Chapter 18

Analysis of Transgenic Glycoside Hydrolases Expressed in Plants: *T. reesei* CBH I and *A. cellulolyticus* EI

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Abstract

Plant cell walls are composed of three basic structural biomolecules: cellulose, hemicellulose, and lignin with cellulose being the most abundant biopolymer on earth. Cellulose is composed of cellodextrins, which are linear polymers of glucose, and considered to be microcrystalline in structure. The conversion of cellulose to free glucose is one of the primary steps in the fermentative conversion of biomass to fuels and chemicals. However, the crystalline nature of this complex, noncovalent structure is highly resistant to enzymatic hydrolysis. Thus, the substantial cost currently associated with biomass saccharification primarily represents the cost of biomass degrading enzymes [1]. Despite the fact that the microbial cellulose hydrolytic "machinery" for the recycling of carbon from plant biomass already exists in nature, the natural enzymatic degradation of plant material is typically a slow and complex process [2]. Thus, if commercial biofuels production is to become a reality, it must be more cost-effective. One method proposed for achieving this objective is to express all or some of the requisite cellulolytic enzymes *in planta*, thus reducing both enzyme and thermochemical pretreatment costs [3, 4].

Key words: Glycoside hydrolase, *In planta* expression, *Trichoderma reesei*, Cellobiohydrolase I, *Acidothermus cellulolyticus*, Endoglucanase EI

1. Introduction

Cellulose is largely degraded by the synergistic action of two general types of cellulases; enzymes that cleave cellulose internally are referred to as endo-1,4-β-D-glucanases (E.C. 3.2.1.4), that serve to provide new reducing and nonreducing chain termini on which more processive enzymes, such as the exo-1,4-β-D-glucanases (cellobiohydrolase, CBH; E.C. 3.2.1.91), can operate. The products of the exoglucanase reactions are typically glucose and/or cellobiose, but other short-chain cello-oligomers are also produced.

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To date, many cellulase genes have been cloned and sequenced from a wide variety of bacteria, fungi, and plants. Subsequently, these genes have been transferred and expressed in model plants (i.e., *Arabidopsis thaliana* and *Nicotiana tabacum*), as well as biomass crops (e.g. *Zea mays*) (3).

Therefore, it is an important prerequisite to be able to assess whether or not the selected plant expressed transgenic enzymes are in fact present in the plant materials and if they are present, whether or not they retain their hydrolytic activities. In this chapter, we describe methods to identify and verify the activity of two important classes of enzymes used for biomass deconstruction: the *Acidothermus cellulolyticus* endoglucanase I (EI) (Cel5A) and the *Trichoderma reesei* cellobiohydrolase I (CBH I) (Cel7A).

The *A. cellulolyticus* EI is a well-characterized glycoside hydrolase family 5 endocellulase known to be thermally tolerant (optimal temperature 80 °C) and generally very stable over broad ranges of pH with an optimum of pH 5 (5). The *A. cellulolyticus* EI has been expressed in both *Nicotiana tabacum* and *Zea mays*, where its expression was targeted to the cell wall under a constitutive promoter (6–8). *T. reesei* CBH I is a major component of most commercial cellulase preparations. It degrades cellulose processively from nonreducing free chain ends. CBH I shows optimal and substantial activity at temperatures up to 50 °C. Expression of CBH I has been reported in corn leaves and kernels (9).

2. Materials and Methods

2.1. Biomass

Several biomass samples and fractions thereof are discussed in this study. Briefly, they include corn (seeds) transformed with the *T. reesei cbh1* gene, corn (stover) transformed with the *A. cellulolyticus e1* gene (8), and tobacco transformed with the *A. cellulolyticus e1* gene (6).

The EI-maize biomass was used as a model lignocellulosic substrate, as corn stover (the nongrain parts of the harvested plant) is one of the leading biofuel feedstock candidates. Ransom and coworkers (10) produced EIcd transformed maize in which EI expression was driven by the 35S CaMV promoter and targeted to the apoplast. EI corn plants were grown to maturity under greenhouse conditions and then allowed to dry in the greenhouse before being harvested (7). Tobacco was transformed successfully according to standard methods using *Agrobacterium tumefaciens*. After identification of stable lines, the biomass was grown under controlled greenhouse conditions. Individual plants were screened for EI activity. Initial studies focused on two transgenic lines designated EI-1 and EI-7, which showed high and low endoglucanase activity, respectively (7). Wild type (WT) maize kernels or

transgenic (TG) maize kernels overexpressing CBH I were created as described by Hood and coworkers, and processed and made into a fine cornmeal by grinding the dried kernels (11).

2.2. Milling

All biomass samples, including both leaves and stalks, were air-dried to a moisture content of approximately 10 %, and milled using a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) to pass a either an 80- or 20-mesh screen. Corn kernels were milled to 20 mesh using a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA).

