Appl Biochem Biotechnol (2010) 161:264–273
DOI 10.1007/s12010-009-8782-x

The Family 1 Glycoside Hydrolase from *Clostridium cellulolyticum* H10 is a Cellodextrin Glucohydrolase

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Received: 29 May 2009 / Accepted: 15 September 2009 /
Published online: 9 October 2009

Abstract The only family 1 glycoside hydrolase in *Clostridium cellulolyticum* H10 (CcGH1) is annotated as a beta-galactosidase but has high sequence homology with many beta-glucosidases. Given the possible importance of beta-glucosidase in cellulose utilization by *C. cellulolyticum*, the encoding open reading frame Ccel_0374 was cloned and expressed in *E. coli* as a soluble fusion protein with thioredoxin. After tag cleavage, the purified enzyme had a molecular mass of 52 kDa and was active in dimeric form on a broad range of substrates, including cellobiose, cellotriose, cellotetraose, *p*-nitrophenyl-beta-glucopyranoside, lactose, and *o*-nitrophenyl-beta-galactopyranoside. The enzyme showed lower K_m and higher catalytic efficiency (k_{cat}/K_m) on cellodextrins with degree of polymerization from 2 to 4 than on lactose, and the k_{cat}/K_m values on cellodextrins increased in the order of cellobiose < cellotriose < cellotetraose, suggesting that CcGH1 was a cellodextrin glucohydrolase (EC 3.2.1.74). The high K_m (69 mM) on cellobiose implies that CcGH1 likely has a minimal role in the intracellular hydrolysis of cellobiose in *C. cellulolyticum*. The three-dimensional structure model of CcGH1 generated by homology modeling showed a typical $(\alpha/\beta)_8$ barrel topology characteristic of family 1 glycoside hydrolases.

Keywords Beta-galactosidase · Beta-glucosidase · *Clostridium cellulolyticum* · Consolidated bioprocessing · Cellodextrin glucohydrolase · Cellulose hydrolysis · Microbial cellulose utilization

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Introduction

Lignocellulosic biomass is the most abundant renewable bioresource on earth [1]. Its utilization for the production of biofuels and biobased products will be important to the development of bioeconomy [2, 3]. Biofuels, such as ethanol, butanol, and hydrogen, can be produced through microbial fermentation or enzymatic biotransformation of the sugars released from lignocellulosic biomass [4–6]. Enzymatic cellulose hydrolysis requires synergistic actions of endoglucanase (EC 3.2.1.4), exoglucanase, or cellobiohydrolase (EC 3.2.1.91), and beta-glucosidase (EC 3.2.1.21) [2, 7]. Hydrolysis of cellobiose by beta-glucosidase drastically decreases product inhibition to endoglucanase and cellobiohydrolase and hence increases overall hydrolysis rate of cellulose [2, 8]. Different from beta-glucosidase, cellodextrin glucohydrolase (exoglucohydrolase, β-1,4-D-glucan glucohydrolase, EC 3.2.1.74) hydrolyzes cellobiose very slowly but acts preferentially on oligosaccharides with longer chains to release glucose [9–11].

Clostridium cellulolyticum produces extracellular multienzyme complexes called cellulosomes, which are highly efficient in the degradation of plant cell wall polysaccharides [12, 13]. It is also considered as one of the model microorganisms for consolidated bioprocessing (CBP) that integrates cellulase production, cellulose hydrolysis, and fermentation in a single step. Another CBP model microorganism—*Clostridium thermocellum* takes up the intermediates of cellulose hydrolysis, i.e., cellodextrins having an average degree of polymerization of four, and hydrolyzes them to glucose-1-phosphate and glucose by intracellular cellobiose phosphorylases (EC 2.4.1.20) and cellodextrin phosphorylases (EC 2.4.1.49) [14, 15]. The fast substrate capture before further extracellular hydrolysis occurs when *C. thermocellum* is closely bound to the surface of cellulose [16].

