Characterization of Chemical Substitution of Hydroxypropyl Cellulose 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 a series of hydroxypropyl cellulose (HPC) derivatives with a high degree of substitution. The HPCs were characterized with cloud-point analysis prior to degradation. The extent of enzymatic degradation was determined with size-exclusion chromatography with online multi-angle light scattering and refractive index detection and also with high-pH anion exchange chromatography with pulsed amperometric detection. 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 depending on structure of the active site. The highly substituted HPCs had different susceptibility to degradation by the endoglucanases. The results show a difference in substituent distribution between HPCs, which would explain the differing cloud-point behaviors. Increased number of regions with low substitution could be correlated with lower polymer cloud point. The study shows the usefulness of enzymatic degradation to study the distribution of substituents in soluble biopolymer derivates.

Introduction

Cellulose is a nontoxic, renewable resource and one of the most abundant polymers on earth.[1] By chemical modification of the polymer, new characteristics can be introduced, e.g., improved solubility in various solvents. This opens up a broad range of applications in which modified cellulose can be used, e.g., in paint, food, and pharmaceutical industry.[2,3] To achieve the desired properties, the hydroxyl groups along the polymer chain are substituted with different groups, e.g., carboxymethyl or methyl, or with a mixture of different groups, e.g., hydroxypropyl and methyl groups. The properties of the modified cellulose will thus not only depend on the chain length but also on the nature of the substituent, the degree of substitution (DS) and for some types of substituents, the length of the side chains. In addition, the distribution of the substituents along the backbone will influence the polymer properties.[4,5] All of these properties may differ between suppliers as well as between batches from one supplier[6] which calls for careful characterization. To fully characterize the modified cellulose, use of several analytical techniques is required. The polymer chain length can be determined quite straightforwardly[7] using size exclusion chromatography with multi angle light scattering and refractive index detection (SEC-MALS−RI). The characterization of the substituent distribution along the polymer chain is still a challenge and requires a multiple analytical approach employing techniques such as nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS), often in combination with chain degradation using acid hydrolysis.[8]

One approach that has been used to investigate the heterogeneity is to use enzymes such as cellulose degrading endoglucanases (i.e., enzymes that selectively hydrolyze the (1→4)-β-D-glycosidic linkages within the cellulose chain).[9−11] Endoglucanases are to some extent hindered by the substituents in cellulose derivatives due to steric interference. This leads to a situation where the enzyme is not capable of degrading the more heavily substituted regions but can manage to degrade less substituted regions, thereby producing fragments that can give information on the distribution pattern, e.g., if the substituents are distributed in blocks or are evenly distributed.[12] A polymer with even distribution is expected to be less degraded than a polymer with blockwise distribution if both have the same average DS.

The extent to which the enzyme is hindered depends on the enzyme active site structure. This gives the opportunity to compare the degradation pattern from several different endoglucanases to obtain more specific information on the substitution pattern.[13] If it was known exactly which substitution a specific enzyme could tolerate and at which position, it would be possible to determine what the polymer would look like at the point where the enzyme cleaves the polymer chain. It is, however, neither known to which degree the enzymes are hindered by substituents nor which of the positions on the glucose residues that are more critical to be unsubstituted. Earlier investigations of this issue have indicated that the C-2 position is most important[14,15] on the glucose which contain the new reducing end, but to what degree the different residues influence hydrolysis is not entirely clear. The sensitivity toward the position of the substituent can also differ between different endoglucanases. Since hydroxypropyl is a quite bulky group, it can be assumed that it will be an obstacle for the enzymes capability to bind the cellulose to its active site both due to the size of the substituent and due to the hindrance to form hydrogen

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bonds. It is also not known how many glucose units in sequence the enzyme requires to allow the hydrolysis of the glycosidic bond. These characteristics will depend on how many subsites constitute the active site in each endoglucanase. Hence, each endoglucanase will produce its unique degradation pattern.

One limitation to this approach is investigation of highly substituted polymers, since there is a risk that the enzymes will be more or less totally hindered and thus unable to degrade the polymer.

