Biochemical Characterization and Structural Analysis of a Biofunctional Cellulase/Xylanase from Clostridium thermocellum*

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Background: CtCel5E can degrade both cellulose and hemicellulose (xylan).

Results: X-ray crystallography and site-directed mutagenesis were used to assess the roles of the active-site residues in CtCel5E.

Conclusion: A flexible loop and other residues participate in substrate discrimination.

Significance: This study provides the mechanisms of substrate recognition and a blueprint for engineering CtCel5E.

We expressed an active form of CtCel5E (a bifunctional cellulase/xylanase from Clostridium thermocellum), performed biochemical characterization, and determined its apo- and ligand-bound crystal structures. From the structures, Asn-93, His-168, His-169, Asn-208, Trp-347, and Asn-349 were shown to provide hydrogen-bonding/hydrophobic interactions with both ligands. Compared with the structures of TmCel5A, a bifunctional cellulase/mannanase homolog from Thermotoga maritima, a flexible loop region in CtCel5E is the key for discriminating substrates. Moreover, site-directed mutagenesis data confirmed that His-168 is essential for xylanase activity, and His-169 is more important for xylanase activity, whereas Asn-93, Asn-208, Tyr-270, Trp-347, and Asn-349 are critical for both activities. In contrast, F267A improves enzyme activities.

Plant lignocellulosic biomass, which is composed of polysaccharides (cellulose and hemicelluloses) and lignin, is a major renewable source of green energy. Cellulose, which is the most abundant component of plant polysaccharides, consists of glucose units linked via β-1,4-glycosidic bonds (1), whereas hemicelluloses are composed of other sugars, such as arabinose, xylose, galactose, glucose, and mannose. Current strategies for the bioconversion of lignocellulosic feedstock into biofuel include three major steps: the pretreatment of biomass, the enzymatic hydrolysis of polysaccharides, and the fermentation of monosugars to bioethanol by yeast or other useful products by engineered microbes. However, the structural diversity of polysaccharides in plant cell walls is a major challenge in cellulose biofuel production because of the rate limiting and high cost of enzymatic hydrolysis that is required to release fermentable monosugars. For example, polysaccharides, such as homo-1,4-β-D-cellulose, hetero-1,4-β-D-xylan, and hetero-1,4-β-D-mannan, need to be hydrolyzed by the endo-/exo-cellulase, xylanase, and mannanase into cellobiose, xylobiose, and mannobiose, respectively. Subsequently, β-glucosidase, β-xylosidase, and β-mannosidase convert the disaccharides into the monosaccharides glucose, xylose, and mannose, respectively.

Clostridium thermocellum is a thermophilic and efficient plant cell wall-degrading bacterium that secretes cellulosome, a highly active cellulosolytic complex (1). Cellulosome functions as a comprehensive enzymatic system that includes cellulases, hemicellulases, and others to synergistically break heterogeneous polysaccharides; it thus has a huge potential in biofuel application. Previous studies of C. thermocellum revealed an interesting cellulosomal celH gene that encodes two functional enzyme domains (Lic26A and Cel5E), a family 11 carbohydrate binding module (CBM11), and two C-terminal type I dockerins (2–4). Lic26A is a glycoside hydrolase (GH)4 family 26 hydrolase that contains β-1,3-1,4-mixed linked endoglucanase activity (4, 5). Cel5E is a bifunctional β-1,4-endoglucanase/xylanase that belongs to the GH5 family (6, 7), and GH5 is the second largest among the 133 GH families (see the CAZY database) (8). Because Cel5E is a bifunctional cellulase/xylanase and xylan is the major component of hemicelluloses in plants, it would be beneficial to understand the structure and function of Cel5E.

To investigate how Cel5E recognizes two different types of substrates, we characterized its substrate specificity and obtained its pH and temperature kinetic profiles. In addition, we determined the crystal structures of apo- and ligand-bound CtCel5E and subsequently carried out site-directed mutagenesis with the aim of understanding the roles of the active-site residues in CtCel5E.

