

Oxidoreductive Cellulose Depolymerization by the Enzymes Cellobiose Dehydrogenase and Glycoside Hydrolase 61^{∇†}

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Several members of the glycoside hydrolase 61 (GH61) family of proteins have recently been shown to dramatically increase the breakdown of lignocellulosic biomass by microbial hydrolytic cellulases. However, purified GH61 proteins have neither demonstrable direct hydrolase activity on various polysaccharide or lignacious components of biomass nor an apparent hydrolase active site. Cellobiose dehydrogenase (CDH) is a secreted flavocytochrome produced by many cellulose-degrading fungi with no well-understood biological function. Here we demonstrate that the binary combination of *Thermoascus aurantiacus* GH61A (TaGH61A) and *Humicola insolens* CDH (HiCDH) cleaves cellulose into soluble, oxidized oligosaccharides. TaGH61A-HiCDH activity on cellulose is shown to be nonredundant with the activities of canonical endocellulase and exocellulase enzymes in microcrystalline cellulose cleavage, and while the combination of TaGH61A and HiCDH cleaves highly crystalline bacterial cellulose, it does not cleave soluble cellobioextrins. GH61 and CDH proteins are coexpressed and secreted by the thermophilic ascomycete *Thielavia terrestris* in response to environmental cellulose, and the combined activities of *T. terrestris* GH61 and *T. terrestris* CDH are shown to synergize with *T. terrestris* cellulose hydrolases in the breakdown of cellulose. The action of GH61 and CDH on cellulose may constitute an important, but overlooked, biological oxidoreductive system that functions in microbial lignocellulose degradation and has applications in industrial biomass utilization.

Cellulose is Earth's most abundant biopolymer, and the biological exploitation of this carbohydrate as an energy source plays a critical role in the carbon cycle and the planet's ecology. Industrial production of fuels and chemicals from this plentiful and renewable resource holds the potential to displace petroleum-based processes, promoting energy self-sufficiency and reducing environmental costs (14, 22, 23, 41). However, the recalcitrance of cellulose to hydrolytic depolymerization is a barrier to both microbial and industrial utilization of lignocellulosic biomass (1, 11, 30, 37, 40).

Lignocellulosic biomass is composed largely of three major components: cellulose, a β -1,4-linked glucose polymer arrayed predominantly in crystalline fibrils; hemicellulose, a mixed carbohydrate polymer; and lignin, a complex polyalkylaromatic compound (11). The heterogeneity and recalcitrance of lignocellulose require the synergistic activities of a number of hydrolytic enzymes for efficient conversion to biologically fermentable monosaccharides. The canonical enzymatic cellulose hydrolysis scheme involves cellobiohydrolases (CBH) which processively degrade cellulose chains from the ends, producing cellobiose (EC 3.2.1.91 for CBHs that proceed from the nonreducing ends of cellulose chains; there is no assigned EC number for cellobiohydrolases that release cellobiose from the reducing ends), endo- β -1,4-glucanases (EG; EC 3.2.1.4) which internally clip cellulose, shortening chains, and generating reactive ends for CBH, and β -glucosidases (BG; EC 3.2.1.21)

which hydrolyze soluble cellobioextrins to glucose, mitigating CBH product inhibition arising from cellobiose (4, 10, 37, 38, 40). Hemicellulases, ligninases, and other secreted accessory proteins, such as swollenin, increase the accessibility of cellulose to these hydrolytic cellulase activities (10, 2, 15, 19, 27, 29, 39).

Another group of proteins broadly expressed in the fungal kingdom and implicated in biomass breakdown is the glycoside hydrolase 61 (GH61) family of proteins. The addition of specific GH61 proteins has been shown to greatly increase the performance of *Hypocrea jecorina* (*Trichoderma reesei*) cellulases in lignocellulose hydrolysis (3, 8). Despite extensive investigation, the mechanism of GH61's enhancement of lignocellulose degradation has remained elusive. These proteins were originally classified as glycoside hydrolases, based on the detection of low levels of endoglucanase activity (17, 18, 26). However, subsequent investigations have revealed that, when purified and within clearly defined assay conditions, the GH61 molecules which enhance lignocellulose breakdown by cellulases do not hydrolyze either cellulose substrates, such as microcrystalline cellulose or phosphoric acid-swollen cellulose (PASC), or hemicellulosic substrates, such as xylan (8). The crystal structures of two GH61 proteins show no evidence of a conventional glycoside hydrolase active site. Both GH61 crystal structures possess a bound metal near a group of planar residues, presenting a putative active site of unknown function (8, 16). A structurally homologous site is found on chitin binding protein-21 (CBP21) (34, 35), a bacterial protein which has recently been shown to cleave chitin into reducing-end oxidized chitin oligomers in a reaction dependent on the presence of O₂ and reducing agent (36). Based on that result, it was proposed that CBP21 degrades chitin by a chitin oxygenase activity in a reaction mechanism which consumes both O₂ and

