Substituent Distribution and Clouding Behavior of Hydroxypropyl Methyl Cellulose Analyzed Using Enzymatic Degradation

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The distribution of substituents along the polymer backbone will have a strong influence on the properties of modified cellulose. Endoglucanases were used to degrade three different batches of hydroxypropyl methyl cellulose (HPMC) derivatives with similar chemical properties. The phase separation of the HPMCs as a function of temperature, i.e., the clouding behavior, was analyzed prior to degradation. The total amount of unsubstituted glucose was determined using total acid hydrolysis followed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The products after enzymatic degradation were analyzed with size-exclusion chromatography with online multiangle light scattering and refractive index detection and also with reducing end determination. To further characterize the formed products, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was employed for analysis of short-chained oligosaccharides. The different endoglucanases showed varying degradation capability of HPMC derivatives, depending on structure of the active site. The investigated HPMCs had different susceptibility to degradation by the endoglucanases. The results showed a difference in substituent distribution between HPMC batches, which could explain the differing clouding behaviors. The batch with the lowest cloud point was shown to contain a higher number of non-degradable, highly substituted regions.

Introduction

Modified cellulose is used in a wide range of industrial applications, for example, in paint, food, and pharmaceuticals.[1−3] The properties of the modified cellulose depend, e.g., upon the chain length of the polymer, the nature of the substituents and the degree of substitution (DS). For substituents that can polymerize into long side chains, e.g., ethylene oxide and propylene oxide, also the length of the side chains influence the polymer properties.[4] In addition, the distribution of the substituents along the polymer backbone as well as the difference in substitution pattern between the polymers will influence the polymer behavior.[5−7] Due to the importance of these characteristics, it is essential to make a thorough characterization of the polymer. This calls for the use of several analytical techniques such as size-exclusion chromatography with multiangle light scattering and refractive index detection (SEC-MALS-RI), which can be used for determination of the molar mass distribution.[8] Nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS), often in combination with chain degradation using acid hydrolysis, can be used for determination of the DS or the molar substitution (MoS) in the case of substituents that introduce new hydroxyl groups and thus can have more than three substituents per glucose units. However, the distribution along the polymer backbone is difficult to determine, due to the lack of suitable analytical methods. One approach has been the use of cellulose degrading endoglucanases that selectively hydrolyze the (1→4)-β-D-glucosidic linkages within the cellulose chain.[9−13] Endoglucanases will degrade regions of modified cellulose where the substituents do not interfere with the enzyme active site. This means that highly substituted regions will be left intact, while low substituted regions will be degraded to short oligosaccharides that can be analyzed with, for example, MS. If the DS is high enough to hinder a total degradation, the consequence when comparing polymers with the same average substitution will be that a polymer with more uniform distribution will be less degraded than a polymer with a more blockwise distribution.[14] The degradation pattern that will be observed will also depend upon the endoglucanase used for degradation of the modified cellulose, due to differences in sensitivity toward substituents between the enzymes. The sensitivity toward substituents will vary between different types of substituents; e.g., a bulky substituent such as hydroxypropyl can be expected to interfere more with the active site of the enzymes than a small substituent such as a methyl group. If the structural basis for the differences in sensitivity could be identified, this could be used as a tool for further characterization of the investigated polymer. So far relatively little is known about the enzyme susceptibility toward substituents or how many consecutive glucose units have to fulfill the enzyme active site requirements regarding substituent positions in order to cleave the linkage. Earlier investigations have shown that the C-2 position on the glucose unit, which contains the new reducing end, is the most critical not to be substituted.[15−17] To what degree the different residues influence hydrolysis can be determined with subsite analysis. For *Bacillus agaradhaerens* (*B. agaradhaerens*) Cel5A the −1 subsite was shown to be the most sensitive.[15−17]

All endoglucanases have an active site with an open binding

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Table 1. Studied Enzymes (Endoglucanases) and Their Abbreviations[a]

enzyme	molar mass (kDa)	abbreviation
Trichoderma reesei Cel5A	42	Cel5A
Trichoderma reesei Cel7B	48	Cel7B
Trichoderma reesei Cel12A	25	Cel12A
Trichoderma reesei Cel45A	23	Cel45A

[a] The molar mass is calculated from the amino acid sequence of the full-length enzyme.

cleft, lined with hydrogen-bonding residues and a few hydrophobic interactions.[18] The filamentous fungi *Trichoderma reesei* (*T. reesei*), which is one of the most efficient cellulose-degrading organisms known, produces endoglucanases from mainly four different glycoside hydrolase families (5, 7, 12, and 45). Theses enzymes, Cel5A, Cel7B, Cel12A, and Cel45A,[18,19] have different hydrolytic properties. These differences make the enzymes interesting to use as tools for characterization of modified cellulose, as it can be expected that they will be hindered to different extent by the substituents.

Of the mentioned enzymes, all except Cel12A consist of a catalytic core coupled with a cellulose binding module (CBM) via a linker region. Cel5A, Cel7B, and Cel12A all have a retaining mechanism for hydrolytic cleavage of the glycosidic bond, while Cel45A has an inverting mechanism.[18] These enzymes differ in the structure of the catalytic core and also in function, where the two most efficient ones, Cel5A andCel7B, have high capability to degrade HPMC, while Cel45A only has a very low activity.