2.3. Extraction of Glycoside Hydrolases

Several methods were employed to extract the enzymes from the ground biomass depending on the desired assay. Briefly, aqueous buffers may be a viable option for extracting small amounts of protein from biomass samples for highly sensitive analyses, such as 4-methylumbelliferyl-β-D-cellobiose. Alternatively for detection using gel electrophoresis and/or subsequent western blotting, typically boiling for 5–10 min with SDS or LDS-containing sample buffer is an effective extraction technique, though it denatures the enzyme. Finally, extraction using a combination of ethylene glycol and detergents can be used to remove glycoside hydrolases containing carbohydrate binding modules from biomass more effectively than with simple buffers by disrupting the CBM/biomass interaction; the recipe for this extraction is as follows: 50 % ethylene glycol, 0.5 % SDS, LDS protein loading buffer, 2 % Brij 30, 2 % Triton X-100, 2 % Tween 80, 2 % non-Ident P-40.

2.4. Identification of Glycoside Hydrolases

Western blotting is among the most commonly used techniques to detect the levels of target proteins when specific antibodies are available (12). Thus, we use this technique (with the respective antibodies) to determine the expression of EI and CBH I in the examined sample.

2.4.1. EI Identification Using Western Blot

To identify the transgenic EI construct we used direct extraction of the plant material into LDS loading buffer followed by western blotting. Briefly, 5 mg milled biomass was added directly to 50 μL NuPAGE LDS sample buffer and boiled for 10 min. After centrifuging for 5 min at 14,000×*g*, 20 μL of the supernatant was loaded onto a NuPAGE 4–12 % gradient gel run in MOPS (3-(*N*-morpholino)-propanesulfonic acid) running buffer (Invitrogen, Carlsbad, CA). The gel was then electroblotted onto polyvinylidene fluoride (PVDF) membrane using the Invitrogen XCell II blot module blotting apparatus. The blot was analyzed for the presence of EIcd using mouse anti-EI monoclonal primary antibody and goat anti-mouse secondary antibody with the Invitrogen WesternBreeze immunodetection kit. EIcd purified from expression in *Streptomyces lividans* was used as a qualitative control for comparison with a dilution series of the EI maize and tobacco extracts.

2.4.2. CBH I Identification Using Western Blot

A western blot of CBH I enzymes obtained from various sources was performed to illustrate the importance of using more than one antibody with different epitopes to identify CBH I or other multi-domain enzymes expressed in plants. Samples (15 µL) of the extracts were loaded onto PAGE/SDS gels then transferred to a PVDF membrane as for EI. Immunodetection was carried out as for EI with either a mouse monoclonal against the CBM/linker domain of CBH I, or a rabbit polyclonal antibody specific to the catalytic domain (CD) portion of CBH I.

2.5. Testing for Transgenic Enzyme Activity

The low EI detection levels indicated by the direct extraction/western blot detection suggested the need for an enzymatic assay with a high sensitivity such as the MUC assay. Other substrates, such as AZCL-hydroxyethyl cellulose and *p*-nitrophenyl-β-D-cellobioside did not exhibit the required sensitivity for quantitation. Additionally, the yellow product from the pNPC assay was lost in the high background color generated by tobacco slurries. The 4-methylumbelliferyl-β-D-cellobiose (MUC) assay takes advantage of the ability of endoglucanases to break down the fluorogenic substrate (MUC) to produce the fluorophore, 4-methylumbelliferone (MU) (13). On the basis of western blot estimates of enzyme concentration and the sensitivity of the assay, the MUC assay was chosen to determine the enzymatic activities of EI in the examined samples.

2.5.1. Fluorogenic Assays for EI Activity

The activity of EI in milled corn stover and tobacco can be estimated on the basis of MUC activity. In a 16 × 100-mm test tube, we added 10 mg of 80-mesh biomass, 200 µL of 1.0 M sodium acetate, pH 5.0, and 1,600 µL of H_2O. After heating the sample to either 60 °C or 84 °C for 5 min, 200 µL of 2.0 mg/mL MUC substrate was added to the reaction mix. The 60 °C assay condition yielded false positives in the untransformed control stover. Presumably, this is due to a native corn β-glycosidase present in the stover. Heating to 84 °C inhibited this activity in wild-type stover.

Each sample was analyzed in triplicate. Samples of 100 µL were taken at 0.0, 0.5, 1.0, and 2.0 h (for stover) or every minute for 15 min (for tobacco), quenched with 200 µL of 1.0 M Na_2CO_3 and fluorescence was measured at 360-nm excitation/465-nm emission in a Tecan GENios microplate reader (MTX Lab Systems, Vienna, VA, USA). EIcd levels in the stover were estimated by comparison of activity to purified *S. lividans*-expressed EIcd activity under the same conditions (Fig. 2).

