New Approaches to the Analysis of Enzymatically Hydrolyzed Methyl Cellulose. Part 2. Comparison of Various Enzyme Preparations

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In this part of our studies, dealing with new approaches to the analysis of enzymatically hydrolyzed methyl cellulose, five different enzymes or enzyme preparations containing endoglucanases (from *Bacillus agaradhaerens Cel 5A*, *Trichoderma reesei*, *Trichoderma viride*, and two obtained from *Trichoderma longibrachiatum*) were used to hydrolyze six different methyl celluloses (MCs). The main goal was to investigate whether enzymes could be used for determination of the heterogeneity of the substituent distribution along the cellulose chain. To obtain information about the heterogeneity, it was necessary to gather information on how the enzymes affect hydrolysis. Size exclusion chromatography with multi-angle light scattering and refractive index detection (SEC-MALS/RI) was used to estimate the molar mass distribution of the MCs before and after hydrolysis. A novel internal standard addition method in combination with electrospray ionization ion trap mass spectrometry (ESI-ITMS) was used to determine the amount of formed oligomers. Two MCs, one with a degree of substitution (DS) of 1.8 and one with DS 1.3, were hydrolyzed with all of the five enzymes. The yield of summarized di- and trisaccharides was approximately 2% of the hydrolysis products for the MC with DS 1.8, whereas the product mixture, obtained from a MC with a DS of 1.3, contained 7−16% di- and trisaccharides. By a novel sample preparation method in combination with ESI-IT tandem MS, outlined in part 1 of this work, it was shown that the enzymes produced oligomers with the reducing end bearing no or only one substituent. Comparison of the methyl pattern at the nonreducing ends of the dimers and trimers indicated that the −2 subsite of the active complex is less tolerant than subsites −3 and +1. All enzymes had similar general selectivity toward the methyl substituents but also showed some differences. From both SEC-MALS/RI and ESI-ITMS, differences with respect to substituent distribution of MCs could be recognized but not for each enzyme used. Basic considerations for enzymatic hydrolysis and analysis of methyl cellulose were listed as a consequence of the results from the work.

Introduction

Cellulose ethers are complex mixtures obtained by usually heterogeneous reactions of alkali treated cellulose with alkyl halides or by addition to oxiranes. Their properties are determined by their structural composition, i.e., molar mass distribution, type and amount of substituents, and their location in the cellulosic material. Although ^{13}C nuclear magnetic resonance (NMR) spectroscopy can be used to get some information on the partial degree of substitution in the various positions of the glucosyl unit (x_2, x_3, x_6)[1,2] where x_i represents the percentage of substituents in position *i*, it is difficult to obtain more detailed structural information on the intact derivatized cellulose polymer chain. Therefore, hydrolysis of the cellulose polymer is often performed to obtain monomers and/or short oligomers that can be analyzed with a variety of analytical techniques. Hydrolysis can be performed usually after permethylation with aqueous acid in a random manner, yielding oligosaccharide mixtures with each fraction of a certain degree of polymerization (DP) representing the pattern of the original sample. Quantitative analysis with mass spectrometry (MS) and comparison with the calculated random pattern give information on the substituent distribution along the cellulose chains.[3,4] Alternatively, hydrolysis can also be performed using cellulose-hydrolyzing enzymes called glucanases. Hydrolysis with glucanases is dependent on the substituent type, amount of substituents, positions of substituents on each glucosyl unit, and distribution of substituents in the oligomeric sequence involved in the active enzyme substrate complex. The general idea when using enzymes for hydrolysis is that low substituted areas are hydrolyzed to a larger extent compared to highly substituted areas. It might therefore be possible to find a correlation between the hydrolysis products and the heterogeneity of the substituent distribution along the cellulose chain.[5,6] However, it has also been shown that modifications on certain positions on the individual glucosyl units as well as on the neighboring glucose units in the chain affect hydrolysis, complicating the interpretation of the product pattern.[7,8] It is also believed that large and bulky substituents such as carboxymethyl substituents are more effective in hindering the enzyme from gaining access to the cellulose chain compared to smaller uncharged substituents such as methyl substituents.[9] Beyond this, it has been stated that at least one unsubstituted anhydroglucose unit is required for the enzyme to gain access inside the cellulose chain.[10]

Enzymes hydrolyzing cellulose can either be exoglucanases, hydrolyzing from the reducing or nonreducing end of the cellulose polymer for production of glucose or cellobiose,[11] or

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endoglucanases, hydrolyzing the interior part of the cellulose chain. β-Glucosidase is another type of exoglucanase cleaving cellobiose (DP 2) to glucose. It is assumed that exoglucanases, with a tunnel shaped active site, can hydrolyze the cellulose derivatives until the enzyme encounters a substituent in the polymer chain that hinders the enzyme from gaining further access.

The hydrolysis pattern associated with endoglucanases, with a cleft shaped active site, is more complex than for exoglucanases. The degree of hydrolysis attained when using enzymes depends to a large extent on the enzyme and the factors described above for hydrolysis of derivatized cellulose. Pure endoglucanases show different selectivity depending on the length of the unsubstituted cellulose chains.[12−14] Typical end products after hydrolysis of unmodified cellulose with pure endoglucanases are cellodextrins with DPs of 1, 2, and 3. Since endoglucanases have easier access to the unmodified parts of the cellulose, it should in principle be possible to obtain information about the substituent distribution along the cellulose chain by studying the products formed.[15−17] However, if the DS of the derivatized cellulose is too high the enzyme cannot hydrolyze the cellulose and no information can be obtained. If hydrolysis is performed using a mixture of enzymes (e.g., a mixture of an endoglucanase and exoglucanase), hydrolysis will generally be more efficient due to synergistic effects. In this work, a detailed investigation has been carried out on how the substituents affect cellulose hydrolysis. Methyl cellulose (MC) was chosen as a substrate for our studies, since the hydrolysis products are easy to detect using mass spectrometry and the methyl substituents are also small and uncharged making them less of a hindrance for the enzymes investigated.

Mass spectrometry (MS) analysis of partially acid hydrolyzed, deuteriomethylated MC yields a constant DS independent of the DP and in agreement with the total average DS of the sample.[3,4] In this paper, we show that this is not the case for enzymatically degraded samples.

In polysaccharide analysis, size exclusion chromatography with multi angle light scattering and refractive index detection (SEC-MALS/RI) is a powerful tool to determine the average molar mass and molar mass distribution of, e.g., cellulose and starch derivatives before and after enzymatic degradation.[17−19] If SEC is employed together with RI and MALS detection, the molar mass distribution of the hydrolysis products can give information on the enzymes' action on the derivatized polysaccharide without any need for the use of calibration standards. However, the sensitivity for MALS in the low molar mass range is generally very poor, and thus, signals for these areas should be analyzed carefully.

In this work, classical and novel methods have been applied to investigate how cellulose-degrading enzymes hydrolyze MC. Detailed and accurate determination of the DS of the produced oligomers (DP 2−DP 8) from hydrolysis of the MCs with the different enzymes was achieved by MS analysis. General information that seems to be valid for most endoglucanases about the enzymes' demands for performing hydrolysis on MC and the accessibility to MC was achieved by means of tandem MS. A novel method for quantitative analysis of the oligomers detectable with MS was applied.

Experimental Section

General. Generally this paper follows the Experimental Section of part 1 of this work.[20] All reagents used were of highest purity available. Five different endoglucanase preparations were used. *Trichoderma viride* (Tr. viride) was purchased from SERVA Electrophoresis GmbH, Heidelberg, Germany (Contr. No.: 10186), *Trichoderma longibrachiatum* 1 and 2 (Tr. longi 1 (Lot nr. 030201) and Tr. longi 2 (Lot. nr. 50201)) from Megazyme, (Wicklow, Ireland), *Bacillus agaradhaerens* (BaCel 5A) was a kind gift from the late Dr. Martin Schülein (NovoZymes, Bagsvaerd, Denmark) and *Trichoderma reesei* (Tr. reesei) was purchased from Fluka (ATCC 26921). Tr. longi 1 was the enzyme used for method development in part 1 of this work.[20] Me-d_3−I was purchased from Deutero (Deutero GmbH, Kastellaun, Germany). MC 1−MC 6 were commercially available cellulose derivatives. D-(+)-Glucose (99,5%), D-(+)-cellobiose (98%), d-(+)-cellotriose (95%), D-(+)-cellotetraose (95%), and D-(+)-cellopentaose (97%) were purchased from Sigma-Aldrich, and D-(+)-cellohexaose (97%) was purchased from Sikagaku Corporation (St. Petersburg, FL).

