Limited proteolysis of the cellobiohydrolase I from *Trichoderma reesei*

Separation of functional domains

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Limited proteolysis of the cellobiohydrolase I (CBH I, 65 kDa) from *Trichoderma reesei* by papain yields a core protein (56 kDa) which is fully active against small, soluble substrates such as the chromophoric glycosides derived from the cellodextrins and lactose. Activity against an insoluble substrate, such as Avicel, is however completely lost and concomitantly decreased adsorption onto this microcrystalline cellulose is observed. The peptide (10 kDa), initially split off during proteolysis, is identified as the heavily glycosylated carboxy-terminal of the native CBH I. Depending on the experimental conditions the core protein is further nicked in between disulfide bonds, but its properties and stability do not appreciably differ from those of intact CBH I. These results lead to the proposal of a bifunctional organisation of the CBH I: one domain, corresponding to the carboxyterminal, acts as a binding site for insoluble cellulose and the other, localised in the core protein, contains the active (hydrolytic) site.

Cellulase *Cellobiohydrolase* (Trichoderma reesei) *Domain* *Enzyme adsorption* *Proteolysis* *Active site*

1. INTRODUCTION

Trichoderma reesei QM 9414 produces large quantities of cellulolytic enzymes with different specificities. Through strong synergism this complex can solubilize crystalline cellulose [1]. CBH I, which is most abundantly present, has been studied best with regard to its structural properties. The gene coding for this enzyme has been cloned [2,3] and its complete nucleic acid sequence been determined [2]. Except for the heavily glycosylated regions the CBH I protein (65 kDa) has been sequenced to near completion [4]. Twelve disulfide bonds confer stability and compactness on the enzyme [5].

CBH I is able to hydrolyse crystalline cellulose, yielding predominantly cellobiose as reaction product. It is characterized by its strong and quasi-irreversible adsorption onto insoluble substrates [6]. This enzyme also catalyses the hydrolysis of small, soluble cellodextrins and their chromophoric glycosides [7]. The possible presence of structural domains in CBH I was discussed in [8].

This study describes the effect of limited pro-

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Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; IEF-PAG, polyacrylamide gel isoelectric focusing; cellobiohydrolase (CBH), 1,4-β-glucan cellobiohydrolase (EC 3.2.1.91); MeUmb(Glc)$_n$ (n = 1–5), 4-methylumbelliferyl β-D-glycosides of cellodextrins (glucose to cellopentaose); MeUmbLac, 4-methylumbelliferyl β-lactoside; HPLC, high-pressure liquid chromatography

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teolysis of the native enzyme on its overall structure and activities. These experiments give new insights into the functional organisation of CBH I.

2. MATERIALS AND METHODS

The liquid culture filtrate of the fungus *T. reesei* was a kind gift from VTT, Technical Research Centre of Finland, Espoo. CBH II was purified by ion-exchange [9] and affinity chromatography [10]. The absorption coefficient at 280 nm is 73000 $cm^{-1} \cdot M^{-1}$ [7]. Total sugar content as D-mannose was measured at 485 nm by the phenol-sulfuric acid reagent [11].

1 mg/ml papain (Boehringer, FRG) was activated for 30 min in 50 mM ammonium acetate, pH 6.5. CBH I:papain ratios (w/w) were either 30:1 or 300:1 and the digests termed 'heavy' or 'light', respectively.

Activity measurements were carried out conventionally (0.1 M sodium acetate, pH 5.0). Avicel suspensions (10 mg/ml) were incubated (40°C) with appropriate amounts of CBH I. The concentration of reducing sugar (as D-glucose equivalents) in the supernatant was determined after 3 h using a colorimetric method (dinitrosalicyclic acid reagent). Activities (25°C) against soluble substrates (1 mM) such as MeUmbLac or MeUmb(Glc)$_n$ (n = 2–5) were determined as described [7].

Adsorption onto microcrystalline cellulose (Avicel) of intact and papain-digested CBH I was measured as the decrease in $A_{280\,nm}$ of the supernatant (after centrifugation) of 30 mg/ml Avicel suspensions incubated (pH 5.0, 25°C) with given amounts of enzyme.

Peptide analysis by reversed-phase HPLC (ProRPC column, Pharmacia, Sweden) was performed with 50 mM ammonium acetate (pH 5.0) and a linear gradient of 70% acetonitrile. Lyophilised samples were first reduced (mercaptoethanol) and alkylated (iodoacetamide) as described [4]. Amino acid analysis of isolated peptides was as reported [4,12].