In this paper HPMC has been investigated with four endoglucanases from *T. reesei* in order to obtain more knowledge about the correlation between the distribution of substituents along the polymer backbone and the polymer clouding behavior in water solution, i.e., to follow how the polymer solution phase separates with increasing temperature. This phase separation will result in a turbid solution ("clouding"). Also, differences in hydrolytic properties of these substrates, between the enzymes, has been studied. In order to correlate the polymer behavior to the distribution of substituents, three batches of HPMC with approximately the same weight average molar mass (M_w), DS_{Me} (with regard to methyl groups), and MoS_{HP} (with regard to hydroxypropyl groups) were studied. The degradation of the polymers was investigated using reducing end analysis in order to quantify the degree of degradation (i.e., the number of cleavages), and SEC-MALS-RI was used to determine the molar mass distribution before and after degradation. These methods give complementary information of the degree of degradation, allowing comparison between both the investigated batches and the endoglucanases studied. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) analysis was performed in order to investigate the differences in sensitivity between the endoglucanases toward substituents as well as to compare the substituent patterns of the investigated HPMC batches. The aim was to correlate polymer clouding behavior to differences in the distribution of substituents.

Experimental Section

Chemicals. Three HPMCs from DOW Chemicals were investigated (Midland, MI). DS_{Me} and MoS_{HP} were determined in-house with ^{1}H NMR (Table 2). NaOH, NaCl, $K_3[Fe^{III}(CN)_6]$, acetic acid (HOAc), and glucose were purchased from Merck (Darmstadt, Germany), and NaN_3 was from Sigma (St. Louis, MO). Sodium carbonate decahydrate was from ICN (Aurora, OH). The MALDI matrix 2,5-dihydroxybenzoic acid (DHB) was from Aldrich (Steinheim, Germany). The water used in all experiments was purified in a Milli-Q system, (18 MΩ cm, Millipore Bedford, MA).

Table 2. Studied Hydroxypropyl Methyl Cellulose Batches[a]

	HPMC-1	HPMC-2	HPMC-3
DS_{Me}	1.68	1.83	1.70
MoS_{HP}	0.26	0.34	0.24
M_w (g*/*mol) [a]	152 500	182 000	164 200
M_w/M_n [b]	1.9	1.9	2.1
unsubstituted glucose[c] (%)	6.1	5.2	7.4

[a] The table shows the degree of substitution with regard to methyl groups (DS_{Me}), molar substitution with regard to hydroxypropyl groups (MoS_{HP}), weight average molar mass (M_w), polydispersity index (M_w/M_n; M_n is the number average molar mass), and the total amount of unsubstituted glucose. [b] The M_w and M_w/M_n were determined using SEC-MALS-RI. [c] The total amount of unsubstituted glucose was determined with acid hydrolysis followed by HPAEC-PAD.

Enzymes. Four endoglucanases from *T. reesei* were used (Table 1): Cel5A, Cel7B, and Cel12A were a kind gift from Dr. Michael Ward and Dr. Colin Mitchinson, Genencor, CA. Cel45Acore was purified as described by Karlsson et al.[19]

Methods. For cloud point analysis, HPMC samples (~250 mg) were dissolved in 10 mM NaCl/3 mM NaN_3 to a final sample concentration of 1.0% (w/w). The samples were slowly stirred at ambient temperature for 4 days. For enzymatic hydrolysis, each HPMC was dissolved to a concentration of 10 g/L in water. Enzyme was added to a concentration of 1 μM, and the hydrolysis continued for 72 h at room temperature. The samples were stored at 4 °C. The hydrolysis was performed at ambient temperature.

For SEC-MALS-RI analysis, the samples were diluted to approximately 1 g/L with 10 mM NaCl solution. Complete degradation of HPMC to monomers was accomplished by hydrolysis of HPMC (5 mg) in 2 M TFA (1 mL) at 120 °C for 16 h. The acid was removed by evaporation. Subsequently, the residue was redissolved in water and finally freeze-dried overnight.

Cloud Point Determination. Cloud point analysis was performed using a Mettler Toledo FP900 Thermo system, consisting of a FP90 central processor connected to a FP810 measuring cell. The temperature interval for analysis was 30−70 °C at a rate of 1 °C/min. The light source for illumination of the samples was a 24-V, 2-W lamp. The data were handled by in-house software, Cloudpoint version 1.0.

HPAEC-PAD Analysis. An HPAEC-PAD system from Dionex (Sunnyvale, CA), consisting of a GS50 gradient pump, a Carbopac PA-100 guard and analytical column, and an ED50 electrochemical detector was used for the glucose determination. The electrochemical detector, with a gold working electrode versus an Ag/AgCl (sat.) reference electrode, was operated at the following waveform: E1 = 0.10 V (td = 0.10 s, t1 = 0.20 s), E2 = −2.00 V (t2 = 0.21 s), E3 = 0.60 V (t3 = 0.23 s), and E4 = −0.10 V (t4 = 0.24 s). The injection volume was 20 μL. Separation was performed at a flow rate of 0.25 mL/min, using a gradient program with water (eluent A), 150 mM NaOH (eluent B), and 150 mM NaOH/500 mM NaOAc (eluent C). The gradient was as follows: 0 min, 75% A and 25% B; 10 min, 25% A and 75% B; 16 min, 25% B and 75% C; 20 min, 25% B and 75% C; 22 min, 75%A and 25% B; 40 min, 75% A and 25% B.

