0273-2289/01/91–93/0099/$12.25

Fingerprinting *Trichoderma reesei* Hydrolases in a Commercial Cellulase Preparation

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Abstract

Polysaccharide degrading enzymes from commercial *T. reesei* broth have been subjected to "fingerprint" analysis by high-resolution 2-D gel electrophoresis. Forty-five spots from 11 × 25 cm Pharmacia gels have been analyzed by LC-MS/MS and the resulting peptide sequences were compared to existing databases. Understanding the roles and relationships of component enzymes from the *T. reesei* cellulase system acting on complex substrates is key to the development of efficient artificial cellulase systems for the conversion of lignocellulosic biomass to sugars. These studies suggest follow-on work comparing induced and noninduced *T. reesei* cells at the proteome level, which may elucidate substrate-specific gene regulation and response.

Index Entries: Cellulase; *Trichoderma reesei*; two-dimensional gel electrophoresis; liquid chromatography–mass spectrometry/mass spectrometry.

Introduction

One key technical challenge to enable the production of sugars and ethanol from lost-cost feedstocks remains the reduction in cost of cellulases acting on pretreated biomass *(1–3)*. Our approach to this dilemma is to increase the specific activity of the enzymes in the cellulase complex. Understanding the roles and relationships of cellulase component enzymes acting on specific substrates is vital to the development of an efficient artificial cellulase system for the conversion of cellulosic biomass to sugars. The *Trichoderma reesei* biomass degrading system consists of many glycosyl hydrolases, of which five β-1,4-endoglucanases (EG I–EG V),

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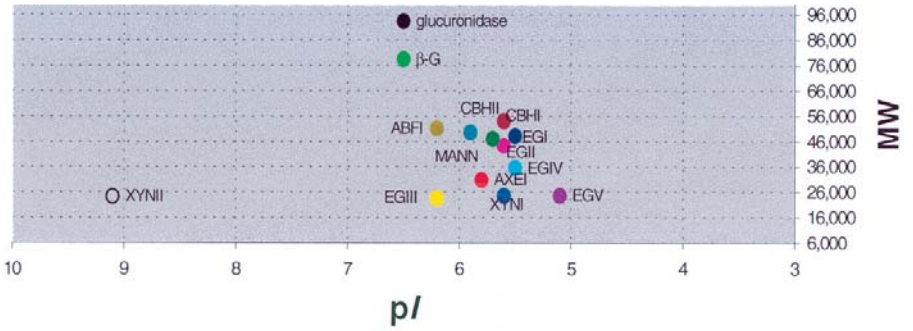

Fig. 1. 2D gel simulation of *T. reesei* hydrolases based on literature values for molecular weight and isoelectric pH.

two β-1,4-exoglucanases (cellobiohydrolase [CBH] I and CBH II), two xylanases (XYN I and XYN II), a β-D-glucosidase, an α-L-arbinofluranosidase, an acetyl xylan esterase, a β-mannanase, and an α-glucuronidase have been sequenced *(4)* (Fig. 1, Table 1).

Application of preliminary proteonomics analysis to a standard commercial cellulase product was judged appropriate for demonstrating fingerprinting methodology *(5)*. Although two-dimensional (2D) protein gels have been used on occasion to follow the expression of selected *T. reesei* cellulase components *(6)* and their glycosylated forms *(7,8)*, a systematic display of the entire system of enzymes found in *T. reesei* culture broth has not been reported. We describe here fingerprinting via 2D gel electrophoresis and internal peptide sequence analysis of a commercial cellulase preparation. The Biomolecular Research Facility at the University of Virginia Medical School processed gels prepared at National Renewable Energy Laboratory for spot sequencing and identification. Forty-five spots were identified by liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS). The experimental results were compared to ~1100 known glycosyl hydrolases from all species, with positive hits only arising from *T. reesei* proteins. Most of these were of known proteins, but several novel proteins were detected.

Materials and Methods

Enzyme Sample and Reagents

Genencor (Palo Alto, CA) generously provided a sample of the commercial cellulase preparation, Laminex, for testing. Buffers, trypsin, dithiothreitol (DTT), and electrophoresis supplies were obtained from Sigma (St. Louis, MO) and Amersham Pharmacia Biotech (Alameda, CA).

