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Ion-exchange chromatographic purification and quantitative analysis of *Trichoderma reesei* cellulases cellobiohydrolase I, II and endoglucanase II by fast protein liquid chromatography

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Abstract

Trichoderma cellulases appear in several isoforms which makes their purification and analysis difficult. We used fast protein liquid chromatography (FPLC) to purify three major cellulases and to quantitate these enzymes in reconstituted mixtures during cellulose hydrolysis studies (in lack of specific substrates and because of the synergism between the enzymes such analysis is very difficult, if at all possible, with conventional activity measurements). For the analysis methods linear calibration was achieved from 10–15 pmol to 0.5–1 nmol (from 0.5–0.8 to 27–64 μg) for the different enzymes. Due to the high resolution chromatographic media used, our purification methods are simpler and quicker than the usual protocols for cellulase purification. Several isoforms of cellobiohydrolase (CBH) I were purified. The isoforms had different isoelectric points (p*I*) but their catalytic and adsorption properties were similar. A remarkable feature of CBH I and endoglucanase (EG) II was that their electrophoretically pure preparations gave double peaks during ion-exchange chromatography in certain pH intervals where the two peaks (probably representing two conformations) were transformed into each other by changing pH. This behaviour of cellulases has never been reported before and further explains the difficulties in cellulase purification.

Keywords: *Trichoderma reesei*; Cellulases; Cellobiohydrolases; Enzymes; Endoglucanases

1. Introduction

Cellulose is the most abundant carbohydrate polymer on Earth and is a potential renewable energy source. Enzymatic cellulose hydrolysis is therefore of great importance from both technical and biochemical point of view. The filamentous fungus *Trichoderma reesei* produces one of the most effective cellulase system for hydrolysis of cellulosic materials. This cellulase system consists of at least six genetically different cellulases: two cellobiohydrolases (CBH I and II; E.C.3.2.1.91) and four endoglucanases (EG I, II, III and V; E.C.3.2.1.4). All *Trichoderma* cellulases, except EG III, have a characteristic two domain structure with a catalytic core and a cellulose binding domain, CBD. The two domains are connected with a flexible and glycosylated hinge region. The enzymes appear in multi-

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ple isoforms in culture filtrates of *T. reesei* and act in synergism with each other during hydrolysis of cellulosic substrates. The adsorption of the cellulases on the solid substrate is an important initial step in the hydrolysis [1,2].

During the past years the understanding of the mode of action of *Trichoderma* cellulases has increased greatly due to structure determinations by X-ray crystallography [3,4] and nuclear magnetic resonance (NMR) [5]. Still, more knowledge needs to be obtained on the reaction mechanisms and the way the enzymes cooperate in hydrolysis of cellulose substrates. Synergism is one of the most important aspects of cellulase action on cellulose and this aspect is not revealed by the structure determinations. An important area of research and our main interest is the study of the relation between the adsorption of the cellulase components and the synergism during cellulose hydrolysis [6]. To perform such studies it is necessary to have reliable purification methods and specific quantitative analysis methods for the enzymes of our interest: CBH I, II and EG II. We used fast protein liquid chromatography (FPLC) for both purposes.

Many authors have reported that purification of *Trichoderma* cellulases is difficult due to the great number of isoforms [7]. Since the genes of the major cellulases were cloned there is no longer any confusion about the number of genetically different cellulases, yet the question of isoforms remains. The difference between isoforms may be due to differences in glycosylation [8] (all *Trichoderma* cellulases, except EG III are glycoproteins). An other reason for confusion about the number of cellulases has been that some catalytic domains may appear in culture filtrates separated from the cellulose binding domain [9]. This diversity combined with the overall similarity of cellulases makes difficult not only their purification but also their quantitative analysis.

We have separated several isoforms of CBH I. The isoforms had different isoelectric points (p*I*) but their catalytic and adsorption properties were similar. An other remarkable property of CBH I and also of EG II was that the electrophoretically pure preparations gave double peaks during ion-exchange chromatography at certain pH intervals, where the two peaks (probably representing two conformations) were transformed into each other by changing pH. Appearance of the double peaks and the pH dependent change in their proportion was observed using columns from different manufacturers and in different pH regions proving that this is not an artefact. This kind of behaviour of cellulases has never been reported before and further explains the difficulties in cellulase purification.

The purification method described here makes use of the latest materials in ion-exchange chromatography. Due to the high resolution power of these new materials the purification scheme is simpler and quicker than former ones. A key feature in the method is that already in the first step a relatively good separation of the major cellulases is achieved. We give protocols both for a small scale purification on 6- and 1-ml prepacked columns (which can be easily automatized in the FPLC system) and a larger scale purification using 50- and 25-ml columns.

The studies of adsorption and synergism are complicated by the great number of cellulases and their isoforms. The methods conventionally used for quantification of cellulases [activity measurements against filter paper, Avicel and carboxymethylcellulose (CMC)] are not applicable when the aim is quantitative analysis of the single enzymes. Chromatographic separation of crude cellulases of *Trichoderma* has earlier been proposed for qualitative and quantitative determination of the enzyme components. [10–15]. According to our experience, chromatography of crude *Trichoderma* culture filtrates yields chromatograms with overlapping peaks and co-elution of several proteins. This makes accurate quantitative determination of individual cellulase components difficult. In the present paper we describe a method we found to be suitable for quantitative analysis of cellulase mixtures during cellulose hydrolysis. First the enzymes are purified to physicochemical homogeneity. Hydrolysis of the cellulose substrate is then studied by using reconstituted mixtures of the pure cellulases. Finally, accurate quantitative determination of enzyme concentrations in the hydrolysate is performed by ion-exchange chromatography. Both the purification and the quantitative analysis of the cellulases is done in an automated FPLC system. Our analysis methods described here proved to be excellent tools in

adsorption and synergism studies to facilitate accurate quantitation of cellulases in binary mixtures of CBH I–CBH II [6] and CBH I–EG II [26].