C. cellulolyticum is hypothesized to have a similar mechanism of cellulose hydrolysis and utilization to *C. thermocellum* [17]. A large number of *C. cellulolyticum* cellulosomal endoglucanases and cellobiohydrolases, such as Cel5A, Cel5D, Cel8C, Cel9E, Cel9G, Cel9M, and Cel48F, have been characterized [12, 13, 18]. However, none of the enzymes involved in intracellular hydrolysis of cellodextrin (including cellobiose) has been characterized so far. Given the possible role of beta-glucosidase in cellulose utilization of *C. cellulolyticum*, we cloned and expressed in *Escherichia coli* the open reading frame (ORF) Ccel_0374 encoding a family 1 glycoside hydrolase from *C. cellulolyticum* H10 and characterized the purified enzyme.

Materials and Methods

Chemicals, Strains, and Enzymes All chemicals were reagent grade, purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Cellotriose and cellotetraose were prepared through mixed acid hydrolysis of cellulose and separated by size-exclusion chromatography [19]. *E. coli* JM109 strain was used for cloning. *E. coli* strains BL21 Star (DE3) used for protein expression were purchased from Invitrogen (Carlsbad, CA). Cell culture of *C. cellulolyticum* H10 ATCC 35319 was a gift from David Yang at the Oak Ridge National Laboratory (Oak Ridge, TN). All the enzymes used in the cloning process as well as enterokinase (EK) were purchased from New England Biolabs (Ipswich, MA).

Cloning of C. cellulolyticum Beta-glucosidase Gene The ORF Ccel_0374 encoding a putative family 1 beta-glucosidase from *C. cellulolyticum* H10 (Gene ID 7309258) was

amplified by using the forward primer bglA.Cc-f-EcoRV (5'-CTGTA TGATA TCTAT GGCAT TCAAA GAAGG, EcoRV restriction site underlined) and the reverse primer bglA.Cc-r-XhoIstop (5'-TCGGT TCTCG AGTTA TTTTA ATACT TCGCC, XhoI restriction site underlined). The polymerase chain reaction product was digested by EcoRV and XhoI and then ligated with the digested expression vector pET-32b(+) (Novagen, Madison, WI), yielding pET32b-bglA.Cc.

Enzyme Production and Purification E. coli BL21 Star (DE3) strain bearing plasmid pET32b-bglA.Cc was grown in the LB medium supplemented with 100 μg/mL ampicillin at 37 °C. When A_{600} reached 0.6–0.7, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added at a final concentration of 50 μM, and the growth temperature was decreased to 18 °C. After overnight cultivation, the cells were harvested and resuspended in about 25 mL binding buffer (pH 8.0) containing 50 mM sodium phosphate and 0.3 M NaCl. The cell suspension was sonicated in an ice bath using a Branson model 102C sonicator (3-s pulse, 5-s pause for 5 min at 40% amplitude) and centrifuged at 24,000×*g* at 4 °C for 20 min. The supernatant was loaded onto a column packed with Bio-Rad Profinity IMAC Resin precharged with Ni^{2+} (Hercules, CA). The column was washed with binding buffers containing 0, 10, and 20 mM imidazole, and the target protein was eluted with 50 and 500 mM imidazole. Imidazole was removed using GE Healthcare PD-10 desalting columns (Piscataway, NJ) with a 20-mM Tris–HCl buffer (pH 7.4) containing 50 mM NaCl. The fusion protein was digested with 0.005% (*w*/*w*) EK overnight at room temperature (*ca.* 22 °C), and then the protein solution was passed through a microcon YM-50 column (Millipore, Billerica, MA) to remove most of the cleaved tags but little EK from the purified CcGH1. Protein concentrations were determined by the Bio-Rad Bradford protein assay with bovine serum albumin as the standard.

Zymogram-PAGE The purified enzyme before and after EK digestion were separated on a precast 4–15% gradient native polyacrylamide gel (Bio-Rad) with HMW-native protein molecular weight marker from GE Healthcare (Piscataway, NJ). The gel slice was incubated at room temperature in a 50-mM sodium phosphate buffer (pH 7.0) containing 2 mM 4-methylumbelliferyl-beta-D-glucopyranoside (4-MUG) for about 10 min until fluorescent protein bands became detectable under UV light [20].

