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SEPARATION AND PARTIAL CHARACTERIZATION OF *TRICHODERMA REESEI* CELLULASE BY FAST CHROMATOFOCUSING

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SUMMARY

The cellulase protein produced by the fungus *Trichoderma reesei* MCG 77 was submitted to a fast protein liquid chromatography separation on the chromatofocusing column Mono-P. This technique offers a means for a rapid and well reproducible screening of the enzyme complex. The individual protein peaks were characterized by their isoelectric points and by measuring different enzyme activities. The presence of one β-glucosidase, one β-xylosidase, two exocellulases, one xylanase and five endocellulases in the extracellular protein of *T. reesei* was shown.

INTRODUCTION

Trichoderma reesei cellulases have received considerable attention in the recent years mainly because such enzymes could be used for bioconversion of cellulose-containing raw materials into glucose[1–5], which in turn can serve as starting material for the production of a great variety of useful products by fermentation processes. Many microbes can produce cellulases[6], but only a few, *e.g.* the different strains of *T. reesei*, secrete enzymes in high yield and in a composition suitable for a rapid saccharification of cellulose. It is known[7] that *T. reesei* cellulase preparations have three major enzyme activities: endoglucanases (1,4-β-D-glucan-4-glucanhydrolase, E.C. 3.2.1.4), exocellulases (exo-cellobiohydrolase, β-D-glucan-cellobiohydrolase, E.C. 3.2.1.91) and β-glucosidase (cellobiase, β-D-glucoside-glucohydrolase, E.C. 3.2.1.21). A synergistic and combined action of these three types of enzyme is necessary for the hydrolysis of crystalline cellulose, as contained in cotton, pulp or native lignocellulose, into glucose[2,8]. In addition to cellulases, minor activities of other enzymes not involved in cellulose hydrolysis were also detected in cellulase preparations. Examples are xylanases[9–11], amylases[12] and proteases[13].

Gong *et al.*[14] used conventional isolation techniques to obtain five endocellulases, differing in molecular weight and/or isoelectric points, from a *T. reesei* QM 9414 cellulase preparation. Other authors have also reported multiple forms of endocellulases[15], exocellulases[16] and cellobiases[17,18]. It has been supposed[14,19] that the occurrence of multiple forms of cellulases might be due to the action of the concomitantly secreted proteases and amylases, which might alter the initially secret-

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ed parent cellulase(s), in particular in old culture mediums[14,19]. A post-secretional modification is unlikely in view of the findings of Labudova and Farkas[20], who resolved the *T. reesei* QM 9414 cellulase complex by means of analytical isoelectric focusing in polyacrylamide, and observed that multiple enzymes of the same type appear at an early stage of the fermentation. Isoelectric focusing was also employed[21] for the detection of differences in enzymes produced by different mutants of *T. reesei*. Bisset *et al.*[22,23] described a high-performance liquid chromatographic (HPLC) method with DEAE glycophase as column material for the separation of *T. reesei* cellulase produced from different mutants grown on different substrates. Shoemaker *et al.*[24] used an anion-exchange column for the HPLC separation of the cellulase complexes of different *T. reesei* mutants. We have shown[11,25] that analytical chromatofocusing by means of fast protein liquid chromatography (FPLC) is an excellent method for a rapid separation of the cellulase enzyme complex into its individual proteins; the method also allowed a one-step isolation of cellobiohydrolase on a preparative scale. Preparative chromatofocusing was also used by Thomas *et al.*[26] for the purification of glucan-hydrolases from a commercial preparation of *T. viride* cellulase.

This paper reports the application of fast protein liquid chromatofocusing to the characterization of the *T. reesei* cellulase complex from the mutant MCG 77. The separation method is highly reproducible and provides a means for the quantitative estimation of the individual cellulase protein constituents.

EXPERIMENTAL

The chromatographic system consisted of a Pharmacia FPLC apparatus equipped with a Mono-P HR 5/10 column (Pharmacia). Peak areas were measured with an integrator (Shimadzu CR1B) connected to the UV detector set to 280 nm. The start buffer was 0.023 *M* bis-tris (4.8 g of bis-tris/l) adjusted with dilute hydrochloric acid (pH 6.5). After column equilibration with the start buffer (45 ml), 2 mg of cellulase protein (prepared as described below) dissolved in 200 μl of start buffer were injected after filtration (0.45 μ) into the chromatograph; elution was performed with 5 ml of start buffer followed by 45 ml of 1:10 diluted Pharmacia Polybuffer 74–hydrocloric acid (pH 4.0) and 35 ml of 1:10 diluted Polybuffer 74–hydrochloric acid (pH 3.0). After each run the column was rinsed with 5 ml of 2 *M* sodium chloride and re-equilibrated with 45 ml of start buffer. The column effluent was fractionated by collecting quantitatively the fourteen peaks (Fig. 1, Table I) shown by the UV trace. The samples were used without any further pretreatment to measure carboxymethylcellulase, β-glucosidase, xylanase and exocellulase activities.

