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Preparative Purification of *Trichoderma reesei* Native and "Core" Cellobiohydrolase I by Electrophoresis and Chromatofocusing

Scientific Note

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INTRODUCTION

The enzymes present in the cellulase complex produced by the fungus *Trichoderma reesei* have been the subject of considerable attention in consequence of their potential for converting cellulosic materials into glucose for further use in fermentation processes (*1*). Cellobiohydrolase I (CBH I) is the major component of crude commercial fungal cellulase preparations and catalyzes the conversion of insoluble cellulose into cellobiose. The primary structure of CBH I is known (*2*), and its tertiary structure, deduced from small-angle X-ray scattering studies, takes the shape of a tadpole (*3*) with a catalytic head region known as "core" CBH I and a C-terminal cellulose-binding tail region. Removal of the tail can be accomplished with the protease papain, resulting in reduced activity towards insoluble substrates but unchanged activity towards soluble substrates (*4,5*).

CBH I possesses 12 disulfide bonds (*6*), two of which reside in the C-terminal region (*7*). We have been interested, recently, in the reduction

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of native and "core" CBH I and have needed to develop a method for their preparative purification. We now report that by using electrophoresis and chromatofocusing, preparative quantities of both native and "core" CBH I have been obtained. Since their pI values are different, milligram quantities of "core" CBH I can be generated and purified from the native enzyme by chromatofocusing within 2 h. This method may have general utility for enzymes that degrade insoluble cellulose.

MATERIALS AND METHODS

Purification of Native CBH I

Preliminary Gel Filtration

A crude cellulase preparation (4 mL; Celluclast 1.5L, NOVO Enzymes, Danbury, CT) was initially separated by gel filtration on a 2.54×94-cm BioGel P-100 column. The fractions containing *p*-nitrophenyl cellobiosidase (PNPCase) activity measured as described in (*8*) were pooled, lyophilized, and reconstituted in nanopure water (5 mL), and the procedure was repeated.

Preparative Native Gel Electrophoresis

The filtered enzyme (4 mL, 17 mg/mL protein) was loaded into a single well (5 mm×125 mm), and electrophoresis (Hoeffer Scientific Instruments, San Francisco, CA) was performed vertically on a 7.5% native polyacrylamide gel (3.75 mm×13 cm×14 cm) at 500 V and 150 mA until the dye marker (0.1% bromophenol blue) had migrated to the bottom of the gel. The band containing CBH I (located by Coomassie blue staining of a gel run in parallel; *see* Fig. 1) was extracted from the gel by maceration in water, followed by centrifugation. After filtering and lyophilization, the enzyme was dissolved in 50 m*M* sodium acetate buffer, pH 5.0. The enzyme (5.2 mL, 22.5 mg) was then subjected to chromatofocusing using fast protein liquid chromatography (FPLC).

Fast Protein Liquid Chromatography (FPLC)

An enzyme sample obtained by electrophoresis (0.5 mL, 4.33 mg/mL) was further purified by chromatofocusing on an FPLC equipped with a Mono P HR 5/20 column and a UV detector (Pharmacia, Piscataway, NJ). A pH gradient was obtained by converting from a 100% 0.025 *M* methylpiperazine/HCl buffer (pH 5.5) to 100% of a 1:10 dilution of polybuffer 74/HCl (pH 3.0) over a period of 45 min. Fractions (0.5 mL) from several runs were collected, and those containing a major peak of protein (107–109; *see* Fig. 2a) were pooled, adjusted to pH 5.0, concentrated by lyophilization, and chromatofocused again (Fig. 2b). The fractions containing pure CBH I (105–109) were pooled and filtered through a PD-10 column with Sephadex G-25 M gel (Pharmacia) equilibrated with 50 m*M* sodium acetate buffer, pH 5.0.

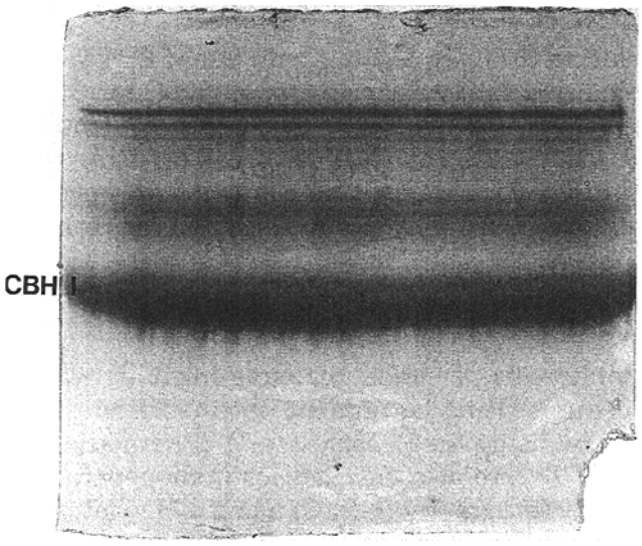

Fig. 1. Preparative gel electrophoresis of crude gel-filtered cellulase. For details, *see* Materials and Methods section.

