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The cellobiohydrolase I from *Trichoderma reesei* QM 9414: action on cello-oligosaccharides*

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ABSTRACT

The action of cellobiohydrolase I [(1→4)-β-D-glucan cellobiohydrolase, EC 3.2.1.91, CBH-I] from *Trichoderma reesei* QM 9414 on cello-oligosaccharides labelled with tritium in the reducing moiety has been investigated. There was a marked preference of CBH-I to attack the first three linkages at the reducing ends of the cello-oligosaccharides (cellotriose–cellohexaose). [1-^{3}H]Cellobiose and 4-methylumbelliferyl β-D-glucopyranoside served as glycosyl acceptors during the degradation of cellotriose by CBH-I.

INTRODUCTION

Cellobiohydrolase [(1→4)-β-D-glucan cellobiohydrolase, CBH, EC 3.2.1.91], a cellulolytic enzyme which degrades crystalline cellulose efficiently but is almost inactive on soluble cellulose derivatives, was identified first[1] in the filamentous fungus *Trichoderma koningii*. Several CBHs have now been described in fungal cellulolytic systems.

Two distinct CBHs with similar catalytic properties are produced by *Trichoderma reesei*[2–5] and *Penicillium pinophilum*[6]. Due to their inability to hydrolyse substituted cellulose derivatives, CBHs have long been considered as exo-(1→4)-β-D-glucanases which attack cellulose and cello-oligosaccharides from the non-reducing end. However, studies of the substrate specifities of the CBHs from *T. reesei* and *P. pinophilum*, using 4-methylumbelliferyl β-D-glycosides of cello-oligosaccharides[7,8], as well as the efficient hydrolysis of mixed (1→3)/(1→4)-β-D-glucans[9,10] indicated that there is no sharp boundary between endo- and exo-(1→4)-β-D-glucanases. The catalytic properties of the products of cloned *T. reesei* genes of CBH-I and CBH-II, which were obtained without contamination from other cellulases, has indicated[11] that (1→3)/(1→4)-β-D-glucans are hydrolysed exclusively by CBH-II but not by CBH-I.

CBH-I remains a candidate for the exo-(1→4)-β-D-glucanase amongst the cellulases[6] of *T. reesei*. However, CBH-I does not behave according to the concept[12] that an exo-glycanase yields products whose anomeric configuration is the opposite of that of the glycosidic bond cleaved, since configuration is retained[13,14]. Thus, CBH-I could exhibit transferase activity[15]. Although not yet documented experimentally, cellulose-

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bound glycosyl–enzyme intermediates of CBH-I are postulated to have an important role in the mechanism of enzymic hydrolysis of cellulose[15].

In seeking to determine the role of CBH-I in the hydrolysis of cellulose, the action of purified CBH-I from *T. reesei* QM 9414 on a series of cello-oligosaccharides, each labelled with tritium at the reducing end, has been investigated. These compounds enabled the initial bond-cleavage frequencies to be determined and the occurrence of resynthesis to be studied.

EXPERIMENTAL

Enzyme. — A lyophilised preparation[2] of purified CBH-I from *T. reesei* QM 9414 was a generous gift from Drs. M. Gritzali and R. D. Brown, Jr., (University of Florida). The purity of the enzyme was checked by isoelectric focusing followed by detection of cellulolytic activities using chromogenic and fluorogenic substrates[16]. The preparation contained two components with pI values of 3.6 and 3.5, detectable with 4-methylumbelliferyl β-lactoside, and traces of endo-(1→4)-β-D-glucanase activity detectable after prolonged exposure, but no corresponding protein was detected by staining with Coomassie Blue.

The molar concentration of CBH-I was calculated on a weight basis and the known[17] molecular mass (65 kDa).

Substrates. — Cello-oligosaccharides, cellotriose (Glc_3)–cellohexaose (Glc_6), were obtained by acid hydrolysis of cellulose[18] and subsequent preparative p.c. on water-washed Whatman No. 3MM paper in 1-butanol–ethanol–water (5:4:4). Cello-oligosaccharides labelled with tritium in the reducing moiety were prepared by catalytic tritiation of a mixture of cello-oligosaccharides from V-Labs Inc. (Covington, U.S.A.) by the method of Evans *et al.*[19] Each [1-^{3}H]cello-oligosaccharide was purified by t.l.c. on Silufol (Kavalier, Czechoslovakia) by one or two developments with 1-butanol–ethanol–water (10:8:3) and detection with the diphenylamine–aniline reagent. [1-^{3}H]Cellobiose had a specific radioactivity of 1.46×10^7 MBq.mol^{-1}; the same value was assumed for the other [1-^{3}H]cello-oligosaccharides since they were isolated from the same reaction mixture.