Enzymatic Degradation of MC. Approximately 30 mg of each MC was dissolved in 3 mL of H_2O at 4 °C overnight. Due to impurities from sugars, buffer, and stabilizers, the enzymes needed to be purified before addition to the MC solutions. The dry enzymes were dissolved in water to a concentration of approximately 30 U mL^{-1} (1 U is defined as the amount of reducing ends after CMC hydrolysis). All enzymes that were obtained in solution were kept in their original concentration. All enzyme solutions were thereafter centrifuged using 5 kDa Millipore Ultrafree centrifuge filtration tubes (Millipore, Bedford, MA). 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 were seen in the filtrates when analyzing the solution with ESI-ITMS. Finally, the supernatant in the filters was diluted in water to a final volume of approximately 400 μL, stirred gently, and added evenly to the six MC solutions. All hydrolyzates were shaken at room temperature for 96 h, then boiled at 95 °C for 15 min, cooled, and finally centrifuged to remove the enzyme. The solution was thereafter freeze-dried overnight in Eppendorf vials.

Perdeuteriomethylation. All methylations were performed according to Ciucanu and Kerek[21] in 1 mL V−Vials. The hydrolyzed sample (4 mg) was solved in 350 μL of DMSO and stirred for 2 h at room temperature. Then 20 mg of pulverized NaOH was added to form the polyanions of the oligomeric mixture. After 30 min, 40 μL of Me-d_3−I was added and the solutions were stirred for at least 6 h at room temperature. For obtaining complete methylation, the reagents were then added for a second time, and the solution was stirred overnight. The samples were cleaned by extraction with dichloromethane and water three times. The combined organic layers were then washed with saturated NaCl solution, then with 5% $Na_2S_2O_3$ solution, and finally water. After that, the organic phase was dried with Na_2SO_4. The solvent was evaporated and the residue was redissolved to approximately 0.5 g L^{-1} in methanol (MeOH). For the tandem MS experiments (see the section Investigation of Enzyme Selectivity to Substituents using MS) performed with Li^+ adducts, the methylated samples were dissolved to a concentration of approximately 0.5 g L^{-1} in 1 mM $LiClO_4$.

Quantification of Oligosaccharides in MS by Internal Standard Addition. A known amount of approximately 4 mg of the enzymatically hydrolyzed MC 1 (DS 1.3) or MC 3 (DS 1.8) was dissolved in 5.0 mL of H_2O. Each solution was distributed into four different vials with 1 mL of solution in each vial. A solution containing a known amount of glucose, cellobiose, and cellotriose (~0.1 g L^{-1} of each) was added in increasing volumes to three of the vials. To the first vial no standards were added. For MC 1 none, 50, 100, or 150 μL of the standard solution was added and for MC 3 no, 25, 50, or 75 μL was added. Thereafter, each vial was dried at 40 °C in a stream of N_2. Subsequently, each sample was deuteriomethylated as described above. To quantify the amount of DP 2 and 3 with respect to the original MC, the signals of a certain DP from the mass spectrum without standard addition were individually normalized to 100%. Then the most intensive peak within each DP in the mass spectrum of the sample where nothing was added was set as a constant. These constant values were used for determining the differences of absolute intensities in the mass spectra after addition. For example in DP 2 and DP 3 the signals with one or two methyl

groups, respectively, (2_1 and 3_2 where the large number is the DP and the superscript is the total number of methyl groups in the oligomer) were the most intense. By correlating the increase in the signal of DP 2_0 with the amount of added standard and the signals of the homologue of DP 2 (2_1, 2_2, etc.), it was possible to determine the amount of DP 2 with respect to the total amount of cellulose material.

End Group-Labeling of Products from Enzymatic Degradation of MC. The method for end groups labeling has been described in detail in part 1 of this work.[20] In short, approximately 2 mg of freeze-dried hydrolyzate of MC was reduced during stirring in $NaBD_4$ (in ammonia) solution. The solution was cooled to room temperature, and the borate was removed by co-distillation (five times) using acetic acid solution in MeOH as its methyl ester. The sample was thereafter acetylated. The products were isolated by liquid−liquid extraction using dichloromethane, evaporated with nitrogen and thereafter deuteriomethylated as described above.

Instrumentation. *Electrospray Ionization with Ion Trap MS Detection.* An Esquire MS equipment (Bruker Daltonics, Bremen, Germany) was employed for acquiring electrospray ionization ion trap (ESI-IT) mass spectra. The mass spectra were recorded in positive ionization mode. The partially degraded samples were introduced directly via a syringe at a flow of 120 μL h^{-1}. For analyzing the lithium ion adducts, samples were dissolved in 1 mM $LiClO_4$ in MeOH. All mass spectra used for quantitative analysis consisted of an average of 200 scans between *m*/*z* of 200 and 2000. Nitrogen was 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.

High-Performance Anion-Exchange Chromatography with Pulse Amperometric Detection (HPAEC−PAD). Cellodextrins of DP 3−6 were hydrolyzed with the enzyme solutions prepared (see above). The cellodextrin concentration was approximately 100 μM, and the enzymes were added with the same concentration as described above. The solutions were incubated overnight at room temperature. The cellodextrin hydrolyzates were analyzed with a Dionex HPAEC-PAD system (Dionex Corp., Sunnyvale, CA) at a flow-rate of 1 mL min^{-1}. The system was controlled by PeakNet software and consisted of a GP40 gradient pump, a Carbopac PA-1 guard and a PA-1 analytical column, and an ED40 electrochemical detector with an Au working and a $Ag|AgCl_{(sat)}$ reference electrode.[22] Injection of the samples was performed using a Spectra-Physics Analytical (San Jose, CA) AS-3000 autosampler with a 10 μL injection loop. Separation of the oligomers was performed using a gradient program with 150 mM NaOH + 250 mM sodium acetate (eluent A) and 150 mM NaOH (eluent B). Between 0 and 0.5 min, eluents A and B were held constant at 30% and 70% respectively. Between 0.5 and 2.5 min eluent A increased linearly from 30% to 50% and then linearly increased up to 100% at 9.5 min. The total flow rate was 1 mL min^{-1}. The column was equilibrated with 30% eluent A and 70% eluent B for 30 min. The ED40 electrochemical detector with an Au working electrode had the following waveform as described by Richardson et al.:[23] $E_1 = 0.10$ V ($t_d = 0.20$ s, $t_1 = 0.20$ s), $E_2 = 0.7$ V ($t_2 = 0.19$ s) and $E_3 = -0.75$ V ($t_3 = 0.39$ s).

Size-Exclusion Chromatography with Multiangle Light Scattering/Refractive Index Detection (SEC-MALS/RI). MCs were analyzed with SEC-MALS/RI detection prior to and after enzymatic hydrolysis. The separation was carried out using a TSKgel $GMPW_{XL}$ column (30 cm × 7.8 mm i.d. TosoHaas, Stuttgart, Germany). The pump in use was a Shimadzu LC-10AD liquid chromatography pump (Shimadzu Corporation, Tokyo, Japan) and the degasser was a Shimadzu DGU-14A. Injection of the polymer solution was carried out by a Waters 717 plus Autosampler (Waters, Milford, MA). MALS and RI detection were performed utilizing DAWN EOS MALS and Optilab RI detectors (Wyatt Technology Corp., Santa Barbara, CA) respectively. The laser wavelength was 690.0 nm. A solution consisting of 10 mM NaCl + 0.02% NaN_3 was used as mobile phase at a flow rate of 0.5 mL min^{-1}, and the sample volume was 100 μL. The analyte concentration was 0.3 g L^{-1}, and double injections were made. A 0.05 μm VM Millipore filter (Millipore, Bedford, MA) was placed between the pump and the injector. A Sartorius CA 0.45 μm filter (Göttingen, Germany) was placed after the column to improve the MALS signal. Astra for Windows version 4.73.04 was used for the data evaluation. Injection of pullulan standards (10 and 400 kDa) was used to verify the measurements. The refractive index increment, d*n*/d*c*, was determined to 0.136 mL/g on MC by the instrument. The weight average and number average molar mass was calculated using the Berry method.[24,25] The recovery was obtained from the ratio of the mass eluted from the channel (determined by intergration of the RI signal) to the mass injected. The recovery for the investigated samples was generally above 80% both before and after hydrolysis. Angles between 35° and 100° were used for the measurements of the molar mass determinations of hydrolyzed samples. For the unhydrolyzed samples angles between 26° and 163° were used.

Results and Discussion

The goal of the work presented was to investigate the potential of enzymes for elucidation of structural differences of MC, which are related to the substituent pattern along the polymer chain. The action of five cellulose degrading enzymes containing mainly endoglucanases on MC was determined using six commercial MCs, one with a DS of 1.32 and the other with a DS around 1.8. All MCs have been investigated, in part 1 of this work,[20] by random degradation and MS as a reference method. Four commercially available enzyme preparations, *Trichoderma longibrachiatum* 1 and 2 (Tr. longi 1 and 2), Tr. reesei, and Tr. viride and one pure noncommercial enzyme, BaCel 5A, were used in the study. To avoid misinterpretations and better understanding of enzymatic hydrolysis, it was first necessary to check the enzymatic purity in terms of extraneous activity by application to defined cellodextrins. SEC-MALS/RI was applied to the MCs prior to and after enzymatic digestion to get an estimation of the degree of hydrolysis. The oligomeric products were quantified using a novel method including standard addition and MS analysis. ESI-ITMS was also applied to investigating the enzymatic action on the MC in more detail. A method involving specific marking of the formed oligmers, developed in part 1 of this work,[20] was used to gain further information about the selectivity of the enzymes.