In this paper, high substituted hydroxypropyl cellulose (HPC) has been investigated in order to characterize the substituent distribution by enzymatic degradation but also to study the hydrolytic properties of several different endoglucanases. Hydroxypropylene oxide can propagate into long side chains due to introduction of new hydroxyl groups, thus the amount of substitution is stated as molar substitution (MoS, defined as the average number of propylene oxide per glucose unit). Due to the high MoS of the investigated polymers, only a small fraction of the respective polymer will fulfill the spatial requirements of the enzyme and be degraded, especially if the polymer is homogeneously substituted. The study was accomplished by comparing HPC samples from two different suppliers using several different endoglucanases in combination with β-glucosidase, which hydrolyses terminal nonreducing β-D-glucose residues releasing D-glucose.[16,17] The latter was used to raise the glucose content to a level where quantification with high pH anion exchange chromatography with pulsed amperometric detection (HPAEC−PAD) is easier and gives results that are more reliable. SEC-MALS−RI was used to determine the average molar mass before and after enzymatic degradation. This analysis allowed quantification of the degree of degradation, which revealed differences between the different polymer batches as well as between the different endoglucanases. It also confirmed that the β-glucosidase did not influence the weight average molar mass (M_w). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) was also performed in order to confirm the release of substituted oligosaccharides. This investigation also gives information about differences in sensitivity between the different endoglucanases toward substituents.

Experimental Section

Chemicals. Ten HPCs from two different manufacturers were investigated: five (HPC LM 1−5) from Nippon Soda Co. (Tokyo, Japan) and five (HPC LF 1−5) from Hercules (Wilmington, DE). The MoS values reported by the manufacturers were 3.3 for HPC LM and 3.5 for HPC LF. Two of these batches have been extensively studied in a previous work in this laboratory.[18]

NaOH, NaCl, acetic acid (HOAc), and glucose were purchased from Merck (Darmstadt, Germany); sodium acetate (NaOAc) was purchased from Acros (Geel Belgium); and sodium azide (NaN_3) was purchased from Sigma (St. Louis, MO). 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).

Enzymes. The following endoglucanases were used (Table 1): *Bacillus agaradhaerens* Cel5A (BaCel5A), *Humicola insolens* Cel5A (HiCel5A), *H. insolens* Cel7B (HiCel7B), *H. insolens* Cel45A (HiCel45A), (all provided purified as monocomponents by Novozymes, Bagsværd, Denmark), and *Trichoderma reesei* Cel45A (TrCel45A) (purified according to Karlsson et al.[19]). β-Glucosidase (*Aspergillus niger*) was purchased from Megazyme (Bray, Ireland). All endoglucanases are full length enzymes consisting of a catalytic module and a cellulose binding module. The concentrations of the enzyme solutions were determined with absorbance measurement at 280 nm.

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

enzyme	molar mass	abbreviation
Bacillus agaradhaerens Cel5A	44 702	BaCel5A
Humicola insolens Cel5A	40 995	HiCel5A
Humicola insolens Cel7B	44 577	HiCel7B
Humicola insolens Cel45A	30 123	HiCel45A
Trichoderma reesei Cel45A	22 799	TrCel45A

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

Methods. For cloud-point analysis, HPC samples (~250 mg) were dissolved in 10 mM NaCl/0.02%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 HPC was dissolved to a concentration of 10 g/L in 50 mM NaOAc (pH 5.0). 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 (to allow comparison with earlier experiments performed at the laboratory[13]).

For SEC-MALS−RI analysis, the samples were diluted to approximately 1 g/L with 10 mM NaCl solution.

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: E_1 = 0.10 V (t_d = 0.10 s, t_1 = 0.20 s), E_2 = −2.00 V (t_2 = 0.21 s), E_3 = 0.60 V (t_3 = 0.23 s), and E_4 = −0.10 V (t_4 = 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.

SEC-MALS−RI. The molar mass distributions of intact and enzymatically hydrolyzed HPCs were determined by SEC-MALS−RI. The analytes were separated on a TSK-GEL GMPWXL 7.8 × 300 mm, particle size 13 μm, 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 mm mixed cellulose ester filter GSWP (Millipore Corporation). The pump was a Shimadzu LC-10AD liquid chromatography pump and the degasser a Shimadzu DGU-14A (Shimadzu Corporation, 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 multi-angle 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 0.138 (determined on HPC 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 recovery for all of the investigated samples were above 90%, both before and after degradation.

