composition and methyl distribution in the polymer chain were analyzed after total or partial random hydrolysis and appropriate derivatization with gas chromatography (GC) and mass spectrometry (MS), respectively, and used as reference data. The same MCs were then hydrolyzed with an enzyme preparation of *Trichoderma longibrachiatum* and further investigated with size-exclusion chromatography with multiangle light scattering and refractive index detection (SEC-MALS/RI) and MS. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) in combination with various MS analyzers were compared with respect to quantification of the degradation products directly and after perdeuteriomethylation. The methyl group distribution in the oligomeric fractions and the average DS as a function of chain length were calculated from ESI mass spectra. With help of the reference analysis, patterns could be corrected for the unspecific contribution of end groups. By labeling and ESI tandem MS, our knowledge about the tolerance of the enzymes' sub-sites with respect to the number of methyl groups could be improved.

Introduction

Cellulose is the most abundant renewable polysaccharide resource; approximately $10^{11}-10^{12}$ tons are synthesized annually by photosynthesis. Substitution of the hydroxyl groups changes the physicochemical properties such as water-solubility, retention, and thickening ability. There are several different cellulose derivatives available, like methyl cellulose (MC), hydroxypropyl- or hydroxyethylmethyl cellulose (HPMC or HEMC), and carboxymethyl cellulose (CMC), which are widely used as thickeners in the building industry or for controlled drug release in the pharmaceutical industry.

The properties of cellulose derivatives depend on the degree of substitution (DS), molar mass distribution, type of the substituent, and its location in the glycosyl unit and along and over the cellulose chain, i.e., whether the substituents are randomly or more heterogeneously distributed. For derivatives with chemically stable, nonionic substituents, methods for the analysis of the substituent distribution on the monomer level are well established.[1] After appropriate sample preparation, various techniques such as nuclear magnetic resonance spectroscopy (NMR), gas−liquid chromatography (GLC), capillary electrophoresis (CE), and mass spectrometry (MS)[2−7] can be applied. Analysis of the substitution pattern along the cellulose chain is more difficult. Quantitative analysis of oligosaccharide mixtures has been achieved after perdeuteriomethylation and random acidic hydrolysis of methyl celluloses or starches by mass spectrometry in combination with soft ionization techniques such as fast atom bombardment (FAB), matrix-assisted laser desorption/ionization (MALDI), or electrospray ionization (ESI) mass spectrometry.[8,9] The experimental results are compared with the calculated random distribution of glucosyl units. This approach has been used as well for hydroxyalkylmethyl ethers,[7,10] silyl ethers,[1] cellulose sulfates,[11] and acetates.[12]

Puls and Saake[13−15] used enzymes as selective tools for polymer chain cleavage followed by size-exclusion chromatography with multi-angle light scattering and refractive index detection (SEC-MALS/RI) and high performance anion exchange chromatography with pulsed amperometric detection (HPAEC−PAD) for analysis of the hydrolyzed cellulose derivatives. This approach is limited by the enzymatic degradability of the sample. Therefore, only a set of samples with the same DS could be compared. Very recently, progress in this field by labeling of the enzymatically degraded samples and tandem MS experiments has been reported.[7,16,17] Another drawback is the incomplete knowledge of the enzymes' tolerance with respect to the substitution patterns of the glucosyl units involved in the active complex and no knowledge about the proportion of the original sample detected as cleavage products by MS. Until now, analysis of the enzymatically degraded cellulose derivative has been performed by mass spectrometry without eliminating the discrimination effects that occur when various ratios of free and methylated hydroxyl groups are present.

There are mainly two types of enzymes that can degrade cellulose. Exoglucanases hydrolyze cellulose either from the reducing or from the nonreducing end of the cellulose polymer, depending on their selectivity, and endoglucanases cleave glycosidic linkages in the inner part of the cellulose chain. Exoglucanases degrade derivatized cellulose until the enzyme encounters a substituent in the polymer chain that hinders its access. The typical hydrolysis product from exoglucanase attack is cellobiose, which in turn could be hydrolyzed to glucose by a β-glucosidase. In contrast, the product pattern from endoglu-

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canase digestion is more complex. Recent studies indicate that endoglucanases can perform hydrolysis even if substituents are present in the part of the polysaccharide chain that binds to the active site of the enzyme.[16,18,19] The degree of enzymatic hydrolysis is influenced by the interplay of the selectivity of the enzyme and the location of substituents in the cellulosic substrate.

Here we report on a new approach, which yields representative quantitative data for enzymatically hydrolyzed MC, thus allowing comparison of samples with different DS. The enzyme used for the hydrolysis was a commercially available preparation from *Trichoderma longibrachiatum* (Tr. longi). Influence of perdeuteriomethylation and instrumental setup was investigated. Thus, all enzymatically hydrolyzed samples were analyzed before and after perdeuteriomethylation with an ESI-ion trap (ESI-IT) instrument, an ESI-triple stage quadrupole (ESI-QqQ) instrument, and a MALDI time-of-flight (MALDI-TOF) instrument. In the work presented here, deviation for the various MS techniques is discussed. For comparison, all MCs were analyzed with respect to monomer composition and distribution in the polymer chain by the established method mentioned above. Furthermore, to get insight into the enzymes' tolerance with respect to the location of methyl groups in MC, ESI-MS^n was applied. The number of substituents in the released di- and trisaccharides was quantified separately for the reducing and the nonreducing end glucosyl units and the internal glucosyl residue, respectively.

Experimental Section

General. All reagents used were of highest purity available and purchased from Fluka, Aldrich, or Merck. The endoglucananase *Trichoderma longibrachiatum* (approximately 500 U mL^{-1}) was purchased from Megazyme International Ireland Ltd., Wicklow, Ireland. MC 1−MC 6 were commercial products. MeI-d_3 was purchased from Deutero GmbH, Kastellaun, Germany.

MonomerAnalysis. For acid hydrolysis MCs (ca. 2 mg) were heated in a 1 mL V−Vial with 1 mL of 2 M trifluoroacetic (TFA) acid for 120 min at 120 °C. After cooling to room temperature (r.t.), the solvent was evaporated in a stream of nitrogen. Residual acid was removed by co-distillation with toluene.

The hydrolyzed sample was reduced with a solution of 0.5 mL of 0.25 M $NaBD_4$ in 2 M NH_3 at 60 °C for 120 min. After cooling to r.t., the solution was coevaporated at 40 °C with 15% methanolic acetic acid in a stream of nitrogen to remove borate as its methyl ester.

After reduction, the residue was dissolved in 50 μL of pyridine, and 200 μL of acetic anhydride was added to acetylate the sample at 90 °C for 3 h. Saturated $NaHCO_3$ solution was added to the mixture and stirred until CO_2-formation ceased. Then the products were extracted four times with dichloromethane. The combined organic layers were first washed two times with saturated $NaHCO_3$-solution, once with cold 0.1 M HCl, once with water, and then dried over $CaCl_2$. After filtration, the solvent was removed at a rotary evaporator. A solution of the residue in 4 mL of CH_2Cl_2 was used for GLC/FID and GLC/MS analysis.

Oligomer Analysis. All samples were alkylated with MeI-d_3 according to Ciucanu and Kerek[20] with NaOH/MeI-d_3, or according to a modified Hakomori procedure[21] with Li-dimsyl/MeI-d_3 in dimethyl sulfoxide (DMSO). Sample cleanup was performed through dialysis of the reaction solution using a dialysis tube with a molecular weight cut off (MWCO) of 12−14 kDa against water for several days. Completeness of the reaction was controlled by means of attenuated total reflectance infrared (ATR−IR) spectroscopy, and yields are between 95 and 100%. The perdeuteriomethylated MCs (3 mg) were partially hydrolyzed in a 1 mL V−Vial with 1 mL of 2 M of trifluoroacetic acid for 15 min at 120 °C. After cooling to r.t., the solvent was evaporated in a stream of nitrogen. Residual acid was removed by co-distillation with toluene (five times). The residue was redissolved in methanol (MeOH) to a concentration of approximately 0.5 mg mL^{-1} and submitted to ESI-MS.