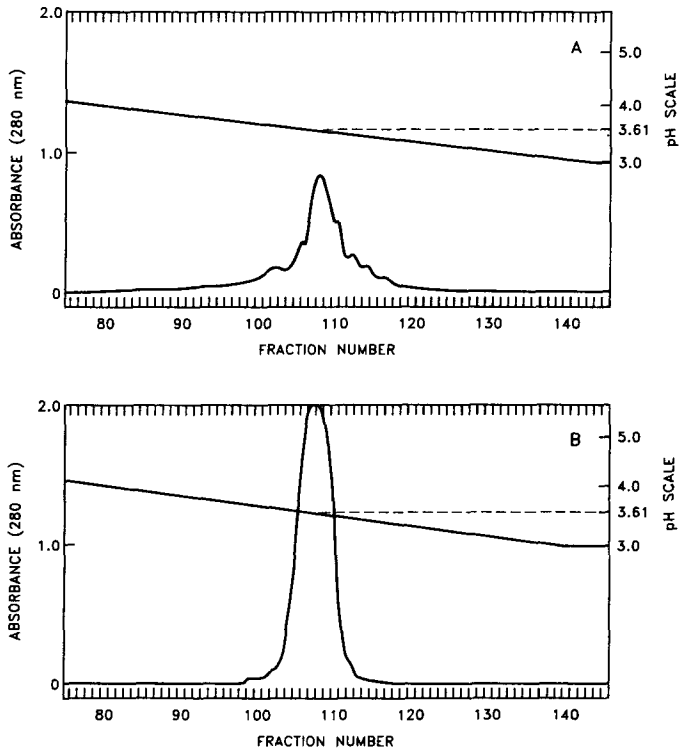

Fig. 2. Chromatofocusing of electrophoretically prepared native CBH I. For details, *see* Materials and Methods section; (A) first chromatofocusing, indicating heterogeneity of preparation; (B) second chromatofocusing, indicating homogeneity.

Purification of "Core" CBH I

Papain solution (32.8 μL; 2.46 mg/mL; 2 m*M* EDTA, 0.2 *M* NaH_2PO_4, 5 m*M* L-cysteine, pH 7.0) was incubated at 37°C for 30 min with 1 mL of CBH I (2.46 mg/mL), according to the method of Abuja et al. (*3*). The "core" CBH I was purified by chromatofocusing using the FPLC, as described above. After 30 min of papain treatment, conversion was complete. A partial digest was obtained by omitting cysteine from the incubation medium.

Analytical Procedures

Gel electrophoresis of various enzyme fractions was carried out by standard procedures using equipment obtained from Hoeffer Scientific Instruments, or by using the Phastsystem™ (Pharmacia). Purified native and "core" CBH I were also checked for homogeneity by high-performance liquid chromatography (HPLC) using a Hewlett Packard HP 1090 liquid chromatograph equipped with a 75×7.5 mm Bio-Gel TSK-DEAE-5W anion-exchange column (Bio-Rad, Richmond, CA) to determine its purity. The mobile phase consisted of 100% 50 m*M* sodium acetate buffer converted to 100% 0.5 *M* NaCl, 50 m*M* sodium acetate buffer (pH 5.0) over 12 min, and elution continued until the 15-min mark with a flow rate of 1 mL/min. Detection was accomplished using a UV diode detector in a range of 260–300 nm with a maximum absorbance occurring at 280 nm. Plots of absorbance against wavelength and time presenting the data in three-dimensional form were achieved using the HP 1040A data evaluation pack II (EVALU2) interfaced with the HPLC.

RESULTS AND DISCUSSION

Native and "core" CBH I were purified from a commercial preparation of cellulase in milligram quantities (Table 1). The low yields refer only to the total PNPCase activity, not to the actual yield of CBH I. PNPC is a substrate for both endoglucanase and β-glucosidase, which cleave the aglycon bond in this substrate (*9*). After preparative gel electrophoresis, the enzyme possessed neither endoglucanase (reducing CMC viscosity) nor β-glucosidase (cellobiase) activities, indicating that these components had been removed. Based on the CBH I activity obtained after preparative gel electrophoresis, the yield of activity of the pure enzyme after the second chromatofocusing step was about 23%. The main reason for this yield of activity after the second chromatofocusing step is that a significant percentage of PNPC-hydrolyzing activity is not associated with the main CBH I peak and is discarded after the first chromatofocusing step.

