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Stereochemical course of hydrolysis and hydration reactions catalysed by cellobiohydrolases I and II from *Trichoderma reesei*

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Cellobiohydrolase I from *Trichoderma reesei* catalyzes the hydrolysis of methyl β-D-cellotrioside (K_m = 48 μM, k_{cat} = 0.7 min^{-1}) with release of the β-cellobiose (retention of configuration). The same enzyme catalyzes the *trans*-hydration of cellobial (K_m = 116 μM, k_{cat} = 1.16 min^{-1}) and lactal (K_m = 135 μM, k_{cat} = 1.35 min^{-1}), presumably with glycosyl oxo-carbonium ion mediation. Protonation of the double bond is from the direction opposite that assumed for methyl β-cellotrioside, but products formed from these prochiral substrates are again of β configuration. Cellobiohydrolase II from the same microorganism hydrolyzes methyl β-D-cellotetraoside (K_m = 4 μM, k_{cat} = 112 min^{-1}) with inversion of configuration to produce α-cellobiose. The other reaction product, methyl β-cellobioside, is in turn partly hydrolysed by cellobiohydrolase II to form methyl β-D-glucoside and D-glucose, presumably the α-anomer. Reaction with cellobial is too slow to permit unequivocal determination of product configuration, but clear evidence is obtained that protonation occurs from the *si*-direction, again opposite that assumed for protonating glycosidic substrates. These results add substantially to the growing evidence that individual glycosidases create the anomeric configuration of their reaction products by means that are independent of substrate configuration.

Cellulase; Cellobiohydrolase; Reaction mechanism; Hydrolysis; Hydration; (*Trichoderma reesei*)

1. INTRODUCTION

The two cellobiohydrolases (CBH I and CBH II) present in culture filtrates of the white-rot fungus *Trichoderma reesei* attack native cellulose individually and in concerted action [1,2]. CBH I and CBH II catalyze further the hydrolysis of cellooligosaccharides (d.p. ≥ 3) and the results [3] indicate preferential release of cellobiose from the non-reducing end at least for the lower members of the series. More complicated reaction patterns exist for the higher homologues (d.p. > 3 for CBH I, d.p. > 5 for CBH II). For CBH I turnover numbers are not influenced by the degree of polymerisation whereas in the case of CBH II these numbers steadily increase. We presently report an analysis of the stereochemical course of the hydrolytic reaction. The hydration reactions, observed for the parent glycals and leading to 2-deoxysugars, are also studied.

Details on the structures of these enzymes, crystallized as their core proteins [4], will soon be available (A. Jones, personal communication), making the results obtained in previous specificity studies [3,5] and the data presented here relevant for the elaboration of structure-activity relationships.

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2. MATERIALS AND METHODS

The methyl β-D-glycosides, derived from the cellooligosaccharides, and the glycals (lactal, cellobial) were synthesised by conventional methods [6,7]. Evidence for purity and structure was obtained by HPLC analysis [3] and proton NMR analysis (see below). The chromogenic substrate, 2′-chloro,4′-nitrophenyl β-lactoside, was prepared as described [3]. All other compounds were of commercial origin and of the highest quality available.

Cellobiohydrolases I and II were purified from crude culture filtrates of *Trichoderma reesei* (QM 9414) by affinity chromatography as described [8]. Purified cellobiose dehydrogenase (cellulase free) from *Sporotrichum pulverulentum* was a generous gift from Dr G. Canavascini (Fribourg, Switzerland).

Cellobiohydrolase activities were determined with the methyl β-glycosides from cellotriose or cellotetraose and a continuous enzymic assay (cellobiose dehydrogenase) was used to measure the cellobiose formed [9]. 2′-Chloro,4′-nitrophenyl β-lactoside was used as chromogenic substrate and the glycals as cosubstrates to assay the hydration of the latter by a method developed by Hwang et al. [10]. Apparent kinetic data were obtained as described by these authors.

For the proton NMR experiments enzymes were lyophilized from aqueous solution and redissolved in 0.6 ml of 0.04 M acetic acid-d_4/NaOD buffer of pD 5.0. The samples (100–200 μM enzyme) were further dialysed at 8°C over a 72 h period against 3 changes of the same buffer to exchange its ^1H atoms for ^2H. After adding the substrate dissolved in the same buffer, the mixtures were immediately transferred to a 5 mm NMR tube and proton NMR spectra recorded (Varian VXR 500) at 25°C after several times of incubation.

