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# ANALYTICAL SEPARATION OF *TRICHODERMA REESEI* CELLULASES BY ION-EXCHANGE FAST PROTEIN LIQUID CHROMATOGRAPHY

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## SUMMARY

The extracellular enzymes produced by *Trichoderma reesei* strain CL847 were submitted to fast protein liquid chromatography on a Mono Q anion-exchange column. Fourteen distinct peaks were separated and characterized by their protein contents and enzymatic activities. The three main peaks were subjected to a second separation on a Mono S cation-exchange column. Except for the cellobiohydrolase II and an *endo*-β-1,4-glucanase which could not be separated, all the components of the cellulase complex, five other *endo*-1,4-β-D-glucanases, the cellobiohydrolase I and the β-glucosidase, were purified almost to homogeneity. The fast and reproducible resolutions obtained allowed the design of a routine test for the characterization of mutant strains of *T. reesei*. Moreover the two-step purification procedure was successfully extrapolated to the preparative scale.

## INTRODUCTION

In spite of much research undertaken during the last decade, plant cellulose valorization via enzymatic hydrolysis has not yet reached the point of economic feasibility. In particular, the cost of the enzyme is still too high. Significant improvements have been achieved in the production yields, by genetic manipulation of the producer strain[1–5], as well as by optimization of the fermentation process[6–8]. Most of these studies concerned *Trichoderma reesei*, a fungus generally considered to be the most potent producer of cellulases. With productivities as high as 200 mg extracellular protein per litre per hour, and final concentrations up to 40 g/l[5], the limit of this technique may soon be reached. However, further improvements can probably be achieved by acting at the level of the individual components of the complex. It is now well established[9–11] that the cellulolytic complex of *T. reesei* includes two *exo*-1,4-β-D-glucanases (cellobiohydrolases), several *endo*-1,4-β-D-glucanases and one β-glucosidase, all of which act synergistically on crystalline cellulose. Whereas the role

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and importance of β-glucosidase are clear, relatively little information is available concerning the exact mode of action of *endo-* and *exo*-glucanases. From a practical viewpoint, the optimum ratio between the different enzymes has not been established. In this context, an analytical procedure allowing a quantitative survey of all the individual enzymes would be of interest.

Several authors have separated cellulases components by using conventional chromatographic techniques[12–15]. However, these techniques cannot be used for routine analysis because they are time-consuming and require large amounts of enzymes. High-performance liquid chromatography (HPLC) has also been applied to cellulases by several authors[16–18], leading to rapid and well defined separations. However, the matrices used in such studies are not usually commercially available. More recently, Hayn and Esterbauer[19] analysed the cellulase complex of *T.reesei* strain MCG77 by using the Pharmacia "Fast Protein Chromatography" system with a Mono P column and polybuffers which allow "chromatofocusing" of the proteins, *i.e.*, elution according to the isoelectric points. We also tried this apparatus and found it well adapted to the rapid resolution of crude protein complexes. For routine analysis, we preferred to use ion exchange instead of chromatofocusing, the former technique being less costly and easier to operate.

This paper reports conditions for the separation of the individual components of the cellulases from *T.reesei* strain CL847 on Mono Q (anionic) and Mono S (cationic) exchange columns.

## MATERIALS AND METHODS

### *Enzyme sample*

The cellulase preparation used was obtained by cultivating the hyperproducing *Trichoderma reesei* mutant strain CL847[5] in a 14-l fermenter. The cultivation conditions and enzyme recovery have been described[20].

### *Electrophoresis*

The most important fractions were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) by using the automated and miniaturized "Phast" system (Pharmacia). The samples were denaturated, applied to ready-to-use acrylamide gradient gels (Phastgel 10-15) with SDS buffer strips and developed by silver staining as described in the manufacturer's manual.

### *Chromatographic separation of proteins*

Medium-pressure ion-exchange chromatography was performed using a fast protein liquid chromatography (FPLC) apparatus (Pharmacia) with an anion-exchange (Mono Q) or a cation-exchange (Mono S) column. Cellulases components were eluted by applying a sodium chloride gradient, the profile of which was designed to optimize peak separation, using a gradient programmer. The flow-rate was kept at 1.0 ml/min. The column effluent was monitored at 280 nm and the UV absorbance *vs.* time was recorded. Fractions (0.5 ml) were collected and analysed for enzymatic activities.