Reducing End Analysis. The amount of reducing sugars released during enzymatic degradation was measured using a ferricyanide method adapted from Kidby and Davidson.[20] A reagent was prepared by dissolving 150 mg of $K_3[Fe^{III}(CN)_6]$, 7 g of sodium carbonate decahydrate, and 200 μL of 50% sodium hydroxide solution in 250 mL of water. Determination was carried out by pipetting 200 μL of reagent and 50 μL of sample into a well of a 96-well microtiter plate (Whatman, Haverhill, MA). After sample deposition the microtiter plate was covered and held at 37 °C for 90 min. Absorbance was measured at 405 nm using a SpectraCount microplate photometer (Packard, Maryland, CT). Calibration was performed using glucose solutions in the concentration range of 0−10 mM.

SEC-MALS-RI. The molar mass distributions of intact and enzymatically hydrolyzed HPMCs were determined by SEC-MALS-RI. The analytes were separated on a TSK-GEL GMPWXL 7.8 × 300 mm, with particle size of 13 μm and a linear mixed bed size-exclusion column (TosoHaas Bioseparation Specialists, Stuttgart, Germany) at a flow rate of 0.5 mL/min. The mobile phase was a 0.10 M NaCl solution filtered with a 0.22 μm mixed cellulose ester filter GSWP (Millipore Corp.). The pump was a Shimadzu LC-10AD liquid chromatography pump and the degasser a Shimadzu DGU-14A (Shimadzu Corp., Tokyo, Japan). Injection of the polymer solution was carried out by a Waters 717 plus Autosampler (Waters, Milford, MA), equipped with a 100 μL sample loop. The injected amount of sample was 100 μg as the polymer concentration in the solution was held at 1.0 g/L. The light scattering photometer was a DAWN-DSP multiangle light scattering instrument (Wyatt Technology, Santa Barbara, CA). Simultaneous concentration detection was performed using an Optilab DSP interferometric refractometer (Wyatt Technology). Both detectors operated at a wavelength of 690 nm. The output signals from the detectors were analyzed by the ASTRA 4.90.07 software (Wyatt Technology). The used refractive index increment (dn/dc) was 0.136 (determined on HPMC with the used instrument). The recovery was obtained from the ratio of the mass eluted from the channel (determined by integration of the refractometer signal) to the mass injected. The theoretical number of cleavages per polymer chain where calculated from the M_w obtained from SEC-MALS-RI. This means that the number of cleavages is underestimated as the MALS is insensitive at M_w below 10 000 g/mol.

MALDI-TOFMS. MALDI-TOFMS experiments were performed using a PerSeptive Voyager-DE STR (Applied Biosystems, Framingham, MA) equipped with an N_2-laser, time-lag focusing, reflector and a tandem coupled microchannel plate detector in reflector mode. Mass spectra were acquired in positive ion mode with the time-lag focusing and the reflector activated at all times. The accelerating voltage was 20 kV, and the reflector voltage was 13% higher. The laser intensity was held slightly above the threshold, the lag time was 150 ns plus instrument offset, and the middle grid voltage in the source was set at 76.0%. To prevent detector saturation, the detector activation gate was set at m/z 300. For most acquisitions, the guide wire was set to 0.01% of the acceleration voltage. Spectra were accumulated for 200 laser shots. Several different positions within the sample spot were used for the acquisition.

DHB was used as MALDI matrix for the analysis of the depolymerized HPMC. The matrix was dissolved to 10 g/L in H_2O. The analyte solutions were filtered through Nanosep 10 kDa Omega filters (Pall, Ann Arbor, MI), thereafter the permeate and the matrix solution were mixed 1:4 (v/v) and then vortexed. A 1 μL aliquot of the mixture was applied on a MALDI sample plate and then allowed to dry at reduced pressure by insertion of the sample plate directly into the loading chamber of the mass spectrometer. When the pressure was lower than 2×10^{-2} Torr, the sample was moved into the source chamber.

Results and Discussion

To link the behavior in solution of modified cellulose to the distribution of substituents, three batches of HPMC with similar M_w, DS_{Me} and MoS_{HP} (Table 2) were studied. These batches were degraded with both acid, in order to determine the total amount of unsubstituted glucose, and endoglucanases, which to a varying degree are hindered by the substituents.

Cloud Point Curves. Given the small differences in M_w, MoS_{HP}, and DS_{Me} (Table 2), it could be anticipated that the investigated batches should behave similarly in solution. One approach to investigate this is to study the clouding behavior which depends both upon the degree of substitution of hydrophobic groups and on the distribution of these groups along the polymer chain. From studies on methyl cellulose it is known that the substitution pattern of methyl groups affects the phase separation. A more heterogeneously substituted sample will have a lower phase separation temperature compared to a more homogenously substituted sample with the same DS_{Me} due to hydrophobic interactions between highly substituted blocks.[7,21] The three investigated batches behave differently in terms of clouding behavior, where the clouding curve of HPMC 2 is positioned at a lower temperature relative to HPMC 1 (Figure 1). This is in line with the expected result, due to the higher DS_{Me} and MoS_{HP} for HPMC 2.