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. The light source for illumination

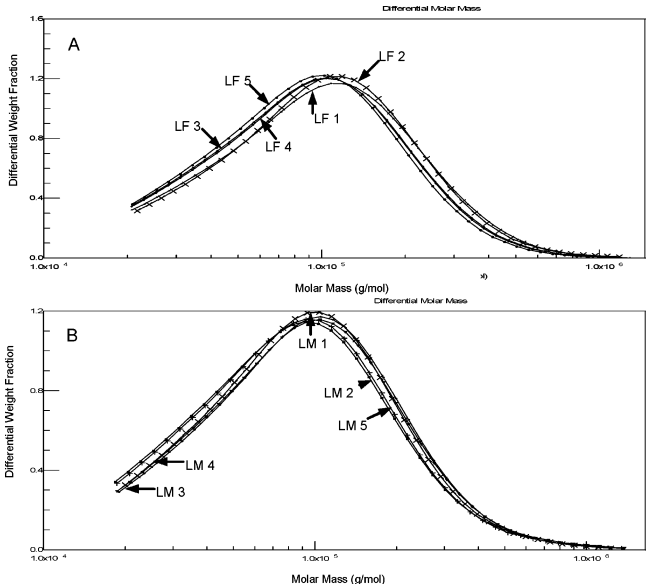

Figure 1. Differential molar mass of intact hydroxypropyl cellulose as measured with SEC-MALS−RI. Batches from Nippon Soda Co. are named HPC LM and batches from Hercules are named HPC LF.

of the samples was a 24-V, 2-W lamp. The predetermined definition of cloud point was the point where the initial transmission of light through the sample suspension was decreased by 4%. The data were handled by an in-house software, Cloudpoint version 1.0.

MALDI-TOF-MS. MALDI-TOF-MS 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 threshold and the lag time was 150 ns plus instrument offset. 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 the MALDI matrix for the analysis of the depolymerized HPC. The matrix was dissolved to 10 g/L in H_2O, and the solutions were filtered through Nanosep 10 kDa Omega filters (Pall, Ann Arbor, MI) whereafter the permeate and the matrix solution were mixed 1:4 (v/v) and then vortexed. A total of 1 μL 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

The distribution of substituents along the polymer backbone will have a strong influence on the properties of the modified cellulose. Thus, it is of great importance to be able to detect and, if possible, quantify the substituent distribution heterogeneity.[4] This is a very challenging task due to the lack of appropriate analytical methods. One approach is to compare different batches resistance toward degradation by endoglucanases and then relate the results to the known properties of these batches, giving a possibility to screen batches for the desired properties.[10] This approach will give a relative measurement between batches rather than an absolute value of the heterogeneity. In this study, ten HPC batches were used (the molar mass distribution of the studied batches before degradation are depicted in Figure 1.)

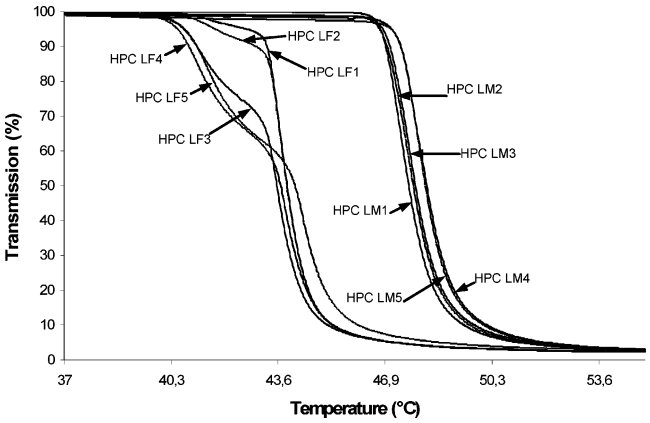

Figure 2. Cloud-point measurement of hydroxypropyl cellulose. Batches from Nippon Soda Co. are named HPC LM and batches from Hercules are named HPC LF.

Cloud Point Determination. One property that can be used to compare different batches is cloud-point temperature, i.e., the temperature when the polymer phase separates from the water phase resulting in formation of a turbid solution ("clouding").[18] The clouding temperature will be dependent upon the degree of substitution of hydrophobic groups on the cellulose, since higher substituted celluloses are expected to have a lower cloud point.[20] The HPC batches from the two suppliers differ both in cloud point temperature and in the shape of the cloud-point curves (Figure 2). The variation between batches from Hercules (HPC LF) is striking compared to the uniform curves given by the batches from Nisso (HPC LM). The cloud-point curves thus indicate some fundamental differences between the batches. HPC LM gives rise to approximately identical curves with a cloud point around 47 °C, whereas HPC LF both gives significant lower cloud point (40−42°C) and has a tendency to form a curve with two distinctly different regions where the low-temperature part of the slope is less steep. This indicates that these batches are not homogeneous but rather consist of at least two distinct polymer populations that differ in properties. The bimodal clouding behavior observed for HPC LF batches cannot be detected in SEC-MALS−RI where all of the batches give rather similar M_w (see below). Thus, the variation in clouding cannot be explained by differing molecular weights.