Enzyme Reaction Conditions The optimum pH and temperature of the recombinant CcGH1 were determined on 2 mM *p*-nitrophenyl-beta-D-1,4-glucopyranoside (*p*NPG). After 5-min incubation, the reactions were stopped by adding an equal volume of 1 M Na_2CO_3 solution, and the absorbance of the hydrolysis product *p*-nitrophenol (*p*NP) at 405 nm was measured. The optimal pH was determined by measuring the enzyme activity at 37 °C in 0.1-M K_2HPO_4–HCl buffer (pH 2.0–4.0 and pH 8.0–9.0), sodium acetate buffer (pH 4.0–6.0), potassium phosphate buffer (pH 6.0–8.0), and Na_2HPO_4–NaOH buffer (pH 10.0–11.0). The temperature profile was obtained between 25 and 80 °C in a 50-mM sodium phosphate buffer (pH 6.5). For determination of thermostability, the enzyme was diluted to 11.4 μg/mL in a prewarmed 50-mM sodium phosphate buffer (pH 6.5) and preincubated at various temperatures between 25 and 80 °C for 30 min. The residual activity was then measured at 37 °C in a 50-mM sodium phosphate buffer (pH 6.5) containing 2 mM *p*NPG for 11 min. CcGH1 activity was also measured at 37 °C in the presence of 5 mM Ni^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+} or EDTA, or 1 mM Pb^{2+}, Cu^{2+}, Zn^{2+} or Hg^{2+} in a 50-mM sodium acetate buffer (pH 6.0) containing 2 mM *p*NPG.

Substrate Specificity The substrate specificity of the recombinant CcGH1 was tested on a number of chromogenic and natural substrates (10 mM) in a 50-mM sodium phosphate buffer

(pH 6.5) at 37 °C. Enzyme activities on chromogenic substrates *p*NPG and *o*-nitrophenyl-beta-galactopyranoside (*o*NPGal) were determined by measuring the absorbance of the liberated hydrolysis product *p*NP and *o*-nitrophenol (*o*NP) at 405 and 420 nm, respectively. For determination of enzyme activities on natural substrates, the reactions were stopped by boiling for 5 min, and the released glucose was determined using the Pointe Scientific liquid glucose (hexokinase glucose-6-phosphate dehydrogenase) reagent set (Canton, MI). One unit of enzyme activity corresponds to release of 1 μmol of *p*NP, *o*NP, or glucose per minute (for cellobiose as the substrate, one unit corresponds to the release of 2 μmol/min).

Kinetic Parameters The initial reaction velocities of the recombinant beta-glucosidase were determined in a 50-mM phosphate buffer (pH 6.5) at 37 °C on *p*NPG (0.2–5.0 mM), cellobiose (12–200 mM), cellotriose (2–25 mM), cellotetraose (2–25 mM), *o*NPGal (0.2–5.0 mM), and lactose (10–192 mM).

Homology Modeling The program MODELLER9v2 [21] was used for generation and analysis of a 3-D structure model of family 1 beta-glucosidase from *C. cellulolyticum* by using homology modeling. The crystal structures used as modeling templates were beta-glucosidases from *Streptomyces* sp. (protein database accession number 1gnx), *Thermus thermophilus* HB8 (1ug6), and *Thermotoga maritima* (1uz1); the A chain was used in all cases.

Results

Protein Expression and Purification

The open reading frame Ccel_0374 from *C. cellulolyticum* H10 was annotated as a beta-galactosidase that belongs to glycoside hydrolase family 1 in the Carbohydrate Active Enzymes (CAZy) database [22]. A protein BLAST search returned 100 hits with *E* values of at least 4e−86, most of which are beta-glucosidases and some of which are beta-galactosidases. Our initial attempt to express the active enzyme from the Novagen vector pET-21c(+) in *E. coli* BL21 Star (DE3) strain was unsuccessful because the recombinant protein formed inclusion bodies regardless of the IPTG concentrations and the expression temperatures we have tested (data not shown). To obtain soluble enzyme, we cloned the encoding gene into the expression vector pET-32b(+) to give plasmid pET32b-bglA.Cc. The resultant recombinant protein was fused to thioredoxin that can help protein folding and improve its solubility [23]. After induction with IPTG, the *E. coli* BL21 Star (DE3) cells bearing plasmid pET32b-bglA.Cc produced the soluble fusion protein at a very high level (*ca.* 145 mg/L cell culture), accounting for about 25% of the total soluble cellular proteins (data not shown).