Enzyme Purity and Enzymatic Mechanisms. The reactivity of each enzyme preparation was investigated by separately hydrolyzing unmodified cellodextrins with a DP of 3−6. All hydrolyses were run to completion; that is, if the enzyme has activity for the cellodextrin, no traces of initial substrates were left in the solution. The hydrolysis products were analyzed by means of HPAEC-PAD. It was concluded that the enzyme preparations producing only glucose (DP 1) as end product from all cellodextrin substrates did not contain only one endoglucanase but also exoglucanase or β-glucosidase (extraneous) activities. This assumption is valid since most (if not all) pure endoglucanases have specific demands on the chain length (DP) of the substrate, and it is also known that the production of only glucose as end product requires a combination of enzymes.[11−13] Even though Tr. longi 1 and Tr. longi 2 are commercialized as pure enzymes, the manufacturer states that they may contain extraneous activity. Tr. viride and Tr. reesei are commercialized as multicomponent enzyme systems containing

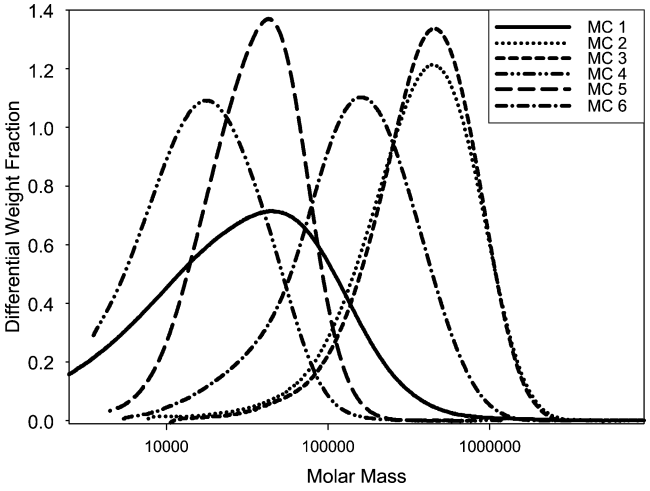

Figure 1. Molar mass distribution of MC 1−MC 6 determined with SEC-MALS/RI.

mixtures of endoglucanases and exoglucanases and should be able to form glucose.

BaCel 5A and Tr. longi 2 seemed to be the only pure endoglucanase preparations since all other enzyme preparations hydrolyzed the cellodextrins completely to glucose (DP 1). In contrast, BaCel 5A showed very low activity for DP 3 (95 mol % remained intact), DP 4 was hydrolyzed to DP 2 (95%), DP 5 was hydrolyzed to DP 2 (48%) and DP 3 (52%), and DP 6 was hydrolyzed to DP 2 (69%) and DP 3 (29%). No glucose formation could be quantified when using BaCel 5A. These results verify our deduction of the mechanism for BaCel 5A in previous work.[14] BaCel 5A can only cleave a site, which is flanked on either side by at least two glucose units. Moreover, all findings are in agreement with Davies et al. in that BaCel 5A has no activity on cellodextrins with a chain length below DP 4.[12,14] Although the Tr. longi 1 and Tr. longi 2 enzyme preparations were sold as the same enzyme, they behaved differently. Tr. longi 1 hydrolyzed all cellodextrin standards to glucose, but Tr. longi 2 did not. It was therefore concluded that Tr. longi 1 was contaminated with species having extraneous activities. These extraneous activities do not seem to influence the degradability of the intact MC (only small difference in SEC) but rather only affect the hydrolysis of small unsubstituted cellooligomers (see the Internal Standard Addition section). Tr. longi 2 hydrolyzed DP 3 to DP 1 (45 mol %) and DP 2 (55%), DP 4 to DP 1 (10%) and DP 2 (90%), DP 5 to DP 1 (30%) and DP 2 (70%), and DP 6 to DP 1 (24%) and DP 2 (75%). That DP 2 is not accepted by the enzyme as a substrate is visible after analyzing the products of the hydrolysis of DP 3, where DP 1 and 2 are produced with a molar ratio of 1:1. Product inhibition, substrate concentration, preference to substrate length and a mixture of enzymes might influence the hydrolysis mechanism investigation in such a way that the products formed will not represent the mechanism entirely.[14,26]

It is expected that the enzymes behave accordingly when they are used for hydrolysis of MC. Thus, no formation of DP 1 is expected when using BaCel 5A for hydrolysis of MC but DP 1 could be formed by the other enzymes if the necessary hydrolysis sites are available.

SEC-MALS/RI. To estimate the molar mass distribution of the intact MC samples, SEC-MALS/RI was employed. The molar mass profiles on intact MCs are shown in Figure 1. In Table 1, the number-average molar mass (M_n), weight-average molar mass (M_w), and polydispersity index (M_w/M_n) are given

Table 1. Number-Average Molar Mass (M_n), Weight-Average Molar Mass (M_w), and Polydispersity Index (M_w/M_n) of MC 1−MC 6 Prior to Enzymatic Hydrolysis[a]

	unhydrolyzed			longi 1			reesei		
sample	M_n	M_w	M_w/M_n	M_n	M_w	M_w/M_n	M_n	M_w	M_w/M_n
MC1	11.6	61.3	5.3						
MC2	221.8	466.5	2.1	22.2	34.7	1.6			
MC3	262.7	506.1	1.9	21.1	33.3	1.6			
MC4	12.1	23	1.9	9	13.1	1.5			
MC5	26.9	42	1.6	10.8	15.1	1.4			
MC6	90.6	201.5	2.2	13.8	22.4	1.6			

	viride			longi 2			BaCel 5A		
sample	M_n	M_w	M_w/M_n	M_n	M_w	M_w/M_n	M_n	M_w	M_w/M_n
MC1									
MC2	13.0	19.8	1.5	22.6	32.5	1.4	10.8	17.2	1.6
MC3	11.6	17.4	1.5	19.4	30.0	1.6	10.6	17.3	1.6
MC4	8.4	12.4	1.5	9.1	13.3	1.5	12.7	15.8	1.2
MC5	8.3	11.0	1.3	10.1	14.1	1.4	13.4	16.2	1.2
MC6	9.9	15.2	1.5	13.2	20.3	1.5	11.6	17.7	1.5

[a] Values are averages of three injections.

for the intact and hydrolyzed samples. For the hydrolyzed samples, the MALS signal was sometimes very low due to low sensitivity at low angles and low amount of hydrolysis products. All MALS signals were calibrated to the linear range. Prior to hydrolysis, MC 2 and MC 3 have approximately the same molar mass distribution, which is shifted to a larger average molecular weight compared to the other MCs. MC 4 has the lowest average molar mass. Due to column restrictions and the extensive hydrolysis, the molar mass of hydrolyzed MC 1 could not be determined.

It can be expected that MCs with a similar substituent distribution will become more resistant to enzymatic attack with increasing DS. Besides DS, regioselectivity of substitution and the probability of sequences able to form an active enzyme−substrate complex will influence the product pattern of enzymatic hydrolysis. If the distribution of methyl substituents is similar in the glucosyl unit and along the chain for all MCs with the same DS, it is expected that the final hydrolysis products will have similar molar mass distributions. Cellulose chains that originally have a higher molar mass will experience more cleavages than shorter ones, until the limit of hydrolysis has been reached. As reported in part 1,[20] differences between the MCs were observed after hydrolysis with Tr. longi 1 in SEC-MALS/RI analysis. However, if the enzyme or enzyme mixture can hydrolyze the MC to too high an extent (very low selectivity of the enzyme) the influence of differences in the substituent distribution will be seen to a much less extent in the SEC profiles. This is indicated in Figure 2 when the BaCel 5A enzyme mixture hydrolyzed five different MCs with similar DS. The apexes of the SEC-elution profiles for five of the MCs are located in a narrow range of molar masses. The same trend could be seen when analyzing the elution volumes using only the RI signal (data not shown). As has been shown in part 1,[20] the range of formed molar masses for Tr. longi 1 are much larger (compared to BaCel 5A and Tr. viride) and as seen in Table 1, the differences between the MCs are much bigger for this enzyme (also seen for Tr. longi 2). As also shown in part 1, the substituent distribution of the five MCs with a DS of 1.8 fall within two groups. This was believed to be due to the fact that MC 2 and MC 3 show nearly random distribution, whereas MC 4, MC 5, and MC 6 are more heterogeneously substituted. These group properties are not reflected to any significant extent

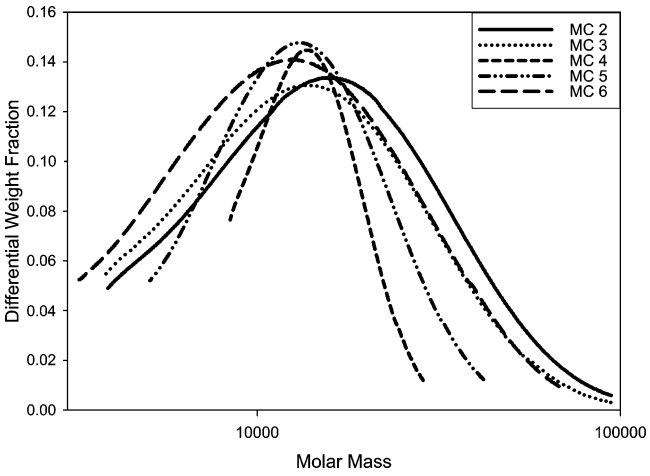

Figure 2. Molar mass distributions of MC 2−MC 6 all with a DS of about 1.8 after hydrolysis with BaCel 5A. Analysis was performed with SEC-RI.

