Endoglucanase Degradation and Enzyme-Aided Characterization of Cellulose Acetates

Bodo Saake, Clemens Altaner, Soo-Jung Lee, Jürgen Puls*

Federal Research Centre for Forestry and Forest Products, Institute for Wood Chemistry and Technology of Wood, Leuschnerstr. 91, D-21020 Hamburg, Germany
Fax: (+49) 040 73962 502; E-mail: bsaake@holz.uni-hamburg.de

Summary: Endoglucanases were used as a selective tool to determine the substituent distribution along the chains of partially substituted cellulose acetates. Unsubstituted segments are intensively fragmented while highly substituted segments are only slightly degraded or not affected at all. Two different procedures were developed to perform a preparative separation of the fragments by size exclusion chromatography. One system is based on a direct separation of the fragmentation products using pyridine : water (9 : 1) as an eluent. The isolated fragments can be analysed regarding to their DS values by ATR/FTIR spectroscopy or by hydrolysis and HPLC. The second system is based on deutero-acetylation of endoglucanase fragmented samples. The complete acetylation of all OH groups enables a chromatographic separation in chloroform. Afterwards the isolated fragments can be analysed regarding to their partial DS values by ^{1}H-NMR spectroscopy.

Keywords: cellulose acetate; endoglucanase; FTIR spectroscopy; NMR spectroscopy; size exclusion chromatography; substituent distribution

Introduction

For all cellulose derivatives one of the most challenging problems is the analysis of the substituent distribution along the polymeric chain. To obtain this information two basic approaches have been pursued, a random degradation of polymers, assisted by mathematical models and a selective degradation by enzymes.[1,2] The random approach compares the real substituent distribution of oligomeric fragments after mild degradation of the polysaccharide with a mathematically calculated distribution. From these data the heterogeneity of the substituent distribution along the molecule chain can be calculated. This method was applied for the analysis of methyl cellulose (MC),[3] cellulose silyl ethers,[4] cellulose sulfates (CS),[5] and cellulose acetates (CA) of higher DS values.[6]

The selective approach takes advantage of the sterical hindrance of chain degrading enzymes by the substituents. Endoglucanases (EGs) need at least one unsubstituted anhydroglucose unit (AGU) to cleave a glycosidic bond.[7] Accordingly the enzymatic

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degradation of cellulose derivatives is not applicable for samples with high DS values. For carboxymethyl cellulose (CMC), CA and CS[9,10] the degradation is limited, when the DS value exceeds 1.6, while MC can be degraded effectively even at a DS of 2.1.[8] The degradation products contain information about the substituent distribution along the molecule chain. For CMC and MC methods for preparative separation and characterisation of the fragments were developed and successfully applied to characterise samples from different synthesis concepts.[10-13]

Iijima, Kowsaka and Kamide were the first to perform an enzyme-aided characterisation of two water soluble CA samples (DS 0.6 and 0.88) with an enzyme preparation. The fragments were separated by aqueous size exclusion chromatography (SEC) and further analysed by liquid chromatography with mass spectroscopy and ^{13}C-NMR spectroscopy.[14] Recently cellulose acetates of DS 2.3 were degraded with EGs and the low molar mass fragments were subjected to MALDI analysis after chemical derivatisation.[15] The authors claimed that mono-, di-, and oligosaccharides were liberated by the enzyme and concluded that regions of low- or unsubstituted fragments are existing within the polymeric chain. Two systems for the enzyme-aided analysis of CA were recently developed in our laboratory, both based on a fragmentation of the polymer with EGs. One procedure is based on the direct preparative separation of fragments by SEC using pyridine : water (9 : 1) as an eluent. Afterwards the isolated fragments can be analysed regarding to their DS values by ATR/FTIR spectroscopy or by hydrolysis and HPLC.[16] The second system is based on deuteroacetylation of EG fragmented samples. The complete acetylation of all OH groups enables a SEC separation in chloroform. Afterwards the isolated fragments can be analysed regarding their partial DS values by ^{1}H-NMR spectroscopy.[17] For both analytical systems the separation and analysis is applicable over a wide DS range provided that the CA samples are accessible to enzymatic degradation.