Enzyme–substrate mixtures. — Aqueous solutions (20 μL; 0.25mM, 1.0mM, and 10mM) of unlabelled cello-oligosaccharides and fixed amounts (2 μL, 0.8–1 kBq) of [1-^{3}H]-labelled substrates were concentrated *in vacuo* and each residue was dissolved in 0.05M pyridine–acetate buffer (20 μL, pH 5.0). Each reaction was started by the addition of an appropriately dilute solution of CBH-I (0.085 μM–1.27 μM) in the same buffer, and the mixture was incubated at 30°. Aliquots (1–4 μL) were taken at intervals and analysed by t.l.c. After the detection of the standards on guide strips, the radioactivity of each oligosaccharide and D-glucose was determined on appropriate pieces of the chromatogram in a dioxane scintillation fluid SLD-31 (Spolana, Neratovice, Czechoslovakia). In order to ensure consistent quenching, each piece of the chromatogram was placed in the bottom of the scintillation vial with the layer of silica gel facing upwards.

Bond-cleavage frequencies. — Initial bond-cleavage frequencies of the cello-

oligosaccharides were determined as product ratios of [1-^{3}H]-labelled substrate referred to zero reaction time[20].

Re-utilisation of products and substrate resynthesis. — Solutions of Glc_3 or Glc_5 (10mM) containing traces of D-[U-^{14}C]glucose or 0.2mM [1-^{3}H]cellobiose were incubated with amounts of CBH-I which hydrolysed, at an appropriate rate, 10mM [1-^{3}H]cellotriose or [1-^{3}H]cellopentaose. Each mixture was subjected to t.l.c. as described above, and the radioactivity of each substrate and product was determined. A decrease in the radioactivity of the added glycosyl acceptors and an increase in that of the substrate or the products of hydrolysis indicated a resynthesis–hydrolysis mechanism.

Transfer to 4-methylumbelliferyl β-D-glucopyranoside (MeUmbGlc). — MeUmb-Glc (2mM) was incubated with CBH-I in 0.05M pyridine–acetate buffer (pH 5.0) in the presence and absence of 10mM cellotriose. Liberation of 4-methylumbelliferone (MeUmb) was followed fluorimetrically[21]. Aliquots of the reaction mixture were each diluted with 0.05M borate buffer (pH 10) and the concentration of MeUmb was determined at 430 nm (emission) using excitation at 365 nm.

Products of the degradation of cellulose. — Microcrystalline cellulose (Lachema, Czechoslovakia) or Solka Floc cellulose (James River Corp., U.S.A.) was suspended in 0.05M pyridine–acetate buffer (pH 5.0) at a concentration of 4 mg/0.1 mL and incubated with 1.5μM CBH-I at 30° under toluene. Each supernatant solution was analysed for hydrolysis products by t.l.c.

RESULTS

Mode of cleavage of cello-oligosaccharides. — Initial products ratios for the [1-^{3}H]-labelled cello-oligosaccharides were determined at 0.25, 1.0, and 10.0mM, as illustrated for the lowest concentration of [1-^{3}H]cellotetraose and [1-^{3}H]cellopentaose (Fig. 1). At the lowest concentration examined, [1-^{3}H]cellotriose was hydrolysed to give mainly labelled D-[1-^{3}H]glucose, indicating that the first linkage was the main target of

