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Mode of action of acetylesterases associated with endoglucanases towards water-soluble and -insoluble cellulose acetates

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Abstract

A screening of commercial enzyme preparations for the capability of cellulose acetate (CA) deacetylation revealed that such enzyme activity is more common than could be anticipated. Enzyme-aided deacetylation of cellulose acetate was clearly a function of the degree of substitution (DS). Cellulose acetates up to a DS of 1.4 were deacetylated by a large number of enzyme mixes. Interestingly, none of the investigated enzyme preparations had the capability for complete cellulose acetate deacetylation. Definitely most hydrolase preparations investigated in our study deacetylated cellulose acetate with no preference for a certain position, as could be confirmed by NMR spectroscopy. However, one acetyl esterase had a clear preference for the C2- and C3-positions, leaving the acetyl substituents at the C6-position intact.

Introduction

Insoluble cellulose is attacked comparatively slowly by a system of cellulolytic enzymes, including endoglucanases, cellobiohydrolases and β-glucosidases (Suurnäkki et al. 2000). A limited introduction of substituents into the cellulose molecule, however, reduces the number of hydrogen bonds of cellulose chains in proportion to the degree of substitution (DS) and the pattern of occurrence along the cellulose chain. Depending on these features and the nature of the substituents, water solubility of cellulose derivatives may already be obtained at DS values ranging between 0.4 and 0.7. Thus cellulose loses its ordered structure and becomes enzymatically highly accessible (Focher et al. 1991). However, when the DS approaches one substituent per anhydroglucose unit the enzymatic hydrolysis rate decreases again and fully substituted cellulose derivatives, e.g. cellulose acetates are regarded as non-degradable (Samios et al. 1996). This finding is partly in contrast to a report by Tominaga et al. (1997), who claimed that the bacterium *Alcaligenes xylosoxydans* could induce the weight loss of cellulose acetate up to a DS of 2.5. According to Gardner et al. (1994) cellulose acetates of DS lower than 2.2 are compostable. A first reference to the existence of enzymes other than cellulases, involved in the degradation of cellulose acetates was given by Gu et al. (1993). The authors reported on a DS reduction of cellulose acetate from 2.5 to 2.2 and 1.7 to 1.3 during 4 days of composting. Sakai et al. (1996) described the same effect, induced by the soil bacterium *Neisseria sicca*. The enzyme activity, responsible for the liberation of acetic acid, was detected in the culture filtrate. Saake et al. (1998) observed an increase in enzymatic cellulose fragmentation by the presence of cellulose acetate deacetylating enzymes. The first purification of an esterase involved in cellulose acetate degradation was reported by Moriyoshi et al. (1999). The enzyme from *Neisseria sicca* also catalysed the hydrolysis of acetyl saccharides and *p*-nitrophenyl esters of short-chain fatty acids and, to a small extent, aliphatic and aromatic esters.

Characterisation and mode of action of acetyl-4-*O*-methylglucuronoxylan and acetyl-galactogluco-

mannan deacetylating esterases have been reported earlier (Biely et al. l985; Puls et al. 1991; Tenkanen et al. 1993). The fractionation of microbial culture filtrates revealed the multiplicity of esterases with different affinities to acetylated xylan-related substrates. One type of esterase could only liberate some acetic acid from xylo-oligomers without deacetylating the polymeric substrate. Together with xylanase, however, it was able to deacetylate 25% of the acetyl substituents in xylan (Poutanen et al. 1990). It also showed high activity towards acetyl xylobiose. The other type of esterase needed no synergy with xylanases. It could efficiently remove the acetyl substituents from polymeric xylan. A complete removal of acetyl substituents, however, was not achieved. 4-*O*-methylglucuronic acid substituents adjacent to acetyl substituents hindered the enzyme from liberating the remaining acetyl substituents. An overview on the significance of esterases in the degradation of xylans was given by Tenkanen and Poutanen (1992). Partially and fully acetylated methyl-glycopyranosides were used in a study on the substrate specificity of purified acetylxylan esterase from *Streptomyces lividans* (Biely et al. 1996a) and *Schizophyllum commune* (Biely et al. 1996b). Whereas the *Streptomyces lividans* esterase regioselectively catalyzed a double deacetylation of methyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside at positions 2 and 3, the *Schizophyllum commune* enzyme preferentially deacetylated the 3-position of this substrate. A slower deacetylation occurred at position 2. Unfortunately acetylxylan was the only polysaccharide to be used in these studies. Meanwhile several genes encoding acetylxylan esterases have been isolated (Margolles-Clark et al. 1996; Kim et al. 1993; Tsujibo et al. 1997; Cybinski et al. 1999). Interestingly, the enzyme encoded by the *axe1* gene of *Trichoderma reesei* carried at its C-terminus a cellulose binding domain, which could be separated from the catalytic domain by limited proteolysis without affecting the activity of the enzyme towards acetylated xylan, but abolishing its capability to bind cellulose (Margolles-Clark et al. 1996). Unfortunately all these enzymes have not been tested for their ability to deacetylate cellulose acetates.