The fusion protein was purified by a Ni^{2+}-based affinity chromatography column. After fusion tag cleavage by EK, the molecular mass of the recombinant protein was reduced from 69 to 52 kDa (Fig. 1a). Removal of the fusion tags had no significant effect on enzyme activity (data not shown). Native PAGE analysis showed that the fusion protein had a molecular mass of 280 kDa, suggesting that it formed tetramers; interestingly, the protein after tag cleavage had a molecular mass of 100 kDa, corresponding to a dimer (Fig. 1b). Both the dimeric and tetrameric forms were active as shown by a zymogram (Fig. 1c).

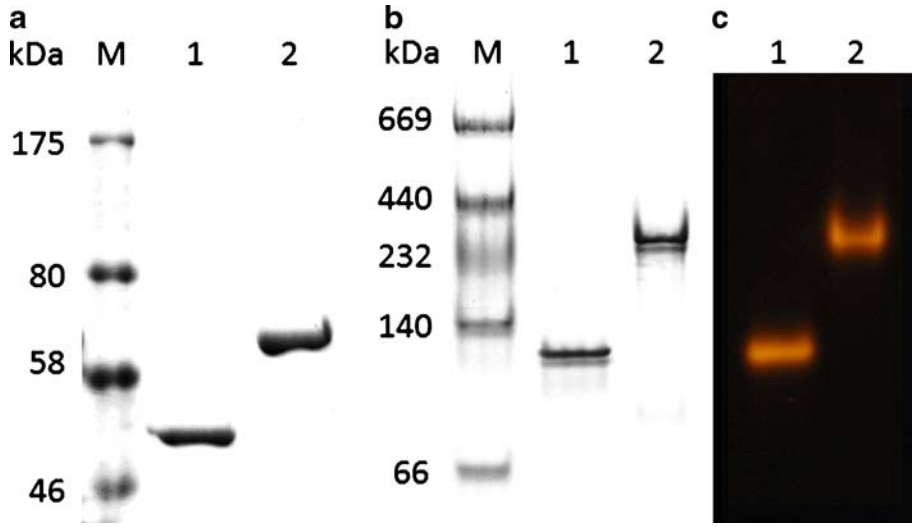

Fig. 1 SDS-PAGE (**a**), native PAGE (**b**), and zymogram (**c**) for the purified CcGH1 after and before fusion tag cleavage by EK. *Lane M* protein marker, *lane 1* after tag cleavage, *lane 2* before tag cleavage

Enzyme Characterization

The enzyme CcGH1 after tag cleavage was characterized on *p*NPG, a chromogenic analog of cellobiose. It showed maximal activity at pH 6.5, over 75% activity at pH 3.5–7.5, and the measured activity decreased rapidly as the pH was decreased below 3.5, but slowly when the pH was increased above 7.5 (Fig. 2a). The maximal enzyme activity was obtained at 60 °C (Fig. 2b). At a concentration of 11.4 μg/mL, CcGH1 was stable at temperatures lower than 30 °C and retained 88%, 84%, 61% of its activity at 37, 40, and 50 °C after 30-min preincubation, respectively (Fig. 2c). No significant change of activity was observed in the presence of 5 mM Mg^{2+}, Mn^{2+}, Ca^{2+}, and EDTA, suggesting that no metal ion was required for enzyme activity (Fig. 3). The presence of Ni^{2+}, Co^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+}, and Hg^{2+} resulted in different extents of loss of enzyme activities: 5 mM Ni^{2+} (33%), 5 mM Co^{2+} (38%), 1 mM Zn^{2+} (92%), 1 mM Pb^{2+} (97%), 1 mM Cu^{2+} (100%), and 1 mM Hg^{2+} (100%).

Kinetic parameters of CcGH1 were determined on various substrates—cellobiose, cellotriose, cellotetraose, *p*NPG, lactose, and *o*NPGal (Table 1). The enzyme released glucose from cellodextrins (cellobiose, cellotriose, and cellotetraose) with increasing catalytic efficiency (k_{cat}/K_m) from 0.17 s^{-1} mM^{-1} for cellobiose to 0.396 s^{-1} mM^{-1} for cellotetraose, a 2.3-fold increase. By comparison, the k_{cat}/K_m value (0.060 s^{-1} mM^{-1}) on lactose was much lower. The high values of K_m on cellobiose (69 mM) and lactose (220 mM) indicated that this enzyme had low affinities for these substrates, but it had higher affinities for the nonnatural chromogenic substrates—*p*NPG (0.36 mM) and *o*NPGal (1.2 mM). Maltose and sucrose were not hydrolyzed by CcGH1 (data not shown).

