NMR, Cloud-Point Measurements and Enzymatic Depolymerization: Complementary Tools to Investigate Substituent Patterns in Modified Celluloses

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Received March 23, 2006; Revised Manuscript Received June 23, 2006

The substituent patterns of some chemically modified celluloses were characterized as a function of their size distribution, using size-exclusion chromatography coupled to both nuclear magnetic resonance spectroscopy (NMR) and cloud-point measurements. Intact and enzymatically hydrolyzed methyl cellulose (MC) was fractionated according to size, and the level of substitution of the fractions was measured off-line using NMR. Clouding behavior was also measured as a function of size. Clear differences between hydrolyzed and nonhydrolyzed samples were observed using both techniques. For samples that had been selectively hydrolyzed using cellulose-degrading enzymes, NMR data showed a direct link between the degree of degradation and the level of substitution. Differences in the clouding behavior highlighted changes in substituent levels and substituent patterns across the size distribution. The techniques gave valuable and somewhat complementary information on the substituent distributions of the samples before and after enzymatic hydrolysis.

Introduction

Modified celluloses have many different practical uses. They are used as thin films, thickeners, binders, and emulsifiers in, for example, the food, cosmetic, construction, paint, and oil industries.[1] In the pharmaceutical world, they have a well-established use as excipients, i.e., neutral carriers for the delivery of active drug substances to the body. They are found in tablet preparations, film coatings, and liquid and semisolid formulations.[2] One area of particular interest is their use in extended-release drug formulations, i.e., drugs that are administered so that the release rate and/or the release site is controlled.[3] Such formulations require excipients with very well-defined properties. They must be able to release the drug to the body in a predictable and reproducible manner. For this, a thorough understanding of the relationship between the chemical nature of the excipient and its dissolution profile in the body is required.

Since these are polydisperse samples, factors such as the molar-mass distribution, substituent type, level, distribution, and pattern can all strongly influence critical properties such as solubility, dissolution profile, viscosity, etc.[4] The molar-mass distribution is generally characterized by size-exclusion chromatography.[5] Average substituent levels can be measured using techniques such as NMR.[6] Characterization of substituent distributions however, requires a separation that can then be related to substituent levels. To date, very few techniques are available for this purpose.

Substituent Groups on Modified Celluloses. Although native cellulose is hydrophilic, it is highly crystalline and is therefore insoluble in water and many organic solvents. Modifying groups added to the cellulose backbone disturb the inter- and intramolecular hydrogen bonds, making the molecule more amorphous and allowing dissolution in water and other solvents.[1] Substituents are added to the glucose units of the cellulose backbone through reaction of the hydroxyl groups on the C-2, C-3, or C-6 positions of the anhydroglucose unit (AGU). Depending on the requirements of the final application, typical substituents include methyl and ethyl groups, as well as propagating substituents (substituents that form chains of their own) such as hydroxyethyl and hydroxypropyl. Often, more than one type of substituent is introduced, for example hydroxypropyl methyl cellulose contains both hydroxypropyl and methyl substituents. Substitution rarely occurs on just one of the hydroxyl positions. Instead, it occurs on all three but to varying extents.[7]

The level to which a particular substituent is present is defined in two ways, i.e., the degree of substitution (DS) and the molar substitution (MS). The DS is the average number of substituted hydroxyl groups per anhydrous glucose unit (AGU) and can range between 0 and 3. The molar substitution (MS) is the average number of substituents per AGU and when the substituent is propagating, i.e., when further substitutions can occur along the substituent chain, it will have no theoretical upper limit.[8] Since methyl substituents cannot propagate, the MS and DS for methyl cellulose is the same. The level of substitution required for the modified cellulose to become water soluble depends on the substituent group, but generally speaking, for commercially produced, nonionic ethers, the DS should be greater than 1 for water solubility at room temperature.[4]

Both the MS and the DS are usually quoted as average values. However, the substituents are present as distributions and since these distributions can strongly affect the final properties of the excipient, the effect of differences in substitution patterns must be established.

Characterization of Distributions for Polydisperse Analytes. Size-exclusion chromatography can be used for the determination of size-based distributions of high-molar-mass

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10.1021/bm060281o CCC: $33.50
Published on Web 09/23/2006

polydisperse analytes.[5] When the separation is coupled to multi-angle light-scattering and refractive index detectors (SEC-MALS/RI), absolute determination of molar mass and molar-mass distributions is possible, since the amount of light scattered by the MALS is directly proportional to the product of the weight-average molar mass and the concentration of the macromolecule.[9] However, since this technique only measures molecular size and conformation, it cannot give any details on chemical differences across the chromatographic peak(s). In this case, techniques that are sensitive to chemical differences, such as NMR, IR, and (within the appropriate mass range) mass spectrometry, are required.

Use of Enzymes for the Characterization of Substituent Distributions. One approach to characterizing substituent distributions in modified celluloses is to use selectively hydrolyzing enzymes (endoglucanases) that can break the internal (1→4)-β-D-glycosidic linkages of the cellulose chain. Cleavage is hindered by the presence of substituent groups on the glucose rings. Therefore, densely substituted areas of the molecule are not hydrolyzed by the enzyme, whereas less substituted regions can be more easily degraded. The extent to which a molecule can be hydrolyzed by a given enzyme is thus an indication of the initial substitution pattern of that molecule. Polymers that are evenly substituted along the backbone should be less hydrolyzed than those with a more blocklike substitution pattern, since the unsubstituted regions are more amenable to degradation.

The extent to which substituent groups hinder a particular enzyme depends on the structure of the enzyme's active site. The number of consecutive glucose units needed (to fulfill the enzyme active-site requirements regarding substituent positions) differs between enzymes. Thus, the composition of the resulting hydrolysate is a factor of both the intact cellulose derivative and the properties of the hydrolyzing enzyme.

It is not just the presence of substituents but also their position on the glucose molecule that affects an enzyme's ability to hydrolyze a cellulose derivative. Studies have indicated that substituents at the C-2 position prevent hydrolysis, whereas cleavage can occur between AGUs when substituents are present on both C-6 positions.[10,11]

The selective nature of these enzymes means that they can be used as analytical tools for the characterization of substituent distributions in modified celluloses. For a given modified cellulose and enzyme, measuring the level of hydrolysis can indicate the level to which the intact cellulose derivative was substituted. By comparing hydrolysates from different enzymes (i.e., enzymes with different prerequisites for cleavage), complementary information regarding substituent levels and patterns can be obtained. However, to take full advantage of selective hydrolysis, the factors affecting the enzyme's ability to cleave must be thoroughly understood.