In this work the action of a variety of commercial enzyme preparations containing acetyl esterases in addition to endoglucanases on a series of cellulose acetates in the DS range between 0.7 and 2.3 is evaluated. Special emphasis is put on a possible regioselective deacetylation mode.

Materials and methods

Cellulose acetates

The DS 0.7 cellulose acetate was a gift of Hoechst-Celanese (Charlotte, NC, USA). The DS 0.9 to DS 2.3 CA were gifts of Rhodia-Acetow (Freiburg, Germany). The DS-values were given by the manufacturer and checked by NMR spectroscopy. For the (DS 0.7) CA the relative proportion of acetyl substituents at the C2, 3 and 6 positions was investigated: 27% C2 acetyl groups, 37% C3 acetyl groups, 36% C6 acetyl groups.

Commercial enzyme preparations

Thirteen enzyme preparations were included in the study. Pectinex 3XL AP-18, SP 249, Novozym 230, Ultraflo, Viscozyme 120L, Pectinex BAN 120L, Gamanase, Novozym 342 were obtained from Novozymes (Bagsvaerd, Denmark). Cellulase AP3 was obtained from Amano Pharmaceuticals (Nagoya, Japan). Meicelase P was from Meiji Seika Kaisha Ltd. (Tokyo, Japan). Pectinol was a gift of Röhm AG (Darmstadt, Germany), Pentosanase was obtained from Solvay Enzymes (Hannover, Germany), Pectinase was purchased from Sigma. Before incubation all enzyme preparations were dissolved in distilled water and ultrafiltrated using collodium bags (Sartorius, Göttingen, Germany) with a molecular weight cut-off of 10000 Da, unless the ultrafiltrates were free of sugars.

Enzyme activities

Acetylesterase

The enzymes (100 μl) were incubated with 0.9 ml of a 0.2% solution of cellulose acetate DS 0.7 in both 0.2 M phosphate buffer pH 7.0 and water, using a thermomixer (Eppendorf, Hamburg, Germany) with 600 rpm at 45 °C for 60 min. The reaction was terminated by boiling for 5 min. After centrifugation the liberated acetic acid was measured against the relevant substrate blank, using the Boehringer acetic acid test combination (Boehringer Mannheim, Germany). The enzyme activity was calculated in nkat from the release of acetic acid. Minor amounts of acetic acid in the substrate blanks were subtracted.

Endoglucanase

The enzymes (200 μl) were incubated with 1.8 ml of a 1% solution of carboxymethylcellulose (Fluka 21900, Buchs, Switzerland, DS 0.6–0.95) in 0.05 M sodium acetate buffer of pH 5 and 37 °C for 5 min. The enzyme activity was calculated from the increase in reducing end groups, as measured by the DNS-reagent (Hostettler et al. 1951).

Enzymatic degradation of cellulose acetates

CA were incubated as 0.2% suspensions in water (for SEC and NMR investigations) or phosphate buffer pH 7 (for evaluation of the deacetylation efficiency) in a laboratory shaker at 45 °C for the indicated time. The reaction was stopped by boiling for 5 min. For NMR characterisation the solutions were freeze-dried and subsequently dissolved in DMSO-d_6 as a 10% solution.