2D Electrophoresis

Characterization of the Laminex cellulase product was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and

Table 1
Critical Values for *T. reesei* Glycosyl Hydrolases[a]

T. reesei enzyme/reference	Molecular weight	Number of amino acids	Calculated p*I* (experimental)	Average hydrophobicity	Hydrophobic (%)/ hydrophilic (%)	Folded/ extended area (native shape)
EG I	48,209	459	5.50 (4.7)	–3.7	1.15	0.23
EG II	44,228	418	5.60 (5.5)	–1.9	1.02	0.24
EG III	23,481	218	6.20 (7.4)	–3.6	0.91	0.28
EG IV *(9)*	35,512	344	5.50	–0.7	0.98	0.25
EG V	24,412	242	5.10	–1.1	0.79	0.28
CBH I	54,075	513	5.60	–4.4	1.12	0.22
CBH II	49,655	471	5.90	–1.2	0.93	0.23
XYN I	24,583	229	5.60	–2.8	1.06	0.28
XYN II	24,173	222	9.10	–4.7	1.04	0.28
β-D-Glucosidase I *(10)*	78,436	744	6.50	–1.6	0.95	0.20
α-L-Arabinofuranosidase I *(12)*	51,117	500	6.20	–1.1	0.84	0.23
Acetyl xylan esterase *(13)*	30,755	302	5.80	–1.5	0.95	0.26
β-Mannanase[b]	47,054	437	5.70	–2.6	0.99	0.23
α-Glucuronidase *(11)*	93,427	847	6.50	–2.5	0.96	0.19

[a]Sequence data are from SwissProt or Genbank and calculated using PepTools (Edmonton, AB, Canada).
[b]Accession no. L25310 NID g506847.

2D gels under denatured conditions. Protein sample preparation was initiated by trichloroacetic acid precipitation followed by acetone washing and drying prior to resolublization in nonionic denaturing loading buffer.

All the 2D gel electrophoreses were performed in a horizontal format utilizing the Pharmacia Multiphor II electrophoresis system and Immobiline Drystrip kit. The first dimension of isoelectric focusing was carried out in an immobilized pH gradient dry strip gel (pH 3.0–10.0 linear). The second dimension was run on 8–18% SDS polyacrylamide gradient gel (ExcelGel) and stained with colloidal blue. The standard methods and procedures were followed directly from the Pharmacia manual.

The precast 180-mm Immobiline Drystrips were hydrated overnight in 8 *M* urea, 0.5% Triton X-100, Pharmalyte 3-10, and DTT and then were loaded into the running tray and overlaid with oil. Approximately 75–100 µg of total protein was added to the sample buffer, loaded into the loading cups, and pulled into the first dimension gel at 500 V and 1 mA for 5 h and then allowed to run at 3500 V and 1 mA for 14 h (total of 55,250 V-h). After the strips were equilibrated in a Tris-HCl, urea, glycerol, and SDS buffer, they were placed on the second-dimension SDS-PAGE gel (8–18% SDS ExcelGel; precast 245 × 110 mm) at a 90° angle to the electrical field. This dimension was run for 1.5–2 h at 600 V and 50 mA. All subsequent gels were stained with either silver stain or colloidal blue.

Liquid Chromatography–Mass Spectrometry/Mass Spectrometry

The gel piece was precisely cut and transferred to a siliconized tube and washed and destained in 200 µL of 50% methanol overnight. The pieces were then dehydrated in acetonitrile, rehydrated in 30 µL of 10 m*M* DTT in 0.1 *M* ammonium bicarbonate, and reduced at room temperature for 0.5 h. The DTT solution was removed and the sample alkylated in 30 µL of 50 m*M* iodoacetamide in 0.1 *M* ammonium bicarbonate at room temperature for 0.5 h. The reagent was removed and the gel pieces were dehydrated in 100 µL of acetonitrile. The acetonitrile was removed and the gel pieces were rehydrated in 100 µL of 0.1 *M* ammonium bicarbonate. The pieces were then dehydrated in 100 µL of acetonitrile, the acetonitrile was removed, and the pieces were completely dried by vacuum centrifugation. The gel pieces were rehydrated in 20 ng/µL of trypsin in 50 m*M* ammonium bicarbonate on ice for 10 min. Any excess trypsin solution was removed and 20 µL of 50 m*M* ammonium bicarbonate added. The sample was digested overnight at 37°C and the peptide formed extracted from the polyacrylamide in two 30-µL aliquots of 50% acetonitrile/5% formic acid. These extracts were combined and evaporated to 25 µL for LC-MS analysis.