Techniques used to characterize enzymatic hydrolysates include SEC-MALS/RI[12] and anionic-exchange chromatography with pulsed-amperometric detection (HPAEC−PAD).[13,14] SEC-MALS/RI measures the change in average molar mass and molar-mass distribution after hydrolysis, and HPAEC−PAD can quantify the amount of unsubstituted glucose and oligosaccharides liberated by hydrolysis. However, neither technique measures changes in the substituent distribution level. Mass spectrometry is also useful, but ionizability and resolution issues limit its use to the low-mass end of the distribution.[11]

For a more complete characterization of the relationship between enzymatic activity and the level of substitution, more comprehensive analytical techniques are required. Saake et al.[15,16] used enzymatic hydrolysis followed by preparative SEC, HPAEC−PAD, and ^{13}C NMR analysis to characterize the substituent distribution of both carboxymethyl cellulose (CMC) and methyl cellulose. They found a direct relationship between size fraction and DS. They also measured the uniformity of the substitution pattern using HPAEC−PAD. In this work, the level of substitution has been measured as a function of size (before and after enzymatic hydrolysis), using SEC coupled to both ^{1}H NMR spectroscopy and to cloud-point measurements.

Coupling Considerations for SEC NMR. Although NMR is not traditionally used as a chromatographic detector, improvements in NMR sensitivity, instrumental interfaces, and solvent suppression techniques over the last number of years have helped to make the two techniques somewhat more compatible.[17,18] High-field instrumentation, improvements in probe design (smaller, cryogenic cooling, etc.), and specialized LC NMR interfaces (such as solid-phase extraction) have all been particularly useful. SEC coupled to NMR shows the relationship between molecular size and chemistry (in this case, level of substitution). For enzymatically hydrolyzed modified celluloses, SEC NMR can indicate the selectivity of the hydrolytic process and highlight differences in the activity of different enzymes.

Various online coupling possibilities are available for LC (SEC)-NMR, including continuous-flow and stopped-flow interfaces, loop-collection systems, and solid-phase extraction (SPE) cartridges.[19] All of these coupling methods, where appropriate, can be very useful. However, when quantitative information is required (such as in this case), continuous-flow systems, which only allow a limited number of transients per unit time, are not usually sensitive enough. Stopped-flow analysis can work well with discreet peaks but is troublesome when differences need to be measured across a continuous peak, such as those found in SEC analyses. Loop collection followed by off-line measurements can also be useful, but sample enrichment through repeat injections is not possible; thus, signal-to-noise ratios can be too low. Another issue with these techniques is that solvent suppression of the nondeuterated SEC solvent signals is required. This can adversely affect the baseline of the NMR spectrum, resulting in inaccurate integrals.

Interfacing LC to NMR using SPE cartridges overcomes many of the problems associated with the coupling[20] and can be performed in a semiautomated fashion. However, when polydisperse samples are being characterized, selective or irreversible retention of some of the molecules on the SPE cartridge becomes a possibility. Therefore, the SPE system was not considered as a first choice. Some further investigations into the suitability of the SPE interface will be carried out.

Sample Preparation. SEC NMR analysis was performed on acetylated derivatives of the modified cellulose samples; that is, all nonsubstituted hydroxyl groups on the cellulose backbone were capped with an acetyl group. Acetylation simplifies NMR interpretation because the methyl unit of the acetyl group gives a discreet signal that can be used for quantitation of the hydroxyl content on the glucose ring. It also improves the solubility of the sample over a broad range of substituent levels.[6,21] Acetylated modified celluloses are insoluble in water but soluble in typical NMR solvents such as chloroform, DMSO, etc.

Clouding Behavior. Measuring clouding behavior is another way to obtain useful information on the substituent distributions of modified celluloses. Clouding, i.e., phase separation, occurs when water molecules associated with the polymer dissociate upon heating, leading to increased polymer−polymer interaction. The rate and temperature of clouding can be related to the substituent pattern on the molecule, with more substituted

molecules clouding at a lower temperature. Clouding can be followed by the formation of a three-dimensional cross-linked network structure. Upon cooling, the process is reversed and the macromolecules return to solution.[22,23] For methyl cellulose, clouding is dependent on both the level of substitution and the uniformity of the substitution pattern (along the backbone).[24] For samples with a very uniform substitution along the backbone, aggregation may not occur at all.[25]

Thermoreversible gelation of methyl cellulose is used in many applications where increased viscosity at elevated temperatures is required. It can also be used as an analytical tool, because the rate and the level to which a solution clouds is a function of various properties, including the type, level, and uniformity of the substitution pattern.

Cloud-point measurements are traditionally bulk measurements, where an average cloud-point or clouding profile is measured for a polydisperse sample. By coupling to a SEC separation, changes in clouding as a function of the size fraction can be seen. Coupling is off-line since clouding behavior is measured in batch mode.

Experimental Details

Enzymatic Hydrolysis. Methyl cellulose (MC) SM-1500 (viscosity type: 1500 cP, with a stated degree of substitution of 1.80) was obtained from Shin-Etsu Chemical Co. (Tokyo, Japan). Family 5A endoglucanase from *Bacillus agaradhaerens* (BaCel5A) was a kind gift from the late Dr. Martin Schülein (Novozymes, Bagsværd, Denmark). Family 12A endoglucanase from *Trichoderma reesei* (TrCel12A) was a kind gift from Dr. Michael Ward, Genencor, CA.

MC was dissolved in water to a concentration of 10 g/L under continuous stirring overnight at 4 °C. After the addition of one of the enzymes to a final concentration of 1 μM, hydrolysis was allowed to proceed for 72 h at room temperature, at which point hydrolysis had reached completion.[26]

Acetylation. Both the intact and the enzymatically hydrolyzed MC samples were acetylated according to a modified version of the procedure outlined by Tezuka.[21] Approximately 50 mg of modified cellulose (intact or enzymatically hydrolyzed) was mixed with 2.5 mL of acetic anhydride and 0.75 mL of pyridine and stirred for 5 h at 90 °C in a sealed vial. The mass of cellulose was reduced if the viscosity of the solution was too high. After reaction, the solution was dialyzed in pure water, using dialysis tubing with a molecular weight cutoff of 10 000 Da. The sample was then dried at 50 °C.

