Investigation of an endoglucanase essential for the action of the cellulase system of *Trichoderma reesei* on crystalline cellulose

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An investigation into the induction of the cellulase complex of *Trichoderma reesei* has shown that crystalline cotton cellulose was effectively degraded by the system induced by scoured cotton but not by the system induced by Solka Floc. A variety of techniques including FPLC, chromatofocusing, SDS-PAGE and isoelectric focusing (IEF) were used to separate and characterize the individual cellobiohydrolases, endoglucanases, cellobiases and glucosidases involved in cellulose degradation. A statistical comparison of the enzymic activities of the differently induced systems after resolution by anion exchange chromatography revealed much lower carboxymethylcellulase activity in one of the pooled fractions from a Solka-Floc-induced preparation, suggesting that one of the endoglucanases is either absent or present at a reduced level. IEF separations indicated that the Solka-Floc-induced system lacked an endoglucanase with an alkaline PI.

**Introduction**

*Trichoderma reesei* is one of the most potent cellulase producers (Eriksson & Johnsrud, 1982; Nummi et al., 1983), the culture filtrates being of a similar cellulolytic activity to those of the intact micro-organism (Wood & McCrae, 1979).

The cellulase system of *T. reesei* is composed of three major types of cellulytic enzyme, categorized according to their action on various substrates. The fungus produces two immunologically distinct 1,4-β-d-glucan cellobiohydrolases (EC 3.2.1.91), CBH1 and CBHII, (Fagerstam & Pettersson, 1979; Nummi et al., 1980), the properties and activities of which are well documented (Berghem et al., 1976; Fagerstam & Pettersson, 1980; Teeri et al., 1983, 1987; Bhikhabhai et al., 1984; Tilbeurgh & Claeyssens, 1985; Nevalainen et al., 1989). In addition to exo-type activity, both 1,4-β-d-glucan cellobiohydrolases exhibit endo-acting characteristics (Nummi et al., 1983; Kyriacou et al., 1987; Biely & Marcovic, 1988).

*T. reesei* also elaborates endo-1,4-β-d-glucan 4-gluconohydrolases (EC 3.2.1.4) (Shoemaker & Brown, 1978; Wood & McCrae, 1979) which exist in multiple molecular forms (Bisaria & Ghose, 1981). The major endoglucanase enzymes secreted by *T. reesei* i.e. EGI, EGII and EGIII are distinct proteins (Teeri, 1987), differing in their molecular masses, isoelectric pH values and carbohydrate contents (Henrissat et al., 1985). A very low molecular mass unglycosylated endoglucanase has also been purified from batch cultures of *T. reesei* (Gong et al., 1979; Ulker & Sprey, 1989).

*T. reesei* also produces one major β-D-glucohydrolase component (EC 3.2.1.21) and at least two minor β-glucosidases (Nummi et al., 1980) which constitute less than 1% of the extracellular protein (Enari et al., 1981). In this study, the multienzyme systems of *T. reesei* elaborated on both scoured cotton and Solka-Floc have been resolved using Fast Protein Liquid Chromatography (FPLC). The recombined peak fractions were further analysed by chromatofocusing, isoelectric focusing and SDS-PAGE, and the activity of the cellulytic components towards various substrates has been examined.

The exoglucanase and endoglucanase activities identified are discussed in the context of cotton cellulose degradation, and the essential role of an alkaline endoglucanase in enzymic hydrolysis of crystalline cellulose investigated.
Methods

Maintenance and growth of the organism. Trichoderma reesei (IMI 192656, QM 9414) was maintained on potato-dextrose agar (PDA) slants at 4°C and subcultured once every two weeks. Flasks (1 l) containing 500 ml culture medium (Eggins & Pugh, 1962) supplemented with 1-0% (w/v) cellulose substrate and glucose (0-2%) were inoculated with a spore suspension prepared from a PDA slant and the cultures shaken at 30°C and 100 r.p.m. for 10 d. The pH of the culture medium was adjusted initially to 5.5 and left uncontrolled thereafter.