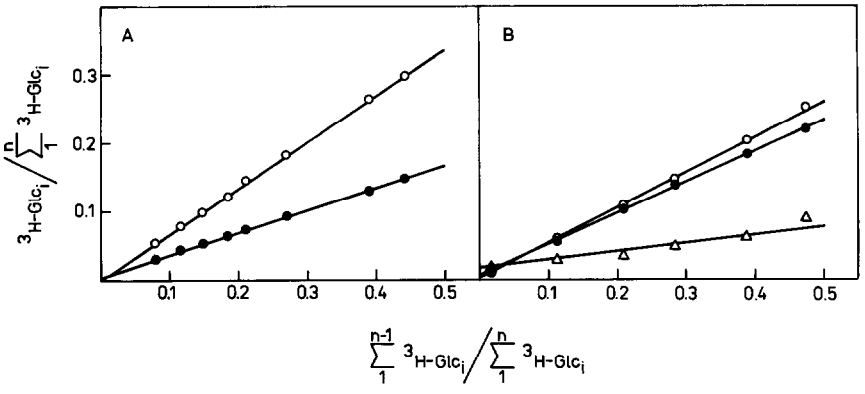

Fig. 1. Initial product ratios of A, [1-^{3}H]cellotetraose (0.25mM) and B, [1-^{3}H]cellopentaose (0.25mM) during hydrolysis by CBH-I (0.13 and 0.21μM, respectively); ●, D-[1-^{3}H]glucose; ○, [1-^{3}H]cellobiose; △, [1-^{3}H]cellotriose; [1-^{3}H]Glc_n, radioactivity in the substrate of d.p.$_n$; [1-^{3}H]Glc_i, radioactivity in a product of d.p.$_i$.

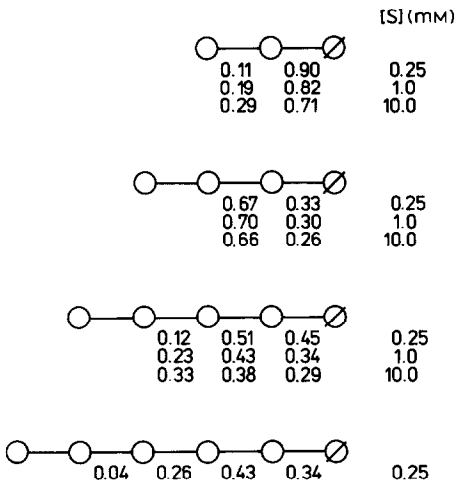

Fig. 2. Initial bond-cleavage frequencies of [1-^{3}H]-labelled cellotriose–cellohexaose by CBH-I at the concentrations [S] of substrates indicated; ○, non-labelled unit; ⊘, labelled unit.

the enzyme (Fig. 2) (the designated positions of glycosidic linkages are relative to the reducing moiety). With increasing concentrations of cellotriose, the proportion of labelled cellobiose increased slightly, whereas that of labelled D-glucose decreased. Products larger than the substrate were not formed, suggesting that the hydrolysis of cellotriose at the second linkage was not accompanied by transglycosylation.

Likewise, for [1-^{3}H]cellotetraose at both low and high concentrations (Fig. 2), labelled cellobiose was the major product of hydrolysis. However, labelled D-glucose was generated to an unexpectedly large extent, giving a ratio of [1-^{3}H]cellobiose to D-[1-^{3}H]glucose of ~ 2:1. A time course of the hydrolysis of [1-^{3}H]cellotetraose by CBH-I published earlier[22] indicated that the above ratio could be somewhat higher with μM substrate.

In contrast to [1-^{3}H]cellotetraose, the initial product ratios for [1-^{3}H]cellopentaose were dependent on the initial concentration of cellopentaose. At the lowest concentration examined (0.25mM), the product ratios of D-[1-^{3}H]glucose and [1-^{3}H]cellobiose attained similar values, whereas there was less frequent hydrolysis at the third linkage. With increasing concentration of cellopentaose, the product ratio of D-[1-^{3}H]glucose and [1-^{3}H]cellobiose decreased as the proportion of [1-^{3}H]cellotriose increased. The fourth linkage was not attacked.

The limited amount of [1-^{3}H]cellohexaose available allowed determination of the bond-cleavage frequencies only at 0.25mM. As in the shorter cello-oligosaccharides, the first three linkages were those hydrolysed most frequently by CBH-I.

Resynthesis–hydrolysis during degradation of the substrate — The incorporation of labelled D-glucose or cellobiose into the substrate and products was followed during degradation of 10mM cellotriose or cellopentaose by CBH-I. The incorporation of D-[U-^{14}C]glucose into the products did not exceed 2.3% of the total radioactivity. On

TABLE I

Incorporation of radioactivity from [1-^{3}H]cellobiose (0.2mM) into the products formed from unlabelled cellotriose (10mM) and cellopentaose (10mM) by CBH-I at concentration [E]