Measurement of enzyme activities

For endocellulase (carboxymethylcellulase, CMCase), 1.4 ml of 1% carboxymethylcellulose sodium salt (Serva, Heidelberg, F.R.G.) in 0.05 *M* citrate buffer (pH 4.8) and 0.1 ml of sample solution were incubated at 50°C for 30 min. The amount of reducing sugars liberated was then estimated by the dinitrosalicylic acid method[34] and calculated as glucose equivalents, using glucose standards for the calibration curve.

Xylanase was assayed by incubating 1 ml of 1% beech wood xylan in 0.05 *M*

citrate buffer (pH 4.8), 0.45 ml of water and 0.05 ml of sample solution at 50°C for 20 min. The reaction was stopped by adding 0.5 ml of 2.5 *M* sodium hydroxide, and reducing sugars were determined with the dinitrosalicylic acid reagent[34]. Calculation of the xylose content was based on the absorbance of xylose standards that had reacted with the dinitrosalicylic acid reagent.

Exocellulase (cellobiohydrolase) activity was measured by following the formation of cellotriose, cellobiose and glucose from Walseth cellulose. A 1.7-ml volume of a 5% suspension of phosphoric-acid-swollen cellulose in 0.05 *M* citrate buffer (pH 4.8) and 0.3 ml of sample solution were incubated at 50°C for 24 h. The sugar mixture was then analysed by HPLC on a Bio-Rad HPX 87P column. Enzymes with an cellobiose/glucose (G_2/G_1) ratio above 10 were defined as exocellulose.

β-Glucosidase was assayed with *p*-nitrophenylglycoside as substrate[25]. The substrate used for the β-xylosidase assay was *p*-nitrophenylxyloside.

The *T. reesei* MCG 77 enzyme used in this study was prepared in a 5-l fermenter with 2% spruce sulphite pulp as carbon source[31]. The clear culture broth was concentrated by ultrafiltration (Amicon hollow-fibre cartridge H1P10-8). The protein was then precipitated by the addition of two volumes of acetone at 4°C and recovered by centrifugation (3000 *g*). After removal of the residual acetone on a rotary evaporator, the protein was redissolved in water and lyophilized.

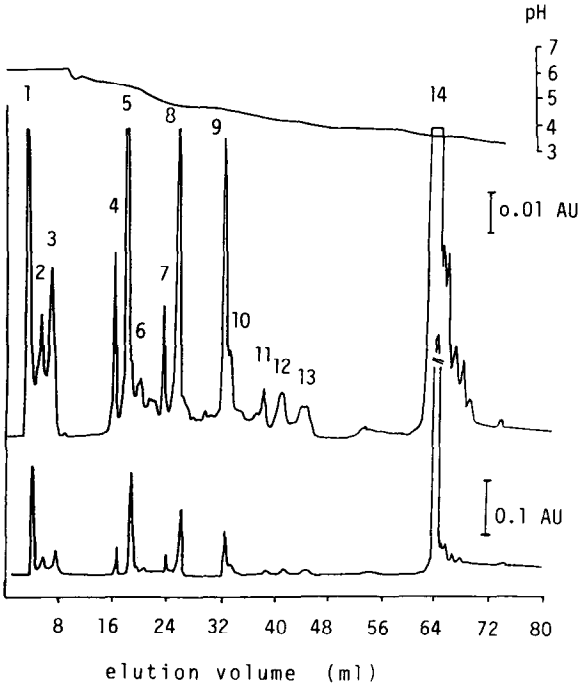

Fig. 1. Separation of *T. reesei* MCG 77 cellulase by chromatofocusing with a pH gradient from pH 6.5 to 3.0 over a total elution volume of 80 ml. Column effluent monitored at 280 nm with 1 absorbance unit (AU) (curve 1) and 0.1 AU (curve 2).