Preparation of cellulase. After 10 d incubation, the culture medium was filtered through Whatman GF/A glass microfiber filters and the proteins precipitating between 20-80% saturation ammonium sulphate were collected by centrifugation. The precipitate was redissolved on 0-1 m-acetate buffer (NaOH-acetic acid) pH 5-4, dialysed against the same buffer in a Sartorius ultrafiltration cell (nominal molecular mass cut-off, 10000 Da), freeze-dried using a Lyolab A bench-top freeze-dryer and stored at 4°C until used.

Ion-exchange chromatography. The partially purified cellulase system was separated by FPLC (Pharmacia) using a Mono Q anion-exchange column coupled to a Pharmacia FPLC system with a UV monitor (Ellouz et al., 1987). Fractions (1-0 ml) were collected at a flow rate of 1-5 ml min⁻¹ by applying a sodium chloride gradient (linear to 0-35 m until 30 ml was collected, and 1-0 m after 32 ml was collected). Fractions 1-4, 5-9, 10-19, 20-30 and 31-40 were combined into five sets of pooled fractions, freeze-dried, resuspended in 0-025 m-imidazole/HCl buffer, pH 7-4, and desalted by ultrafiltration before being applied to a chromatofocusing column.

Chromatofocusing. A column (15 cm × 1-0 cm) containing Polybuffer exchanger 94 (PBE 94) was equilibrated with starting buffer, 0-025 m-imidazole/HCl, pH 7-4. Samples were then applied, and the column eluted at a flow rate of 0-8 ml min⁻¹ with Polybuffer 74, pH 4-0 (Pharmacia), creating a linear pH gradient of 7-4 to 4-0.

Enzyme assays. Solubilization of cotton was determined using the method described by Wood (1968), measuring residual cellulose after 7 d incubation at 37°C. Endoglucanase activities were measured using a method based on that of Brown & Boston (1961) and Bitner & McLeary (1963) with 0-5% (w/v) carboxymethylcellulose (CMC, degree of substitution not less than 0-4) in 0-2 m-acetate buffer, pH 5-4 as substrate. Liberated reducing sugars produced after 60 min were assayed using a cuprous neocuproine complex) using a Technicon AutoAnalyser 1 system (Technicon Instrument Company). Enzymic hydrolysis of phosphoric-acid-swollen cellulose was measured according to the method of Wood & McCrae (1977), reducing sugars produced after 18 h being measured using the Technicon AutoAnalyser 1 system. β-D-Glucosidase activities were measured using o-nitrophenyl β-D-glucopyranoside as substrate (Wood, 1968); 1 unit of activity is defined as the amount of enzyme required to increase A₄₀₅ by 1-0 in 60 min. Cellobiase activity was measured using the method described by Wood (1969) and monitoring the amount of glucose liberated from cellobiase using glucose oxidase. Protein was determined by the Lowry method.

Electrophoresis. Samples were analysed by SDS-PAGE on 7-5% (w/v) polyacrylamide gels using a modification of the technique of Weber & Osborne (1969). Samples were concentrated by ultrafiltration and desalted with 0-025 m-Tris/HCl, pH 8-8, and dissociated in the same buffer containing 12-0% urea, 4% (w/v) mercaptoethanol, 2% (w/v) SDS and 0-2% bromophenolblue at 100°C for 5 min. The gel was pre-conditioned for 10 min at 120 V, the sample (10 μl) was applied to the gel and electrophoresis was done at 250 V for 3 h. After electrophoresis the gels were stained with Page Blue 83 (0-125% in a solution of glacial acetic acid (9%, v/v) and methanol (45%, v/v)). Gels were destained using 7-5% (v/v) glacial acetic acid and 35% (v/v) methanol and preserved in glacial acetic acid and methanol, both 2% (v/v).