2. Experimental

2.1. Chemicals

Triethanolamine (TEA) and 4-methylumbelliferyl-β-D-cellobiopyranoside [MeUmb$(Glc)_2$] were purchased from Sigma (St. Louis, MO, USA). Tris-(hydroxymethyl)aminomethane (Tris), sodium sulphate, sodium chloride, sodium acetate (NaOAc), acetic acid (HOAc) and hydrochloric acid were from Merck (Darmstadt, Germany). Other chemicals were of analytical grade.

2.2. Enzymes

For the enzyme purification Celluclast (Novo, Bagsvaerd, Denmark), a commercial cellulase from *Trichoderma reesei*, was used. It is a dark brown, syrup-like liquid with a protein content of approximately 12% (w/w, estimated from A_{280}). CBH I (the major protein in Celluclast), comprises about 6% of the liquid (w/w, estimated from FPLC chromatogram) i.e., approximately 50% of the protein is CBH I. Pure cellulases from *T. reesei* QM 9414, which were used as standards, were a kind gift from Dr. Göran Pettersson (Dept. of Biochemistry, Uppsala, Sweden). Enzyme concentration in the standard solutions was determined from the UV absorbance at 280 nm using the following extinction coefficients (M^{-1} cm^{-1}): CBH I, 78 800; CBH II, 92 000 and EG II, 78 000 [16]. When the amount of enzymes is given in mg the following molecular masses were used for the calculation: CBH I, 64 000 g/mol; CBH II, 53 000 g/mol and EG II, 48 000 g/mol [16].

2.3. Sample preparation

Buffer exchange (and concentration) before chromatography of certain samples was performed by means of repeated ultrafiltration in 10- or 150-ml Omegacell (Filtron Technology Corporation, MA, USA) units with M_r 10 000 cutoff membranes. If it was necessary, prior to isoelectric focusing (IEF), 3.5-ml Microsep (Filtron) ultrafiltration units with M_r 3000 cutoff membranes were used to perform buffer exchange and to concentrate the samples.

In the scaled up purification method the buffer exchange of large sample volumes was done with a 1.4-l column packed with Sephadex G-25 Superfine gel filtration media (Pharmacia Biotech, Uppsala, Sweden). Crude Celluclast (50 g diluted in 200 ml water) was buffer exchanged to 10 m*M* TEA–HCl, pH 7.6 by this means. The same column was used to buffer exchange the CBH I pool from the first ion-exchange step to 50 m*M* NaOAc–HOAc, pH 3.7 prior to separation of the isoforms.

Samples for quantitative analysis were filtered through a small diameter (4 mm) 0.2 μm low-protein binding syringe filter (Milex-GV4, Millipore, Bedford, MA, USA). Crude, diluted Celluclast was filtered with a 13 mm diameter PTFE-based syringe filter (Minidisc T, Filtron) before buffer exchange.

2.4. Chromatography instruments and columns

The enzyme purification was performed in an automated FPLC system (Pharmacia Biotech). The system consisted of a LCC-501-plus chromatography controller; two high-precision pumps (P-500); two 7-port injection valves (MV-7), one of which was used to inject samples from conventional sample loops, and the other to collect fractions in, and to inject samples from a 10-ml SuperLoop; two 8-port selection valves (MV-8), to switch between columns; a fixed-wavelength UV monitor (UV-M II) with analytical flow cell (5 mm path length) and Hg optics with 280 nm filter; a fraction collector (FRAC-200); to facilitate automated large volume sample injection a peristaltic pump (P-1) was used; a solenoid valve (PSV-100) was used to direct fractions to the SuperLoop; the chromatograms were recorded on a REC 102 recorder.

For the quantitative analysis of cellulases the chromatography system above was supplemented by an autosampler (Pharmacia Biotech, Model 2157) equipped with a 100-μl syringe. This facilitated easy and accurate injection of different sample volumes. (In the FPLC system sample loops of certain volumes are normally used for sample injection). The

signal from the UV detector was collected by a PE Nelson 900 Series Interface (Perkin-Elmer Nelson Division, San Jose, CA, USA) and evaluated by the ''Turbochrom'' program (version 4.1, PE Nelson). Integration of the peak areas by this software was simpler and more reliable than the built in integrator of the LCC-501-plus or the integration achieved by the ''FPLC Director'' (Pharmacia Biotech) software.

Studies on pH dependent appearance of chromatographically different conformations of CBH I and EG II were performed on a BioCAD Perfusion Chromatography Workstation (PerSeptive Biosystems, Cambridge, MA, USA) as ''pH scans'' using the following buffer stock solutions: A: 100 m*M* Tris–HCl, pH 7.7; B: 100 m*M* Tris–HCl, pH 8.7; C: water; D: 3 *M* NaCl solution. The BioCAD was then programmed to mix the desired eluent: 20 m*M* Tris–HCl with the required pH and salt content. A Pharmacia Biotech SuperLoop was used in the system and the instrument was programmed in ''direct control'' configuration to facilitate on-line dilution of the enzyme samples with the actual start buffer.