*Enzyme assays*

The protein concentration was determined by the method of Lowry *et al.*[21], or in samples with low protein contents by the direct spectrophotometric method of Vernon *et al.*[22].

The polysaccharide hydrolases were assayed according to Mandels *et al.*[23]. The general procedure was as follows: 0.5 ml of substrate solution or suspension (1%, w/v) in 50 m*M* citrate buffer, pH 4.8 and 0.5 ml of enzyme solution were incubated at 50°C. The amount of reducing sugars liberated was estimated by the 3,5-dinitrosalicylic acid method[24], using glucose as a standard. Enzyme dilutions and the reaction time were adjusted in each case to obtain a final sugar concentration in the range 0.2–1 mg glucose equivalent.

The following substrates were used: carboxymethylcellulose (CMC) 7L (Hercules) for *endo*-1,4-β-D-glucanases; phosphoric acid-swollen cellulose ("Walseth")[25] for cellulases; xylan (Fluka) for xylanases; soluble starch (Sigma) for amylases; pectin (Sigma) for pectinases; laminarin (Sigma) for 1,3-β-D-glucanases.

Other hydrolytic activities were assayed using nitrophenyl derivatives (Sigma) as substrates: *p*-nitrophenyl-β-D-glucopyranoside for β-glucosidase; *p*-nitrophenyl-β-D-xylopyranoside for β-xylosidase; *o*-nitrophenyl-β-D-galactopyranoside for β-galactosidase; *p*-nitrophenyl-α-D-galactopyranoside for α-galactosidase; *p*-nitrophenyl phosphate for acidic and alkaline phosphatases. A 0.5-ml volume of 5 m*M* substrate solution plus 0.5 ml enzyme sample were incubated at 50°C (37°C for phosphatase), then the reaction was stopped by the addition of 2 ml of a sodium carbonate solution (20 g/l in water) and the amount of nitrophenol liberated was determined by measuring the absorbance at 405 nm. All these assays were performed at pH 4.8 (50 m*M* citrate buffer) except for alkaline phosphatase, which was assayed at pH 8.5 (50 m*M* Tris–HCl). 10 m*M* magnesium sulphate was added to the buffer for phosphatase assays.

Proteolytic activity was evaluated using a chromogenic substrate, hide powder azure (Sigma). To 0.4 ml of a 2% (w/v) substrate suspension in 0.1 *M* morpholinoethanesulphonic acid (MES) pH 6.5 buffer, 0.1 ml of enzyme sample was added. After incubation at 37°C for 60 min with continuous shaking (New Brunswick gyrotary shaker), the undigested substrate was precipitated by adding 0.5 ml of a 10% (w/v) trichloroacetic acid solution and after centrifugation (5 min, 10 000 *g*) the absorbance at 630 nm of the supernatant was correlated with the extent of proteolysis (release of soluble dyed oligopeptides).

The analysis of the sugars liberated by different cellulases fractions acting on Walseth cellulose were performed by HPLC using an Amino column (Brownlee Labs.) and acetonitrile–water (75:25 v/v) as the mobile phase. The flow-rate was 1 ml/min. The refractive index of the effluent was recorded and the nature and the concentration of sugars in the sample were determined by reference to glucose and cellobiose standard solutions.

## RESULTS AND DISCUSSION

*Separation on anion-exchange column*

The major part of the component of the cellulolytic complex from *T. reesei* have an acidic isoelectric point[10,11,16]. Thus it could be expected that the use of

TABLE I

PROTEIN CONTENTS AND ENZYME ACTIVITIES ASSAYED IN EACH PEAK COLLECTED AFTER SEPARATION OF *T. REESEI* CL847 CRUDE CELLULASES ON A MONO Q COLUMN

Peak numbers refer to Fig. 1. Experimental conditions as in Materials and Methods.