RESULTS AND DISCUSSION

Standard procedure and its reproducibility

Analytical chromatofocusing by FPLC on a Mono-P HR 5/10 column (Pharmacia) leads to an excellent separation of the complex protein mixture contained in the cellulase enzyme. The proteins are eluted from the column in the order of decreasing isoelectric points, and the protein pattern can be obtained by monitoring the column effluent at 280 nm. As an example, Fig. 1 depicts the separation of an enzyme as produced by the mutant *T. reesei* MCG 77 when grown under standard conditions on 2% spruce sulphite pulp[31]. At low detector attenuation the chromatogram showed one large protein peak with a retention time of 63.3 min, and eight medium-sized peaks with retention times ranging from 3 to 32 min. At high detector attenuation a great number of additional minor peaks were also clearly detectable. The total number of protein peaks was *ca.* 24; this includes most of the minor peaks not completely resolved from the accompanying major peaks. This number of individual proteins agrees well with the results obtained by analytical isoelectric focusing in polyacrylamide, where cellulases from five different mutants gave between 22 and 32 protein bands[21]. Long-term reproducibility of the standard separation method in respect of peak retention times and protein peak areas was tested by separating a cellulase from one batch three times at weekly intervals (Table I). For all peaks except peak 3 the standard deviation of the retention time was less than 2.5%. Reproduci-

TABLE I

ENZYME ACTIVITIES, RETENTION TIMES, ISOELECTRIC POINTS AND PERCENTAGE DISTRIBUTION OF THE INDIVIDUAL PROTEINS OF THE *TRICHODERMA REESEI* MCG 77 CELLULASE COMPLEX SEPARATED BY FAST CHROMATOFOCUSING

The separation was performed as shown in Fig. 1. Means ± standard deviation of retention time and percentage distribution were obtained from three runs, performed at weekly intervals. Peak numbers refer to Fig. 1, percentage of whole protein refers to peak areas, which is (see text) very close to weight percent. Endo = endocellulase, carboxymethylcellulase; Exo = exocellulase, cellobiohydrolase; Xyl = xylanase; β-Glu = β-glucosidase; β-Xyl = β-xylosidase.

Peak	*Retention time (min) (mean ± S.D.)*	*Isoelectric point*	*Percentage of whole protein (mean ± S.D.)*	*Enzyme activity*
1	2.99 ± 0.03	>6.5	9.1 ± 0.7	Xyl, β-Glu, β-Xyl, Endo
2	4.98 ± 0.04	*ca.* 6.5	2.6 ± 0.1	Endo
3	5.99 ± 0.55	*ca.* 6.5	3.5 ± 0.4	Endo
4	15.62 ± 0.38	5.85	2.0 ± 0.2	?
5	17.46 ± 0.29	5.70	6.9 ± 0.2	Exo, Endo
6	19.61 ± 0.26	5.60	0.6 ± 0.3	?
7	22.90 ± 0.27	5.10	1.0 ± 0.2	Endo
8	24.99 ± 0.04	4.95	5.0 ± 0.4	Xyl, Endo
9	31.84 ± 0.08	4.80	3.4 ± 0.3	Endo
10	32.68 ± 0.13	4.75	0.66 ± 0.45	Endo
11	37.82 ± 0.06	4.50	0.46 ± 0.06	?
12	40.44 ± 0.14	4.40	0.77 ± 0.15	?
13	44.28 ± 0.28	4.25	0.50 ± 0.26	?
14	62.55 ± 0.74	3.60	61.9 ± 2.6	Exo

bility of the peak areas of medium and large peaks was good, with standard deviations in the range 3–10%. The very small peaks, Nos. 6, 7, 10, 11, 12 and 13, showed standard deviations of *ca.* 50%: however, these peaks contain only *ca.* 9% of the total protein.

A great advantage of chromatofocusing lies in the fact that the chromatogram gives not only the qualitative composition of the enzyme mixture in the form of a protein profile but also an accurate estimation of the relative amount of the individual proteins. The factors determining the relative peak area of an individual protein component are its proportion in the whole cellulase complex and its specific absorption coefficient at 280 nm, the wavelength used for monitoring the protein profile. The major protein of the cellulase complex, peak 14 (cellobiohydrolase) has a specific absorption coefficient of 1.27 l g^{-1} cm^{-1} at 280 nm[30]; a purified xylanase (peak 8) gave a value[11] of $A = 1.07$. For the whole cellulase purified by ultrafiltration and acetone precipitation, the A value at 280 nm was found to be 1.2. The close agreement of the specific absorption coefficients indicates that the relative peak areas expressed as a percentage of total peak area deduced from the protein profile are an accurate indication of the weight percentages of each protein in the cellulase complex (Table I). It was shown that all the protein material is completely eluted by the standard procedure employed: when the column was rinsed with 2 *M* sodium chloride after a complete run (80 ml) no residual protein was eluted, as evidenced by the absorption of the effluent at 280 nm.