SEC System. The molar-mass distributions of the intact cellulose sample and its enzymatically hydrolyzed equivalents were determined using aqueous phase SEC-MALS/RI. The system consisted of a TSKgel $GMPW_{XL}$ mixed-bed column 300 × 7.8 mm i.d. (TosoHaas Bioseparation Specialists, Stuttgart, Germany) with an aqueous mobile-phase (10mM NaCl). SEC separations of the acetylated MC samples were carried out in THF with a PLgel 5 μm mixed-D column (300 × 7.5 mm) at a flow rate of 0.5 mL/min. Detection was with a Waters RI detector. The acetylated samples were dissolved in the mobile phase (1 mg/mL) and 100 μL was injected via a Waters 717plus autosampler. When fractions were being collected for NMR analysis, a Gilson FC203B fraction collector was connected to the system. To couple the SEC separation to the NMR spectrometer, the chromatogram was fractionated into 30-s portions (0.25 mL) across the peak. The signal-to-noise ratio of the NMR measurements was enhanced by repeating the separation (and subsequent fractionation) a number of times (usually 10) and by using a 3 mm probe in the NMR experiments.

Solvent Impurity Issues. Impurities in the LC eluent can cause problems in LC NMR analysis, even after the solvent has been evaporated. Although LC mobile phases are typically high-purity solvents, impurities that do not impact separation or detection with typical LC detectors (UV, RI, ELSD, etc.) can have a detrimental effect on NMR measurements. In this work, THF was used as the chromatographic solvent and this is known to be problematic for NMR analysis.[17] Solvent evaporation under N_2 did not always remove THF-based impurities. This was particularly the case when a significant amount of polymer was present in the fraction, since the polymeric material impeded evaporation. Gentle heating of the dry fractions (12 h at 50 °C) was required as an additional step.

NMR Analysis. The dry fractions were redissolved in deuterated chloroform and transferred to a 3 mm NMR tube. ^{1}H NMR measurements were carried out on a Varian 600 MHz INOVA instrument. The experiments were run at 50 °C. The pulse sequence included an acquisition time of 3 s followed by a delay time of 4 s. A line-broadening factor of 0.3 Hz was used. The chemical shift values were referenced to the solvent signal of $CDCl_3$ (7.26 ppm).

Because the fractions were measured in a batch mode, analysis time was not limited and the number of transients measured in each case was determined by the signal-to-noise ratio. Typically, 128 transients were measured, but for fractions with a lower concentration (tailing and leading edges of the peak), up to 10 000 transients were recorded. The spectra were phased and background corrected prior to integration.

Calculation of Substituent Levels. A typical NMR spectrum for acetylated MC is shown in Figure 1. The broad signal labeled (a) results from the substituent methyl protons and protons on the glucose ring. Signal (b) is due to protons on the acetyl group. To calculate the number of substituent methyl groups per glucose unit, signal (a) must be deconvoluted, so that the signal from the substituent methyl content can be extracted from the total signal. The number of acetate groups plus substituent methyl groups on each glucose ring must equal 3 (9 protons in total). Therefore, a correction factor was used to force the integrals of the portion of signal (a) due to the substituent methyl protons plus the integral of signal (b) to be equal to 9 (i.e., one unit per proton). The signal from the 7 glucose protons in signal (a), which then corresponded to 7 units relative to the corrected integral of signal (a), can then be subtracted, leaving the signal due to the substituent methyl protons. Dividing this value by 3 gives the total number of methyl groups per glucose unit.

The correction factor x was calculated according to

$$(ax - 7) + bx = 9$$

$$x = 16/(a + b).$$

Clouding Behavior. Nonacetylated methyl cellulose samples were used in the clouding measurements. The enzymatically hydrolyzed samples were size-fractionated on a FPLC system (Pharmacia, Uppsala, Sweden) equipped with a Superdex 75 HR 10/30 column, connected in series with a Superdex 200 HR 10/30 column (Amersham Bioscience, Uppsala, Sweden) and a refractive index detector ERC-7510 (ERMA Inc., Tokyo, Japan). A 500 μL sample loop was used, and the eluent was water (0.5 mL/min). Fractions of 2 mL were collected between the elution volumes of 15 and 45 mL. A total of 20 fractionations were made. The collected fractions were pooled and freeze-dried. The fractions were then redissolved in a buffer solution (0.1 M phosphate buffer, pH 6.5), such that all fractions had the same final concentration (i.e., 10 mg/mL).

Analysis was carried out using a Mettler Toledo FP 81C clear-point and cloud-point measuring cell and a Mettler Toledo FP90 central processor. The data were processed using in-house software (IPCLab, version 3.2.1). A programmed run increased the temperature of the solution from 30 °C to 80 °C at a rate of 1 °C per minute.

Results and Discussion

SEC. The molar-mass distributions (MMD) of the intact and enzymatically hydrolyzed MC (nonacetylated) were calculated from SEC-MALS/RI analysis of nonacetylated MC. The dn/dc for methyl cellulose in aqueous solution was taken to be 0.141

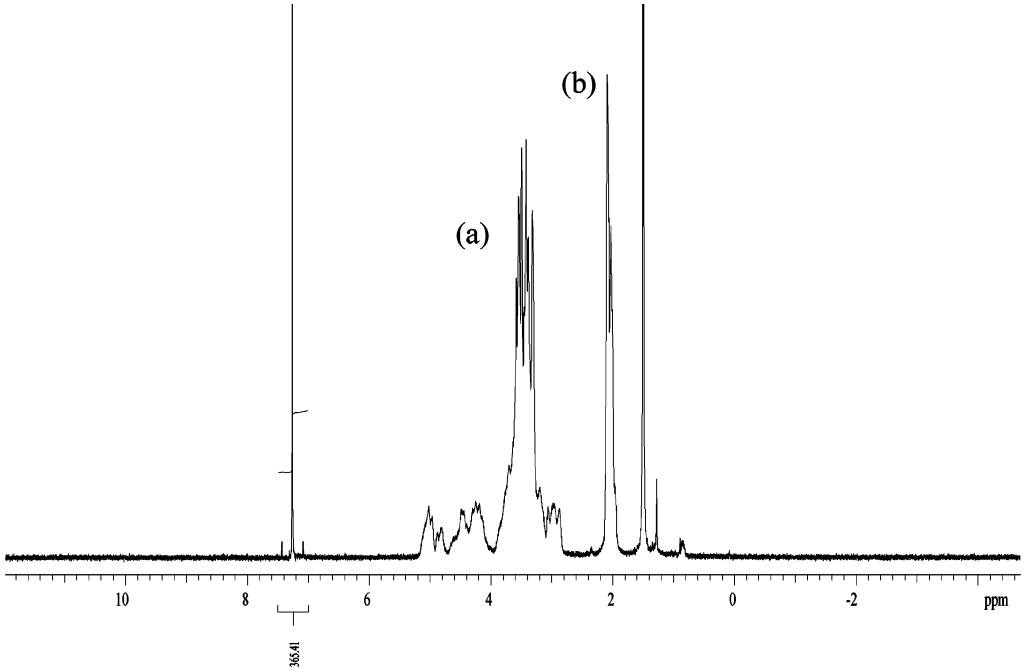

Figure 1. NMR spectrum of acetylated methyl cellulose. Signal (a) results from protons on the glucose ring and the methyl substituents, signal (b) is due to protons on the acetyl group.