3. RESULTS AND DISCUSSION

3.1. *Hydrolysis of cellooligosaccharides*

The methyl β-D-glycosides of cellotriose and

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cellotetraose are hydrolyzed by CBH I and CBH II, respectively, yielding in both cases cellobiose and methyl β-D-glucoside, respectively methyl β-D-cellobioside, as reaction products [3]. The relevant catalytic constants are reported in Table I.

The stereochemical course of the reactions is followed by proton NMR spectroscopy and the relevant data for the anomeric protons of substrates and products are gathered in Table II. Fig. 1A illustrates the anomeric region of the spectrum of 20 mM methyl β-cellotrioside in deuterated buffer, showing doublets centered at 4.52, 4.49 and 4.39 ppm referable, respectively, to the H''-1, H'-1, and H-1 protons of the substrate. Spectrum (B) of the enzymic mixture, comprising methyl β-cellotrioside and CBH I after 9 min incubation, shows new signals centered at 4.66 ppm ($J_{1,2}$ ~ 8 Hz) and 4.37 ppm ($J_{1,2}$ ~ 8 Hz) assignable to the H-1 of β-cellobiose and H-1 of methyl β-D-glucoside, respectively; the H-1 doublet of the substrate, at 4.39 ppm, is diminished relative to the control spectrum (A). These changes are more pronounced in spectrum (C) (15 min reaction). In spectra (B) and (C) the H'-1 resonance (of the non-reducing D-glucose residue of cellobiose) is part of the multiplet at 4.50 ppm that includes the H''-1 and H'-1 resonances of residual substrate, methyl β-cellotrioside (compare spectrum (D)).

The results indicate that the CBH I catalyses the hydrolysis of this substrate to form β-cellobiose plus methyl β-D-glucoside. The reaction is stereospecific; formation of α-cellobiose was not detected under the reaction conditions.

Fig. 2A illustrates part of the spectrum of a control solution of methyl β-cellotetraoside in pD 4.98 buffer. Evident are the resonance doublets of the anomeric protons H'''-1 and H''-1 centered at 4.52, of H'-1 at 4.50 and of H-1 at 4.39 ppm. Spectrum (B) of the enzymic test mixture, comprising this substrate and CBH II after 7 min at 25°C, shows that the substrate's H'''-1 plus H''-1 resonance at 4.52 ppm has almost disappeared and that only a small multiplet remains in its place. New resonances include a large doublet at 5.21 ppm ($J_{1,2}$ ~ 3.7 Hz) referable to the H-1 equatorial proton of α-cellobiose. Also new are two large overlapping doublets centered at 4.50 and 4.49, each with a coupling constant of ca. 8 Hz, assignable to the H'-1 resonance of cellobiose and methyl β-cellobioside, respectively. The large H-1 resonance of α-cellobiose relative to that of β-cellobiose is again observed in spectrum (C) at 27 min.

The presence in spectrum (B) of a small doublet at 4.36 ppm ($J_{1,2}$ ~ 8 Hz) assignable to the H-1 resonance of methyl β-D-glucoside – and especially its increase with time relative to the doublet at 4.39 ppm assigned to H-1 of the primary hydrolytic product, methyl β-cellobioside – indicates the occurrence of some secondary hydrolysis of the latter compound. (From the weights of sectioned 4.36 ppm and 4.39 ppm resonance areas from enlarged copies of successive NMR spectra, the extent of methyl β-cellobioside hydrolysis was estimated to be 13.4% at 7 min and 24.5% at 16 min, but thereafter to rise only slightly to 30.6% after 24 h incubation.) That D-glucose is produced in the reaction is apparent from the presence of the H-1 resonance of β-D-glucose at 4.63 ppm in spectra (C) and (D). Its delayed appearance and low intensity relative to the H-1 of methyl β-D-glucoside at 4.36 ppm, however, suggests that α- rather than β-D-glucose is the primary

Table I

Kinetic parameters of some substrates of CBH I and CBH II

Reaction substrate	CBH I		CBH II	
	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})
Hydrolysis[a] (pH 6.3, 37°C)				
methyl β-D-cellotrioside	48	0.7	–[d]	–[d]
methyl β-D-cellotetraoside	4	1.7	7.2	112
Hydration[b] (pH 5.6, 25°C)				
cellobial	116	1.16	–[d]	–[d]
lactal	135	1.35		
		(1.5[c])	–[e]	–[e]

[a] Kinetic parameters obtained graphically by double reciprocal plotting of the initial velocities of cellobiose formation [9] against substrate concentration

[b] Reactions followed by competitive spectrophotometry [10] using 2'-chloro,4'-nitrophenyl β-D-lactoside as substrate

[c] Value obtained by measuring the decrease in glycal concentration by quantitative HPLC analysis [3] at 3 mM substrate concentration