The baseline given by the polybuffer used for elution did not interfere with the quantitation of the peak areas, since it was a straight line even at a high detector sensitivity. Histidine–HCl buffer, which was previously used by us[25,30] for sample application and as start buffer, in later experiments gave an unsatisfactory baseline with a broad and unreproducible peak with an isoelectric point of *ca.* 4.8, which was probably due to UV-absorbing contaminants contained in some batches of commercially available analytical grade histidine. Histidine was therefore replaced by bis-tris; in more than 50 separations, this buffer gave no problems caused by UV-absorbing contaminants, even when different batches were used.

Assignment of specific enzyme activities to individual proteins

The column effluent was fractionated by collecting the peak materials and intermediate fractions. These fractions were used without any pretreatment for the estimation of endocellulase (carboxymethylcellulase), xylanase, exocellulase, β-glucosidase and β-xylosidase in order to obtain a more complete characterization of the enzyme composition (Fig. 2). Carboxymethylcellulase activity (Fig. 2A) was highest in peaks 3, 5, 8, 9 and 10; some activity was also present in peaks 1, 2, 4 and 7. Virtually free of any carboxymethylcellulase activity were peaks 11, 12 and 13 and the major protein peak 14. Farkas *et al.*[21], using isoelectric focusing in polyacrylamide, obtained six to eight bands with endocellulase activity with *T. reesei* mutants. Other authors studying mutants of the same fungus found four[27] or five[22] different endocellulases. β-Glucosidase was present only in the protein fraction not retained by the column (start fraction) indicating that the enzyme has an isoelectric point above pH 6.5. This is in contrast to other reports, according to which *Trichoderma* cellulase contains three different β-glucosidases[18,21]. It is likely that our β-glucosidase is identical with the β-glucosidase component with an isoelectric point of 8.8 found

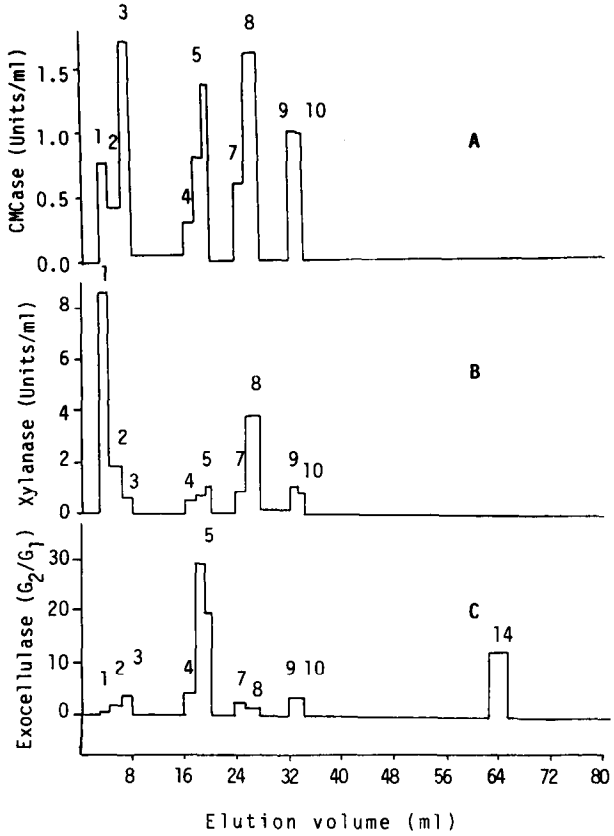

Fig. 2. Distribution of enzyme activities in fractions of a separation of *T. reesei* MCG 77 cellulase by chromatofocusing. Separation conditions were as used for Fig. 1. Peak numbers refer to Fig. 1 and Table I. The column effluent was fractionated by hand according to protein peaks, and each fraction was assayed for endocellulase activity (A), xylanase activity (B), and exocellulase activity (C). G_2/G_1 in curve C is the ratio of cellobiose to glucose fromed from Walseth cellulose after 24 h of incubation. β-Glucosidase and β-xylosidase activity was present only in peak 1 (data not shown).

by Farkas *et al.*[21] and Berg and Petterson[28]. The start peak also contained the β-xylosidase activity. Xylanase activity (Fig. 2B) was high in two peaks, the start peak with an isoelectric point of 6.5 and peak 8 with an isoelectric point of 4.95. A small xylanase activity was also associated with most of the endocellulase peaks, suggesting that endocellulases have a broad substrate specifity. Dekker[32] reported the occurrence of five proteins with xylanase activity in a *T. reesei* QM 9414 cellulase. Biely *et al.*[34] recently reported the occurrence of three major xylanases and ten minor xylanases in a *T. viride* cellulase preparation.