Enzymatic Hydrolysis of Methyl Celluloses. Approximately 30 mg of each MC was dissolved in 3 mL of H_2O at 4 °C overnight. The enzyme was dissolved in water to a concentration of approximately 30 U mL^{-1} at r.t. It was necessary to purify the enzyme before the addition due to contaminations from sugars and buffer components. This was done by centrifugation of the enzyme through a 5 kDa Millipore Ultrafree centrifuge filtration tube (Millipore, Bedford, USA). When the centrifuge filters were half dry, water was added and the samples were centrifuged again. This procedure was repeated until no traces of sugars or buffer components were seen in the filtrates when analyzing the solution with ESI-ITMS. Finally, the supernatant in the filters was dissolved in water to a final volume of approximately 400 μL, stirred gently, and added evenly to the six MC solutions at r.t. All enzymatic batches were shaken at r.t. for 96 h, and then they were boiled at 95 °C for 15 min and centrifuged. Thereafter, the solution was freeze-dried overnight in Eppendorf vials.

Perdeuteriomethylation of Enzymatically Hydrolyzed Samples. All methylations were performed according to Ciucanu and Kerek[20] in a 1 mL V−Vial. The hydrolyzed sample (4 mg) was solved in 350 μL of DMSO and stirred for 2 h at r.t. Then 20 mg of pulverized NaOH was added to form the poly-anions of the oligomeric mixture. After 30 min, 40 μL of MeI-d_3 was added and the solution was stirred for 6 h at r.t. To achieve quantitative methylation, addition of reagents was repeated, and the solution was stirred overnight. Samples were cleaned by extraction with dichloromethane three times. The combined organic layers were washed with saturated NaCl solution, 5% $Na_2S_2O_3$ solution, and water, and subsequently dried over Na_2SO_4. The solvent was evaporated, and the residue was redissolved in MeOH.

Analysis of Enzymes' Selectivity. The enzymatically degraded sample (4 mg) was centrifuged through a 5 kDa Millipore Ultrafree centrifuge filtration tube (Millipore, Bedford, MA). The solvent of the obtained filtrate was evaporated in a stream of nitrogen. The residue was reduced with a solution of 0.5 mL of 0.05 M $NaBD_4$ in 2 M NH_3 for 2 h at 60 °C. The sample cleanup and the acetylation and the perdeuteriomethylation of the enzymatically hydrolyzed sample were performed using the same procedure as described above. The deuteriomethylated samples were directly submitted to ESI-ITMS. For ESI-MS/MS, $LiClO_4$ was added to the sample solution, and Li adducts fragmented according to Adden et al.[22]

Instrumentation. Gas chromatographic analysis with flame ionization detection (GC-FID) was carried out with a Carlo Erba GC 6000 Vega Series 2 instrument with a CPSil 8 column (25 m), a retention gap (1.5 m), and H_2 as carrier gas. Injection was carried out on-column.

The temperature program starts at 60 °C for 1 min, heats with 20 °C min^{-1} to 130 °C, and subsequently with 4 °C min^{-1} to 290 °C, keeping isothermic for 30 min, giving a total run length of 57.5 min. Data were recorded with a Merck Hitachi D 2500 Chromato-Integrator. For peak identification, mass spectra were recorded with GC-MS performed with an Agilent 6890 GC equipment and a JEOL GCmate II benchtop double-focusing magnetic sector mass spectrometer. The gas chromatograph was equipped with a HP-5 column (30 m). A split injection port at 250 °C was used for sample introduction, and the split ratio was set at 5:1. Helium carrier gas was set to 1.5 mL min^{-1} flow rate (constant flow mode). The transfer line was kept at 250 °C. The mass spectrometer was operating in electron impact ionization (EI) mode at 70 eV with an ion source temperature of 180 °C. Low-resolution mass spectra were acquired at a resolving power of 650 (20% height definition) and scanning from *m*/*z* 39 to *m*/*z* 650 at 1.0 s/scan with a 0.2 s inter-scan delay.

An Esquire LC equipment (Bruker Daltonics, Bremen, Germany) was used for acquiring ESI-IT mass spectra. The mass spectra were recorded in positive mode. The partially degraded samples were introduced directly via a syringe at a flow of 120 μL h^{-1}. For analyzing the lithium adducts, samples were dissolved in 1 mM $LiClO_4$ in MeOH.

All mass spectra used for quantitative analysis consist of an average of 200 scans. Nitrogen is used as drying gas (4 L min^{-1}, 300 °C) and as nebulizer gas (10 psi). Two different voltage settings were used, one optimized on *m*/*z* 900 and one on *m*/*z* 1500: For *m*/*z* 900: capillary 4500 V, end plate offset −500 V, capillary exit 120.0 V, skim 1 40.0 V, skim 2 10.0 V and trap drive 55.0. For *m*/*z* 1500: capillary 4500 V, end plate offset −500 V, capillary exit 101.3 V, skim 1 77.4 V, skim 2 6.0 V and trap drive 77.8. The amplitude of the resonance frequency, which excites the ions for fragmentation in the ion trap was optimized for every ion and was between 0.85 and 0.95 V. The isolation width for MS^n experiments was 1 *m*/*z*-unit.

ESI-QqQ mass spectra were recorded using a Thermo Finnigan TSQ 7000 (Thermo Electron Corporation, San Jose, CA) in positive mode. The sample was injected through direct infusion into the ESI interface with a flow-rate of 100 μL min^{-1}. Sheath gas pressure of nitrogen was set at 40 psi, capillary temperature was set to 250 °C, and the spray of the needle was set to 5.0 kV. Peaks with *m*/*z* between 200 and 2000 were collected for 2 min. The deuteriomethylated samples were measured in a concentration of approximately 1 g L^{-1} in MeOH and the nondeuteriomethylated in water of approximately 1 g L^{-1}. For quantitative analysis, data were acquired for 2 min and averaged to obtain one mass spectrum.

For obtaining MALDI-TOF mass spectra, a PerSeptive Voyager-DE STR from Applied Biosystems (Framingham, MA) with a N_2 Laser 337 nm was used. The mass spectra were acquired in positive mode with a reflector and delayed extraction activated. DHB was used as the matrix for all samples with a concentration of 10 g L^{-1}. Preparation of the nonmethylated samples was carried out by mixing an analyte solution (~3 g L^{-1}) with a matrix solution in water at a ratio of 1:9 (v/v). A certain volume, 1 μL of each mixture, was dropped on the target spot, and the spots were dried in the vacuum chamber.[23] The deuteriomethylated samples were prepared using a higher concentration of the analyte (~10 g L^{-1}) in MeOH. The matrix was dissolved in ethanol (EtOH) at the same ratio as used for the nonmethylated samples. Here, 0.5 μL of each mixture was dropped on the target spot, and the spots were dried at atmospheric pressure. For quantitative analysis, 200 shots from each spot were averaged to obtain one mass spectrum.

Results and Discussion

Six methyl celluloses were treated with an enzyme as selective tool and subsequently analyzed with soft ionization/mass spectrometry. In contrast to previous enzymatic work, appropriate sample preparation allowed quantitative evaluation of the MS data.[16,23] For comparison, random degradation of the perdeuteriomethylated MC and monomer analysis have been performed as well.[8,9]

Since the ratio of free and modified hydroxyl groups influences the ion yields of oligosaccharides, these discrimination effects are investigated with respect to different techniques of mass spectrometry. Results obtained with ESI-IT, ESI-QqQ, and MALDI-TOF are compared.

Analysis of the Discrimination Effects Depending on the MS Instrumentation. When analyzing cellulose derivatives by mass spectrometry after any partial degradation, quantitative data are not acquired unless the sample is modified prior to analysis. Without proper modification, relative information is only obtained for a set of samples with the same DS. However, as mentioned above, no absolute data for an individual sample can be obtained. These discrimination effects are especially important, when oligomers are analyzed after enzymatic degradation. Misinterpretation of the enzymes' activity and the extent of hydrolysis due to overestimation of signals in the mass spectra can occur.