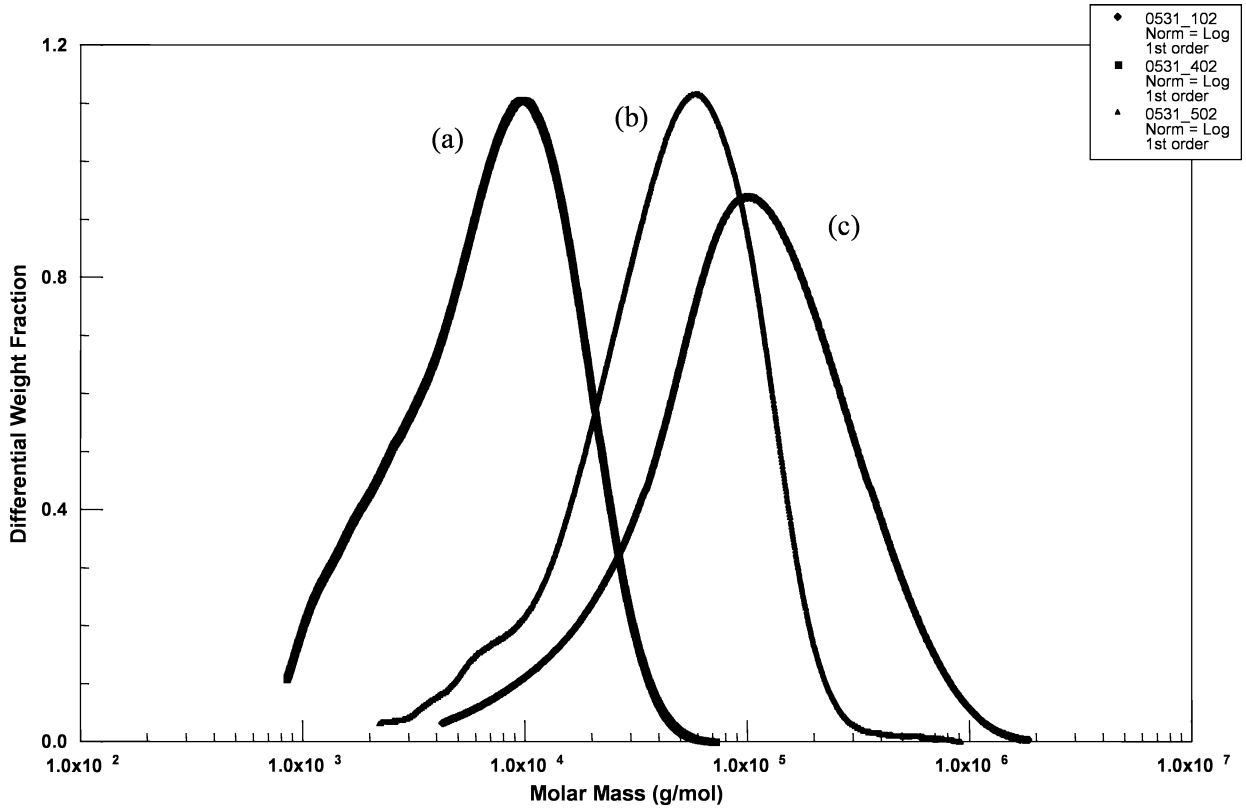

Figure 2. Molar-mass distribution of methyl cellulose before and after hydrolysis. (a) BaCel5A hydrolyzed, (b) TrCel12A hydrolyzed, and (c) intact polymer.

mL/g and was assumed to be constant across the molar-mass and substituent-level distributions.[27] The resulting MMDs are shown in Figure 2.

The hydrolyzing action of the two enzymes can be seen from changes in the molar-mass distribution of the depolymerized samples, relative to their intact equivalent (Figure 2). BaCel5A hydrolyzes the sample to a greater degree than TrCel12A, resulting in a lower average molar mass. For both enzymes, a high-molar-mass fraction remained in the sample. The amount of nonhydrolyzed MC in the TrCel12A hydrolyzed sample is greater than for the BaCel5A hydrolysate (Figure 3). This indicates that the substituent pattern on some of the molecules is such that enzymatic degradation cannot occur.[15,28]

NMR Measurements. The SEC NMR profile of intact MC, showing calculated substituent values as a function of size fraction, is given in Figure 4. A slight increase in the DS was seen as the average size decreased. This indicates that smaller molecules in the distribution are somewhat more amenable to substi-

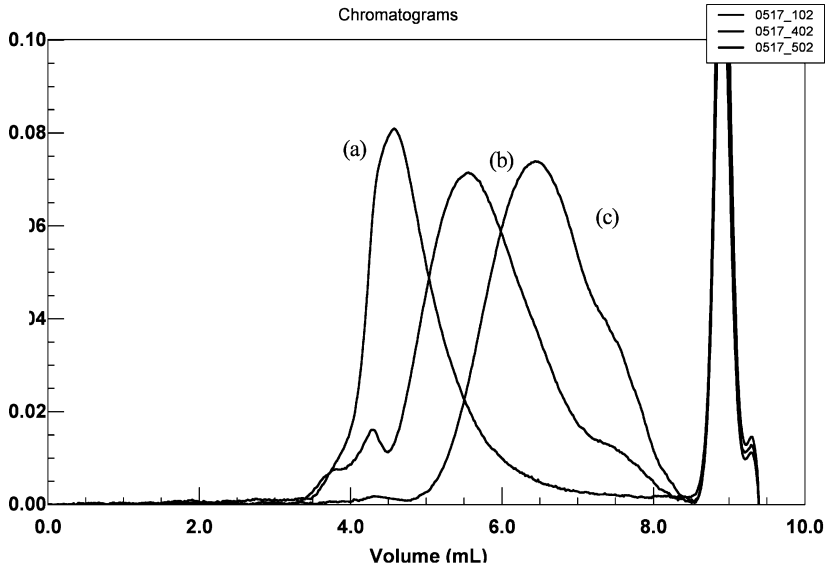

Figure 3. SEC−RI profile for acetylated intact and hydrolyzed methyl cellulose (THF based separation). (a) Intact (b) TrCel12A hydrolyzed, and (c) BaCel5A.