All the purification and quantitative analysis were performed using ion-exchange media from Pharmacia Biotech: 1-ml Mono Q (50×5 mm I.D.) and 6-ml Resource Q (30×16 mm I.D.) columns are prepacked with strong anion exchanger with quaternary ammonium groups on 10 and 15 μm monodisperse poly(styrene–divinylbenzene) matrix, respectively. Source 15Q is a bulk media, same as in Resource Q columns. We packed a Pharmacia XK 26/20 column (26 mm I.D.) with 50 ml resin. The 1-ml Mono S column is prepacked with strong cation exchanger with methyl sulphonate groups on 10 μm monodisperse poly(styrene–divinylbenzene) matrix. Source 15S is the corresponding bulk media with 15 μm beads. We packed a Pharmacia XK 16/20 column (16 mm I.D.) with 25 ml resin.

For studies on pH dependent appearance of chromatographically different conformations of CBH I and EG II a 1.7-ml Poros HQ/M (100×4.6 mm I.D.) column from PerSeptive Biosystems was also tested (as well as the 1-ml Mono Q column). This column is also a strong anion exchanger: 20 μm cross-linked poly(styrene–divinylbenzene) support matrix surface-coated with quaternized polyethyleneimine.

All solutions used for the chromatography runs were prepared by dissolving the substances in water obtained from a Millipore Milli-Q water purification system. All eluents were filtered through a 0.2-μm membrane filter (Schleicher and Schuell, Dassel, Germany) in a 1-l Nalgene (Rochester, NY, USA) filter holder unit and were degassed by stirring for at least 10 min under vacuum.

For all the ion-exchange chromatography steps used for purification and quantitative analysis of cellulases the running parameters (column, buffer system, elution profile, etc.) are summarised in Table 1.

2.5. Activity measurement of CBH I

Methylumbelliferyl-cellobiosidase activity of fractions from chromatographic separation of CBH I isoenzymes were determined as follows: 200 μl of the appropriately diluted sample mixed with 800 μl substrate [0.5 m*M* MeUmb$(Glc)_2$ in 0.1 *M* sodium acetate buffer, pH 4.8] were thermostatted at 50°C for 10 min. The reaction was stopped by addition of 1 ml 12.5% Na_2CO_3 solution. After dilution with 8 ml distilled water, the fluorescence in the samples was measured in a Perkin-Elmer LS-50 Luminescence Spectrometer (Perkin-Elmer, Norwalk, CT, USA) using 2.5 nm slits, and 360 nm and 450 nm as the excitation and the emission wavelengths, respectively. Activity is given in arbitrary units.

2.6. IEF and activity staining (zymogram)

IEF of samples from the purification was performed using Ampholine PAGplates (pH 3.5–9.5) and the Multiphor II electrophoresis system (Pharmacia Biotech) according to the manuals. Methylumbelliferyl cellobiosidase and CMCase activity in the gel was detected by means of a special overlay gel containing both substrates (see composition further down). After the electrophoresis step, the polyacrylamide gel was immediately placed in contact with the thin zymogram gel. After 20 min of incubation at room temperature, the gels were separated. The IEF gel was placed into fixative (trichloroacetic acid solution) and later stained with Coomassie and destained, according to the methods described in the Ampholine PAGplate instruction manual. To detect CBH I and EG I activity, the

Table 1
Ion-exchange chromatography methods used for purification and quantitative analysis of cellulases

Chromatography step	Column[a] and buffer[b] system	Sample preparation[b,c]	Elution profile	Flow (ml/min)
Purification methods, "small scale"				
1. Separation of major cellulase pools (not shown)	Resource Q, 6 ml A: 20 m*M* TEA–HCl, pH 7.0 B: 1 *M* NaCl in A	Celluclast: Buffer exchanged by ultrafiltration to 10 m*M* TEA–HCl, pH 7.6	3 ml sample 17 ml A 120 ml 0–33% B	3 3
2a. Purification of EG II and CBH II (not shown)	Mono S, 1 ml A: 10 m*M* NaOAc–HOAc, pH 4.0 B: 250 m*M* NaOAc–HOAc, pH 4.0	Respective pool from step 1: Buffer exchanged by ultrafiltration to A	1 ml sample 6 ml A 16 ml 0–25% B 8 ml 25–50% B	1 1
2b. Separation of CBH I isoforms (not shown)	Resource Q, 6 ml A: 100 m*M* NaOAc–HOAc, pH 3.5 B: 250 m*M* NaOAc–HOAc, pH 3.5	Pool from step 1: buffer exchanged by ultrafiltration to 50 m*M* NaOAc–HOAc, pH 3.7	5 ml sample 3 ml A 80 ml 0–100% B	6 6
Purification methods, "scaled up"				
1. Separation of major cellulase pools (Fig. 1A)	Source 15Q, 50 ml A: 20 m*M* TEA–HCl, pH 7.0 B: 1 *M* NaCl in A	Celluclast: Buffer exchanged by gel filtration to 10 m*M* TEA–HCl, pH 7.6	50 ml sample 120 ml A 400 ml 0–40% B	3 8
2a. Purification of EG II and CBH II (Fig. 1B Fig. 1C)	Source 15S, 25 ml A: 10 m*M* NaOAc–HOAc, pH 4.0 B: 250 m*M* NaOAc–HOAc, pH 4.0	Respective pool from step 1: Diluted ten times in A	500 ml dil. EG II or 900 ml dil. CBH II 25 ml A 150 ml 0–30% B 100 ml 30–100% B	3 6
2b. Separation of CBH I isoforms (Fig. 1D)	Source 15Q, 50 ml A: 100 m*M* NaOAc–HOAc, pH 3.5 B: 300 m*M* NaOAc–HOAc, pH 3.5	Pool from step 1: Buffer exchanged by gel filtration to 50 m*M* NaOAc–HOAc, pH 3.7	100 ml sample 250 ml 0–30% B 350 ml 30–100% B	3 8
Quantitative analysis				
CBH I–CBH II (Fig. 1 in Ref. [6])	Mono Q, 1 ml A: 20 m*M* TEA–HCl, pH 7.6 B: 1 *M* NaCl in A	10–300 μl sample from hydrolysis experiment: On-line diluted with A to 6 ml in a 10 ml SuperLoop	10 ml dil. sample 8 ml A 12 ml 0–50% B	1 1
CBH I–EG II (Fig. 5)	Mono Q, 1 ml A: 10 m*M* Tris–HCl, pH 8.1 B: 0.3 *M* Na_2SO_4 in A	10–300 μl sample from hydrolysis experiment: On-line diluted with A to 6 ml in a 10 ml SuperLoop	10 ml dil. sample 18 ml A 14 ml 0–25% B 14 ml 25–100% B	1.5 1.5