Isoelectric focusing. Samples were dialysed against deionised water and concentrated before application to Ampholine PAG plates (pH 3-5-9-5). The cathode strip was soaked in 1 m-sodium hydroxide and the anode in 1 m-orthophosphoric acid. Gels were run at 1 W cm⁻¹ gel length for 1 h. After electrophoresing, the PAG plate was fixed (57-5 g trichloroacetic acid and 17-25 g sulphosalicylic acid in 500 ml distilled water) for 1 h, washed in destain (500 ml ethanol and 160 ml acetic acid in 21 distilled water) for 5 min and then stained for 10 min (0-46 g Coomassie Blue in 400 ml destain at 60°C). The PAG plate was then destained until the background was clear.

Statistical analysis. Two sample statistical analyses were done using the Statgraphics program, v. 2.0 (Serial No. 957313) from the Statistical Graphics Corporation, USA.

Chemicals. PBE 94, Polybuffer 74 and Ampholine PAG plates were from Pharmacia. All other chemicals and substrates were from Sigma, except the ammonium sulphate and carboxymethylcellulose, which were from BDH.

Results and Discussion

Chromatographic separation of the components of the cellulase system

Fig. 1 shows a typical A3₄₀ trace of a 10 d partially purified culture filtrate elaborated on scoured cotton after application to a Mono Q anion exchange column and elution with 0-06 M-Trizma/HCl pH 7-4 together with a 1 M-NaCl gradient. Assay of the fractions for activity to CMCase, phosphoric-acid-swollen cellulase, cellolobiose and o-nitrophenyl β-D-glucoside (Fig. 2) indicated the presence of cellobiohydrolases, endoglucanases and β-glucosidase, in agreement with the results of Ellouz et al. (1987).

Chromatofocusing of the first major protein peak (fractions 1-4, Fig. 3) revealed at least 1 β-glucosidase, 1 endoglucanase and possibly 1 exoglucanase. β-Glucosidase activity was found only in this peak, and the alkaline pH value of the enzyme (Hayn & Estebauer, 1985) was confirmed. Enzymic activity on phosphoric-acid-swollen cellulose but low CMCase activity indicates an exoglucanase (Wood, 1985). Further separation of the second protein peak (fractions 10-19, Fig. 4) resulted in three, possibly four peaks with endoglucanase activity and one peak with phosphoric-acid-swollen cellulase activity only (possibly cellobiohydrolase II, Ellouz et al., 1987). The largest protein peak from the ion-exchange chromatography (fractions 20-30) was resolved into four peaks by chromatofocusing (Fig. 5). The first peak eluted had both CMCase and phosphoric-acid-swollen cellulase activities. The second and third peaks also showed activity to both these substrates, but CMCase activity was much reduced. The final peak had exoglucanase activity only. The remaining two pooled fractions (5-9
Alkaline endoglucanase of *T. reesei*

Fig. 1. (a) Elution profile of the cellulase complex of *Trichoderma reesei*. The fungus was grown for 10 d on scoured cotton in submerged culture and culture supernatants were separated on a Mono Q column coupled to a Pharmacia FPLC system, with Trizma/HCl (0.06 M, pH 7.4) as the eluent. Dotted line, salt gradient. (b) FPLC elution profile of the cellulase complex of *T. reesei* grown on Solka Floc for 10 d in submerged culture, eluted under the same conditions as in (a).

Fig. 2. Enzymic profiles of a partially purified 10 d culture filtrate from *T. reesei* induced on scoured cotton, after resolution by anion FPLC using a Mono Q column. Symbols: ○, protein; ●, cellulase (assayed on H₂PO₄-swollen cellulose); □, CMCase; ■, cellobiase; △, β-glucosidase.

and 31–40) showed no enzyme activity to any of the selected substrates.

Solka Floc, a wood pulp cellulosic substrate, was also used to induce extracellular cellulolytic enzymes of *T. reesei*. However, while a similar elution profile to that of the scoured-cotton-induced preparation was obtained upon resolution of the cellulolytic system by FPLC (Fig. 1), the Solka-Floc-induced system was unable to degrade scoured cotton as effectively as the scoured-cotton-induced system (12% compared to 95% solubilization after 7 d).

