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Determination of the substituent distribution along cellulose acetate chains as revealed by enzymatic and chemical methods

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Abstract

Eight cellulose acetate samples with degree of substitution (DS) in the range from 0.4 to 2.7 were characterised regarding their DS, substituent distribution in the anhydroglucose units, molar mass distribution and intrinsic viscosity. Samples were intensively hydrolyzed with a purified endoglucanase and the degradation monitored by analytical size exclusion chromatography (SEC). A preparative SEC system using pyridine:water was developed, suitable for the analysis of samples over a wide range of DS. Two methods were established to determine the DS in milligram scale for further analysis of isolated fragments. For cellulose acetate samples with DS 0.4, 0.7 and 1.2 endoglucanase fragmentation followed by preparative chromatography and analysis of isolated fragments was performed. The procedure was suitable to determine differences in the substituent distribution along the polymeric chain of cellulose acetates susceptible to endoglucanase hydrolysis.

Keywords: Cellulose acetate; Substituent distribution; Endoglucanase; Size exclusion chromatography; FT-IR-spectroscopy

1. Introduction

The physical and chemical properties of polysaccharide derivatives are influenced not only by their molecule size and type of substituents but also by the amount and distribution of the substituents within the monomer units as well as along the molecule backbone (Kamide & Saito, 1994). While the degree of substitution (DS) is determined fairly easily by various methods, the substituent distribution within the monomeric units and especially along the polysaccharide chain is more difficult to assess. To obtain information on the substituent distribution along the polymer chain two basic approaches have been pursued, a random degradation of polymers, assisted by mathematical models and a selective degradation by enzymes (Mischnick, 2001). 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. Requirements are the statistical degradation of the polymer and analytical methods, suitable for the analysis of degradation products (Arisz, Kauw, & Boon, 1995). This method was applied for the analysis of cellulose ethers, e.g. methyl cellulose (MC) (Arisz et al., 1995) and cellulose silyl ethers (Mischnick, Heinrich, Gohdes, Wilke, & Rogmann, 2000). Within the group of cellulose esters cellulose sulfates (CS) (Gohdes & Mischnick, 1998) and cellulose acetates (CA) of higher DS values (Heinrich & Mischnick, 1999) have been investigated.

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 (Wirick, 1968). Accordingly the enzymatic degradation of cellulose derivatives is not applicable for samples with high DS values. For CMC, CA and CS (Saake, Horner, & Puls, 1996; Saake, Puls, & Wagenknecht, 2002) the degradation is limited, when the DS value exceeds 1.6, while MC can be degraded effectively even at a DS of 2.1 (Saake et al., 1996). The degradation products yield information about the substituent distribution along the molecule chain. For CMC a method for preparative separation and characterisation of the fragments was developed and successfully applied to characterise samples from different synthesis

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concepts (Heinze et al., 2000; Horner, Puls, Saake, Klohr, & Thielking 1999; Saake et al., 2000).

Iijima, Kowsaka, and Kamide (1992) were the first to perform enzyme-aided characterisation of CA. The authors fragmented two water-soluble CA samples (DS 0.6 and 0.88) with an enzyme preparation and separated the fragments by aqueous size exclusion chromatography (SEC). The fragments were further analysed by analytical SEC, liquid chromatography with mass spectroscopy and ^{13}C NMR-spectroscopy. These methods can provide detailed information. However, the necessary sample amount and instrument time restricted this procedure to a small number of samples and accordingly to an application for fundamental research only.

Compared with many other cellulose derivatives the analysis of CA is accompanied by various problems. First the acetate groups are quite sensitive to harsh reaction conditions both, in alkaline and acidic media. Prior to chemical degradation the methylation of free hydroxylic groups is the preferred method to create an inverse picture with stable ether substituents. In this context mild methylation using methyl triflate (Prehm, 1980) is superior to various other methods (Mischnick, 1991). Complete permethylation of all hydroxyl groups could be achieved for CA of DS around 2.5. However, even in various modifications, with this procedure complete methylation was not achieved for CA samples of lower DS, which limits the application of this procedure (Heinrich, 1999). Accordingly, it could be assumed that the enzymatic approach might be more suitable for samples of lower DS up to 1.6, while the chemical degradation is more effective for higher DS ranges. For enzymatic fragmentation of CA one has to consider that acetyl groups are as well easily cleaved off by acetyl esterases (Saake et al., 1996). These enzymes are present in many cellulolytic enzyme preparations (Altaner, Saake, & Puls, 2001), which therefore are not suitable for the determination of the substituent distribution of CA.

A further feature of CA is the dependence of solubility on the DS. Accordingly an aqueous SEC system, as applied by Iijima et al. (1992), is only suitable for a narrow DS range. After fragmentation, especially of heterogeneously substituted samples, one can expect fragments of different substitution and solubility. In the present paper an analytical system is introduced to extend the analytical separation to a broader DS range. Additionally a methodology for the analysis of fragments is introduced, which is applicable to small sample amounts. This should enable the application of the analytical procedure in a more routine manner.

