Chapter 15

Progress in the Enzymatic Hydrolysis of Cellulose Derivatives

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Single enzymes of the cellulase complex are efficent tools for understanding the biodegradation of cellulose derivatives. Sensitive size exclusion chromatography makes it possible to detect minor fragmentations. The accessibility of cellulose derivatives is clearly a function of the degree of substitution (DS). In addition, charge and size of the substituents and the substituent distribution play major roles for enzymatic attack. Whereas carboxymethyl cellulose of a certain DS was fully resistant against endoglucanase action, a methylcellulose of the same DS was markedly fragmented. Cellulose acetate was taken as an example for demonstration of the impact of esterases besides cellulases in biodegradation. The presence of acetyl esterase enabled the endoglucanase to degrade cellulose acetate much faster than when this type of enzyme was absent.

There is a continuous interest in the enzymatic hydrolysis of cellulose derivatives, starting with the pioneering work of Husemann (*1*) and Reese (*2*). Whereas Husemann was mainly interested in the location of the hydrolysis, Reese's main focus was to prevent a microbial attack to cellulose. One of the means to prevent an enzymatic degradation was identified to consist of an exchange of the free OH groups of the anhydroglucose units by bulky substituents. In contrast to the early research, today's concern is at least partly the conservation of the biodegradability of cellulose derivatives, i.e., cellulose should be substituted by certain derivatives only to the extent that the biological attack is not impaired. However, early papers as well as recent publications discuss the action of cellulases against cellulose derivatives in order to draw conclusions about the location of the substituents within the anhydroglucose units, as well as about the substituent distribution along the cellulose chain. Unfortunately, the early work was exclusively performed using enzyme cocktails of different compositions and origins; for this reason this overview was supplemented by additional practical experiments conducted in the authors' laboratory. In these experiments a mono-component cellulase, namely a fungal endoglucanase. was used.

Heinze and Glasser; Cellulose Derivatives
ACS Symposium Series; American Chemical Society: Washington, DC, 1998.

Analytical Methods for Measuring the Enzymatic Degradation of Cellulose Derivatives. The analytical methods mainly used to follow the enzymatic hydrolysis of water-soluble cellulose derivatives are based on measuring changes in viscosity, the amounts of glucose, and reducing sugars. From these data only indirect conclusions about the length of cleaved chains and the relative amount of derivatized cellulose fragments can be drawn.

In more recent investigations the enzymatic action was visualized by changes in the molecular weight distribution, as revealed by size-exclusion chromatography (SEC). The main problem with SEC-characterization of polyelectrolytes such as CMC and the separation of their enzymatic degradation products was the selection of an appropriate eluent. The nature of the buffer and the ionic strength were essential factors in avoiding unwanted interaction with the stationary phase material (*3*). In order to differentiate the DS, ^{13}C-NMR spectroscopy was used (*4*).

Demeester et al. could demonstrate the usefulness of light scattering for the detection of the enzymatic action (*5*). The cellulolytic degradation of cellulose was also considered as a sample pretreatment prior to the structural characterization of cellulose derivatives (*6*). This method, however, could not be applied for high DS samples.

The Cellulose Degrading Enzyme System. The complete hydrolysis of natural cellulose demands the action of exoglucanases (also called cellobiohydrolases, CBH), endoglucanases (EG), and ß-glucosidases. Only a few microorganisms produce the complete set of cellulases for efficient degradation of insoluble cellulose. The general term cellulase usually refers to this complete set of cellulolytic enzymes. According to the current hypothesis, EGs initialize the attack on cellulose by randomly hydrolyzing internal bonds in amorphous regions of the cellulose. The action of EG thus produces new chain ends, which then become available for CBHs, which are believed to hydrolyze the chains from both the reducing (CBH I) and the non-reducing end (CBH II), whereby cellobiose is liberated (*7,8*). Finally, ß-glucosidases hydrolyze cellobiose into glucose. The different cellulases have been shown to hydrolyze cellulose synergistically.

The proposed degradation pathway, as well as the synergy, has been the subject of extensive discussion during recent years. Thus the present classification of specific cellulases as being either CBH or EG seems dubious. Today cellulases are classified for their amino acid sequence, followed by a classification by means of hydrophobic cluster analysis, HCA, in addition to classification according to their action against specific substrates (*9*). The HCA considers the different charges of amino acids and indentifies hydrophobic clusters along the two-dimensional sequences. Based on this information a three-dimensional structure can be predicted. This method is especially useful for the detection of similar folds in different enzymes with low sequence identity. Of the 11 cellulase families currently classified (*10*), fungal cellulases are found in six.

