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The Applicability of Enzymes in Cellulose Ether Analysis

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Summary: Six methyl cellulose (MC) samples, one with a DS of 1.32 and five with a DS between 1.83 and 1.88, were degraded with five different enzymes or enzyme preparations containing endoglucanases. The main goal was to investigate whether enzymes could be used for determination of heterogeneity of the substituent distribution along the cellulose chain. To obtain information about the heterogeneity it was necessary to gather information on how the enzymes affect hydrolysis. Monomer composition and methyl distribution in the polymer chain were analyzed after total or partial random hydrolysis and appropriate derivatization by GC and MS, respectively, and used as reference data for the evaluation of the enzymatic hydrolysis. Size exclusion chromatography with multi angle light scattering and refractive index detection (SEC-MALLS/RI) was used to estimate molar mass distribution of the MCs before and after hydrolysis. Electrospray and matrix assisted laser desorption/ionization (ESI and MALDI) in combination with various MS analyzers were compared with respect to quantification of the degradation products directly and after perdeuteromethylation. Methyl group distribution in the oligomeric fractions and the average DS/DP were calculated from ESI mass spectra. With help of the reference analysis, patterns could be corrected for the unspecific contribution of end groups. By labelling and ESI-MS^n, our knowledge about the tolerance of the enzyme's sub-sites with respect to the number of methyl groups could be improved. A novel standard addition method in combination with electrospray ionization ion trap mass spectrometry (ESI-IT MS) was used to determine the amount of formed oligomers.

Keywords: cellulose degrading enzymes; endoglucanases; enzyme specificity; ESI-MS; MALDI-MS; methyl cellulose; quantitative mass spectrometry

Introduction

Cellulose ethers like methylcellulose (MC), methylhydroxyethyl cellulose (MHEC) or hydroxypropylmethyl cellulose (HPMC) have a wide field of applications in the food, the pharmaceutical or the construction industry. They are important due to their thickening and water-retention ability. Other important features of cellulose ethers are their property to form thermoreversible gels (MC, HPMC) or their flocculation behaviour (MHEC). These properties are related to the degree and the distribution of the substituents in the polymer.

Analyzing cellulose derivatives with respect to their substitution pattern is a challenging task. The distribution of the substituents in the monomer units and along the polymer chain has to be investigated. Besides of this, there is a distribution of the substituents over the polymer chain, leading to a DS-gradient. New methods to

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analyze parts of these substituent distributions will be explained.

Already in the sixties Wirick[1] and later Gelman[2,3] used cellulases for the degradation of cellulose ethers and tried to estimate the theoretical and observed number of cleavages. Lately, enzymes have especially been used by the groups of Gorton and Tjerneld[4–7] and of Puls and Saake[8–11] in cellulose ether analysis. Degraded samples have been investigated by SEC-MALLS/RI and fractions obtained have been studied with respect to their monomer composition. MS techniques have been applied to the low molecular weight fraction. The general idea behind using enzymes, in this particular case cellulases, is that lower or unsubstituted areas of the cellulose derivative are hydrolyzed to a higher extent than higher substituted regions of the polymer, since substituents like methyl, hydroxyethyl, or carboxymethyl groups are expected to interfere with the formation of an active complex and thus hinder the enzyme from hydrolyzing the cellulose chain.

Enzymes commercially available are mixtures of endo- and exo-cellulases, thus improving degradation due to synergistic effects, but making the interpretation of the product pattern more complicated.

Enzymatic degradation has been followed using different approaches. The decrease in viscosity with time gives information about the extent and the kinetics of hydrolysis. However, this is a rather unspecific parameter which only provides an estimation of the enzymes activity related to the given substrate.[12,13] The parameters that mainly influence the decrease in viscosity are the degree of substitution (DS) and the original degree of polymerization (DP) of the sample.

Determination of the increase of reducing ends is another method frequently employed to assess enzymes' activity. However, the reducing-end assays[14] often suffer from substituent pattern depending sensitivity and non-stoichiometric transformations, especially for partly substituted oligosaccharides.