2. Materials and methods

2.1. Cellulose acetates

The DS 0.7 CA was a gift of Hoechst-Celanese (NC) produced in a homogeneous synthesis procedure. All other CAs (DS 0.4 and DS 0.9 to DS 2.6) were gifts of Rhodia-Acetow (Germany) and prepared by acid catalysed deacetylation of cellulose triacetate.

2.2. Enzymatic methods

2.2.1. Enzyme preparation

The endoglucanase (EG) preparation Novozym 476 from a *Humicola* strain, obtained from Novozymes (Denmark) was used as enzyme source. In order to receive an EG free of acetyl esterase activity the protein of Novozym 476 was precipitated by addition of $(NH_4)_2SO_4$ (100% satn.). The precipitate was resuspended after centrifugation in dest. H_2O, desalted on a Bio-Gel P-2 column (Bio-Rad Laboratories, California) and freeze-dried. This treatment was sufficient to remove all traces of acetyl esterase activity.

2.2.2. Endoglucanase activity

The enzyme solution (200 μl) was incubated with 1.8 ml of a 1% solution of CMC (Fluka 21900, Switzerland) at pH 5 in a 0.05 M sodium acetate buffer at 37 °C for 5 min. The enzyme activity was calculated from the increase of reducing end groups, as measured by the DNS-reagent (Hostettler, Borel, & Deuel, 1951).

2.2.3. Enzyme-aided fragmentation

Solutions of 0.2% CA (w/v) in de-ionised water were incubated with 100 nkat of EG per mg of sample for 92 h at 45 °C. No buffers were used in order to prevent salt impurities, which would impede carbanilation or SEC separation experiments. The reaction was stopped after 92 h by boiling for 10 min to inactivate the enzyme.

2.3. Carbanilation and analytical size exclusion chromatography

The CA samples were saponified with 20% ammonia (4 h, 30 °C). The ammonia was removed by repeated water addition and roto-evaporation followed by freeze-drying of the samples. Carbanilation was performed according to a previously published procedure (Saake, Patt, Puls, Linow, & Philipp, 1992).

Separation was performed in THF injecting 100 μl of 0.1% solutions onto thermostatically controlled columns (40 °C; Chrompack Microgel 50, 100, 1000, 10,000, and 100,000 Å, each 250 × 7.7 mm). The flow rate was 1 ml/min resulting in a pressure of 0.84 Kpsi. A multi-detected system with refractive index (Shodex RI-71, Japan), light scattering (Precision Detector PD 2000, MA), and viscosity detector (Viscotek H502, TX) was used. Data collection and calculation was performed using the PSS 6.0 software (PSS, Germany). Molar masses were calculated by the 'triple detection method' using the 90° light scattering signal and calculating the angular correction from intrinsic viscosity by an iterative process applying a dn/dc of 0.163 cm^3/g.

2.4. Preparative size exclusion chromatography

Five-hundred microlitres of a 1% CA solution were separated at 30 °C with a flow of 2.5 ml/min on PSS GRAM 10 μm columns (pre-column: 20 × 50 mm; main-columns: GRAM 100 and GRAM 3000, each 20 × 300 mm; PSS, Germany). Pyridin:water 9:1 (v/v) was used as eluent. Samples were dissolved overnight on a laboratory shaker at room temperature and filtered (0.45 μm regenerated cellulose) prior to analysis. The elution profile was followed using an evaporative light scattering PL-ELS 1000 detector (Polymer Laboratories Ltd, UK), after the main flow was directed to a fraction collector with a micro splitter valve. The fractionation was performed at least five times to obtain sufficient CA material within the individual fractions. The CA was then recovered by roto-evaporation of the individual fractions. Samples were dissolved or suspended in distilled water and freeze-dried.

2.5. DS determination

2.5.1. IGATED ^{13}C NMR-spectroscopy

A 400 MHz spectrometer (Varian-Mercury) with a 5 mm probe was used at 60 °C.

Quantitative ^{13}C NMR-spectroscopy of the starting materials was performed under inverse gated decoupling (IGATED) with a puls angle of 45° and a relaxation delay of 4 s. Samples were analysed at a concentration of approximately 10% in DMSO-d_6. The central DMSO-d_6 signal at 39.43 ppm was used as reference. The DS values determined by ^{13}C NMR-spectroscopy are used as sample names in the course of this study.

2.5.2. Hydrolysis and HPLC

The hydrolysis method was developed by investigating various hydrolysis times on the starting materials with DS 0.7, 0.9, 2.3, and 2.7. A post-hydrolysis time of 40 min yielded maximum glucose recoveries without any cellobiose serving as indicator for incomplete hydrolysis. The method was then subsequently adapted to the following micro-hydrolysis procedure suitable for sample sizes of 1 mg CA.