The differences in cloud point were surprisingly large in proportion to the rather small differences in MoS and M_w (Table 2). This might be caused by differences in the distribution of substituents along the polymer chain between the two suppliers where more or less extended low substituted regions can give rise to an unexpected behavior. These differences are, however, not detected by ^{13}C NMR.[18] Therefore, use of specific enzymes (endoglucanases) may be attractive due to the high selectivity toward unsubstituted or low substituted regions in the polymer during enzymatic degradation.

Effect of Different Enzymes. To be able to investigate differences between different HPCs it was necessary to identify one or more endoglucanases with the capability to degrade HPC. Therefore, a study of various enzymes ability to degrade HPC was performed. The studied enzymes were endoglucanases from three different families (Table 1). One batch from each supplier (HPC LF1 and HPC LM1) was used in this screening experiment. The investigated polymers have approximately the same MoS (HPC LF, MoS = 3.54; HPC LM, MoS = 3.35)[18] and M_w (approximately 120 000 g/mol) but differ in behavior such as cloud point (Figure 2). If these polymers were entirely homogeneously substituted, they would probably be virtually nondegradable by endoglucanases since the enzyme would be

Table 2. Enzymatic Degradation of Two Different HPC Batches[a]

enzyme	M_w	degradation (%)	glucose released (%)
HPC LF-1 (M_w = 125 500, MS = 3.54)			
BaCel5A	109 700	12.6	0.169
HiCel5A	110 050	12.3	0.176
HiCel7B	106 750	14.9	0.184
HiCel45A	115 900	7.6	0.151
TrCel45A	117 600	6.3	0.158
HPC LM-1 (M_w = 120 500, MS = 3.35)			
BaCel5A	120 500	*b*	0.020
HiCel5A	112 800	6.4	0.015
HiCel7B	114 700	4.8	0.048
HiCel45A	116 000	3.7	0.007
TrCel45A	115 250	4.3	0.007

[a] The table shows the weight average molar mass (M_w) of degraded polymer, reduction of M_w in relation to intact polymer (in %), and released glucose determined with HPAEC−PAD after β-glucosidase addition. [b] Since the MALS signal consists of two different peaks, the calculated M_w will be an average of both leading to a higher area than before hydrolysis. See Figure 3b.

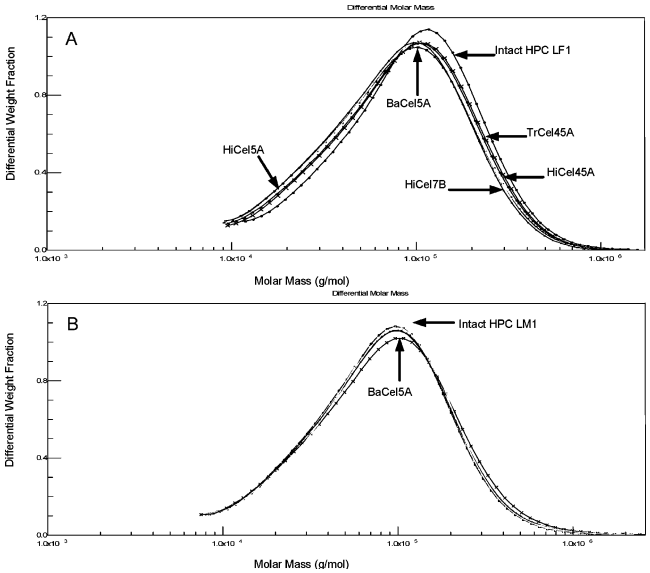

Figure 3. Differential molar mass of intact and enzymatically degraded HPC polymers as measured with SEC-MALS−RI. Enzymes (endoglucanases) are indicated in the figure. (A) HPC LF1, (B) HPC LM1. Due to the small difference between the enzymatic degradation curves, only BaCel5A and the intact HPC curves are indicated.

hindered by the substituents that prevent the necessary interaction between the enzyme active site and the substrate.