2.5.2. Assay of Transgenic Cornmeal Extracted CBH I Against Microcrystalline Cellulose: Extraction of CBH I from Transgenic Cornmeal

Six aliquots of ground transgenic corn meal, averaging 4.8 g each for a total of 28.8 g of ground corn kernels, were each extracted for 80 min (room temperature with constant mixing by inversion at 20 revolutions per minute in VWR-Brand 50-mL centrifuge tubes) with 37.0 mL each of 6 mM acetate, pH 4.5, containing 0.02 %

sodium azide (7.71 mL buffer per g cornmeal). After pellets were centrifuged, supernatants were decanted and filtered using Nalgene filter units (0.2-μm polyethersulfone membrane, Cat # 568-0020) to remove remaining fine particulates. An 8.0 mL aliquot of the total of 182 mL of clarified solution was then exchanged into fresh acetate buffer using two Vivaspin 4 (Vivascience, 10,000 MWCO) centrifugal ultrafiltration units (4.0 mL each). After three concentration steps, the retentate was rinsed out into a total volume of 8.0 mL (returning retained species to their original concentrations) in pH 5.0, 20 mM acetate buffer. The ultrafiltered sample, from which possible inhibitors and other small molecules that might interfere with HPLC analysis have been effectively removed (dilution factor of 1,400), was then assayed for activity against Sigmacell-20.

2.5.3. Sigmacell Enzyme Assay

Assays intended to measure the actual cellulolytic capabilities of the transgenic CBH I extract utilized microcrystalline cellulose as substrate (Sigmacell, Type 20, Sigma-Aldrich Corp, St. Louis, MO). As shown in Table 2, the buffer extract was assayed at two different loadings of extract, acting against Sigmacell-20 (20.83 mg/mL) at 40 °C in 20 mM acetate buffer, pH 5.0, to which sodium azide had been added at 0.02 % (w/v) to retard microbial growth, and which also contained "protease-free" bovine serum albumin (Product No. A3294, Sigma-Aldrich Corp, St. Louis, MO) at 0.16 mg/mL to reduce the possibility of target proteins being adsorbed on glass walls of the reaction vessels. Assay digestions were carried out in a 1.2-mL reaction volume in crimp-sealed HPLC vials (nominally 2.0-mL volume) with constant mixing by inversion at 10 revolutions/min. After a 72-h digestion, all vials were centrifuged at room temperature and 0.5 mL of supernatant from each was diluted with 0.5 mL of acetate buffer in a termination vial, which was sealed, mixed by vortexing, and then immersed in a boiling water bath for 10 min to terminate enzyme activity. After cooling and vortexing, the contents of these termination vials were filtered into HPLC vials for determination of released soluble sugars. Sugar analysis was carried out by chromatography on a BioRad HPX-87H column operated at 65 °C with 0.01N H_2SO_4 (0.6 mL/min) as eluent in an Agilent model 1100 chromatograph with refractive-index detection.

2.5.4. Assay for Total Cellulase Activity in Cornmeal (Tightly Bound to Biomass Tissues As Well As That Readily Extractable)

For "in situ" assays of cellulase activity from cornmeal, the "enzyme" was un-extracted transgenic cornmeal, weighed directly into HPLC-vial reaction vessels at 100 mg/vial, where it was mixed with 25.0 mg of Sigmacell, type 20 as substrate, the Sigmacell being delivered as a calibrated slurry. Control (enzyme-only) digestion vials contained only the transgenic biomass, with no added microcrystalline cellulose. The mixtures in all vials were adjusted to a total volume of 1.0 mL, in the same pH 5.0, 20 mM acetate buffer used for the assay of the extracts.

All vials were incubated for 168 h (7 day) at 40 °C, with continuous mixing by inversion at 20 revolutions/min. At the end of the incubation period, all vials were centrifuged at room temperature and 0.5 mL of supernatant from each was diluted with 0.5 mL of acetate buffer in a termination vial, which was sealed, mixed by vortexing, and then immersed in a boiling water bath for 10 min to terminate enzyme activity. After cooling and vortexing, the contents of these termination vials were filtered into HPLC vials for soluble-sugar analysis. Sugar analysis was carried out by chromatography on a BioRad HPX-87H column operated at 65 °C with 0.02N H_2SO_4 (0.6 mL/min) as eluent in an Agilent model 1100 chromatograph with refractive-index detection.

3. Results

3.1. EI Identification

Western blotting clearly identifies EIcd as being present in both the tobacco and EI-1 corn-stover extracts. The EI-7 sample was very weakly detectable by western (data not visible) but later showed activity against fluorogenic substrate (Fig. 1).

3.2. CBH I Identification

The results of the western blotting (Fig. 2) are highly dependent on the primary antibody used. In the case of a monoclonal antibody that recognizes the CBM/linker portion of CBH I, detection was strong for several positive controls (i.e., CBH I purified from *T. reesei* broth or commercial cellulase, rCBH I expressed in

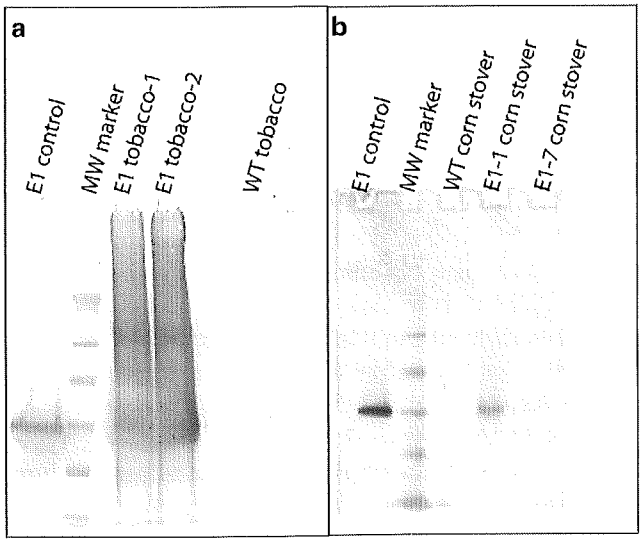

Fig. 1. EI expression *in planta*. Western blot analysis of wild-type (WT) and EI-transformed tobacco and corn stover. (Reproduced with permission ref. 7).