Vacuum-dried solid 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. The DS was calculated from the amount of glucose and acetic acid in this solution, considering a glucose loss factor of 0.931, which was determined experimentally for these reaction conditions.

Glucose was determined by borate complex anion exchange chromatography as reported earlier (Puls, 1993). Acetic acid was determined by HPLC on a Biotek system with a HPLC 540 Diode array detector (detection: 205 nm) and KromaSystem 2000 software (Biotek, Germany). Separation was performed on a PL Hi-Plex H column (300 × 7.2 mm; PL, UK) using 0.005 M H_2SO_4 (0.6 ml/min) as eluent.

2.5.3. FT-IR-spectroscopy

CA powders were inspected with a Vector 33 FT-IR 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 FT-IR investigations was performed correlating this band ratio of starting materials with DS-values from ^{13}C NMR-spectroscopy. After measurements of CA samples derived from preparative SEC the material could be recovered from the ATR diamond and used for micro-hydrolysis experiments.

2.6. AEC-PAD analysis of oligomeric products

Cellooligomers were analysed by high pH anion exchange chromatography with pulsed amperometric detection (AEC-PAD) using a Dionex System, a Carbo Pac™ 100 column (4 × 45 mm) and Chromelion software (Dionex, CA). A gradient of 0.1 M NaOH (A) and 1 M CH_3COONa in 0.1 M NaOH (B) was applied as follows: 0 min: 90% A; 5 min: 70% A; 10 min: 70% A; 10.1 min 90% A, 18 min: 90% A (Puls & Dokk Glawischnig, 1997).

3. Results and discussion

3.1. Characterisation of polymeric substrates

The CA were analysed regarding their partial substitution by IGATED ^{13}C NMR-spectroscopy (Table 1). The partial substitution was calculated from the carbonyl signals according to the assignment by Buchanan, Edgar, Hyatt, and Wilson (1991) and Kowsaka, Okajima, and Kamide (1988). The analysis revealed that the saponification of substituents can result in different substitution patterns. Within the group of high DS CAs the DS 2.7 sample had highest substitution at C_3 position ($C_3 > C_6 \sim C_2$). For

Table 1
Degree of substitution and substitution pattern of cellulose acetate as determined by IGATED ^{13}C NMR-spectroscopy

Sample DS	Pattern of substitution			
	C_2	C_3	C_6	
2.7	0.87	0.93	0.89	$C_3 > C_6 \sim C_2$
2.3	0.66	0.84	0.81	$C_3 \sim C_6 > C_2$
1.8	0.59	0.59	0.59	$C_2 = C_3 = C_6$
1.4	0.49	0.42	0.45	$C_2 > C_6 > C_3$
1.2	0.60	0.31	0.29	$C_2 > C_3 \sim C_6$
0.9	0.26	0.28	0.32	$C_6 > C_3 \sim C_2$
0.7	0.15	0.31	0.23	$C_3 > C_6 > C_2$
0.4	0.11	0.11	0.22	$C_6 > C_3 = C_2$

the DS 2.3 sample the substitution in C_3 and C_6 position were approaching equal proportions ($C_3 \sim C_6 > C_2$), while for the DS 1.8 sample the substitution in all three positions was identical ($C_2 = C_3 = C_6$). For the DS 1.2 and DS 1.4 samples C_2 was the dominating position. While C_6 was the second recurrent position for CA DS 1.4 ($C_2 > C_6 > C_3$), C_3 and C_6 were of similar magnitude in sample DS 1.2 ($C_2 > C_3 \sim C_6$). For samples with DS 0.9 ($C_6 > C_3 \sim C_2$) and DS 0.4 ($C_6 > C_3 = C_2$) the C_6 position was predominant. The sample DS 0.7 excelled by the pattern $C_3 > C_6 > C_2$. Therefore it was the only low DS sample with a predominance of the C_3 position. This sample was obtained from a homogeneous derivatisation procedure, while all other samples were derived from controlled saponification of cellulose triacetate.

The results show clear differences regarding the substitution pattern, which should have a certain influence on EG hydrolysis. Nevertheless, all samples were substituted at all three position and the differences described cannot be considered as a regioselective modification. Accordingly the action of endoglucanse should mainly be dependent on the DS and not on the substitution pattern within this set of samples.

3.2. Effect of the degree of substitution on the endoglucanase fragmentation of cellulose acetate

All CA samples were subjected to EG fragmentation in an aqueous system. CA is soluble in water only in a narrow DS range (around 0.6–0.9) depending on the substitution pattern. Therefore the enzymatic hydrolysis took place under heterogeneous conditions for most of the CA samples. The intensity of fragmentation was monitored by analytical SEC. To overcome the effect of different DS values on solubility all samples were saponified under mild conditions, subjected to carbanilation, and analysed in THF.