| *Peak number* | *Volume (ml)* | *Protein content (mg/ml)* | *Percentage of total protein* | *endo-1,4-β-D-Glucanase activity (I.U.)* | *Walseth cellulose activity* | | *β-Glucosidase (I.U.)* | *Xylanase (I.U.)* | *Protease (arbitrary units)* |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | *I.U.* | *Cellobiose/glucose* | | | |
| 1 | 2.5 | 0.15 | 11.5 | 0.45 | 0.45 | 0 | 198 | 1.70 | – |
| 2 | 1 | 0.05 | 2.1 | 0.06 | 0.08 | – | – | – | – |
| 3 | 0.5 | 0.64 | 12.7 | 0.46 | 0.28 | 16 | – | – | – |
| 4 | 0.5 | 0.15 | 3 | – | 0.09 | 15 | – | – | 187 |
| 5 | 1 | 0.07 | 2.7 | 0.06 | 0.09 | 14 | – | – | 495 |
| 6 | 1 | 0.04 | 1.6 | 0.07 | – | – | – | – | 238 |
| 7 | 1.5 | 0.03 | 1.9 | 0.11 | 0.10 | 3 | – | – | 156 |
| 8 | 1 | 0.08 | 3.2 | 0.12 | 0.12 | 2 | – | 0.39 | – |
| 9 | 1 | 0.13 | 5.2 | 0.11 | 0.16 | 1 | – | 0.37 | – |
| 10 | 0.5 | 0.06 | 1.2 | – | – | – | – | – | – |
| 11 | 1 | 0.05 | 1.8 | 0.08 | – | 2 | – | – | – |
| 12 | 1 | 0.03 | 1 | – | – | – | – | – | – |
| 13 | 1 | 0.02 | 0.8 | – | – | – | – | – | – |
| 14 | 1 | 1.02 | 40.6 | – | 0.35 | 11 | – | – | – |

anion exchange under slightly alkaline conditions would allow the binding of the majority of the proteins.

The nature, concentration and pH of the buffer and the profile of the salt gradient were selected after several trials to optimize the resolution. Under the conditions chosen, about 80% of the proteins bind to the column and are gradually eluted in well separated peaks (Fig. 1). The elution is completed and the column is ready for a new injection after 42 min, including washing and re-equilibration steps. Repeated cycles gave well reproducible results (data not shown). The fractions collected were assayed for enzymatic activities and protein content (Table I). The latter was found to be well correlated to the area of the peaks measured on the recorded profile. The sum of the protein content of the fractions was similar to the total amount of proteins in the sample. This amount (2.5 mg) is sufficient to allow the detection in the effluent of all the enzymatic activities originally present. The main enzymatic activities detected in each peak are summarized in Table II.

$\beta$-Glucosidase activity was found only in the first peak, indicating that the enzyme has an isoelectric point above 7.6. This is in agreement with the results of Hayn and Esterbauer[19] and Farkas *et al.*[26].

Cellobiohydrolase activity, as shown by the high cellobiose/glucose ratios in Walseth cellulose hydrolysates, was present in two major peaks, the most important one being the last eluted, indicating a very low isoelectric point. These results are in accordance with previously published data, *e.g.*, in ref. 14. The cellobiohydrolases