Use of size exclusion chromatography (SEC) in combination with MALLS or RI detection has been reported in various publications[15–18] for the determination of the extent of the hydrolysis, which causes a significant shift of the molecular mass distribution.

Davies et al. suggest a model with different subsites in the active site[19], the number of which depends on the enzyme used. The DP of the oligomeric products can be determined by HPAEC-PAD. By incubating the enzymes with cellodextrins (DP 2–6) and analysis of the products released, some conclusion on the structural requirements of the enzymatic hydrolysis can be deduced. Depending on the enzyme about 4–8 glycosyl units are involved in the active complex. Melander et al. performed some respective studies using an immobilized enzyme micro reactor (IMER) online coupled to a HPAEC-PAD system.[4]

In this summary we report on new approaches, which yield representative quantitative data for enzymatically hydrolyzed MC, thus allowing comparison of samples with different DS. Influence of perdeuteromethylation and instrumental setup was investigated. Thus, all enzymatically hydrolyzed samples were analyzed before and after perdeuteromethylation with an ESI-ion trap (ESI-IT) instrument, an ESI-triple stage quadrupole (ESI-QqQ) instrument, and a MALDI time-of-flight (MALDI-TOF) instrument. In the work presented here, deviation for the various MS techniques is discussed. Furthermore, to get insight into the enzymes' tolerance with respect to the location of methyl groups in MC, ESI-MS^n was applied. The number of substituents in the released di- and trisaccharides was determined separately for the reducing and the non-reducing end glucosyl units and the internal glucosyl residues, respectively. A novel method for quantitative analysis of the oligomers detectable with MS was applied.

For comparison, all MCs were analyzed with respect to monomer composition and distribution in the polymer chain by established methods. After appropriate

sample preparation, various techniques such as nuclear magnetic resonance spectroscopy (NMR), gas-liquid chromatography (GLC), capillary electrophoresis (CE), and mass spectrometry (MS)[11,20–24] can be applied for analysis of the substituent distribution in the monomer unit. Analysis of the substitution pattern along the cellulose chain is more difficult. Quantitative analysis of oligosaccharide mixtures has been achieved after perdeuteromethylation and random acid hydrolysis of methyl celluloses or starches by mass spectrometry in combination with soft ionization techniques such as fast atom bombardment (FAB), matrix-assisted laser desorption/ionization (MALDI), or electrospray ionization (ESI) mass spectrometry.[25,26]

Experimental Part

General

All reagents used were of highest purity available and purchased from Fluka, Aldrich, or Merck. Five different endoglucanase preparations were used. *Trichoderma viride* (*T. viride*) was purchased from SERVA Electrophoresis GmbH, Heidelberg, Germany (Contr. No.: 10186), *Trichoderma longibrachiatum* 1 and 2 (*T. longi 1* (Lot nr. 030201) and *T. longi 2* (Lot. nr. 50201)) from Megazyme, (Wicklow, Ireland), *Bacillus agaradhaerens* (*BaCel 5A*) was a kind gift from the late Dr. Martin Schülein (NovoZymes, Bagsvaerd, Denmark) and *Trichoderma reesei* (*T. reesei*) was purchased from Fluka (ATCC 26921). MC 1 - MC 6 were commercial products. MeI-d_3 was purchased from Deutero GmbH, Kastellaun, Germany.

Reference Analysis

The substituent distribution on the monomer level has been analyzed after hydrolysis, reduction and acetylation of the polymer sample with GLC. The distribution of the substituents along the polymer chain has been determined after deuteromethylation and subsequent partial methanolysis by mass spectrometry as has been described. For more detailed information see ref [27,28].

Enzymatic Hydrolysis of Methyl Celluloses

Approximately 30 mg of each MC was dissolved in 3 mL of H_2O at 4 °C overnight. The enzyme was purified[27,28] dissolved in water to a concentration of approximately 30 UmL^{-1} at r.t. All enzymatic batches were shaken at r.t. for 96 h, and then they were boiled at 95 °C for 15 min and centrifuged. Thereafter, the solution was freeze-dried overnight in Eppendorf vials.