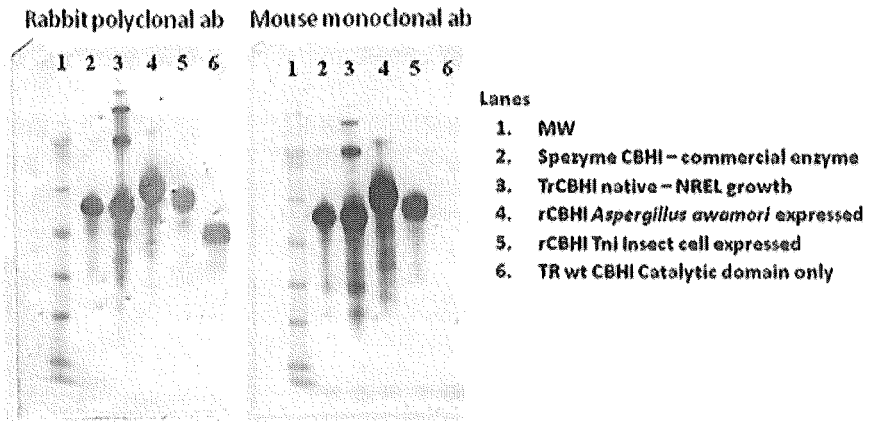

Fig. 2. Detection of CBH I using either mouse monoclonal or rabbit polyclonal antibodies.

Aspergillus awamori, or insect cells) but nonexistent for the truncated CBH I_{cd} enzyme. A polyclonal antibody was used in the second western blot experiment. The polyclonal was raised in rabbit against purified CBH I catalytic domain. The polyclonal anti-CBH I_{cd} exhibited strong detection of all of the CBH I variants.

3.3. EI MUC Assay

The MUC assay demonstrated that active EI enzyme was present in both transgenic maize and in tobacco samples (Fig. 3). It also showed native plant enzyme activity from corn stover when the samples were incubated at 60 °C. As EI is thermal tolerant, samples were preincubated at 84 °C and then assayed. This higher temperature apparently denatured the native corn enzyme, allowing EI activity only to be measured. The MUC assay also allows us to estimate the EIcd levels based on activity compared to the control EI. In the case of EI-1, this is approximately 0.3 ng/mg biomass. Although the EI-7 sample was not detected by the antibody method, the MUC assay estimated the EI-7 level to be about ten-fold less than that of EI-1 approximately 0.03 ng/mg biomass for EI-7 (Fig. 3). Wild-type stover showed no presence of EI by either method. For tobacco, the EIcd expression level was approximately 1,000-fold higher than that in the EI-1 transgenic stover.

3.4. Sigmacell Enzyme Assay Results Activity in Extracts

Tables 1 and 2 describes the release of soluble sugars from Sigmacell-20 by activity found in the acetate extracts of CBH-transgenic cornmeal. The data in Table 1 are those used to construct a standard curve (shown in Fig. 4) for the activity of wild-type *T. reesei* CBH I against Sigmacell-20 under the same conditions.

In assay B1 in Table 2 was loaded with 0.8 mL of the diafiltered buffer-extract and 0.352 mg/mL of total soluble *anhydro*-sugars were released over the course of a 72-h digestion. This release of soluble