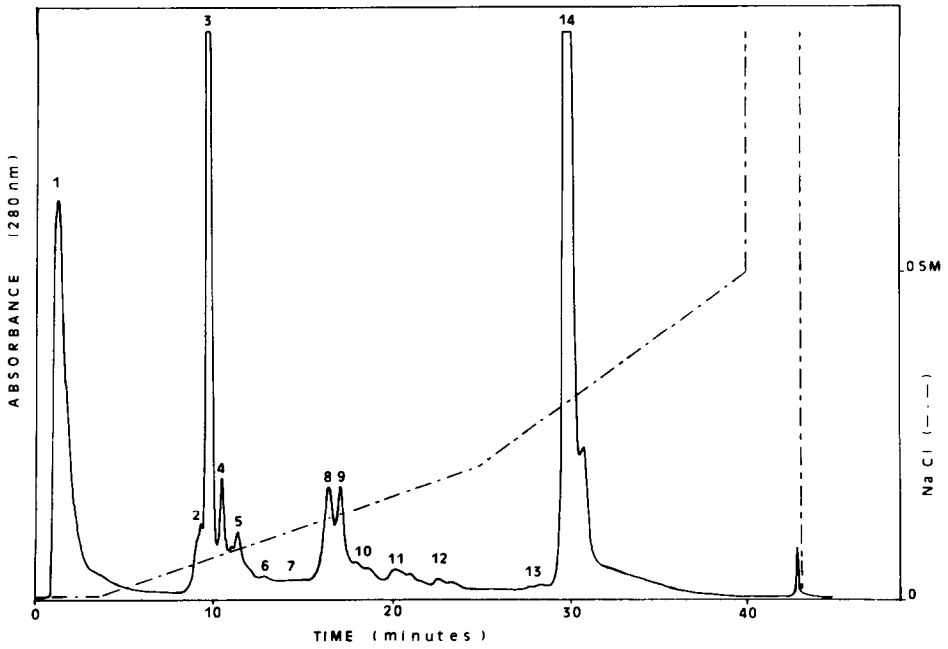


Fig. 1. Chromatographic profile of *T. reesei* CL847 enzymes on a Mono Q anion-exchange column. A 500-$\mu$l sample of crude cellulases (5 mg/ml) dissolved in 20 m*M* Tris–HCl buffer pH 7.6 was applied to the column and eluted at 1 ml/min using a 0–0.5 *M* sodium chloride gradient in the same buffer.

TABLE II

MAIN ENZYMATIC ACTIVITIES DETECTED IN THE PEAKS OBTAINED AFTER CHROMATOGRAPHY OF CRUDE *T. REESEI* CL847 CELLULASES ON A MONO Q COLUMN

Peak numbers refer to Fig. 1.

| *Peak number* | *Enzymatic activities* |
|---|---|
| 1 | *endo*-1,4-β-D-Glucanase, β-glucosidase, xylanase, laminarinase |
| 2 | |
| 3 | *endo*-1,4-β-D-Glucanase, *exo*-1,4-β-D-glucanase |
| 4 | |
| 5 | Pectinase, protease |
| 6 | |
| 7 | *endo*-1,4-β-D-Glucanase |
| 8 | *endo*-1,4-β-D-Glucanase, xylanase, amylase |
| 9 | *endo*-1,4-β-D-Glucanase, xylanase, acidic phosphatase |
| 10 | |
| 11 | *endo*-1,4-β-D-Glucanse, xylanase |
| 12 | |
| 13 | β-Galactosidase |
| 14 | *exo*-1,4-β-D-Glucanase, β-xylosidase, alkaline phosphatase |

present in peaks 3 and 14 (Fig. 1) are probably identical to CBH II and CBH I purified by Petterson *et al.*[14] and by Shoemaker *et al.*[15].

*endo*-1,4-β-D-Glucanase, characterized as enzyme hydrolysing CMC and Walseth cellulose, the latter with a low cellobiose/glucose ratio, was detected in several

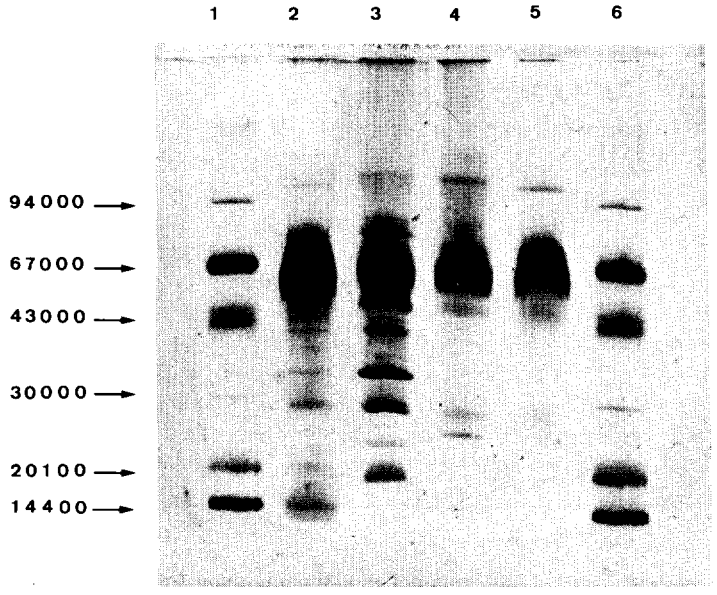


Fig. 2. Electrophoresis of the main fractions obtained after anion-exchange chromatography. Lanes: 1 and 6 = molecular weight markers; 2 = crude sample; 3 = peak 1; 4 = peak 3; 5 = peak 14.