Substrate	*Time of incubation (min)*	*Radioactivity (%) of [1-^{3}H]Glc_2 in*			*Extent of hydrolysis[a] (%)*
		Glc	*Glc_2*	*Glc_3*	
Glc_3	0	0	100	0	100
[E] 1.27μM	5	1.2	96.1	2.3	90.8
	30	4.8	84.6	8.9	60.4
	60	7.9	84.7	5.7	45.0
	90	8.8	82.8	6.5	34.7
Glc_5	0	0	100	0	100
[E] 0.085μM	5	0.0	97.6	1.3	92.6
	30	0.13	99.8	0.0	75.3
	60	0.04	98.3	0.5	61.6
	90	0.36	95.1	1.8	57.0

[a] Determined in separate experiments in which 10mM [1-^{3}H]-labelled compounds were incubated with CBH-I at concentration [E].

following the fate of [1-^{3}H]cellobiose during the degradation of cellotriose (Table I), radioactivity appeared transitorily in cellotriose and increased progressively in D-glucose as a result of hydrolysis of newly formed [1-^{3}H]cellotriose at the first linkage. However the extent of incorporation of radioactivity from [1-^{3}H]cellobiose into the products of hydrolysis of cellopentaose was much lower.

Additional evidence for the transferring activity of CBH-I was obtained in the reaction of 10mM cellotriose in the presence of 2mM MeUmbGlc. As a result of the

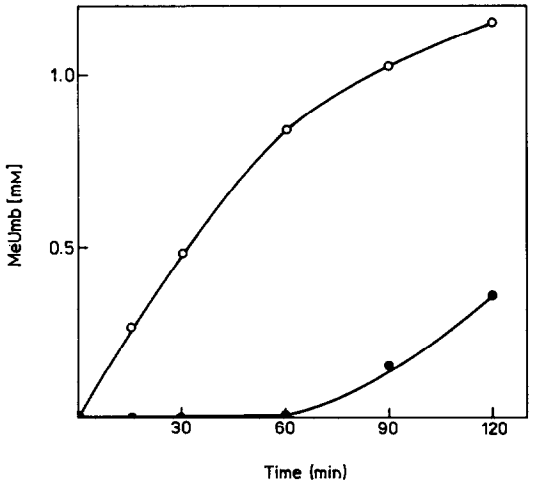

Fig. 3. Liberation of MeUmb from 2mM MeUmbGlc by CBH-I (8.0μM) in the presence (o) and in the absence (•) of 10mM cellotriose.

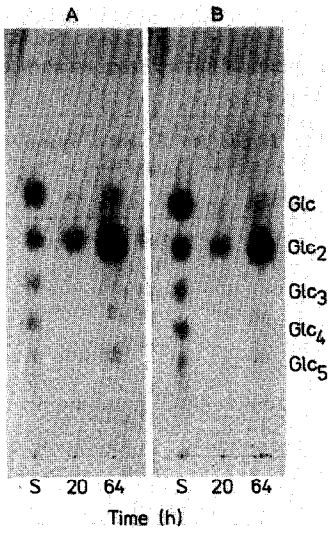

Fig. 4. T.l.c. of the products liberated by CBH-I (1.3μM) from A, microcrystalline cellulose; and B, Solka Floc cellulose: S, standards.

formation of 4-methylumbelliferyl β-cellobioside (MeUmGlc$_2$), which is a good substrate for CBH-I[7,21], liberation of MeUmb took place. The fluorescent aglycon was formed only after a long lag phase when MeUmbGlc was incubated with CBH-I in the absence of cellotriose (Fig. 3)*.

Products of the hydrolysis of cellulose by CBH-I. — In agreement with data in the literature[2–4,23,24], the main product of the action of CBH-I on microcrystalline cellulose or Solka Floc cellulose was cellobiose. T.l.c. of the products of hydrolysis (Fig. 4) revealed D-glucose to be among the initial products and cellotriose–cellopentaose appeared later.

DISCUSSION

In spite of extensive studies, the mode of action of CBH-I of *T. reesei*, which plays a crucial role in the hydrolysis of cellulose, remains to be characterised in detail. The ideal substrate to test various hypotheses about the mode of action of CBH-I[4,15,25] would be cellulose in which the individual chains were radio-labelled either on the reducing or non-reducing ends. The results with [1-^{3}H]-labelled cello-oligosaccharides now reported may be relevant to the mechanism of the interaction of CBH-I with soluble fragments of cellulose and with highly hydrated parts of the cellulose molecule.