NMR spectroscopy

^{13}C NMR spectroscopy was performed using a Varian Mercury 400 MHz instrument. Samples were measured in DMSO-d_6 at 80 °C. The enzyme-degraded CA samples were investigated in 5 mm probes under routine conditions with broadband decoupling. The starting materials were analysed in a quantitative mode using a pulse angle of 45°, a relaxation time of 3 s and inverse gated decoupling. For referencing of spectra the centre signal of DMSO-d_6 was taken as 39.43 ppm.

SEC of water-soluble cellulose acetate

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$_{XL}$, 300×7.8 mm each, and a G2500PW$_{XL}$ guard-column, 40×6 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 nitrate 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.

Results and discussion

Occurrence of acetyl esterase activity in commercial enzyme preparations

In Table 1 acetyl esterase and endoglucanase activities of thirteen commercial enzyme preparations are given. These enzyme preparations are normally used for juice and beer clarification, processing of cereals and vegetable material, softening of tissue paper or stone washing of textiles. Accordingly many of them contain a wide range of carbohydrase activities, including pectinase, xylanase, and arabinanase. Interestingly, nearly all enzyme preparations were able to catalyse the deacetylation of cellulose acetate. The acetyl esterase activity was determined with the soluble CA DS 0.7 in phosphate buffer pH 7.0 as well as in distilled water. The latter solvent was used for CA incubations in order to avoid problems in the NMR investigations, caused by salt accumulation after drying the samples for re-dissolution in DMSO-d_6. When water was used the most active enzymes reduced the pH from 7 to 3.7. This was the reason for duplicate experiments in phosphate buffer pH 7.0. Novozym 342 (194 nkat/ml), Ultraflo (178 nkat/ml), Novozym 230 (95.9 nkat/ml) and Viscozyme (72.5 nkat/ml) were highest in acetylesterase activity when phosphate buffer was used. At least partly, this activity varied considerably when CA (DS 0.7) was dissolved in water. Activity differences in both systems were certainly caused by more or less favourable pH conditions for acetyl esterase activity. Testing Novozym 230 this enzyme activity increased from 95.9 nkat/ml (buffer pH 7) to 249 nkat/ml (water). Except acetylesterase, the enzymes contained substantial endoglucanase activity. Accordingly it had to be accepted that both deacetylation and depolymerization would occur to the substrate, although this investigation was preferentially aiming at an enzyme-aided reduction of the degree of substitution.

Degradation of water-soluble CA by commercial enzyme preparations

Aqueous solutions of water-soluble CA (DS 0.7) have been incubated for 20 h with all enzyme preparations listed in Table 1. Sugars and other fermentation residues had been removed from the enzymes by ultrafiltration in collodium bags in order to avoid problems in the chromatographic and spectroscopic evaluation of the incubated substrate.

Table 1. Cellulose acetate (DS0.7) degrading activities in commercial enzyme preparations

Commercial name	Producer	Enzyme source	Acetyl esterase (nkat)		Endoglucanase (nkat)
			in dist. water	in phosphate buffer pH 7	in phosphate buffer pH 7
Pectinex AP 18[1]	Novozymes		68.5	10.2	1926
SP 249[1]	Novozymes	*Aspergillus aculeatus*	64.2	57.3	4427
Novozym 230[1]	Novozymes	*Aspergillus niger*	249	95.9	1183
Ultraflo[1]	Novozymes	*Humicola insolens*	169	178	3891
Viscozyme[1]	Novozymes	*Aspergillus* spp.	61.1	72.5	25858
Pectinex BAN 120L[1]	Novozymes	*Bacillus amyloliquefacien*	77.5	49.8	4685
Gammanase[1]	Novozymes	*Aspergillus niger*	43.6	61.6	710
Novozym 342[1]	Novozymes	*Humicola insolens*	119	194	3522
Cellulase AP 3[2]	Amano	Unknown	2.6	62	411
Meicelase[2]	Meiji seika kaisha	Unknown	1.6	7.4	204
Pectinol[2]	Röhm Enzyme	Unknown	1.6	5.1	79
Pentosanase[2]	Solvay Enzymes	Unknown	1.2	6.6	600
Pectinase[1]	Sigma	*Aspergillus niger*	249	88	8381

[1]nkat/ml; [2]nkat/mg.

