RESEARCH COMMUNICATION

The stereochemical course of reactions catalysed by the cellobiohydrolases produced by *Talaromyces emersonii*

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Three forms of exocellobiohydrolase (EC 3.2.1.91), CBH IA, CBH IB and CBH II, were isolated to apparent homogeneity from culture filtrates of the aerobic fungus *Talaromyces emersonii*. CBH IA and CBH II appear to be native forms of these enzymes, while CBH IB may represent a proteolytic degradation product of the CBH IA enzyme. The hydrolysis of β -cellobiosyl fluoride by each form was monitored by ^1H -n.m.r. spectroscopy. The reactions catalysed by CBH IA and CBH IB proceed with retention of the anomeric configuration, whereas that catalysed by CBH II is one of inversion. Thus one may deduce that CBH IA (or CBH IB) and CBH II operate double and single displacement reactions respectively during catalysis of substrate. On the basis of these findings and the observed substrate specificities of the various forms, one may conclude that CBH IA (and CBH IB) is a family C enzyme, while CBH II belongs to family B [Henrissat, Claeysens, Tomme, Lemesle & Mornon (1989) *Gene* **81**, 83–95].

INTRODUCTION

A variety of aerobic fungi and aerobic bacteria produce complete cellulase systems, i.e. systems that catalyse the extensive degradation of crystalline cellulose (Coughlan & Ljungdahl, 1988). Hydrolysis of the substrate by such systems is brought about by the synergistic action of endo- β -1,4-glucanases (1,4- β -D-glucan 4-glucanohydrolase; EC 3.2.1.4) and exocellobiohydrolases (1,4- β -D-glucan cellobiohydrolase; EC 3.2.1.91). The former catalyse the random cleavage of cellulose, thereby producing chain ends from which the latter remove cellobiose units (Wood, 1985). The reaction is completed by extracellular or cell-bound β -glucosidases that cleave the resulting cellobiose to glucose.

It is generally found that the endo- and exo-acting enzymes in question exist in culture filtrates in a number of different forms (see e.g. Coughlan, 1985). One reason put forward to explain this multiplicity is that, since each glucosyl unit in a cellulose chain is rotated 180° with respect to its neighbours, an enzyme approaching the surface of the crystalline substrate will encounter two stereochemically different β -1,4-glycosidic linkages (Wood, 1985). Thus hydrolysis of internal linkages will require the operation of at least two types of endoglucanase differing in stereospecificity. Depending on which type of glycosidic linkage is hydrolysed by the endo-acting enzymes, two different types of chain ends would be produced and would require two stereospecifically different types of cellobiohydrolase to effect removal of cellobiose.

While the stereospecificity of any cellulase has yet to be examined, the stereochemical course of the reactions catalysed by a few such enzymes has been investigated. Cellobiohydrolase type I (CBH I) and endoglucanase type I (EG I) from *Trichoderma reesei* (Knowles *et al.*, 1988; Claeysens *et al.*, 1990) are retaining enzymes, i.e. the anomeric configuration of the glycosidic linkage cleaved is retained. Cex, a cellobiohydrolase, and CenB, an endoglucanase, from *Cellulomonas fimi* (Withers *et al.*, 1986; Meinke *et al.*, 1991) are also retaining enzymes. The *Trich. reesei* and *C. fimi* enzymes (the specificity of CenB has yet to be determined) can utilize artificial oligosaccharides, including lactosides and cellobiosides, as substrates (Gilkes *et al.*, 1988; Claeysens & Tomme, 1989; Kilburn *et al.*, 1990).

By contrast, CBH II and EG III from *Trich. reesei* (Saloheimo *et al.*, 1988; Knowles *et al.*, 1988; Claeysens *et al.*, 1990) and CenA, an endoglucanase from *C. fimi* (Withers *et al.*, 1986) are inverting enzymes and, while they cannot use lactosides and cellobiosides as substrates or do so very poorly, they can hydrolyse higher oligosaccharides (Gilkes *et al.*, 1988; Claeysens & Tomme, 1989; Kilburn *et al.*, 1990). A cellobiohydrolase isolated from *Phanerochaete chrysosporium* was also shown to be inverting (Streamers *et al.*, 1975). Since then, the genes encoding two cellobiohydrolases have been isolated from this source (Sims *et al.*, 1988). Thus it is possible that one of these genes encodes a retaining enzyme. That this might be the case is supported by the fact that the gene in question was isolated using as a probe the gene sequence from *Trich. reesei* that encodes CBH I, a retaining enzyme.