SEC-MALS−RI. The hydrolyzed samples were analyzed with SEC-MALS−RI. As can be seen in Figure 3 and in Table 2, the polymers are only slightly degraded even though HPC LF is clearly degraded to a higher extent than HPC LM. The fact that degradation occurred at all is noteworthy, because of the high MoS of the polymers, and indicates that the polymers contain low substituted regions accessible for enzymatic degradation. However, a difference can be seen between the enzymes in the degree of degradation (Table 2). Even though the differences between the batches are small, the relative order of degradation between the enzymes is constant. This was expected due to the structural difference in active site that exists between the different enzymes.

BaCel5A, HiCel5A, and HiCel7B are more capable to degrade the substrate than HiCel45A and TrCel45A. Of the studied endoglucanases, BaCel5A,[21,22] HiCel7B,[23,24] and HiCel45A[25] have been crystallized and structurally determined, also with substrate or analogue in the active site. When comparing structures of enzymes with substrate in its active site, it can be observed that all of the enzymes have an active site formed like a cleft, but the accessibility for the substrate varies according to the width and depth of the cleft. Both the BaCel5A[26] and HiCel7B[23] enzyme active sites are relatively shallow clefts. In contrast, HiCel45A has an active site with steep edges and with few hydrophobic interactions.[27] This means that for HiCel45A hydrogen bonding is more important, and thus, the enzyme is more sensitive toward substituents that hinder hydrogen bonds to be formed. HiCel45A has the most narrow active site and will thus be more hindered than both BaCel5A and HiCel7B, especially when a bulky substituent is introduced.

Also, the number of subsites influences degradation capability due to interactions involving a larger number of glucose units. BaCel5A has 5 subsites,[26] whereas HiCel7B has 8 subsites whereof 4 are most significant.[24] The active site of HiCel45A has 7 subsites[25] with the consequence that HiCel45A needs longer regions with low substitution for cleavage of the cellulose chain. In this respect, the lower degradation by endoglucanase HiCel45A can be understood. TrCel45A which structure has not been determined has the lowest capacity for HPC hydrolysis compared to the other endoglucanases investigated. Also, in recent studies on carboxymethyl cellulose (CMC) with a DS of 0.7[13] TrCel45A had lower capability to degrade the substituted cellulose compared with HiCel45A. The latter enzyme was however slightly more efficient than HiCel7B in CMC degradation. When HPC was degraded, both the family 45 enzymes were more hindered than the other investigated enzymes.

This indicates that the enzymes relative sensitivity toward substitution on cellulose cannot be transferred between different substrates. For HPC and CMC, both types of substituents are quite bulky, but CMC has a substituent that is charged at neutral or alkaline pH, whereas HPC is uncharged at the same pH. The side chains in HPC can be elongated into chains of polypropylene oxide thereby increasing the sterical hindrance.

Released Glucose Determination. As a complement to the characterization of chain length degradation with SEC-MALS−RI, the amount of released glucose from the enzymatic hydrolysis was also measured (Table 2). The amount of liberated sugar after hydrolysis with the different endoglucanases was small; hence, β-glucosidase, which degrades soluble cello-oligosaccharides to glucose monomers, was added to the samples. When analyzing these samples with SEC-MALS−RI it could be verified that the β-glucosidase does not have a detectable effect on the size of the longer polymer chains. When degrading the substrates with β-glucosidase in the absence of endoglucanases, no released glucose can be detected. From the obtained glucose content in the hydrolysates, it could be shown that approximately 10 times more glucose was released from HPC LF than from HPC LM (Table 1). The result was expected, although not in this magnitude, since in a previous study[18] it was shown that approximately 2.5 times more glucose was released from HPC LF than from HPC LM when performing a total acid hydrolysis of the polymer. This is in contradiction to the higher MoS of HPC LF (3.5) compared to 3.3 for HPC LM.