The highest molar mass and intrinsic viscosity was found for the starting material with a DS value of 2.7 while for lower DS samples a continuous reduction of molar mass occurred. This can be explained by the mode of preparation (acid saponification of cellulose triacetate). Under these conditions not only a cleavage of ester groups, but as well a depolymerisation of the polysaccharide chain occurs. Accordingly the DP_w was reduced from 326 (DS 2.7) to 34 (DS 0.4). Only the sample with DS 0.7, which already excelled by an unusual substitution pattern, was deviating to this tendency (Table 2). Considering the low DS this sample had a comparatively high DP_w of 169 due to its manufacture by the direct homogeneous synthesis method.

The EG hydrolysis was clearly dependent on the DS of the samples. While CA within the DS range from 0.4 to 1.4 were intensively EG fragmented, samples of DS 1.9 and higher were not affected by this treatment.

Table 2
Effect of endoglucanase fragmentation on intrinsic viscosity, molar mass, and DP_w of cellulose acetates with different degree of substitution

Sample DS_{NMR}	Before EG-fragmentation			EG-fragmented material		
	$[\eta]$ (ml/g)	M_w (g/mol)	DP_w	$[\eta]$ (ml/g)	M_w (g/mol)	DP_w
2.7	107	169,000	326	116	168,500	325
2.3	93	158,000	305	102	155,800	300
1.8	57	78,500	151	59	79,900	154
1.4	50	65,100	125	19	17,000	33
1.2	34	42,400	82	13	15,000	29
0.9	24	36,400	70	7	9000	18
0.7	62	87,500	169	5	6000	12
0.4	15	17,600	34	4	4130	8

The result obtained for DS 1.9 was somehow surprising, since in previous CA investigations a sample of DS 1.9 was degraded by EG, although to a small extent (Saake et al., 1996). This deviation between the two samples might be explained by the fact that the fragmentability of cellulose derivatives is dependent on the substituent distribution along the polymeric chain (Saake et al., 2000, 2002). For high DS samples a more homogeneous distribution may limit the susceptibility towards endoglucanase action.

3.3. FT-IR/ATR-spectroscopy of cellulose acetates

All starting materials were investigated by FT-IR-spectroscopy using the ATR technique. The intensity ratio of different spectral bands representative for the anhydroglucose rings and the acetyl side groups were correlated with the DS values determined by ^{13}C NMR-spectroscopy. Best results were obtained using the C–O acetyl stretching (1173–1302 cm^{-1}) divided by the C–O skeletal stretching (916–1138 cm^{-1}, Fig. 1a). The integration was performed by drawing the baseline between the minima of the neighbouring bands. A high correlation coefficient of 0.993 was obtained for a polynomial fit of third order (Fig. 1b). It is noteworthy that Harada et al. (1992) who have used FT-IR/ATR-spectroscopy for depth profiling in CA films chose the C=O ester stretching signal at 1735 cm^{-1} for the quantitative determination of the acetyl groups. However, using intensity ratios of different bands for the FT-IR/ATR analysis of powders a better correlation could be expected for bands that are close to each other, since the band intensity is changing drastically over the spectral regions with this method. This might be considered as a disadvantage compared with the KBr technique but for this study the ATR system proved to be superior. It could be better adapted to smaller sample quantities, since no grinding procedures had to be applied for pellet preparation and samples were even available for further experimentation.