The only true exoglucanase structures solved are the two CBHs of the fungus *Trichoderma reesei*. The three-dimensional structures of the catalytic domain of *T. reesei* CBH II (family 6) and CBH I (family 7) revealed that the active site of both enzymes was identified to be situated in a tunnel ranging through the whole domain and formed by stable surface loops (*11,12*). It therefore seems that the active site tunnel is a general feature of exoglucanases. Indeed, exoglucanases are believed to be unable to attack cellulose derivatives, and the existence of the active site inside a tunnel could explain why especially bulky cellulose derivatives could get stuck inside the

tunnel. Thus, EGs should have similar overall folds, but the active site should be more open, allowing a random hydrolysis of the cellulose (*11*). The three-dimensional structure of the *Thermomonospora fusca* EG E2 (*13*), belonging to the same family 6 as CBH II of *T. reesei*, confirmed this hypothesis. A similar structure was found for EG I (family 7) from *Humicola insolens* (*14*). EG V from *Humicola insolens* belongs to family 45 and cleaves 1,4-ß-glucosidic linkages with inversion of configuration, whereas EG I catalyses cleavages with retention of configuration. As a general feature, the active sites of endoglucanases were found to be located in long open grooves.

The structure of EG V has been solved, and it was found that this protein has 7 subsites (A to G) for binding with its substrate, the cleavage taking place between subsites D and E. On the basis of mutation experiments of the active site of EG V, there was strong evidence that an aspartate near subsite E, sitting in a predominantly hydrophobic environment, acts as the proton donor in the hydrolysis mechanism, whereas another aspartate functions as the base, activating the nucleophile.The active site residues of the *H. insolens* endoglucanase I are two glutamates and an aspartate, the latter functioning as the proton donor (*14*). This enzyme has the negative charge of its active site in common with EG V of *Humicola insolens*. In all cases investigated so far, the major role of the carboxylic amino acids glutamate and aspartate for scisson of the polysaccharide has been identified (*15*).

Accessibility of Cellulose Derivatives to Enzymes. It is well known that the accessibility of cellulose to enzymatic hydrolysis is dependent upon physical and structural features of the polymer. The relevant features are crystallinity, degree of swelling, solubility, and presence of other structural components (lignin and hemicelluloses). Cellulose derivatives are prepared by exchanging hydrogen atoms on primary and secondary hydroxyl groups with various functional groups such as methyl, carboxymethyl, diethylaminoethyl. Modification of cellulose usually makes it non-crystalline and in many cases soluble. Both factors increase the susceptibility of the cellulose to enzymatic hydrolysis. The susceptibility of cellulose derivatives to enzymatic hydrolysis increases as the substrate becomes less crystalline and more water-soluble. Water-solubility is dependent upon the degree of substitution (DS). The DS value at which complete water-solubility is achieved ranges from DS 0.4 to 0.7, depending on the solvation capacity and on the pattern of substitution (*16*). However, when on the average more than one substituent occurs per each anhydroglucose unit, the enzymatic hydrolysis rate decreases, and higher DS values result in a complete inertness of the polymer (*16*).

When the degree of substitution approaches an average of one substituent per glucose unit, steric factors become important, and enzymatic hydrolysis is retarded. The International Union of Pure and Applied Chemistry has published recommendations on the measurement of endoglucanase activities. This commission recommended substrates such as carboxymethyl cellulose (CMC) of DS 0.7 and hydroxyethyl cellulose (HEC) of DS 0.9 - 1.0 (*17*). In connection with the use of CMC as a test substrate for endoglucanase activity measurement, it has been stated that besides the DS, the same origin (i.e., the same method for the preparation of the derivative) is absolutely necessary in order to ensure a reproducible distribution of the substituents (*18*).

Reasons why HEC is preferable to CMC or to other water-soluble substrates have been discussed (*19*). According to earlier reports (*20*) two or more contiguous non-

substituted anhydroglucose units in CMC are necessary for an enzymatic scission of the polysaccharide chain. In contrast Ach claims that cellulose acetate of DS 2.5 is biodegradable, although degradation proceeds extremely slowly (*21*). For these reasons it seems advisable to differentiate the accessibility of cellulose ethers and cellulose esters.

Enzymatic Degradation of Insoluble Cellulose Derivatives. Only a few papers deal with the heterogeneous enzymatic hydrolysis of cellulose derivatives. Highly substituted and water-insoluble cellulose derivatives (i.e., cellulose triacetate) are regarded to be completely resistant to enzymatic attack, because of a combination of factors - a lack of hydrophilicity, reduced swelling, and the hindrance caused by spatial substituents (*22*).

Enzymatic Degradation of Cellulose Ethers. Most of the work published on the enzymatic degradation of cellulose derivatives was performed with HEC and CMC (*20, 23-26*). Some results have also been obtained with MC (*1*). From Husemann, it may be assumed that the small methyl substituents inhibit degradation to a lesser extent than do the more bulky carboxymethyl groups (*1*). Intensive studies on the enzymatic degradation of cellulose ethers have established that the degree and the uniformity of substitution are decisive factors for their accessibility (*2, 20, 26, 27*).

According to Reese (*2*), a scission of monosubstituted MC is possible. This finding was confirmed by Schuseil (*28*), whereas CMC requires a sequence of three non-substituted anhydroglucose units (*24*). Wirick (*20, 26*) came to the conclusion that glycosidic bonds within cellulose ethers are only enzymatically cleaved in the case that two neighbouring non-substituted anhydroglucose units or that eventually a non-substituted and a C-6 substituted anhydroglucose unit are present. This result is in accordance with Bhattacharjee and Perlin (*25*), who pointed out that a scission between non-substituted units as well as between a substituted and a non-substituted anhydroglucose unit seems possible depending on the position of the substituent.