In the interests of finding further examples, we report on the stereochemical course of the reactions catalysed by three cellobiohydrolases isolated from the culture filtrates of *Talaromyces emersonii*. This moderately thermophilic aerobic fungus produces a complete cellulase system when grown on appropriate media (McHale & Coughlan, 1980, 1981; Moloney *et al.*, 1983).

MATERIALS AND METHODS

Organism and materials

Talaromyces emersonii, CBS814.70, obtained from Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands, was routinely cultured on Sabouraud dextrose/agar medium. Liquid-state cultivation on medium containing Solka floc as growth and inducing substrate and extraction of enzymes were performed as described earlier (Moloney *et al.*, 1983; Tuohy & Coughlan, 1990).

Abbreviation used: CBH I etc., cellobiohydrolase I etc.

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1992). 4-Methylumbelliferyl β -cellobioside and 4-methylumbelliferyl β -cellotrioside were obtained from Lambda Probes and Diagnostics (Graz, Austria); Avicel was from Merck (Darmstadt, Germany); CM-cellulose, *p*-nitrophenyl β -lactoside and chloronitrophenyl β -cellobioside were from Sigma Chemical Co. (Poole, Dorset, U.K.). Cellobiosyl β -fluoride was prepared as described by Micheel *et al.* (1955).

Enzyme purification and assay procedures

Three cellobiohydrolases, termed CBH IA, CBH IB and CBH II, were isolated from crude extracts of liquid cultures by procedures to be detailed elsewhere (M. G. Tuohy & M. P. Coughlan, unpublished work; details available on application to the authors). Since contamination of cellobiohydrolase preparations with endoglucanase activity renders difficult the interpretation of results (Wood *et al.*, 1989; Coughlan, 1990), the final steps in each purification process involved hydrophobic chromatography on phenyl-Sepharose, followed by affinity chromatography on a Sepharose 4B carrier substituted with *p*-aminobenzyl 1-thio- β -D-cellobioside (van Tilbeurgh *et al.*, 1984). Thus the final preparations had no detectable contaminating activities and yielded single peaks or bands on chromatofocusing, electrophoresis under denaturing conditions and isoelectric focusing (M. G. Tuohy & M. P. Coughlan, unpublished work).

Cellobiohydrolase activity was assayed, using the dinitrosalicylate procedure of Miller (1959), by measuring the release of reducing sugar on incubation of an aliquot of enzyme with Avicel (1.0%, w/v) in 50 mM-sodium acetate buffer, pH 5.0, for 1 h at 50 °C. In addition, activity was measured by monitoring at 410 nm the release of *p*-nitrophenol or chloronitrophenol from *p*-nitrophenyl β -lactoside or chloronitrophenyl β -cellobioside respectively, following incubation (15 min at 50 °C) of 0.5 mM substrate with an aliquot of enzyme in 100 mM-sodium acetate buffer, pH 5 (final volume 0.6 ml) (Claeysens, 1988). Some assays were carried out using microtitre plates with the filter appropriate to 405 nm (Claeysens, 1988). Reaction mixtures contained 100 μ l of 100 mM-sodium acetate buffer, pH 5, and 5 μ l of appropriately diluted enzyme. Reactions, in either type of assay, were stopped before reading by the addition of 1 vol. of 1.0 M-sodium carbonate. The absence of endoglucanase activity in the purified preparations was demonstrated by the lack of activity against CM-cellulose. Protein concentration was determined after precipitation with 5% (w/v) trichloroacetic acid and redissolution in 0.1 M-NaOH containing 2.0% (w/v) Na₂CO₃. Standard BSA was treated in the same way and protein concentration was determined by the method of Lowry *et al.* (1951).

The products of hydrolysis of 4-methylumbelliferyl β -cellobioside and 4-methylumbelliferyl β -cellotrioside were fractionated by h.p.l.c. on a column (25 cm $\times$ 0.43 cm) of Polyol RSil (10 μ m; Bio-Rad) (van Tilbeurgh *et al.*, 1982). Reaction mixtures, incubated at 25 °C, contained 200 μ l of 50 mM-sodium acetate buffer, pH 5.6, plus 20 μ l of substrate (1.0 mM) and an aliquot of enzyme. Aliquots (10 μ l) of reaction mixture were mixed with 100 μ l of acetonitrile/water (7:3, v/v). A 50 μ l sample of this mixture was applied to the column, and fractions were eluted at 1.5 ml $\cdot$ min⁻¹ using acetonitrile/water (7:3, v/v) and monitored spectrophotometrically at 313 nm.