Beside others, the discrimination effects in mass spectrometry depend on the ability to form adducts with cations. This ability

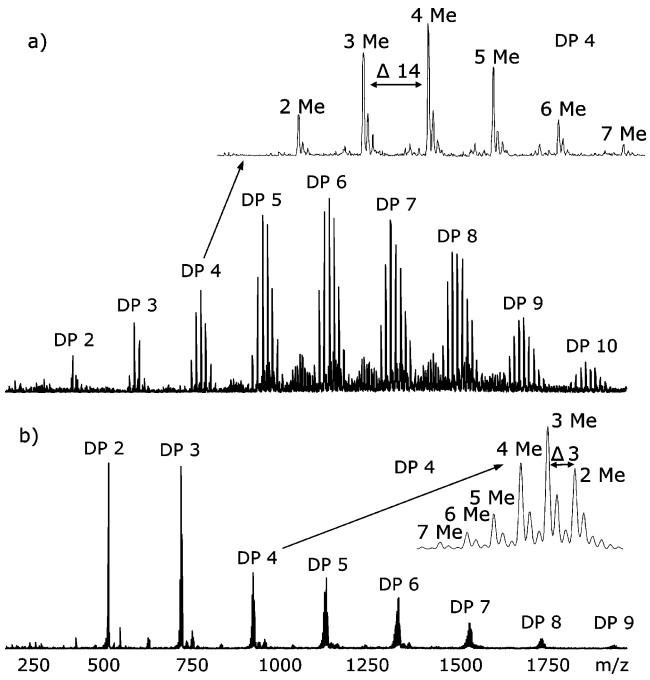


Figure 1. 1. ESI-IT mass spectra of MC 1 after enzymatic degradation by *Trichoderma longibrachiatum*. Before (a) and after (b) perdeuteriomethylation. DP 4 is shown in detail.

is increased by methyl and even more by hydroxyalkyl groups.[10] To measure the extent of discrimination in ESI-ITMS, ESI-QqQMS, and MALDI-TOF−MS, methyl derivatives were selected, since they can easily be transformed to isotopically labeled chemically uniform oligomers, giving equal response in MS. In Figure 1, ESI-IT mass spectra of a partially methylated and a perdeuteriomethylated sample are shown in comparison. The *m*/*z* range is narrower within each DP for the perdeuteriomethylated sample compared to the partially methylated one. In addition to the chemical uniformity and therefore polarity, making the analyte monodisperse (only one isotopic distribution remains) additionally contributes to comparable ion yields.

In Figure 2, results are shown for enzymatically hydrolyzed MC 1 (DS 1.32, Tr. longi). DS values were calculated and plotted against DP 2−8 (Figure 2a). Unfortunately the data obtained for DP 7 and 8 of the perdeuteriomethylated sample by ESI-QqQ could not be evaluated due to very low signal intensities. Methyl distributions for DP 5 are shown for the three instruments applied (Figure 2b). Results are in good agreement for the perdeuteriomethylated sample for all MS techniques, as has been reported for FAB-MS, ESI-MS, and MALDI-TOFMS[24] after random acidic cleavage. DS values for the nondeuteriomethylated oligomers varied with the strongest deviation for ESI-IT, followed by ESI-QqQ, and nearly none for MALDI-TOF. A shift of relative intensities to the higher methylated oligosaccharides for the nondeuteriomethylated samples is observed to a different extent for each technique. Obviously the polarity of the oligomers has a big influence in electrospray ionization. To see whether the voltage settings influence the extent of discrimination in ESI-IT, two different voltage settings were used in the analysis of the sample before and after perdeuteriomethylation. For optimization, the smart parameter settings (SPS) from the instrument's software were used, which does not only change the voltages of the skimmers, but the trap drive as well. The first setting optimized for an area around *m*/*z* 900 and the second one for an area around *m*/*z* 1500. Spectra obtained under both conditions show the same relative signal intensities for a certain DP, indicating that there is no influence

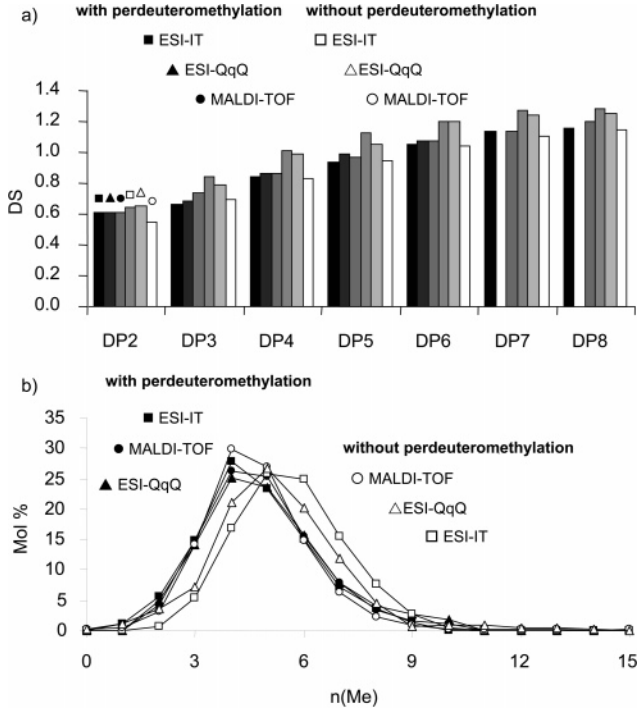


Figure 2. 2. Influence of the different MS techniques on the analysis of the methyl distribution of MC 1 after degradation with *Trichoderma longibrachiatum* prior and after perdeuteriomethylation. (a) DS calculated for each DP; (b) methyl distributions for DP 5 (for details see text); ■/□ ESI-IT, ▲/△ ESI-QqQ, ●/○ MALDI-TOF; black areas: with perdeuteriomethylation; white areas: without perdeuteriomethylation.

of the chosen voltage on the relative ion yields of chemically uniform oligosaccharides of a certain DP.

Discrimination of lower methylated constituents levels off with increasing DP and DS, but has not completely been equalized until DP 8 (Figure 2a). The MALDI-TOF mass spectra of the original sample clearly differ from those of the ESI instruments. The maximum of the methyl distribution matches the deuteriomethylated sample. During MALDI-TOF analysis, occasionally different DS values were obtained, creating not entirely reproducible data. The reproducibility might be influenced by sample preparation, solvents and the matrix.[23,25] In contrast, very good reproducibility was obtained for the deuteriomethylated samples.

The results indicate that in MALDI-TOF, the polarity of constituents is of less influence compared to electrospray ionization and/or that mass analysis by time-of-flight does not discriminate ion transfer with respect to their *m*/*z* value. MALDI-TOFMS has been found to be superior to ESI-IT with respect to quantitative evaluation in the analysis of hydroxyalkyl methyl celluloses.[7,10] However, MALDI-TOFMS suffers from the disturbing signals from the matrix at low *m*/*z*.

As has been shown, perdeuteriomethylation is crucial for accurate analysis of enzymatically hydrolyzed MC. Otherwise, when the enzymes are studied, their tolerance with respect to certain methyl patterns can be overestimated. If perdeuteriomethylation is not possible, MALDI-TOF is the preferred technique, otherwise all techniques can be used.

Analysis of Methyl Cellulose. To assess the potential of enzymes for the analysis of cellulose ethers, reference data for the substituent distribution along the polymer chain were determined after random degradation as has been described.[8,9] First, the monomer compositions of six commercial methyl celluloses MC 1−MC 6, were analyzed with GLC after transformation to partially methylated glucitol acetates. In Table 1, the results are listed, where s_i denotes the normalized percentage of each substitution pattern i; x_i the partial DS values in the positions 2, 3, and 6; and c_i the percentage of the total amount of substituents in the glycosyl unit (c_0, c_1, c_2, c_3). Included as well is a comparison with the statistical models of Spurlin[26] and Reuben.[27] These models are known for estimating the heterogeneity of the substituent distribution. Reuben states that the probability of a substitution in position 3 of the glycosyl unit is enhanced if position 2 is already derivatized, in contrast to Spurlin, whose basic model does not consider any influence of substitution on still unsubstituted OH groups during the reaction. For the MCs studied, the model of Reuben better fits the experimental data. The heterogeneity parameters (H_1) are generally low, for MC 1−MC 3 around 3 and for MC 4−MC 6 around 1. However, it must be considered that the H_1 values, defined as the root of the sums of the squared deviations of each substitution pattern, are only averaged deviations. The data are listed in more detail in Table 1. The compositions of MC 2 and MC 3 on one hand, and that of MC 4−MC 6 on the other hand, are very similar. Between these two sets of MCs with nearly the same DS, the main difference is an enhanced 2,6-di-*O*-substitution for the latter, compensated by a decrease in 3,3,6-di-*O*-methylation and most pronounced 2,3,6-tri-*O*-methylation. The slightly higher preference for 2-*O*-methylation of MC 4−MC 6 is also obvious from the partial DS values x_i given in Table 1, because x_2 is increasing at the expense of x_3 compared to MC 2 and MC 3. However, as mentioned above, these differences are rather small.