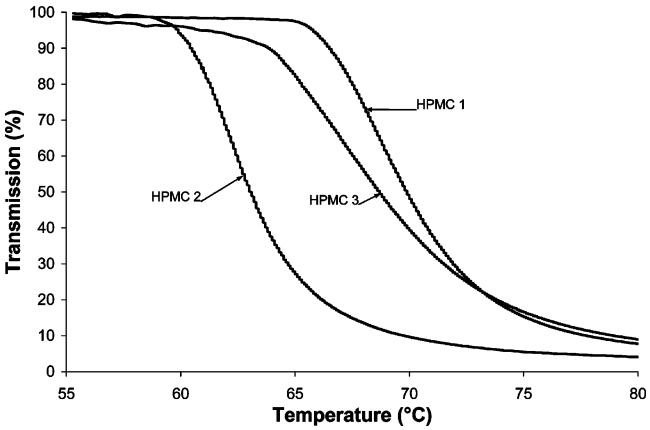

Figure 1. Cloud point analysis of three batches of hydroxypropyl methyl cellulose. Data on the polymers are given in Table 2.

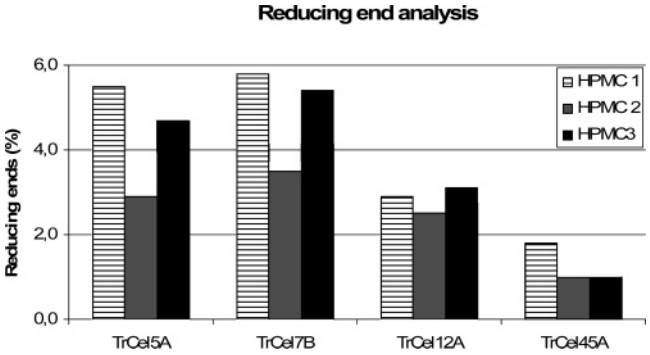

Figure 2. Reducing end analysis: amount of reducing ends produced by degrading HPMC batches for 72 h with Cel5A, Cel7B, Cel12A, and Cel45A. The reducing ends were determined using a ferricyanide method adapted from Kidby and Davidson[20]

Determination of Unsubstituted Glucose. In order to determine the total amount of unsubstituted glucose within the polymer chains, the samples were hydrolyzed using TFA; thereafter, the glucose content was determined using HPAEC-PAD. HPMC 3 (7.4%) had the highest amount of unsubstituted glucose, followed by HPMC 1 (6.1%) and HPMC 2 (5.2%) that had the lowest amount (Table 2). Even though HPMC 1 and 3 had approximately the same MoS_{HP} and DS_{Me}, they differed in the content of free glucose, which indicates that HPMC 3 has a more heterogeneous substituent distribution. HPMC 2 had higher DS_{Me} and MoS_{Hp}, which is in correlation to the low amount of unsubstituted glucose.

Reducing End Analysis. Reducing end analysis was used to estimate to what degree the different endoglucanases were able to degrade the samples. This method gives an estimation of the production of new reducing ends, i.e., how many β-1−4 linkages that have been hydrolyzed by the enzymes. When comparing to what degree the different HPMCs have been degraded by the endoglucanases, it can be observed that Cel45A and Cel12A degrade HPMC to a lesser extent than Cel5A and Cel7B (Figure 2). Cel7B has been shown to degrade cellulose substrates to glucose and cellobiose, while Cel5A also produces

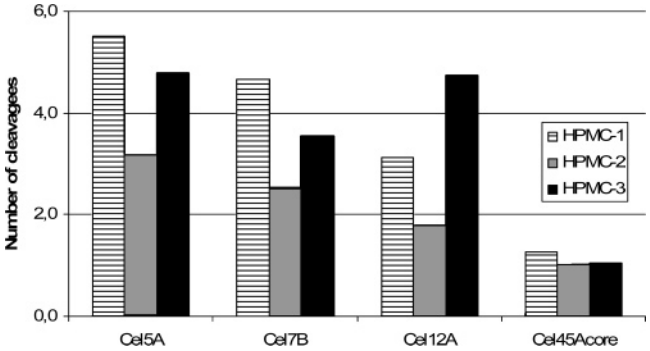

Figure 3. Number of enzymatically hydrolyzed (1→4)-β-D-GLUCOSIDIC LINKAGES PER POLYMER CHAIN. THE NUMBER WAS CALCULATED FROM THE M_w OF UN-DEGRADED AND DEGRADED POLYMERS OBTAINED FROM SEC-MALS-RI.

cellotriose.[19] This will have the effect that Cel7B produces more reducing ends even if both enzymes are equally hindered by substituents.

Cel5A and Cel7B showed a large difference between the studied HPMCs, where degradation of HPMC 2 liberated less reducing ends than when degrading the other two HPMCs. This difference is probably caused by the higher DS_{Me} and MoS_{HP}. Cel12A is not as effective as Cel5A and Cel7B. However, it is noteworthy that Cel12A has almost the same degradation capability on all three substrates, indicating that Cel12A has a qualitatively different tolerance toward substituents relative to Cel5A and Cel7B. Cel45A had the lowest capability to degrade HPMC, which is consistent with earlier results on carboxymethyl cellulose,[13,14] where it was shown that Cel45A required a block of low substituted glucose units in order to hydrolyze the glycosidic bond.