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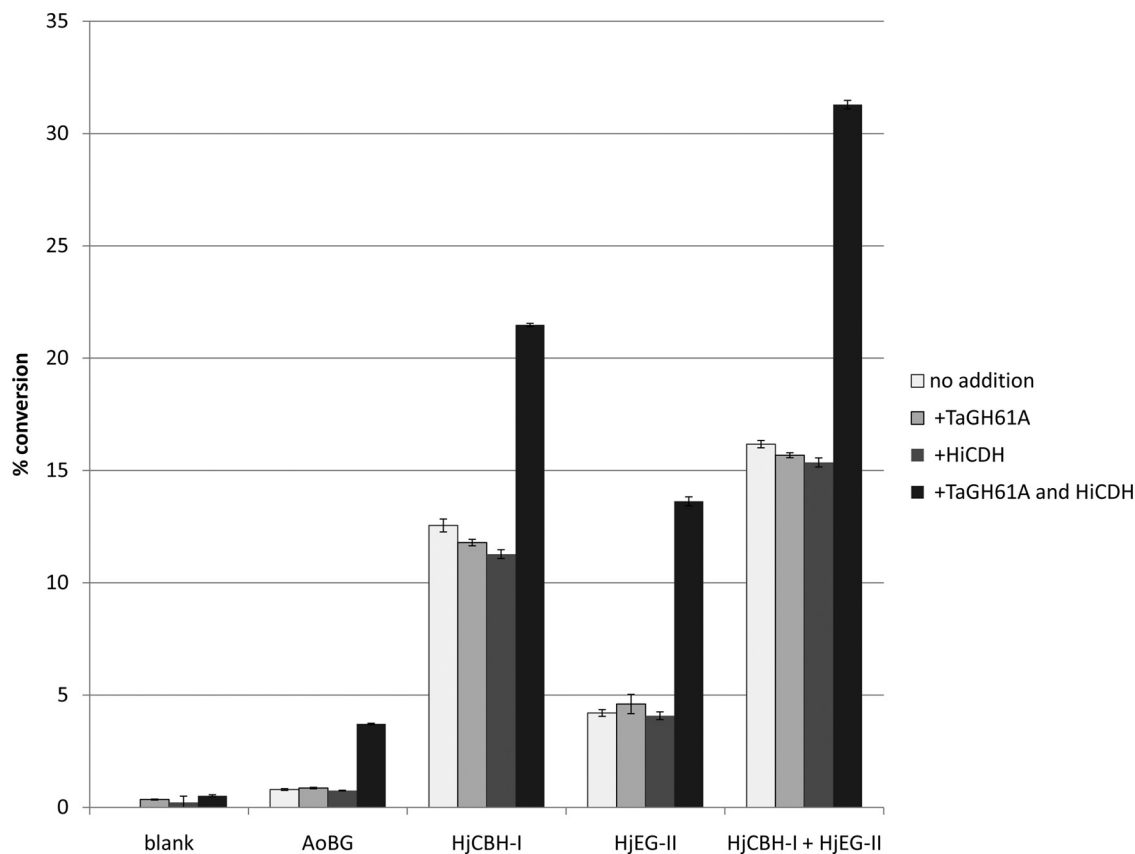


FIG. 1. Microcrystalline cellulose conversion assay. HiCDH was loaded at 1 mg protein per gram cellulose, TaGH61A was loaded at 5 mg protein per gram cellulose, and HJCBH-I, HJEG-II, and AoBG were loaded at 10 mg protein per gram cellulose. Assay conditions were 50 mM NaOAc at pH 5, 1 mM MnSO₄, 50°C, and a 72-h time course. Monosaccharide and disaccharide were quantified by RID-HPLC using an Aminex HPX-87H column, with percent cellulose conversion calculated compared to a limit digest by a high loading of cellulase. Error bars are for triplicate cellulose cleavage reactions, each measured once.

reducing agent as cosubstrates (36). Very recently, CelS2, another bacterial homolog of GH61, was shown to cleave cellulose into a mixed population of reducing-end oxidized and reduced cellodextrin in a reductant-dependent reaction (6). Given the structural similarity of both CBP21 and CelS2 to GH61 (6, 8, 16, 34), it was further proposed that GH61 attacks cellulose by a similar reductant-dependent oxygenase mechanism (6, 36).

Cellobiose dehydrogenase (CDH; EC 1.1.99.18), the only known example of a secreted flavocytochrome, is another fungal enzyme which has no clearly defined biological function despite extensive study over the last 30 years (5, 43). The suggested roles for CDH are diverse, including activating hydrolytic cellulases by relief of cellobiose product inhibition, being a component of the respiratory chain, preventing cellulose or lignin repolymerization after cleavage, acting as an antimicrobial agent, supporting manganese peroxidase, acting in the reduction of toxic quinones, or initiating Fenton-type hydroxyl radical-mediated cleavage of cellulose, hemicellulose, or lignin (5, 9, 43). Of these, the hydroxyl radical cleavage of the cellulose hypothesis is most widely proposed in current literature. What is known is that CDH both binds cellulose and catalyzes the oxidation of cellodextrins, lactose, and maltodex-

trins to their corresponding lactones using a diverse selection of electron acceptors (9).

Evidence of a linkage between the activities of CDH and GH61 has previously been shown in the patent literature (32, 33). With a range of pure cellulose substrates, the combination of both GH61 and CDH increased cellulose conversion by hydrolytic cellulases and cellulase mixtures. In this study, the nature of GH61-CDH stimulation of hydrolytic cellulase activity is examined, demonstrating that the combination of GH61 and CDH cleaves cellulose and that the oligosaccharides released in this cleavage differ from those produced by the activities of canonical hydrolytic cellulases. Further, we show that the *in vitro* combination of CDH and GH61 coexpressed by *Thielavia terrestris* enhances cellulose breakdown by canonical *T. terrestris* cellulose hydrolases, demonstrating evidence of a microbial oxidoreductive cellulose depolymerizing enzyme system active in parallel with the better-described hydrolytic cellulase enzyme system.

MATERIALS AND METHODS

Substrates and reagents. Reagents and buffers were commercial products of reagent grade or better. PASC was prepared from Avicel (FMC BioPolymer; PH