Aqueous size exclusion chromatography demonstrated that the cellulose acetate (DS 0.7) sample was intensively degraded by each of the enzymes (Figure 1). The reference sample had a flat, broad elution curve with a peak maximum at an elution volume of 25 ml, indicating polymeric material, whereas the major peaks of the incubated material eluted after 31 ml. The improved RI intensity, in conjunction with the intense degradation, especially as compared to the reference material, can be explained by the improved water solubility resulting in reduced filtration losses prior to SEC analysis. The preparation Cellulase AP3 seemed to contain the most efficient mixture of cellulose acetate degrading enzymes, whereas some polymeric and oligomeric material was left when CA was incubated with Novozym 230 and Ultraflo (Figure 1). The material remained water-soluble due to the fact that the polysaccharide was deacetylated and depolymerised, both at the same time.

The incubated (DS 0.7) cellulose acetate samples were also inspected by ^{13}C NMR spectroscopy, and the spectrum of the reference material in comparison to the most interesting spectra of the degraded material is given in Figure 2. The Cellulase AP3- and Ultraflo-degraded CA have been selected due to their special deacetylation pattern, whereas the Novozym degraded CA was a typical example of relatively uniform degradation.

The carbon atoms of the carbonyl groups are well suited to follow the cleavage of acetyl substituents (Kowasaka et al. 1988; Buchanan et al. 1991) The ^{13}C NMR spectrum of the reference material (bottom)

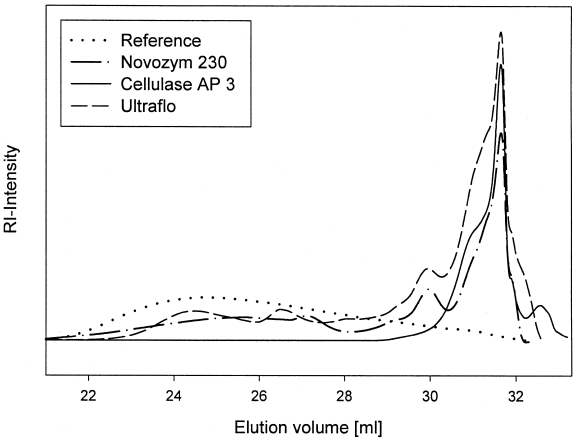

Figure 1. SEC analysis of DS 0.7 cellulose acetate after depolymerisation by commercial enzyme preparations.

gives the carbonyl signals of the acetyl substituents in the C6 (169.2–169.8 ppm), C3 (168.5–169.2 ppm) and C2 (167.7–168.5 ppm) positions. In comparison to this spectrum it can be interpreted that the acetyl substituents at the C3 and C2 positions have been preferentially removed by Cellulase AP3, whereas the C6 substitution seems to remain intact. This appeared to be the same deacetylation regioselectivity, reported by Biely et al. (1996a) for the purified acetylxylan esterase from *Streptomyces lividans* and methyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside, as the model compound. A preferential deacetylation of the 3-position, as reported by the same group (Biely et al. 1996b) for the acetylxylan esterase from *Schizophyllum commune*, was not found in this study. An-