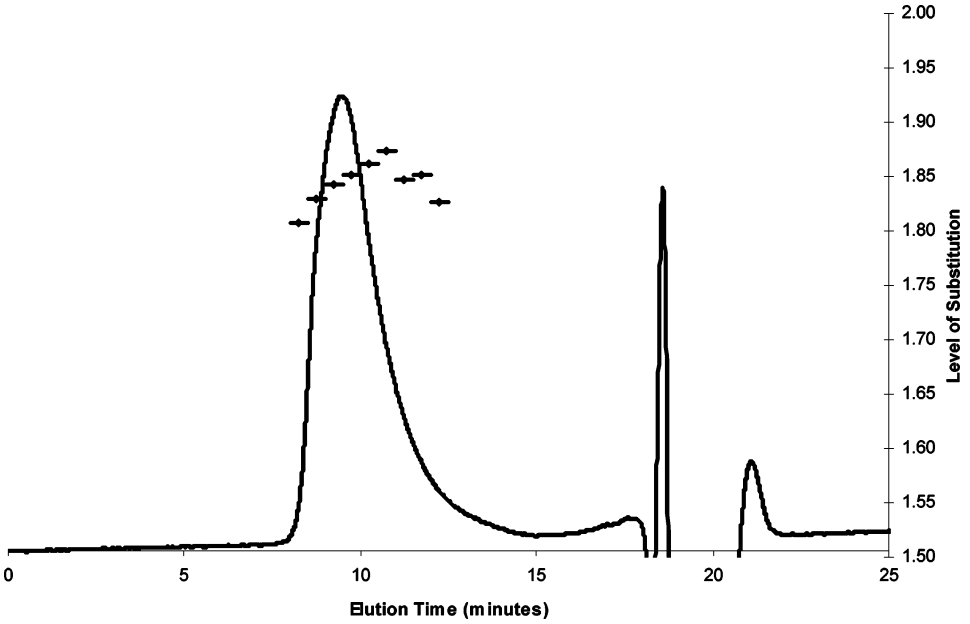

Figure 4. SEC NMR profile for non-hydrolyzed MC. Markers across the peak indicate the substituent level of the various fractions.

tution than larger ones. However, the variation is only slight, increasing from 1.81 to 1.87 methyl groups per glucose unit, across the chromatographic peak. The precision of the measurement depends on the signal-to-noise ratio, which changes from fraction to fraction across the distribution. In this case, the gradual increase in the substitution level suggests that the measurements for fractions up to 10.5 min are precise as given. Later eluting fractions are less concentrated and so less precise. This can be seen in the deviation of the data for these fractions.

For the enzymatically hydrolyzed samples, the change in DS values across the size distribution was much more pronounced (Figure 5). Substitution levels decreased sharply with decreasing size, ranging from above 2 for the early eluting fractions (largest molecules in the distribution) to below 0.7 for the latest eluting fraction in the chromatographic peak. This pattern can be understood in terms of the hydrolyzing action of the enzyme (see the Introduction) and has been seen by others.[15] Generally, the more substituted the molecule is, the less the enzyme can hydrolyze it. When a region along the polymer chain has a sufficiently low level of substitution, the enzyme is less hindered, and thus degradation can occur to a greater extent. The molar-mass distribution of the hydrolysate is then closely linked to substituent level. The largest molecules in the hydrolysate MMD are then the most substituted molecules, whereas the smallest molecules are also the least substituted. The size-based separation is thus an indirect separation based on substituent levels as well as a measure of an enzyme's hydrolyzing ability.

An interesting aspect of the study is the comparison between the selectivity of two different enzymes. The SEC profile showed that BaCel5A hydrolyzes the sample to a greater extent than TrCel12A. For a given size fraction, the level of substitution is higher for the BaCel5A hydrolysate than for the TrCel12A, though the differences becomes less significant as the average molecular size decreases. This indicates differences in the selectivity of the two enzymes, with BaCel5A less sensitive toward substituents and thus better able to hydrolyze more highly substituted molecules.

The molar-mass distribution for the TrCel12A hydrolysis was bimodal, with the high-mass peak corresponding to nonhydrolyzed methyl cellulose. Some nonhydrolyzed cellulose was also

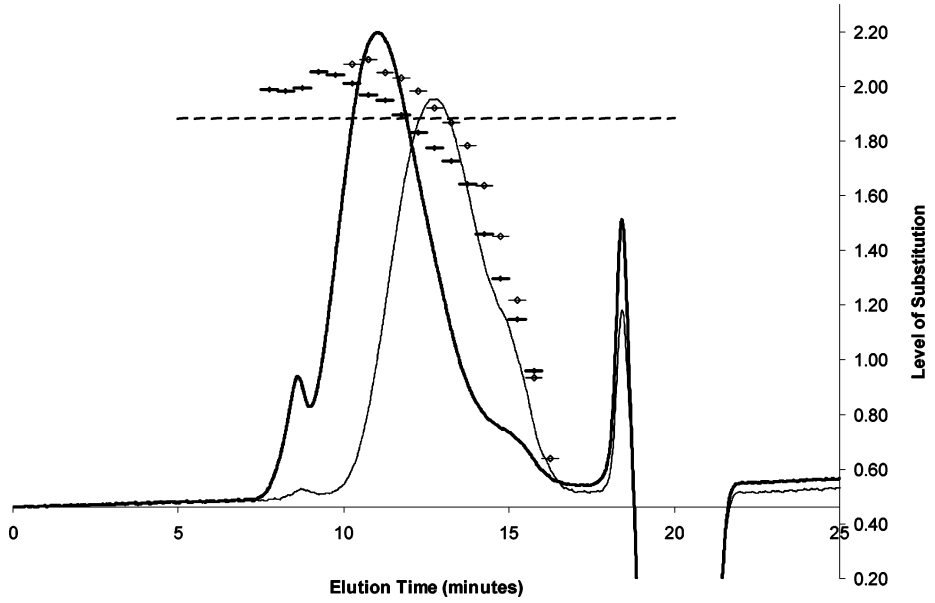

Figure 5. SEC NMR profile for enzymatically hydrolyzed MC. Dashed line corresponds to the average substitution level (bulk measurement). Heavy-line chromatogram and bars correspond to TrCel12A hydrolyzed MC (SEC profile and substitution level of fractions). Lighter-line chromatogram and bars correspond to BaCel5A.

