Detection and differentiation of cellulase components using low molecular mass fluorogenic substrates

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The 4-methylumbelliferyl β-D-glycosides of glucose, cellobiose, cellotriose and lactose are used to differentiate several exo-cellobiohydrolase, endocellulase and β-glucosidase activities in crude cellulase from *Trichoderma reesei*. Spectrophotometric or fluorimetric assays allow simple detection and quantitative measurements of eluate activities from ion-exchange chromatography and after analytical gel electrofocusing. Using the fluorophoric glucoside several β-glucosidases can be visualised after isoelectric focusing on polyacrylamide gels. The use of the lactoside and of the same substrate supplemented with cellobiose as inhibitor allows a clearcut distinction to be made between endocellulase II and exo-cellobiohydrolase I. Both enzymes are present as 'iso-enzyme' mixtures. With the cellotrioside only one fraction is detectable (endocellulase III). The same methods could be used in culture growth experiments.

Cellulase *Exo-cellobiohydrolase* *Endocellulase* *Assay* Trichoderma reesei *Fluorogenic substrate* *Isoelectric focusing*

1. INTRODUCTION

The complexity of the multi-component cellulolytic system secreted by microorganisms such as *Trichoderma* species, is the subject of current investigation and speculation [1–3]. The need for appropriate, chromogenic substrates and differentiation methods has only partially been filled. The majority of the methods are based on measurements of reducing sugars released from soluble or insoluble substrates of these enzymes: carboxymethyl-cellulose, filter papers or microcrystalline cellulose (Avicel). Alternatively viscosimetric measurements have been adapted. Methods for the detection and differentiation of cellulase components after electrophoretic separation in polyacrylamide gels have also been reported [4].

We introduced the soluble 4-methylumbelliferyl glycosides derived from a series of cello-oligosaccharides and lactose as valuable tools in the study of the enzymes from *T. reesei* QM9414 [5]. The cellobioside and lactoside were characterised as fluorogenic substrates of the purified exo-cellobiohydrolase I (EC 3.2.1.91). Here we report on the use of the same compounds in the detection of endocellulase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21) activities and the differentiation of the cellulolytic components in separated fractions after analytical isoelectric focusing in polyacrylamide gels (PAG-IEF). Further applications in enzyme purification and detec-

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Abbreviations: PAG-IEF, polyacrylamide gel isoelectric focusing; endocellulase, 1,4-β-glucan glucanhydrolase (EC 3.2.1.4); exo-cellobiohydrolase (CBH), 1,4-β-glucan cellobiohydrolase (EC 3.2.1.91); MeUmbβGlc, 4-methylumbelliferyl β-D-glucopyranoside; MeUmbβ-(Glc)$_n$ (n = 2,3), 4-methylumbelliferyl β-D-glycosides of cellobiose and cellotriose; MeUmb β-Gal β(1→4)Glc, 4-methylumbelliferyl β-lactoside; MeUmb, 4-methylumbelliferone; HPLC, high-pressure liquid chromatography

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tion of activities in agar-grown cultures are demonstrated.

2. MATERIALS AND METHODS

Commercial culture filtrate from *T. reesei* (Celluclast, NOVO, Denmark) was subjected to gel filtration (Biogel P-6) before fractionation by DEAE-Trisacryl (IBF, France) ion-exchange chromatography at pH 5.0 (5–250 mM ammonium acetate, room temperature). Homogeneous exo-cellobiohydrolase I and cellobiohydrolase II were obtained by affinity chromatography [6]. Endoglucanase II and III from *T. reesei* QM 9414, purified by ion-exchange chromatography, were kindly donated by Dr G. Pettersson (Uppsala, Sweden). Estimated p*I* values for each enzyme are as reported [7].

The 4-methylumbelliferyl β-glycosides of the cello-oligosaccharides (MeUmbβ(Glc)$_n$ n = 1–5) and of lactose (MeUmb β-Gal $\beta(1\rightarrow4)$Glc) were prepared as described [5,8]. Cellobiose, lactose and gluconolactone are commercial products (Koch Light, England).

The release of 4-methylumbelliferone (MeUmb) from the cellobioside or lactoside was measured continuously by difference spectrophotometry at 347 nm [5]. Detection by HPLC of the chromophoric reaction products (313 nm) resulting from the action of the enzymes on MeUmbβ(Glc)$_n$ was as reported [5].