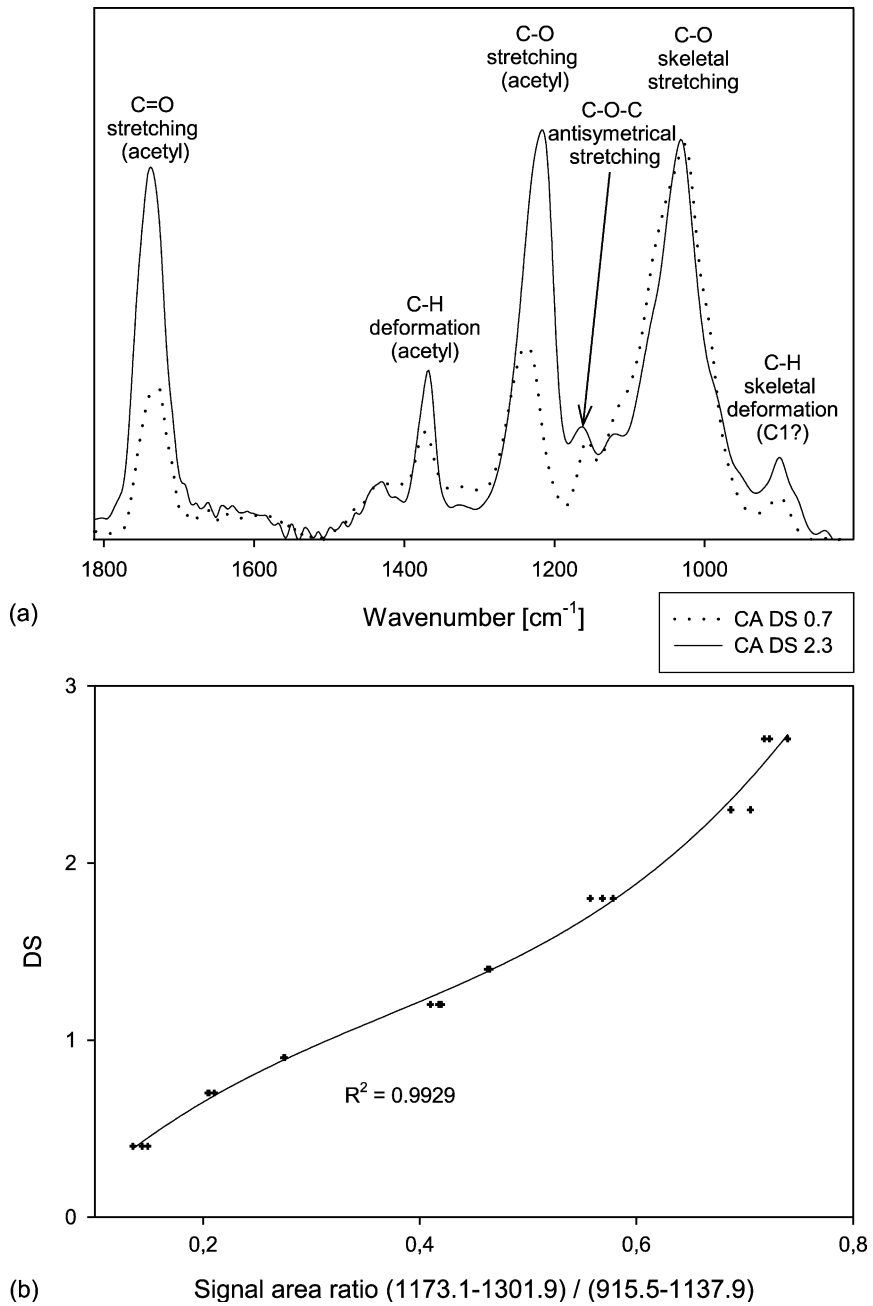

Fig. 1. Annotation of bands for FT-IR/ATR spectra of selected cellulose acetates (a) and calibration curve obtained by correlating the quotient of C–O acetyl stretching and C–O skeletal stretching with the DS values determined by ^{13}C NMR-spectroscopy (b). Assignment according to O'Connor (1971).

3.4. Preparative separation and analysis of the resulting fragments

3.4.1. Substituent distribution of the individual fractions

For the preparative separation several eluent systems were 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. It turned out that pyridin: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 in the eluent provided a good solubility of low DS fragments. High DS starting materials could be solubilized at least up to DS 2.3 (results not shown). Considering that samples of such a DS are not susceptible to EG fragmentation this restriction seemed to be acceptable for the analytical approach. Considering previous studies on carboxymethyl cellulose (Saake et al., 2000) one could expect that even for very heterogeneously or block-wise substituted samples the fragments should be soluble in the eluent system.

CA samples with DS 0.4, 0.7, and 1.2 were subjected to EG fragmentation and separated by preparative SEC. The effect of the DS on the intensity of the fragmentation was

apparent from the preparative chromatograms (Fig. 2a–c) analogously to the analytical SEC of the corresponding carbanilated sample (Table 2). This congruence between both SEC systems confirms that the preparative separation is performed according to SEC principles. It should be mentioned that small but well-defined pre-humps in the chromatograms of the starting materials indicated a certain amount of aggregation (results not shown). This effect is diminishing after EG fragmentation, showing only a slight distortion of the baseline around 25–30 ml elution volume for samples DS 0.7 and 1.2 (Fig. 2b and c). Accordingly endoglucanase hydrolysis improved the solubility of cellulose derivatives as it was already described for MC (Saake et al., 1996) and CS (Saake et al., 2002).