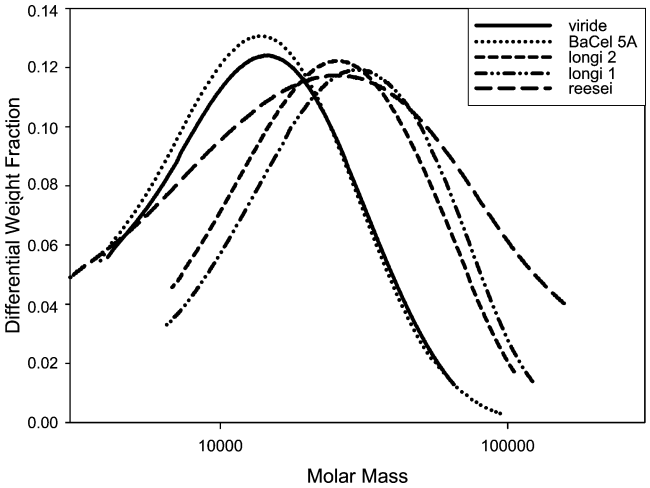

Figure 3. Molar mass distribution of MC 3 (DS 1.83) hydrolyzed with different enzymes. Analysis was performed with SEC-MALS/RI.

from the SEC analysis after degradation with Tr. viride or BaCel 5A but, as mentioned above, can be recognized more clearly for the Tr. longi 1 and Tr. longi 2 digest. MC 4 deviates with a slightly more narrow distribution. The order of M_w of the original samples (MC3 > MC 2 > MC6 > MC 5 > MC 4) is nearly retained after enzymic hydrolysis (MC 2/MC 3 > MC 6 > MC 5/MC 4) with all enzymes except Tr. reesei. This enzyme preparation gives a broad mass distribution profile completely different from the other enzymes as can be seen in Figure 3. This might be considered as surprising since it is expected that mixtures of enzymes such as Tr. reesei and Tr. longi 1 should also be less selective like Tr. viride since they contain extraneous activities. MC 1 is not included in this picture since it has a lower DS of 1.32 and is therefore hydrolyzed to such a large extent that it is not comparable with the other MCs. The SEC-MALS/RI system was not capable of distinguishing any differences for MC 1 hydrolyzed with different enzymes. Thus, another approach had to be used (see the Internal Standard Addition section below).

As can be seen in Figure 3, where the molar mass distributions of MC 3 (DS 1.83) after degradation with each enzyme are compared, the distribution profiles differ. This means that the enzymes degrade the MC with different efficiencies. The lower the molar mass of the products in comparison to the value of the intact MC, the more the enzyme has been able to attack the derivatized cellulose chain. MC 3 was used for this comparison since the differences between the enzymes were most pronounced. The Tr. viride and BaCel 5A enzymes seemed to hydrolyze MC 3 to approximately the same extent. However, Tr. longi 1 and Tr. longi 2 hydrolyzed MC 3 to a much lower extent with the apex more shifted toward higher molar mass. The observation that the Tr. longi 1 and the Tr. longi 2 enzymes curves did not coincide, confirms that these two enzyme batches do not share the same activity. A very broad mass range of hydrolysis products are produced with Tr. reesei with even some intact material left after hydrolysis completion. The data from Tr. reesei indicated that there were aggregates in the sample and the data was very unreliable. Also, due to extremely bad signals, these data was therefore excluded from Table 1.

Internal Standard Addition. Quantification of the oligomeric hydrolysis products derived from MC has to the best of our knowledge not been performed using MS. Until now quantification of the hydrolysis products has only been possible after extensive sample pretreatment and cleanup using HPAEC−PAD. It also requires transformation of the oligomers to monomers.[6] Furthermore, the response in PAD is strongly decreased by OH protection, and standards are not available for longer oligomers.

In this study, a novel approach using internal standard addition and perdeuteriomethylation was applied to determine the amount of oligomers formed after enzymatic hydrolysis with respect to the starting material. Pure cellodextrin standards with DP 1 (1_0), DP 2 (2_0), and DP 3 (3_0) were added in different linear amounts to the enzymatic hydrolyzates, whereafter perdeuteriomethylation was performed. It is thereby possible to obtain a correlation between the peak area and the amount of the compound. No production of DP 1 could be measured due to a very low signal. This can be explained since enzymatic degradation with β-glucosidase would require two adjacent unsubstituted glucosyl units, which is most unlikely in these samples (<0.3% for MCs with DS 1.8). It is seen from the spectra in Figure 4 that the peak area corresponding to 2_0 increased with added amount of cellobiose. By correlating the increase in signal of DP 2_0 with the amount of added standard and the signals of the DP 2 homologous (2_1, 2_2, etc.), it was possible to determine the amount of DP 2 with respect to the total MC material. The quantification of DP 3 was performed in the same manner. The method proposed does not require standards of each individual constituent of a certain DP. Since the response within each fraction of a certain DP is linear for all peaks, it is possible to summarize all peaks and obtain the amount for that DP.

MC 1 (DS 1.32) and MC 3 (DS 1.83) were investigated according to the procedure described above after hydrolysis with each enzyme. As seen in Table 2 there is a clear difference between MC 1 and MC 3. As expected, higher amounts of DP 2 and DP 3 were produced for MC 1 by all enzymes. Hydrolysis of MC 3 produces approximately 1% of each DP, whereas for MC 1, the formation of these oligosaccharides in summary ranges from 7 to 16% with respect to the starting material and enzyme. The linear regression correlation coefficients for the amount added vs signal increase are given and they are high in all cases (r^2 > 0.99). Nevertheless, there might be errors due to influences from scaling and sample contaminates that could influence the outcome. From these results the question arises whether any conclusion can be drawn from the pattern of oligosaccharides representing only 1 or 10% of the entire sample.

Determination of the amount of oligosaccharides longer than DP 3 would be of interest. However, sufficient pure cellodextrin

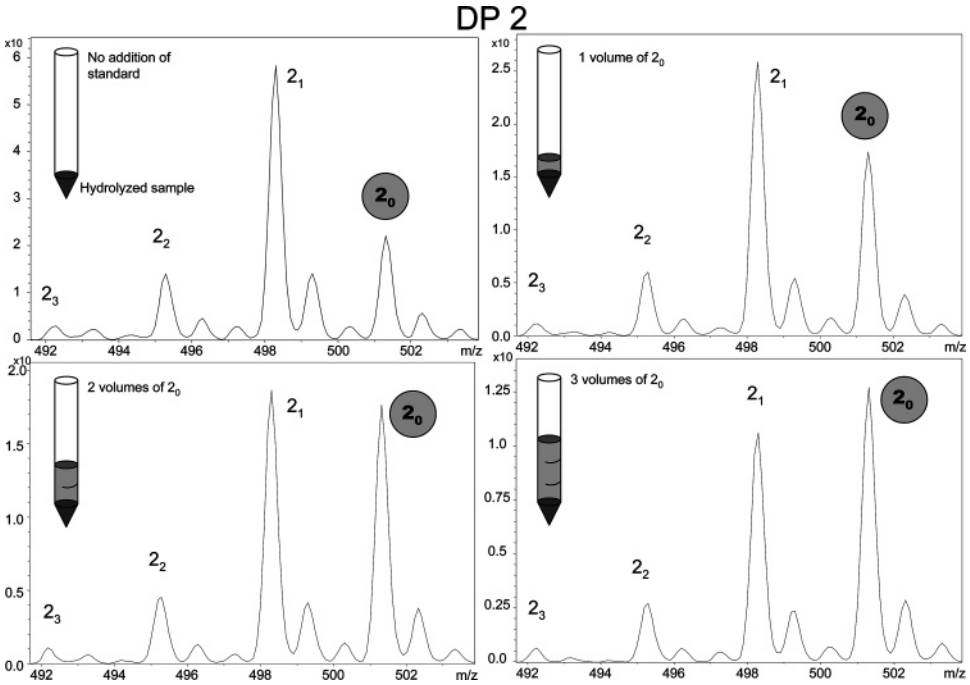

Figure 4. Mass spectra (ESI-ITMS) of the DP 2 region of MC 3 after enzymic hydrolysis with BaCel 5A, addition of increasing amounts of standard (unsubstituted cellobiose, 2_0) and subsequent perdeuteriomethylation. Peaks are assigned with respect to DP (=2) and the number of methyl groups originally present in the sample (=0−3).