Experimental

Substrates and enzymes. The DS 0.7 CA was a gift of Hoechst-Celanese (N.C.) All other CAs were gifts of Rhodia-Acetow (Germany) and prepared by acid catalysed deacetylation of cellulose tri-acetate. The DS values were determined by ^{13}C-NMR spectroscopy as published previously.[16] The *Aspergillus niger* acetylesterase (AE) was purified from the commercial enzyme preparation Cellulase AP3.[19] EG free of acetyl esterase was obtained

from the *Humicola* strain preparations (Novozymes, Bagsvaerd, Denmark). Enzymatic fragmentation was perfomed by incubating 0.2% CA (w/v) solution/suspension in de-ionised water with 100 nkat of EG per mg of sample for 92 hours at 45 °C.[16]

Carbanilation and analytical size exclusion chromatography. After saponification with ammonia carbanilation was performed according to a previously published procedure.[19] Separation was performed in THF at 40 °C on Chrompack Microgel columns (50, 100, 1000, 10000, and 100000 Å, each 250x7.7 mm) using a flow rate of 1 ml/min. Molar masses were calculated by the "triple detection method" (Shodex RI-71, Japan; Precision Detector PD 2000, MA; Viscotek H502, TX) using the 90° light scattering signal, applying a dn/dc of 0.163 cm^3/g and calculating the angular correction from intrinsic viscosity.[16]

Preparative size exclusion chromatography. Separations were performed on PSS GRAM 10 µm columns (pre-column: 20 x 50 mm; main-columns: GRAM 100 and GRAM 3000, each 20 x 300 mm; PSS, Germany) using an evaporative light scattering PL-ELS 1000 detector (Polymer Laboratories Ltd., UK). The main flow was directed to a fraction collector with a micro splitter valve. In pyridine : water 9 : 1 (v/v) 500 µl of a 1% CA solution were separated at 30 °C with a flow of 2.5 ml/min.[16] The separation of deutero-acetylated samples was performed in freshly distilled chloroform separating 500 µl of a 1% CA solution at 30 °C with a flow of 5 ml/min.[17]

DS determination by micro-hydrolysis procedure. 1 mg of vacuum-dried CA samples were hydrolysed with 20 µl 72% H_2SO_4 for 1 h at 30 °C. After addition of 560 µl H_2O a post-hydrolysis was performed for 40 min at 120 °C. Glucose was determined by anion exchange chromatography considering a loss factor of 0.931. Acetic acid was determined on a Hi-Plex H column (300 x 7.2 mm; PL, UK) using 0.005 M H_2SO_4 as eluent.[16]

DS determination by ATR/FTIR spectroscopy. CA powders were inspected with a Vector 33 FTIR instrument (Bruker, Germany) using the ATR technique. The ratio between the signal areas of the C-O signals from the acetyl substituents (1173.1-1301.6 cm^{-1}) and the anhydroglucose ring (915.5-1137.9) of the CA samples were calculated. Calibration of the FTIR investigations was performed correlating this band ratio of starting materials with DS values from ^{13}C-NMR spectroscopy.

DS determination by deutero-acetylation and ^{1}H-NMR spectroscopy. Deutero-acetylation was performed using 30-50 mg of CA, 3-5 ml of dried pyridine and 620 µl of acetic acid anhydrid–d_6.[20] The partial DS of deutero-acetylated samples was determined in $CDCl_3$ by ^{1}H-NMR. Fractions from preparative separation were analysed at 25 °C using a 400 MHz spectrometer (Varian-Mercury, CA), 5 mm tubes with microcell inserts, a pulse angle of 90° and a relaxation delay of 11 s.[17]

AEC-PAD analysis of oligomeric products. Cellooligomers were analysed by high pH anion exchange chromatography using a Carbo Pac™ PA 100 column (4 x 45 mm) and a gradient of 0.1 M NaOH (A) and 1 M CH_3COONa in 0.1 M NaOH (B) (0 min: 90% A; 5 min: 70% A; 10 min: 70% A; 10.1 min 90% A, 18 min: 90% A).

Endoglucanase Fragmentation of Cellulose Acetates

The enzyme-aided analysis of cellulose derivatives is in general strongly dependent on the DS of the samples. Limitations occur for samples of rather high DS values due to the sterical hindrance for the enzymatic hydrolysis at highly substituted fragments. In earlier studies the EG hydrolysis of CAs derived from acidic saponification of commercial CAs was investigated. For *Humicola insolens* EG from the family Cel7B a hydrolysis up to DS 1.9 could be achieved.[8] While samples of DS 0.9 and 1.2 were fragmented to oligosaccharides the DP_w of CA DS 1.6 was still reduced by a factor of 5.1 (Tab. 1). For samples of DS 1.7 and 1.9 the EG caused a reduction of DP_w by a factor of 1.8 and 1.9. Although these reductions are smaller they still prove a significant effect of the enzyme. For the DS 1.9 sample the high polydispersity index of 11.0 indicates a very heterogenous fragmentation pattern.