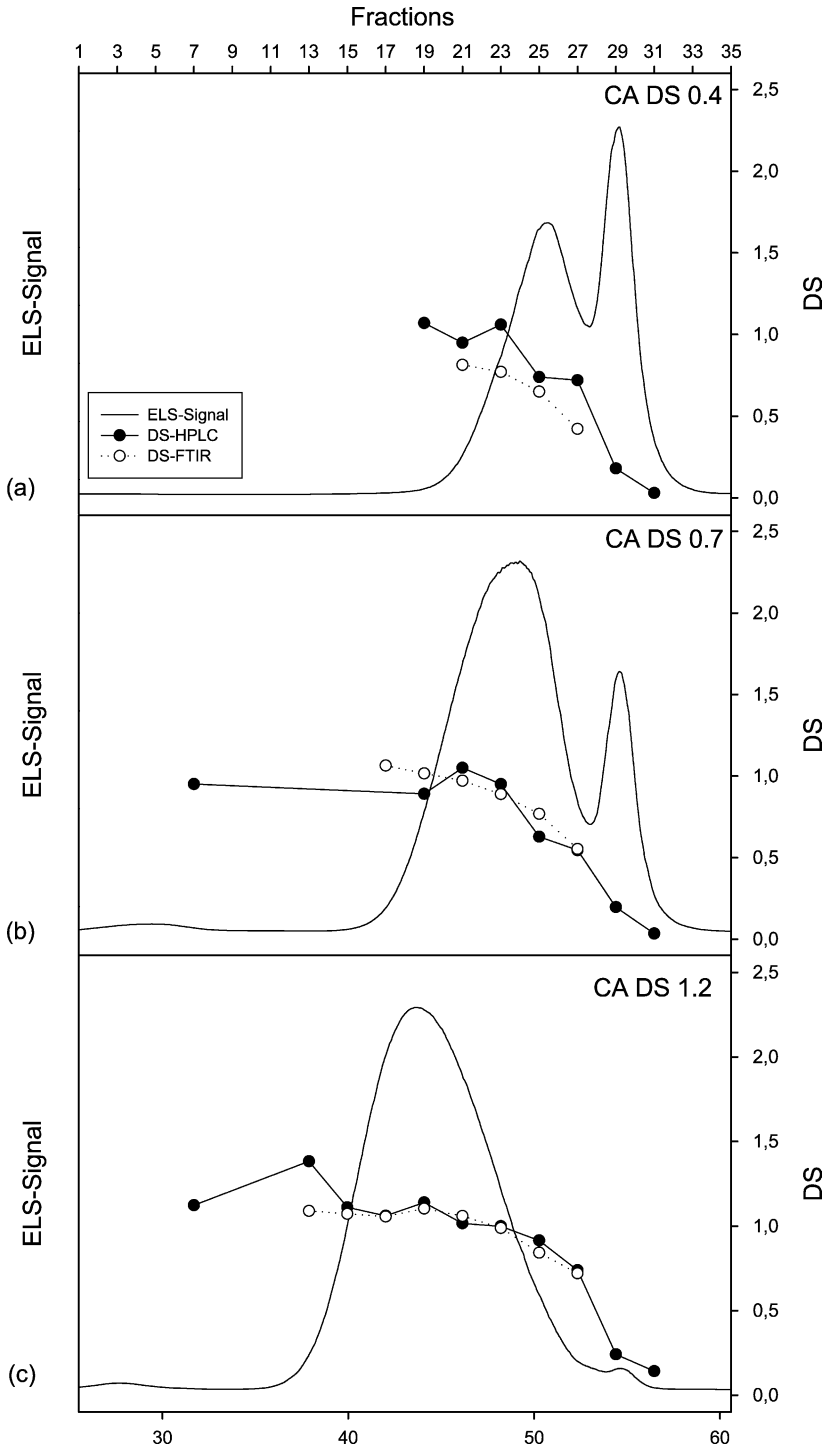

Fig. 2. Preparative SEC separation of EG fragmented CA samples depicting the degree of substitution (DS) of individual fractions determined by FT-IR/ATR (DS-FT-IR) and hydrolysis HPLC (DS-HPLC) determination. (a) CA DS 0.4; (b) CA DS 0.7; (c) CA DS 1.2.

The DS values of individual fractions were determined by FT-IR/ATR-spectroscopy and by HPLC of the hydrolysed material. The DS values obtained by both methods were plotted in Fig. 2a–c referring to the right *y*-axis. Both methods showed the same tendency, following the elution profile along the preparative SEC separation. Only small differences between DS values for individual fractions were observed. In addition it became apparent that both methods include specific advantages and disadvantages. The FT-IR method was much easier to perform and less time consuming. However, this method was 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 and not by FT-IR/ATR.

Regarding the substituent distribution the most heterogeneous pattern was obtained for sample DS 0.4. The fragments eluting first from the column (fractions 17–21, Fig. 2a) had DS values around 0.8–1.0, which was at least twice as high as the average DS of the sample. Along the course of the preparative separation the DS values decreased steadily down to 0.4–0.7 for fractions 25–27 (Fig. 2a). A high proportion of the sample were small oligomeric products in fractions 28–31. Here almost no substituents were found. Accordingly the acidic saponification to the very low DS 0.4 yielded a product which had large segments of the polymer bearing roughly 150–250% of the starting material's average DS. From the concentration detector signal the proportion of these fragments amounted to 63% of the total sample. The remaining polymer was made up by segments, which were basically un-substituted, resulting in intensive enzymatic hydrolysis.

The high molar mass fractions from preparative SEC of the DS 0.7 sample excelled by DS values around 0.9–1.1 (fractions 17–24, Fig. 2b), which was higher than average. Only a slight decrease of the DS could be observed in this range. For fractions of lower molar mass a significant decrease of DS occurred down to about DS 0.5 for fraction 28. Again the final oligomeric fractions 29–31 were made up mainly by unsubstituted material. Evaluating the intensity of the concentration signal this sample was constituted to 81% of segments, which were bearing 70–160% of the average DS value of the initial sample. In addition about 19% of the polymer was made up by practically unsubstituted fragments. Although the fragmentation pattern of the sample was still heterogeneous the deviation with the polymeric chain was much smaller as for the DS 0.4 sample.