Perdeuteromethylation of Enzymatically Hydrolyzed Samples

All methylations were performed according to Ciucanu and Kerek[29] in a 1 mL V-Vial. The hydrolyzed sample (4 mg) was solved in 350 μL of DMSO and stirred for 2 h at r.t. Then 20 mg of pulverized NaOH was added to form the poly-anions of the oligomeric mixture. After 30 min, 40 μL of MeI-d_3 was added and the solution was stirred for 6 h at r.t. To achieve quantitative methylation, addition of reagents was repeated, and the solution was stirred overnight. Samples were cleaned by extraction with dichloromethane three times. The combined organic layers were washed with saturated NaCl solution, 5% $Na_2S_2O_3$ solution, and water, and subsequently dried over Na_2SO_4. The solvent was evaporated, and the residue was redissolved in MeOH.

Analysis of Enzymes' Selectivity

The enzymatically degraded sample (4 mg) was centrifuged through a 5 kDa Millipore Ultrafree centrifuge filtration tube (Millipore, Bedford, MA). The solvent of the obtained filtrate was evaporated in a stream of nitrogen. The residue was reduced with a solution of 0.5 mL of 0.05 M $NaBD_4$ in 2 M NH_3 for 2 h at 60 °C. The sample cleanup and the acetylation and the perdeuteromethylation of the enzymatically hydrolyzed sample were performed using the same procedure as described above. The deuteromethylated samples were directly submitted to ESI-ITMS. For ESI-MS/MS,

Table 1.
Heterogeneity parameter for MC 1–6 determined as reference data for enzymatic analysis.

	MC 1	MC 2	MC 3	MC 4	MC 5	MC 6
DS	1.32	1.88	1.83	1.83	1.84	1.84
H_1 Spurlin (Monomer)	9.0	4.8	5.4	3.8	2.9	3.5
H_1 Reuben (Monomer)	3.0	3.3	2.9	1.0	0.8	1.3
H_2 (Dimer)	6.2	3.3	2.1	5.7	8.7	6.8
H_3 (Trimer)	9.0	4.3	4.2	7.3	11.8	8.4

$LiClO_4$ was added to the sample solution, and Li adducts fragmented according to ref. [30].

Results and Discussion

Six MCs (DS 1.32–1.88) have been analyzed in this study, the main goal of which was to investigate whether enzymes can be used for determination of heterogeneity of the substituent distribution along the cellulose chain. As a reference information about the samples and the results of their analysis after random acidic degradation are given in Table 1. The H_1 values represent the average deviation of the experimentally determined monomer composition from the calculated data for a random reaction which are based on an ''independence model'' (Spurlin) or a ''dependence model'' (Reuben, reactivity of 3-OH is increased if 2-OCH_3 is present). Our finding that the model of Reuben fits better is in agreement with literature.[31] The H_2 and H_3 values represent the deviation of the experimentally obtained distribution in the dimers and trimers from the calculated random distribution and therefore reflect the heterogeneity of the substituent distribution along the polymer chain. The lower DS sample MC 1 shows a higher heterogeneity. MC 2 and 3 seem to be rather randomly substituted while MC 5 and 6 show a pronounced heterogeneity.

The six MCs were then independently degraded with five different enzymes. Details about the used enzymes are listed in Table 2. Since most of commercially available enzymes are mixtures of enzymes, the activity towards different cellooligosaccharides (DP 3–DP 6) was tested first. If the enzymes hydrolyze an oligomer of DP6 to glucose they clearly contain some exoglucanase and/or β-glucosidase activity. As can be seen in the Table only one batch of the *T. longi 2* and the *BaCel 5A* are pure endoglucanases.