All reactions were at pH 5.0 (0.1 M sodium acetate buffer) and 25°C. Protein was estimated from absorption coefficients at 280 nm as determined by dry weight measurements (H.v.T., unpublished).

Analytical isoelectric focusing (PAG-IEF) was performed on an LKB Multiphore apparatus and Ampholine PAG plates (pH 3.5–9.5). Samples were first dialysed against distilled water using an Amicon dialysis apparatus (PM-10 filters). Isoelectric point standard proteins were from LKB. After the focusing run gels were flooded with a solution of the appropriate MeUmbβ-glycoside (0.5 mM in sodium acetate buffer, pH 5.0) in the presence or absence of an inhibitor (5 mM cellobiose, or 1 mM gluconolactone). After approx. 2 min incubation (room temperature) the gels were rinsed once with distilled water. Enzyme active fractions became visible as blue fluorescent bands upon transillumination with a 6 × 15 W UV lamp (302 nm) and could be photographed (Polaroid 53) (emission maximum of MeUmb at 450 nm). The gels were then stained conventionally with Coomassie blue.

T. reesei QM 9414 (gift from Dr M. Mandels, MA) was grown (29°C) on 1% agar, containing the minimum medium [9] and either glucose (1%) or lactose (1%) as carbon source. Enzymic activity of the colonies (after 48 h growth) was detected similarly as described above using MeUmb β-Gal $\beta(1\rightarrow4)$Glc (0.5 mM) as substrate.

3. RESULTS

3.1. *Specificities of the endo- and exo-type cellulases for the MeUmb glycosides*

The specificities of the purified cellulase components from *T. reesei* were studied qualitatively by HPLC as reported [5].

Thus with the MeUmbβ(Glc)$_n$ (n = 1–3) and MeUmb β-Gal $\beta(1\rightarrow4)$Glc as substrates a differentiation between the several components of the cellulase complex becomes possible: MeUmb is a reaction product when: (i) the cellobioside or lactoside are used as substrates with either CBHI or endocellulase II. These activities however can be differentiated since the CBHI is strongly inhibited by cellobiose (K_i = 2 mM at 25°C for endocellulase II; K_i = 0.02 mM at 25°C for CBHI); (ii) the MeUmbβ(Glc)$_3$ is used as substrate for endocellulase III (this specificity is unique since with all the other enzymes the initial reaction product is MeUmbβGlc); (iii) the β-glucosidases act on MeUmbβGlc. Gluconolactone is a potent inhibitor.

Formation of MeUmb is never observed when CBHII acts on the above substrates: as reported [10] MeUmbβ(Glc)$_n$ is hydrolysed for $n \geqslant 3$, with formation of cellobiose. Further quantitative data (K_m, V) for these enzymes and substrates have been estimated and will be published elsewhere.

3.2. *Fractionation of the cellulase complex by DEAE-anion exchange chromatography and analytical isoelectric focusing*

DEAE-Trisacryl ion-exchange chromatography at pH 5.0 (5–250 mM ammonium acetate) yields 4 main fractions (fig.1). The column eluates were tested for their activity with MeUmbβGlc and with

MeUmb β-Gal β(1→4)Glc (0.5 mM) in the presence or absence of 0.01 M cellobiose. β-Glucosidase activity can be detected mainly in the fractions eluted before the application of the gradient; they also contain the bulk of endocellulase III and of CBH II as reported [7]. Upon application of the gradient, separation of endocellulase II and CBHI fractions is obtained. The 2 activities can readily be differentiated by their activity against MeUmb β-Gal β(1→4)Glc in the absence or presence of 5 mM cellobiose (fig.1). These fractions were further analysed by PAG-IEF and the activities present detected (fig.2).

The complexity of the crude cellulase preparation and the partial fractionation achieved by DEAE can be judged from the protein pattern after isoelectric focusing (fig.2A). For comparison pure CBHI (lane 5) (p*I* 3.95), endocellulase II (lane 7) (p*I* 4.0–4.6) and CBHII (lane 8) (p*I* 5.9) are shown. Lane 9 represents the 'iso-enzyme pattern' observed for CBHII. The respective p*I* values coincide with those reported [7].