[a] Column descriptions: see Section 2.4.
[b] TEA: Triethanol amine, Tris: tris(hydroxymethyl)aminomethane, NaOAc: sodium acetate, HOAc: acetic acid.
[c] Description of ultrafiltration and gel filtration methods: see Section 2.4 Section 2.6.

zymogram gel was immediately placed over a UV lightboard to visualise fluorescent bands indicating methylumbelliferone release, and photographed (with proper yellow filter). The substrate gel was then immersed in 0.1 *M* NaOH for 10 min to stop the reactions. To visualise CMCase activity, the substrate gel was subsequently immersed in 0.1% Congo Red stain for several hours. Hollows on the red background indicating break down of CMC become visible after washing the gel with 1 *M* NaCl for a few times. Finally the zymogram gel was immersed in 5% acetic acid to improve contrast between the

bands and the background colour (the CMC–Congo Red complex turns blue at low pH). The gel was dried and/or placed over a light board and photographed.

The composition of the activity staining gel as follows: 1.2% agar, 0.5% CMC and 0.1 m*M* MeUmb(Glc_2) dissolved in 100 m*M* sodium acetate buffer pH 4.8. The components were dissolved in the buffer under heating and stirring until the mixture boiled. The solution was allowed to cool to about 75°C, and poured quickly onto a GelBond film's hydrophilic side (FMC Bioproducts, Rockland, ME, USA), which was placed in a Ultro Mould gel casting unit (Pharmacia Biotech) with 1 mm spacers while sliding the glass plate (treated with Repelsilane, Pharmacia) over the top. After about 2 h the glass plate was carefully removed. To store, a second GelBond film was placed over the gel (hydrophobic side should face the gel). Wrapped in plastic wrap the zymogram gels could be stored at 4°C for weeks.

3. Results and discussion

3.1. Purification of cellulases

Separation of the main cellulases of *Trichoderma* using different methods has earlier been described by several groups. ion-exchange chromatography has been the preferred method for purification [7,17]. The method described here is also based on ion-exchange chromatography but is quicker and simpler than those described in the literature. The purification scheme consists of only three different chromatography steps and yields three pure enzymes. The simplification was possible to achieve by utilising the better resolution power of the modern ion-exchange resins and the possibility of simple and quick method development offered by the FPLC system. Also, the broad pH stability of the cellulases allowed relatively free choice of separation conditions [18]. For the method development IEF analysis of the fractions was used to identify the cellulase containing peaks; for the identification pure cellulases were applied on the gels as standards.

Repeated runs are very reproducible and can be easily programmed in the FPLC system, thus, even with the small scale method described below, the enzymes can be purified in large quantities despite the small size of the columns. For larger scale purification we successfully scaled up the methods. In Figs. 1–3 chromatograms and IEF gels from the scaled up method are shown. The small scale method resulted in very similar chromatograms and gels. Protocols for all chromatography steps are shown in Table 1.

3.1.1. Separation of the major cellulase pools

Under the conditions chosen (see Table 1), the major cellulases (CBH I, CBH II and EG II) can be relatively well separated from each other already in the first chromatography step (Fig. 1A). IEF analysis of fractions 1–8 (marked by arrows in Fig. 1A) is shown in Fig. 2. An important detail in the first step is the isocratic separation of the breakthrough material into two major peaks (as marked in Fig. 1A). The EG II pool contains almost pure EG II (Fig. 2, lane ''Fr 1''). The CBH II pool contains mainly CBH II and two other proteins of which one is probably an isoform of EG I (Fig. 2, lanes ''Fr 6–8''). The good separation of these two peaks during isocratic conditions, despite the big sample volume, is assured by setting the sample pH higher and the ionic strength lower (10 m*M* TEA–HCl, pH 7.6) than the starting buffer used for elution (20 m*M* TEA–HCl pH 7.0). This part of the separation can not be called specially ''robust'', it is sensitive for small changes in ionic strength and pH in the sample buffer and the start buffer and also for the sample volume. Yet in our hands, using the same chemicals and enzyme source, the method was reproducible. The CBH I pool contains exclusively CBH I isoforms (Fig. 1D, Fig. 3).