For all samples, data for the substituent distribution along the chain were obtained after perdeuteriomethylation, subsequent partial hydrolysis, and analysis with ESI-ITMS. The results were compared with two calculated models. In the first one (M_0), the interaction between glycosyl units during derivatization is not accounted for. Thus, the monomer units all react with the same probability independent of each other. The deviation between this model and the experimental data was defined as H_i, i denotes the DP of the oligomer, and was used as a measure for the heterogeneity in the sample. The DS values calculated for each DP from the mass spectra and the heterogeneity parameters H_i are listed in Table 2, indicating the averaged deviation of the determined pattern from the calculated random distribution of substituents (M_0). H_i values are small for MC 2 and MC 3 ($H_2 = \sim 2.7$; $H_3 = \sim 4.3$), and rather high for MC 1 and MC 4−MC 6 ($H_2 = \sim 6-9$; $H_3 = \sim 9-12$). The distribution for higher DPs of each sample supports the same trend (not shown). MCs with the same DS around 1.8 again form two groups: One is represented by MC 2 and MC 3 and the other one by MC 4−MC 6. In contrast to the results of monomer analysis (Table 1), MC 2 and MC 3 deviate significantly less from the calculated random distribution than MC 4−6 (Figure 3, exp. and M_0), which show deviations in the opposite direction. Since the latter ones show a higher amount of sequences with high and low substitution indicating a slight heterogeneity, the former set represents a slightly higher regularity of monomers in the chain. This is very uncommon for MCs and means that methylation at a certain glucosyl unit had reduced the probability of methylation at the neighboring unit. Compared to the calculated distribution of methyl groups, MC 1 shows the same trend as MC 4−MC 6.

However, the interpretation of this model follows a more qualitative course; no absolute values are obtained. Mischnick and Hennig introduced an extended model, including a neighbor dependence of the reactivity of glycosyl units during the course of the derivatization.[28] With this model, it is possible to deduce

Table 1. Distribution of Methyl Substituents of MC 1−MC 6[a]

substituent pattern	GC	Spurlin	Δ	Reuben	Δ	GC	Spurlin	Δ	Reuben	Δ	GC	Spurlin	Δ	Reuben	Δ
			MC 1					MC 2					MC 3		
s_0	21.1	16.4	4.7	19.4	1.7	5.6	4.2	1.4	4.8	0.8	6.3	4.8	1.5	5.6	0.7
s_2	16.9	19.8	−2.9	16.8	0.1	13.7	13.0	0.7	12.4	1.3	14.1	13.8	0.3	13.0	1.1
s_3	2.7	6.2	−3.5	3.2	−0.5	2.2	3.1	−0.9	2.5	−0.3	2.2	3.3	−1.1	2.4	−0.2
s_6	17.8	16.5	1.3	19.5	−1.7	10.4	9.8	0.6	11.2	−0.8	11.8	10.6	1.2	12.5	−0.7
s_{23}	9.3	7.5	1.8	10.5	−1.2	8.5	9.7	−1.2	10.3	−1.8	8.6	9.3	−0.7	10.2	−1.6
s_{26}	16.8	19.9	−3.1	16.9	−0.1	27.6	30.3	−2.7	28.9	−1.3	27.4	30.4	−3.0	28.6	−1.2
s_{36}	3.8	6.2	−2.4	3.2	0.6	6.3	7.3	−1.0	5.9	0.4	5.6	7.2	−1.6	5.4	0.2
s_{236}	11.7	7.5	4.2	10.5	1.2	25.7	22.6	3.1	24.0	1.7	24.0	20.6	3.4	22.4	1.6
c_0	21.1	16.4	4.7	19.4	1.7	5.6	4.2	1.6	4.8	0.8	6.3	4.8	1.5	5.6	0.7
c_1	37.3	42.5	−5.2	39.5	−2.2	26.3	25.9	0.4	26.1	0.2	28.1	27.6	0.5	27.8	0.3
c_2	29.9	33.6	−3.7	30.6	−0.7	42.4	47.3	−4.9	45.1	−2.7	41.6	47.0	−5.4	44.1	−2.5
c_3	11.7	7.5	4.2	10.5	1.2	25.7	22.6	3.1	24.0	1.7	24.0	20.6	3.4	22.4	1.6
H_1[b]			9.0		3.0			4.8		3.3			5.4		2.9
x_2			0.547 (41.35%)					0.756 (40.15%)					0.741 (40.43%)		
x_3			0.275 (20.79%)					0.427 (22.68%)					0.404 (22.04%)		
x_6			0.501 (37.87%)					0.700 (37.17%)					0.688 (37.53%)		
DS			1.32					1.88					1.83		
			MC 4					MC 5					MC 6		
s_0	5.3	4.4	0.9	5.1	0.2	5.0	4.2	0.8	4.8	0.2	5.3	4.3	1.0	4.9	0.4
s_2	14.9	15.1	−0.2	14.4	0.5	15.5	16.0	−0.5	15.4	0.1	14.3	14.3	−0.0	13.7	0.6
s_3	1.3	2.4	−1.1	1.7	−0.4	1.3	2.4	−1.1	1.8	−0.5	1.4	2.5	−1.1	1.9	−0.5
s_6	11.7	10.2	1.5	11.9	−0.2	10.1	9.0	1.1	10.4	−0.3	11.4	10.2	1.2	11.7	−0.3
s_{23}	8.7	8.2	0.5	9.0	−0.3	9.8	9.1	0.7	9.7	0.1	8.7	8.4	0.3	9.1	−0.4
s_{26}	32.8	35.0	−2.2	33.3	−0.5	33.1	34.6	−1.5	33.3	−0.2	32.0	34.1	−2.1	32.6	−0.6
s_{36}	4.3	5.6	−1.3	3.9	0.4	4.3	5.1	−0.8	3.8	0.5	5.0	6.0	−1.0	4.5	0.5
s_{236}	21.0	19.1	1.9	20.8	0.2	20.9	19.7	1.2	21.0	−0.1	22.0	20.1	1.9	21.6	0.4
c_0	5.3	4.4	0.9	5.1	0.2	5.0	4.2	0.8	4.8	0.2	5.3	4.3	1.0	4.9	0.4
c_1	27.9	27.7	0.2	27.9	−0.0	26.9	27.4	−0.5	27.5	−0.6	27.1	27.1	0.0	27.3	−0.2
c_2	45.8	48.8	−3.0	46.2	−0.4	47.2	48.8	−1.6	46.8	0.4	45.7	48.5	−2.8	46.2	−0.5
c_3	21.0	19.1	1.9	20.8	0.2	20.9	19.7	1.2	21.0	−0.1	22.0	20.1	1.9	21.6	0.4
H_1[b]			3.8		1.0			2.9		0.8			3.5		1.3
x_2			0.774 (42.39%)					0.793 (43.12%)					0.769 (41.70%)		
x_3			0.353 (19.33%)					0.362 (19.68%)					0.371 (20.12%)		
x_6			0.699 (38.28%)					0.684 (37.19%)					0.704 (38.18%)		
DS			1.83					1.84					1.84		

[a] Monomer composition (mol %) and comparison with models according to Spurlin and Reuben. [b] Heterogeneity parameter (H_i) = $\sqrt{\Sigma(\Delta^2)}$.

Table 2. DS_{Me} Values and Heterogeneity Parameters H_i Calculated after MS Analysis for DP2 and DP3 Fragments from Partial Hydrolysis of MC 1−MC 6[a]

	DS (MA)	DS_{Me} DP2	H_2	DS_{Me} DP3	H_3
MC 1	1.32	1.33	6.2	1.34	9.0
MC 2	1.88	1.84	3.3	1.88	4.3
MC 3	1.83	1.81	2.1	1.89	4.2
MC 4	1.83	1.86	5.4	1.86	7.3
MC 5	1.84	1.81	8.7	1.80	11.8
MC 6	1.84	1.85	6.8	1.80	8.4

[a] The DS values of the total samples (DS_{MA}) determined by complete hydrolysis and GLC of the monomers are listed for comparison.

the interdependence of the substitution at two adjacent glucosyl units. In model M_1, which was applied to MC 1 (DS < 1.5), unsubstituted (c_0) and anyhow substituted (c_1, c_2, c_3) neighbors are differentiated. In model M_2, which was used for MC 2−MC 6 (DS >1.5), trisubstituted and less substituted units are differentiated. M_1 is more appropriate for samples with a DS below 1.5 and M_2 for samples with a higher DS, because these models are most accurate when the abundance ratio of the two groups of monomer units is as close as possible to 1. For details see Mischnick et al.[28]