Analytical SDS gel electrophoresis of CBH I after preparative gel electrophoresis showed it to be apparently homogeneous (Fig. 3); however,

Table 1
Summary of the Purification of Native and "Core" CBH I

Fraction	Volume (mL)	Protein (mg)	Activity (munits)	Activity (munits/mg)	Yield (%)
Gel Filtration	4.0	68.0	2638	38.8	100
Electrophoresis	5.2	22.5	448	19.9	17
FPLC (2nd run) (native CBH I)	5.0	12.3	102	8.4	4
"Core" CBH I	4.9	4.3	36	8.4	1.4

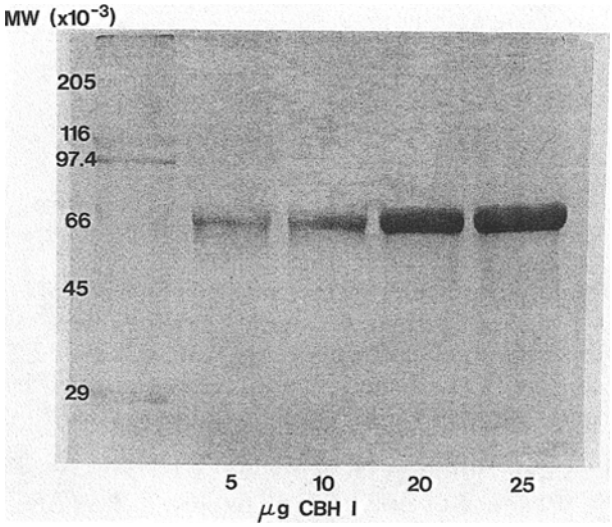

Fig. 3. SDS gel electrophoresis of native CBH I obtained after preparative gel electrophoresis. The electrophoretic system used was that obtained from Hoeffer, and the samples were reduced prior to electrophoresis. Acrylamide gels were 7.5% [w/v].

when subjected to chromatofocusing using FPLC, this was clearly not the case (Fig. 2a). The CBH I preparation consisted of at least five different proteins, the pI values of which varied between 3.5 and 3.7 (*see also* Fig. 4, lane 3). After rechromatofocusing fractions 107–109, one peak of protein was obtained (Fig. 2b) that was at least 99% homogeneous, as shown by isoelectric focusing, (IEF), *see* Fig. 4, lane 6. This was confirmed by a densitometric analysis of the IEF gel (data not shown). From Fig. 2b, the pI of CBH I was calculated to be 3.62, in agreement with Hayn and Esterbauer (*10*). CBH I eluted after 9.1 min using analytical HPLC and was also >99% homogeneous (Fig. 5). The reason why the specific activity of pure CBH I was 42% of that obtained by preparative gel electrophoresis is not known

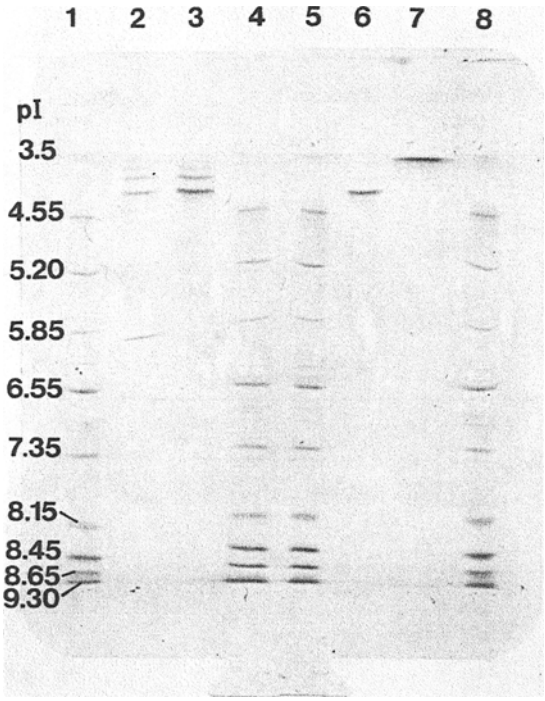

Fig. 4. Analytical isoelectric focusing (pH 3–9) of CBH I using the Phastsystem. Lanes 1, 4, 5, 8: pI markers; Lane 2: crude-filtered cellulase; Lane 3: CBH I after preparative gel electrophoresis; Lane 6: native CBH I after second chromatofocusing by FPLC; Lane 7, "core" CBH I purified by chromatofocusing. One microliter samples (1–3 μg protein) were applied to the gels.

at the present time. Some evidence suggested that lyophilization of the enzyme in the presence of polybuffer resulted in loss of activity and that, if the fractions containing the enzyme were subjected to gel filtration prior to lyophilization, the specific activity of homogeneous CBH I was similar to that of heterogeneous CBH I obtained after preparative gel electrophoresis.