The enzyme activities of the pooled peak fractions of the Solka-Floc-induced system were determined, and a comparison made with those from a scoured-cotton-induced system. Enzyme separations and analyses were carried out on the cellulase systems obtained from four different fermentation experiments with each substrate; representative results are presented in Table 1.

A comparison of the activities of peak 4 showed that the CMCase activity from the Solka-Floc-induced preparation was significantly lower (*P* = 0.002) than that of the scoured-cotton-induced preparation. There was no significant difference between the activities in the other peaks to the various substrates or in peak 4 to substrates other than CMC.

These results indicate a reduction in endoglucanase activity in the Solka-Floc-induced preparations, which may account for the much lower crystalline-cotton-
Fig. 3. Separation of pooled fractions 1–4 from a scoured-cotton-induced preparation on a chromatofocusing column (15.0 cm × 1.0 cm) containing Polybuffer exchanger 94 equilibrated with imidazole/HCl (0.025 M, pH 7.4). The sample was eluted with Polybuffer 74 (pH 4.0) at a flow rate of 0.8 ml min⁻¹. Symbols: ○, protein; ●, cellulase (assayed on H₂PO₄-swollen cellulose); □, CMCase; ■, cellobiase; △, β-glucosidase.

degrad ing activity. El Gogary et al. (1989) reported that the *T. reesei* cellulase system is composed of two cellbiohydrolases, two endoglucanases and a β-glucosidase. Wood (1985) hypothesized that each of the cellbiohydrolases requires a specific endoglucanase, which cleaves the cellulose chain to expose two different types of end group, which are subsequently attacked by the relevant stereospecific cellbiohydrolases. If one of these endoglucanases was absent from the system, or both were present but at a reduced level, the ability of the organism to degrade crystalline cellulose would be greatly reduced. The CMCase activities in the remaining peaks are fairly consistent irrespective of induction substrate, which may imply that only one of the endoglucanases is absent or present at a reduced level.

**Electrophoretic separations**

Peak 4 was analysed further by isoelectric focusing (Fig. 6). Two bands were observed for the Solka-Floc-induced
peak 4 which were tightly focused together with pI values of 4.5 and 4.6. These two bands were also present in the scoured-cotton-induced peak 4, with a third band with pI 7.1. A low-molecular-mass endoglucanase (12.5–20 kDa) has been purified from *T. reesei* by various researchers (Berghem et al., 1976; Håkansson et al., 1978; Gong et al., 1979). Ulker & Sprey (1989) have described it as an unglycosylated endoglucanase with molecular mass of 25 kDa and a pI of 7-5. This is in contrast to most *Trichoderma* endoglucanases which are acidic and show extreme heterogeneity after separation by IEF (Sprey, 1987). After electrophoretic separation on a 7.5% polyacrylamide gel (SDS-PAGE) two faint bands were observed in the region 20–24 kDa for the dissociated peak 4 (scoured-cotton-induced preparation). The enzymic activities and electrophoretic separations for peak 4 from a scoured-cotton-induced preparation suggest the presence of a cellobiohydrolase and an alkaline endoglucanase.

**Recombination experiments**

After separation by FPLC, the resulting fractions were recombined in a ratio consistent with their protein ratio in the unresolved systems and assayed for their ability to solubilize crystalline cotton (Table 2). A total unresolved
Fig. 5. Separation of pooled fractions 23-28 from a scoured-cotton-induced preparation on a chromatofocusing column (15.0 cm x 1.0 cm) containing Polybuffer exchanger 94 equilibrated with imidazole/HCl (0.025 M, pH 7.4). The sample was eluted with Polybuffer 74 (pH 4.0) at a flow rate of 0.8 ml min⁻¹. Symbols: O, protein; ●, cellulase (assayed on H₂PO₄-swollen cellulose); □, CMCase.