According to Kasulke et al. (*29*), three non-substituted glucose units were required in order to achieve the liberation of glucose. In this case, both hydrolysis products carried a non-substituted anhydroglucose unit at the end.

Enzymatic Degradation of Cellulose Esters. In the hydrolysis of water-soluble cellulose acetates Kamide et al. (*30*) found this polymer to be degradable up to DS 1. The authors observed a gradual precipitation of high DS cellulose acetate in the course of enzyme incubation, indicating that short highly-substituted cellulose acetate blocks can exist in water-soluble cellulose acetate chains (*4*). According to Buchanan et al. (*31*), the biodegradable DS range of cellulose acetates is even wider. The authors found cellulose acetates with DS values between 1.7 and 2.5 to biodegrade by mixed culture systems, at least partly following deacetylation. A series of other esters besides acetates was synthesized by Glasser et al. (*32*). The biodegradability of cellulose esters using cellulolytic enzymes was found to depend on two factors: degree of substitution and substituent size. The cellulose esters had acyl substituents ranging in size from propionyl to myristyl and DS values between 0.1 and nearly 3.0. The maximum degree of acylation, that the enzyme could tolerate before the polymer became undegradable and that resulted in degradation in excess of 10 % by weight, ranged from DS 0.5 to at least 1.0, depending on the ester type. It became evident that the larger the substituent

of the cellulose ester, the harder it became for the enzyme to recognize the macromolecule and degrade it.

Influence of Substituents and Degree of Substitution. A set of CMC of DS, ranging from 0.6 to 2.1 and different molecular weights were incubated with three cellulase preparations of different origin and purity (*33*). A decrease in enzymatic hydrolysis with increasing DS was observed for all enzyme activities. This decrease could be explained by the difficult access of the enzyme to the 1,4-ß-glycosidic linkage due to the steric hindrance from the carboxyl groups, located mainly at C-2. The action of cellulase enzyme preparations on CMC samples of DS 0.7 and varying MW ranging from 0.55 to 8.71 x 10^6 g/mol was not affected by the large difference in molecular size. Complete hydrolysis was obtained with a purified cellulase at hydrolyzing CMCs with DS up to and including 0.9. Philipp et al. (*18*) reported on two different phases in the enzymatic degradation of CMC, both of them first-order reactions. The first (quick) reaction was explained by the combined action of endo- and exo-enzymes, which was favoured by the presence of longer sequences of non-substituted anhydroglucose units. The second (slower) phase was explained by sporadic EG-mediated "hits", which were made possible by shorter (possibly two) non-substituted units.

Influence of Derivatization Procedure. Cellulose derivatives are obtained mainly by heterogenous derivatization, which is normally considered to yield a statistic distribution of substituents. Cellulase hydrolysis yields large fragments from the more highly substituted regions, whereas small fragments are obtained from those regions which had a lower degree of substitution (*16*). Philipp et al. (*34*) could clearly show that the enzymatic hydrolysis of CMC in the DS range of 0.5 to 0.8 depends not only on the DS, but also on the procedure applied for preparing samples, with different distributions of substituents along and between the polymer chain. A CMC sample, which has been prepared after solubilization of the cellulose, excels by low values for liberated glucose in comparison to a CMC sample prepared in a heterogenous reaction system. Beyond a DS of 0.7 this value seems to be the determining factor for the liberation of reducing sugars viz. glucose (in case sufficient ß-glucosidase activity is present). In this higher DS range factors derived from the procedure applied for the production of the derivatives seem to be of minor importance. Philipp et al. (*34*) draw the conclusion that longer sequences of non-splitable linkages were present in higher DS CMCs. For the DS region 0.5 to 0.7, average sequences of derivatized anhydroglucose units of 4 to 6 were given, whereas in the DS region >1 averages of up to 40 carboxymethyl-glucose blocks were calculated.

Recently Heinze at al. stated that CMC samples, which were synthesized via an induced phase separation, contained a significantly higher amount of both tricarboxymethylated and unsubstituted units than those obtained in a slurry of cellulose in isopropanol/water at comparable DS values (*35*). This finding pointed out that it is not adequate to automatically assume a homogeneous substituent distribution from a homogenous reaction mixture nor a heterogenous distribution from a heterogenous system. Since the substituent distribution along the polymer chain is difficult to access, detailed insight on its impact on enzymatic degradation is still missing.