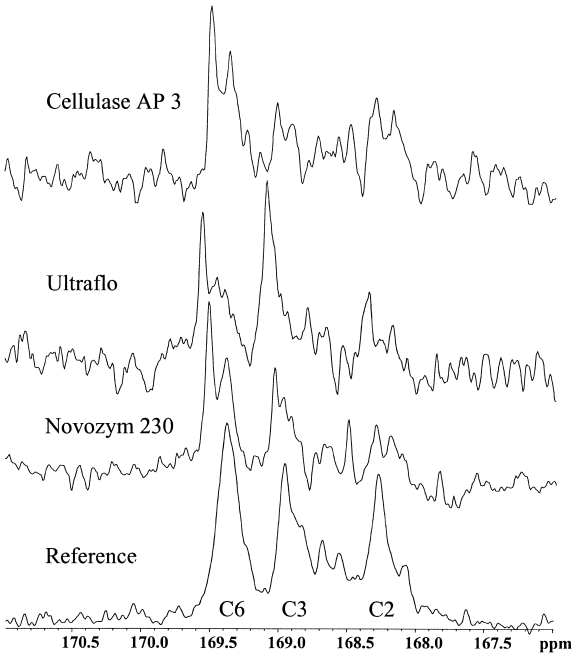

Figure 2. Carbonyl range of the ^{13}C NMR spectra of DS 0.7 cellulose acetate after incubation with commercial enzyme preparations.

other deacetylation mode became apparent from the NMR spectrum of the Ultraflo-modified (DS 0.7) CA. The substituents at the C2 position and the C6 position have been partly removed, with the effect that the acetyl groups at the C3 position seemed to be predominant. The substitution pattern of the Novozym 230 treated material has been equally reduced at all three positions. This non-specific deacetylation mode, which was also typical for most of the other enzyme preparations in this study, could be due to the presence of more than one acetyl esterase in the commercial enzyme preparations. From the results obtained so far Cellulase AP3 and Ultraflo could be considered as interesting enzyme sources for the isolation of regioselectively deacetylating esterases. Both enzyme preparations contain a huge variety of different hydrolases, including endoglucanases. Accordingly, it cannot be excluded that the acetyl esterases identified in this work only act in synergy with endoglucanases.

Deacetylation efficiency of enzyme preparations towards water-insoluble CA

Cellulose acetates in the range between DS 0.7 and 2.3 have been incubated with the enzyme preparations from Figure 2, and the time course of acetic acid liberation has been determined (Figures 3–5). The three enzyme preparations contained acetyl esterase activity capable to deacetylate CA in the range of DS 0.7 to 1.4. Accordingly, this enzyme activity was not only restricted to the water-soluble (DS 0.7) CA substrate. However, none of the enzyme preparations were able to deacetylate cellulose acetate DS 2.3. In a comparison of the three enzyme sources the Ultraflo derived enzyme was most powerful, deacetylating more than 80% of the existing acetyl substituents from CA (DS 0.7 and 0.9) after 100 h. The deacetylation rate was reduced to 50% when the DS 1.4 CA was used (Figure 3). In the early stages of incubation substantial quantities of acetyl substituents have been removed, whereas in the later stages of incubation the amount of liberated acetic acid levelled off. A similar effect was already observed in acetylxylan degradation, when the *Streptomyces lividans* acetylesterase rapidly deacetylated monoacetylated xylopyranosyl residues, but attacked doubly acetylated residues much more slowly (Biely et al. 1996a). Ultraflo was the only preparation capable to slightly deacetylate (10% within 220 h) CA DS 1.8 (Figure 3).

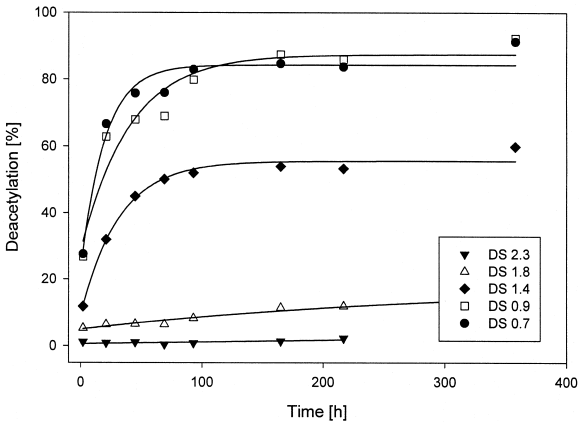

Figure 3. Acetic acid liberation as a function of DS by the 'Ultraflo' enzyme preparation.