Table 2. Composition of the DP 2 and DP 3 Fractions of MC 1 (DS 1.32) and MC 3 (DS 1.83) Obtained by Degradation with Various Enzymes[a]

MC 1	BaCel 5A	longi 1	longi 2	viride	reesei	MC 3	BaCel 5A	longi 1	longi 2	viride	reesei
2_6	0.00	0.00	0.00	0.00	0.00	2_6	0.00	0.01	0.00	0.02	0.02
2_5	0.00	0.00	0.00	0.12	0.00	2_5	0.04	0.01	0.00	0.09	0.03
2_4	0.11	0.13	0.00	0.31	0.21	2_4	0.04	0.01	0.01	0.11	0.07
2_3	0.19	0.28	0.26	0.77	0.68	2_3	0.04	0.02	0.02	0.13	0.11
2_2	0.39	0.61	0.93	1.90	1.96	2_2	0.10	0.08	0.07	0.34	0.32
2_1	1.49	2.00	3.57	3.25	4.00	2_1	0.20	0.28	0.20	0.42	0.31
2_0	1.16	0.26	1.37	0.29	0.29	2_0	0.06	0.03	0.08	0.08	0.22
Σ	3.34	3.28	6.13	6.64	7.15	Σ	0.48	0.45	0.37	1.19	1.07
r^2	0.995	0.997	0.999	0.999	0.994	r^2	1.000	0.994	0.999	1.000	0.997
3_9	0.00	0.00	0.00	0.00	0.00	3_9	0.00	0.00	0.00	0.00	0.00
3_8	0.00	0.00	0.00	0.00	0.00	3_8	0.00	0.00	0.00	0.05	0.01
3_7	0.00	0.06	0.00	0.11	0.08	3_7	0.02	0.00	0.00	0.09	0.03
3_6	0.04	0.16	0.00	0.26	0.21	3_6	0.11	0.03	0.02	0.18	0.05
3_5	0.17	0.21	0.32	0.50	0.57	3_5	0.19	0.00	0.04	0.29	0.08
3_4	0.38	0.41	0.92	0.89	1.28	3_4	0.32	0.09	0.07	0.36	0.14
3_3	0.89	0.89	2.95	1.50	2.75	3_3	0.41	0.30	0.16	0.63	0.25
3_2	1.29	1.72	4.30	1.43	2.91	3_2	0.27	0.31	0.21	0.45	0.15
3_1	0.97	1.03	1.71	0.12	0.14	3_1	0.09	0.11	0.06	0.07	0.01
3_0	0.04	0.01	0.11	0.00	0.07	3_0	0.01	0.07	0.02	0.10	0.14
Σ	3.78	4.49	10.32	4.81	8.02	Σ	1.41	0.91	0.58	2.22	0.86
r^2	0.994	1.000	1.000	0.999	0.993	r^2	0.998	0.994	0.998	0.993	0.992

[a] The values given are in weight % referred to the amount of the original MC. R^2 values refer to the calibration curve of standard addition for each sample.

standards were not available for standard addition. Applying the method on other cellulose derivatives such as hydroxyethyl-, hydroxypropyl-, or ethyl-cellulose, or mixed ethers bearing methyl groups as well should be possible as long as a quantitative response is achieved.[3,27] This makes the method proposed superior to for instance HPAEC-PAD, where identification is difficult and, furthermore, the response is not linear for compounds with different degrees of substitution making quantification of the oligomers impossible.

Investigation of oligosaccharide profile by ESI-ITMS for enzyme characterization. In the first part of this work,[20] we investigated how the products formed after partial acidic and enzymatic degradation of MCs were discriminated in different soft MS techniques due to polarity and molecular size. To remedy these effects, we performed perdeuteriomethylation of the MC after partial degradation. In this paper, the focus has been on the investigation of the mass spectral profiles of the deuteriomethylated hydrolysis products with respect to differences between the hydrolytic performances of the enzymes. The six MCs were therefore hydrolyzed individually by the enzymes, and the hydrolysis products were analyzed using MALDI-TOFMS, ESI-QqQMS, and ESI-ITMS. Since it has already been

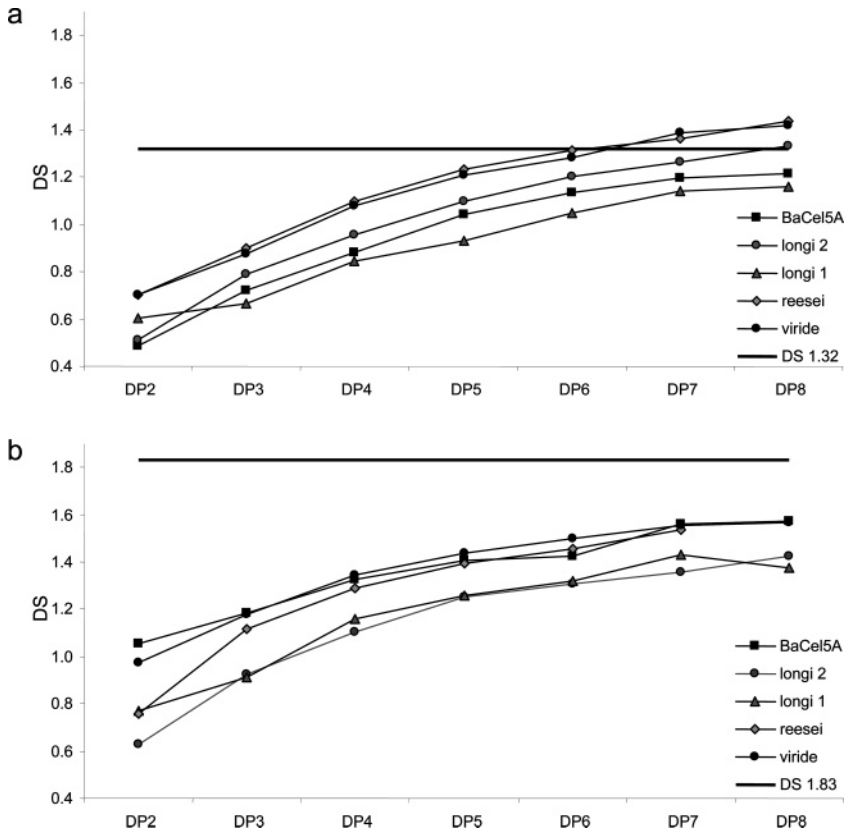

Figure 5. DS per DP calculated from signal intensities in mass spectra of perdeuteriomethylated oligomers produced from (a) MC 1, DS 1.32 and (b) MC 3, DS 1.83, with all endoglucanases. The horizontal lines give the average DS for the MCs.

shown in part 1[20] of this work that after perdeuteriomethylation the variation between these methods is eliminated, ESI-ITMS data is only presented in this work. For illustration, results obtained for MC 1 and MC 3 are presented in Figure 5, where DS was calculated and plotted versus DP.

As shown in part 1 of this work, DS increases with DP of enzymatically depolymerized MC.[28] The DS/DP profiles were influenced by the original molar mass of the intact MCs and their DS. As shown in Figure 5, the DS/DP curves also depend on the enzyme applied, with Tr. viride and Tr. reesei being the most efficient with respect to DS for both MCs. As DP increases, the oligomer can bear more substituents without affecting the cleavage site.

For MC 1 (DS 1.3, Figure 5a), the DS increases from 0.5 (Tr. longi 2 and BaCel 5A) to 0.7 (Tr. reesei and Tr. viride) at DP 2 to 1.1 (Tr. longi 1) to 1.4 (Tr. reesei and Tr. viride) at DP 8. For MC 3 (DS 1.8, Figure 5b) the DS starts at 0.6 (Tr. longi 2) to 1.05 (BaCel 5A) and seems to level off at a DS of about 1.35 for both Tr. longi 1 and 2 and 1.55 for the other enzymes, only increasing slightly thereafter. This was observed for all MCs with a DS of 1.8 (data not shown). As seen with both, SEC-MALS/RI and the standard addition method, MC 1 with a lower DS was hydrolyzed to a higher extent producing a significantly higher amount of oligosaccharides. For this MC, the DS increases for some enzymes (Tr. reesei and Tr. viride) to a value above the average already at DP 6−8. From the abundance ratios of DP 2 and DP 3 with respect to the intact sample (15% for Tr. reesei) and the DS/DP plot, it can be roughly estimated that the oligomers detected in MS represent about 25−40% of the sample. Thus, the average DS is met at a much lower DP (6 or 8 for three of the enzymes) than for the more highly substituted MCs, which is expected at a much higher DP.