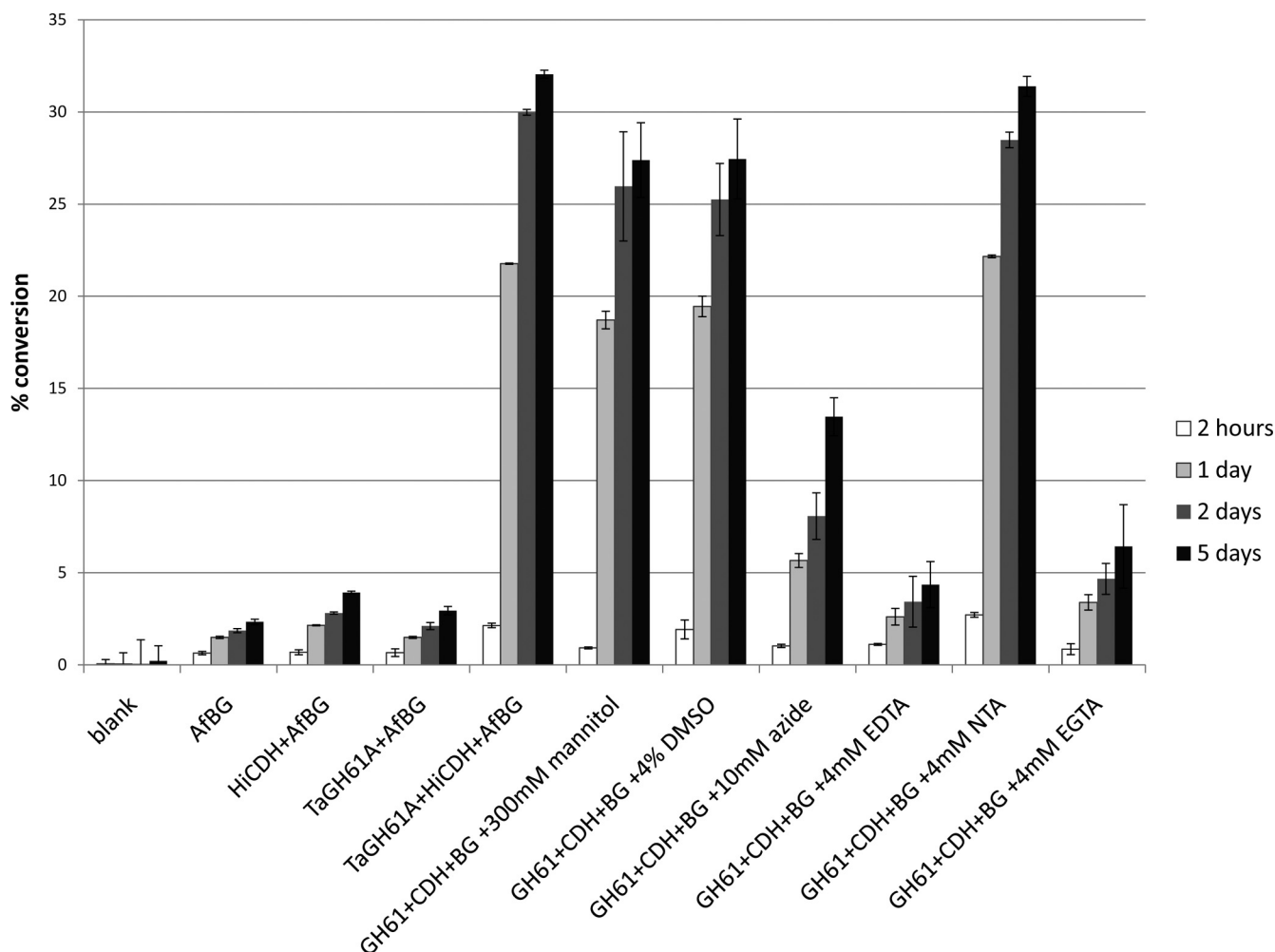


FIG. 2. PASC conversion assay (5 g/liter PASC digestion). HiCDH and AfBG were loaded at 5 mg protein per gram cellulose, and TaGH61A was loaded at 20 mg protein per gram cellulose. Assay conditions were 50 mM NaOAc at pH 5, 1 mM MnSO₄, 50°C, and a 2- to 120-h time course. Monosaccharide release was determined by RID-HPLC using an Aminex HPX-87H column, with percent cellulose conversion calculated compared to a limit digest by a high loading of cellulase. Error bars are for triplicate cellulose cleavage reactions, each measured once.

101) (44). Bacterial cellulose was prepared as described previously (32). All reagents and substrates were prepared in high-purity Milli-Q (Millipore) water.

Preparation of enzymes. A cellulase preparation, Celluclast Plus (Novozymes), was used as a control for complete cellulose hydrolysis. The mix contained *Hypocrea jecorina* cellulases and *Aspergillus oryzae* Cel3 BG (AoBG). Native *H. jecorina* Cel7A (HjCBH-I), recombinant *H. jecorina* Cel5A (HjEG-II), recombinant *T. terrestris* Cel6A (TtCBH-II), recombinant *T. terrestris* Cel7E EG (TtEG), recombinant *T. terrestris* GH61E (TtGH61E), recombinant *T. terrestris* Cel3A beta-glucosidase (TtBG), recombinant AoBG, recombinant *Aspergillus fumigatus* Cel3 BG (AfBG), recombinant *Humicola insolens* CDH (HiCDH), recombinant *T. terrestris* CDH (TtCDH), and recombinant *Thermoascus aurantiacus* GH61A (TaGH61A) were expressed and purified as previously reported (7, 8, 20, 32, 33). Protein quantification was performed using bicinchoninic acid (BCA) analysis (Pierce) or the Bio-Rad protein assay (Bio-Rad; for *T. terrestris* enzymes only).

Instrumentation. A Powerwave X (Biotek Instruments) or SpectraMax (Molecular Devices) plate reader was used for spectrophotometric measurement using Costar 96-well microplates. Ultra-high-performance liquid chromatography (UPLC) electrospray ionization mass spectrometry (UPLC-ESI-MS) and tandem mass spectrometry (MS/MS) were performed on a Waters Micromass Technologies Q-ToF microhybrid orthogonal quadrupole mass spectrometer, coupled to an Acquity nano-flow HPLC system with a bridged ethyl hybrid (BEH) amide column.

Enzymatic cellulose cleavage assays. Cellulose conversion assays were carried out at 25 g/liter microcrystalline cellulose (Avicel), 5 g/liter PASC, or 2.3 g/liter bacterial cellulose in Milli-Q (Millipore) water buffered with 50 mM sodium acetate [pH 5], 1 mM MnSO₄, at 50°C for 1 to 5 days (unless otherwise noted in figure legends), using protein loadings (depending on substrate, stated in figure legends) of 10 to 50 mg GH61 protein per gram cellulose, 1 to 10 mg CDH per gram cellulose, and 1 to 50 mg BG per gram cellulose. Control incubations excluding either GH61 or CDH alone were performed in the same manner. Triplicates were run in each experiment. Samples were filtered using a 0.45- μ m Multiscreen 96-well filter plate (Millipore) prior to further analysis. Glucose and cellobiose concentrations were quantified in the filtrates by HPLC with an Agilent 1100 system equipped with a refractive index detector, using an Aminex HPX-87H analytical column (Bio-Rad), eluting with 5 mM H₂SO₄ at 0.6 ml/min, at 65°C. The extent of hydrolysis, based on percent cellulose conversion, was calculated from the ratio of measured glucose plus cellobiose in a sample relative to a limit digest by a high loading of cellulase (100 mg Celluclast Plus per g cellulose).