The results are displayed in Figure 3. In the left column. the experimental values and the substituent distributions calculated according to the models M_0 and M_1 or M_2, respectively, are compared for the dimer fraction. In all cases, the standard deviation of the models M_1 and M_2 is lower than the deviation of the model M_0. The right column depicts the experimental data of the un-, mono-, di-, and trisubstituted monomer fractions. It can be seen that averaged mol fractions are divided into two sub-patterns. In the case of MC 1 these reflect the probabilities that glucosyl units with a certain number of methyl groups are located next to an unsubstituted (c_0) or an anyhow substituted residue (c_1, c_2, and c_3) and correspond to the fitting in the extended model (M_1). For the un- and monosubstituted glucosyl units (c_0 and c_1), the probability to be neighbored to an unsubstituted unit is enhanced compared to the random model, whereas on the other side, the probability of di- and trisubstituted units to be located next to unsubstituted glucosyl units is lowered. These result in a partial DS of 0.85 and thus 36% lower for the neighbors of the unsubstituted glycosyl units compared to a random distribution (1.32). This fraction contributes with 21.1% to the total DS. Consequently, the DS of the remaining glucosyl residues, contributing with 78.9% to the entire DS, is 1.44. The probability of lower substituted sequences and of higher substituted sequences is increased compared to a random distribution, which can be seen in the left column of Figure 3 where for MC 1 the experimental distribution is broader than the model M_0. If the derivatization of a glucosyl unit is

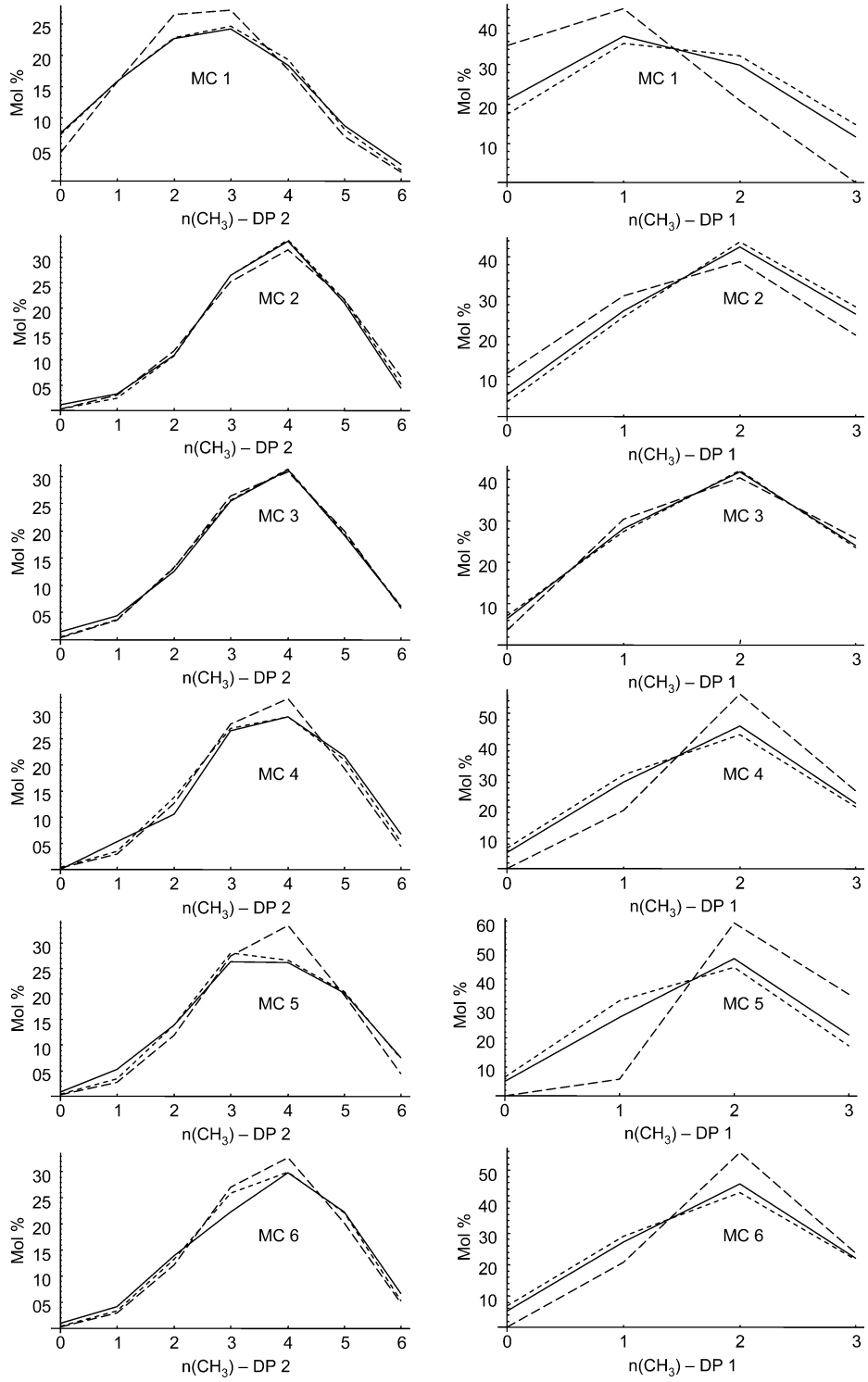


Figure 3. 3. Left column: Mol fractions [%] of dimers with *n* methyl groups. Comparison of the experimental data (black line) with those calculated by models M_0 (random, long dashes) and M_2 (MC 2−MC 6) (random comprising conditional probabilities, short dashes); right column: Mol fractions [%] of un-, mono-, di-, and trisubstituted monomers: Experimental data (black line), sub-pattern for monomers with a trisubstituted neighbor (MC 2−MC 6, M_2, long dashes), and sub-pattern for the monomers with a less than trisubstituted neighbor (MC 2−MC 6, M_2, short dashes). For MC 1 the corresponding model M_1 for DS > 1.5 was applied (for details, see text).

independent of the situation at the neighbor unit, i.e., if it follows the random model M_0, coincidence of the three curves should be observed (for dimers and monomers as well). That this is not always the case is also obvious for MC 4, MC 5, and MC 6. The comparison with random model M_0 also indicates a broader distribution, i.e., a more heterogeneous pattern along the polymer chain. The probabilities estimated by fitting to model M_2 now illustrate that the fraction of di- and trisubstituted monomers, adjacent to a trisubstitued glucosyl residue, is enhanced compared to the independence model M_0. These result for MC 6 in a partial DS of 2.05 and thus 11% higher for the neighbors of the trisubstituted glycosyl units compared to a random distribution (1.84). This fraction contributes with 22.0% to the total DS. The DS of the remaining glucosyl residues, contributing with 78.0% to the entire DS, is 1.79.

On the other hand, MC 2 and MC 3 show a rather low intercorrelation of adjacent glycosyl units. Especially for MC 3, all curves in Figure 3 are in very good agreement. Only a slight

Table 3. M_n, M_w, and Polydispersity (M_w/M_n) of MC 1−MC 6 Prior and After Degradation with Tr. longi[a]

	original sample			digested with Tr. longi		
sample	M_n	M_w	M_w/M_n	M_n	M_w	M_w/M_n
MC 1	11.6	61.3	5.3			
MC 2	221.8	466.5	2.1	22.2	34.7	1.6
MC 3	262.7	506.1	1.9	21.1	33.3	1.6
MC 4	12.1	23.0	1.9	9.0	13.1	1.5
MC 5	26.9	42.0	1.6	10.8	15.1	1.4
MC 6	90.6	201.5	2.2	13.8	22.4	1.6

[a] M_n and M_w are given in kDa.

deviation toward a more regular pattern compared to the statistical distribution can be recognized and are more pronounced for MC 2. Here, the probability of trisubstitution in neighborhood to di- or trisubstituted residues is lower compared to the independence model. This results in a partial DS of only 1.67 and thus 11% lower for the neighbors of the trisubstituted glycosyl units compared to a random distribution (1.88). This fraction contributes with 25.7% to the total DS. The DS of the remaining glucosyl residues, contributing with 74.3% to the entire DS is 1.95.

Thus, fitting of the experimental data with the models M_1/M_2 gives a more detailed and quantitative picture of the deviations from the random model and additional quantitative data.

Enzymatic Hydrolysis. In the next step, the MCs were degraded with a commercially available enzyme preparation from Tr. longi. Tr. longi has previously shown hydrolytic activity on derivatized cellulose.[29] This enzyme preparation hydrolyzes cellodextrins to glucose[30] indicating that it does not only contain endoglucanase but also β-glucosidase (and/or exoglucanase) activity.[31] The purpose of enzymatic hydrolysis was to find out whether it allows us to observe the differences in the substitution pattern found by the "random approach" and if even more data could be extracted. Different enzymes possess different abilities to hydrolyze the derivatized cellulose.[31] Therefore, it is important to gain knowledge on the enzymes' selectivity.