Compared to Ultraflo the Novozym 230 deacetylation curves ran less steep and the maximum values for CA (DS 0.7 and 0.9) did not exceed 75% (Figure 5). A maximum of 50% of acetyl groups from CA (DS 1.4) could be removed by this preparation, whereas in this case the deacetylation rate of Ultraflo was nearly 50%.

The flattest deacetylation curves were observed after incubation of the DS 0.7, 0.9 and 1.4 CA with the Cellulase AP3 preparation (Figure 4). The deacetylation rates for the first two substrates did not exceed 60%, whereas 20% acetyl groups were released from

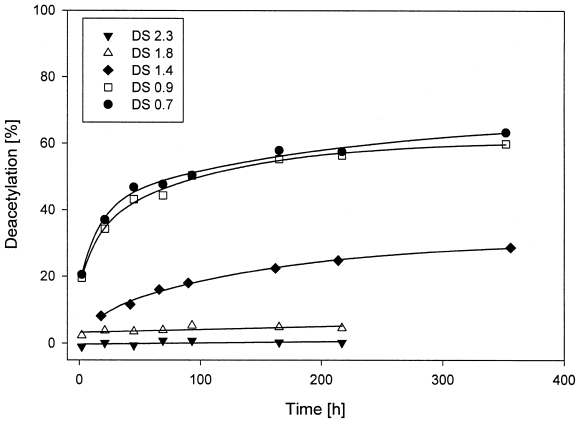

Figure 4. Acetic acid liberation as a function of DS by the 'Cellulase AP3' enzyme preparation.

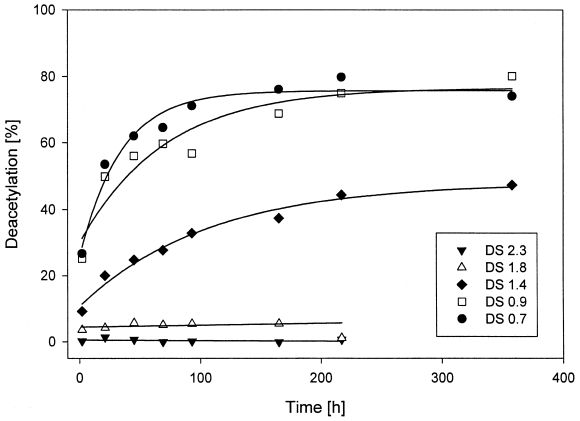

Figure 5. Acetic acid liberation as a function of DS by the 'Novozym 230' enzyme preparation.

the DS 1.4 CA. The comparatively low deacetylation rates can partly be explained from the NMR result, which suggested a regioselective deacetylation of the enzyme preparation, leaving the acetyl substituents at the C6 position completely preserved. This conclusion is in agreement with the proportion of the C6 acetylated substituents in the initial material, amounting to 37%.

A deacetylation rate of more than 80% by the Ultraflo preparation would point to a non-selective deacetylation behaviour of this preparation. Accordingly, the acetyl substituents from the C2 and C6 positions might have been preferentially removed in the initial phase of incubation, as has been found in the NMR investigations (Figure 2). However, in the later stages of incubation removal of the 2- and 6-acetyl group was accompanied by a slower deacetylation at position 3.

Conclusions

Acetylesterases deacetylating cellulose acetate have a wider distribution than could be anticipated. They seem to be present in nearly every fungal enzyme preparation being commercialised for plant cell wall modification. However, the action of acetylesterases seems to be limited to cellulose acetates of DS $\leqslant$ 1.4. One of the enzyme preparations under investigation shows an interesting feature by preferentially deacetylating a certain position within the substituted anhydroglucose units. A principal deacetylation at the C2 and C3 positions would suggest that the enzymes described here would be real acetylxylan esterases. However, most esterases were also able to cleave the acetyl substituents at the C6 position, which is not present in naturally occurring xylan. Accordingly, the most interesting enzymes described here must be purified in order to explore their real potential.

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