GH61-CDH reactions prepared for UPLC-ESI-MS were similarly performed with 5 g/liter PASC in Milli-Q (Millipore) water buffered with 20 mM ammonium acetate, 0.1 mM MnSO₄ at pH 5.0 treated with 50 mg TaGH61A per gram cellulose, and 5 mg HiCDH per gram cellulose and with or without 5 mg AfBG per gram cellulose, at 50°C for 4 days. All reaction mixtures, prior to refractive index detection (RID)-HPLC or UPLC-ESI-MS analysis, were filtered as de-

scribed and then passed through a 10-kDa-molecular-mass-cutoff membrane using ultrafiltration (Vivacell [Sartorius-Stedim] or VivaSpin [GE Healthcare]).

RESULTS

Influence of TaGH61A and HiCDH on hydrolytic cellulase conversion of cellulosic substrates. In order to test the effects of TaGH61A and HiCDH on cellulase hydrolase activity, a microcrystalline cellulose conversion assay was conducted using the monocomponent cellulases HJEG-II, HiCBH-I, and AoBG or the combination of HJCBH-I and HJEG-II. The detection of glucose and cellobiose released was used as a metric for conversion. Shown in Fig. 1, the combination of both TaGH61A and HiCDH greatly increased cellulose conversion by all hydrolytic cellulases tested compared to addition of TaGH61A or HiCDH individually. An increase in cellulose conversion to glucose was also observed for AoBG, which is otherwise inactive on insoluble cellulose. This increase in glucose release in the presence of BG may indicate the release of soluble cellodextrin from cellulose, which serves as a BG substrate.

In order to confirm that the combined enzymatic activities of TaGH61A and HiCDH, rather than an activation of one protein by a contaminant in either preparation, were responsible for the increase in microcrystalline cellulose conversion by cellulase, experiments were conducted in which either heat-inactivated TaGH61A and fresh HiCDH or fresh TaGH61A and heat-inactivated HiCDH were added to individual cellulases in microcellulose conversion assays similar to those shown in Fig. 1. No synergistic effect was seen when either HiCDH or TaGH61A was previously heat inactivated prior to the assay (data not shown). Similarly, pretreatment of microcrystalline cellulose with either HiCDH or TaGH61A individually, followed by heat inactivation of the material and a cellulose cleavage assay with addition of cellulase and either TaGH61A or HiCDH, demonstrated that both active TaGH61A and HiCDH must be present simultaneously in order to increase cellulase conversion of cellulose (data not shown).

A PASC cleavage assay was conducted in order to test the activity of TaGH61A and HiCDH on a highly amorphous cellulose substrate. AfBG was added to hydrolyze any released cellodextrins, allowing glucose detection as a conversion metric. Shown in Fig. 2, treatment of PASC with AfBG, the binary combination of AfBG and TaGH61A, or the binary combination of AfBG and HiCDH resulted in very low PASC conversion, while the ternary combination of TaGH61A, HiCDH, and AfBG resulted in significant cellulose conversion (30% at 2 days). The addition of radical quenching agent mannitol or dimethyl sulfoxide (DMSO) (25) was only slightly inhibitory to cellulose conversion by the combination of TaGH61A, HiCDH, and AfBG. The activity of this enzyme mixture was strongly inhibited by the addition of the chelating agents EDTA and EGTA but not inhibited by the chelator nitrilotriacetic acid (NTA). The addition of the O₂ mimic sodium azide also resulted in the reduction of cellulose conversion by the combination of TaGH61A, HiCDH, and AfBG.

Shown in Fig. 3, the activity of a combination of TaGH61A, HiCDH, and AoBG was tested on highly crystalline bacterial cellulose. The ternary combination of TaGH61A, HiCDH, and

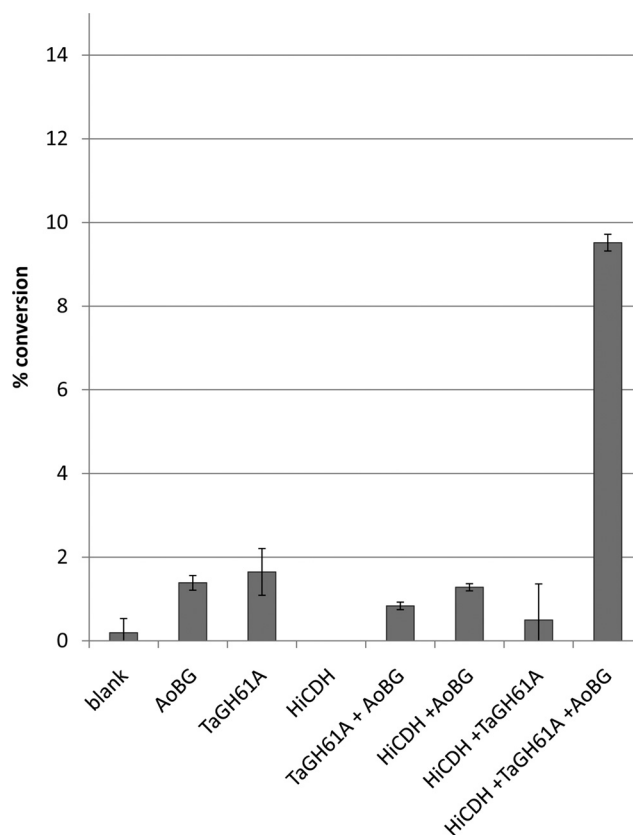


FIG. 3. Bacterial cellulose conversion assay (2.3 g/liter bacterial cellulose). HiCDH and AfBG were loaded at 5 mg protein per gram cellulose, and TaGH61A was loaded at 20 mg protein per gram cellulose. Assay conditions were 50 mM NaOAc at pH 5, 1 mM MnSO₄, 50°C, and 3-day time course. Monosaccharide release was determined by RID-HPLC using an Aminex HPX-87H column, with percent cellulose conversion calculated compared to a limit digest by a high loading of cellulase. Error bars are for triplicate cellulose cleavage reactions, each measured once.

AoBG resulted in significant conversion of bacterial cellulose, while treatment with individual enzyme components and binary combinations resulted in little glucose formation from this substrate.

In similar assays using hemicellulosic substrates (e.g., birch wood xylan), the combination of TaGH61A and HiCDH was tested for their effect on hemicellulose conversion by various hemicellulose hydrolases. For all hemicellulose substrates tested, hemicellulose conversion was not increased by individual addition of TaGH61A, HiCDH, or the combination of TaGH61A and HiCDH (data not shown), indicating cellulase specificity for TaGH61A and HiCDH.