Influence of the Position of the Substituents. Little is known about a possible toleration of endoglucanase activity for a certain position carrying a substituent. The three possible positions for derivatization in cellulose are the hydroxyl groups at C-2, C-3, and C-6. The experimentally determined molar distribution of carboxymethyl groups in CMC has been reported to be 2:1:2.5 for C-2, C-3, and C-6 hydroxyls (*36*). While the C-6 primary hydroxyl is generally thought to be the most reactive in this particular system, this is not always so. There is considerable evidence that the C-2 hydroxyl group is the most acidic in cellulose (*37*). As a result, many equilibria and rate-controlled reactions that involve cellulosic alkoxide ions appear to favour this site. NMR studies of substituent distribution indicate C-2 > C-6 > C-3 (*6*). The finding that reducing end residues liberated during enzymatic hydrolysis of cellulose were never substituted at the 2-position (*6*) is a strong indication that this position seems to be needed for binding of the enzyme protein to the substrate.

The Influence of the Type of Substituent. The susceptibility of cellulose derivatives to cellulose hydrolysis is also dependent on the type of the substituent (*25*). The derivatization will certainly modify the charge of the polysaccharide and thus influence the interaction of the enzyme's active site with its substrate. Wirick (*20, 26*) investigated the enzymatic hydrolysis of CMC, MC, HEC, and hydroxypropyl cellulose. In a comparison of the above-mentioned cellulose derivatives, and in accordance with Husemann, CMC was the most resistant substrate.

Interestingly, Philipp and Stscherbina (*22*) reported a shift in the pH-optimum from pH 4 (DS 0.7) to pH 7.5 (DS 1.0) in the degradation of CMC using the enzyme system of *Penicillium citrioviride*. The nature of CMC to be a polyelectrolyte was also pointed out by Nicholson and Merritt (*38*). According to Philipp and Stscherbina (*22*) only the reaction velocity was reduced compared to the degradation of non-ionic cellulose derivatives. The extent of hydrolysis was not impaired by the charge of the substrate.

An increase in the negative charge resulted in a reduction of the hydrolysis rate, whereas the presence of positive charges markedly increased the enzyme activity (*39*). This finding could be verified and explained by recent investigations on the reaction mechanism (*15*) and the fine structure of endoglucanases (*14*).

Experimental

Substrates. All CMCs were gifts of Wolff-Walsrode (Walsrode/ Germany). The MC samples came from Kalle, a subsidary of Hoechst AG (Wiesbaden / Germany). The DS 0.7 CA was a gift of Hoechst-Celanese (Charlotte, N.C. / USA). The DS 0.9 to DS 2.9 CAs were gifts of Rhône-Poulenc Rhodia (Freiburg / Germany). The DS-values were determined by the manufacturers.

Enzymes. The mono-component endoglucanase preparation was an experimental product from a genetically modified *Aspergillus* strain, obtained from Novo Nordisk (Bagsværd / Denmark) and used after removal of low-molecular weight components by ultrafiltration. The *Aspergillus* enzyme mix was obtained from Nagase & Co (Tokyo / Japan) and used after ammonium sulfate precipitation and desalting.

Enzyme Treatments. 0.2 % solutions of CMC, MC, and the water-soluble DS 0.7 CA in 0.1 M sodium nitrite were incubated with 10000 nkat per mg substrate. The incubations were performed in an Eppendorf Thermomixer at 45 °C and 1200 rpm for 3 or 6 days. After completion, the samples were boiled for 3 minutes for protein denaturation. The precipitated enzyme protein was removed by centrifugation at 2000 g for 10 minutes. Substrate blanks were incubated for 3 days and treated correspondingly. The incubation of the water-soluble DS 0.7 CA with the enzyme mix was performed as a 1 % solution in bi-destilled water at 40 °C and 1200 rpm. The enzyme dosage was 500 nkat/mg. Enzyme protein was removed according to the procedure mentioned above. For SEC analysis, samples were diluted to a 0.2 % solution using sodium nitrite. Liberated acetic acid was determined using the Boehringer Test Combination Kit (Cat. No. 148 261 Boehringer, Mannheim / Germany).

For endoglucanase treatment of cellulose acetate higher in DS than 0.7, 1 % solutions or suspensions in bi-destilled water were incubated with 10000 nkat *Aspergillus* endoglucanase / mg substrate. The incubation was finalized by ammonium hydroxyde addition and incubation at 30 °C overnight for protein denaturation and acetyl saponification. The solutions were freeze-dried and afterwards stored in vacuum over P_2O_5. Blanks underwent the same procedure.

Carbanilation. 100 mg of saponified samples were suspended in 100 ml of pyridine and derivatized at 80 °C using 7 ml phenylisocyanate. After 48 h the reaction was stopped. A refinement procedure by direct evaporation of the pyridine (*46*) was applied as published previously (*40*).

SEC of Water-Soluble Derivatives. SEC was performed using sample concentrations of 0.2 % and sample volumes of 100 µl injected into three SEC columns, coupled in line (TSK G5000PW_{XL}, G4000PW_{XL}, G3000PW_{XL}, 300x7.8 mm each, and a G2500PW_{XL} guard-column, 40x6 mm, TosoHaas, Stuttgart / Germany). The column temperature was kept constant at 40 °C. The mobile phase (0.4 ml/min) was 0.1 M sodium nitrite in water. The elution profiles were detected by changes in refractive index. The WINGPC 3.0 software (Polymer Standard Service, Mainz / Germany) was used for data aquisition.