N.m.r. spectroscopy

The stereochemical course of the enzyme-catalysed hydrolysis of β -cellobiosyl fluoride (5.8–12.0 mM) by CBH IA, CBH IB or CBH II in ²H₂O buffer (100 mM-sodium acetate, p²H 5.64) was monitored by ¹H-n.m.r. spectroscopy using a Jeol GX-270 instrument at a probe temperature of 293 K. For each experiment 200–400 transients were collected with a spectral width of 3000 Hz

and 16000 data points. The recovery delay between transients was 1 s. Resolution enhancement, when used, was achieved by applying a Gaussian weighting function to the time-domain data followed by Fourier transformation. Chemical shifts are expressed in p.p.m. downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured by reference to internal acetone (δ = 2.225 p.p.m.) with an accuracy of 0.005 p.p.m.

RESULTS AND DISCUSSION

Three cellobiohydrolases were isolated to apparent homogeneity, as determined by chromatofocusing, SDS/PAGE and isoelectric focusing, and absence of contaminating activities (M. G. Tuohy & M. P. Coughlan, unpublished work). *M_r* and *pI* values were determined to be: CBH IA (66 100, 4.54); CBH IB (56 330, 3.70); CBH II (56 240, 6.09). The corresponding values for the *Trich. reesei* enzymes are: CBH I (*M_r*, 65 000; *pI* 3.9; van Tilbeurgh *et al.*, 1986); CBH I catalytic domain (*M_r*, 56 000; *pI* 3.5; van Tilbeurgh *et al.*, 1986) and CBH II (*M_r*, 58 000; *pI* 5.9; Abuja *et al.*, 1988).

None of the purified enzymes were active against CM-cellulose, β -glucan, laminarin, xylan, *p*-nitrophenyl β -D-galactoside, *p*-nitrophenyl β -D-glucoside or *p*-nitrophenyl β -D-xyloside. CBH IA and CBH IB each hydrolysed Avicel (although the specific activity of CBH IB was considerably lower than that of CBH IA), β -cellobiosyl fluoride, *p*-nitrophenyl β -D-lactoside, chloronitrophenyl β -D-cellobioside, 4-methylumbelliferyl β -cellobioside and 4-methylumbelliferyl β -cellotrioside. Each form of the enzyme bound to the affinity gel, although CBH IB was the more readily bound. Each form was eluted from the gel using 0.1 M-lactose. CBH II was active against Avicel, β -cellobiosyl fluoride and 4-methylumbelliferyl β -cellotrioside, but was inactive against the other substrates listed above. It also bound to the affinity gel and could be desorbed by 0.01 M-cellobiose, but not by 0.1 M-lactose.

In each of the above properties, including behaviour on affinity chromatography, forms CBH IA and CBH II resemble the native CBH I and CBH II from *Trich. reesei*, while the *Tal. emersonii* CBH IB resembles the catalytic domain of CBH I from *Trich. reesei* (van Tilbeurgh *et al.*, 1984, 1986, 1988; Abuja *et al.*, 1988).

The stereochemical course of the hydrolysis of β -cellobiosyl fluoride by these enzymes was monitored using ¹H-n.m.r. spectroscopy. Fig. 1(a) shows the spectrum of β -cellobiosyl fluoride with the anomeric hydrogen signal [δ (5.187) [dd, *J*₁₋₂ 7.3 Hz, *J*_{1-F} 53.1 Hz] and H1' [δ (4.433) [d, *J*₁₋₂ 7.7 Hz]]. Signals due to the presence of 1,6-anhydrocellobiose [δ (5.379) [bs] and δ (4.531) [d, *J*₁₋₂ 7.7 Hz]] were used as internal standards. During incubation with CBH IA (Fig. 1b) or CBH II (Fig. 1c), the anomeric H signals from the substrate were replaced by those due to the anomeric H of β -cellobiose [δ (4.578) [d, *J*₁₋₂ 8.1 Hz]] and α -cellobiose [δ (5.142) [d, *J*₁₋₂ 3.7 Hz]] respectively. The products in each reaction readily mutarotated to yield, finally, an equilibrium mixture of α - and β -cellobiose (Fig. 1d).