Analysis of cellulose cleavage products of TaGH61A and HiCDH by LC-MS and MS/MS. In order to further explore the nature of the TaGH61A and HiCDH action upon cellulose, a mass spectrometric analysis of TaGH61A- and HiCDH-treated PASC samples was undertaken. Analytes were separated by UPLC with a BEH amide column, and electrospray ionization and detection by a quadrupole/time-of-flight mass spectrometer followed. Shown in Fig. 4 is the LC elution profile, using total ion count (TIC) as a bulk readout, for PASC treatment by either the binary combination of TaGH61A and HiCDH or the

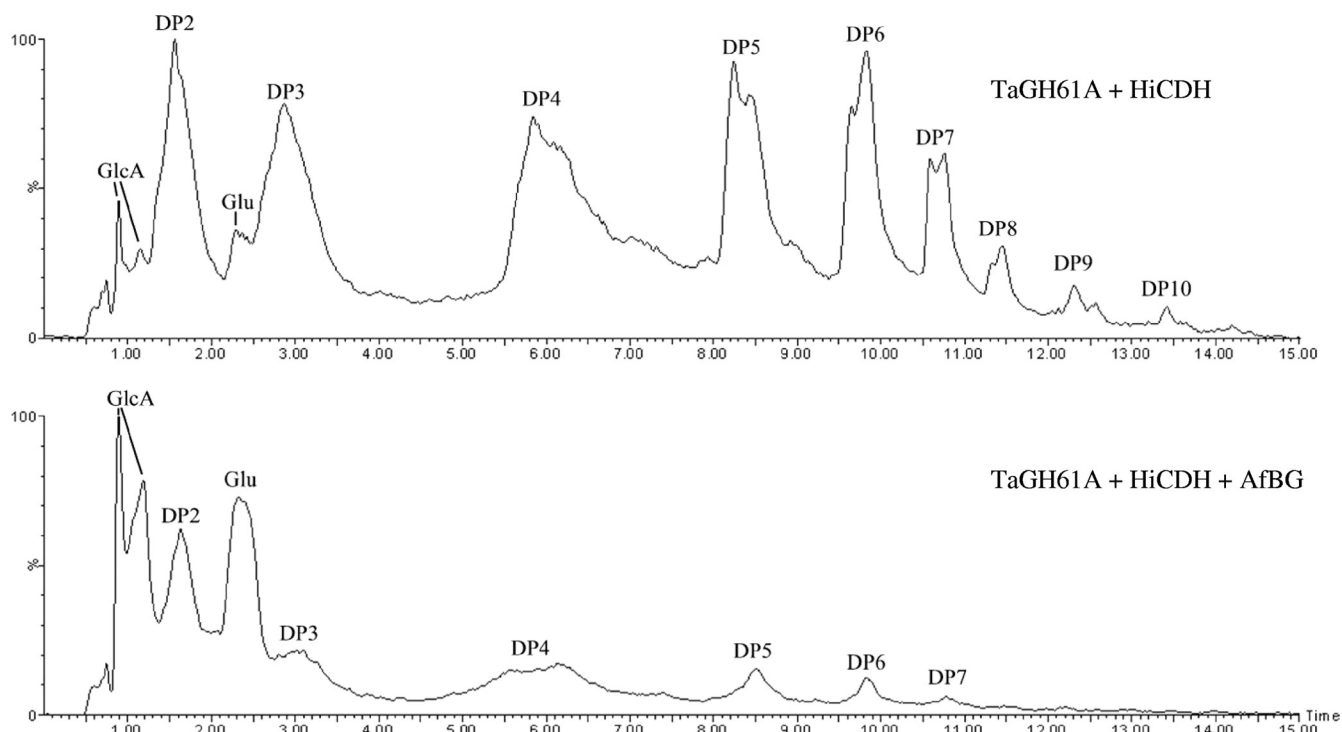


FIG. 4. LC-MS TIC profiles of UPLC separation of samples with a BEH amide column. (Top) PASC, 5 g/liter, treated with 50 mg TaGH61A per gram cellulose and 5 mg HiCDH per gram cellulose in 20 mM ammonium acetate at pH 5, 0.1 mM MnSO_4 , for 4 days at 50°C. (Bottom) PASC, 5 g/liter, treated with 50 mg TaGH61A per gram cellulose, 5 mg HiCDH per gram cellulose, and 5 mg AfBG per gram cellulose in 20 mM ammonium acetate at pH 5, 0.1 mM MnSO_4 , for 4 days at 50°C. Note that all cellulodextrins in this sample set were observed as cellulodextrin acids; e.g., DP4 marks cellotetraonic acid.

ternary combination of TaGH61A, HiCDH, and AfBG. This data set shows that the combination of TaGH61A and HiCDH cleaves PASC, resulting in the release of soluble oligosaccharide. Elution times for these oligosaccharides corresponded with those of cellulodextrin aldonic acid standards (not shown), which would be expected in the presence of CDH which oxidizes the reducing end of cellulodextrins. AfBG-treated samples show the conversion of most oligosaccharides to glucose and some quantity of gluconic acid (GlcA), though a minority of oligosaccharides are resistant to BG cleavage.

Figure 5A shows the mass spectra for the degree of glucose polymerization 2 (DP2) time window of the elution profile, and Fig. 5B shows the mass spectra for the DP3 region of the elution profile. In the cases of both DP2 and DP3 oligomers, three masses were detected: a +16-Da mass predicted for a reducing-end C-1-oxidized cellulodextrin aldonic acid which was BG labile and also +14-Da and +32-Da species which were both BG resistant. This pattern of +14 Da, +16 Da, and +32 Da repeats itself at all oligosaccharide lengths (not shown). BG cleaves the terminal glucose from the nonreducing end of cellulodextrin (7), as such a BG-resistant oligosaccharide population may result from modification of a cellulodextrin at any point other than the reducing end.