Another issue in the interpretation of the results after enzymatic degradation is to what extent the MC has been hydrolyzed. Information with respect to the distribution of the substituents along the cellulose chain is difficult to achieve, due to the generally high DS. From starch analysis it is known[32−35] that representative data can be obtained, because these polysaccharides are normally derivatized to a low level with a DS $\leq$ 0.1.

The extent of hydrolysis of the polymer was estimated with SEC-MALS/RI. The results are presented and discussed in detail in part 2 of this work.[30] In Table 3, the results of SEC-MALS/RI analysis are summarized. Prior to hydrolysis, the MCs covered a broad range of molecular weights M_w from 23 to 506 kDa. After enzymatic degradation, M_w and the polydispersity (M_w/M_n) had decreased for all samples. Low molar mass oligomers are discriminated in this analysis due to detection and column limitation of the instrumental setup applied (elution with the salt peak) as well as the low concentration used. From comparison of the molecular mass distribution before and after enzymatic hydrolysis with Tr. longi, it is evident that degradation of MC 4, MC 5, and MC 6 (see Figure 4) stops at about the same average M_n and M_w, independent of the starting situation. This is a second indication that these MCs have a similar substituent distribution. MC 2 and MC 3 originally have a high M_n and M_w and are hydrolyzed to a different level-off M_n and

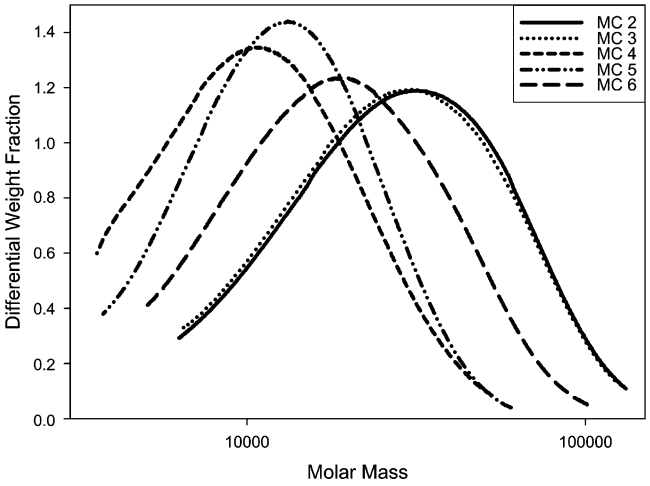


Figure 4. 4. SEC-MALS/RI profile of MC 2−MC 6 hydrolyzed with enzyme preparation from *Trichoderma longibrachiatum*.

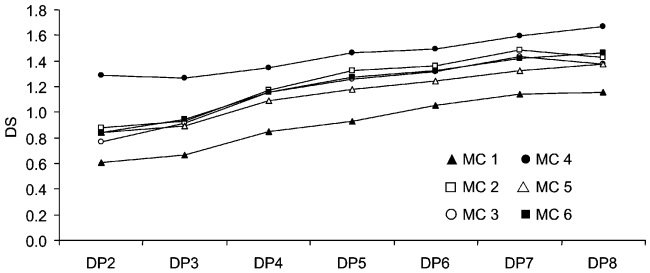


Figure 5. 5. DS/DP plot for MC 1−MC 6 calculated from ESI-ITMS after hydrolysis with enzyme preparation from *Trichoderma longibrachiatum* and perdeuteriomethylation.

M_w compared with that of MC 4, MC 5, and MC 6. However, the reason for the large end products of MC 2 and MC 3 is not the size of the intact polymer but rather the more regular or nearly random distribution of the various glucosyl residues for MC 2 and MC 3, which apparently allows the enzyme only limited access to the substrate. It is also obvious that significant hydrolysis has occurred for MC 2, MC 3, and MC 6, since M_w has decreased considerably. For MC 4 and MC 5, only few cleavages have occurred per chain. Due to its lower DS, MC 1 has been hydrolyzed the most (see Table 3).

ESI-IT mass spectra were recorded after enzymatic digestion and subsequent perdeuteriomethylation. DS values were calculated for each DP. In Figure 5, they are shown up to DP 8 for all MCs. For all samples, DS increased with the DP. This is expected due to the fact that the smaller the oligomers, the higher the proportion of monomer units, which have been involved in the active complex and thus limited by the tolerance of the enzymes' cleavage site. At a DP lower than or equal to the number of sub-sites, some monomers have even been involved twice in the active complex. The differences between various enzymes are further investigated in part 2 of this work.[30] The higher the DP of the product of enzymatic digestion, the lower the relative contribution of the monomer constituents involved in the active complex. Therefore, an asymptotic approach toward a certain DS above the average DS is expected. If the MC is degraded to a large extent, the DS should reach a level-off DS higher than the average already at lower DP. The DS/DP plots (Figure 5) of MC 2, MC 3, MC 5, and MC 6 show nearly the same features. The course for MC 1 is shifted to lower DS values, whereas MC 4 deviates toward higher DS values. This means that although the degradability increases with decreasing DS, this does not result in the same limiting DS/DP, since the

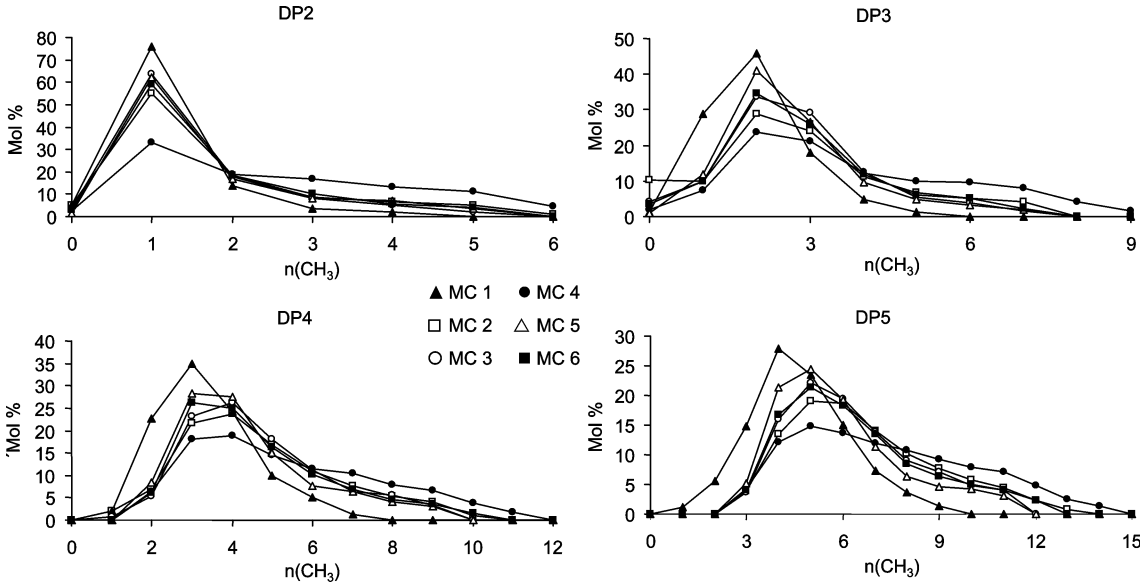


Figure 6. 6. Distribution of methyl groups in DP 2−5 of MC 1−MC 6 after degradation with the enzyme preparation from *Trichoderma longibrachiatum*, perdeuteriomethylation and analysis with ESI-ITMS.

inhibition by methylation at a certain position is active for the corresponding mono-, di-, and tritsubstituted glucosyl units as well.