The reason for the differences between the enzymes in the DS/DP plots is believed to be the same as explained for the SEC-MALS/RI analysis, i.e., due to the sensitivity of the enzymes to the substituents. A higher DS per DP for a certain MC indicates that the enzyme is more tolerant against substituents. Differences between the activities of the enzymes are also shown in Figure 6, where two enzymes are compared. In this figure, DS/DP plots are compared for all MCs hydrolyzed with Tr. viride and Tr. longi 1. MC 1 with the lower DS differs in the already described manner for all enzymes. Consistent with the SEC results, no larger differences between MC 2−6 are observed after digestion with Tr. viride (Figure 6a) and BaCel 5A (not shown). However, the results for Tr. longi 1 (Figure 6b) do not show the same difference between MC 2 and 3 on one hand, and MC 4−6 on the other hand as visible in SEC-MALS/RI shown in part 1 of this work.[20] This again indicates that the qualitative product composition is determined by the enzyme's restrictions and requirements, whereas the amount of such suitable sequences depends on the distribution of methyl groups in the substrate, i.e., the MC. Thus, the differences are better reflected by the molar proportions of oligomers than by their DS. For MC 4 hydrolyzed with Tr. longi 1, the DS/DP plot is significantly shifted toward higher values, the reason for which has been explained in detail in part 1 of this work. Although the DS/DP plots provide information about the enzymes, they are not sufficient to give detailed information on the differences between the MCs or the enzymes. Instead one has to investigate the methyl distribution for each DP individually. This is presented in Figures 7−9, where the distributions of the methyl substituents of DP 3 (Figures 7−9) and DP 5 (Figure 8) of MC 1 and MC 3 (Figure 7), MC 2, 3, and 6 (Figure 8) and MC 4, 5, and 6 (Figure 9) are compared. All DPs showed similar trends. The influence of DS on the

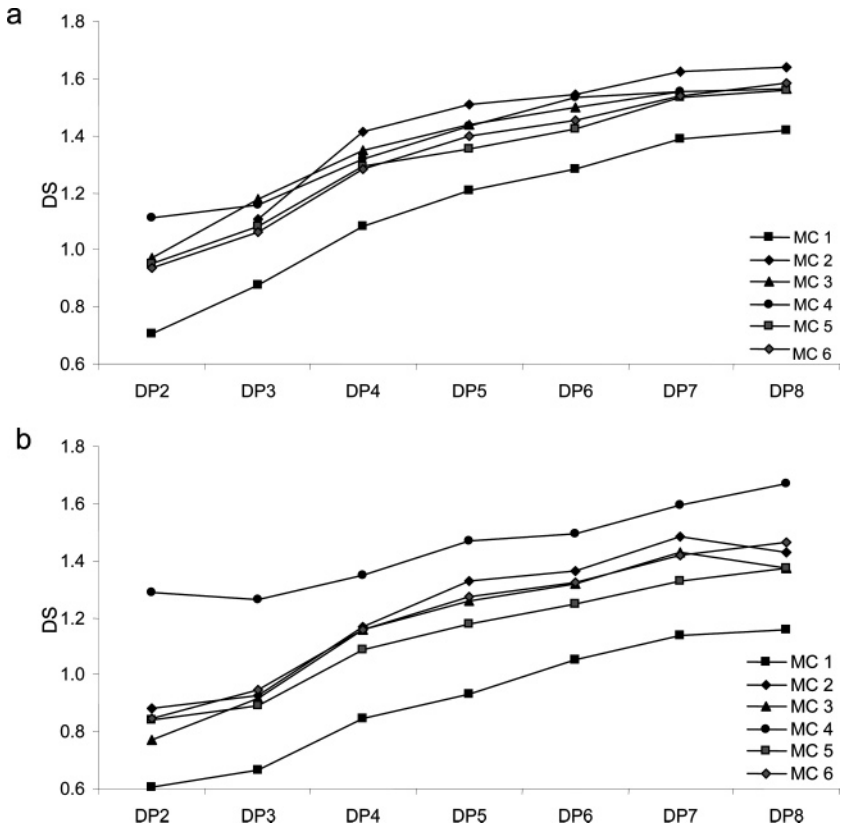

Figure 6. DS per DP calculated from signal intensities in mass spectra of perdeuteriomethylated oligomers produced from MC 1−MC 6 by (a) Tr. viride and (b) Tr. longi 1.

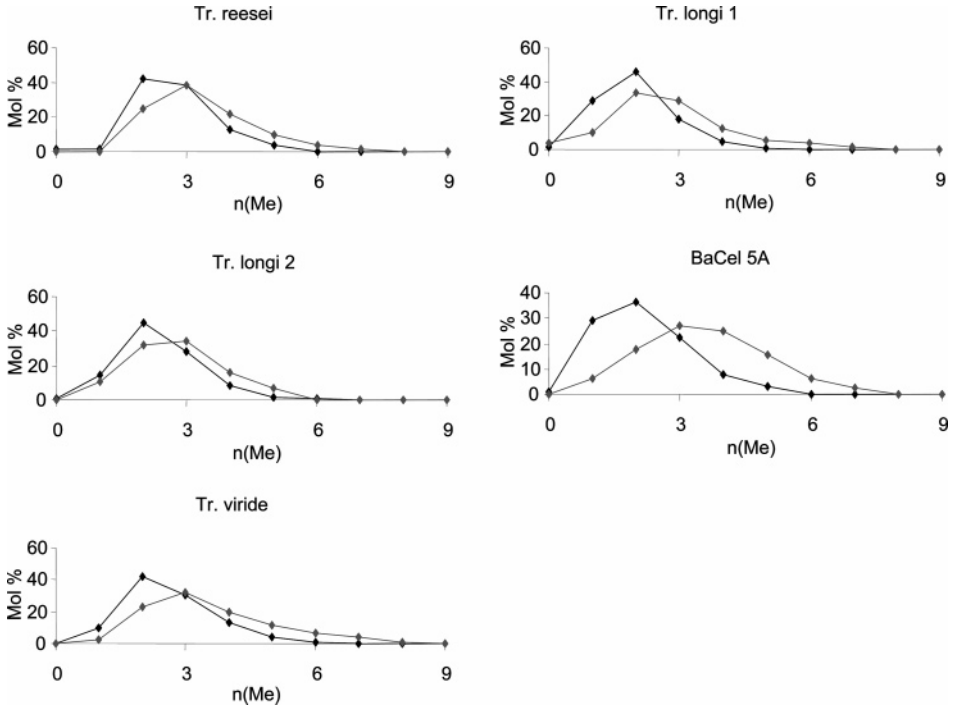

Figure 7. Comparison of methyl distribution in the DP 3 fraction obtained from MC 1 and MC 3, with various enzymes. Black diamond, MC 1; gray diamond, MC 3.

distribution pattern produced with each enzyme can be deduced from Figure 7, where MC 1 and MC 3 are compared. All enzymes show the same trend: the distribution of the substituents in MC 1 is shifted toward the regions of lower methyl content after degradation in comparison to MC 3. This is expected and can also be deduced from the DS/DP plots, where the DS values of MC 1 are generally lower compared to the values for MC 3 after degradation with the same enzyme. Unfortunately, no information can be extracted with respect to the distribution of the substituents in the original polymer chain.

Figure 8 shows a comparison of DP 5 of MC 2, 3, and 6. There are no significant differences in the distribution pattern of these MCs after hydrolysis with Tr. reesei, Tr. longi 1, and Tr. longi 2. However, as can be seen in Figures 7−9, Tr. reesei

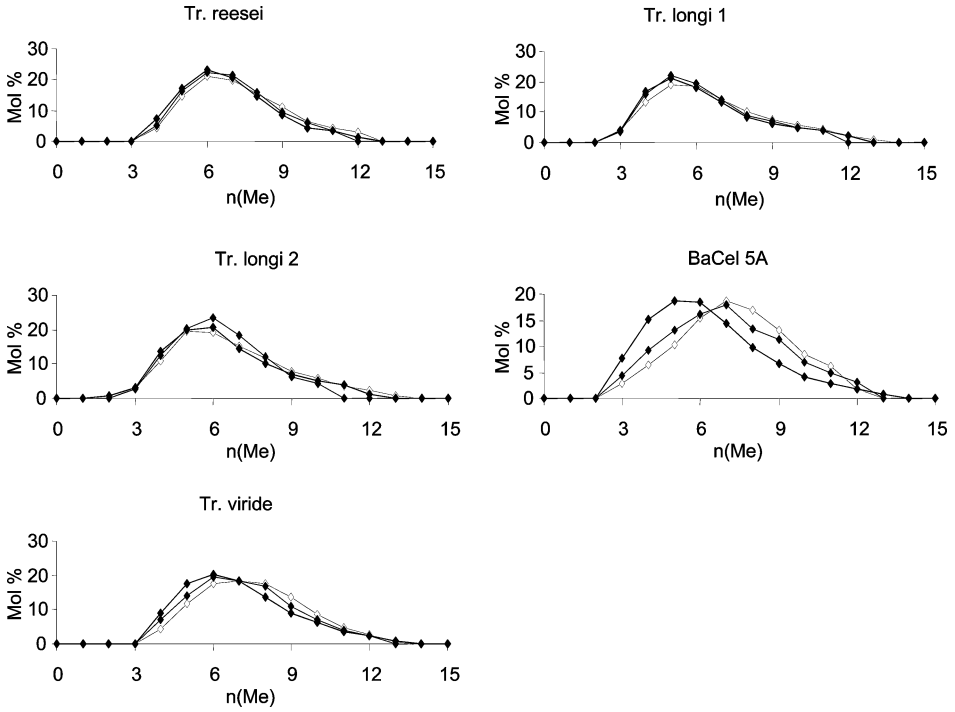

Figure 8. Comparison of methyl distribution in the DP 5 fraction obtained from MC 2, MC 3, and MC 6 with various enzymes. Light-gray diamond, MC 2; dark-gray diamond, MC 3; black diamond, MC 6