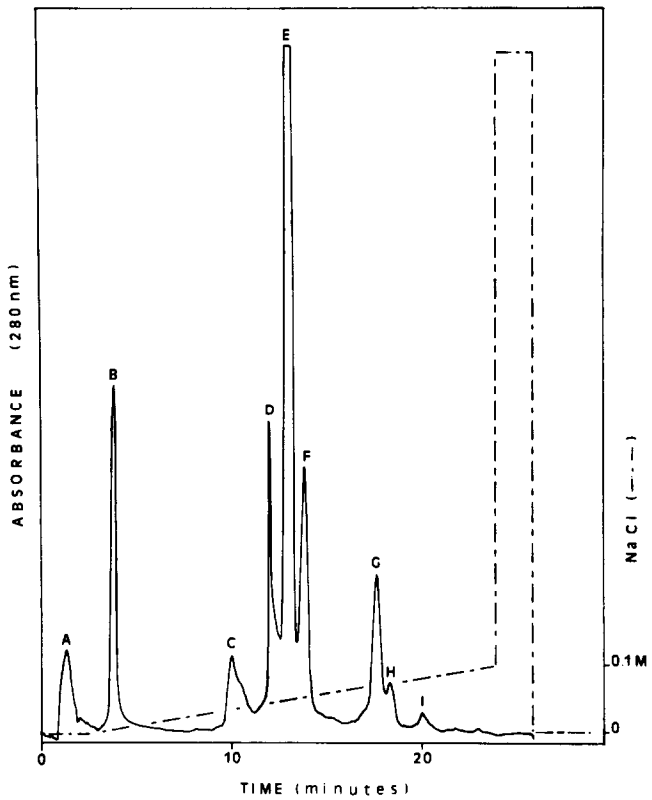


Fig. 3. Separation on a Mono S cation-exchange column of the non-adsorbed fraction (peak 1) obtained after chromatography of the crude preparation on Mono Q. Fractions corresponding to peak 1 collected after chromatography on Mono Q were pooled and the proteins precipitated by two volumes of cold acetone were dissolved in 200 $\mu$l of 20 m*M* acetate buffer pH 3.6. The sample was applied to the Mono S column and eluted at 1 ml/min using a 0–0.1 *M* sodium chloride gradient in the same buffer.

peaks (Table II). In peak 3, CMCase activity and an high cellobiose/glucose ratio on Walseth were found, probably indicating the presence of both CBH II and an *endo*-1,4-$\beta$-D-glucanase.

Xylanase activities were also found in several peaks, whereas all other enzymes present in the crude preparation were found in only one peak.

All the collected fractions contained several enzymatic activities. In particular, the proteins in peaks 1, 3 and 14, the most important ones qualitatively as well as quantitatively, were analysed by PAGE. Several bands (9, 6 and 5 respectively) were detected after silver staining (Fig. 2). In order further to separate and characterize these proteins, a second separation step on an ion-exchange column was performed.

*Chromatography on cation-exchange column*

Peak 1 corresponds to proteins non-adsorbed on the Mono Q column, indicating that their isoelectric points are higher than 7.6. Consequently it was decided to investigate the possibility of separating them on a cation exchanger at acidic pH.

TABLE III

MAIN ENZYMATIC ACTIVITIES PRESENT IN THE DIFFERENT PEAKS OBTAINED AFTER SEPARATION ON MONO S OF THE NON-ADSORBED FRACTION (PEAK 1) FROM MONO Q CHROMATOGRAPHY

Peak symbols refer to Fig. 2.

| *Peak* | *activity* |
|---|---|
| A | – |
| B | Laminarinase |
| C | – |
| D | – |
| E | *endo*-1,4-β-D-Glucanase |
| F | *endo*-1,4-β-D-Glucanase |
| G | Xylanase |
| H | Xylanase |
| I | β-Glucosidase |

After several trials, 20 m*M* acetate buffer (pH 3.6) and a linear salt gradient from 0 to 0.1 *M* were found adequate for resolving these proteins into nine major peaks, probably corresponding to the nine bands observed by electrophoretic analysis (Fig. 3). Each enzymatic activity present in the initial sample (see Table II) was found in