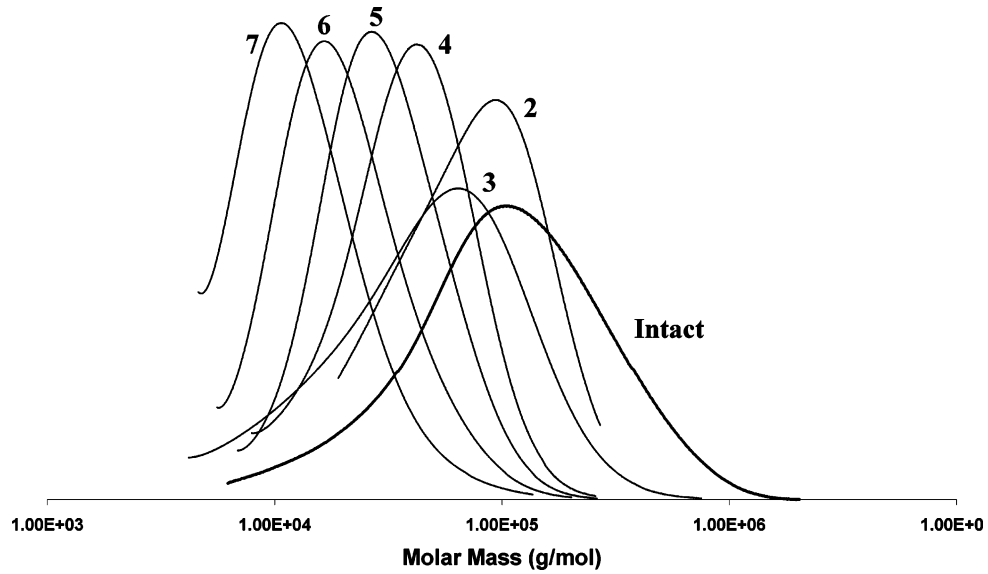

Figure 6. Molar mass distributions for TrCel12A hydrolyzed fractions of methyl cellulose.

seen in the SEC−RI profile for the BaCel5A hydrolyzed sample, but the amount present was too small to be measured by NMR. Saake et al.[16] also noted the presence of a bimodal molar-mass distribution in some CMC fractions. They assumed that the bimodal distribution was due to inefficient preparative SEC fractionation, although they also concluded that aggregated or gel like particles were present in the hydrolyzed sample.

Although it may have been assumed that the nonhydrolyzed portion of the sample had a prohibitively high substituent level, SEC NMR data showed that the average substituent level was lower than that of hydrolyzed molecules that eluted later in the separation. This may suggest that the uniformity of substitution across the molecule has a significant impact on the hydrolyzing ability of the enzyme. For example, when substituents are randomly positioned along the cellulose chain, there are fewer positions where the enzyme can cleave and the molecules will not be hydrolyzed to the same extent as ones with a similar DS but a more blocklike substitution pattern. An alternative explanation for the high molar-mass fraction remaining in the sample may be that remaining crystalline regions on these molecules prevented enzymatic hydrolysis. Some further work will be required to clarify this.

Clouding Behavior. Clouding behavior is dependent on a number of factors, including molecular size and structure, sample concentration, solvent, rate of heating, etc.[20] When experimental factors are kept constant, differences in clouding can be related to molecular differences. By size-based fractionation of both intact and hydrolyzed MC samples (preparative SEC), the effect of substitution level on clouding can be established. The molar-mass distributions of the various size fractions (TrCel12A hydrolyzed fractions) are given in Figure 6. Fraction numbers correspond to a fixed elution-volume range in the SEC fractionation, and so equivalent fractions numbers have the same size profile for all three samples (intact and hydrolyzed MCs).

The effect of molar mass on clouding was seen with nonhydrolyzed MC, where there appears to be no significant relationship between size and substitution level across the molar-

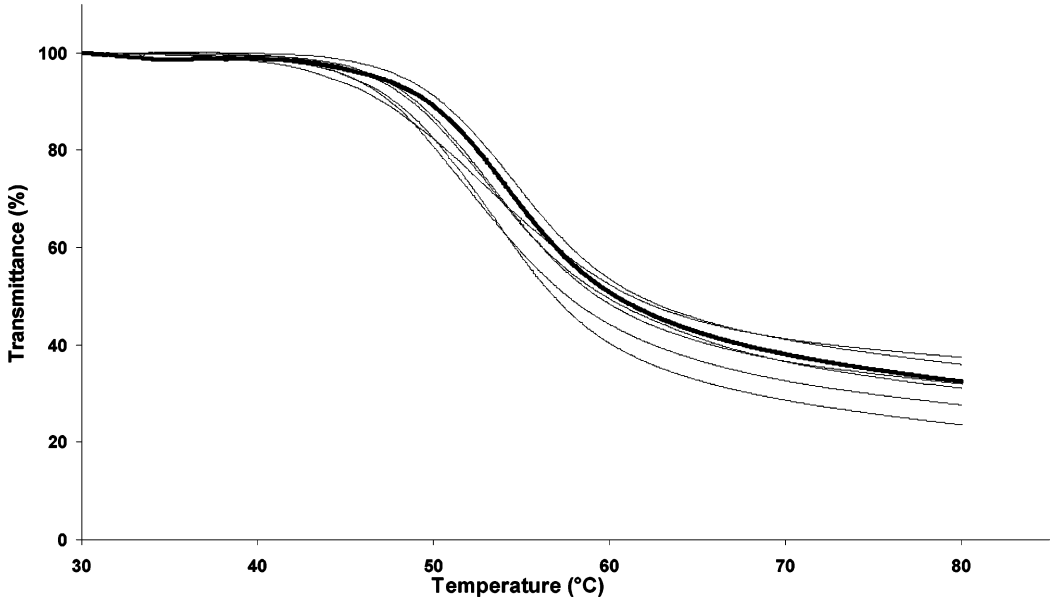

Figure 7. Clouding curves for SEC fractions of non-hydrolyzed methyl cellulose. The heavy line corresponds to the bulk (nonfractionated) measurement. Fraction order (decreasing transmittance at 80 °C): 3 > 2 > bulk > 4 > 6 > 5 > 1.