As can be judged from the activity tests performed on these gels, by immersion in the appropriate substrate or substrate-inhibitor solutions (fig.2B), the MeUmbβGlc active fractions have a considerably higher p*I* than the other components of the complex and appear in the first fractions eluted from the DEAE column (lanes 2 and 3). These β-glucosidases can be completely inhibited by 1 mM gluconolactone (not shown). The endocellulase III activity (fig.2C) as measured with MeUmbβ$(Glc)_3$ is detectable in the first eluates (lane 2). Its p*I* (5.7) corresponds with the reported value [7].

The activity tests with the MeUmb β-Gal

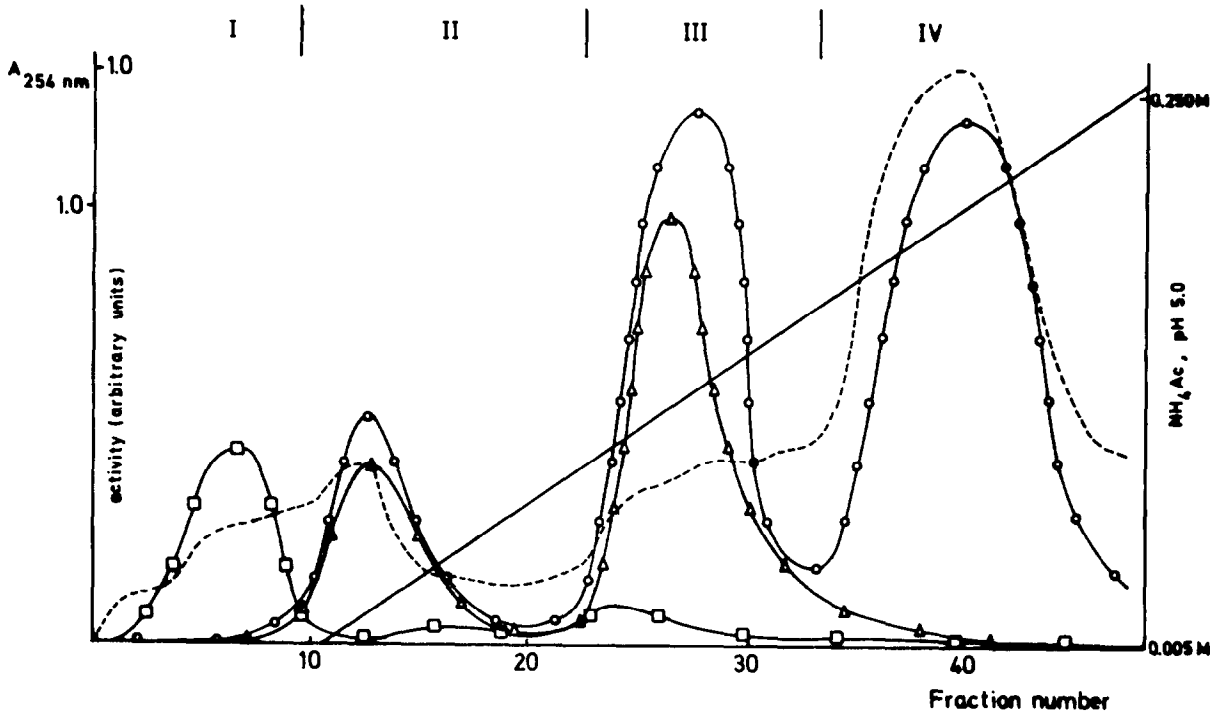
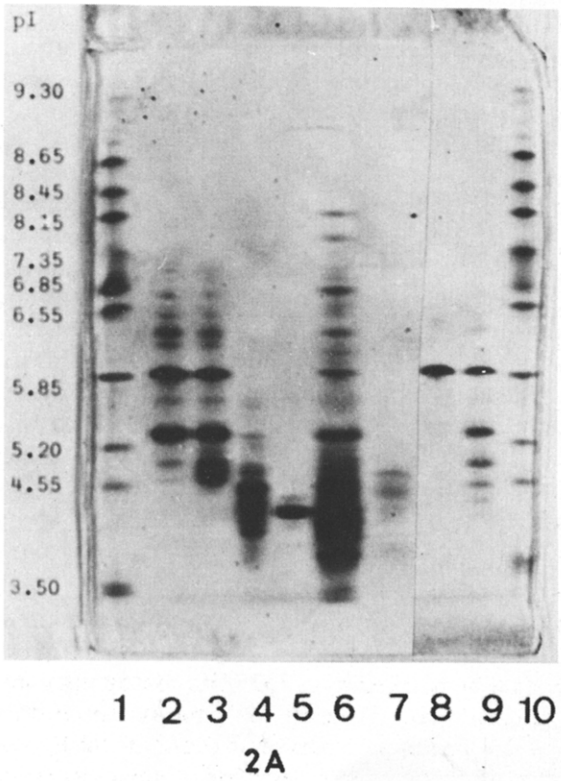
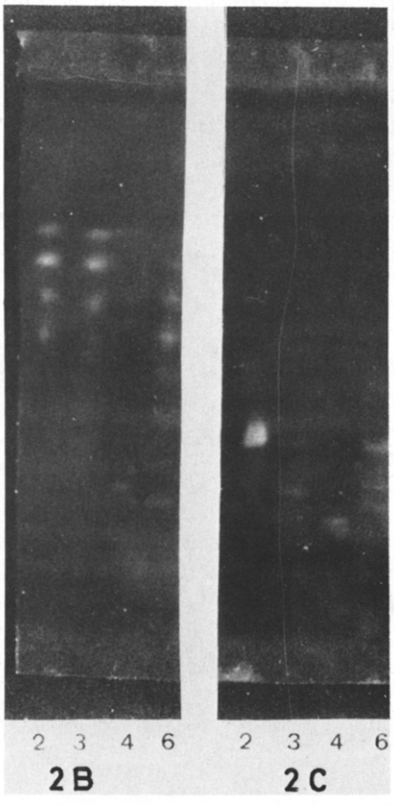