In later studies Cel45A, a *Humicola insolens* EG from another family, was tested for a similar but not identical set of CA samples.[16] It turned out that this enzyme could only hydrolyse samples up to DS 1.4 (Tab. 2). Again low molar mass samples were transferred into oligomeric products. Nevertheless, it became apparent that even for samples of low DS the Cel45A always produced larger fragments compared to Cel7B. Of course this comparison has some limitation since the two enzymes were not tested on the same set of samples. Nonetheless, the enzyme Cel 7B is clearly more effective towards CA samples. This was surprising because both enzymes had a similar performance towards carboxymethyl cellulose (CMC) which was reported by Karlson *et al.*[21] and which was

Table 1. Effect of EG hydrolysis with Cel7B on CA samples of varying DS as revealed by SEC of the corresponding carbanilates.[8]

DS	Starting material		EG-fragmented		$\left[\frac{DP_w \text{ before EG}}{DP_w \text{ after EG}}\right]$
	DP_w	M_w / M_n	DP_w	M_w / M_n	
2.9	387	4.1	394	3.3	1.0
2.5	316	3.2	306	3.0	1.0
1.9	189	2.0	100	11.0	1.9
1.7	92	1.7	50	1.2	1.8
1.6	138	1.7	27	5.1	5.1
1.2	85	1.8	5	1.2	17.0
0.9	31	1.5	4	1.1	7.8

Table 2. Effect of EG hydrolysis with Cel45A on CA samples of varying DS as revealed by SEC of the corresponding carbanilates.[16]

DS	Starting material		EG-fragmented		$\left[\frac{DP_w \text{ before EG}}{DP_w \text{ after EG}}\right]$
	DP_w	M_w / M_n	DP_w	M_w / M_n	
2.7	326	2.3	325	2.3	1.0
2.3	305	2.2	300	2.0	1.0
1.8	151	1.6	154	1.5	1.0
1.4	125	1.4	33	1.8	3.8
1.2	82	1.6	29	1.5	2.8
0.9	70	1.6	18	1.9	3.9
0.7	169	1.6	12	1.3	14.1
0.4	34	1.3	8	1.1	4.3

found as well in unpublished studies in our laboratory. A major difference between the two enzymes is the lack of a carbohydrate binding module for Cel7B. It might be that this is an advantage for the hydrolysis of CA which is mainly taking place under heterogeneous reaction conditions due to the limited water solubility of those samples. Both EGs were unable to hydrolyse CA samples with a DS above 2.0. Therefore, it is most likely that samples above those DS are not suitable for an enzyme-aided analysis. However, this conclusion is in contrast to findings of Bashir and co-workers.[15] The authors reported that low substituted fragments were liberated from a CA sample with DS 2.3 by EG treatments with Cel45 and Cel7B, while the enzyme Cel5A was unable to depolymerise the sample. The authors did not perform a direct analysis of the enzymatical fragmented samples regarding their molar mass distribution or oligosaccharide content. Before the analysis of

the sample, the enzymatically degraded samples were freeze-dried followed by derivatisation reactions and extraction of the water soluble parts. These oligosaccharides were subjected to MALDI analysis. The relevance of these fragments for the polymer properties is difficult to judge since their weight percentage was not reported. It might be possible that some low molar mass fragments result from degradation side reactions during the sample preparation. This could be one explanation for the deviation to the work performed by our group. Of course a very heterogeneous substituent distribution of the CA under investigation could be responsible for this finding as well.

Enzyme-Aided Analysis of Cellulose Acetates

The first step of the enzyme-aided analysis was an intensive EG fragmentation of the samples with the EG Cel45A. The enzyme was purified in larger quantities from its residual acetyl esterase activity at the beginning of the project.[16] The previously discussed result that Cel7B is more suitable for CA fragmentation could not be foreseen at this point, since no differences were observed between the two enzymes in the previous work on CMC. This difference does not affect the development of the separation and analysis procedures presented here. However, one should keep in mind that a change of EG can further improve the resolution and extend the application range of the procedures.