Each enzymatically hydrolyzed sample was submitted to SEC-MALLS/RI to monitor the extent of the hydrolysis and consequently whether MS analysis of oligomeric products allows any conclusion with respect to the entire MC. One can see quite clearly, that the loss in molecular weight for e.g. MC 2 is significant so that mass spectrometry can be used for general data analysis in principle. However, for MC 4 the decrease of molecular weight is rather small, since the raw material already has a rather low M_w. This will be an important

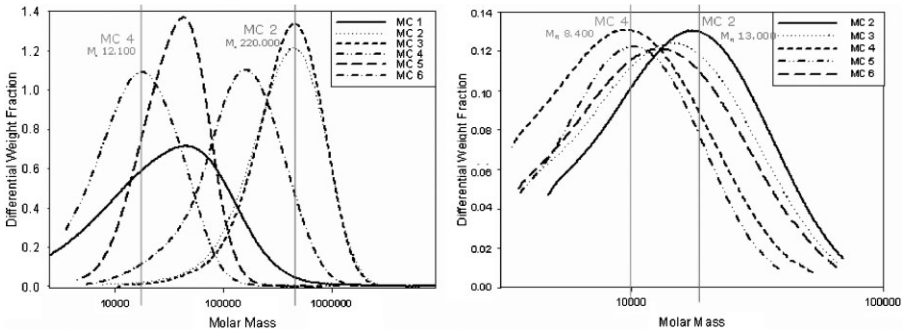

Figure 1.
Molecular weight distribution of the original MCs (left) and the enzymatically degraded MCs (right, only results for hydrolysis with *T. viride* are shown).

Table 2.
Enzymes and their activities towards cellooligosaccharides. (not pure: contains exoglucanases and/or β-glucosidases; pure: only endoglucanases) DP of educts (heading of columns) and products are given.

Educt	DP3	DP4	DP5	DP6	comment
Trichoderma Longibrachiatum 1	DP1	DP1	DP1	DP1	not pure
Trichoderma Longibrachiatum 2	DP1/2	DP1/2	DP1/2	DP1/2	pure
Bacillus agaradhaerens	DP3	DP2	DP2/3	DP2/3	pure
Trichoderma viride	DP1	DP1	DP1	DP1	not pure
Trichoderma reesei	DP1	DP1	DP1	DP1	not pure

point in the data analysis and discussion later on Figure 1.

To obtain information about the enzymes' selectivity and the distribution of the substituents in the oligomeric fractions quantitative analysis of the hydrolysis products after perdeuteromethylation was performed as well. The degradation products were analyzed by means of different mass spectrometric techniques (ESI-IT, ESI-QqQ, MALDI-TOF) and SEC-MALS/RI. The results of this analysis can be seen in Figure 2.

DS values for the non- and deuteromethylated oligosaccharides varied with the strongest deviation for ESI-IT, followed by ESI-QqQ, and nearly none for MALDI-TOF. For the non-deuteromethylated samples bias of relative signal intensities with decreasing DS is observed to a different extent for each technique. Obviously the polarity of the oligomers has a big influence on relative ion yield in electrospray ionization. The deuteromethylated oligomers for all techniques are superposing. The results indicate that in MALDI-TOF the polarity of constituents is of less influence compared to electrospray ionization and/or that mass analysis by time-of-flight does not discriminate ion transfer with respect to their *m/z* value. Thus, perdeuteromethylation is crucial for accurate quantitative analysis of MCs hydrolyzed by enzymes. Otherwise their tolerance with respect to certain methyl patterns can be overestimated. If perdeuteromethylation is not possible, MALDI-TOF is the preferred technique, otherwise all techniques can be used. All data analysis and interpretation in this work have been based on the analysis of