HPMC 1 was significantly more degraded by Cel45A compared with the other batches (Figure 2), indicating that this batch contains longer low substituted regions. The amounts of reducing ends produced by enzymatic degradation were approximately the same for both HPMC 1 and 3 (Figure 2) for Cel5A and Cel7B even though HPMC 3 had 20% more free glucose than HPMC 1 (Table 2). Thus, we can conclude that HPMC 3 has a higher number of short low substituted regions compared with HPMC 1.

SEC-MALS-RI. In order to determine the molecular weight distribution prior to and after degradation, the samples were analyzed with SEC-MALS-RI. The intact HPMC batches had slightly different M_w (Table 2); furthermore, HPMC 3 had a broader distribution than HPMC 1 and 2. The theoretical number of cleavages per polymer chain is calculated from the M_w obtained from SEC-MALS-RI. However, this means that the number of cleavages is underestimated as the MALS detection is insensitive at M_w below 10 000 g/mol.

The different enzymes had a varying effect on the samples, as can be seen in Figures 3 and 4, where Cel45A degraded the samples least and Cel5A and Cel7B were the most effective enzymes. It can anew be observed that Cel12A did not degrade the samples to the same degree as Cel7B and Cel5A. In the SEC-MALS-RI analysis, Cel5A and also, to some extent, Cel7B gave rise to a peak of short fragments (Figure 5). This peak can also be seen from Cel12A on HPMC 3 but not on the other two studied HPMCs. This indicates that parts of HPMC 3 had such low DS_{Me} and MoS_{HP} that the enzymes efficiently degrade these parts to a significantly higher degree than the rest of the sample. It is also observed that Cel12A has a significantly higher capability to degrade this batch compared to the other two investigated batches (Figure 3). This difference, however, cannot be observed in the reducing end analysis (Figure 2), strengthening the indication that HPMC3 has many short low substituted regions which enables the enzyme to cleave one hydrolytic bond, but effectively hindering further degradation to occur. In Figure 6 it is seen that parts of HPMC 2 remains intact after degradation; thus, it can be concluded that this batch has regions with a substitution pattern that effectively hinders all the studied enzymes to degrade it. This is also reflected in an *increase* in polydispersity after degradation (Figure 4b) rather than the expected decrease. This resistant part of the cellulose is also noticed for the other investigated batches but to a very small extent. In HPMC 3 only Cel45A leaves a fraction intact to a significant degree (Figure 5), indicating that part of the polymer has a relatively homogeneous substituent distribution, which is enough to hinder Cel45A but not the other less sensitive enzymes.

MALDI-TOFMS. To get more detailed information of the low molecular weight products, the samples were investigated using MALDI-TOFMS. The samples degraded with Cel45A gave a low concentration of products, resulting in low peak intensity which gave a higher degree of uncertainty when the level of substitution was calculated. The detected oligosaccharides had a degree of polymerization (DP) in the range of 1−10 glucose units. However, due to interference between matrix peaks and some of the peaks from DP 1 and 2, these oligosaccharides were excluded from analysis (Figure 7). Also DP 9 and 10 were excluded as the low intensities made the analysis less reliable. For comparison the corresponding intact samples were also analyzed. Because no peaks were observed for these samples, it could be concluded that the species detected for the degraded HPMCs were indeed products of the enzymatic degradation.

The analyzed oligosaccharides had a level of substitution (including both methyl and hydroxypropyl groups) substantially lower than on the intact polymer (Table 2). The measured levels of substitution are most probably somewhat overestimated as the ionization efficiency is lower for the less substituted oligosaccharides.[22−24] The level of substitution in the analyzed products also differed between the different enzymes. While Cel5A and Cel7B could tolerate more substituents close to the hydrolytic cleavage, Cel45A was the least tolerant enzyme of the four (Figure 7). For HPMC 2 the investigated enzymes gave a product that reflects the higher MoS_{HP}. When comparing the studied batches considering the DS_{Me} with the same degrading enzyme, it can be observed that the products formed had approximately the same DS_{Me} per DP irrespective of the batch, indicating that the final product was less influenced by the polymer than by the enzyme.

Substituent Effect on Enzyme Activity. An overall observation is that Cel5A is slightly more efficient than Cel7B in the degradation of HPMC. From the MALDI-TOFMS data (Figure 7) it can be observed for the products formed by Cel5A and Cel7B the DS_{Me} is DP dependent up to DP 5 and thereafter the DS_{Me} per DP levels out, indicating that the enzymes are not affected by substituents three glucose units or longer from the substrate cleavage site. A possible explanation for the lower DS_{Me} in DP 5−8 (1.1−1.2) than on the intact polymer (1.8) is the fact that the end parts of the oligosaccharides have a lower DS due to the hindrance of the hydrolytic cleavage observed on DP 3 and 4.