SEC Separation in Pyridine : Water

For the direct preparative separation of the fragments a variety of solvents was tested with the starting materials and the EG fragmented samples. Simultaneously solutions were tested for the formation of acetic acid to monitor CA stability under the conditions applied. Pyridine : water in a ratio of 9 : 1 (v/v) was suitable over a wide DS range without cleavage of substituents under the experimental conditions. The presence of water provided a good solubility for low DS fragments. High DS starting materials could be solubilised at least up to DS 2.3 (Fig. 1). Considering that samples of such a DS are not susceptible to EG fragmentation this restriction seemed to be acceptable. Considering previous studies on CMC one could expect that even for very heterogeneously or block-wise substituted samples the fragments should be soluble in the eluent system.[11]

The preparative SEC separation of CAs before EG fragmentation showed beside of the main peak two more peaks eluting in the high molar mass separation range at 20 min and

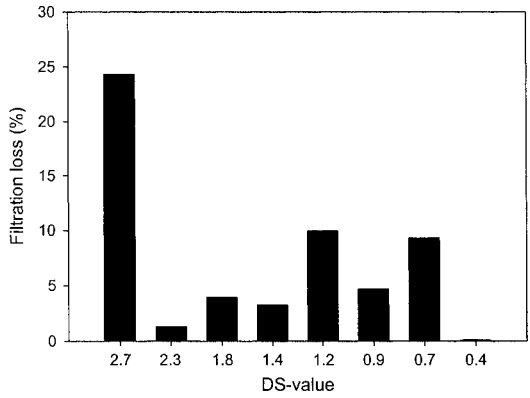

Figure 1. Effect of sample DS on the solubility in pyridine : water (9 : 1) determined by filtration loss on 0.45 μm regenerated cellulose membranes.

30 min. These peaks should be mainly due to aggregated materials and were most pronounced for the DS 0.7 and DS 2.3 samples (Fig. 2A). Similar prehumps were described repeatedly for technical CA DS 2.5 samples in acetone.[22] For the main peaks eluting between 35 and 55 min the sequence of the peak maxima correlated well with the sucession of molar masses determined by analytical SEC. After EG fragmentation the elution profiles were shifted into the elution range of lower molar masses (Fig. 2B). No significant prehumps could be detected for these samples indicating that those materials were fully accessible to the enzyme. The sequence of the peak maxima correlated again with the molar masses determined by analytical SEC. For DS 0.7 and DS 0.9 samples a significant fraction of monomeric and oligomeric degradation products occurred in the chromatograms. The variation of sheer force by higher sample concentration and flow rate did not affect the chromatograms of EG fragmented samples. This supported the assumption that no significant aggregation existed in these samples. The quality of preparative separation was further evaluated by anion exchange chromatography (AEC) of the individual fractions. Due to the alkaline eluent in this system the acetyl groups are saponified and the chain length of the original fragments can be determined. AEC chromatograms of some fractions of the EG fragmented DS 0.7 samples are depicted in Figure 3 as an example. Fragments which were obtained in the SEC after 44-45 min in the acceding first part of the chromatogram showed a broad peak (5-7 min) in the

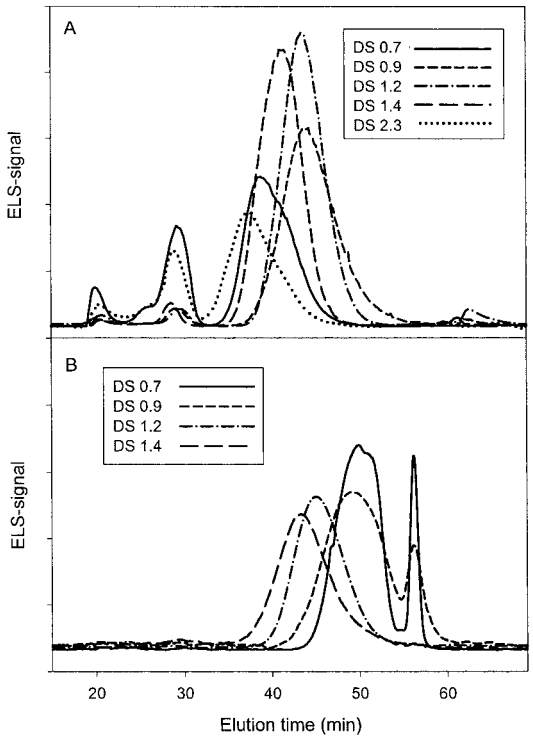

Figure 2. Preparative SEC separation of CAs in pyridine : water (9 : 1) before (A) and after fragmentation (B) with EG Cel45A.