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1 The abbreviations used are: GH, glycoside hydrolase; CtCel5E, C. thermocellum Cel5E; TmCel5A, T. maritima Cel5A; CMC, carboxymethyl cellulose; BW, beechwood; PASC, phosphoric acid-swollen cellulose; TLC, thin layer chromatography; PDB, Protein Data Bank.
Characterization and Structures of CtCel5E

TABLE 1
Primers used in generating wild-type and mutant CtCel5E enzymes

The mutated codons are underlined.

| Primer | Nucleotide sequence |
|--------|---------------------|
| P1     | 5’-GGAAATTTCCAGATTTCCGAAGCCCTTGCAG-3’ |
| P2     | 5’-CCCGTCGACATCCTGCAAGCTGCGGTTGTTTGCAG-3’ |
| P3     | 5’-GTTTTTGTTCCATAGGCTGGAATTCTTTTGTTTCCGAG-3’ |
| P4     | 5’-TTTTTGTTCCATAGGCTGGAATTCTTTTGTTTCCGAG-3’ |
| P5     | 5’-CCCGTCGACATCCTGCAAGCTGCGGTTGTTTGCAG-3’ |
| P6     | 5’-GTTTTTGTTCCATAGGCTGGAATTCTTTTGTTTCCGAG-3’ |
| P7     | 5’-GTTTTTGTTCCATAGGCTGGAATTCTTTTGTTTCCGAG-3’ |
| P8     | 5’-TTTTTGTTCCATAGGCTGGAATTCTTTTGTTTCCGAG-3’ |
| P9     | 5’-CTCTTCGATTTCCGAAGCCCTTGCAGGTACACCTGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
Characterization and Structures of CtcCel5E

Expression and Characterization of Different Constructs Containing Cel5E Catalytic Domain—Four different constructs that contained the Cel5E catalytic domain were cloned and expressed (Fig. 1). CtCel5E-T1 containing the catalytic domain Cel5E without the N- and C-terminal linkers and CtCel5E-T2 with the C-terminal linker were expressed primarily in the inclusion bodies (data not shown). CtCel5E-T3 that contained the N-terminal linker and CtCel5E that contained both the N- and C-terminal linkers were expressed as soluble proteins, but only CtCel5E showed activities toward CMC (a cellulase substrate) and BW (a xylanase substrate). Thus, this construct was chosen for further studies.

The optimal temperatures for CtCel5E to hydrolyze CMC and BW were 50 and 60 °C, respectively (Fig. 2A). At the optimal temperatures, CtCel5E showed the best cellulase and xylanase activities at pH 5 and 6, respectively (Fig. 2B). Subsequently, the TLC analysis shown in Fig. 2C revealed cellobiose and cellotriose to be the major products from cellotetraose, cellopentaose, cellohexaose, and polymeric CMC and PASC, whereas xylobiose and xylotriose were produced from polymeric BW. Moreover, CtCel5E was active toward birchwood, a xylan that lacks the 6-CH2OH side chain, and arabinoxylan, but it had no mannanase activity on mannan and locust bean gum with C2-OH at the axial position. The specific activities in IU (μmol of product/min/μmol of protein) of CtCel5E toward different substrates under the respective optimal conditions are

pH 7.5, and 150 mM NaCl. A French press instrument (Constant Cell Disruption System) was used to disrupt the cells at 20,000 p.s.i., followed by centrifugation at 10,000 × g for 1 h. The supernatant was loaded onto a 20-ml nickel-nitrotriacetic acid column, which was equilibrated with the lysis buffer. The column was washed with the lysis buffer containing 10 mM imidazole and eluted with 300 ml of a 10–300 mM imidazole linear gradient in the same buffer. Fractions of 3 ml were collected and were analyzed by SDS-PAGE. Fractions containing the recombinant proteins were combined and further purified by a HiLoad 26/60 Superdex 75 column (GE Healthcare) using lysis buffer for elution. The protein concentrations were determined by the Bradford method (18). Protein purification was performed at 4 °C.

**Enzyme Activity Assays**—Reducing sugars formed through enzyme catalysis were determined with 3,5-dinitrosalicylic acid reagent, using D-glucose and D-xylose as standards (19). Total cellulase activities were measured with the modified soluble celluloses, CMC and PASC, as substrates. BW was used to determine xylanase activity. Assay mixtures containing 1% CMC, 1% PASC, or 1% BW in 10 mM CGH buffer (10 mM citric acid, 10 mM glycine, and 10 mM HEPES) were incubated for the given time periods under optimal conditions, and the reactions were stopped by adding 3,5-dinitrosalicylic acid reagent and then heated at 100 °C for 5 min. Absorbance at 540 nm was measured using a Spectra Max Plus 384 (Molecular Device) to determine enzyme activities. The optimal conditions for cellulase and xylanase activities of wild-type and F267A mutant enzymes were determined under a range of different temperatures and pH levels using suitable buffers.