The DS distribution of the DS 1.2 sample showed a very homogeneous course. For the major part of the polymer eluting in fractions 13–23 (Fig. 2c) DS values between 1.4 and 1.0 were determined, representing a deviation of only $\pm 17\%$ 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 this segment constituted 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 minimal percentage. Considering the mode of endoglucanase action, the liberation of cellobiose would require at least two neighbouring unsubstituted glucose units. Accordingly one can postulate that such sections 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.

For both the DS 0.7 and 1.2 sample a pre-hump in fractions 1–3 indicated the existence of some aggregated material. The DS analysis of this material resulted in DS 1.0 for the DS 0.7 sample and 1.2 for the DS 1.2 sample. Accordingly this aggregated material did not excel by a DS value deviating strongly from the first polymer fractions of the corresponding samples. The amount was not sufficient for a more detailed analysis of these fractions. Nevertheless an isolation of aggregated pre-humps might be an interesting option especially for the starting material prior to EG fragmentation. A separated analysis of theses fraction regarding their partial substitution and substituent distribution might be an additional approach to learn about solution phenomena of cellulose acetates.

3.4.2. AEC-PAD of individual fractions

It should be further confirmed that separation in the preparative SEC system was performed according to size exclusion principles and not affected by the DS value of the fragments. Analytical SEC after carbanilation was not suitable for this investigation, since this procedure requires various reaction steps and is difficult to apply to small samples quantities and low DP fragments. Therefore the individual fragments of samples with DS 0.4 and 0.7 were analysed by AEC-PAD chromatography. The strong alkaline eluent used for the dissolution of samples saponifies the acetyl groups and delivers information on the various oligomeric products contained in a fraction. Examples for the composition of different fractions isolated from both samples are depicted in Fig. 3. The superposition of the chromatograms obtained from fractions 24–25 for both samples showed a practically identical composition regarding the fragments obtained from both samples. These fractions were obtained around the main peak of these low DS samples. Due to the relatively broad collection mode in the SEC the fragments of major importance vary from DP 3 to DP 11. Nevertheless one has to consider that the detector response in amperometric detection is dependent on the presence of OH-groups to be oxidised. At the same time it is reduced for higher DP products, apparently due to a less effective contact to the electrodes surface. Therefore the importance of the low molar mass fragments is strongly overestimated by this analysis. For comparison the superposition of the AEC-PAD chromatograms of SEC-fractions 28–29 is depicted in Fig. 3b. These fractions are dominated by cellobiose still giving significant amounts of