AEC separation (Fig. 3A). This is representative for polymeric fragments. The AEC analysis of products from the peak maximum of SEC separation (48-59min, Fig. 2B) showed oligomeric fragments varying between DP 5 and 13 (Fig. 3B). After 52-53 min a minimum between the main peak and the smaller second peak of the SEC separation was found (Fig. 2B). The AEC separation revealed that these products consisted mainly of fragments with DP 2 to 5 but contained as well some glucose (Fig. 3C). Finally the major part of the low molar mass SEC peak consisted mainly of glucose and cellobiose (results not shown here). The results confirmed that a good separation could be achieved in the preparative SEC. It was somehow surprising that glucose was found among the degradation products since normally it is assumed that EGs are only producing oligomeric but no monomeric products. However Karlsson *et al.*[21] reported that EG Cel7B can cleave cellotriose to cellobiose and glucose. For the Cel 45A EG used in this work we

found also a degradation of low molar mass model compounds to glucose.

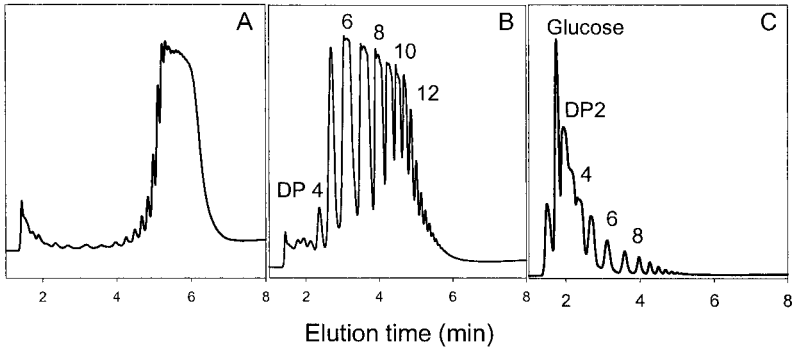

Figure 3. AEC analysis of isolated fractions from the preparative SEC separation of EG fragmented CA DS 0.7. Fractions: A: 44-45 min; B: 48-49 min; C: 52-53 min.

EG Fragmentation and Preparative SEC in Pyridine : Water Followed by DS Determination on Individual Fractions

Various samples in the DS range from 0.4 to 1.4 were analysed regarding their substituent distribution by EG fragmentation, preparative SEC and DS determination on the individual fractions.[16,23] The results obtained for a DS 0.9 and 1.2 sample are depicted in Figure 4. DS values were determined by HPLC after hydrolysis of the material and for the DS 1.2 sample also by ATR/FTIR spectroscopy. The results of both methods agreed very well with each other (Fig. 4B). It became apparent that both methods include specific advantages and disadvantages. The FTIR method was much easier to perform but not suitable for very low molar mass materials due to their syrup-like consistency. Therefore the fractions 28-31 were only analysed by the hydrolysis HPLC method. The substituent distribution of sample DS 0.9 turned out to be slightly more heterogeneous as for sample DS 1.2. The fragments eluting first from the column (fractions 15-20, Fig. 4A) had DS values around 1.1-1.2, which was about 20-33% higher compared to the average DS of the sample. Along the course of the preparative separation the DS values decreased steadily down to 0.6-0.5 for fractions 25-27 (Fig. 4A). A high proportion of the sample were small oligomeric products in fractions 28-31. Here almost no substituents were found (DS 0.06-0.1). The DS distribution of the DS 1.2 sample showed a very homogeneous

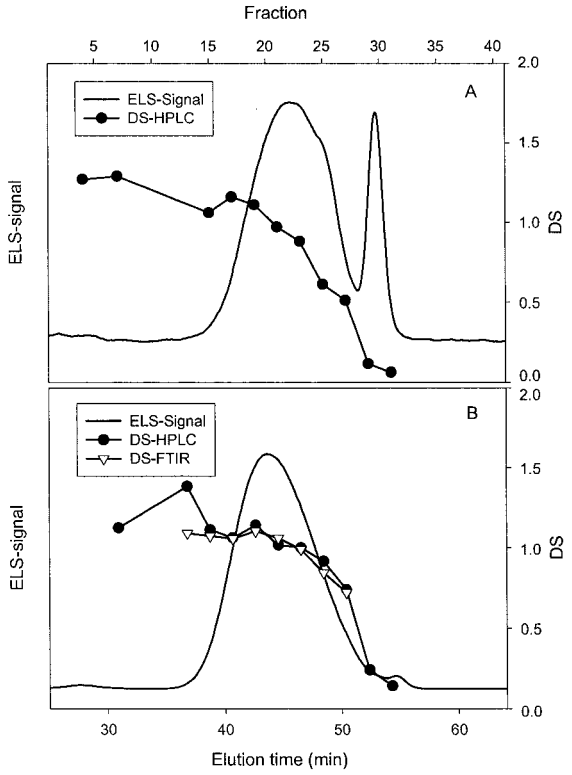

Figure 4. Preparative SEC separation of EG fragmented CA samples DS 0.9 (A) and DS 1.2 (B) in pyridine : water (9 : 1) depicting the DS of individual fractions.