The $K_m$ and $k_{cat}$ values of CtCel5E and its mutants toward CMC and BW were determined by fitting the initial velocities at different substrate concentrations with the Michaelis-Menten equation. The assays were run in triplicate with at least five substrate concentrations, varying from approximately half to 5-fold the $K_m$ values, under the optimal conditions.

**End Product Determination**—Purified CtCel5E was mixed with G2–G6 cello-oligosaccharides, 1% CMC, 1% PASC, or 1% BW in 10 mM CGH buffer (10 mM citric acid, 10 mM glycine, and 10 mM HEPES) and incubated for the given time periods under optimal conditions, and the reactions were stopped by adding 3,5-dinitrosalicylic acid reagent and then heated at 100 °C for 5 min. Absorbance at 540 nm was measured using a Spectra Max Plus 384 (Molecular Device) to determine enzyme activities. The optimal conditions for cellulase and xylanase activities of wild-type and F267A mutant enzymes were determined under a range of different temperatures and pH levels using suitable buffers. The $K_m$ and $k_{cat}$ values of CtCel5E and its mutants toward CMC and BW were determined by fitting the initial velocities at different substrate concentrations with the Michaelis-Menten equation. The assays were run in triplicate with at least five substrate concentrations, varying from approximately half to 5-fold the $K_m$ values, under the optimal conditions.

**Site-directed Mutagenesis**—Mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Agilent) and confirmed by DNA sequencing. The mutagenic forward/reverse primers were P9/P10 for E209A, P11/P12 for E314A, P13/P14 for N93A, P15/P16 for H168A, P17/P18 for H169A, P19/P20 for N208A, P21/P22 for P267A, P23/P24 for P267E, P25/P26 for P267K, P27/P28 for Y270A, P29/P30 for Y270E, P31/P32 for H277A, P33/P34 for V299A, P35/P36 for P30A, P37/P38 for W347A, P39/P40 for N349A, and P41/P42 for V299A/W302A. The mutant recombinant proteins were expressed and purified by following the same procedures as described above for wild-type CtCel5E.

**RESULTS**

Expression and Characterization of Different Constructs Containing Cel5E Catalytic Domain—Four different constructs that contained the Cel5E catalytic domain were cloned and expressed (Fig. 1). CtCel5E-T1 containing the catalytic domain Cel5E without the N- and C-terminal linkers and CtCel5E-T2 with the C-terminal linker were expressed primarily in the inclusion bodies (data not shown). CtCel5E-T3 that contained the N-terminal linker and CtCel5E that contained both the N- and C-terminal linkers were expressed as soluble proteins, but only CtCel5E showed activities toward CMC (a cellulase substrate) and BW (a xylanase substrate). Thus, this construct was chosen for further studies.

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The recombinant CtCel5E wild type and E314A inactive mutant were concentrated to 25 mg/ml in 25 mM Tris (pH 7.5) and 150 mM NaCl and crystallized from 0.2 M ammonium acetate, 0.1 M sodium acetate (pH 4.6), and 21% (w/v) PEG 4000 with by the sitting drop vapor diffusion method at 25 °C. The wild-type CtCel5E crystals were transferred to a reservoir solution supplemented with 10% (v/v) glycerol. The xylobiose- and cellobiose-bound crystals were obtained by soaking the CtCel5E_E314A crystals with the cryoprotectant solution (0.2 M ammonium acetate, 0.1 M sodium acetate (pH 4.6), 21% (w/v) PEG 4000, and 10% (v/v) glycerol) that contained 10 mM xylobiose and cellobiose, respectively. All crystals were flash-frozen in liquid nitrogen prior to data collection. The diffraction data were collected at beam lines BL13B1 and BL13C1 of the National Synchrotron Radiation Research Center (Hsinchu, Taiwan) at 100 K. The data were processed using the HKL2000 program suite (21). The apo-form structure of CtCel5E was determined by the molecular replacement method, using the program Molrep and based on the structure of TnCel5A (PDB entry 3AMC) as the search model. The xylobiose- and cellobiose-bound structures were solved by molecular replacement with the refined CtCel5E structure as a search model. The models were refined using Coot (22) and Refmac5 (23).
Characterization and Structures of CtCel5E

736.2 ± 12.8 for CMC, 165.2 ± 4.8 for PASC, 351.6 ± 2.9 for BW, 255.8 ± 7.2 for birchwood, and 153.1 ± 3.2 for arabinoxylan. Our results thus confirmed that CtCel5E is a bifunctional β-1,4-endoglucanase/β-1,4-xylanase. Under optimal conditions, the $K_m$ of CMC is 2.1 ± 0.2 (mg/ml), and the $k_{cat}$ is 1564.0 ± 69.1 min$^{-1}$, whereas the $K_m$ of BW is 4.6 ± 0.2 (mg/ml), and the $k_{cat}$ is 883.5 ± 13.2 min$^{-1}$ (Table 2).