MS/MS analysis, shown in Fig. S1 in the supplemental material, confirmed that the +16-Da species were reducing-end C-1 oxidized (gluconic acid equivalent at the reducing end) for both DP2 and DP3, based on a comparison to gluconic acid and cellobionic acid standards. We were unable to unambigu-

ously identify the +14-Da and +32-Da species based on this MS/MS datum set, though reducing-end C-1 oxidation coupled with a non-reducing-end modification is consistent with the observed fragmentation pattern and BG resistance of these species.

Cellulose cleaving activity by GH61 and CDH from *T. terrestris* and synergy with *T. terrestris* cellulose hydrolases. Recombinant TtCDH was tested for activity with recombinant TtGH61E in a PASC cleavage assay, with AfBG present in order to allow monosaccharide quantification. Shown in Fig. 6, the ternary combination of TtGH61E, TtCDH, and AfBG resulted in substantial, dose-dependent conversion of PASC, while the binary combinations of either TtGH61E or TtCDH with AfBG did not result in significant monosaccharide release.

In order to investigate the effect that the interaction of TtGH61E and TtCDH may have on the canonical cellulose hydrolase enzyme system, several purified recombinant *T. terrestris* cellulose hydrolases, including TtCBH-II, TtEG, TtBG, and combinations thereof, were assayed for microcrystalline cellulose-degrading activity in the presence of TtGH61E, TtCDH, and the combination of TtGH61E and TtCDH. Shown in Fig. 7, the addition of both TtCDH and TtGH61E to TtBG resulted in an increase in the conversion of microcrystalline cellulose, relative to TtBG alone or TtBG with either TtGH61E or TtCDH alone, of ~2%. The addition of TtGH61E and TtCDH to TtCBH-II, TtEG, or the combination of TtCBH-II and TtEG, all in the presence of TtBG, resulted in increases in microcrystalline cellulose conversion of

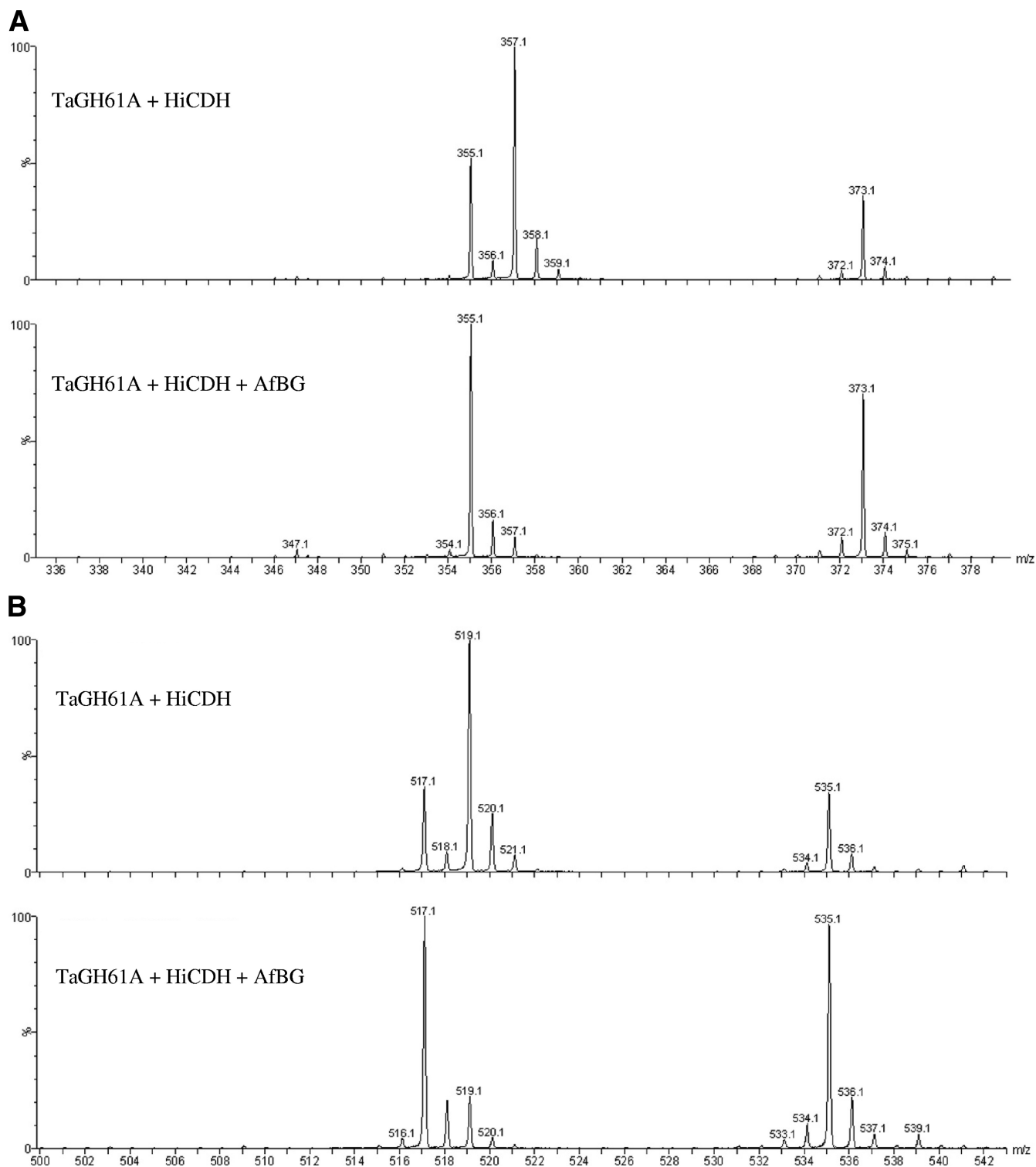


FIG. 5. (A) MS-1 spectra of cellobionic acid (DP2) region of LC elution profile. (Top) PASC, 5 g/liter, treated with TaGH61A and HiCDH. (Bottom) PASC, 5 g/liter, treated with TaGH61A, HiCDH, and AfBG. (B) MS-1 spectra of cellotrionic acid (DP3) region of LC elution profile. (Top) PASC, 5 g/liter, treated with TaGH61A and HiCDH. (Bottom) PASC, 5 g/liter, treated with TaGH61A, HiCDH, and AfBG.

~12%, ~6%, and ~14%. Compared to the addition of the binary combination of TtGH61E and TtCDH, the addition of either TtGH61E or TtCDH individually did not result in an increase of microcrystalline cellulose conversion by *T. terrestris*

cellulose hydrolases under these assay conditions. Similar results, that the addition of both TtGH61E and TtCDH enhance cellulose conversion by *T. terrestris* cellulose hydrolases, were seen in the absence of TtBG (not shown).