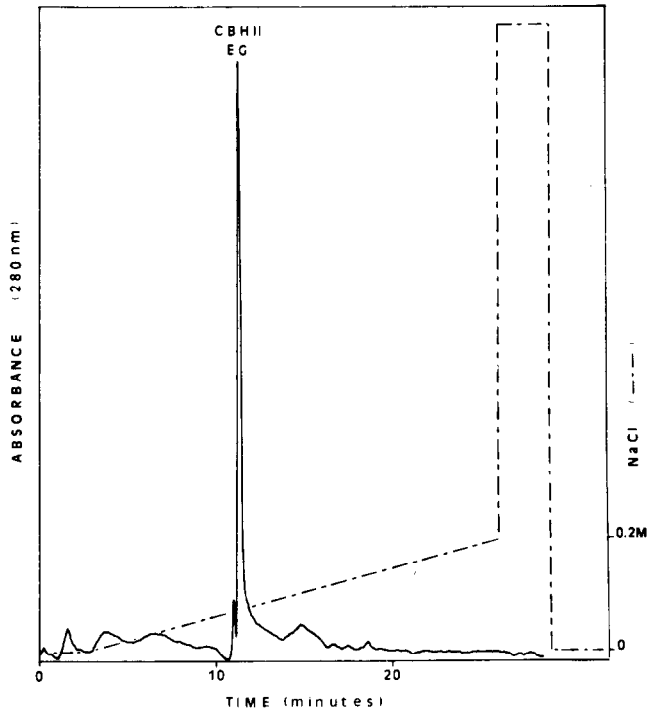


Fig. 4. Separation on a Mono S column of the proteins contained in the peak 3 of the Mono Q chromatogram. Operating conditions as in Fig. 2. CBH II = Cellobiohydrolase II; EG = *endo*-1,4-β-D-glucanase.

one or two of these peaks (Table III). *endo*-1,4-β-D-Glucanase activity was detected in two contiguous peaks (E and F) indicating very little difference between the physicochemical properties of these two enzymes. Laminarinase, xylanase and β-glucosidase peaks were well separated from each other and from the *endo*-1,4-β-D-glucanase group. Three additional peaks (A, C and D) were not identified.

β-Glucosidase was found only in the last eluted small peak. From the area of this peak, it appears that β-glucosidase represents a very low proportion (less than 1%) of the proteins present in the crude preparation. This finding is in accordance with other results[13,27] and confirmed that *T. reesei* is a poor β-glucosidase producer, although the CL847 strain has been markedly improved as regards the specific activity of this enzyme compared to the parent strain QM9414 (data not shown).

Several authors[15,27] have reported the occurrence of two distinct β-glucosidases in cellulase preparations from *T. reesei*. Shoemaker *et al.*[15] suggested that the second enzyme, with a high molecular weight and acidic isoelectric point, is similar to the intracellular β-glucosidase purified by Inglin *et al.*[28]. In some cases, this enzyme could be liberated in the external medium due to partial lysis of mycelium. The absence of this latter enzyme from the preparation used in the present study could be explained by the fact that the proteins were extracted from the supernatant of a young culture.

The Mono S cation-exchange column, with the same starting buffer as for peak

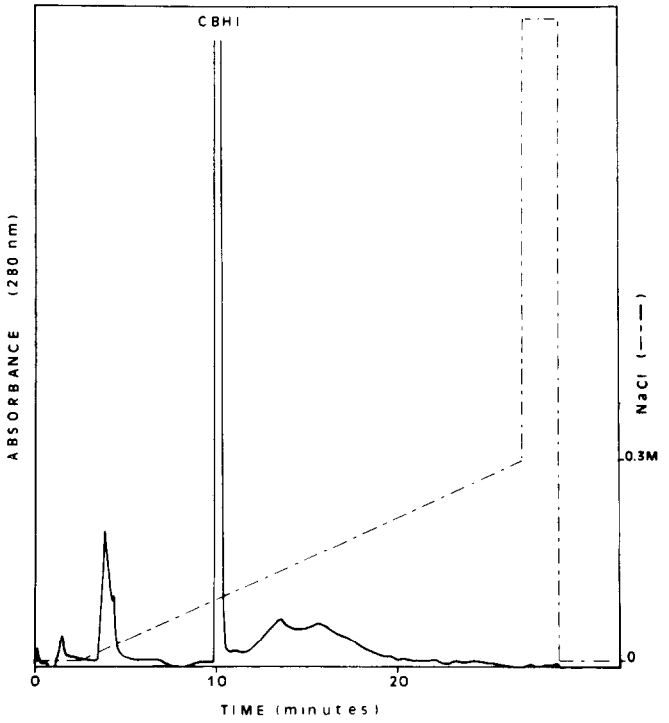


Fig. 5. Separation on a Mono S column of the proteins contained in peak 14 of the Mono Q chromatogram. Operating conditions as in Fig. 2. CBH I = Cellobiohydrolase I.