T. reesei Cel5A has not been structurally determined as opposed to Cel7B,[25] but family 5 endoglucanases from for example *B. agaradhaerens*[26−28]and *Thermoascus aurantiacus* (*Th. Aurantiacus*)[28] have been structurally determined. From

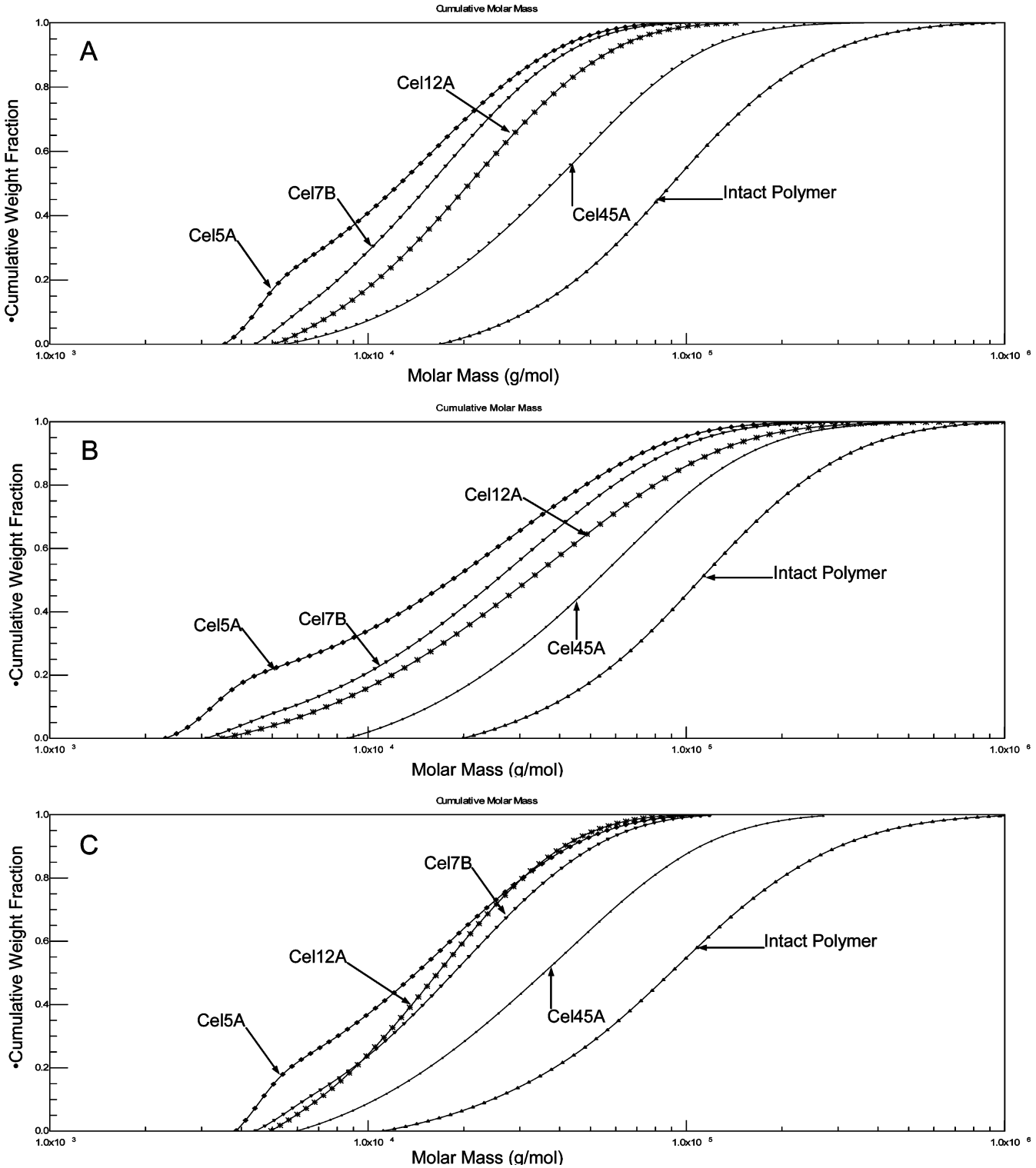

Figure 4. Molar mass distribution of intact and enzymatically degraded HPMC polymers: (A) HPMC-1, (B) HPMC-2, and (C) HPMC-3 from the SEC-MALS/RI analysis. The enzymes are indicated in the figure.

these structures it can be observed that family 5 and, to less extent, family 7 have open and shallow active sites where the catalytic residues are placed on one side of the groove and at the bottom, respectively.[25,28,29] The active sites of families 5 and 7 enzymes also have relatively few subsites (5 and 4, respectively), resulting in fewer interactions with the substrate that can be hindered and thus a shorter segment compatible with the active site is needed.

T. reesei Cel12A has been structurally determined,[29] and it can be observed that it has a narrow active site consisting of six sugar binding subsites (−4 to +2) with the catalytic residues placed on both sides of the cleft. The narrow active site with hydrophobic sides and a polar bottom gives a more restricted access for a substrate with a bulky substituent such as hydroxypropyl. The narrow active site can be correlated to lower degradation compared with Cel5A and Cel7B.

Cel12A produces approximately the same amount of reducing ends independently from which batch is hydrolyzed. If measured with SEC-MALS-RI, Cel12A can, however, degrade HPMC 1 and 3 to a higher degree, indicating that the reason behind the