SEC of cellulose carbanilates. 0.5 mg cellulose derivative per ml of stabilized tetrahydrofurane (THF) was shaken for 3 days for dissolution. Samples were then centrifuged for 2 h at 20 °C (16000 to 22000 g), and the supernatant was used for further analysis. SEC was performed using a Waters 510 pump, a Kontron 360 autosampler (100 µl), Waters Ultrastyragel 10^3 Å and 10^4 Å columns (300x7.8 mm each), a Spectra Physics SP8400 UV detector, a Shodex RI-71 detector, and WINGPC 3.0 software. The eluent was THF with a flow rate of 1 ml/min at 20 °C. Polystyrene standards were monitored at 254 nm and cellulose tricarbanilates at 235 nm. A calibration curve was obtained by a broad fit of two cellulose tricarbanilate samples with a molar mass of 210000 g/mol and 1000000 g/mol (*40*) to a polystyrene standard curve. The Mark-Houwink constants obtained by this procedure were K: 1.756 $+10^{-3}$ ml/g and α: 0.890.

Results and Discussion

Endoglucanase Degradation of CMC. CMCs in a DS range from 0.6 to 2.4 were incubated for 3 and 6 days with an overdosage of a mono-component *Aspergillus* endoglucanase (10000 nkat/mg substrate), in order to reach the final possible stage of fragmentation. Substrate blanks were incubated for 3 days. Figure 1 demonstrates the hydrodynamic volume of the blank samples to be too high for a perfect separation in the chromatographic system. This is especially the case for the DS 0.6 and DS 0.8 samples, starting at an elution volume of 13.75 ml, with a pronounced steep shoulder. While most blanks had a narrow elution curve, the DS 1.2 and 1.6 samples showed a tailing in the lower molar mass region.

The intensity of endoglucanase degradation was strongly dependent on the DS. DS 0.6 and 0.8 samples gave a strong shift of the elution profile to the low-molecular region and displayed distinct signals for oligomeric compounds. The fragments which were washed from the columns at the elution volume of around 29.6 ml can be attributed to monomeric degradation products. It becomes evident from Figure 1 that the maximal possible fragmentation of the polymer was completed after 3 days of incubation.

DS 0.9 and 1.2 CMCs were also markedly fragmented by the endoglucanase, which is visualized by pronounced shifts of the elution profile to longer elution volumes (Figure 1). However, the degradation of these derivatives was less intense in both regions, the higher molar mass region and the oligomeric range. In the degradation of these samples, a prolongation of the reaction time gave rise to an additional fragmentation. Possibly the prolonged and slower phase can be explained by sporadic cuts between shorter non-substituted or less substituted regions, according to the explanation given by Kasulke et al. (*27*). In addition, tertiary conformation effects of the charged polymer could limit or slow down the accessibility of the enzyme to positions which principally can be cleaved.

The DS 1.6 sample shows only a minor shift in the elution curve and a small bimodality in the lower molar mass range after endoglucanase treatment. For the DS 2.4 CMC sample, the effect of the treatment on the elution curve was even more reduced. However, a small but significant bimodality demonstrates the availability of some positions along the CMC chain to the endoglucanase. This minor change had no effect on the elution curve of the main polymer peak. For both the DS 1.6 and DS 2.4 samples only minor changes occurred even after prolonged incubation times. These results are in accordance with Philipp et al. (*34*), who could demonstrate a reduction in viscosity for DS 1.7 CMC after incubation with a *Gliocladium* culture filtrate. Due to the close involvement of the carboxylates of two amino acids in its active site, the reduced activity of endoglucanase against the negatively charged CMC becomes explainable.

It is noteworthy that the intensity of the elution curve of the enzyme treated DS 2.4 sample was slightly higher than the substrate blank. It could be excluded that this phenomenom was derived from experimental errors. However, it was noted that the number of visible gel particles in enzyme-incubated samples was significantly reduced; this effect explains the higher yield of enzyme-treated samples. It was even more pronounced for the treatment of both MC and water soluble CA, discussed in the following paragraphs.