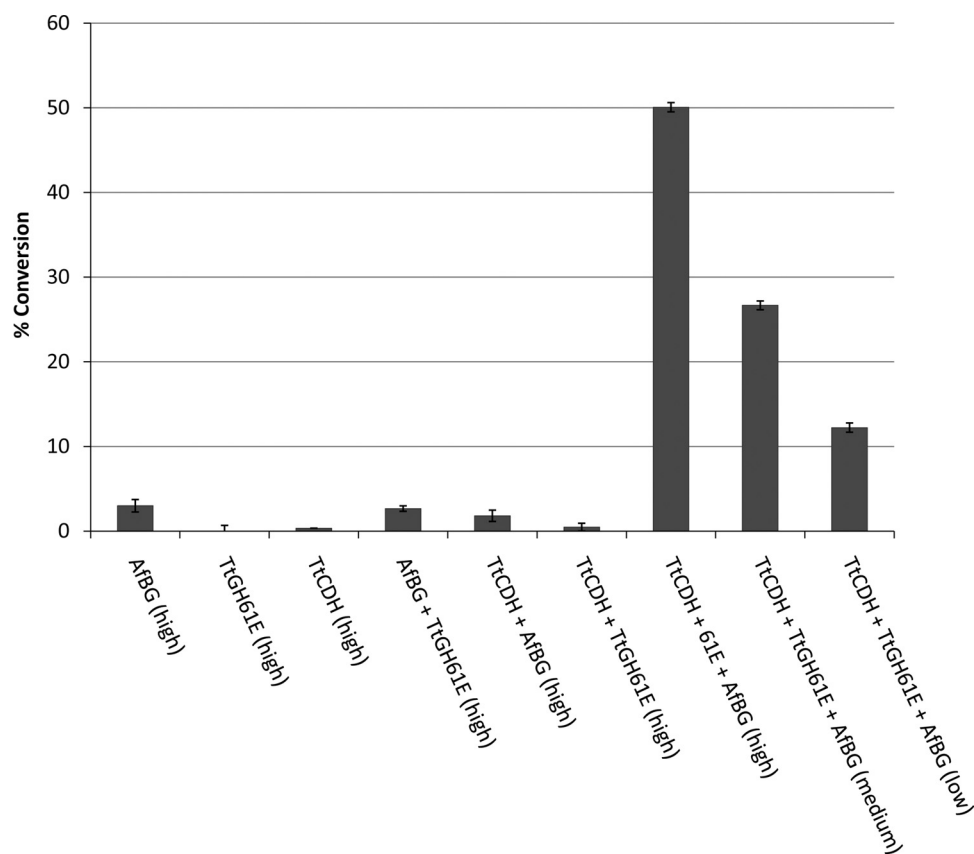


FIG. 6. PASC conversion to monosaccharide by TtCDH, TtGH61E, and AfBG. Reactions were carried out with 5 g/liter PASC in 100 mM sodium acetate at pH 5.0 with 1 mM CaCl_2 for 3 days at 50°C, using single enzymes as well as binary and ternary combinations. Enzyme loadings at highest concentrations (high) were (in mg protein per gram cellulose) 5 mg TtCDH per gram cellulose, 50 mg TtGH61E per gram cellulose, and 5 mg AfBG per gram cellulose. Medium protein loadings (medium) were 2.5 mg TtCDH per gram cellulose, 25 mg TtGH61E per gram cellulose, and 5 mg AfBG per gram cellulose. Low protein loadings (low) were 1 mg TtCDH per gram cellulose, 10 mg TtGH61E per gram cellulose, and 5 mg AfBG per gram cellulose. Error bars are for triplicate cellulose cleavage reactions, each measured once.

DISCUSSION

We have shown that, in the absence of hydrolytic cellulases, the binary combination of TaGH61A and HiCDH catalyzes cellulose cleavage into soluble oligosaccharides and that this activity is highly synergistic with cellulose breakdown by canonical cellulose hydrolases. Soluble cellulose cleavage products of TaGH61A and HiCDH are further shown to have a length that extends from DP2 to DP10 and are mixed species, including reducing-end oxidized species as well as non-reducing-end modified species.

The ascomycete *T. terrestris* secretes a number of canonical cellulose hydrolases when grown in the presence of cellulose (8, 20); formerly, these cellulases have been presumed to account for the majority of cellulose-degrading activity by this fungus. However, cellulose-induced secretion of GH61 and CDH by *T. terrestris* results in an additional cellulose-degrading enzyme activity. The combined increases in conversion, which are significantly greater than the simple addition of conversions from TtGH61E and TtCDH with canonical *T. terrestris* hydrolases, indicate synergy between the canonical cellulose hydrolase components and the cellulose cleavage system of GH61 and CDH. A very recent report also reveals coproduction of CDH and GH61 by the distantly related basidiomycete

Phanerochaete chrysosporium in response to cellulose and xylan, indicating that a GH61-CDH-based oxidative carbohydrate depolymerization system may not be uncommon (12).

The synergy of the GH61-CDH activity with canonical cellulose hydrolase activities argues for a lack of redundancy in the substrates and activities of these systems and suggests that the combination of GH61 and CDH may be opening up new sites for hydrolase action. One possibility is that they act by attacking highly crystalline surfaces, upon which canonical cellulose hydrolases show low activity (28, 31). Canonical hydrolytic endocellulases (endoglucanases) show high activity on amorphous, cellodextrin-like strands of cellulose, while canonical hydrolytic exocellulases (cellobiohydrolases) processively degrade crystalline cellulose but require a cellodextrin chain end to initiate attack. GH61 and CDH may be hypothesized to have an endocrystalline cellulose cleaving activity, unknown with canonical cellulose hydrolases, accounting for the observed degree of synergy with canonical endo- and exocellulose hydrolases. This hypothesis is consistent with the persistence of long oligosaccharide products in the presence of both TaGH61A and HiCDH and is supported by the inability of TaGH61A and HiCDH to cleave DP2 to DP5 soluble cellodextrin standards