As mentioned above, the M_w of MC 4 is significantly lower than the values of the other samples. Therefore, it is likely that cleavage products involving one original end group significantly contribute to the oligomers detected with MS. Since these compounds are not or less influenced by the enzymes' selectivity, the average DS increases. These oligosaccharides cause a bimodal distribution of the methyl distributions in each DP as will be outlined as follows. In Figure 6, the methyl distributions of DP 2, 3, 4, and 5 are displayed, and all MCs are compared to give a more detailed picture. Again MC 2, MC 3, MC 5, and MC 6 show nearly the same distribution, whereas MC 1 and MC 4 deviate. The methyl pattern observed for MC 1 is simply shifted toward the lower substituted region, due to the lower DS (1.32) of this sample. However, for MC 4 not only a shift but a bimodal distribution is visible from the plots for the methyl distribution. Figure 7 illustrates the assumption that the pattern of MC 4 is caused by the low molecular mass of the original sample. Thus end groups can no longer be neglected. The substituent distribution after enzymatic cleavage and after random cleavage is compared for DP 3 of MC 4 (Figure 7a). The maximum after random cleavage with trifluoroacetic acid (TFA) matches the second maximum of the distribution after enzymatic cleavage. When taking a contribution of 45% of the random hydrolysis products and subtracting this part from the bimodal curve, the bimodal distribution can be separated into two overlaying patterns (Figure 7b). The left sub-pattern with a low average DS (0.77) represents products from selective cleavage, whereas the right one with the average DS of the sample is interpreted as the contribution of oligomers comprising one original end unit. The influence of the original ends of the polymer in these calculations is now removed after subtracting the contribution of random cleavage from the pattern after enzymatic cleavage. Thereby, a curve representative for the enzymatic cleavage is generated. As can be seen, the enzyme accepts only 4−5 methyl groups at DP 3 instead of the otherwise misinterpreted nine methyl groups. After applying this method to all MCs and subsequent normalization, it is possible to compare the representative enzymatic curves of all MCs (Figure 7c). As seen in this figure, the distribution pattern is independent

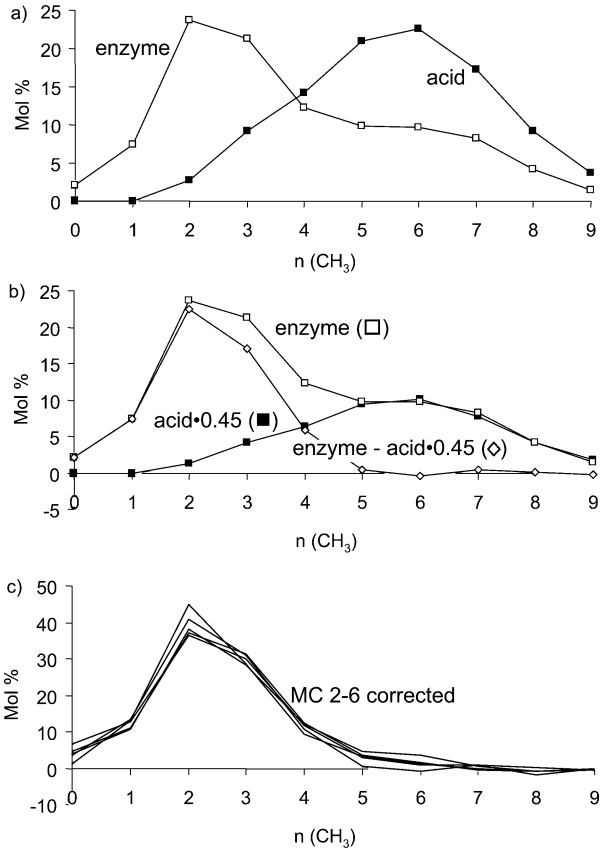


Figure 7. 7. Influence of the samples' original molar mass on the results after enzymatic hydrolysis of MC 4 with *Trichoderma longibrachiatum*. DP 3 is shown for illustration. (a) Methyl distribution after enzymatic hydrolysis and after random degradation; (b) subtraction procedure to correct for unspecific cleavages (see text); (c) methyl pattern of MC 2−MC 6 corrected for unspecific end groups contribution as illustrated for MC 4 in panel b.

of the MC used, when the contribution of the original terminal residues is eliminated. This means that within the sensitivity of this analytical approach the methyl distribution for a certain DP is determined by the selectivity of the enzyme (Tr. longi) and not by the pattern of the MC, since only degradable sequences are selected by the enzymes. However, in part 2 of

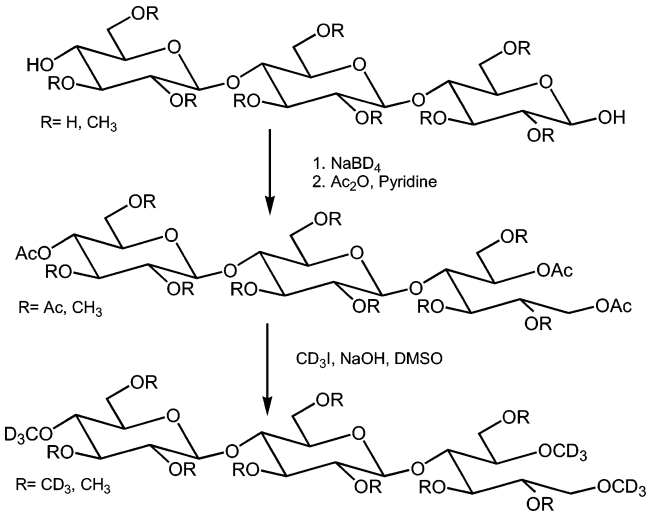
this study, we will show that it is possible to see differences between the MCs if enzyme preparations with different selectivity are used. The difference in the heterogeneity of various MCs should thus be reflected by the quantitative composition of the degraded MC with respect to DP. As already discussed, the used SEC-MALS/RI-instrumentation is not sufficient to separate the low molecular fractions. HPLC could fill this gap between MS and SEC analysis.

Analysis of the Enzymes' Selectivity. Apparently, substitution of glucosyl units interferes with the formation of the active enzyme−substrate complex. Although it can be assumed that the enzyme is most sensitive against the glucosyl units on either side of the cleavage point, the activity of the enzyme also depends on the cooperation of the neighboring glucosyl units. All monomeric units of the oligomeric sequence involved in the active complex can hinder the enzyme in an individual manner. Attempts have been made to determine how the different monomer patterns hinder the enzyme using regioselectively substituted cellulose derivatives.[18] In these investigations 6-*O*- and 2,3-di-*O*-methyl celluloses were analyzed with respect to their degradability with an endoglucanase (*Trichoderma viride*), and it has been found that the enzyme could only cleave glycosidic bonds of the 6-*O*-methyl but not of the 2,3-di-*O*-methyl derivative. In some cases, the enzyme−substrate complex (unsubstituted cellooligomers) could be crystallized, and X-ray structure analysis was performed.[36] With this structural information, molecular modeling calculations could give indication on the special requirements of the active site.

To get an overall picture of the enzyme activity on methyl cellulose, one has to consider that the cellulose derivative contains multiple sequences of possible substrates for the enzyme. It also has to be considered that hydrolysis is achieved either with a pure enzyme or a mixture of several. By a labeling strategy similar to the one described for the analysis of enzymatically hydrolyzed methyl amyloses,[33] the reducing and the nonreducing glycosyl unit of DP 2 and DP 3 and the internal glucosyl unit of DP 3 could be individually analyzed, and their DS could be calculated independently. Furthermore, the contribution of un-, mono-, di-, and trisubstituted residues, c_0, c_1, c_2, and c_3, to the reducing end of DP 2 and 3, and to the nonreducing end of DP 3 (but not of DP 2, see below), could be determined with ESI-MS/MS. Since the end groups of the original sample influence the pattern of hydrolysis products and therefore might cause erroneous interpretation (see above), MC 3 with the highest molecular mass has been selected, since the end groups can then be neglected. As summarized in Scheme 1, the products from enzymatic hydrolysis with Tr. longi endoglucanase were reduced, acetylated (for isolation by extraction), and after exchange of acetyl groups to deuteriomethyl groups submitted to ESI-MS/MS analysis. By this procedure, oligomers are obtained that contain, besides the three either methylated or deuteriomethylated groups (O-2, O-3, and O-6), two deuteriomethyl groups at the reduced terminal glucitol (O-1 and O-5) and one additional at the nonreducing end (O-4). By means of ESI-MS/MS analysis of all oligomers belonging to one DP, quantitative structural information about the individual residues was obtained (Figure 8), in a similar way as has previously been described by Adden and Mischnick et al.[22]

In Figure 8a, the mother mass spectrum of the labeled oligomers (as $[M+Na]^+$) is displayed. DP 3 is shown in an enlarged scale. The original number of methyl groups is assigned to the signals. Figure 8b shows one daughter ion mass spectrum from the trisaccharide fraction originally containing two methyl

Scheme 1. Sample Preparation for the Investigation of the Enzymes Selectivity[a]



[a] DP 3 is shown as an example.