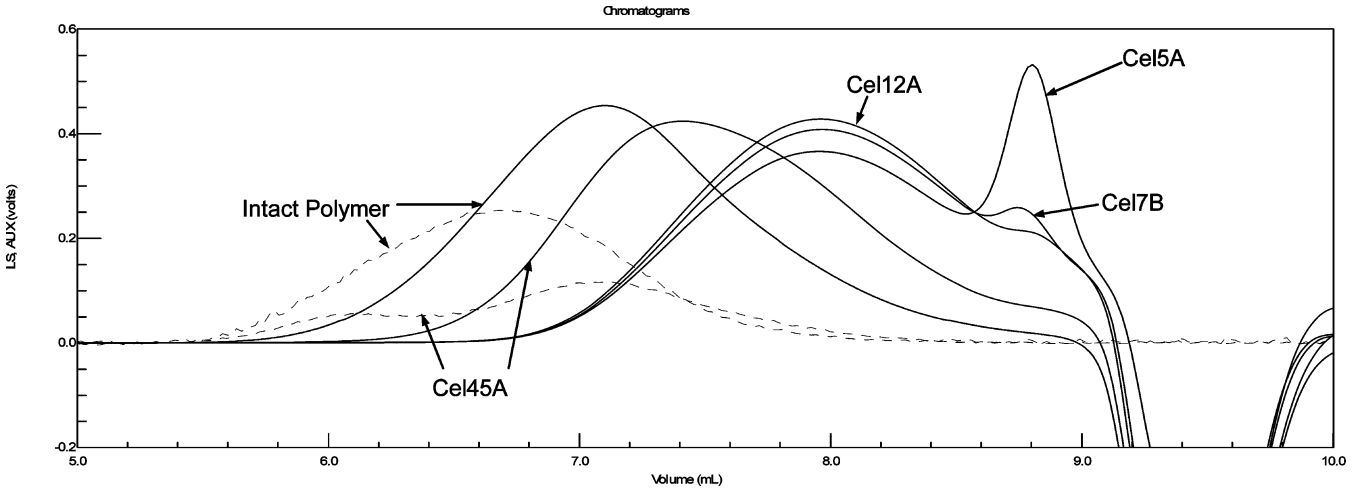

Figure 5. SEC-MALS-RI chromatogram of intact and enzymatically degraded HPCM 3. The solid curves represent the RI signal, and the dashed curves represent the MALS signal. The enzymes are indicated in the figure.

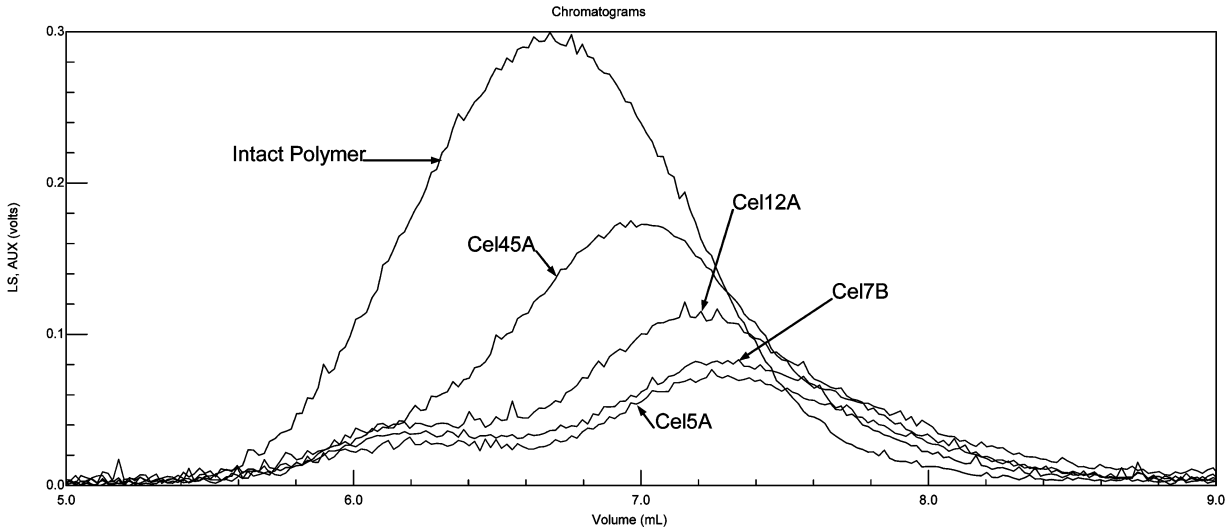

Figure 6. SEC-MALS chromatogram of intact and enzymatically degraded HPMC 2. The enzymes are indicated in the figure.

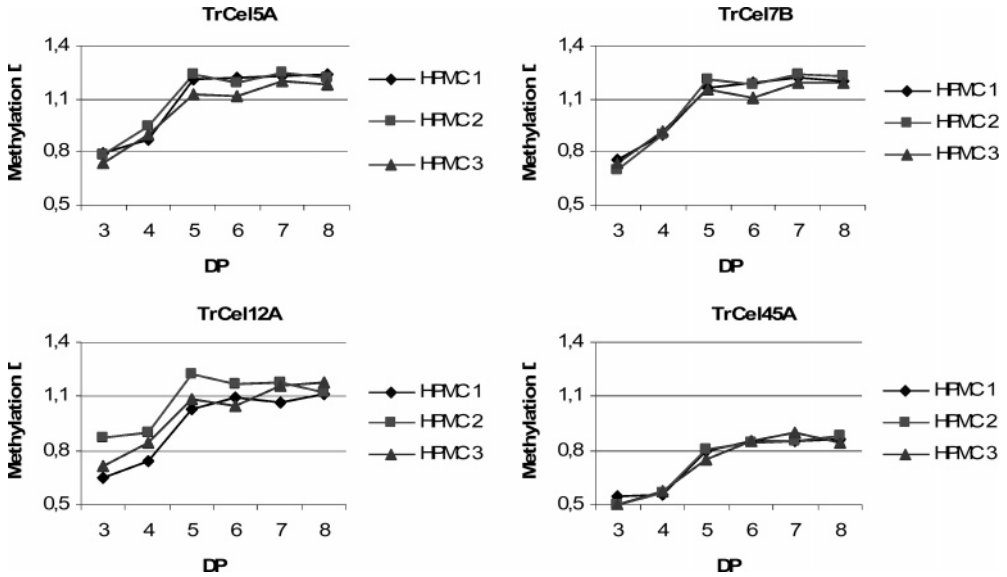

Figure 7. Average degree of substitution (DS_{Me}) as a function of DP in degradation products after enzymatic hydrolysis. The determinations were performed with MALDI-TOFMS.