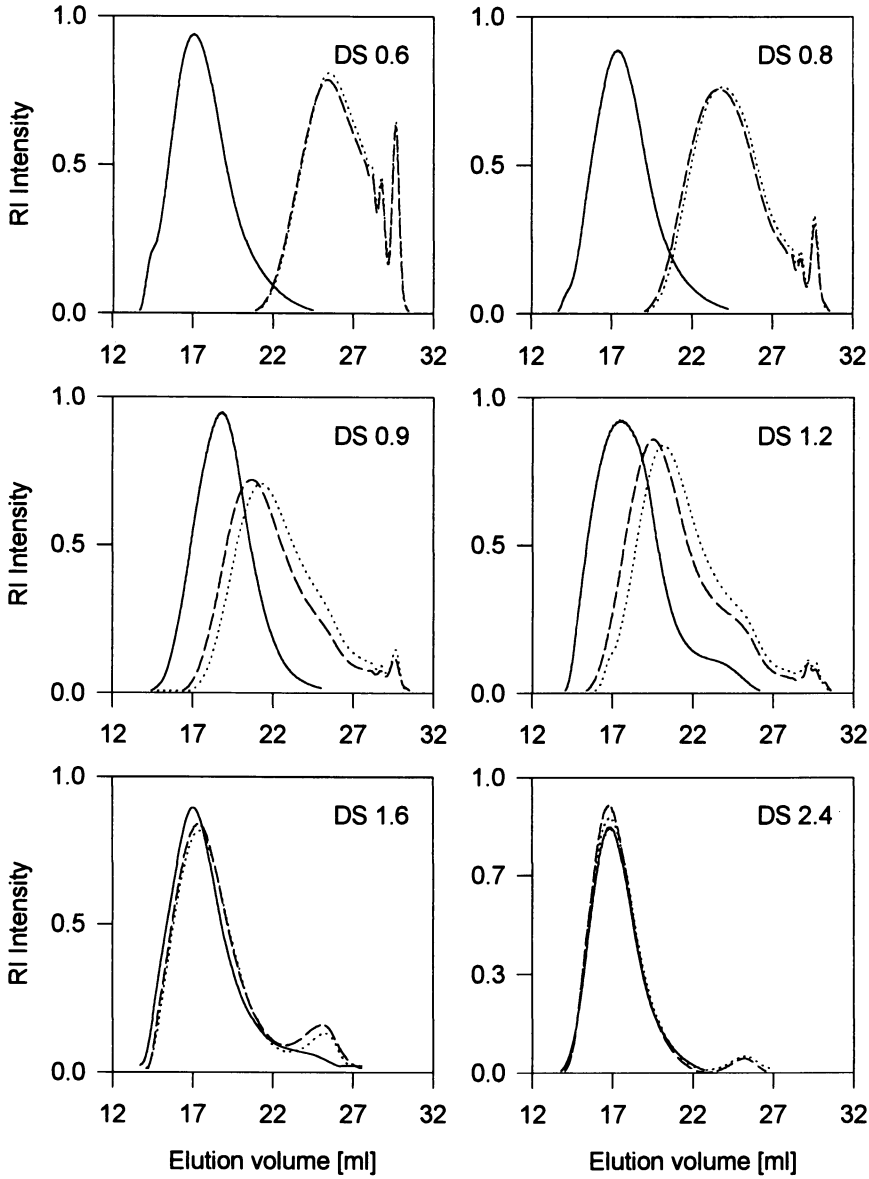

Figure 1. Influence of DS on the fragmentation of carboxymethyl-cellulose by *Aspergillus* endoglucanase, after 3 days and 6 days incubation, monitored by aqueous SEC.
blank: ____ 3 days: ---- 6 days: .....

Endoglucanase Degradation of MC. It could be verified that endoglucanase fragmentation of MC in the range from DS 0.5 to 2.1 was much more effective then that of CMC. The DS 0.5 sample was more or less completely degraded into oligomeric products (profile not shown). After 3 days of degradation, the fragmentation of the DS 1.5 MC in Figure 2 was comparable to the DS 0.6 CMC in Figure 1. The MC curve had an even more intensive shift to low molar mass and oligomeric products. At 31.2 to 31.3 ml, the elution curve falls almost rapidly, since monomeric MC products elute into the separation limits of the column.

In another comparison, the DS 2.1 MC was significantly fragmented, whereas the elution curve of the DS 2.0 CMC was only slightly modified, compared to the substrate blank.

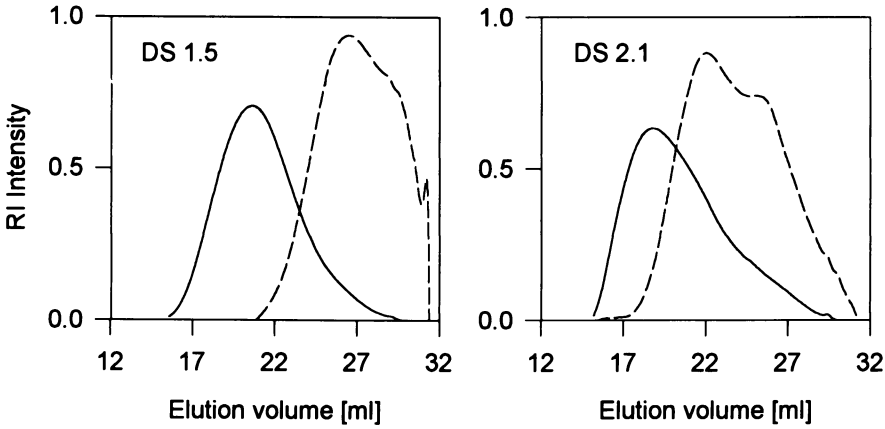

Figure 2. Influence of DS on the fragmentation of methyl cellulose by *Aspergillus* endoglucanase (3 days incubated) monitored by aqueous SEC. blank: —— enzyme treated: ----

All endoglucanase-treated and centrifuged MC samples have in common that the intensities of the elution curves were by far higher than the substrate blanks. This phenomenon underlines the possibility of endoglucanase-mediated improvements in solubility.