The new quantitative data on the bond-cleavage frequencies of cello-oligosaccha-

* After this manuscript was submitted, the transglycosylation activity of CBH-I from *Trichoderma longibrachiatum* was reported [A. V. Gusakov, O. V. Protas, V. M. Chernoglazov, A. P. Sinitsyn, G. N. Kovalysheva, O. V. Shpanchenko, and O. V. Ermolova, *Biochim. Biophys. Acta*, 1073 (1991) 481–485].

rides by CBH-I considerably weaken the generally accepted view that the enzyme attacks the cellulose chain or cello-oligosaccharides from the non-reducing end. CBH-I of *T. reesei* preferentially attacked the substrates from the reducing end and did not attack exclusively the second linkage. The first three linkages were hydrolysed at rates which were dependent on the concentration of the substrate. Changes of the cleavage frequencies with changes in the concentration of the substrate were observed mainly with cellotriose and cellopentaose. The most striking observation concerned the hydrolysis of the first linkage, which released considerable amounts of D-glucose. There was a decrease in the proportion of D-[1-^{3}H]glucose with increasing concentration of the substrate, accompanied by an increase in the frequency of cleavage of the second and third linkages. The low proportion of D-glucose in the enzymic hydrolysate of cellulose (Fig. 4) suggests that this tendency continues with the insoluble polysaccharide on which CBH-I operates under constant saturation with the substrate. The large proportion of cellobiose in the CBH-I hydrolysate of cellulose also supports the idea[25] that CBH-I, once adsorbed onto the insoluble substrate, effects the cleavage of many linkages. The cleavage occurs mainly at every second linkage. The presence of larger cello-oligosaccharides in CBH-I hydrolysates of cellulose could be a consequence of the enzyme acting as an endo-glucanase.

According to a theoretical analysis[15], CBH-I could form enzyme–glycosyl intermediates and this proposal has been confirmed. Clear evidence for the reversibility of some partial reactions during degradation of the substrate, due to the existence of such intermediates, was obtained in experiments with 10mM cellotriose. Both [1-^{3}H]cellobiose and MeUmbGlc served as glycosyl acceptors during the degradation of cellotriose. The reaction sequences leading to the resynthesis of cellotriose and to the liberation of MeUmb from MeUmbGlc are depicted in Fig. 5. Cellobiose was not efficient as the glycosyl donor in the reaction of 10mM cellopentaose. This is the main difference to the transferring ability of endo-(1→4)-β-D-glucanase I (EG-I) of *T. reesei*, which incorporates radioactive cellobiose efficiently into the substrate and products for both cello-oligosaccharides[26]. This comparison proves that the transferring activity of CBH-I was not due to contamination with EG-I. The differences in the extents of glycosyl transfer reactions during the degradation of cellotriose and cellopentaose suggest that the mode of action of CBH-I may depend on the length of the substrate. That the mechanism of hydrolysis changes qualitatively with the length of the substrate has been reported for serine proteinases[27]. Interaction of remote subsites, in addition to specific interactions in the vicinity of the catalytic site, may be required for full catalytic capability of a hydrolase acting on a polymeric substrate[27]. This consideration may be even stronger for CBH-I since the enzyme has a binding domain responsible for its adsorption on to cellulose[28,29]. Such an interaction, of course, does not take place with soluble cello-oligosaccharides. Despite the fact that CBH-I is the enzyme of *T. reesei* that is particularly responsible for the hydrolysis of insoluble and crystalline cellulose, the results of the present work demonstrate, to some extent, the interaction of the enzyme with loose, hydrated reducing ends of cellulose chains.

Patterns of degradation of cello-oligosaccharides, essentially the same as those

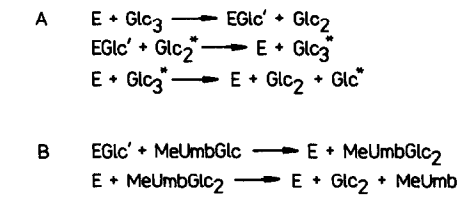

Fig. 5. Schematic representation of A, the incorporation of [1-^{3}H]cellobiose (Glc_2) into cellotriose; and B, the liberation of 4-methylumbelliferone (MeUmb) from 4-methylumbelliferyl β-D-glucopyranoside (MeUmbGlc) during the degradation of cellotriose by CBH-I.

described above, were recorded[30] with a preparation of CBH-I purified from the cellulolytic system of a different strain of *T. reesei* (VTT-D-80133)[31].

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