Treatment of pure CBH I with papain resulted in its complete conversion to "core" CBH I within 30 min, as judged by IEF (Fig. 4, lane 7). Chromatofocusing by FPLC of a partial digest of native CBH I showed this technique to be useful for the separation and purification of "core" CBH I, the pI if which was determined to be 3.23 (Fig. 6). After purification, the "core" was reconstituted in 50 m*M* sodium acetate buffer, pH 5.0, and its homogeneity checked by HPLC. Elution occurred after 8.9 min, and the "core"was >99% pure (data not shown). The finding that "core" CBH I as more acidic than native CBH I is in agreement with van Tilbeurgh et al. (*5*). The specific activity of "core" CBH I with respect to PNPC hydrolysis was identical to that of the native enzyme.

The molecular size of native and "core" CBH I was analyzed by SDS electrophoresis using the Phastsystem (Fig. 7). The "core" enzyme is, as

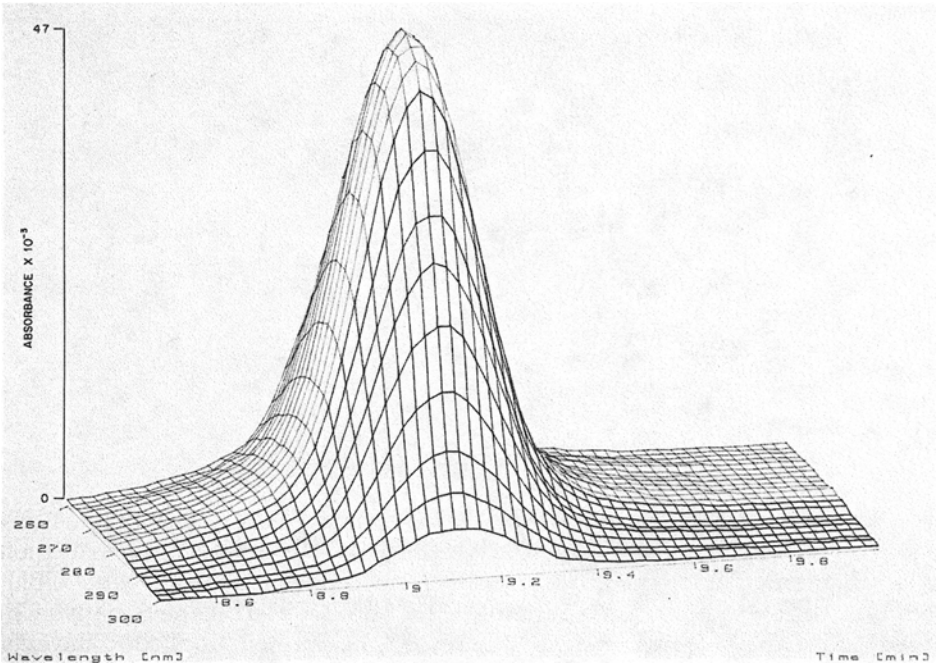

Fig. 5. Analytical HPLC of CBH I. For details, *see* Materials and Methods section. The peak of absorbance represents 47 mA units.

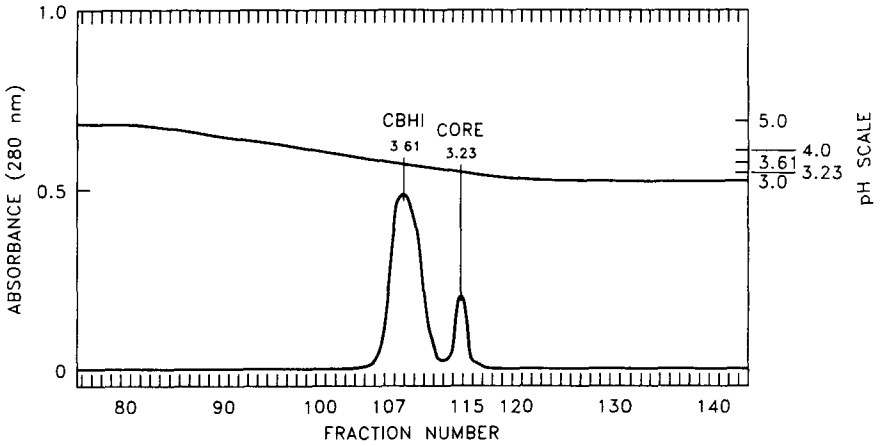

Fig. 6. Chromatofocusing by FPLC of a partial papain digest of native CBH I. For details, *see* Materials and Methods section.