Fig.1. DEAE-Trisacryl ion-exchange chromatography of the cellulase complex from *T. reesei* QM 9414. Crude culture filtrate (5 ml) was dialysed against 5 mM ammonium acetate buffer, pH 5.0, by gel filtration (Biogel P-6), adsorbed onto a column (1 × 10 cm) of DEAE-Trisacryl and eluted with a 5–250 mM gradient of ammonium acetate, pH 5.0 (room temperature). UV absorbance of the eluates (– – –) was followed continuously at 254 nm. Activity measurements (arbitrary units) on 50 μl aliquots of the fractions (5 ml) were performed with solutions of the appropriate 4-methylumbelliferyl glycoside (1 ml): (—□—) β-glucosidase activity; (—○—) activity against the lactoside; (—△—) activity against the same substrate but in the presence of 5 mM cellobiose. Fractions I–IV were pooled as indicated. All measurements were at pH 5.0 and 25°C.





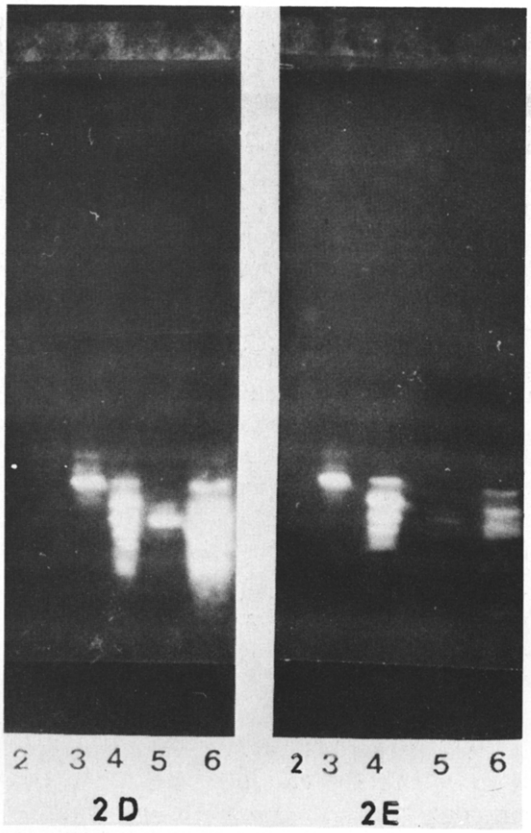


Fig.2. Analytical isoelectric focusing. The pH gradient was 3.0 (bottom) to 9.5. (A) Protein staining: reference proteins have IP as indicated (lanes 1 and 10). Lanes 2–4 show fractions I–III (fig.1). Crude culture filtrate is shown in lane 6, pure CBH I, endocellulase II and CBH II are present in lanes 5, 7 and 8, respectively. Lane 9 contains the CBH II iso-enzymes. 1–10 μg of the proteins was applied. (B) β-Glucosidase activity and (C) endocellulase III activity in fractions I and II and in crude celluclast, using MeUmbβGlc (B) and MeUmb$\beta(Glc)_3$ (C) as substrate, respectively. (D,E) CBH I and endocellulase II activities in the same fractions as detected with MeUmbβGal$\beta(1\rightarrow4)$Glc, respectively in the absence (D) or presence (E) of cellobiose. Pure CBH I is present in lane 5.