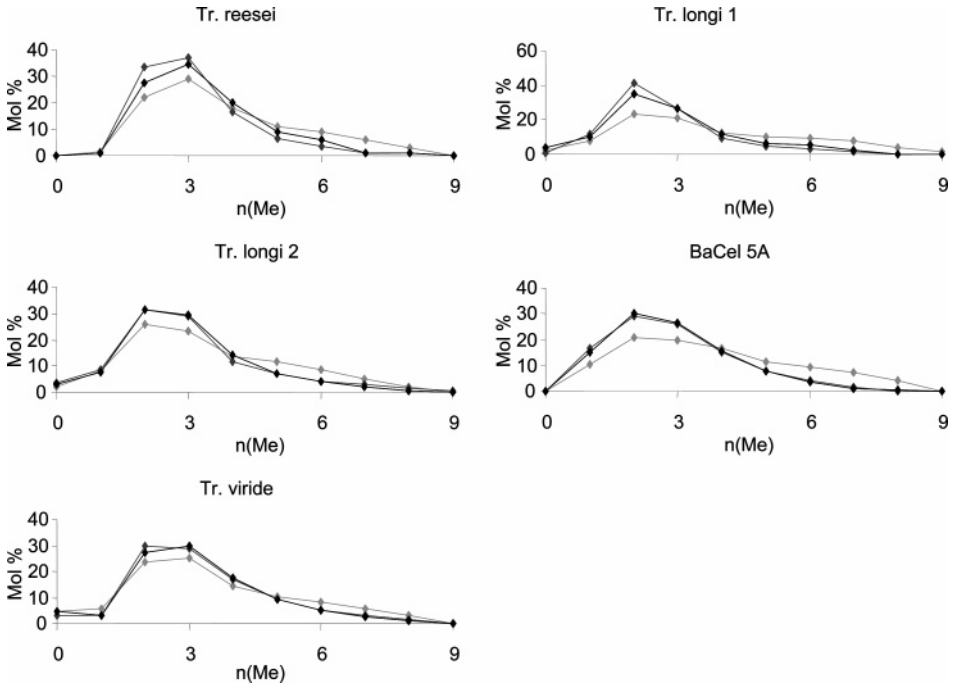

Figure 9. Comparison of methyl distribution in the DP 3 fraction obtained from MC 4, MC 5, and MC 6 with various enzymes. Light-gray diamond, MC 4; dark-gray diamond, MC 5; black diamond, MC 6

and Tr. viride do not produce any DP 3 with less than two substituents and no DP 5 with less than four methyl groups, whereas the other enzymes do. Interestingly, one can see differences in the plots obtained with the other two enzymes (BaCel 5A and Tr. viride). The DP 5 patterns of MC 2 and 3 are significantly shifted toward the higher methylated constituents (maximum at $n_{Me} = 7$, corresponding to DS 1.4) compared to MC 6 (maximum at $n_{Me} = 5$, corresponding to DS 1.26) after hydrolysis with BaCel 5A. The same deviation, but only to a small extent, is visible in the plot of Tr. viride. This can also be seen in DS/DP plots for the BaCel 5A, where MC 2 and MC 3 express a higher DS compared to MC 5 and MC 6 (data not shown). Before these differences are discussed, one has to look back on the results of the analysis of the substituent distribution along the polymer chain after random degradation in part 1.[20] Here the distribution determined by ESI-ITMS after perdeuteriomethylation and partial hydrolysis is compared with a calculated random distribution for DP 2. The monomeric units with their different methyl patterns are nearly randomly distributed in MC 2 and 3, only deviating slightly from the calculated pattern, whereas MC 6 as well as MC 4 and 5 show more significant deviations to a slightly heterogeneous pattern.

This means that there are fewer and more substituted regions in the polymer on the expense of regions with average substitutions; that is, there is a heterogeneity concerning the DS in and/or between the chains. Thus, the lower substituted areas are degraded to a higher extent and consequently form more oligosaccharides with a lower DS. On the other side, the more substituted areas are less accessible to the enzymes, thus reducing the probability that short, more highly methylated oligosaccharides are delivered.

Figure 9 shows a comparison of the substituent distribution after enzymatic cleavage of MC 4, MC 5, and MC 6. As just mentioned, a slightly heterogeneous methyl pattern has been found for these MCs by the established random degradation method.[20] However, after enzymatic degradation, differences are observed for these samples. The methyl distributions of MC 5 and MC 6 are in very good agreement, but MC 4 shows nearly a bimodal distribution with a shoulder in the distribution plot (Figure 9). It is originated by highly methylated oligomers, which include one terminal residue of the polymer and therefore less reflect the selectivity of the enzymes compared to oligosaccharides formed by two cleavages from the internal part of the chain. This finding is very important. It indicates that the oligosaccharide profiles obtained by enzymatic cleavage can be overlaid by nonselective contributions of the original end group. The extent depends on the average molecular weight of the original samples in relation to the degradability. For a certain enzyme, this is itself related to the DS in combination with partial DS values, i.e., the regioselectivity of substitution, and the amount of sequences able to form an active complex with the enzyme. Without correction of this nonselective contribution the profiles may be misinterpreted (for details see part 1).[20]

In contrast to SEC investigations, MS studies of the low DP region did only show differences between the MCs after degradation with BaCel 5A and Tr. viride but not with any of the batches from Tr. longi 1, Tr. longi 2, and Tr. reesei. This might reflect a different selectivity of BaCel 5A with respect to certain methyl positions and thus being sensitive to the differences in regioselectivity of methylation in MC 2 and MC 3 with less 2- and more 3-O-substitution compared to MC 4−MC 6 (as seen in part 1).

Investigation of Enzymes' Sensitivity to Substituents. To determine the sensitivities of the enzymes to methyl substituents, the hydrolysis products of MC 3 produced with all enzymes were investigated by the novel method described in part 1 of this work. Briefly, the reducing and nonreducing ends are marked specifically and then analyzed by MS/MS enabling the determination of the degree of methylation for each type of residues and thus the tolerance for methyl groups at certain subsites of the enzymes. 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 could be determined. For calculating the enzyme selectivity toward the methyl substituents, the procedure explained in part 1 of this work was used.

Figure 10 summarizes the results of these MS/MS studies for DP 2 and 3 of MC 3. The nomenclature for endoglucanases used in this figure states that the left glucose unit next to an enzymatic cleavage site, containing the newly formed reducing end, is the −1 subsite, the second to the left is the −2, and so on. The newly formed nonreducing is called the +1 subsite and so on.[29] It is seen in Figure 10 that the glucosyl unit at the reducing end is mainly unsubstituted but can also be monosubstituted. In rare cases, it can hold two substituents, but this is most likely due to the contribution of reducing end groups of the original sample. No trisubstituted glucosyl units are observed at the −1 subsite for any of the enzymes. For the reducing end of DP 2, two different trends were observed. BaCel 5A, Tr. longi 1, and Tr. longi 2 accepted a lower amount (∼5% of the total substituent distribution) of monosubstituted glucosyl units compared to Tr. viride and Tr. reesei (∼30%). At DP 3, the situation is more differentiated with BaCel 5A showing ∼30% monosubstituted residues, followed by Tr. viride and Tr. reesei with 20% and ending with Tr. longi 1 with a contribution of 10% monosubstituted glucsoyl residues at the reducing end. In the polymer chain, the average of unsubstitued residues is 6.3 mol % compared to 28.1% monosubstituted. Thus, the probability that an unsubstituted residue is located at subsite −1 compared to a monosubstituted is enhanced by a factor of 10−30. This is probably an image of the relative rate constants for the respective cleavage reactions. The differences for DP 2 and 3 might be related to the fact that the glucosyl units of these small degradation products have been involved in two cleavages. Thus, the reducing end of DP 3 has been located at subsites −1 and +3 and in DP 2 at −1 and +2. The nonreducing glucosyl units represent subsites −3 and +1 at DP 3. For all enzymes, they are mainly mono- and disubstituted and for BaCel 5A even 37% is trisubstituted. For the internal residue of DP 3 (subsites −2 and +2) and the nonreducing residue of DP 2 (subsites −2 and +1), only the average DS could be calculated, which lies between 0.7 and 1. Therefore, the requirements of subsite −2 are more restrictive than those of subsite +1, for which the DS was ≥1.5 for all samples. Attempts were made to get information about the position of the substitution in the monosubstituted glucosyl units at the new reducing end by MS^3 experiments. However, fragmentation of monomeric fragments from MS^2 was too poor to get any reliable results. Therefore, at present, it cannot be deduced which position (2, 3, or 6) affects hydrolysis the most, but according to recent studies performed by Momcilovic et al., it is most likely that the substitution at *O*-2 is not tolerated at subsite −1.[7] It has also been shown by Nojiri et al. that for Tr. viride the substitution on position *O*-6 does not influence the hydrolysis to any significance and that 2,3-di-*O*-methyl-cellulose blocks the hydrolysis completely.[8]