[d] Not determined

[e] No reaction

Table II

NMR data for the anomeric protons of β-D-glucose, α- and β-cellobiose and the methyl β-D-glycosides of glucose, cellobiose, cellotriose and cellotetraose[a] (500 MHz, D_2O)

Chemical shift[b] δ (ppm)	Coupling constant $J_{1,2}$ (Hz)	Assignment
5.21	3.7	H-1: α-D-glucose, α-cellobiose
4.65	8.0	H-1: β-cellobiose
4.63	8.0	H-1: β-D-glucose
4.52	7.9	H'''-1: MeG_4
		H''-1: MeG_4, MeG_3
4.50	7.5	H'-1: α-, β-cellobiose
4.49	7.5	H'-1: MeG_4, MeG_3, MeG_2
4.39	8.0	H-1: MeG_4, MeG_3, MeG_2
4.36	8.0	H-1: MeG

[a] MeG_4 = methyl β-cellotetraoside; MeG_3 = methyl β-cellotrioside; MeG_2 = methyl β-cellobioside; MeG = methyl β-D-glucoside. H-1, anomeric proton resonance of D-glucose and the D-glucosyl residue linked to -OMe; H'-1, that of the D-glucosyl residue linked to the 4-OH position of methyl β-D-glucoside or D-glucose; H''-1, that of the D-glucosyl residue linked to the 4-OH position of methyl β-cellobioside; H'''-1, that of the D-glucosyl residue linked to the 4-OH position of methyl β-cellotrioside

[b] Relative to 3-(trimethylsilyl)propanesulfonic acid sodium salt

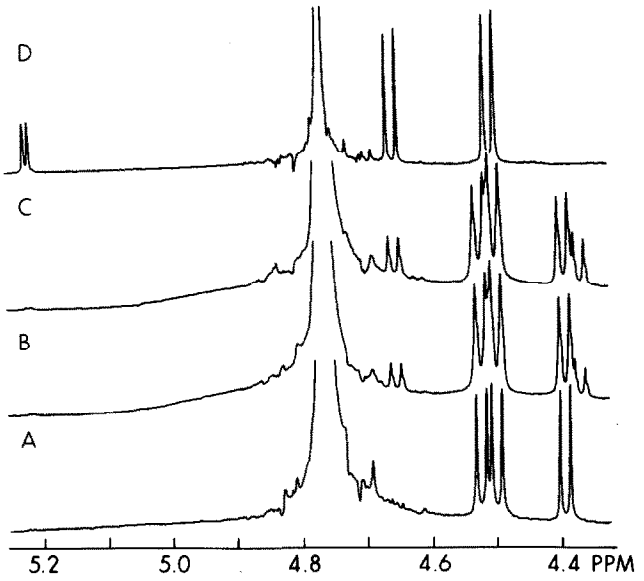

Fig. 1. ^{1}H NMR spectra (500 MHz) of methyl β-cellotrioside and enzymic digest with CBH I. (A) Reference spectrum of methyl β-cellotrioside (20 mM). (B) Enzyme digest comprising 20 mM substrate and 5.5 mg·ml^{-1} (110 μM) enzyme in pD 5.09 buffer (25°C) after 9 min; (C) after 15 min. (D) Reference spectrum of anomerically equilibrated cellobiose (20 mM).