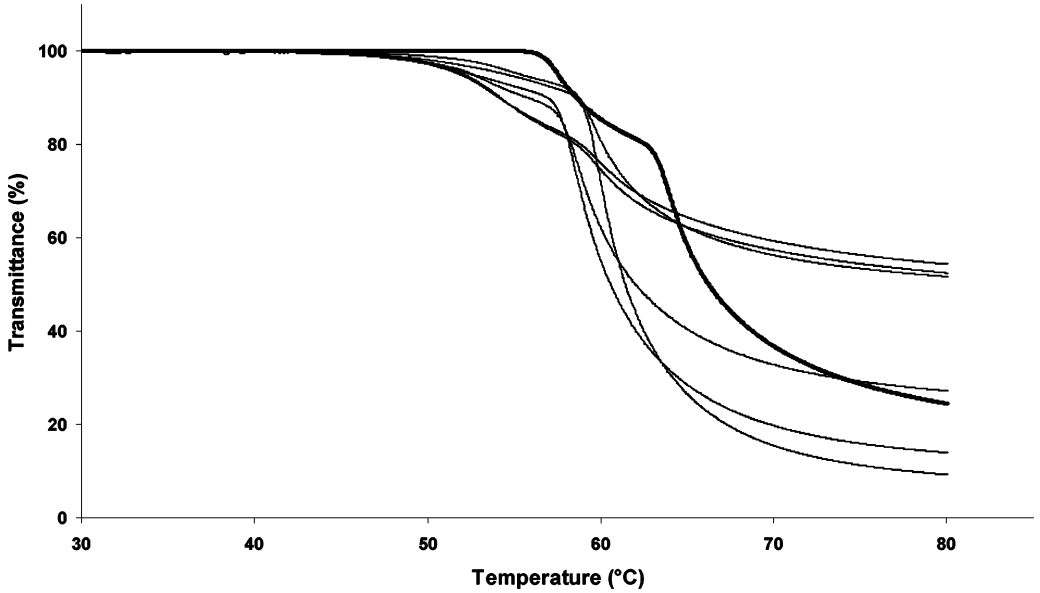

Figure 8. Clouding curves for SEC fractions of TrCel12A hydrolyzed methyl cellulose. The heavy line corresponds to the bulk (nonfractionated) measurement. Fraction order (decreasing transmittance at 80 °C): 2 > 3 > 4 > bulk > 5 > 6 > 7.

mass distribution (as illustrated in Figure 4). The clouding profiles for the fractions are given in Figure 7. In general, clouding did not vary dramatically between fractions and no molar-mass related trends were seen; that is, in this case, it is reasonable to suggest that clouding behavior is independent of polymer size. Clouding of the bulk MC sample (i.e., non fractionated) showed a similar profile. These results also indicate that the substitution level (and the substituent pattern) does not vary significantly across the size-distribution, since differences in substitution levels are known to affect clouding behavior.[22] This is in agreement with the NMR results shown above and other studies.[29,30] In contrast to nonhydrolyzed MC, clouding of the enzymatically depolymerized samples changed significantly from fraction to fraction. Curve shapes were quite heterogeneous; that is, the rate of decrease in transmittance changed as temperature increased. Although clouding behavior is often defined in terms of a single point (e.g., temperature at 96% or 50% transmittance), the shape of the clouding curves is also important and in this case gave much more information than a single point measurement. SEC NMR data showed a strong relationship between size and substituent level for the enzymatically hydrolyzed samples, with the DS decreasing with decreasing molar mass. Since clouding of the nonhydrolyzed sample did not vary dramatically with size fraction, it can be concluded that the differences in the clouding curves of the hydrolyzed fractions are related to differences in the substitution level and substitution pattern.

The steepness of the clouding curve can be related to the uniformity of the substituent pattern within the batch, since for a homogeneously substituted sample, all of the molecules will behave similarly and cloud at a similar temperature. Shallower curves imply nonuniform substitution levels, since a broader range of temperatures are required for the entire sample to phase separate.

The early fractions of the TrCel12A hydrolyzed sample (high mass, high substituent level) had shallow clouding curves with high levels of transmittance at the final temperature (Figure 8). This suggests that these fractions were hydrolyzed to a very

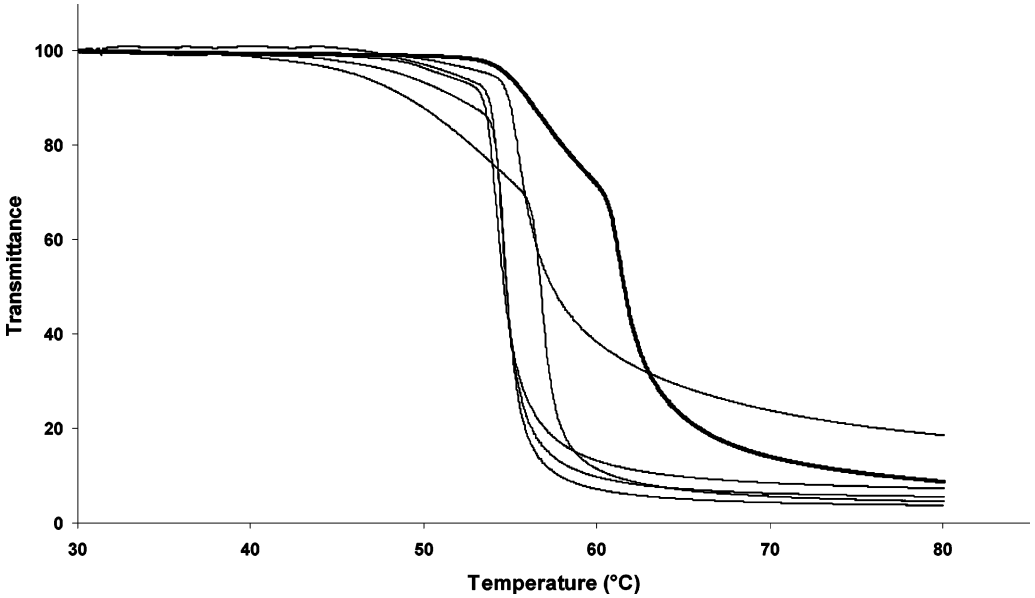

Figure 9. Clouding curves for SEC fractions of BaCel5A hydrolyzed methyl cellulose. The heavy line corresponds to the bulk (nonfractionated) measurement. Fraction order (decreasing transmittance at 80 °C): 3 > bulk > 4 > 5 > 7 > 6. Fraction 2 did not dissolve in the buffer.