A conclusion that can be drawn from the result with enzymatic degradation is that HPC LF has a higher degree of low substituted regions compared to HPC LM. It has been shown that high substituted regions in methyl cellulose enhances gel formation due to hydrophobic interactions.[28] Thus, the variation in low substituted regions might explain the different behavior in cloud point analysis where HPC LF has a shallower

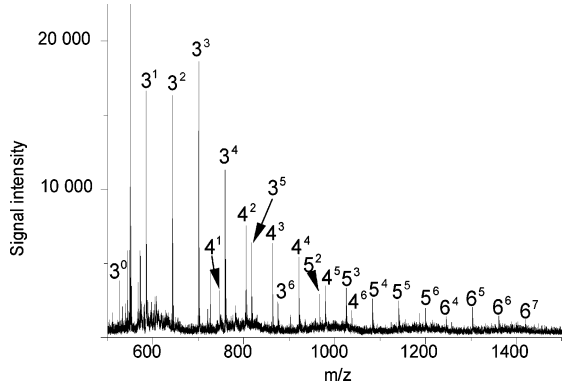

Figure 4. MALDI-TOF mass spectrum of HPC LF 1 degraded with BaCel5A. The larger numbers indicate the DP of the formed oligomers, and the superscript indicates the number of hydroxypropyl groups. Fragments in the range of DP 2−6 could be detected, but due to matrix interference DP 2 was excluded from the analysis.

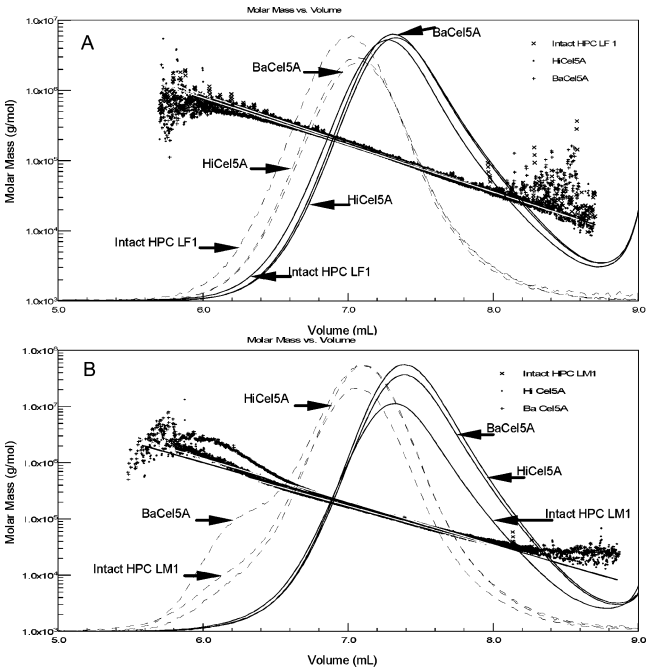

Figure 5. SEC-MALS−RI chromatogram of intact and enzymatically degraded HPC LM1. The solid curves represent the RI signal and the dashed curves represent the MALS signal. (A) HPC LF1, (B) HPC LM1.

curve (Figure 2). The bimodal clouding behavior in batches from Hercules could also be explained by two distinct populations of polymers within the batch with different properties, one of them more susceptible to enzymatic degradation. This can however not be observed in the SEC-MALS−RI chromatogram (Figure 5A) indicating that the most probable explanation is variations in low substituted regions. Since HPC LF has a higher molar substitution it can also be concluded that HPC LF has more elongated polypropylene oxide side chains.

MALDI-TOFMS. To deduce to what extent the enzymes are able to break glycosidic bonds close to substituents and to confirm that the substituted cellulose was degraded to oligosaccharides, the samples were analyzed using MALDI-TOFMS (Figure 4). The detected oligosaccharides are in the range of DP 2−6, but because of interference between matrix peaks and some DP 2 peaks, the latter were excluded from the analysis. For comparison the corresponding intact samples were also analyzed. Since no peaks were observed for these samples, it could be concluded that the species detected for the degraded

Table 3. Comparison between Different Batches of HPC Using the Enzymes BaCel5A and HiCel5A[a]

HPC LM		BaCel5A		HiCel5A	
batch	M_w	degradation (%)	glucose released (%)	degradation (%)	glucose released (%)
1	120 500	*b*	0.031	6.4	0.030
2	114 950	*b*	0.028	7.7	0.035
3	111 800	*b*	0.033	5.3	0.041
4	120 700	*b*	0.035	5.4	0.048
5	120 600	*b*	0.046	4.8	0.051

HPC LF		BaCel5A		HiCel5A	
batch	M_w	degradation (%)	glucose released (%)	degradation (%)	glucose released (%)
1	125 500	13.5	0.166	12.3	0.180
2	127 300	14.7	0.180	14.7	0.195
3	116 900	11.9	0.188	11.0	0.205
4	119 700	13.8	0.216	13.1	0.228
5	111 600	12.9	0.235	12.1	0.247

[a] The table shows the weight average molar mass (M_w) of degraded polymer, reduction of M_w in relation to intact polymer (in %), and released glucose determined with HPAEC−PAD after β-glucosidase addition. [b] Since the MALS signal consists of two different peaks, the calculated M_w will be an average of both leading to a higher area than before hydrolysis. See Figure 3b.