Sinnott (1990) states that establishment of inversion requires quantities of enzyme sufficient to make the total flux to product comparable with that from accumulated product to the mutarotational equilibrium mixture, and that the analytical system must be capable of responding on a time scale that is short compared with the mutarotational constants. The n.m.r. experiments reported here were carried out at 20 °C, partly to slow the rate of equilibration of cellobiose released from β -cellobiosyl fluoride. By contrast, the temperature optima of the purified cellobiohydrolases from *Tal. emersonii* are around 70 °C (D. J. Walsh, M. G. Tuohy & M. P. Coughlan, unpublished

Fig. 1. ^1H -n.m.r. spectra of the anomeric region of β -cellobiosyl fluoride and its enzymic hydrolysis products

(a) Reference spectrum of β -cellobiosyl fluoride (12 mM in 100 mM-sodium acetate buffer, p^2H 5.64 at 20°C). (b) Spectrum of reaction mixture after 55 min of incubation of β -cellobiosyl fluoride (12.2 mM) with CBH IB ($5.9\ \mu\text{M}$) under the above conditions. (c) Spectrum of reaction mixture after 90 min of incubation of β -cellobiosyl fluoride (5.8 mM) with CBH II ($1.4\ \mu\text{M}$) under the above conditions. (d) Reference spectrum of anomerically equilibrated cellobiose obtained by hydrolysis of β -cellobiosyl fluoride (12.0 mM) as above and standing. *Signals due to 1,6-anhydrocellobiose.

work). Thus the enzyme-catalysed reactions were operating at some considerable disadvantage. Nevertheless, the time course of the reactions catalysed by CBH IA or CBH IB clearly showed that hydrolysis of β -cellobiosyl fluoride by CBH IA or CBH IB was accompanied by retention of the β -configuration, while that

Fig. 2. Time course of hydrolysis of β -cellobiosyl fluoride measured by n.m.r.

Hydrolysis by CBH IA ($0.1\ \mu\text{M}$; a), CBH IB ($5.9\ \mu\text{M}$; b) or CBH II ($1.4\ \mu\text{M}$; c) was measured as the changes in the intensities of the ^1H -n.m.r. anomeric signals H-1 ($\square$), H-1 α ($\circ$) and H-1 β ($\bullet$) of the non-resolution enhanced spectra. Experimental conditions were as described in Fig. 1.

catalysed by CBH II proceeded with inversion of the configuration (Fig. 2). That the anomeric configuration of the newly formed reducing end produced by CBH II action was inverted with respect to that of the substrate was confirmed by ^1H -n.m.r. analysis of the hydrolysis of 4-methylumbelliferyl β -cellobioside (results not shown).

Koshland (1953) stated that glycoside hydrolases that acted with retention of an anomeric configuration operated via a double displacement mechanism involving an enzyme nucleophile, while inverting glycosidases operated via single displacement of the aglycon by a nucleophilic water molecule. In his review, Sinnott (1990) claims that 37 years of subsequent investigation have produced no evidence to the contrary. It is reasonable to suppose that this is also true of the cellobiohydrolases from *Tal. emersonii*. Moreover, on the basis of substrate specificity and the stereochemistry of the reactions they catalyse, one may classify CBH IA (and CBH IB) as family C enzymes, while CBH II belongs to family B (Henrissat *et al.*, 1989). However, the relationship, if any, between the stereochemistry of the reactions catalysed by all of these enzymes and the need, imposed by the structure of cellulose (see the Intro-

duction section), for enzymes differing in substrate stereospecificity, remains to be elucidated.