The liquid chromatography–mass spectrometry system consisted of a Finnigan LCQ ion trap mass spectrometer system with a Protana nanospray ion source interfaced to a self-packed 8 cm × 75 µm id Pnenomenex Jupiter 10-µm C18 reversed-phase capillary column. Volumes of the extract (0.5–2 µL) were injected and the peptides eluted from the column by an acetonitrile/0.1 *M* acetic acid gradient at a flow rate of 0.25 µL/min. The

nanospray ion source was operated at 2.8 kV. The digest was analyzed using the double play capability of the instrument acquiring full-scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in sequential scans. This mode of analysis produces approx 400 collisionally associated desorption (CAD) spectra of ions ranging in abundance over several orders of magnitude. Not all CAD spectra are derived from peptides.

The data were analyzed by database searching using the Sequest search algorithm. Peptides that were not matched by this algorithm were interpreted manually and searched vs the expressed sequence tag databases using the Sequest algorithm. Experimental data were compared to a database of ~1100 known glycosyl hydrolases plus the NCBI database. Proteins were positively identified by 100% identity to two or more peptide fragments per protein.

Results and Discussion

Recent advances in protein chemistry tools including rapid separation techniques such as 2D electrophoresis and LC-MS/MS afford unique opportunities to examine complex protein mixtures, such as the multicomponent mixture of hydrolytic enzymes secreted by *T. reesei*. These techniques permit fingerprinting of mixtures of proteins found in cellulase preparations and their degradation products over a wide range of pHs and molecular weights. The capability of 2D electrophoresis to separate several hundred proteins in a single experiment can ultimately be used to identify products expressed by different genomes as well as to identify unique proteins that may be expressed in response to induction by different biomass substrates. The data generated from the analysis of complex cellulase mixtures not only gives important information about this complex biologic system relevant to biomass conversion, but also may yield information about degradation of proteins in commercial preparations.

2D gel electrophoresis of a commercial *T. reesei* cellulase preparation yielded approx 70 discernible spots on a 245×110 mm gel (Figs. 2–4). Fifty-five to 60 gel species were observable in the pH and molecular weight window we considered appropriate for secreted fungal hydrolases (pH 2.5–9 and 20–180 kDa). Thirty-four of these spots were identified as known glycosyl hydrolases by LC-MS/MS. Eleven spots contained unknown proteins.

Although a number of spots were found by LC-MS/MS to contain a single protein component (i.e., EG I, EG IV, EG V, CBH I, CBH II, AXE, and β-D-glucosidase), analysis of many of these spots yielded multiple identities (Table 2). We feel that the sampling and identification conditions applied to these gels were stringent enough to rule out contamination or misidentification of spots. The likely explanations for this observation lie in both the methodology used and the inherent nature of the sample. Some proteins may still be unresolved under the electrophoretic conditions applied (Fig. 3). For example, some improvement in the separation win-