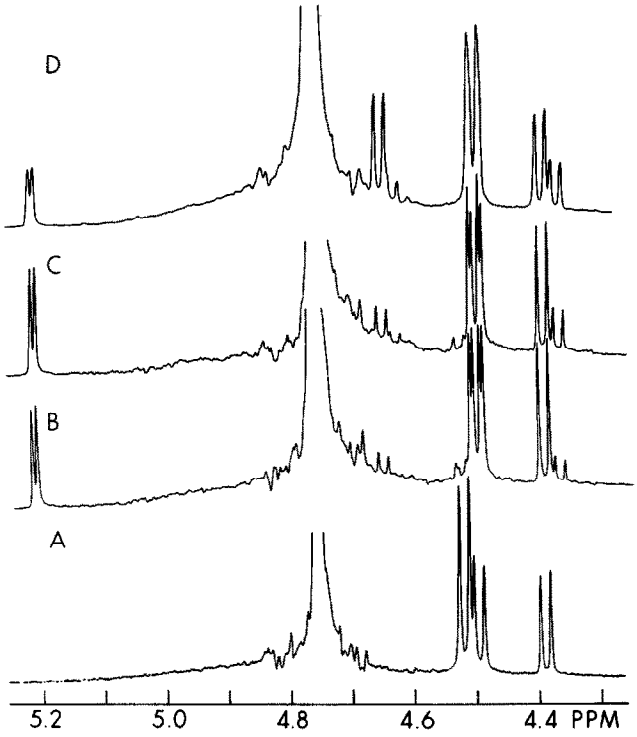

Fig. 2. ^{1}H NMR spectra (500 MHz) of methyl β-cellotetraoside and enzymic digest with CBH II. (A) Control of 20 mM methyl β-cellotetraoside. (B) Reaction mixture comprising 20 mM methyl β-cellotetraoside plus 7.2 mg·ml^{-1} (144 μM) CBH II in pD 4.98 buffer, incubated at 25°C for 7 min; (C) 27 min; (D) 24 h.

product of methyl β-cellobioside hydrolysis, in keeping with the enzymic inversion reaction of methyl β-cellotetraoside. Direct evidence is lacking, but the observed doublet at 5.21 ppm could represent both the H-1 equatorial resonance of α-D-glucose and that of α-cellobiose (see Table II).

3.2. *Hydration of glycals*

Hydration reactions of cellobial and lactal catalyzed by CBH I were followed by a competitive spectrophotometric method [10]. The resulting apparent kinetic constants are gathered in Table I. Turnover numbers are in the range observed for cellobial hydrated by *Irpex lacteus* Ex-1 cellulase [7] and are in fact similar to values observed for the hydrolysis of the cellooligosaccharides by CBH I [3]. K_m values are, however, much higher.

The cellobial proton NMR reference spectrum with H-1 resonance at 6.48 ppm ($J_{1,2}$ ~ 5.7 Hz), and the H′-1 doublet of the anomeric proton of the β-D-glucosyl moiety at 4.61 ($J_{1,2}$ ~ 8 Hz) is shown in Fig. 3A. Signals referable to the H-1, H′-1 or H-2 resonances of β-2-deoxy-cellobiose are absent. Spectrum (B) is of a digest of cellobial and CBH I after 15 min incubation (pD 5.09) and shows clear doublets at 4.95 ppm ($J_{1,2}$ ~ 9 Hz) and 4.52 ppm ($J_{1,2}$ ~ 7.9 Hz) representing the H-1 and H′-1 resonances, respectively, of β-2-deoxy-cellobiose. In addition, a multiplet is evident at 1.5 ppm, attributable to the H-2 axial proton of β-2-deoxy-cellobiose. These three resonances are seen to be much increased in spectrum (C) of the CBH I digest after 43 min incubation. At this time, small signals at 5.38 ppm and at 1.7 ppm – the H-1 and H-2 axial resonances of α-2-deoxy-cellobiose – are evident. A barely detectable multiplet at 2.3 ppm, assignable to the H-2 equatorial proton of β-2-deoxy-cellobiose, is present. The data show that CBH I catalyses *trans*-hydration of cellobial with deuteration from the *si*-face and production of β-[2(e)-^{2}H]cellobiose. The slow formation of a little α-[2(e)-^{2}H]cellobiose is attributed to non-enzymic anomerisation of the primary product.

The results with the lactal (not shown) are very similar to those obtained with cellobial and the same conclusions regarding the stereochemical course of the hydration to the β-anomer of 2-deoxylactose are valid. In both cases protonation of the double bond is from a direction opposite that assumed for protonating cellulosic substrates, but product configuration matches that of products from methyl β-cellotrioside (see above) or β-cellobiosyl fluoride [5].

Spectrum (D), of a digest of cellobial and CBH II incubated for 65 min, shows comparable small resonances at 5.38 and 4.95 ppm, representing the H-1 signals of α- and β-2-deoxy-cellobiose, respectively. That CBH II, in slowly catalysing the hydration of cellobial, protonates the enolic bond from the *si*-direction is substantiated by the findings (spectrum (E)) obtained with the 2-deoxy-cellobiose-d_1 isolated from