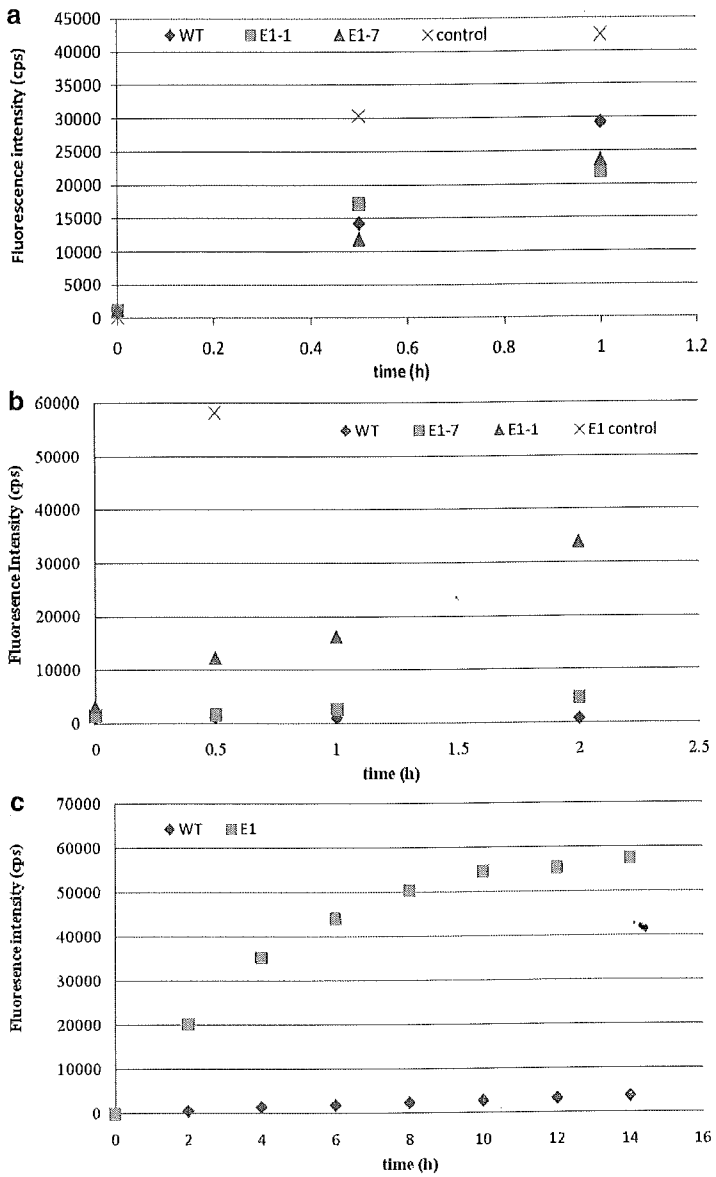

Fig. 3. EI activity from plant extracts. (**a**) WT and EI corn stover at 60 °C versus WT and EI corn stover (**b**) tobacco at 84 °C were extracted and assayed for activity on 4-methylumbelliferyl-β-D-cellobiose (MUC) substrate and (**c**) WT and E1 tobacco compared at 84 °C (Reproduced with permission ref. 7).

sugars may be the result of action of either intact CBH I, or CBH I catalytic domain, or a mixture of the two on the Sigmacell substrate. We can estimate the amount of apparent CBH I *active site* present in the sample by a linear interpolation between the "zero CBH I"

Table 1
Standard curve of wild-type *Trichoderma reesei* CBH I activity against Sigmacell

	CBHI (mg/mL)	AH-CB (mg/mL)	AH-Glc (mg/mL)	Total AH-sugar (mg/mL)	% Cellobiose in product	% Conversion of substrate
A1	0.417	2.217	0.116	2.451	95.04	11.77
A2	0.156	1.271	0.076	1.392	94.35	6.68
A3	0.063	0.715	0.054	0.789	93.01	3.79
A4	0.021	0.390	0.034	0.424	91.97	2.04
A5	0.000	0.000	0.000	0.000	0.00	0.00

Table 2
Activity of acetate-extract of cornmeal against Sigmacell

	Extract (fractional volume in assay)	AH-CB (mg/mL)	AH-Glc (mg/mL)	Total AH-sugar (mg/mL)	% Cellobiose in product	% Conversion of substrate
B1	0.667	0.015	0.337	0.352	4.34	1.69
B2	0.333	0.017	0.246	0.262	6.33	1.26

and the "0.0208-mg/mL CBH I" points. From this, we might conclude that this level of sugar release corresponds to an intact CBH I concentration of 0.017 mg/mL in assay B1 and a concentration of 0.0253 mg/mL in the diafiltered extract used to set up the assay.

The perpendicular red lines in Fig. 4 illustrate the method of this estimate. A similar estimation using the sugar-release value of 0.0262 mg/mL for assay B2 yields the higher estimate of 0.0388 mg/mL for the CBH I concentration in the extract. This B1 value, based on sugar release closer to that of the nonzero "standard-curve" CBH I loading of 0.0208 mg/mL, is probably the more meaningful value. Because the response curve is almost certainly nonlinear (significantly concave-downward) in this region, both estimates are likely to be; however, overestimates. Even if all of the sugar-releasing activity seen in assay B1 (Table 2) were ascribable to intact CBH I, the total amount of CBH I available in the extract of 28 g of "CBH I corn" would be relatively low, i.e., 4.4 mg or ~0.015 % by weight.

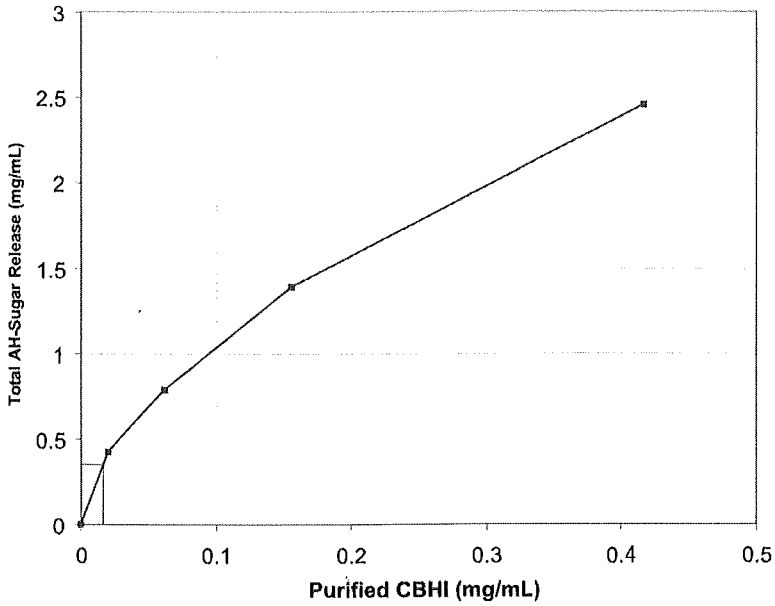

Fig. 4. Digestion of Sigmacell 20 (20.83 mg/mL) by purified *Trichoderma reesei* CBHI at pH 5.0, 40 °C, at 72 h.