Gel filtration on Biogel P-100 (Biorad, USA), SDS-PAGE and IEF-PAG (LKB, Sweden) were carried out conventionally. Enzymic activities on the IEF-PAG gels were visualised as in [13].

3. RESULTS

3.1. *Effects of limited and extensive proteolysis on the molecular properties and activity of CBH I*

Aliquots of heavy and light CBH I:papain digests were analysed at regular time intervals for residual MeUmbLac and Avicel activities (fig.1). Whereas Avicelase decreases rapidly no loss of activity against the soluble substrate is observed even after 24 h incubation.

By SDS-PAGE analysis under reducing conditions patterns were obtained as shown in fig.2A. For the light digest (ratio 300:1) a band corresponding to intact CBH I (65 kDa) is still present next to a major band (56 kDa). In the heavily degraded sample (ratio 30:1) only low molecular mass bands can be observed. It seems that a fragment (~10 kDa) is initially split off and that with high papain concentrations further 'nicking' of a core protein (56 kDa) occurs.

SDS-PAGE, without prior reduction of the samples (fig.2B), proves that even after extensive proteolysis, the higher molecular mass fraction

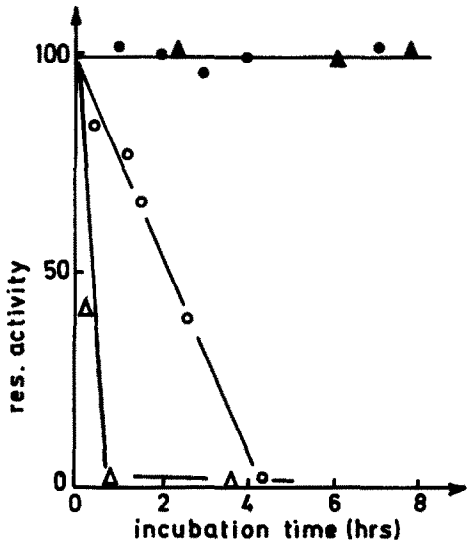

Fig.1. Residual activities of papain-digested CBH I. CBH I (180 M) was incubated (pH 5.0, 25°C) with 0.6 μM (300:1) or 6 μM papain (30:1). Aliquots (50 μl) were removed at appropriate times to measure the Avicelase activities (△,○) or the activities against MeUmbLac (▲,●). Residual activities are given as % of the original. (△) Heavy digest, (○) light digest.

persists, suggesting that peptides in the core protein are held together by disulfide bridges.

Proteolytic digests were also analysed by Biogel P-100 gel filtration (fig.3) under native conditions (100 mM NH_4Ac buffer, pH 5.0). Both limited and extensive proteolysis caused a similar reduction in molecular mass (fraction I). The carbohydrate content of this main fraction (38 kDa) was compared to that of the peptide (10 kDa; fraction II). The latter was clearly more heavily glycosylated. The poor agreement of the molecular masses as determined by the two different techniques (SDS-PAGE and gel filtration) is undoubtedly due to the fact that the core protein is still appreciably glycosylated (~5%, as D-mannose residues).

IEF-PAG analysis (fig.4) of these digests yields further information on the molecular properties of the proteolysed CBH I. The enzyme sample subjected to limited proteolysis (300:1) shows two protein bands, one (p*I* 3.9) corresponding to the intact CBH I and a second more acidic fraction (p*I* 3.5). Both bands show MeUmbLac activity. In completely digested (30:1) samples only the more acidic fraction (p*I* 3.5) persists.

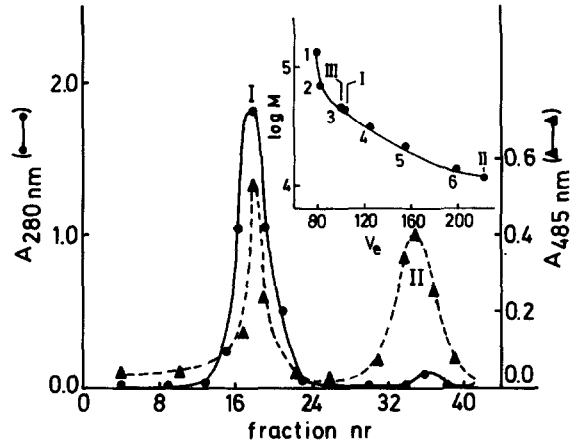

Fig.3. Biogel P-100 gel filtration of papain-digested CBH I. 1 ml CBH I (200 μM) was digested (0.7 μM papain, 6 h) and applied to the column (2 × 100 cm), run at 12 ml/h (30°C, pH 5.0). Eluates were analysed for protein (280 nm) and carbohydrate content (485 nm). Inset: calibration curve of standard proteins (1, human globulin; 2, bovine serum albumin; 3, ovalbumin; 4, carbonic anhydrase; 5, trypsin inhibitor; 6, lysozyme). Arrows indicate maxima of elution peaks (carbohydrate content, as D-mannose, in brackets): I, CBH I core protein (5%); II, glycopeptide (40%); III, native CBH I (7%).