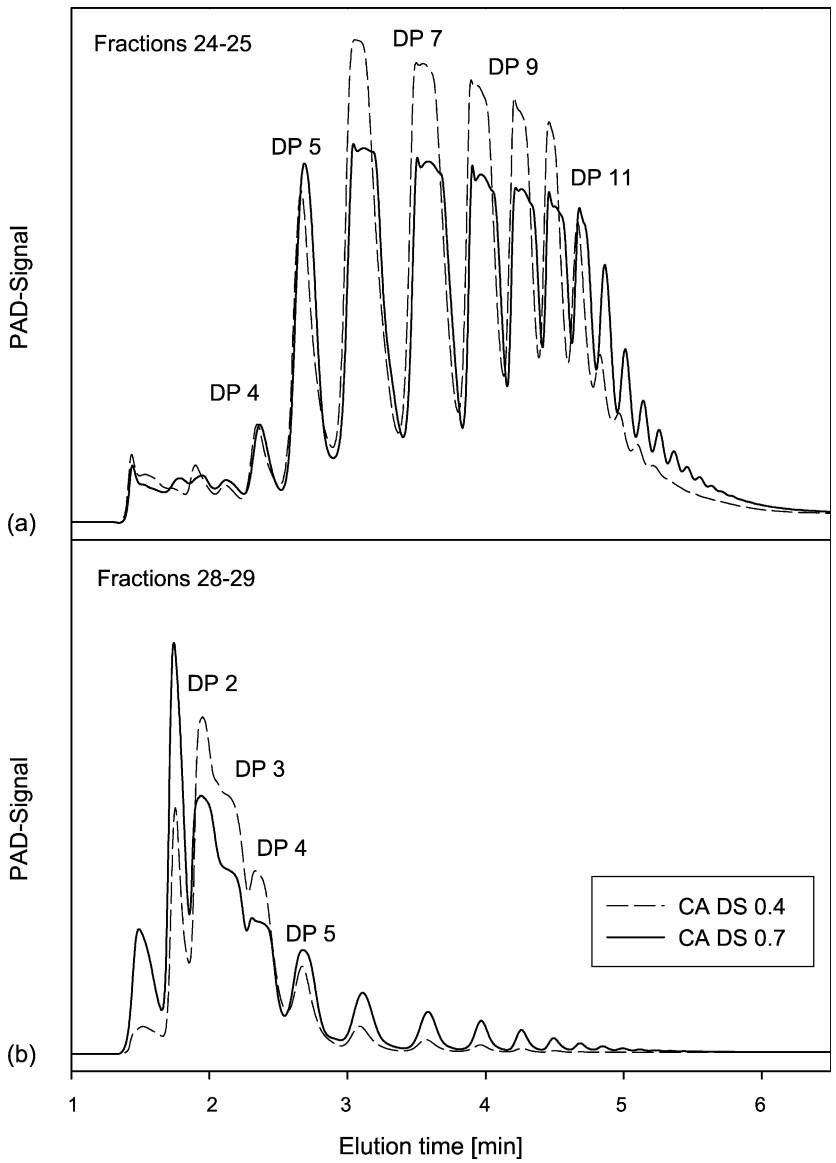

Fig. 3. AEC-PAD chromatograms of individual fractions obtained after EG fragmentation and preparative SEC from samples DS 0.4 and 0.7. (a) Fractions 24–25; (b) fractions 28–29.

cellohexaose products. A small amount of glucose was found which is normally not a side product in EG hydrolysis. Nevertheless, when cellulose derivatives are hydrolyzed a longer time a certain amount of glucose is often formed, presumably by secondary degradation of small unsubstituted fragments when no other substrate is available.

The AEC-PAD analysis underlines that fractions were indeed separated according to chain lengths. Identical fragments were obtained in corresponding fractions of different samples. The resolution obtained in the fragmentation was limited which is due to the number of fractions collected along the elution profile of the SEC-separation. However, a more precise separation would make it difficult to collect sufficient material for further analysis. Larger columns or repeated separations per sample could enable a more exact fractionation mode. However, in both cases the cost for solvents, column investment or analysis time increases, while the overall information on the substitution pattern seems to be sufficient by the method introduced her.

4. Conclusion

The CAs investigated in this study could be intensively hydrolyzed at least up to DS 1.4 by EG. For heterogeneously substituted samples a fragmentation of higher DS samples can be postulated based on earlier results. Pyridin:water in a ratio of 9:1 (v/v) was an appropriate solvent system to enable the SEC separation of CA over a wide DS range at least up to DS 2.3. The comparison of preparative

SEC curves with the results of analytical molar mass determination indicated that the separation was performed according to SEC principles. This could be confirmed by AEC-PAD chromatography of corresponding fractions from different samples. After saponification the cellooligomers eluting after the same elution time from the column had similar DP values. Therefore the preparative SEC system introduced in this study is suitable for the enzyme-aided characterisation of CA over the whole DS range susceptible to endoglucanase fragmentation.

The two methods introduced for DS determination showed a good correlation. Both could be adapted to sample sizes in mg scale, a prerequisite for routine operation. While the FT-IR/ATR technique was less time consuming it had the disadvantage that fragments of lowest molar mass could not be analysed by this method, at least when samples should be recovered for further analysis. The DS determination based on hydrolysis and HPLC was suitable for the analysis of all fragmentation products. However, for all samples analysed in this study it could be confirmed that those fragments not suitable for the FT-IR were not bearing any significant amounts of substituents. Therefore one could consider deciding in favour of the spectroscopic methods. The final fractions containing mainly cellobiose could be evaluated by the weight proportion calculated from the preparative chromatogram.

Regarding the substituent distribution the most heterogeneous pattern was obtained for sample DS 0.4 containing fragmentation products with up to 250% substitution based on the average DS of the total sample. This indicates that acidic saponification to very low DS contains a risk of extremely heterogeneous distribution.

For the DS 0.7 sample 81% were segments bearing 70–160% of the average DS of the total sample, while 19% of the polymer were made up by un-substituted fragments. The fragmentation pattern of the sample was rather heterogeneous, especially considering the homogeneous synthesis. However, the deviation within the polymeric chain was much smaller compared with the DS 0.4 sample.

For the DS 1.2 sample a very homogeneous picture was obtained. For the major part of the polymer only a deviation of $\pm 17\%$ based on the DS of the starting material was determined.

These results show that the EG-aided analysis can provide information on the heterogeneous or homogeneous nature of CA samples. The procedure, at least based on FT-IR/ATR for DS analysis, can be applied for routine analysis. It should be possible to further simplify the method using FT-IR interfaces. After passing the concentration detector the eluate would be sprayed directly on geranium discs, which can subsequently be analysed in FT-IR spectrometers using an automatic mode. This would not only lower the work time drastically but should improve the resolution of analysis. Due to a high sensitivity for direct sample recovery the resolution of a fraction collector could be surpassed by far. A general limitation of the technique introduced in this paper is that no information on the partial degree substitution is obtained for the fragmentation products. A further adaptation of the method to provide this more detailed information is currently under investigation.

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