Table 3
Release of sugar upon incubation of microcrystalline cellulose (Sigmacell-20) with transgenic (CBH I) cornmeal

Sample	Peak area	AH-Glc (mg/mL)	Average	Std. Dev.	Difference (mg/mL)
G-CBH I meal + cellulose	12,500	12.30	12.50	0.24	
TG-CBH I meal + cellulose	12,838	12.70			
					2.85
TG-CBH I meal alone	9,925	9.80	9.65	0.17	
TG-CBH I meal alone	9,684	9.50			

3.5. Results of the Assay for Cellulase Activity Tightly Bound to Biomass Tissues

Table 3 compares the concentrations of soluble sugar detected in the liquid phases of acetate-buffer co-slurries of transgenic (CBH I) corn-kernel material and Sigmacell-20, after a 168-h incubation at 40 °C with constant mixing, with the HPLC-measured soluble-sugar levels in identically treated slurries of the ground corn-kernel material alone. The soluble sugar concentrations are reported as *anhydro*-glucose (AH-Glc), in order to facilitate comparison with the starting concentration (25 mg/mL) of Sigmacell-20 in the mixture. All soluble sugar was in fact detected as glucose, with no detectable amounts of cellobiose or other sugars. The Sigmacell-only controls showed no detectable soluble sugars at all and are not included in the table.

4. Discussion

4.1. EI Identification and MUC Activity

4.1.1. Identification

A. cellulolyticus EI was readily extracted from ground biomass samples using LDS sample buffer as described and then subjected to western-blot analysis. This is the simplest method reported so far to extract and identify recombinant proteins, such as EI, in biomass samples. Extraction with buffer or buffer followed by ethylene glycol gave low protein yield (data not shown). This indicates that the EI expressed in maize was either tightly bound to the cell wall (presumably the cellulose fraction) or was localized deeply enough in the cell wall matrix to make extraction difficult. While extraction with detergent (boiling LDS-sample buffer) yielded measurable protein, activity is abolished using this method from general protein denaturation. This technique is useful for qualitative evaluation of expression levels; however, quantitative determination of expressed protein can only be achieved by comparison with known levels of protein measured by band intensity on a western blot (and possibly by ELISA) or direct mass spectral fingerprinting. This estimation assumes that both proteins (purified control and transgenic) are equally antigenic, which may not be true given that they are expressed in different host systems. It also assumes that all of the transgenic protein is released from the biomass and moves into the gel, which may not be the case.

4.1.2. Activity

To test for active EI enzyme or other highly thermostable recombinant enzymes, we recommend the use of fluorogenic substrates. As EI was not readily extracted from ground biomass in an active form, assays were carried out using milled biomass as the "enzyme." Heating to 84 °C was required to denature native plant glycosyl hydrolases that interfered with the MUC assay conducted at 60 °C. This same technique could presumably also be used with cellulose as substrate. Incubation of EI-stover at 84 °C with AZCL-hydroxyethyl cellulose as a substrate yielded enzymatically released dye (data not shown). Although this hydrolysis result was not quantified, the results suggest that incubation with cellulose would yield new reducing ends. However, because EI is primarily an endo-acting cellulase, the production of detectable, soluble sugars might be low. Inclusion of a β-glucosidase and/or exocellulase would enhance this activity evaluation.

In addition to verifying that the plant expressed EI was in fact active, we can also estimate the amount of EI present in the plant material using a known amount of control WT purified EI. It is possible to estimate the amount of EI present in the biomass samples by comparing the MUC digestions to the control during the linear phase of the digestion curve. However, as can be seen in Fig. 3a, native plant glycosyl hydrolases can interfere with the MUC assay at lower incubation temperatures. This possibility must be carefully considered if one is testing for enzymes that do not have high thermostability.

4.2. CBH I Identification and Activity

Extraction and western blotting of CBH I from various sources illustrates a critical point: that when examining CBH I enzymes, or multidomain enzymes in general, one must consider the possibility of truncation or changes in the antigenicity of CBH I (Fig. 2). Given the possibility that truncation of CBH I can occur when expressed in plants, one must be careful when selecting antibodies to the protein of interest; that is, it must be known what the actual epitope of the antibody is targeted to. In our case, the monoclonal mouse antibody failed to detect certain truncated variants of CBH I, because it binds only to the CBM/linker region of this protein. However, by using the polyclonal rabbit antibody, we ascertained that at least the catalytic domain of CBH I was present in all cases.