Table 1. Representative specific activities of pooled fractions (after separation by FPLC anion exchange chromatography) against various substrates

The protein concentration and specific enzyme activities to the various substrates in peak 1 are arbitrarily put equal to 100 to allow easy comparison to activity levels in other peaks and between the Solka-Floc-induced system and the scoured-cotton-induced system. SC, scoured cotton; SF, Solka Floc.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Protein (µg ml⁻¹)</th>
<th>10⁶ × CMCase (mmol glucose equiv. min⁻¹ µg⁻¹)</th>
<th>10⁶ × Cellulase* (mmol glucose equiv. µg⁻¹)</th>
<th>10⁴ × Cellobiase (mg glucose equiv. µg⁻¹)</th>
<th>10⁴ × β-Glucosidase (A₄₉₀ µg⁻¹)</th>
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<tr>
<td></td>
<td>SC</td>
<td>SF</td>
<td>SC</td>
<td>SF</td>
<td>SC</td>
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<tr>
<td>1-4 (peak 1)</td>
<td>610.0 (100)</td>
<td>370.0 (100)</td>
<td>3.9 (100)</td>
<td>16.2 (100)</td>
<td>4.0 (100)</td>
</tr>
<tr>
<td>5-9 (peak 2)</td>
<td>190.0 (31)</td>
<td>215.0 (58)</td>
<td>0.4 (2)</td>
<td>0.4 (2)</td>
<td>0.5 (13)</td>
</tr>
<tr>
<td>10-19 (peak 3)</td>
<td>315.0 (52)</td>
<td>240.0 (68)</td>
<td>1.4 (36)</td>
<td>20.8 (128)</td>
<td>5.4 (135)</td>
</tr>
<tr>
<td>20-30 (peak 4)</td>
<td>900.0 (148)</td>
<td>2200.0 (595)</td>
<td>0.4 (10)</td>
<td>0.02 (0.12)</td>
<td>1.9 (48)</td>
</tr>
<tr>
<td>31-40 (peak 5)</td>
<td>175.0 (29)</td>
<td>170.0 (46)</td>
<td>0.4 (1)</td>
<td>0.4 (2)</td>
<td>0.3 (8)</td>
</tr>
<tr>
<td>Recovery</td>
<td>97%</td>
<td>101%</td>
<td>98%</td>
<td>117%</td>
<td>114%</td>
</tr>
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</table>

* Assayed on H₂PO₄-swollen cellulose.
The results of the chromatographic and electrophoretic separations, and the reconstitution experiments, suggest that an alkaline endoglucanase is an essential part of a T. reesei cellulase system capable of efficiently degrading crystalline cellulose. Much of the early discussion on the mechanism of cellulase action centred on the role of a C1 component as defined by Reese et al. (1950), who suggested that it was the microorganism's ability to elaborate this unique factor which enabled it to solubilize the strongly hydrogen-bonded areas of the crystalline cellulose. The observation that the combined action of C1 and Cx components appear to be confined only to the pair of faces of the crystalline elementary fibrils containing the 2,3 and 2,3,6 hydroxyl groups (accessible on alternate anhydroglucose units along the cellulose chains), led Sagar (1985) to suggest that a component of the cellulase system has a specific affinity for the cellulose molecules in this pair of surfaces associated with the unique spatial configuration of their free hydroxyl groups. If such a component were an endoglucanase, the resulting chain scission would lead to the production of free cellulose chain ends accessible to the cellobiohydrolases. Alternatively, disruption of the fine structure on the surface of the cellulose crystallites may involve the formation of an enzyme–enzyme system of cellobiohydrolase and certain endo-β-1,4-glucanases (Sagar, 1985; Wood & McCrae, 1978). The more effective synergism observed between the cellobiohydrodase and endoglucanase components of certain fungi supports such a hypothesis (Wood et al., 1980). The question now arises, ‘how important is this alkaline unglycosylated endoglucanase in the degradation of crystalline cotton?’. Could it possibly be Reese's C1?

References