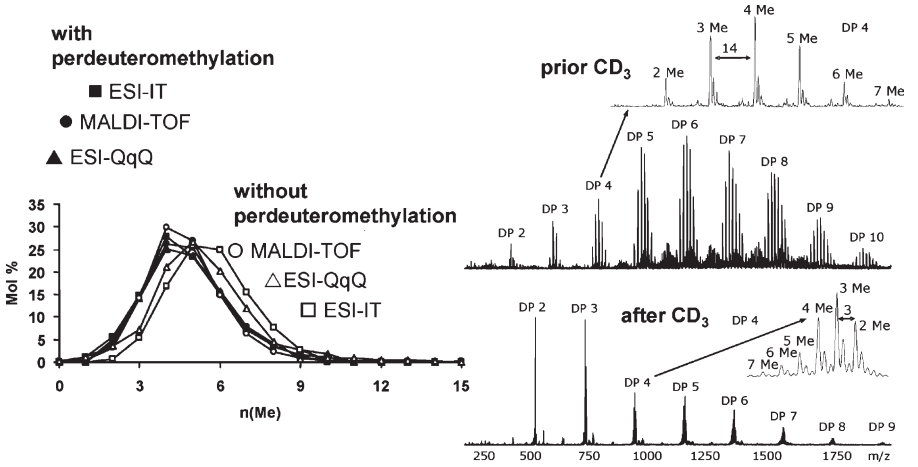

Figure 2.
Comparison of MS analysis of an enzymatically degraded MC (*T. longi 1*) prior to and after perdeuteromethylation submitted to different MS techniques.

perdeuteromethylated samples submitted to ESI-ITMS.

One very important result of enzymatic degradation studies is given by the fact that samples with a minor number of chain cleavages due to a relatively low original molecular weight give a rather unspecific cleavage pattern. This presumably is due to the significant contribution of chain ends. It is understood that the oligosaccharides obtained by only one cleavage are on average higher substituted than oligosaccharides resulting from two specific enzyme cleavages within the chain. These higher substituted oligomers from chain ends effect a bimodal substitution pattern and therefore might cause misinterpretations of the enzymes' specificity and the substituent pattern of the cellulose ether. This effect is shown in more detail in Figure 3, which shows the trimer fraction of MC 4 (starting $M_n = 12.1$ obtained after enzymatic degradation. Methyl groups show a bimodal distribution with one maximum at low content of methyl groups (expected, since the enzymatic cleavage is hindered by too many substituents), and another maximum at a very high content of methyl groups. The latter maximum is superposing to the data obtained after random cleavage. These trimers present the products from less selective cleavages at or near an original chain end.

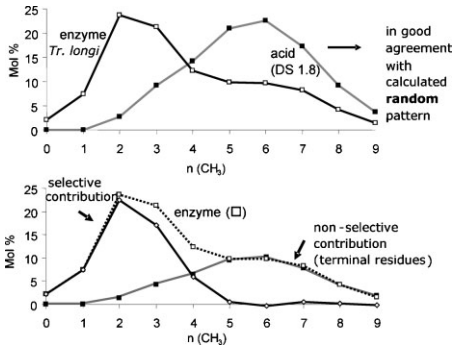

Figure 3.
Top: Methyl pattern in the DP3 fraction of perdeuteromethylated MC4 after random hydrolysis (red) and enzymatic hydrolysis (blue). Bottom: Non-selective cleavages are subtracted from the bimodal pattern to obtain only the selective contribution.

The data obtained needs to be corrected by these non-selective cleavages to get a full understanding about the specificity of the enzyme.

Analysis of the substituent distribution of cellulose ether after enzymatic degradation is a challenging task. As pointed out before, the degradation has to be performed to an extent where a sufficient amount of small oligomeric products are formed so that conclusions can be drawn especially from mass spectrometrical analysis. The strategy of MS analysis of methyl cellulose is based on the interpretation of the DS/DP pattern obtained. Prerequisite for this is to obtain quantitative data from the mass spectrum. As just shown on the example of MC 4, a sufficient high starting molecular weight is required to justify neglecting of products from chain ends.

For the analysis of the substituent distribution along the polymer chain several plots can be analyzed. An overall picture over the results can be obtained by plotting the DS values of the oligomeric products against DP.