course. For the major part of the polymer eluting in fractions 13-23 (Fig. 4B) DS values between 1.4-1.0 were determined, representing a deviation of only ± 17% based on the average DS of the starting material. In fractions 24-27 a further reduction of the DS down to 0.7 was observed. However, these segments represent only a minimal proportion of the sample. A separated peak of unsubstituted material (fractions 29-31) was observed but was not of practical importance due to its minor amount. Considering the mode of endoglucanase action one can postulate that at least sections with two unsubstituted glucose units were not present in the polysaccharide chain of this sample to any significant extent. This conclusion highlights again the homogenous substituent distribution pattern of the DS 1.2 sample. The material eluting in the prehump range around 30 min was also collected and analysed. Although this material made up only a tiny fraction of the samples

it indicated the existence of some aggregated material. The analysis resulted in a DS of 1.3 for the DS 0.9 sample and 1.2 for the DS 1.2 sample. Accordingly this aggregated material did not deviate by DS from the first polymer fractions of the corresponding samples.

EG Fragmentation, Deutero-Acetylation and Preparative SEC in Chloroform Followed by ^{1}H-NMR Spectroscopy on Individual Fractions

The CA samples with DS 0.9 and 1.2 were also analysed by EG fragmentation followed by deutero-acetylation and ^{1}H-NMR spectroscopy. This procedure assured that all fractions, independent of their original DS, could be solubilised in $CHCl_3$ for the chromatographic separation. In addition the deutero-acetylation enabled a direct determination of the proton bearing acetyl groups by ^{1}H-NMR spectroscopy giving a good resolution of the substituents in positions C-2, C-3, and C-6.[17,20,24] The chromatograms from preparative SEC in $CHCl_3$ are presented in order to depict the total DS and partial DS values obtained by ^{1}H-NMR spectroscopy of the individual fractions (Fig. 5). A slightly more heterogeneous distribution was found for CA DS 0.9 (Fig. 5A) and a more even distribution for the CA DS 1.2 sample (Fig. 5B). These results were in accordance with the

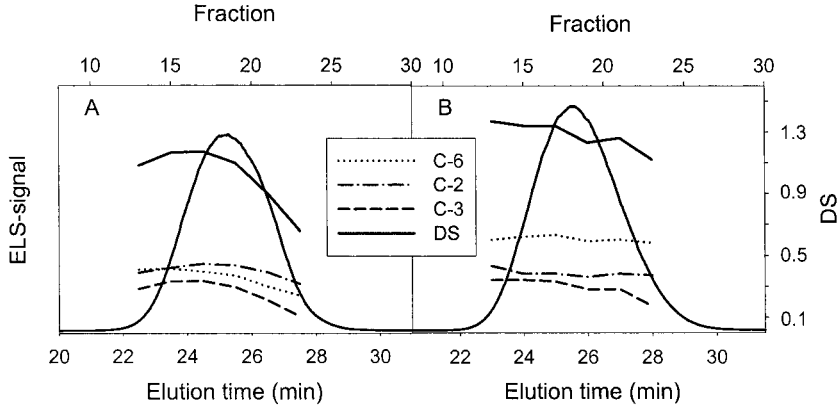

Figure 5. Preparative SEC of EG fragmented and deutero-acetylated CA samples DS 0.9 (A) and DS 1.2 (B) in chloroform depicting the DS and partial DS of individual fractions.

results obtained by the procedure based on preparative separation in pyridine : water. Larger differences between the two samples existed when not only the DS of the

individual fractions but the partial degree of substitution at the different positions (C-2, C-3, C-6) was considered. A clear preference for the C-6 position was determined for the CA with DS 1.2 followed by the substitution in C-2 and C-3. For the DS 0.9 sample the partial substitution in C-2 and C-6 were of similar magnitude with a slight preference for the substitution in position C-2. For the DS 0.9 it should be pointed out that the preparative separation of the deutero-acetylated sample did not characterise the total sample but only its major polymeric components. The mono- and oligosaccharides found in fraction 29-31 of the pyridine : water system (Fig. 4A) are missing after the deutero-acetylation procedure due to sample loss in the refinement step.