3.1.2. Purification of EG II and CBH II

The EG II and CBH II pools from the first step were treated exactly in the same way (cation-exchange chromatography at pH 4.0, details are given in Table 1). Since this step assures good separation of CBH II, EG II and the contaminating peaks (see Fig. 1B,C), it is not a problem if the separation in the first step is not so good or even the whole break through material from the first step could be treated together. In this case the pools of CBH II and EG II can be rechromatographed at the same conditions to increase the purity. Ultrafiltration was used as sam-

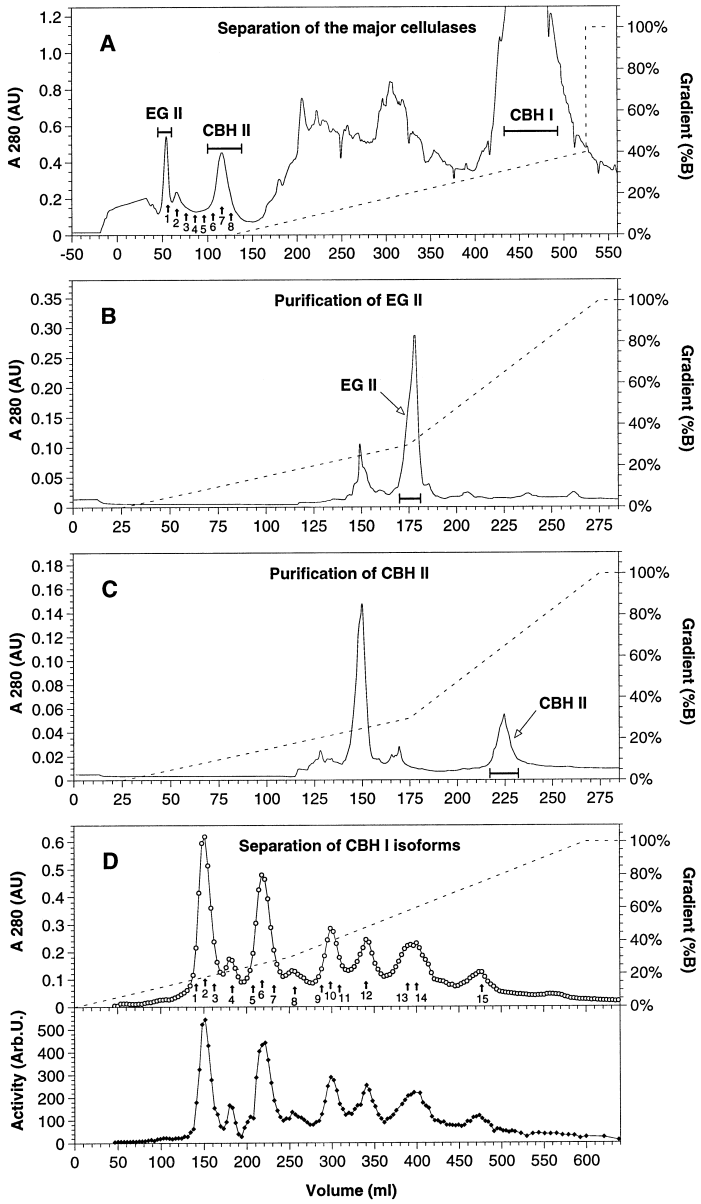

Fig. 1. Purification of three major cellulases from Celluclast. (A) Separation of the major cellulase pools; (B) purification of EG II; (C) purification of CBH II; (D) separation of CBH I isoforms: upper plot shows the UV absorbance of the fractions (at 280 nm), lower plot shows the methylumbelliferyl cellobiosidase activity of the fractions (in arbitrary units). Conditions for the separations are shown in Table 1.

ple preparation in the small scale method to perform buffer exchange and to concentrate the sample (so ordinary sample loops could be used for injection). In the scaled up method the pools were diluted instead, with the start buffer.

3.1.3. Purification of CBH I

CBH I has rather low p*I* (3.6–3.9) [17] and according to IEF analysis all the proteins in the CBH I pool from the first step have similar low p*I* values (Fig. 3, lane ‘‘Crude’’). Anion-exchange chromatog-

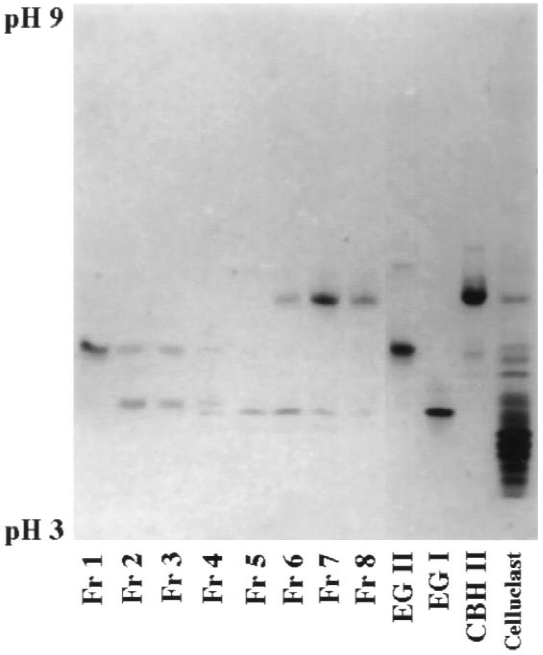

Fig. 2. IEF of fractions from Fig. 1A. Gel: Ampholine PAGplate (pH 3.5–9.5). Staining method: Coomassie staining. Lanes: Fr 1–8: fractions as indicated by arrows in Fig. 1A; EG II (p*I* 5.5), EG I (p*I* 4.5) and CBH II (p*I* 5.9) are purified standards; Celluclast: buffer exchanged material before separation.