Table 4. Composition (Mol %) and DS Values of the Constituents of the DP 2 and DP 3 Fraction of MC 1 and MC 3 Obtained by Enyzmatic Hydrolysis with Enzyme Preparation from Tr. longi

		MC 1	MC 3
DP 2			
reducing end	c_0	97.48	85.80
	c_1	2.52	13.82
	c_2	0.00	0.38
	c_3	0.00	0.00
	DS	0.03	0.15
nonreducing end	DS	1.03	1.04
	DS_Σ	0.53	0.59
DP 3			
reducing end	c_0	94.71	89.05
	c_1	5.29	10.44
	c_2	0.00	0.51
	c_3	0.00	0.00
	DS	0.05	0.11
nonreducing end	c_0	1.32	2.48
	c_1	56.40	50.09
	c_2	40.38	34.48
	c_3	1.90	12.95
	DS	1.43	1.58
internal residue	DS	0.46	0.69
	DS_Σ	0.65	0.79

groups (as $[M+Li]^+$). Fragment ions are assigned according to the nomenclature of Domon and Costello.[37] Direct information on the contribution of c_0, c_1, c_2, and c_3 to the nonreducing and the reducing ends are obtained from the fragments C_1 and Y_1, respectively. The DS of the internal 1,4-linked glucosyl residues could be calculated using the data of the mother spectrum and those deduced from the intensities of C_1 and Y_1 fragments. Each group of glucosyl units contributes by one-third to the average DS in DP 3. By fragmentation of the homologous belonging to DP 2, Y_1 fragments were exclusively obtained as characteristic ions, providing only information about the reducing end. The DS of the nonreducing end was calculated using the same procedure as has been described for the internal residue of DP 3.

One important issue to prove the accuracy of this method is the agreement of the DS values obtained by this approach and directly after perdeuteriomethylation. On the basis of this

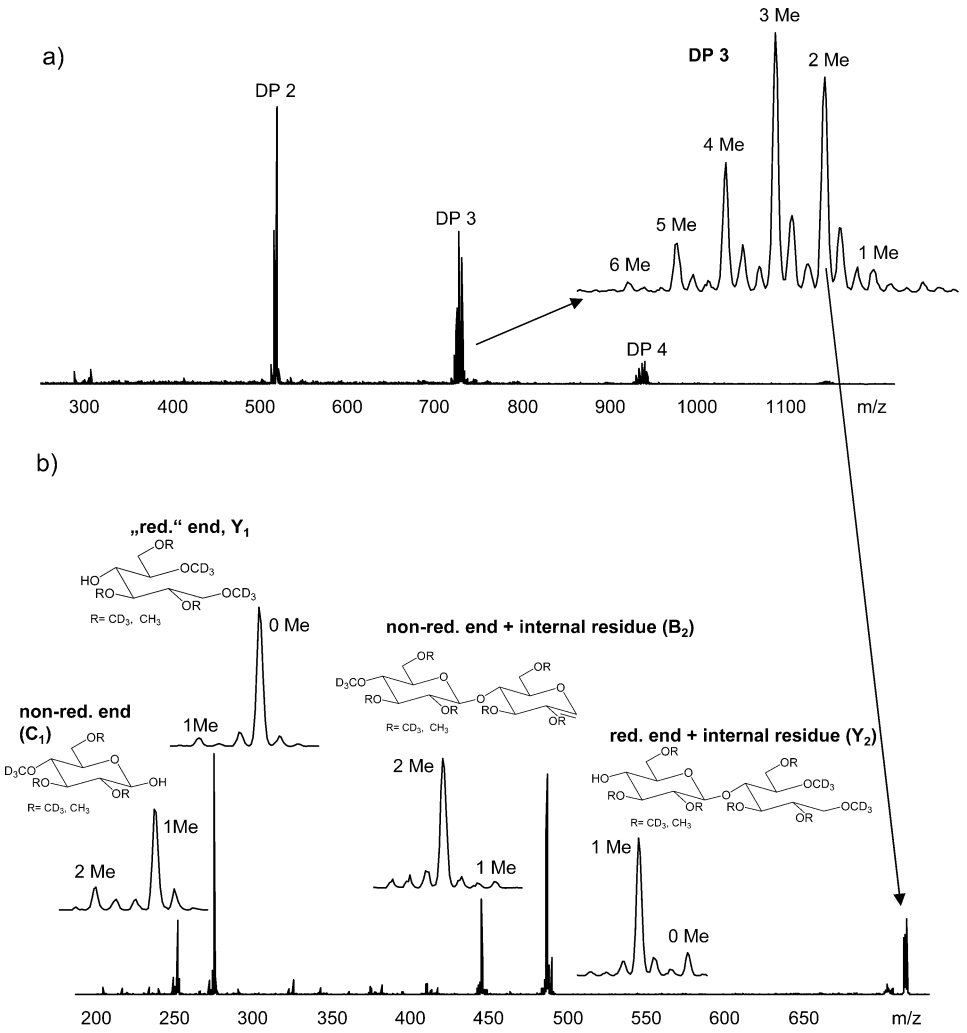


Figure 8. 8. (a) ESI mass spectrum of MC 1 after enzymatic degradation and sample preparation according to Scheme 1. DP 3 is shown in detail. (b) Daughter mass spectrum of the trimer with two CH_3 groups. Enlarged are the *m*/*z* areas of all fragments.

method, the values of MC 1 and MC 3 are just approximately 0.1 and 0.15 lower, showing that the method is valid; that is, the calculated DS of DP 2 (DP 3) for MC 1 after degradation with Tr. longi is 0.61 (0.67) after perdeuteriomethylation and 0.53 (0.65) after performing the sample preparation described here.

Results for enzymatically degraded MC 1 and MC 3 are listed in Table 4. The nomenclature for endoglucanases[38] states that the newly formed reducing end was located at the −1 sub-site of the active center, the next at glycosyl unit −2, and so on. The newly formed nonreducing end was located at sub-site +1 and so on. As shown in Table 4, the new reducing ends are mainly unsubstituted but can bear one substituent. In rare cases, it can hold two, but this is most likely due to the original reducing end of the mother polymer prior to the enzymatic hydrolysis. The DS values for the individual residues are only slightly higher for MC 3 (0.15) than for MC 1 (0.03). The new formed nonreducing end in DP 3 of MC 1 (DS 1.32) refers to sub-site +1 and after another cleavage to sub-site −3. This new nonreducing end is even higher substituted (DS 1.43) than MC 1 on average, whereas for MC 3 (DS 1.83), a DS of 1.58 is observed. In contrast, the nonreducing end of the new formed dimers (DP 2), which refers to sub-sites +1 and −2 of the active site, only reaches a DS of 1.0 for both MCs. The DS of the internal residues of DP 3, which combines relation to sub-sites −2 and +2, is even lower (0.46 for MC 1 and 0.69 for MC 3). Therefore, the influence of sub-site +2 seems to be more pronounced than that of subsite +1. The reducing ends for DP 2 and DP 3 are very similar in composition for both MCs. It is concluded that it is sufficient to choose MC 3 for this analysis. MC 1 does not offer further data.

Conclusion

In part 1 of our studies, it has been shown that perdeuteriomethylation prior to analysis with any MS technique is a prerequisite to evaluate quantitative data. In the investigation of five MCs with a DS of about 1.8, two sets have been found with respect to the monomer composition and the distribution in the polymer chain (heterogeneity). After hydrolysis of these MCs with an enzyme preparation of Tr. longi, differences could be observed for these sets with SEC-MALS/RI. In contrast, the methyl pattern in the oligomeric products and the DS vs DP did not reflect these differences in heterogeneity but were much more influenced by the DS and the M_w of the MC. At a low M_w, oligomers containing one end group of the original polymer strongly contribute and thus overlay the methyl pattern and cause too high a DS. This will results in misinterpretations with respect to the tolerance of the enzyme against methyl groups at certain positions. The methyl distributions/DP could be corrected for this unspecific contribution (random), thus showing that there were no differences for the MCs of the same DS. Thus, the differences in degradability, visible in SEC-MALS/RI, are not reflected in the methyl patterns of the oligomeric degradation

products with Tr. longi that could be analyzed with MS. However, information bearing differences between the samples might be contained in the larger degradation products, which are not accessible by MS analysis. The enzyme cuts out only sequences compatible with its active site. Thus, the results indicate that the amount of these products will be more characteristic for a certain methyl pattern than the pattern itself. To gain information about the selectivity of the enzyme, the end groups representing certain sub-sites of the active complex were labeled by reduction and perdeuteriomethylation. By means of ESI-MS/MS, the methyl pattern or at least the average DS could be analyzed for each individual residue. The reducing end, related to sub-site −1, was nearly completely unsubstituted, whereas the nonreducing end showed the highest DS (>1) and the internal residue of DP3 an average DS value. The methods presented in this study were applied to five cellulose degrading enzymes, which is reported in part 2.

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