Further on the CA samples DS 0.9 and 1.2 were regioselectively modified with an acetyl esterase (AE). The AE is able to selectively remove substituents from the position C-6 resulting in a product with a preferred substitution in C-2 and C-3.[18] These samples were analysed regarding their substituent distribution along the polysaccharide chains to investigate if the AE is acting randomly along the polysaccharide chain. Both samples showed a relatively uniform DS and partial DS distribution within the individual fractions (Fig. 6). This finding demonstrated that neither the formation of block-wise structures within a chain nor the formation of chains with significantly higher or lower substitution occurred. It proved as well that the enzymatic modification was not influenced by differences in sample solubility. The CA DS 1.2 was water insoluble and therefore modified under heterogeneous reaction conditions, while the CA DS 0.9 was almost completely water soluble. Nevertheless, the enzymatic modification of the DS 1.2 sample resulted in an even more homogeneous product. For both incubated samples, the substitution in C-6 was now clearly predominating while C-2 and C-3 were preferentially reduced by the AE enzyme (Fig. 6). In accordance with the higher C-6 substitution of the starting material the modified DS 1.2 sample retained its higher substitution in this position. A comparison of Fig. 5B and Fig. 6B demonstrated that the partial DS C-6 was not changed by the enzymatic reaction and the analytical protocol. Surprisingly after AE modification the substitution in C-2 and C-3 position was lower for the CA DS 1.2 compared to the DS 0.9 sample. This finding indicated that the total DS of the samples was not the limiting factor to hinder the access of AE to the polysacchride chain. Besides its lower DS the DS 0.9 starting material had a higher partial substitution in positions C-2 and C-3. Additionally the distribution of the substituents along the polymer chain was more heterogeneous. It is most likely that the access of AE to AGUs was influenced by the

partial substitution in C-2 and C-3 of the neighbouring units.

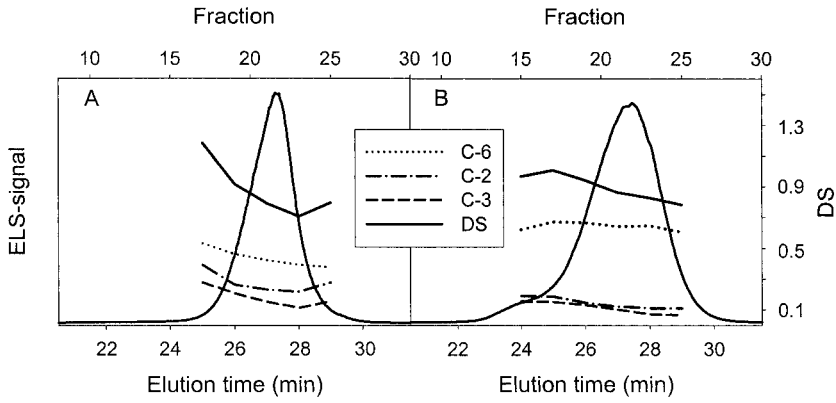

Figure 6. Analysis of regioselectively modified CA samples by EG fragmentation, deutero-acetylation, preparative SEC and ^{1}H-NMR. A: CA DS 0.9 AE modified B: CA DS 1.2 AE modified.

Conclusions

The EG fragmentation of CAs is clearly not only dependent on the sample DS but also on the enzyme family. EG Cel7B could hydrolyse samples up to DS 1.9 while Cel 45A could only access samples up to DS 1.4. This was remarkable, because in previous studies no difference was found regarding the activity of those enzymes towards CMC. It is not unlikely that other EGs might have even higher activities towards CA. Nevertheless, for commercial cellulose-2,5-acetates and tri-acetates it is most unlikely that the EG fragmentation can make a significant contribution to the analysis of the substitutent distribution along the polysaccharide chain. This conclusion is somehow in contrast to observation reported by Bashir *et al.*[15] and deserves further attention in future studies.