4.2.1. Sigmacell Enzyme Assay

One consideration when interpreting the data on digestion of Sigmacell with buffer-extracted rCBH I (Table 3) which in this case was identified using mass spectrometry techniques (data not shown) is that these estimates arise from the low overall extents of conversion shown by the extract assay. It is well known that in nearly all microcrystalline celluloses, a significant portion of the material is present as amorphous content (14). This material is less ordered than the more crystalline material, and is much more readily hydrolyzed by either enzymes or chemical catalysts. And at <2 % conversion, the extract activity is likely to still be working on this more digestible fraction of the material. The shape of the response curve of purified *T. reesei* CBH I in Fig. 4 would support this view. Below 4 % or so conversion, (approximately 0.087 mg/mL *anhydro*-sugar released) the steeper slope of the curve indicates that the enzyme is working on much more readily hydrolysable material (i.e., amorphous cellulose) compared to later digestion where the rate is much shallower. Presumably, this later hydrolysis is on more crystalline material. Hydrolysis of this amorphous material does not require an intact CBH I; CBH I catalytic domain alone has significant activity against such material. This suggests that the low extent of conversion of Sigmacell by extracted rCBH I is probably due to the activity of either catalytic domain only CBH I or CBH I with a truncated or modified CBM that does not readily bind to crystalline cellulose.

Also present in (Table 3), we see a clear difference between the compositions of the sugar mixtures released from Sigmacell-20 by *T. reesei* CBH I (chromatographically purified from commercial cellulase) and the mixtures released by the extract of transgenic (CBH I) cornmeal, in that the purified CBH I releases predominantly cellobiose, whereas the extract of maize kernel material releases predominantly glucose. The low percentage of cellobiose as product from this reaction is not typical of CBH I activity. Presumably, the cellobiose produced by the extracted CBH I is being hydrolyzed by a native β-glucosidase-like enzyme in the biomass. This conclusion is supported by results from the MUC assays applied to EI-stover, which clearly showed MUC activity in the wild-type plant material. As the activity of the extracted rCBH

I is low, it would not take much glycosidase activity to convert the majority of cellobiose to glucose. The fact that the higher loading of the extract (B1) shows a lower proportion of cellobiose is in agreement with this explanation.

For an assay to give strong indication of the presence of the complete, active enzyme, it is necessary for a large enough fraction of the crystalline cellulose be removed. This condition must be met before one can be confident that the enzyme has removed the "easy," presumably less-ordered or amorphous cellulose, and is working its way through the much more refractory substrate that is presumed to be crystalline.

4.2.2. Assay for CBH I Activity that is Tightly Bound to Biomass

In the previous Sigmacell digestion assay, we found relatively small amounts of cellulose-depolymerizing activity in buffer extracts of several transgenic corn-kernel tissues expected to be expressing *T. reesei* CBH I. Given the low overall extent of conversion of these digestions, it is possible that much of the activity that we observed in the extracts might be attributable to CBH I molecules having functional catalytic domains, but lacking functional cellulose-binding modules; these CBH I_{cd} molecules were detected by mass-spectrometry (data not shown). This non-functional CBM phenotype could be explained by truncated protein lacking a complete CBM or by altered glycosylation of the linker peptide and/or CBM which could interfere with optimal binding of the CBM to cellulose.

Based on this idea, significant quantities of complete, cellulolytically competent CBH I molecules, possessing not only active catalytic domains, but also linker regions and CBMs properly attached, might be bound through the CBMs to the cellulose component of the corn-kernel cell walls. These putative molecules are contrasted with the catalytic-domain-only molecules actually found, which could be extracted rather quickly using aqueous buffers. Thus, the holoenzymes (those with CBMs) may be extracted at a much slower rate, because of the presumed tighter binding of the intact CBH I. In addition, intact CBH I would be expected to have an equilibrium between being free in solution and bound, possibly very heavily in favor of bound molecules, whereas for CBH I_{cd}, the opposite behavior is expected. Simple buffer extractions, therefore, wash out only a very small fraction of the whole CBH I enzyme in each washing, but should remove most of the truncated CBH I_{cd}.

This in situ assay is designed to take advantage of a possible two-step transfer of CBH I. First, corn-kernel-bound CBH I dissociates from the kernel cell-wall cellulose, into the solution. Second, solution CBH I diffuses to, and binds to, microcrystalline cellulose that has been added to the slurry of milled corn-kernel material and is intimately mixed with it. In this scheme, even though production of free (solution) CBH I may be slow, and although there is not much of the free species present at any one time, complete, active CBH I may be funneled through the free population to accumulate on another CBH I-sink such as clean

cellulose (Sigmacell 20), provided CBH I has adequate affinity for the second sink and the sink is in significant excess to the corn cellulose. This process is seen as an equilibrium process, not a one-way transfer, but if the process is given sufficient time, it can be expected that a significant time-averaged population of CBH I molecules will appear on the coincubated microcrystalline cellulose, and measurable sugar-release from the microcrystalline cellulose will occur as a result.