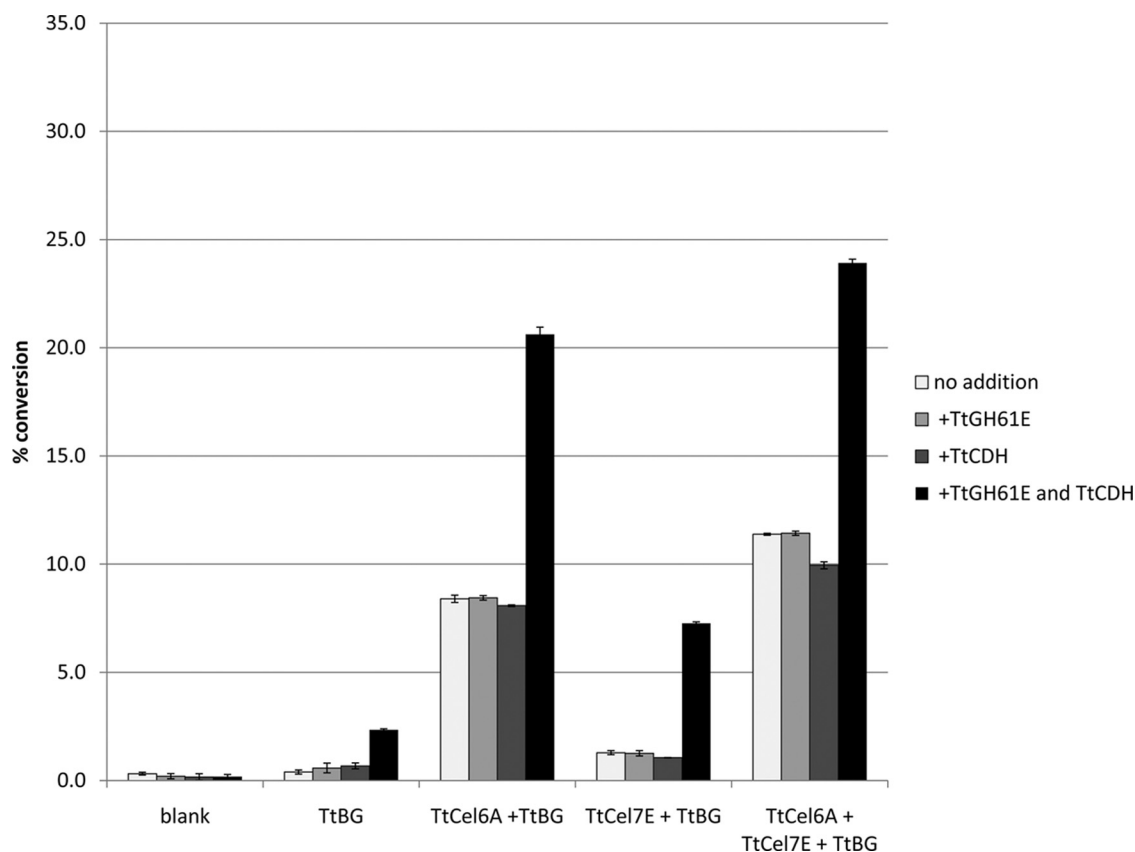


FIG. 7. Microcrystalline cellulose conversion assay by combinations of TtGH61E, TtCDH, TtBG, TtCel6A, and TtCel7E. Reactions were carried out with 25 g/liter microcrystalline cellulose (Avicel) in 100 mM sodium acetate at pH 5.0 with 1 mM CaCl₂ for 3 days at 50°C, using single enzymes as well as enzyme combinations. Enzyme loadings were 5 mg TtGH61E per gram cellulose, 0.5 mg TtCDH per gram cellulose, 1 mg TtBG per gram cellulose, 5 mg TtCel6A per gram cellulose, and 0.75 mg TtCel7E per gram cellulose. Error bars are for triplicate cellulose cleavage reactions, each measured once.

(data not shown). The structure of GH61, which has one flat surface bearing a metal binding site and putative carbohydrate binding tyrosyl residues, but no active site cleft that could accommodate a cellodextrin strand, is consistent with this hypothesis (8, 16). If the crystalline surface is the target of GH61-CDH activity, two potential modes of cellulose attack would be indicated: most likely is a random nicking of the surface that opens new sites for hydrolase attack, but also possible is a semiprocessive attack either parallel with or perpendicular to the grain of the cellulose fibril.

The specific biochemical mechanism by which GH61A and CDH cleave cellulose remains unknown. GH61 homology to the chitin oxygenase CBP-21 (36) and cellulose cleaving CelS2 (6) suggests that GH61 is the species responsible for cellulose cleavage and that CDH is somehow supporting GH61 activity. Indeed, in the absence of CDH and upon addition of ascorbate, TaGH61A has been shown to have cellulose cleaving activity (13), though the specific details of the mechanism by which GH61 cleaves cellulose are beyond the scope of this study and are being actively pursued by ongoing studies. Mixed-product species argue for radical involvement, possibly from cleavage by diffusive Fenton-type radical species (e.g., hydroxyl radical). Arguing against diffusive, unshielded, radical species is the observation that

hydroxyl radical quenching agents only slightly inhibit the reaction; further, no cross-ring cleavage products are detected, and no cleavage of noncellulose carbohydrates is observed (data not shown). The analysis of TaGH61A and HiCDH cellulose cleavage products is complicated by CDH cellodextrin oxidase activity on possible cleavage products, making it unclear if oligosaccharide products are reduced end C-1 oxidized prior to, during, or subsequent of cleavage. However, if deprived of C-1-reduced oligosaccharide substrate as an electron donor, CDH activity in this system would be dissimilar to any function shown in the previous literature (5, 9, 43), presenting the possibility that some reduced cellodextrin may be produced in this cleavage reaction, as seen in the products of CelS2 (6), which later serves as a CDH donor. In addition to CBP21, CelS2, and GH61, oxidoreductive carbohydrate cleavage has also been shown in the NAD⁺-dependent mechanisms of the GH4 and GH109 families (21, 24, 30, 42). GH4 activity is divalent cation dependent, similar to results shown for GH61, CelS2, and CBP21 (6, 8, 36), but it is unclear if further similarities exist within these enzyme systems. Further study will be required to answer these questions and allow full mechanistic elucidation as well as industrial exploitation of this microbial oxidative cellulose depolymerization enzyme system.

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