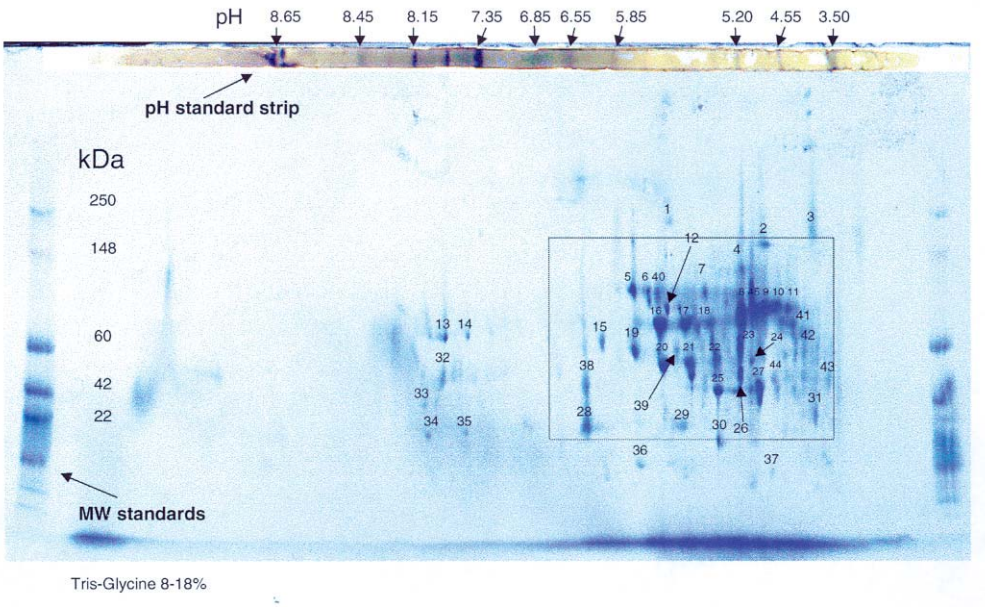

Fig. 2. Pharmacia 2D gel of *T. reesei* cellulase preparation (Laminex; Genencor) visualized with Coomassie blue. MW, molecular weight.

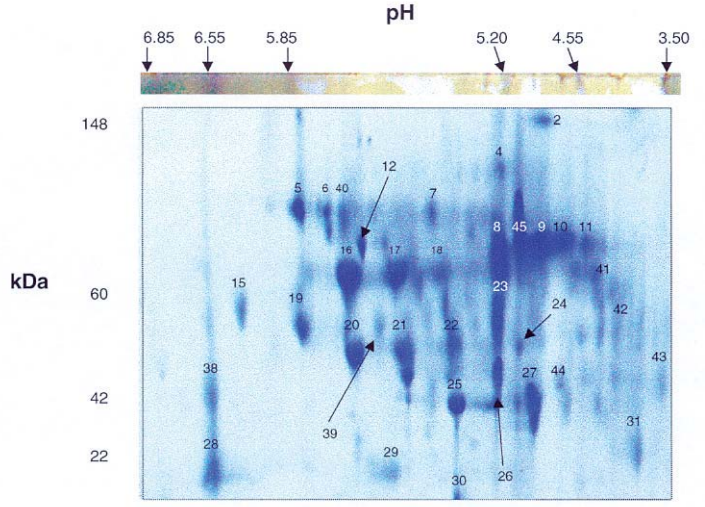

Fig. 3. Pharmacia 2D gel of *T. reesei* cellulase preparation (Laminex; Genencor). Closer view of the gel zone defined by pH 3.5–6.85 and molecular weight (MW) of 20–150 kDa.

dow may be possible with new recently available "narrow" pH range ampholines. Also, isozymes or variably glycosylated forms of the proteins may explain small changes in molecular weight or isoelectric point (p*I*);