The investigation of CA DS 0.9 and CA DS 1.2 revealed analogous results for the two analytical systems developed. In both procedures it turned out that the DS 1.2 sample had a slightly more homogenous distribution. A comparison of the two analytical systems showed that the direct separation of EG fragmentation products in pyridine : water is easier to perform. However, the individual fractions can only be characterised regarding to their DS value. This can be pursuit either by ATR/FTIR or by hydrolysis and HPLC. The method based on deutero-acetylation, SEC in chloroform and ^{1}H-NMR of the individual fractions is more time consuming. In addition possible losses of mono- and dimeric

products in the refinement procedure have to be avoided or should be monitored. An advantage of this procedure is the additional information on the partial degree of substitution for the isolated fragments. This could be demonstrated by the analysis of the CA samples modified by an AE. The enzyme-aided analysis revealed that the esterase modification occurs evenly along the polysaccharide chain. The substitution in position C-6 was not effected by the esterase, while C-2 and C-3 were regioselectively cleaved to a major extend. By comparison of the two samples it became evident as well that a high substitution in position C-2 seems to limit the access of the esterase to the polymer. Although the EG fragmentation might be limited for commercial samples, a greater potential of enzyme-aided analysis exists for new products with lower DS, and samples with block-wise or regioselective substitution pattern.

For the enzymatic fragmentation of CA one has to consider that acetyl groups are easily cleaved off by AE.[8] These enzymes are present in many cellulolytic enzyme preparations which therefore are not suitable for the determination of the substituent distribution of CA without further purification.[25]

[1] P. Mischnick, *J. Carbohydr. Chem.* **1991**, *10*, 711.
[2] P. Mischnick, *Cellulose* **2001**, *8*, 245.
[3] P.W. Arisz., H.J.J. Kauw , J.J. Boon, *Carbohydr. Res.* **1995**, *271*, 1.
[4] P. Mischnick, J. Heinrich, M. Gohdes, O. Wilke, N. Rogmann, *Macromol. Chem. Phys.* **2000**, *201*, 1985.
[5] M. Gohdes, P. Mischnick, *Carbohydr. Res.* **1998**, *309*, 109.
[6] J. Heinrich, P. Mischnick, *J. Polym. Sci., Part A: Polym. Chem.* **1999**, *37*, 3011.
[7] M. G. Wirick, *J. Polym. Sci., Part A-1* **1968**, *6*, 1965.
[8] B. Saake, S. Horner, J. Puls, in: " Cellulose Derivatives. Modification, Characterization and Nano-structures", Th. Heinze, W.G. Glasser, Eds., ACS Symp. Ser. 688, American Chemical Society, Washington D.C. **1998**, p. 201ff.
[9] B. Saake, J. Puls, W. Wagenknecht, *Carbohydr. Polym.* **2002**, *48*, 7.
[10] S. Horner, J. Puls, B. Saake, E.-A. Klohr, H. Thielking, *Carbohydr. Polym.* **1999**, *40*, 1.
[11] B. Saake, S. Horner, T. Kruse, J. Puls, T. Liebert, T. Heinze, *Macromol. Chem. Phys.* **2000**, *201*, 1996.
[12] U. Heinze, J. Schaller, T. Heinze, S. Horner, B. Saake, J. Puls, *Cellulose* **2000**, *7*, 161.
[13] B. Saake, S. Lebioda, J. Puls, *Holzforschung* **2004**, *58*, 97.
[14] H. Iijima, K. Kowsaka, K. Kamide, *Polym. J. (Tokyo)* **1992**, *24*, 1077.
[15] S. Bashir, P. Critchley, P. J. Derrick, *Cellulose* **2004**, *8*, 81.
[16] S.-J. Lee, C. Altaner, J. Puls, B. Saake, *Carbohydr. Polym.* **2003**, *54*, 353.
[17] C. Altaner, J. Puls, B. Saake, *Cellulose* **2003**, *10*, 391.
[18] C. Altaner, B. Saake, J. Puls, *Cellulose* **2003**, *10*, 85.
[19] B. Saake, R. Patt, J. Puls, K. J. Linow, B. Philipp, *Makromol. Chem. – Macromol. Symp.* **1992**, 61, 219.
[20] C. Deus, H. Friebolin, E. Siefert, *Makromol. Chem.* **1991**, *192*, 75.
[21] J. Karlsson, D. Momcilovic, B. Wittgren, M. Schülein, F. Tjerneld, G. Brinkmalm, *Biopolymers* **2002**, *63*, 32.
[22] Y. Funaki, K. Ueda, S. Saka, S. Soejima, *J. Appl. Polym. Sci.* **1993**, *48*, 419.
[23] S.-J. Lee, Diploma theses, University of Hamburg 2002, pp. 79.
[24] V. W. Goodlett, J. T. Dougherty, H. W. Patton, *J. Polym. Sci., Part A* **1971**, *9*, 155.
[25] C. Altaner, B. Saake, J. Puls, *Cellulose* **2001**, *8*, 259.