raphy at pH≤4.9 was tried as a second purification step. Optimisation experiments showed that the best separations are achieved at pH values even lower than the p*I* of CBH I (where theoretically the protein should not even bind to the anion exchanger). Finally pH 3.5 was chosen. The buffer in the CBH I pool was exchanged to 50 m*M* ammonium acetate pH 3.7 by gel filtration as described in Section 2.6. Buffer exchanged material was separated as given in Table 1. To check the enzyme activity of the peaks, methylumbelliferyl-cellobiosidase activity of the fractions were determined. MeUmb$(Glc)_2$ is a relatively specific substrate for CBH I [19–21]. (It is also cleaved by EG I, but EG I contamination in the CBH I pool was not expected based on IEF analysis – Fig. 3, lane ''Crude''). The chromatogram and the activities are shown in Fig. 1D. The strict correlation between activity and UV absorbance for all fractions indicates that all the separated peaks contain isoforms of CBH I with the same specific activity.

Fig. 3 shows the IEF analysis of some fractions (indicated by arrows in Fig. 1D). The several bands in the IEF gel in Fig. 3 support the multiplicity of peaks in Fig. 1D, but only partly. Based on the gel one can identify five isoforms with different p*I* values while there are eight peaks in the chromato-

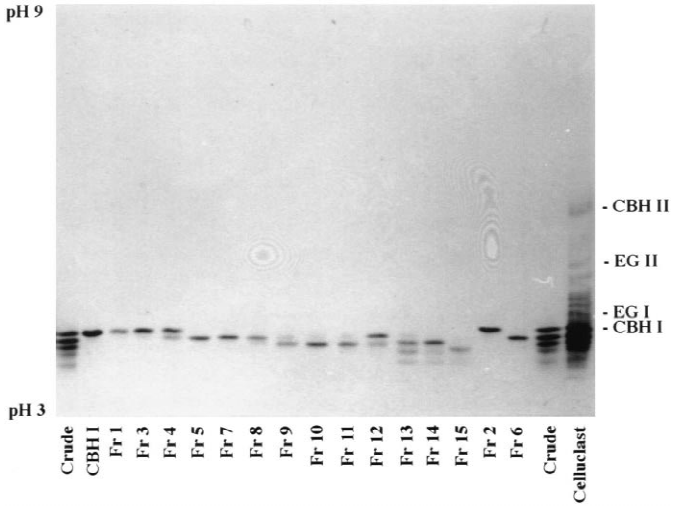

Fig. 3. IEF of fractions from Fig. 1D. Gel: Ampholine PAGplate (pH 3.5–9.5). Staining method: Coomassie staining. Lanes: Fr 1–15: fractions as indicated by arrows in Fig. 1D; Crude: buffer exchanged material before separation in this step; Celluclast: buffer exchanged material before separation in Fig. 1A. The bands of the major cellulases in this sample are also indicated.

gram (all with same specific activity). The explanation is that most of the later peaks in the chromatogram are ''ghosts'' of the early ones. Appearance of ''ghost'' peaks of CBH I and its possible reason is discussed below.

3.2. Electrophoretically identical but chromatographically different conformations of CBH I and EG II

During our chromatographic work with cellulases we often experienced ''ghost'' peaks in the chromatograms. These were especially disturbing in the quantitative analysis, since they made doubtful the purity of our preparations and destroyed the resolution between the ''real'' peaks. Therefore we examined the origin of these ''ghost'' peaks more closely. We conclude that at certain pH intervals CBH I and EG II may exist in two conformations which bind with different affinity to the columns thus eluting as two peaks. As Fig. 4 illustrates for electrophoretically pure CBH I, by increasing pH the enzyme appears more and more in a form which binds stronger to the anion-exchanger media. This