Homology Modeling

The *C. cellulolyticum* beta-glucosidase CcGH1 has 450 amino acids. Three crystal structures of beta-glucosidases of similar sizes were used as the templates for homology modeling (Fig. 4). The amino acid sequences of the structure templates 1gnx, 1ug6, and

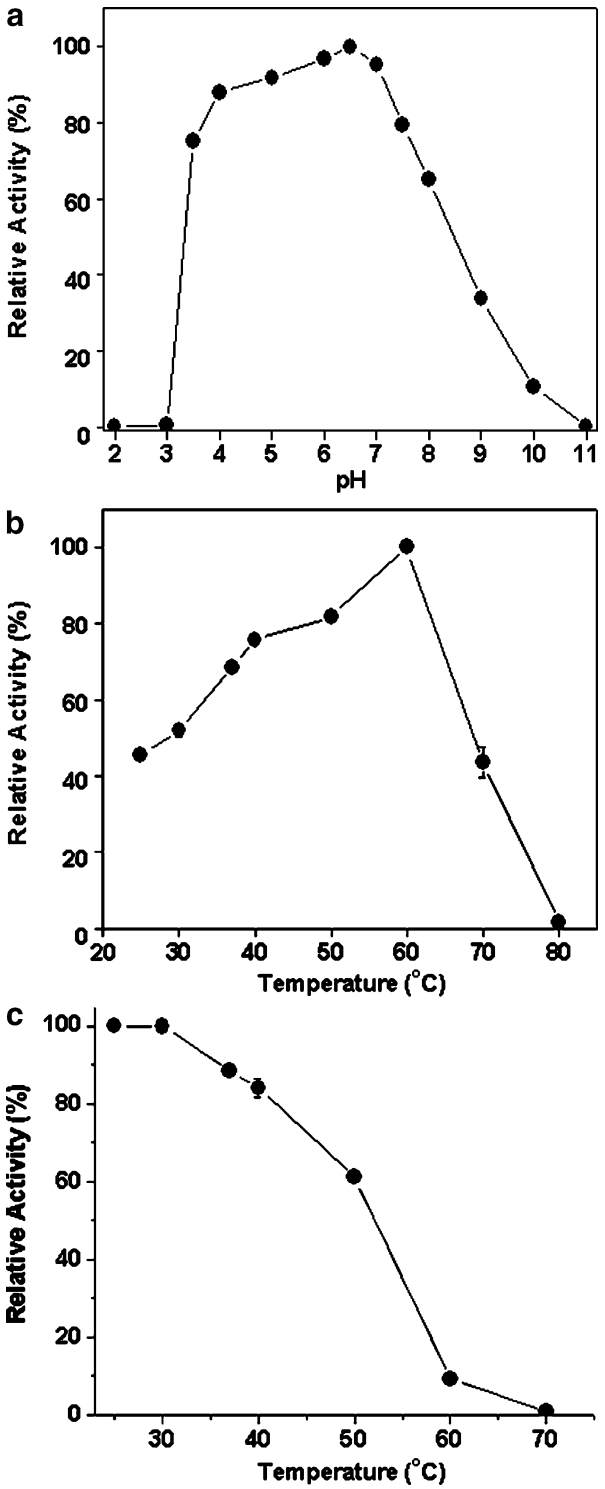

Fig. 2 The pH profile (**a**), optimal temperature (**b**), and thermostability (**c**) of the purified CcGH1. For thermostability determination, residual activities were measured after 30-min preincubation at various temperatures. Each point is the mean of triplicate experiments