Despite the necessity of subtracting out a large blank of glucose contributed by the (previously unextracted) corn-meal added to the coincubation mixtures; we see that the incubations containing Sigmacell-20 in addition to the cornmeal show sugar-release greater than that in the cornmeal-alone controls (Table 3). With the reasonably good reproducibility shown by the duplicates, it appears that the difference is statistically significant. Whether the "extra" sugar release can be ascribed to the action of intact CBH I on the Sigmacell-20 is still under study.

While the product of the coincubation does not show the characteristic digestion profile of CBH I acting alone on cellulose; in that the detected sugar is entirely glucose, rather than the expected mixture: predominantly cellobiose containing a small fraction of glucose. However, as in the case of the extract assays described above, the absence of cellobiose from the product mixture is not a serious objection to ascription of the observed release to a rCBH I species, in that there is generally even more endogenous cellobiase retained on the cornmeal pellets than is extracted in simple buffer-extractions such as these. The presumed extent of conversion of the Sigmacell in this experiment (approximately 10.1 % based on 2.8 mg/mL anhydro-glucose in the calculated 0.895-mL liquid-phase of the 1.0-g assay mixture) is considerably higher than that seen in the assays of extract.

The example results described here for the cellulose-conversion assays unfortunately did not give definitive answers to the question of whether or not the putative rCBH I responsible for the sugar-release is actually cellulolytically competent (i.e., capable of attacking crystalline cellulose), or if the activity belongs to holoenzyme, isolated catalytic domain, or a mixture of the two. However, demonstrating "cellulolytic competence" would require larger extents of conversion than the higher of the two extents seen here. The assay designs themselves could, in fact, be greatly improved. The "solid-to-solid extraction" assay in particular needs a corresponding control utilizing WT control corn-meal, and would benefit from a procedure in which the cornmeal solids were preextracted to remove both the readily extractable cellulase and other endogenous MUCase/MULase activities (with these activities in the extractions/washes carefully accounted for, so that the results of the "pellet" assays would reflect only the more tightly bound activities corresponding to intact rCBH I). This pre-extraction would also avoid the necessity of making a large correction for extractable

sugars and other substances potentially by interfering with the HPLC analysis, as was necessary for the results in Table 3. The procedures and results presented here are simply intended to provide a starting point for important considerations of the questions and concerns encountered in such work.

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References

1. Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315:804–807
2. Wei H, Xu Q, Taylor L, Baker J, Tucker M, Ding S-Y (2009) Natural paradigms of plant cell wall degradation, Curr Opin Biotechnol 20:330–338
3. Taylor LE, Dai Z, Decker SR, Brunecky R, Adney WS, Ding S-Y, Himmel ME (2008) Heterologous expression of glycosyl hydrolases in planta: a new departure for biofuels, Trends Biotechnol 26:413–424
4. Sticklen MB (2008) Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol, Nat Rev Genet 9:433–443
5. Baker JO, Adney WS, Nieves RA, Thomas SR, Wilson DB, Himmel ME (1994) A new thermostable endoglucanase, Acidothermus cellulolyticus E1, Appl Biochem Biotechnol 45:245–256
6. Ziegelhoffer T, Raasch JA, Austin-Phillips S (2001) Dramatic effects of truncation and subcellular targeting on the accumulation of recombinant microbial cellulase in tobacco, Mol Breed 8:147–158
7. Brunecky R, Selig MJ, Vinzant TB, Himmel ME, Lee D, Blaylock MJ, Decker SR (2011) In planta expression of A. cellulolyticus Cel5A endocellulase reduces cell wall recalcitrance in tobacco and maize, Biotechnol Biofuels 4:1
8. Ransom C, Balan V, Biswas G, Dale B, Crockett E,Sticklen M(2007)HeterologousAcidothermus cellulolyticus 1, 4- -endoglucanase E1 produced within the corn biomass converts corn stover into glucose, Appl Biochem Biotechnol 137–140: 207–219
9. Harrison MD, Geijskes J, Coleman HD, Shand K, Kinkema M, Palupe A, Hassall R, Sainz M, Lloyd R, Miles S, Dale JL (2011) Accumulation of recombinant cellobiohydrolase and endoglucanase in the leaves of mature transgenic sugar cane, Plant Biotechnol J 9(8):884–896
10. Ransom C, Balan V, Biswas G, Dale B, Crockett E, Sticklen M (2007) Heterologous Acidothermus cellulolyticus 1,4-beta-endoglucanase E1 produced within the corn biomass converts corn stover into glucose, Appl Biochem Biotechnol 137:207–219
11. Hood EE, Love R, Lane J, Bray J, Clough R, Pappu K, Drees C, Hood KR, Yoon S, Ahmad A, Howard JA (2007) Subcellular targeting is a key condition for high-level accumulation of cellulase protein in transgenic maize seed, Plant Biotechnol J 5:709–719
12. Burnette WN (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A, Anal Biochem 112:195–203
13. Heptinstall J, Stewart JC, Seras M (1986) Fluorimetric estimation of exo-cellobiohydrolase and [beta]-d-glucosidase activities in cellulase from Aspergillus fumigatus Fresenius, Enzyme Microb Technol 8:70–74
14. Park S, Baker J, Himmel ME, Parilla P, Johnson D (2010) Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance, Biotechnol Biofuels 3:10