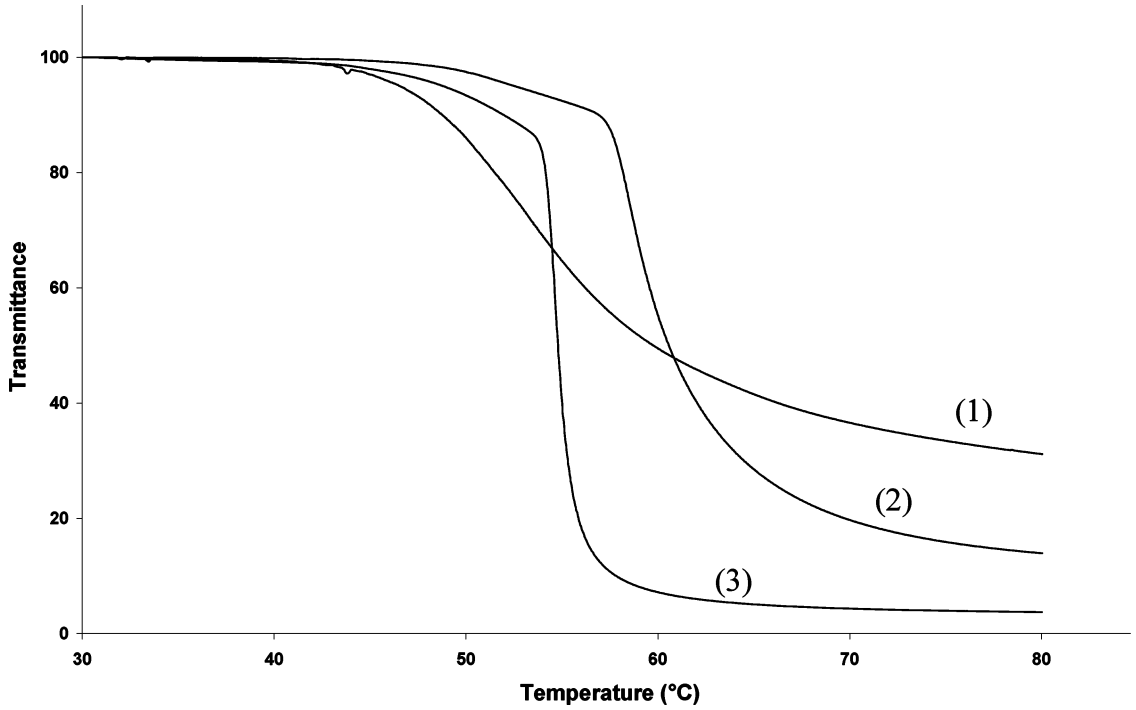

Figure 10. Clouding curves for SEC fraction number 6 from (1) intact, (2) TrCel12A, and (3) BaCel5A hydrolyzed methyl cellulose.

low extent, presumably because this enzyme had more stringent conditions associated with cleavage. Later eluting fractions (low mass, low substituent level) began with a gradual decrease in transmittance, but at a certain temperature (around 60 °C), the curves became much steeper and transmittance fell off sharply. It seems that these fractions mainly consisted of uniformly substituted MC (steep curve). Since these were low-mass fractions that had been hydrolyzed to a greater extent by the enzyme, a more uniform hydrolysate would be expected, in accordance with other studies.[29,30] The gradual decrease at the beginning of the curve can be due to remaining low molar-mass parts of the original sample that were not hydrolyzed by the enzyme.

For the BaCel5A hydrolyzed fractions (Figure 9), the clouding curves were much steeper, indicating that the resulting hydrolysate has a much more uniform substitution pattern. This suggests that BaCel5A is less sensitive and thus a more efficient enzyme in this case. Fraction 2 did not dissolve in the buffer, indicating that the DS was high, since MC with a DS of 2 or higher is not water soluble.[1] The high DS was also observed in the SEC NMR data.

Figure 10 is a comparison of the clouding behavior of one fraction (fraction 6) from all three samples (same size distribution in all three cases). Intact MC has a broad, shallow slope, similar to its bulk equivalent. Clouding starts early (96% transmittance at 46 °C) but progresses slowly (50% transmittance at 60 °C). Of the two hydrolyzed samples, the BaCel5A fraction clouded earliest and had the steepest slope. This indicates a higher DS and a more efficient enzymatic hydrolysis relative to the TrCel12A hydrolysate. This is again in agreement with the SEC NMR data. A similar pattern was seen for other (equivalent) size fractions.

Conclusion

The effect of enzymatic degradation of methyl cellulose was investigated. The hydrolysates formed were characterized using SEC NMR, and the results were correlated to the clouding behavior of SEC fractions of (hydrolyzed) MC. SEC NMR data of intact and enzymatically hydrolyzed methyl cellulose clearly showed the selective nature of enzymatic hydrolysis for modified celluloses. A clear relationship between the degradability of the cellulose derivative and level of substitution was established and a comparison between two enzymes with different hydrolyzing selectivites was made. The SEC NMR data highlighted that the substituent pattern is the most important factor controlling hydrolysis, since the nonhydrolyzed fraction was not especially highly substituted. Cloud-point analysis of the same hydrolysates showed large differences in the clouding behavior as a function of enzymatic degradation. Fractions that have been degraded to a greater extent exhibited lower clouding temperatures as well as steeper clouding curves. This indicates that enzymatic degradation results in molecules with more homogeneous substituent patterns. This will be valid up to a certain DS, above which the enzymes are no longer capable of hydrolyzing the polymer. When this is the case, shallower clouding curves (indicative of a more heterogeneous material) will be observed. The DS at which the polymer becomes unhydrolyzable reflects the enzymes ability to cope with substituents. Differences in the uniformity of the substituent patterns of different fractions were highlighted by the profiles of the clouding curves.

Acknowledgment. This work was supported by the Swedish Centre for Amphiphilic Polymers from Renewable Resources (CAP). F.F. also acknowledges the scientific support of AstraZeneca R&D.

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BM060281O