Basic Consideration. There are some basic considerations as a result from this work that are summarized in a few points:

- The enzymes investigated are hindered by the substituents to different extents. The degradability decreases with an increase in DS. The number of cleavages of the MC depends on the enzymes, DS, DP, distribution of substituents in the glucosyl unit, and the heterogeneity along the chain.
- The enzymes are sensitive to the number and location of substituents in the polymer chain bound to the active site. However, all investigated enzymes share the same basic selectivity toward the methyl distribution in the active site.
- The selectivity of the enzymes is reflected by the composition of DP 2 and DP 3 products, which are the results of two cleavages of glucosidic linkages. From these it can be deduced that the restrictions for subsite −1 are very high, in principle at most monsubstitution is tolerated, followed by subsite −2, whereas for +1, +3, and −3, apparently a broad range of patterns up to trisubstituted moieties are tolerated.
- MS analysis of the formed oligomers can give misleading values of the DS and methyl pattern if the intact MC has a low molar mass. This is due to the influence of the original terminal residues of the cellulose derivative. Thus a certain number of cleavages of the cellulose molecule are necessary to obtain a negligible contribution of these less specific products, which do not reflect the selectivity of the enzyme.

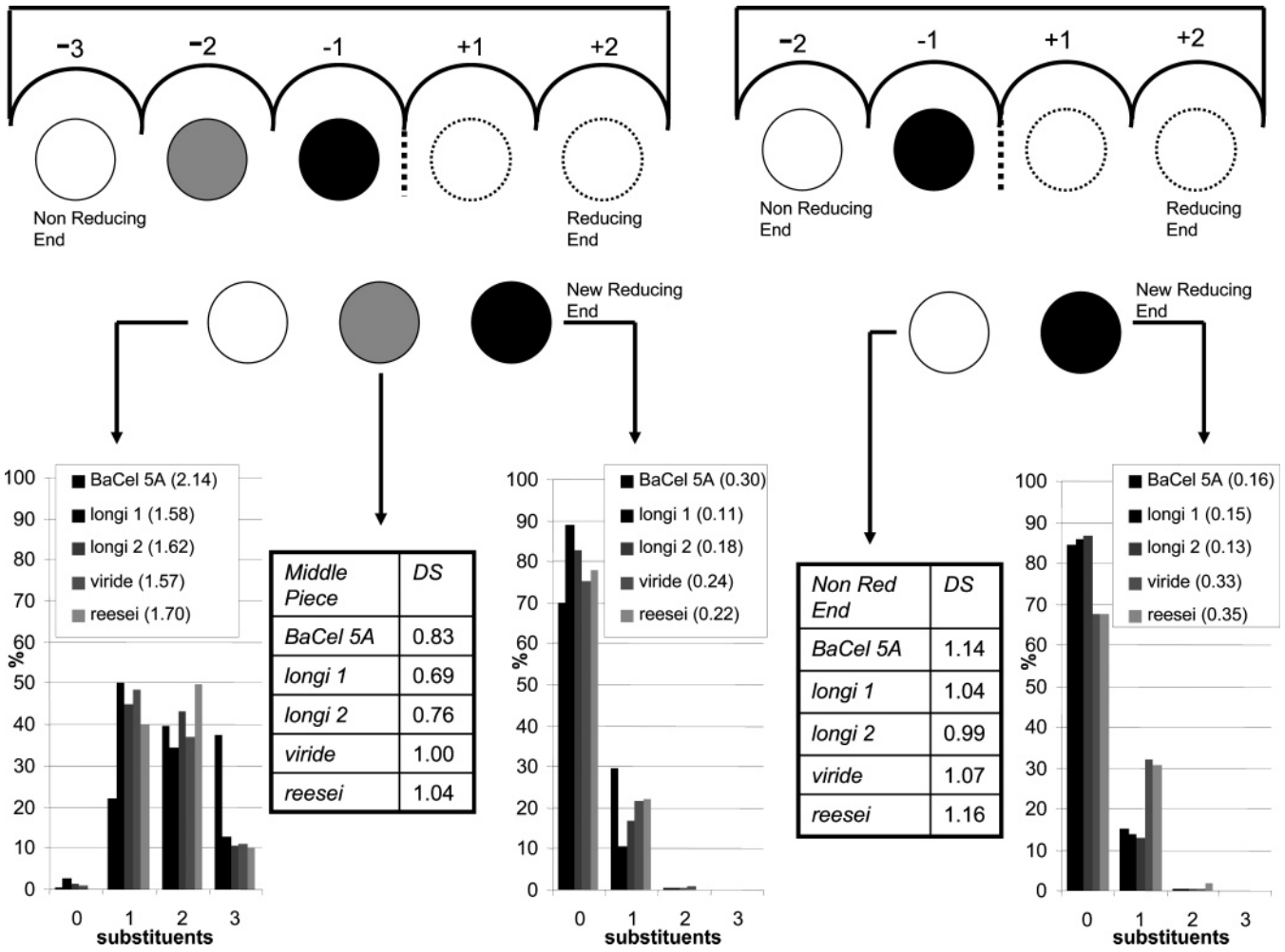

Figure 10. Methyl pattern of di- and trisaccharides obtained by hydrolysis with various enzymes. The scheme on the top illustrates the former position of the reducing (black), nonreducing (white), and internal residue (gray) of a di- or trisaccharide with respect to the subsites in the active enzyme−substrate complex for one of the cleavages these oligomers have been involved in. The values within parentheses are the calculated DS value for that glucose unit.

•Independent of the starting molar mass, the limit of degradation should be the same for a sample with a certain degree and type of methyl pattern when hydrolyzed with the same enzyme. With different enzymes or for cellulose derivatives with different substituent distributions in either the monomeric unit or along the polymer chain, differences in the product patterns can be observed with appropriate methods.

•After perdeuteriomethylation of the degradation products of the MCs and by addition of cellodextrins as internal standards, the amount of oligosaccharides formed from the original MC can be quantified with MS (ESI or MALDI).

•Although not generally, one can see differences between MCs and/or enzymes, but it is difficult to interpret them without any reference method or material.

•By further investigations of the enzymes' restrictions with respect to the methyl pattern, it should in principle be possible to estimate the number of appropriate sequences for a random distribution of monomer units present and to compare the experimental data with this model.

•It is assumed that the qualitative and quantitative composition of the enzymatic degradation products in the DP range, which until now is neither presented by MS nor by our SEC studies, will be of characteristic expressiveness for the substituent distribution over and in the polymer chains (heterogeneity of first and second order).

Conclusions

In this work, several important features when using enzymes for hydrolysis of methyl cellulose were investigated. It was found that different cellulose degrading enzymes hydrolyze particular MCs to an average molar mass, which is specific for the enzyme. It was also found that if MCs with similar heterogeneity are hydrolyzed with the same enzyme the hydrolysis products will have about the same average molar mass regardless of the size of the intact MC. However, if the heterogeneity is different, it might only be possible to see this using SEC-MALS/RI after hydrolysis with an appropriate not too efficient enzyme. A novel method for quantitative analysis of the formed hydrolysis products using MS shows that, for an MC with a DS of 1.3, 7−15% of the original polymer is represented by di- and trisaccharides formed by enzymic hydrolysis, but only 1−2% of an MC with a DS of 1.8. By analyzing the methyl distributions within the oligomers formed by enzymatic hydrolysis, it was possible to find correlations between the results from random degradation in comparison with statistical models and the results obtained here. It was thereby shown that it is possible to obtain information about the heterogeneity from enzymatically hydrolyzed samples with an appropriate enzyme. It was also found that it is of importance to investigate the efficiency of the enzymes before any conclusions can be drawn about the heterogeneity.

Some limitations for the enzymes with respect to methyl patterns involved in the active complex have been found. All investigated enzymes are very sensitive to substituents at subsite −1 and somewhat less at subsite −2, whereas they are very tolerant to substituents at subsite +1, whereas at −3, they can accept even up to trisubstituted residues.

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