HPCs were indeed products of the enzymatic degradation. The enzymes give products with estimated MoS values close to 1 for both HPC LM and LF, a number that is substantially lower than the MoS measured on acid degraded polymer by MALDI-TOFMS or with NMR on intact polymer.[18] The measured MoS is most probably overestimated since the ionization efficiency is lower for the less substituted oligosaccharides.[11,29,30]

It can be concluded from the mass spectrometry measurements that some parts of the cellulose are very low substituted since, for instance cellopentaose with only 2 substituents can be detected (Figure 4).

From MS data, it can also be confirmed that the family 45A endoglucanases are hindered to a higher degree than the other enzymes that were studied. For family 45A, only a few peaks from products could be detected (data not shown) and it was impossible to calculate a MoS value from these spectra. Enzymatic degradation of both batches gave rise to spectra with low intensities even though HPC LF in general gave higher signal intensity than HPC LM. The spectra from HPC LM also contain fewer peaks than the spectra from HPC LF. This indicates that both batches have regions of low substituted glucose units, even though they are fewer or shorter in HPC LM.

Batch Comparison. To evaluate if the approach with enzymatic degradation is suitable as a tool for characterization of highly substituted HPC, the study continued with eight more batches, four from each supplier. Based on the achieved results two efficient enzymes were selected BaCel5A and HiCel5A.

The results were in agreement with the two previously studied batches. According to SEC-MALS−RI (Figure 5), the HPC LF batches are degraded to a higher extent compared with HPC LM (Table 3). The difference in M_w after degradation between batches from the same supplier is small.

In addition, more glucose is released from HPC LF after enzymatic hydrolysis (Table 3). Furthermore, the variation in released glucose after degradation, in HPC LF batches, is rather high and indicates a variation in low substituted regions between

the different batches. The difference in low substituted regions can be an explanation to the differing clouding behavior between batches from this supplier. In contrast to these results, HPC LM batches have rather homogeneous clouding behavior, and a similar amount of glucose is released after degradation. This indicates that the batches have a more uniform substituent pattern than the HPC LF batches.

Degradation of the HPC LM batches by BaCel5A (Figure 5) gave rise to chromatograms with a shoulder, where a small part of the peak in the MALS corresponds to an apparent molar mass up and above 10^6 g/mol. This peak can only be observed when degrading HPC LM with BaCel5A. Furthermore, the peak cannot be detected by RI, indicating that the concentration is very low. The peak may be caused by aggregates of intact or depolymerized HPC. One possible explanation is that the BaCel5A enzyme is incapable of degrading a part of HPC LM that in the intact sample is concealed by the rest of the sample (an indication to a shoulder can be seen in the undegraded sample). When the enzyme degrades the rest of the sample, this shoulder will be more pronounced in the chromatogram. Another explanation could be that some aggregated fractions are present in the intact HPC LM and have been removed by filtration prior to SEC analysis. After enzymatic degradation by BaCel5A these aggregates might be solubilized (and thus not removed by the filter) and show up in the high molar mass range.

Conclusions

Surprisingly, endoglucanases are able to degrade even very highly substituted HPC to some extent, making it possible to extract information concerning the substituent distribution. The investigation clearly shows the advantages of using a selective hydrolysis as a tool to obtain information of the low substituted regions of a substituted polymer, thereby giving complementary information. A previous study by Richardson et al.[18] indicated a difference between the two suppliers. In the present study, the results from the used analytical techniques correlate well and indicate that the HPC LF from Hercules has more regions with low substitution than HPC LM from Nippon. This might explain the difference in cloud-point behavior between the HPCs from the two suppliers indicating that small varations in substituent distribution can have a significant impact on the polymer behavior. The study also shows the usefulness of enzymatic degradation to compare the extent of low substituted regions between different batches, especially when used in combination with acid hydrolysis, making it possible to select batches with similar properties. It is interesting that the enzymatic degradation of the highly substituted polymer could be correlated with the structure of the active site of the endoglucanases.

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