Figure 4 shows the DS-values for DP2 - DP8 after enzymatic hydrolysis and perdeuteromethylation of MC 1 and 3 for all enzymes (left), and from *T. viride* and *longi 1* digestion for all MCs (right). One clear trend is the influence of DS on the enzymatic degradation. While for MC 1 the larger oligosaccharides show a higher DS than the average value (MC1: 1.32), no higher values than 1.6 were observed for MC 3 (average value 1.83).

The DS-values for each DP only display the average substitution. The distribution of the substituents in each DP is also available from MS measurements and can be plotted for each DP individually giving a more differentiated picture of enzymatic hydrolysis. This more detailed analysis is shown in Figure 5 for MC 2, 3 and 6 for three different enzymes.

There are no significant differences in the distribution pattern of these MCs after hydrolysis with *T. reesei, T. longi 1*, and *T. longi 2*, therefore only *T. reesei* is included in this plot.

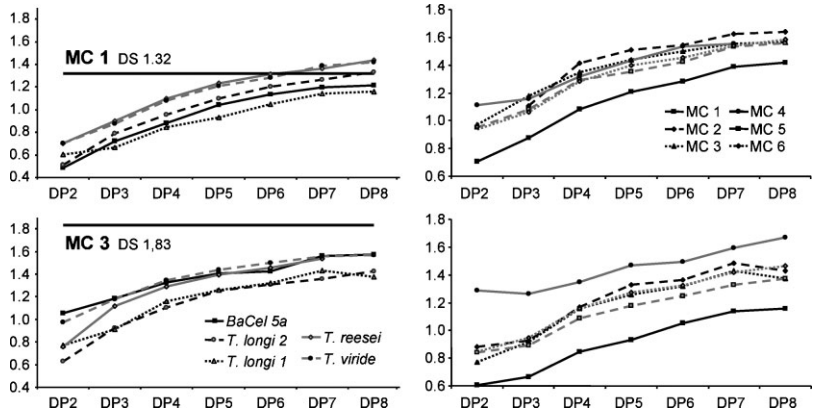

Figure 4.
DS-values *versus* DP calculated for DP2–8 after enzymatic hydrolysis. Left: Results for MC 1 and 3 for all enzymes; Right: Results for all MCs analyzed with *T. viride* (top) and *T. longi. 1* (bottom).

Interestingly, one can see differences in the plots obtained with the other two enzymes *(BaCel 5A* and *T. viride*). The relative intensity patterns for DP 5 of MC 2 and 3 are significantly distorted in favour of the higher methylated constituents (maximum at n(Me) = 7, corresponding to DS 1.4) compared to MC 6 (maximum at n(Me) = 5, corresponding to DS 1.26) after hydrolysis with *BaCel 5A*. The same deviation, but only to a small extent, is visible in the plot of *T. viride*.

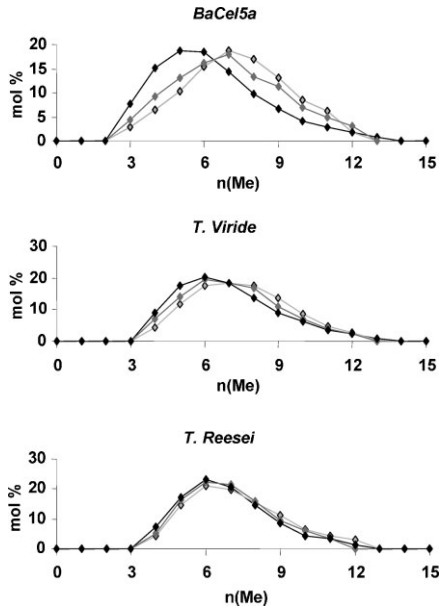

Figure 5.
Distribution of substituents in DP5 fraction obtained after enzymatic hydrolysis with *B. agaradhaerens (BaCel5A), T. viride* and *T. reesei* from MC2 (grey, black frame), MC3 (grey) and MC6 (black).