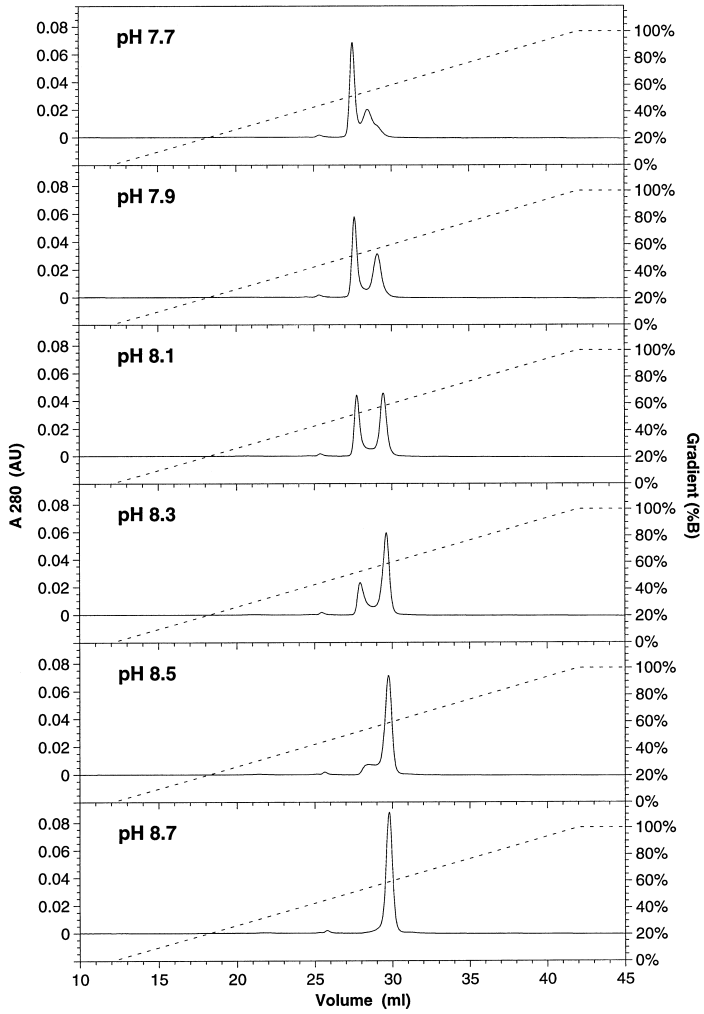

Fig. 4. Chromatographic behaviour of CBH I as a function of pH. Electrophoretically pure CBH I was chromatographed on a strong anion-exchange column (Mono Q, see Section 2.4) at various pH values, as indicated in the plots. With increasing pH CBH I probably gradually turned into a conformation which bound stronger to the column.

behaviour may be explained by shielding of a positively charged surface or by exposing a negatively charged surface as a result of some conformational changes at higher pH. Similar behaviour of EG II was observed in the pH range 7.8–9.0. (The pH scan is not shown, but in Fig. 5 the double peaks of EG II can be observed).

To make sure that we do not see an artefact we did the following: (i) adequacy of column equilibration was checked by equilibrating the column with different volumes of buffer A (pH 8.1) in the range of 2–80 column volumes. Although minor differences could be seen in the proportion of the two peaks no correlation with the equilibration volume could be found, i.e., appearance of the double peaks is not due to problems with column equilibration. (ii) Both peaks of CBH I were collected at pH 8.1 (Fig. 4) and each peak was reinjected at the same conditions. The results were two peaks in the same proportion as in the original chromatogram for both peaks (not shown). (iii) Another column (Poros HQ/M, see Section 2.4) with a chemistry similar to that of Mono Q was tested using the same buffer system. Two peaks, similar to those shown in Fig. 4 were observed (not shown). Also here, each individual peak was split into two peaks when reinjected. ''Ghost'' peaks of CBH I were observed also at low pH (3.5), as mentioned above in context with the separation of CBH I isoforms. This behaviour of cellulases explains some of the difficulties in their purification.

The reason for using relatively high pH buffers for the quantitative analysis of CBH I and EG II was that preservation of the enzyme activity was not necessary, but strong binding of the analyte to the column was desired.

3.3. Quantitative analysis of cellulases in reconstituted mixtures

The most common methods for quantification of cellulases are based on measuring activity against substrates like filter paper, microcrystalline cellulose or CMC. These methods are very useful when the aim is to get an overall information about the cellulase activity of the sample in question, but if the aim is to get specific information about the single enzymes they fail, since these substrates are not selective against either of the enzymes. Further complication is caused in the evaluation of the results by the synergism of the enzymes. Synthetic substrates, like methylumbelliferyl derivatives of cellooligosaccharides are also used and reported to be more specific; e.g., in combination with inhibitors they can be used to identify purified cellulases [21]. Recently Nidetzky and Claeyssens [22] reported methods for quantitative determination of the major cellulases in reconstituted mixture.

A straightforward idea was to separate the enzyme components in a chromatographic run. This has been suggested for characterisation of *Trichoderma* culture filtrates or commercial enzyme preparations [10,12,14]. *Trichoderma* culture filtrates contain, beside the number of cellulases and isoforms, hemicellulases, amylases, proteases and others, al-

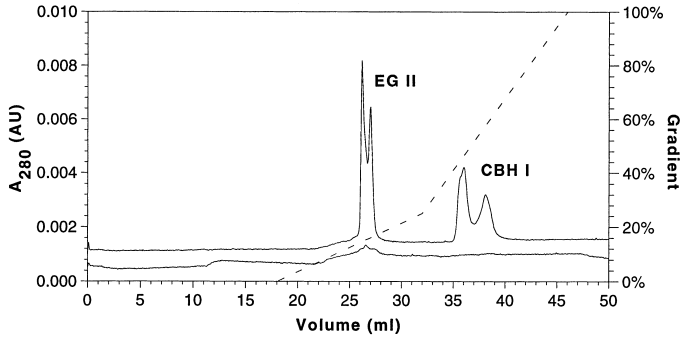

Fig. 5. FPLC chromatogram of a sample containing 190 pmol of CBH I and 190 pmol of EG II (12 and 9.3 μg, respectively). The sample was applied on a Pharmacia Mono Q HR 5/5 column in 10 m*M* Tris–HCl buffer at pH 8.1 and eluted by a 28 ml salt gradient as indicated in the Figure (100%=0.3 *M* Na_2SO_4, further conditions for the separation are described in Table 1). The baseline is a result of a blank run. Under the given conditions both cellulases appeared as double peaks as discussed in Section 3.2.

together dozens of proteins. Naturally they can not be resolved in a single chromatographic run, thus the chromatogram will contain overlapping peaks and electrophoretic analysis of the fractions will prove co-elution of several proteins in most of the peaks. A good example for this is the first purification step described in the previous section (Fig. 1A). Chromatofocusing, as suggested by Hayn and Esterbauer [13] and Yu et al. [15], seems to give better resolution of crude cellulases than ion-exchange chromatography, but overlapping peaks and co-elution still could cause a problem in the evaluation. A drawback with chromatofocusing is that Polybuffer is rather expensive compared to the simple eluents used for ion-exchange, also the runs are usually longer. Ellouz et al. [11] suggested a multi-step separation method based on ion-exchange chromatography where the cellulase containing peaks are resolved in subsequent chromatography steps. That method, we believe, is good for more detailed characterisation of cellulase preparations but too complicated to perform with great number of samples e.g., in an enzyme adsorption experiment. Also, they had problems with separating a CBH and an EG fraction. We conclude that although these methods are good for rough or more detailed characterisation of enzyme preparations or mutant strains, they are not relevant if the aim is accurate quantitative measurement of the enzymes in numerous samples. Since the aim of our research project is to study the adsorption and synergism of the major cellulases during hydrolysis of cellulose, both simplicity and accuracy were important requirements for the analysis method.