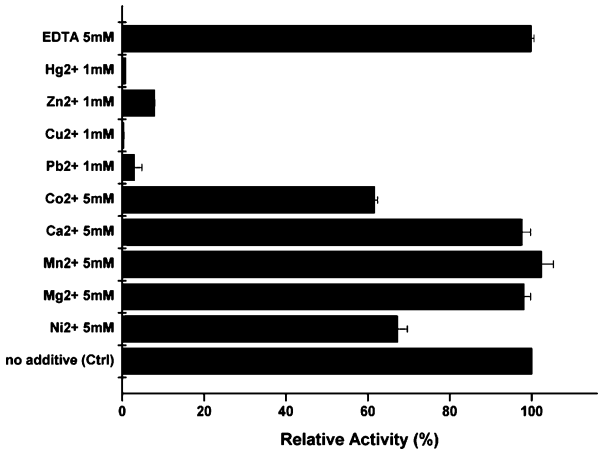

Fig. 3 The effects of metal ions and EDTA on the purified CcGH1. Each point is the mean of triplicate experiments

1uz1 have respective sequence identities of 44%, 44%, and 45% relative to CcGH1. The predicted 3-D structure model resembles the template structures very closely, especially at the core regions (Fig. 4a–c). The monomer displayed the $(\alpha/\beta)_8$ barrel topology characteristic of family 1 glycoside hydrolases. A Ramachandran plot generated by PROCHECK showed that 99.7% of residues are in allowed regions, with 89.5% in the most favored regions, 8.7% in additional allowed regions, and 1.5% in generously allowed regions (Fig. 4d). Verify-3D analysis [24] indicated that the model was of good quality, with only two residues in loops being in a slightly unfavorable region. Furthermore, PROSA analysis returned a favorable result, with a Z score of −9.03, which is a good value for a protein of this size.

Discussion

The enzyme CcGH1 had substrate specificities and action pattern characteristics of cellodextrin glucohydrolase (EC 3.2.1.74), beta-glucosidase (EC 3.2.1.21), and beta-galactosidase (EC 3.2.1.23; Table 1). The kinetics data suggested that CcGH1 was a cellodextrin glucohydrolase rather than a beta-galactosidase, as annotated in the genome sequence of *C. cellulolyticum* H10, or a beta-glucosidase [25]. So far, the glycoside hydrolase family 1 in the CAZy database does not include any enzyme EC 3.2.1.74 [22]. The characterization of CcGH1 expanded the known activities of the family 1 glycoside hydrolases.

Table 1 Kinetic parameters of the recombinant CcGH1 at pH 6.5 and 37 °C.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3 s^{-1} M^{-1}$)
*p*NPG	0.36±0.02	6.74±0.05	19.0±0.9
Cellobiose	69±5	11.5±0.4	0.17±0.01
Cellotriose	15.7±0.5	4.9±0.1	0.312±0.007
Cellotetraose	16.1±0.3	6.36±0.09	0.396±0.005
*o*NPGal	1.2±0.1	27±1	22±2
Lactose	220±14	13.1±0.8	0.060±0.002