Before these differences are discussed, one has to look back on the results of the reference analysis of the substituent distribution along the polymer chain after random degradation above. The monomer units with their different methyl patterns are nearly randomly distributed along the polymer chain in MC 2 and 3, only deviating slightly from the calculated pattern, whereas MC 6 as well as MC 4 and 5 show more significant deviations and thus a slightly heterogeneous pattern.

The interpretation of the enzymatic digested MCs allows a similar conclusion, however as pointed out before only for some of the used enzymes and only comparatively within the set of MCs. If more low and high substituted areas are present in the polymer the obtained small oligosaccharides will be lower substituted compared to the randomly substituted polymer since the enzyme will not tolerate the highly substituted polymer regions in its active center.

Comparing now MC 2, 3 and 6 in Figure 5 one can see that MC 2 and 3 shows especially for the *BaCel5A* and (somewhat less pronounced) for the

T. viride more higher substituted pentamers compared to MC 6.

This means that there are less low and high substituted regions in these polymer but more regions with average substitutions. The lower substituted areas as present in MC 6 are degraded to a higher extent and consequently form more oligosaccharides with a lower DS. On the other side, the more densely substituted areas are less accessible to the enzymes, thus reducing the probability that short, more highly methylated oligosaccharides are delivered.

However this interpretation can only be applied if all other relevant parameters like molecular weight distribution, regioselectivity and average DS are similar.

From the results after enzymatic degradation the following conclusions can be drawn: Data for MC 2 and 3 are in better agreement with a random substitution pattern than for MC 6. Only certain enzymes can reflect these differences in their degradation products that are available to MS analysis. One further point that might lead to misinterpretations of enzymatic digests of polysaccharide derivatives has been mentioned in the beginning.

The quantitative contribution of the total amount of oligosaccharides analyzed afterwards in ESI-MS is important to give a representative and diagnostically valuable information for the entire sample. Therefore a new standard addition method using ESI-MS was introduced.[28] By this method it could be shown that degradation of a common MC with DS 1.3 yields approx. 7–15% DP 2 and 3 referred to the original polymer, while digestion of a MC with DS 1.8 degrades about 1–3% of the polymer to DP 2 and 3 oligomers. This indicates as well, that not all samples can be analyzed satisfactorily. The results of the enzymatic degradation depend on original molecular weight and extent of degradation and DS as well as on the enzyme used.

Future work in this area should focus on the analysis of the higher DP oligomers obtained after enzymatic cleavage. On the one hand it would be very interesting to obtain quantitatively the amounts of DP 4–8, which was not possible in this work to get even more information about the extent of the degradation. Data about the high DP fraction that cannot be analyzed directly via mass spectrometry would be of interest since these parts should be enriched on e.g. highly substituted oligomers/polymers, and reflect the assumed higher substituted domains of more heterogeneously methylated samples. In addition, more knowledge on the selectivity of the enzymes is required, since the tolerance against certain methylated positions will significantly influence the product pattern from digestion and might otherwise be misinterpreted with respect to heterogeneity.

Conclusion

The main question addressed by our enzymatic studies was, whether enzymes are capable of showing differences between the substituent patterns of MCs. As reference method random hydrolysis of perdeuteromethylated MCs and monomer analysis by GLC of corresponding alditol acetates have been performed. As a matter of course, a low DS favours degradation with a high yield of oligomers appropriate for MS analysis. At a certain DS, a heterogeneously substituted sample with enlarged low- as well as high-substituted regions will yield an increased amount of low-substituted oligomers compared to a sample with the same DS but a random pattern. A high selectivity of the enzyme is more valuable with respect to interpretation of the product pattern than an ''aggressive'' less selective enzyme. As shown, from all enzymes tested, only *BaCel 5A* and *T. viride* were able to image differences between the samples. Reference data obtained by well established methods for all MCs under investigation were essential for the interpretation of the results from enzymatic degradation. However, enzymes can be very helpful if substituents interfere with a random partial hydrolysis or if the DS is very low, so that product patterns from random cleavages of various samples

look more or less the same while at the same time, a high yield of oligomeric degradation product available to MS studies are obtained.

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