Our idea was to first purify the enzymes then remix them and study the hydrolysis with such preparations. Similar studies were made by Tomme et al. [23] using reconstituted mixtures of CBH I and CBH II. For the analysis they used total protein measurement combined with activity measurements. Kyriacou et al. [24,25] used radio-labelled enzymes in their adsorption studies to trace the two enzymes in a mixture. We use chromatographic separation of the enzymes which is a simple method and gives accurate information on concentration of the different cellulases in solution. An analysis method based on chromatographic separation, in principle, facilitates analysis of mixtures with more than two of the cellulases, which is an objective for our future research.

The sample preparation before the chromatography is simple. The samples are first filtered through a 0.22-μm low-protein binding syringe filter then diluted with start buffer. The dilution step is performed on-line by injecting the desired volume (10–300 μl) of the sample from an autosampler into a 10 ml SuperLoop where it is diluted up to 6 ml. This dilution was sufficient for rebuffering the samples and assure their proper binding on the column when the samples were containing 50 m*M* sodium acetate buffer, pH 4.8 and the sample volume was not larger than 500 μl.

Fig. 5 shows a chromatogram of a mixture containing 190 pmol of both CBH I and EG II. Both peaks appear as double peaks (as explained in Section 3.2) but accurate evaluation of the chromatograms is still possible, since under the chosen experimental conditions peaks from CBH I and EG II are baseline separated. The whole separation takes 50 min including on-line sample dilution, washing and re-equilibration of the column. Separation of CBH I and CBH II at pH 7.6 results in chromatograms with two sharp and distinctly separated peaks [6].

Calibrations were made for CBH I, CBH II and EG II using both peak heights and peak areas. Since CBH I and EG II at pH 8.1 appeared as double peaks, and the proportion of the peaks changed with small variations in the chromatographic conditions, peak heights could not be used for quantification at this pH, but the integrated areas gave reproducible results.

As an example of the results Fig. 6 shows the calibration curves achieved for CBH II. On the *x* axis injected amount is showed because both the concentration of the standard and the injected volume was varied (0.1–25 μ*M*, 10–300 μl). All calibration data could be fitted with a straight line with good correlation indicating that the injection volumes do not influence neither the peak height nor the integrated area if the injected amount is the same. (Because of the on-line dilution step before the separation, in fact, the actual sample volume that has been injected on the column was 6 ml independent of the initial sample volume). This is quite convenient since it is possible to expand the concentration range

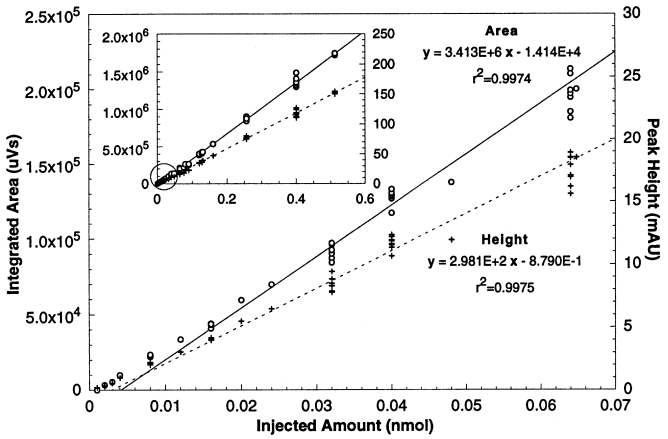

Fig. 6. Calibration curves for CBH II achieved by anion-exchange chromatography as described in Table 1. UV absorbance of the effluent was detected at 280 nm. (○) Integrated area; (+) peak height. The figure shows results of calibrations performed during a five month period. The insert shows the results from the whole range tested while on the main chart the region of low injected amounts is enlarged.

where the method can be used by injecting larger volumes (up to 500 μl) at low protein concentration and small volumes (down to 10 μl) at high concentrations. At injection volumes greater than 500 μl the on-line dilution with start buffer may not assure proper binding; at injection volumes less than 10 μl the reproducibility decreased considerably.

Fig. 6 shows results from calibrations performed in a five month period. The standard deviation (7–9 parallels) was normally less then 6% for peak areas and less then 3% for the retention times. This indicates that the overall reproducibility of the method is fairly good. The insert shows calibration data for the whole range examined. It is seen that the linear range is probably wider, but analysis of higher enzyme concentrations were not of interest in our studies. The main figure shows the region of low injected amounts. At amounts below 10–15 pmol a deviation from the line fitted to all data points can be seen. When measuring such low amounts an other calibration line, fitted only to the data obtained in this region, should be used.

The limit of detection (LOD) was 2, 1 and 1.5 pmol (128, 53 and 100 ng) for CBH I, CBH II and EG II, respectively. Linear calibration was achieved in the range of 10 pmol–1 nmol (0.64–64 μg); 15 pmol–0.5 nmol (0.8–27 μg) and 10 pmol–1 nmol (0.5–50 μg) for the three enzymes.

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