Each point is the mean of triplicate experiments

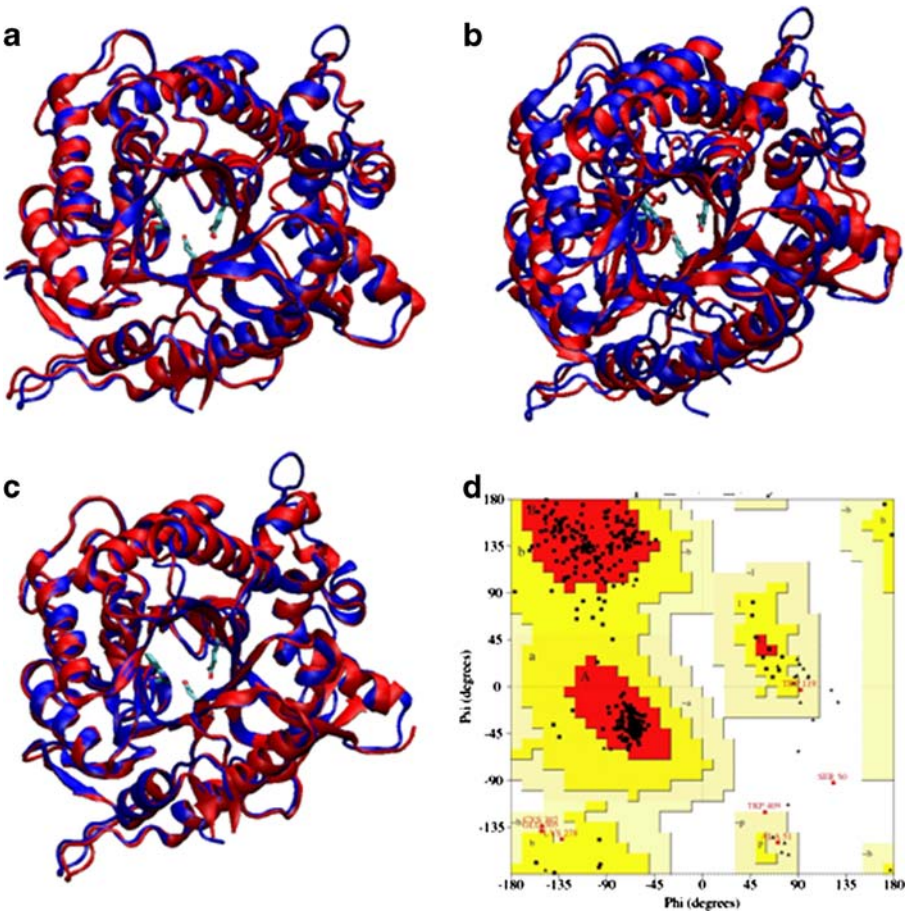

Fig. 4 Overlay of the 3-D structure model of CcGH1 (*blue*) with the template structures (*red*; **a**–**c**) and Ramachandran plot for the model (**d**). The templates are the A chains of structures 1gnx (**a**), 1ug6 (**b**), and 1uz1 (**c**). The overlay was based on the two catalytic glutamate residues as well as a conserved tryptophan residue in the active site (bond rendering, colored by atom). **d** *Red shaded areas* are the most favored regions, *yellow* areas are additional allowed regions, *tan areas* are generously allowed regions, and *white areas* are disallowed regions. The *black dots* are the phi, psi values from the enzyme

The enzyme CcGH1 showed comparable catalytic efficiencies for *p*NPG (k_{cat}/K_m= 19.0 s^{-1} mM^{-1}) and *o*NPGal (k_{cat}/K_m=22 s^{-1} mM^{-1}), the chromogenic analogs of cellobiose and lactose, respectively; yet the k_{cat}/K_m values on cellobiose (0.17 s^{-1} mM^{-1}) and lactose (0.060 s^{-1} mM^{-1}) were different by 2.8-fold. These results suggested that the analog substrates good for fast cellulase assays were not necessarily reliable for understanding the natural roles and classification of cellulase. Similar situations were observed for endoglucanase on natural cellulose and the analog substrate carboxymethyl cellulose [8, 26].

The *C. cellulolyticum* CcGH1 had a high K_m value of 69 mM on cellobiose, implying that this enzyme might serve as an accessory enzyme for intracellular cellobiose hydrolysis, similar to the *C. thermocellum* beta-glucosidase A (glycoside hydrolase family 1), which had a K_m of 83 mM on cellobiose [27, 28]. Nevertheless, CcGH1 showed much higher

substrate affinities and catalytic efficiencies on cellotriose and cellotetraose, implying that it may be responsible for converting a fraction of long-chain cellodextrins to cellobiose.

Various metal ions were observed to reduce the enzyme activity of CcGH1. This might be attributed to complexation of the amino acids with metal ions. Such interactions could change the microenvironment of the catalytic center of the enzyme, which may cause activity reduction.

The recombinant CcGH1 was shown to be active in a multimeric form. In fact, most of the family 1 beta-glucosidases with known 3-D structures occur as multimers. However, it is unclear whether multimerization is required for enzyme activity or stability, or both. Considering that the active center of our structure model of CcGH1 is in the middle of each monomer rather than at the multimer interface, the formation of multimers might not be essential for enzyme activity. Nonetheless, multimerization might help improve enzyme stability against heat and pH denaturation.

This study clearly suggested that the putative *C. cellulolyticum* beta-galactosidase annotated based on bioinformatic analysis was a cellodextrin glucohydrolase with significant beta-galactosidase and beta-glucosidase activities and that the enzyme most likely played an accessory role in intracellular cellobiose hydrolysis by *C. cellulolyticum*.

Acknowledgments This work was supported mainly by the DOE BioEnergy Science Center and partially by the USDA Bioprocessing and Biodesign Center as well as the DuPont Young Professor Award.

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