expected, smaller than the native enzyme. The reduced enzymes (samples boiled with mercaptoethanol prior to electrophoresis) were larger than the nonreduced samples, because, presumably, the latter are more streamlined and move faster through the gel. The estimated molecular weights for both the reduced and nonreduced enzymes are given in Table 2.

The nature of the minor bands observed during chromatofocusing of CBH I (after preparative gel electrophoresis) is not known. These bands

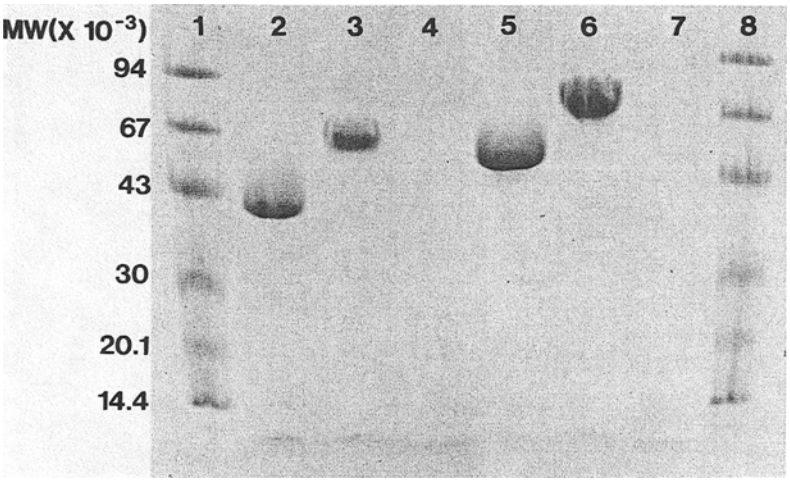

Fig. 7. SDS gel electrophoresis of native and "core" CBH I by the Phast-system. Polyacrylamide gels (10–15%) were used in the analysis. Lane 1: molecular weight markers; Lane 2: "core" CBH I, not reduced; Lane 3: "core" CBH I, reduced; Lane 4: empty; Lane 5: native CBH I not reduced; Lane 6: native CBH I, reduced; Lane 7: empty; Lane 8: markers (1–3 μg protein applied to the gels).

Table 2
Estimated Molecular Weight of Reduced and Nonreduced Native and "Core" CBH I*

	Native CBH I	"Core" CBH I
Reduced	67,000	55,000
Nonreduced	47,000	36,000

*Samples boiled with 2.5% SDS ± 5% β-mercaptethanol prior to electrophoresis.

could be subforms of CBH I differing in their levels of glycosylation (*11*). In experiments where treatment of these proteins with papain did not result in the generation of "core" CBH I, they are clearly not identical with pure native CBH I. Glycosylation could also be responsible for the stability of these forms against proteolysis by papain (*12*). The finding that the specific activity of CBH I does not increase upon purification using FPLC suggests that the subforms are in fact CBH I, not inactive protein. Indeed, they possessed the ability to hydrolyze PNPC with similar specific activity towards this substrate as the homogeneous preparation of CBH I. No synergism between the subforms with respect to PNPC hydrol ysis was observed, indicating that separation of "apparently homogeneous" CBH I into its subforms by FPLC was not responsible for the loss in specific activity.

The results of this study shows that extremely pure native and "core" CBH I can be obtained quickly and in quantities large enough to allow more accurate studies on their structure and properties. In this regard, "core" CBH II, but not its native form containing the glycosylated C-terminal region, has been crystallized (*13*). It is also of interest to note that the four major cellulase components of *Trichoderma reesei* (CBH I, CBH II, EG I, EG II) and cellulases from the cellulolytic bacterium *Cellulomonas fimi* appear to have a common structural organization that consists of a catalytic "core" and cellulose binding domain (*14,15*). The method described in this paper could, therefore, have general applicability for the isolation of the catalytic "cores" of these cellulases, necessary to study their structure and function.

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