Exocellulase or cellobiohydrolase activity is conventionally assayed by using phosphoric-acid-swollen cellulose (Walseth cellulose) as substrate, and enzymes releasing cellobiose from this substrate are named cellobiohydrolases provided that they minimal or no capacity to hydrolyse carboxymethylcellulose. We have investigated all protein fractions collected from a chromatofocusing separation, in respect

of their ability to hydrolyse Walseth cellulose to glucose, cellobiose and cellotriose. Interestingly enough, all fractions were able to hydrolyse Walseth cellulose although the relative abundance of the three sugars was very different. The ratio of cellobiose to glucose was used as an index to define the degree of specificity of the enzyme activities (Fig. 2C). The protein from peaks 5 and 14 gave the highest ratios, 28.8 and 12.3 respectively, whereas all other fractions (peaks 1–4, and 6–13) gave ratios between 0.33 (peak 1) and 4.1 (peak 7). These results strongly suggest that peaks 5 and 14 are cellobiohydrolases and that the cellulase from *T. reesei* MCG 77 contains two forms of cellobiohydrolase, one with an isoelectric point of 5.70 (peak 5) and one with an isoelectric point of 3.6 (peak 14). The enzyme of peak 5 also showed a high capacity to hydrolyse carboxymethylcellulose, whereas the enzyme of peak 14 was virtually free of any carboxymethylcellulose activity.

Modification of the standard separation procedure

The pH gradient used for the standard separation (Fig. 1) ranged from pH 6.5 to 3.0 in a total elution volume of 80 ml, corresponding to a run time of 80 min for one separation. The criteria for selecting this pH profile were in principle the requirements for a rapid and reproducible separation of all major proteins contained in the cellulase complex, since the method should be suitable for fast screening of cellulases from various sources including their possible alteration during fermentation and cellulose hydrolysis. A flatter pH gradient than that used in the standard separation shown in Fig. 1 led to a somewhat better ressolution of all proteins eluting between

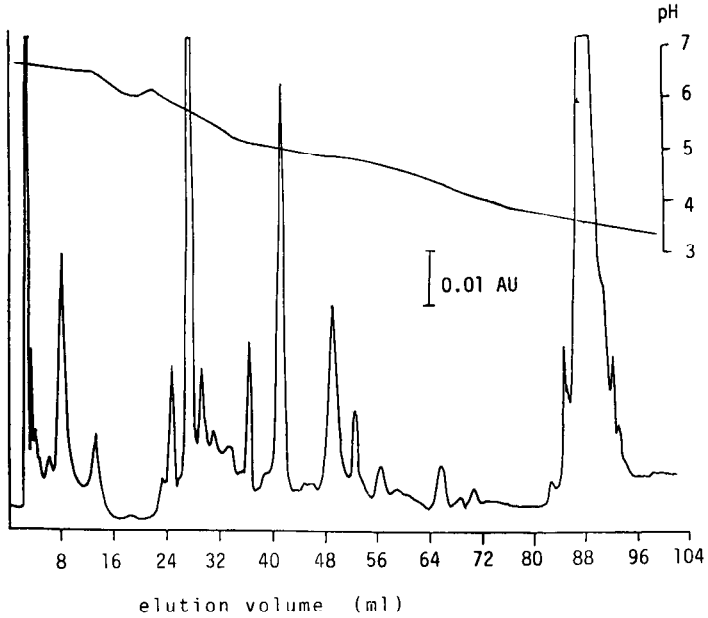

Fig. 3. Separation of *T. reesei* MCG 77 cellulase by chromatofocusing with a pH gradient from pH 6.5 to 3.0 over a total elution volume of 100 ml. After equilibration of the column with 0.025 *M* histidine–HCl 2 mg of cellulase protein in 0.2 ml of start buffer were applied to the column. Elution was performed with 10 ml of start buffer followed by 60 ml of 1:15 diluted Polybuffer 74 (pH 4.0) and 40 ml of 1:10 diluted Polybuffer 74 (pH 3.0).

pH 6.5 and 4.5. As an example, Fig. 3 shows the protein profile of a separation with a pH gradient from 6.5 to 3.0 over a total elution volume of 100 ml, which is equal to a run time of 100 min. For occasional analysis, where optimal separation is required and total run time is of minor importance, these separation conditions are certainly preferable to the standard conditions, which is a compromise between assay time and peak resolution. Steeper pH gradients would further reduce the elution time and accelerate the analysis. Our experiments, however, showed that in such cases peak resolution is not satisfactory.

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