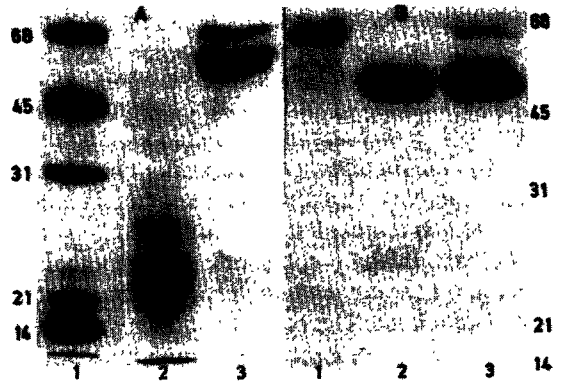

Fig.2. SDS-PAGE of papain-digested CBH I. (A) Under reducing conditions: CBH I (180 μM) was partially or completely digested (6 h) (papain concentrations as in fig.1), denatured (1% SDS, 0.1% mercaptoethanol) and run on a 15% gel. Staining with Coomassie blue. Lanes: 1, molecular mass markers; 2, heavy digest, 3, light digest. (B) Under non-reducing conditions: same samples as in A but denatured in the absence of mercaptoethanol; lanes: 1, intact CBH I; 2,3, as in A. Positions of molecular mass markers as indicated.

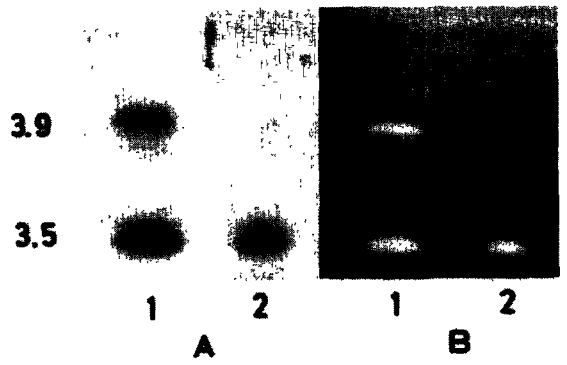

Fig.4. IEF-PAG of papain-digested CBH I. CBH I (180 μM) was treated with papain as described in fig.2. Samples (2 μl) were analysed by IEF-PAG (pH 3–10 gradient). (A) Protein staining (Coomassie blue). Lanes: 1, light digest; 2, heavy digest. (B) MeUmbLac activity [13]. Before staining with Coomassie blue the gel was immersed in 0.5 mM of the fluorogenic substrate (pH 5.0, room temperature) and transilluminated with UV to locate the enzymically active bands; lanes 1 and 2 as in A.

To characterise further the CBH I core protein and the smaller peptide, the 300:1 digest was analysed by reversed-phase HPLC (fig.5). Two peaks were observed (A and B) the latter increasing with longer incubation time. Peak B was isolated and its amino acid composition corresponds to that of the carboxyl-terminal peptide (59 amino acid residues) of intact CBH I [12]. The molecular mass of this fragment, taking into account its high carbohydrate content should be about 10 kDa.

3.2. *Enzymic properties of the proteolysed CBH I*

Specific activities against MeUmbLac and MeUmb(Glc)$_3$ are virtually the same for the core protein (turnover numbers at 1 mM substrate 12 and 8.3 min^{-1}, respectively) as for the native CBH I (11.4 and 9.2 min^{-1}). The activity against MeUmb(Glc)$_4$ and MeUmb(Glc)$_5$ is also unaffected by papain treatment.

As already pointed out, proteolysed CBH I is not active on insoluble cellulose (Avicel). Binding of intact CBH I, proteolysed mixture and isolated core protein are compared in table 1. The core protein binds 50% less effectively to the insoluble cellulose. More extensive nicking of the CBH I (30:1) does not affect the binding capacity further.

Since the loss of Avicel activity of the proteolysed CBH I is apparently due to its changed binding properties, the interaction with the affinity column (*p*-aminobenzyl 1-thiocellobioside ligand) [10] was investigated. The core protein was totally bound and could be specifically eluted with 0.1 M lactose, a competitive inhibitor.