$\beta(1\rightarrow4)$Glc show considerable complexity (fig.2D, E). In the presence of cellobiose (fig.2E) the CBHI active bands are nearly completely inhibited (cf. lanes 5 and 6). Thus a qualitative distinction between endocellulase II and CBHI active fractions becomes possible.

As shown (fig.1) fraction II eluted early in the DEAE-column experiments contains mainly en-

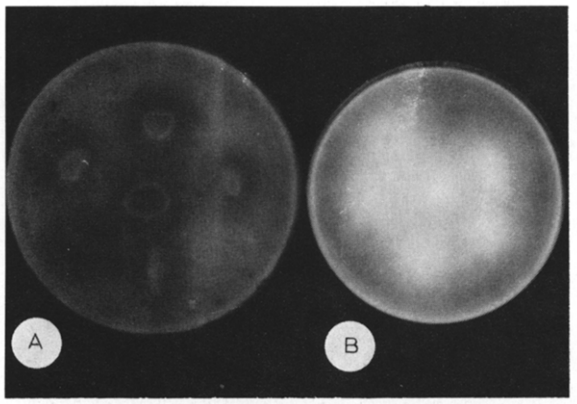


Fig.3. Colonies of *T. reesei* QM 9414 grown in the presence of 1% glucose (A) or 1% lactose (B). Activity tests with MeUmbβGal$\beta(1\rightarrow4)$Glc (0.5 mM, pH 5.0, 20 min at 30°C).

docellulase II activity (lane 3), whereas in fraction III (lane 4) some CBHI active bands can be detected.

3.3. *Detection of cellulolytic activities in colonies of* T. reesei *QM 9414*

Certain oligosaccharides, such as cellobiose, sophorose and lactose act as inducers of the cellulolytic system of *T. reesei* [9]. In contrast glucose is reported to act as a repressor of the same system [11]. The fluorophoric substrates described here are potentially adaptable for the detection of the cellulolytic activity in agar grown colonies of the microorganism. Thus, colonies grown in the presence of 1% glucose are unable to hydrolyse the MeUmb β-Gal $\beta(1\rightarrow4)$Glc in contrast to the colonies induced with 1% lactose (fig.3).

4. DISCUSSION

Recently strong evidence has been obtained to show that the presence of multiple enzyme forms is an inherent property of the cellulase of *Trichoderma* species and cannot be considered solely as the result of proteolytic, postsecretional modifications [1–3]. In an attempt to delineate more clearly the specificities of the component enzymes of the cellulase complex we propose the use of small, chromophoric substrates, i.e. the 4-methylumbelliferyl β-glycosides of glucose, cellobiose, lactose and cellotriose. Several activities present (β-glucosidases, endocellulases II and III, CBHI and II) can be detected and differentiated. Since the fluorescent MeUmb can be detected at very low concentrations, enzyme activities could be measured at nanomolar level. Visualisation of cellulolytic activities on gels (PAG-IEF) proved to be equally sensitive. The method is essentially non-destructive and photographic reproductions show activity patterns readily comparable with the protein staining. No blotting procedures are necessary and screening of the gels is done within seconds without further derivatisation of the reaction products. Here DEAE-ion-exchange chromatography was used in the preliminary purification of crude cellulase from *T. reesei*. Under these conditions partial separations of the enzymes present are observed: β-glucosidase activities are eluted before the bulk of the fractions containing enzymes active on the test substrate MeUmb β-Gal $\beta(1\rightarrow4)$Glc and 2 main fractions are characterised by their differential inhibition by cellobiose (figs 1 and 2). They can further be purified by affinity chromatography [6].

Considerable progress has been made recently [12–15] in sequence determinations of these proteins and their genes. Striking homologies have been found between the endocellulase II and CBHI and their genetic relationship seems to be confirmed by the present findings regarding their specificities.

The potential use of these substrates in following enzymatic activity during culture growth (fig.3) is also demonstrated.

Applications to other cellulolytic systems from different microorganisms are under investigation.

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