1, was also used for further analysis of the two other main peaks (3 and 14, see Fig. 1) obtained after chromatography of the crude preparation on the Mono Q column. Proteins from fraction 3 could be separated into one sharp major peak and several very small ones (Fig. 4). Enzymatic assays showed that the main peak still contains both *endo*-1,4-β-D-glucanase (CMCase) and cellobiohydrolase activities. Several attempts to separate these enzymes by varying the pH and/or the gradient profile did not succeed. However, analytical chromatofocusing and electrophoresis (not shown) confirm the presence in this fraction of two distinct proteins with very similar physicochemical properties.

Re-chromatography of fraction 14 on Mono S gave a profile similar to that of fraction 3 with a main peak and several small ones (Fig. 5). In this case, the major fraction was found to contain only the *endo*-1,4-β-D-glucanase activity. Although some contaminating activities were present in the starting sample (see Table II) they could not be detected in any fraction: this could be explained by a deactivation of the enzymes, possibly due to the rather drastic pH conditions used in the two steps. CBH I seems to be practically pure at this step, as only one band was detected by staining of polyacrylamide gel loaded with concentrated samples.

## CONCLUSIONS

The use of FPLC with the ion-exchange columns Mono Q and Mono S provides a rapid and reproducible procedure for the separation of different components of the cellulolytic complex of *T. reesei*. The loading capacity of the columns (*ca.* 2.5 mg protein) and quantitative recovery allow easy subsequent analysis of the fractions by enzymatic assays, re-chromatography or electrophoresis. This technique proved to be valuable as a routine test for the characterization of enzymes produced by different mutant strains of *T. reesei*.

On the anionic exchanger, the cellulolytic complex of *T. reesei* CL847 strain was resolved into at least fourteen distinct peaks, among which one β-glucosidase, six *endo*-1,4-β-D-glucanases and two *exo*-1,4-β-D-glucanases were revealed. The chromatographic profile is grossly similar to that obtained by Bissett[16] with enzymes from the wild type strain QM6a on a DEAE-glycophase column in HPLC. The results are also in accordance with those published by Hayn and Esterbauer[19] who separated the cellulolytic complex of MCG77 mutant strain by chromatofocusing on a Mono P FPLC column. More generally, the results of this study were found to be in good agreement with most of the previously published data on the same topic. In particular, the presence in the cellulolytic complex of *T. reesei* of one β-glucosidase with an high isoelectric point, p*I*, and representing a very low portion of total proteins, and of two *exo*-1,4-β-D-glucanases, one with a very low p*I* and representing about 40% of total proteins (CBH I) and one with intermediate p*I* (CBH II), were clearly shown. The situation concerning *endo*-1,4-β-D-glucanases is more confused. We found six peaks displaying this activity, which could be classified into two groups of three: one group with relatively high p*I* (near neutrality) and from which xylanase activity could be separated; another group with low p*I*, and always associated with a xylanase activity. In the latter case, several attempts to separate the CMC and xylan hydrolysing activities were unsuccessful (data not shown). It appears that each group of *endo*-glucanases could be derived from an unique protein, with some vari-

ations in postranslational events, *e.g.*, glycosylation which may explain the slight differences observed in physicochemical properties. The first group might correspond to the enzyme purified by Petterson *et al.*[14] as "*endo* I" and by Shoemaker *et al.*[15] as "*endo* III", the second group to "*endo* II" (Petterson) or "*endo* I" (Shoemaker). Both these sets of authors mentioned that the latter enzyme displays a xylanase activity.

Except for CBH II and one of the endoglucanases, most of the individual components of the cellulase complex were purified after two steps, *i.e.*, anion-exchange followed by cation-exchange chromatography. The rapidity of the technique greatly facilitated the optimization of the separation conditions, which were further easily extrapolated to the preparative scale.

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