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REFERENCES

- Abuja, P. M., Pilz, I., Claeysens, M. & Tomme, P. (1988) *Biochem. Biophys. Res. Commun.* **156**, 180–185
- Claeysens, M. (1988) in *Biochemistry and Genetics of Cellulose Degradation* (Aubert, J.-P., Béguin, P. & Millet, J., eds.), pp. 393–397, Academic Press, London
- Claeysens, M. & Tomme, P. (1989) in *Enzyme Systems for Lignocellulose Degradation* (Coughlan, M. P., ed.), pp. 37–49, Elsevier Applied Science, London
- Claeysens, M., Tomme, P., Brewer, C. F. & Hehre, E. J. (1990) *FEBS Lett.* **263**, 89–92
- Coughlan, M. P. (1985) *Biotechnol. Genet. Eng. Rev.* **3**, 39–109
- Coughlan, M. P. (1990) in *Microbial Enzymes and Biotechnology*, 2nd edn. (Fogarty, W. M. & Kelly, C. T., eds.), pp. 1–36, Elsevier Applied Science, London
- Coughlan, M. P. & Ljungdahl, L. G. (1988) in *Biochemistry and Genetics of Cellulose Degradation* (Aubert, J.-P., Béguin, P. & Millet, J., eds.), pp. 11–30, Academic Press, London
- Gilkes, N. R., Warren, R. A. J., Miller, R. C., Jr. & Kilburn, D. G. (1988) *J. Biol. Chem.* **263**, 10401–10407
- Henrissat, B., Claeysens, M., Tomme, P., Lemesle, L. & Mornon, J.-P. (1989) *Gene* **81**, 83–95
- Kilburn, D. G., Gilkes, N. R., Miller, R. C., Jr. & Warren, R. A. J. (1990) in *Biotechnology in Paper and Pulp Manufacture: Applications and Fundamental Investigations* (Kirk, T. K. & Chang, H.-M., eds.), pp. 551–557, Butterworth-Heinemann, Boston
- Knowles, J. K. C., Lehtovaara, P., Murray, M. & Sinnott, M. L. (1988) *J. Chem. Soc. Chem. Commun.* 1401–1402
- Koshland, D. E. (1953) *Biol. Rev.* **28**, 416–436
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- McHale, A. & Coughlan, M. P. (1980) *FEBS Lett.* **117**, 319–322
- McHale, A. & Coughlan, M. P. (1981) *Biochim. Biophys. Acta* **662**, 145–151
- Meinke, A., Braun, C., Gilkes, N. R., Kilburn, D. G., Miller, R. C., Jr. & Warren, R. A. J. (1991) *J. Bacteriol.* **173**, 308–314
- Micheel, F., Klemer, A., Baum, G., Ristic, P. & Zumbülte, F. (1955) *Chem. Ber.* **88**, 475–478
- Miller, G. L. (1959) *Anal. Chem.* **31**, 426–428
- Moloney, A. P., Considine, P. J. & Coughlan, M. P. (1983) *Biotechnol. Bioeng.* **25**, 1169–1173
- Salohelimo, M., Lehtovaara, P., Nevalainen, H., Bhikhabhai, R. & Knowles, J. K. C. (1986) *Gene* **63**, 11–21
- Sims, P. F. G., James, C. & Broda, P. (1988) *Gene* **74**, 411–422
- Sinnott, M. L. (1990) *Chem. Rev.* **90**, 1171–1202
- Streamier, M., Eriksson, K.-E. & Pettersson, B. (1975) *Eur. J. Biochem.* **59**, 607–613
- Tuohy, M. G. & Coughlan, M. P. (1991)
- Tuohy, M. G., Buckley, R. J., Griffin, T. O., Connelly, I. C., Shanley, N. A., Filho, E. X. F., Hughes, M. M., Grogan, P. & Coughlan, M. P. (1989) in *Enzyme Systems for Lignocellulose Degradation* (Coughlan, M. P., ed.), pp. 293–312, Elsevier Applied Science, London
- van Tilbeurgh, H., Claeysens, M. & de Bruyne, C. K. (1982) *FEBS Lett.* **149**, 152–156
- van Tilbeurgh, H., Bhikhabhai, R., Pettersson, L. G. & Claeysens, M. (1984) *FEBS Lett.* **169**, 215–218
- van Tilbeurgh, H., Tomme, P., Claeysens, M., Bhikhabhai, R. & Pettersson, G. (1986) *FEBS Lett.* **204**, 223–227
- van Tilbeurgh, H., Loontjens, F. G., DeBruyne, C. K. & Claeysens, M. (1988) *Methods Enzymol.* **160**, 45–59
- Withers, S. G., Dombroski, D., Berven, L. A., Kilburn, D. G., Miller, R. C., Jr., Warren, R. A. J. & Gilkes, N. R. (1986) *Biochem. Biophys. Res. Commun.* **139**, 487–494
- Wood, T. M. (1985) *Biochem. Soc. Trans.* **13**, 407–408
- Wood, T. M., McCrae, S. I. & Bhat, K. M. (1989) *Biochem. J.* **260**, 37–43

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