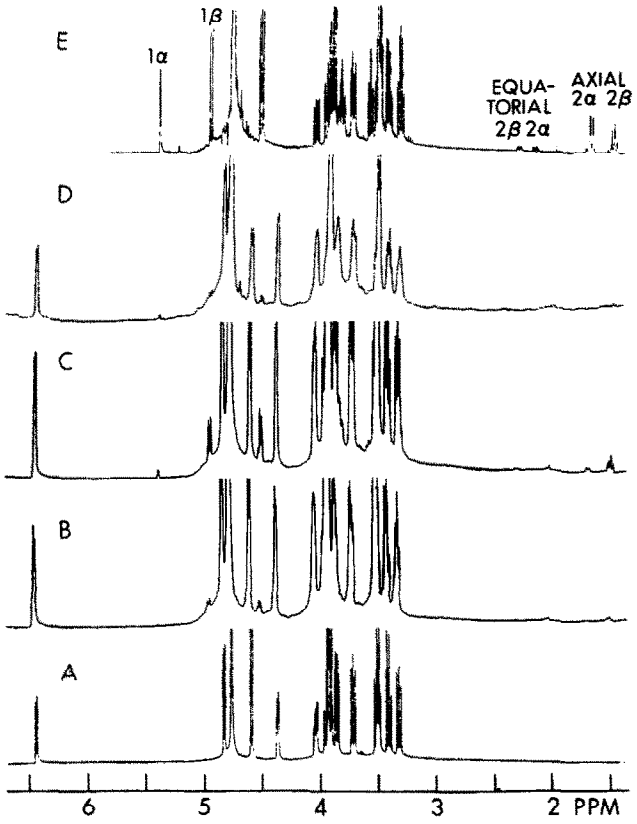

Fig. 3. ^{1}H NMR spectra (500 MHz) of cellobial and enzymic digests of the glycal with CBH I, respectively CBH II. (A) Control spectrum of 100 mM cellobial in D_2O buffered at pD 4.99. (B) Test mixture comprising 162 μM CBH I and 80 mM cellobial after 15 min incubation (pD 5.09, 25°C). (C) Same digest after 43 min. (D) Test mixture comprising 692 μM CBH II and 100 mM cellobial in pD 5.00 buffer and incubated at 25°C for 65 min. (E) Anomerically equilibrated 2-deoxy-cellobiose-d_1 isolated from previous digest (24 h incubation at 25°C) by preparative paper chromatography [7].

the digest incubated at 25°C for 24 h. The anomerically equilibrated product is characterised by prominent H-2 (a) proton resonances (multiplets) of α- and β-2-deoxy-cellobiose at 1.7 and 1.5 ppm; only small H-2 (e) resonances are seen at 2.15 and 2.3 ppm, revealing that a deuteron is present at the equatorial position at C-2. Findings are further suggestive that α-2-deoxy-cellobiose is initially formed as in the case of CBH I.

In conclusion, the conventional assumption that glycosidases operate either by 'inversion' or 'retention' (single or double nucleophilic displacements) [11] cannot account for the ability of an individual enzyme to impress the same specific configuration on the product(s) of its reaction with both prochiral and chiral substrates. This is clearly demonstrated in the case of CBH I and CBH II from *Trichoderma reesei*, both enzymes' catalytic groups responding differently in protonating cellooligosaccharides and glycals. These as well as several other findings, e.g. in [12], strongly suggest that product configuration is determined by inherent structural features of the enzymes as will hopefully become clear when more three-dimensional data become available.

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REFERENCES

[1] Fagerstam, G. and Pettersson, G. (1979) FEBS Lett. 98, 363–367.
[2] Henrissat, B., Driguez, H., Viet, C. and Schulein, M. (1985) Bio/Technology 3, 722–726.
[3] Claeyssens, M., Van Tilbeurgh, H., Tomme, P., Wood, T. and McRae, S. (1989) Biochem. J. 261, 819–825.
[4] Bergfors, T., Rouvinen, J., Lehtovaara, P., Caldentey, X., Tomme, P., Claeyssens, M., Pettersson, G., Teeri, T., Knowles, J. and Jones, T.A. (1989) J. Mol. Biol. 209, 167–169.
[5] Knowles, J., Lehtovaara, P., Murray, M. and Sinnott, M. (1988) J. Chem. Soc. Chem. Commun. 1988, 1401–1402.
[6] Wolfrom, M.C. and Haq, S. (1964) Tappi 47, 183–185.
[7] Kanda, T., Brewer, C.F., Okada, G. and Hehre, E.J. (1986) Biochemistry 25, 1159–1165.
[8] Tomme, P., McRae, S., Wood, T. and Claeyssens, M. (1988) Methods Enzymol. 160, 187–193.
[9] Canevascini, G. (1988) Methods Enzymol. 160, 112–116.
[10] Hwang, S.Y., Brown, K.S. and Gilvarg, C. (1988) Anal. Biochem. 179, 161–167.
[11] Sinnott, M.L. (1988) in: Enzyme Mechanisms (Page, M.I. and Williams, A. eds) pp.259–296, Royal Society of Chemistry, London.
[12] Weiser, W., Lehman, J., Chiba, S., Matsui, H., Brewer, C.F. and Hehre, E.J. (1988) Biochemistry 27, 2294–2300.