Endoglucanase Degradation of Cellulose Acetate. Cellulose acetate fragmentation was performed with DS 0.7 to DS 2.9 samples, which included heterogeneous hydrolysis of water-insoluble cellulose acetate powder of DS > 1 and homogeneous hydrolysis of water-soluble samples DS < 1. The substrates have been prepared by acid saponification of cellulose triacetate. This is the reason that the molecular weight of the starting material was reduced with decreasing DS (Table I). Due to the heterogeneous hydrolysis of most of the cellulose acetate substrates, the material could not be directly analyzed by gel permeation chromatography, but had to be gently saponified using ammonium hydroxide prior to tricarbanilation and SEC analysis in tetrahydrofurane (*40*).

Table I. Homogeneous and heterogeneous endoglucanase hydrolysis of cellulose acetate samples as revealed by SEC of the corresponding carbanilates

DS	Starting material			Endoglucanase treated material		
	M_W	M_W/M_N	DP	M_W	M_W/M_N	DP
0.9	16 000	1.5	31	1 860	1.1	4
1.2	44 000	1.8	85	2 500	1.2	5
1.6	72 000	1.7	138	14 000	5.1	27
1.7	48 000	1.7	92	26 000	1.2	50
1.9	98 000	2.0	189	52 000	11.0	100
2.5	164 000	3.2	316	159 000	3.0	306
2.9	201 000	4.1	387	204 000	3.3	394

Due to the surplus of endoglucanase activity used in this study, the DS 0.9 sample was extensively degraded. Caused by acid saponification prior to the hydrolysis step, the partly deacetylated material was already considerably reduced in degree of polymerization (DP = 31); the endoglucanase treatment, however, caused a further degradation by homogeneous hydrolysis to DP = 4. A similar result was obtained for the DS 1.2 sample, which was degraded from molecular weight (M_w) 44000 to M_w 2500, equivalent to an average DP of 5.5, indicating a reasonable accessibility of both cellulose acetate samples to endoglucanase action. The accessibility was a clear function of the DS. From DS 1.6 on, the degradation by endoglucanase action was considerably retarded, which was certainly caused by two facts: the water-insolubility of the material and the shielding by increasing amounts of acetyl substituents. In spite of the unfavourable conditions, the material was still markedly degraded up to a DS of 1.9 (from M_w 98000 to M_w 52000). Cellulose acetate of DS 2.5, which is normally used for technical applications, was more or less resistant.

On first glance, these results might be not fully consistent with the results given by Buchanan et al. (*31*), who found cellulose acetate up to DS 2.5 to be biodegradable. However, these authors used a mixed culture system instead of a single, cell-free enzyme. It was noted in their investigations that cellulose acetate degradation was followed by deacetylation. In our own experiments, no acetyl was released by the *Aspergillus* endoglucanase.

Acetyl Esterase Involvement in Cellulose Acetate Fragmentation

In the search for suitable enzyme preparations, it was found that most commercial cellulase preparations had the capability of releasing varying amounts of acetyl groups. Due to the fact that the same endoglucanase dosage (tested with HEC as substrate) was used, it could be concluded that acetyl release is not a common feature of endoglucanase activity, but must be deduced to a separate enzyme. It is known from earlier studies that acetyl xylan esterase (*41*) and acetyl mannan esterase (*42*) are common features in hemicellulolytic enzyme systems. From more detailed work it is known that some esterases are highly specific, whereas others are not (*43*). Due to the man-made origin of cellulose acetate, nature will not have provided microorganisms with specific cellulose acetate esterases, but will probably have provided non-specific

esterases capable of deacetylating cellulose as well as other polymers such as pectin and hemicelluloses.

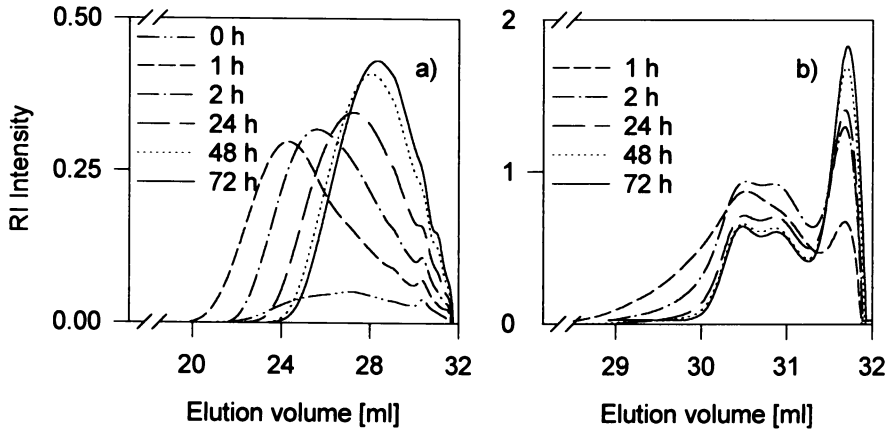

Figure 3. Elution profiles of cellulose-acetate (DS 0.7) monitored by aqueous SEC, fragmented by *Aspergillus* endoglucanase (a) and an *Aspergillus* enzyme mix (b).