equal amount of reducing ends is a more extensive degradation of the produced short oligosaccharides from HPMC 2, which is not accounted for in the SEC-MALS-RI.

T. reesei Cel45A has not been structurally determined, but the structures of Cel45 from *Humicola insolens* (*H. insolens*)[30−32] and *Melanocarpus albomyces* (*M. albomyces*)[33] are available.

Cel 45A from *T. reesei* has been compared to Cel45A from *H. insolens* in previous studies on carboxymethyl cellulose (CMC)[13] and HPC[34] where HiCel45A were more efficient on CMC degradation, while on HPC the enzymes had similar degradation capability. This calls for caution in making too extensive conclusions from structures from other family 45 enzymes, although some observations can be made.

The active site from family 45 has, in contrast to the other studied endoglucanases, very few hydrophobic stacking interactions to the substrate.[33] Thus, it can be anticipated that substituents that hinder hydrogen bond formation between the substrate and the active site would render it difficult for hydrolysis to occur.

Cel45A is more hindered than Cel5A and Cel7B by the substituents. In previous studies on CMC by Karlsson et al.,[13,19] Cel45A was shown to need a substrate longer than DP5, thus demanding more than five glucose units in sequence which fulfills the enzyme active site requirements regarding substituent positions. Since this enzyme leaves relatively long oligosaccharides as products, i.e., up to cellopentaose, the amount of reducing ends will be lower than for an enzyme which is capable of degrading oligosaccharides further.[13] This can, however, not fully explain the observed low amount of reducing ends, as Cel45A also shows a lower degradation capability when measured with SEC-MALS-RI. The higher sensitivity toward substituents is also pronounced in the observed DS_{Me} per DP from MALDI-TOFMS, where Cel45A has a significantly lower DS_{Me} per DP and a very low MoS_{HP} per DP. The latter observation correlates with the results from the previous study on HPC, where very few degradation products with hydroxypropyl groups could be detected.[34] This indicates that Cel45A cannot tolerate hydroxypropyl groups in the active site and is severely hindered by methyl groups, indicating that only almost unsubstituted regions are degraded.

Clouding Behavior and Substituent Distribution. The clouding curve of HPMC 2 is positioned at a lower temperature relative to HPMC 1 (Figure 1). This sample has a higher proportion of un-degraded regions after enzymatic degradation, indicating the existence of highly substituted regions responsible for clouding. HPMC 3, which has almost identical M_w, DS_{Me}, and MoS_{HP} as HPMC 1 (Table 2), has a more extended clouding curve starting at a lower temperature than HPMC 2 and continuing for more than 20 °C in contrast to the sharper slope of the HPMC 1 and 2 clouding curves.

Characterization through enzymatic degradation gives knowledge about low substituted regions. The highly substituted regions can be isolated after enzymatic cleavage, allowing characterization by acid hydrolysis and for example NMR analysis.[35,36]

The data from the enzymatic degradation suggest that HPMC 1 has fewer but longer regions of low substituted regions, while HPMC 3 has more but shorter low substituted regions. The shallower clouding curve for HPMC 3 might be caused by heterogeneity between the individual polymers of the batch. The data from HPMC 2, which has higher DS_{Me} and MoS_{HP} as well as the lowest clouding temperature, indicate that this batch contains regions that are inert to degradation. It can be assumed that the un-degraded parts have a higher substitution level, but this can also be caused by a more homogeneous distribution of substituents. The parts with high substitution of hydrophobic groups can be assumed to be responsible for the lower clouding temperature.[7,21]

Conclusions

Three HPMC batches with a DS_{Me} of 1.7−1.8, MoS of 0.24−0.34, and divergent clouding behavior were investigated using four pure endoglucanases. The enzymes had differing sensitivity toward substituents and their ability to degrade the cellulose derivative where analyzed. From the obtained results, it could be observed that the three HPMC batches had divergent substitution patterns. The results showed a difference in substituent distribution between HPMC batches, which could explain the differing clouding behaviors. The batch with the lowest cloud point was shown to contain a higher number of non-degradable highly substituted regions.

From the MALDI-TOFMS data a relationship between the active site structure of the enzyme and the substitution level of the oligosaccharide degradation products was observed. The MS data are, however, not reflecting the small differences between the used substrates but instead demonstrate the enzyme specificity.

The use of several pure enzymes with different sensitivity toward substituents gives a more detailed picture of the substituent distribution when compared to the information given by degradation either with only one endoglucanase or with a commercially available enzyme mixture. This shows the advantage of using several pure endoglucanases with different sensitivity toward substituents. It also emphasizes the need for further characterization of the enzyme selectivity for cellulose derivatives in order to uncover more details in substituent distributions.

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