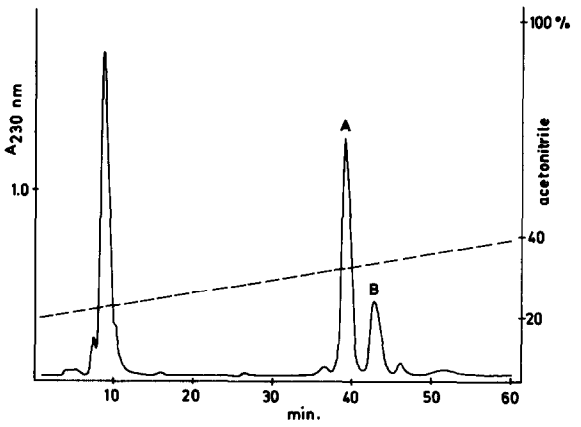

Fig.5. Reversed-phase HPLC analysis of CBH I:papain digest (300:1). Elution on the reversed-phase ProRPC 5/10 column (Pharmacia) was achieved by a linear acetonitrile gradient (– – –). Fraction A corresponds to core protein; fraction B is the glycopeptide representing the C-terminal region 439–497 of CBH I.

Table 1

Adsorption of native and papain-treated CBH I onto Avicel

Sample	% adsorption
Native	88
Native + 0.5 M cellobiose	88
CBH I:papain digest (30:1, 6 h)	38
Core protein	36

Enzyme samples were incubated (10 μM, pH 5.0, 25°C) with a 30 mg/ml Avicel suspension and shaken occasionally. % adsorption was calculated from the original and residual absorbances (280 nm) in the supernatant after centrifugation

The thermal stability of the core protein is the same as for the intact enzyme: both retain full activity (against MeUmbLac) when kept for several hours at 50°C and irreversible denaturation occurs above 60°C.

4. DISCUSSION

The most striking results from this study are that the hydrolytic activity of the CBH I from *T. reesei* against low molecular mass substrates is unaffected by incubation with papain, whereas the Avicelase activity rapidly decreases (fig.1). From the evidence presented it appears (figs 2–5) that this protease initially splits off the carbohydrate rich carboxyl-terminal while the core protein is further nicked (fig.6). The stability and active conformation of this core protein are maintained most probably through disulfide bridges present [5]. On the other hand, the loss in activity towards cellulose (Avicel) is paralleled by a decrease of the enzyme adsorption onto this insoluble substrate (table 1).

Therefore a bifunctional organisation of the CBH I is proposed: one domain, corresponding to the carboxyl-terminal of the enzyme, is implicated in the adsorption process, whereas the other contains the hydrolytic function. The relative ease with which the terminal peptide is split off suggests

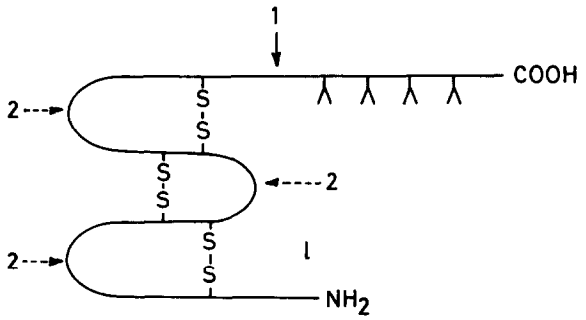

Fig.6. Sequence of events during limited proteolytic degradation of CBH I by papain. The heavily glycosylated (⅄) C-terminal is initially split off by papain (→1) and the core protein is further nicked (→2) at several cleavage sites in between the 12 disulfide bonds in native CBH I [5].

that these two parts of the protein molecule are themselves structural domains probably linked by some hinge region susceptible to proteolytic attack. Cellobiose, although a very effective competitive inhibitor (K_i = 5 × 10^4 M^{-1}, 25°C) [13], does not influence the binding of CBH I onto Avicel (table 1). We also obtained strong independent evidence for a restricted binding site (cellobiosyl residue) for soluble substrates which is different from the cellulose-adsorption site (Van Tilbeurgh, unpublished). Since adsorption on the affinity column [10] is not affected by proteolysis further evidence is provided for the fact that in this type of binding the active site present in the core protein is involved.

Thus, a cellulose adsorption site as distinct from the active (hydrolytic) site should be considered. The former could be identified with the carbohydrate-rich carboxyl-terminal of the enzyme.

The present results demonstrate the potential of proteolysis in the study of the cellulase. In the light of the observed homologies in the primary structures of these enzymes [12] this study will be extended to the other components of the complex from *Trichoderma*.

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