In order to learn more about this phenomenon, a DS 0.7 cellulose acetate sample was comparatively incubated with the mono-component *Aspergillus* endoglucanase and another commercial *Aspergillus* enzyme mix (Celluzyme), which had previously been used in the authors' laboratory as a source for the isolation of mannanase activity (*44*) and acetyl mannan esterase activity (*42*). The incubated samples were directly analyzed by aqueous SEC. The time course of the degradation is illustrated by the elution curves from 0 to 72 hours incubation (Figure 3 a and b). The improving RI intensity, in conjunction with the increasing degradation rate, especially as compared to the substrate blank (0 h) with the sample after 1 hour incubation, can be explained by the improved water-solubility of the material. The shift to smaller fragments equivalent with longer elution times is particularly obvious within the first 24 hours of incubation. There was almost no change between 48 hours and 72 hours of incubation. The substrate blank was not included in Figure 3b in order to allow an improved resolution of the low molecular weight fragment (note the different ml-scale in Figures 3a and 3b). Indeed the polymeric material was already drastically reduced in chain length after one hour of incubation. After 24 hours only minor additional changes occurred.

This difference in speed and extent of degradation has been made possible by the presence of an acetyl esterase, in addition to the endoglucanase activity. Unambiguously, the presence of this enzyme was established by the release of acetic acid into solution (Figure 4). The speed of acetic acid liberation was going hand in hand with the fragmentation of the polysaccharide and became slower after 24 hours of incubation. About 50 % of the total acetyl-content of the material was cleaved off after 72 hours. There could be various reasons for the limited release of acetic acid: 1. The acetyl esterase could be a specific enzyme acting exclusively on intact polysaccharides. With increased endoglucanase action, the enzyme could have lost its specificity due to

the presence of shorter fragments. 2. The acetyl esterase could be restricted in its catalytic capacity to certain positions within the anhydroglucose unit. 3. The enzyme could be restricted in its action due to the presence of a non-accessible, highly substituted region within the cellulose chain.

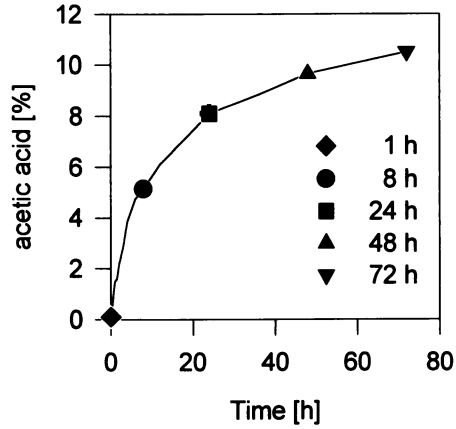

Figure 4. Time course of acetic acid release from cellulose acetate (DS 0.7) during incubation with the *Aspergillus* enzyme mix.

There is some probability for the third possibility, because no acetic acid was released from the DS 2.5 cellulose acetate, incubated with the enzyme mix. However, a similar phenomenon was found for the acetyl xylan esterase, which acted preferentially on acetylated xylan and not on xylan fragments (*45*). The question of the existence of a specific acetyl cellulose esterase or a rather unspecific acetyl esterase could not be solved in this study, but will be the subject of further investigation.

Conclusion

The accessibility of cellulose derivatives clearly was a function of the degree of substitution (DS), in that the material became less degradable with increasing DS. In addition to this factor, charge and size of the substituents play major roles for enzymatic attack. Whereas CMC of DS 1.6 was almost resistant against endoglucanase action, a MC of DS 2.1 was markedly fragmented into shorter chains. Although low DS cellulose ethers were quite markedly degraded by the mono-component endoglucanase, they could not be completely converted into very short fragments. This is a strong indication for the occurrence of non-degradable, highly substituted regions. Consequently, the allocation of the substituents within the anhydroglucose unit and along the cellulose molecule are additional impacts on the availability of the polysaccharide chain for enzymatic fragmentation.

Cellulose acetate was more degradable than could be anticipated from the literature. Cellulose acetate was taken to demonstrate of the impact of esterases, besides endoglucanases, in biodegradation. The presence of acetyl esterase enabled the

endoglucanase to degrade cellulose acetate much faster and intensively, and with less enzyme protein. Until now this class of enzymes has been neglected in the consideration of biodegradability of cellulose esters. The specificity of this enzyme is not yet understood and will take our attention in the near future.

The comparison of different authors' work was complicated by the fact that most researchers have drawn their conclusions from viscosity measurements as well as from the determination of nonsubstituted anhydroglucose values and reducing sugars, using culture filtrates containing different ratios of endoglucanases, cellobiohydrolases, ß-glucosidases, and possibly acetyl esterases. It is clear that these catalysts contribute to a different extent of fragmentation, leading to the increase in reducing sugars and liberated anhydroglucose from cellulose derivatives.

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