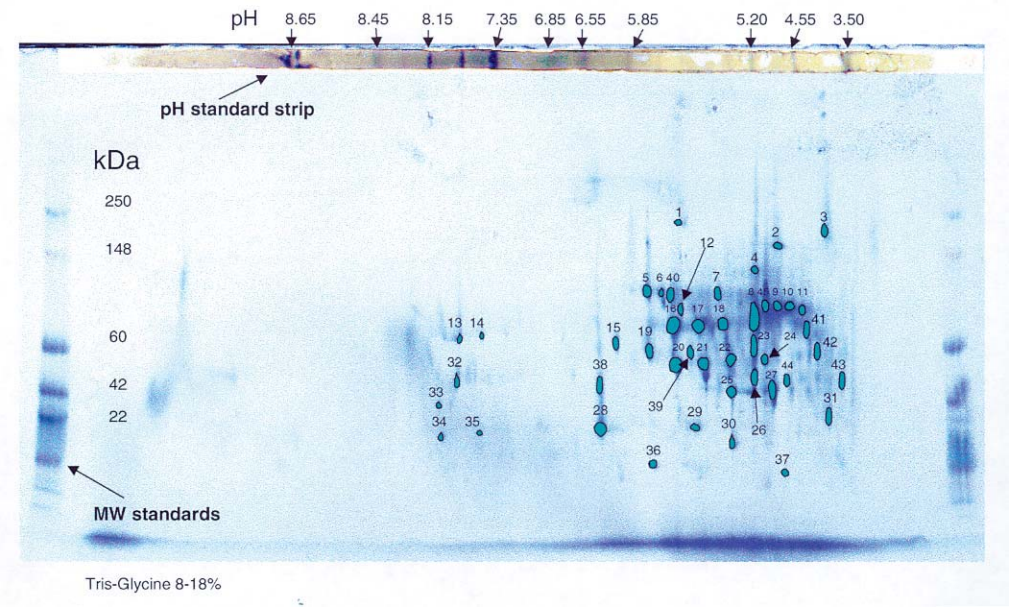

Fig. 4. Pharmacia 2D gel of *T. reesei* cellulase preparation (Laminex; Genencor). Analysis of the 45 spots shown was made by LC-MS/MS. MW, molecular weight.

however, this explanation would not account for the large alterations in the properties observed in the case of EG I, CBH I, and CBH II. Severe proteolysis of some proteins during production, storage, or sample preparation may provide the answer. Except for sample preparation alteration, the presence of these altered proteins raises several interesting possibilities. Can overall system-specific activity be increased through prevention of these altered enzymes? Can purified cellulases be used to reconstitute a cellulase mixture that is more active than native? The phenomenon of multiple electrophoretic forms was most obvious with CBH II. Having many CBH II spots widely distributed on the gel raises two obvious questions. Is CBH II really needed for efficient hydrolysis? and Can the addition of holo-CBH II (the intact enzyme) to complex cellulase mixtures enhance activity?

It is now logical to compare compositions of variably induced cellulase preparations from *T. reesei* by high-resolution 2D electrophoresis and note the differences as a function of the individual enzyme species present and the overall activity of the cellulase complex on pretreated biomass. Special assays such as the diafiltration saccharification assay can be used to assess overall cellulose digestibility. The results of these comparisons may lend yet another perspective to the biochemical interactions of the individual enzyme species within the *T. reesei* cellulase complex needed for the rapid and total saccharification of cellulose in biomass.

Table 2
2D Gel Component Analysis
of *T. reesei* Preparation by LC-MS/MS[a]

Spot no.	Spot assignment
1	CBH II
2	CBH II
3	EG I, CBH II
4	Unknown 1, same as spot 31
5	Unknown 2, same as spot 6
6	Unknown, same as unknown 2
7	EG IV
8	CBH I, CBH II, EG I, EG II
9	CBH I, EG I
10	CBH I, EG I
11	CBH I, EG I
12	Unknown 3
13	β-D-Glucosidase
14	β-D-Glucosidase
15	CBH II, β-D-glucosidase
16	CBH II
17	CBH II
18	CBH II, CBH I
19	CBH II
20	CBH II, CBH I
21	CBH II, CBH I
22	EG II, CBH II, CBH I
23	EG II, CBH II, CBH I
24	EG II, CBH II, CBH I
25	CBH II
26	EG II, CBH II, CBH I
27	EG II
28	Endo
29	Endo, CBH II, EG II
30	Acetyl xylan esterase
31	Unknown, same as unknown 1
32	Unknown 4, same as spot 33
33	Unknown, same as unknown 4
34	Unknown 5, same as spot 35
35	Unknown, same as unknown 5
36	Unknown 6
37	Unknown 7
38	Endo
39	β-D-Mannanase
40	EG IV, CBH II
41	CBH I
42	EG I, CBH I
43	EG V
44	Unknown 8
45	EG I

[a]EG I: 8–11, 42, 45; EG II: 3, 8, 22–24, 26, 27, 29; EG IV: 18, 24, 40; EGV: 43; β-glucosidase: 13–15; CBH I: 8–11, 20–24, 26, 41, 42; CBH II: 1–3, 8, 15–26, 40; AXE I: 30; β-mannanase: 39; novel: 5–7, 30–36, 44.

Acknowledgments

This work was partially funded by the Biochemical Conversion Element within the Biofuels Systems Program of the Office of Fuels Development of the US Department of Energy. This work was also funded by the University of Virginia Pratt Committee.

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