Structures of Apo-form of CtCel5E—The crystal structure of CtCel5E-apo was determined at 2.4 Å resolution, with data processing and refinement statistics summarized in Table 3. Two CtCel5E molecules are present in the asymmetric unit of the crystal structure (Fig. 3A). The electron density map indicates that CtCel5E is ordered from Pro-74 to Ala-378, except for two missing loop regions (residues 276–280 and residues 353–357), thus indicating their flexibility (Fig. 3B). The overall structure of CtCel5E displays a classical (α/β)$_8$-TIM barrel fold. The TIM barrel consists of a parallel eight-strand β sheet and seven α helices and is surrounded by two short strands. In addition, one α helix at the N terminus is located at the bottom of the barrel, and one 3$_{10}$ helix is located at the top of barrel.

GH5 enzymes contain two invariant catalytic glutamate residues: the acid/base and nucleophile (24). Site-directed mutagenesis studies (Table 2) indicate that Glu-209 and Glu-314 are these two catalytic residues. In addition, Arg-124, Asn-208, His-268, and Trp-347 are strictly conserved and posited similarly in the active site compared with those in other GH5 enzymes (10, 11, 25) (Fig. 3C).

The overall structure of CtCel5E is similar to those of the other GH5 family enzymes, such as Clostridium cellulolyticum endoglucanase CelCCA (PDB entry 1EDG) and TmCel5A (PDB entry 3AMC) (10). The root mean square deviation between CelCCA and CtCel5E is 1.4 Å over 204 Cα atoms, and the root
Characterization and Structures of CtCel5E

The His-169 side chain forms a hydrogen bond with the C3-OH of (−1)-xylose. The Trp-347 side chain probably contributes a hydrophobic stacking interaction with the carbohydrate skeleton of (−1)-xylose and N1 of Trp-347 hydrogen-bonds to the C2-OH of (−2)-xylose. Asn-349 also probably forms a weak (3.42 Å) hydrogen bond with the C2-OH in chain B.

Structures of CtCel5E_E314A-Cellobiose—Because CtCel5E also exhibits cellulase activity, we further determined the crystal structure of CtCel5E_E314A-cellobiose. This crystal structure was determined at 2.7 Å resolution, and two CtCel5E molecules are present in the asymmetric unit cell (data not shown). The data processing and refinement statistics are summarized in Table 3. The cellobiose (electron density shown in Fig. 4C) is also bound as a product located at the −1 and −2 subsites. The binding interactions for cellobiose are primarily contributed by residues His-168, His-169, Asn-208, and Glu-209 (Fig. 4D), which are similar to those used for binding with xylobiose (Fig. 4B). Interestingly, the flexible loop (residues 276–280) in subunit A becomes visible. Overall, the structures of apo- and xylobiose- and cellobiose-bound CtCel5E are almost identical (Fig. 4E), indicating no major conformational changes upon product binding. However, this does not exclude the possibility that the flexible loop needs to be relocated during catalysis.

Structural Comparison with TmCel5A to Rationalize Substrate Specificities—CtCel5E, excluding the N- and C-linker regions, shares 36% sequence identity with TmCel5A (Fig. 5A). However, they possess different substrate specificities. The sequence alignment shows 10 + 1 residues missing in CtCel5E, around the first flexible loop (Fig. 5A), which may be the key for determining substrate specificity. Compared with the structure of TmCel5A_E253A-mannotriose (Fig. 5B), where the side chain of Tyr-198 forms hydrogen bonds with C1-OH and C5-O of (−1)-mannose and both His-205 and Trp-210 form hydrogen bonds with the C6-OH, the corresponding residues (Tyr-270 and His-277) in CtCel5E, which are near or located in the flexible loop, make no contact with the (−1)-xylose, and there is no residue

Table 2

| CtCel5E substrate | CMC | k_{cat} | kW | k_{cat} |
|-------------------|-----|---------|----|---------|
|                   | mg/ml | min |    | mg/ml | min |
| WT                | 2.1 ± 0.2 | 1564.0 ± 69.1 | 4.6 ± 0.2 | 885.3 ± 13.2 |
| E209A             | ND* | ND |     | ND | ND |
| E314A             | ND | ND |     | ND | ND |
| N93A              | ND | ND |     | ND | ND |
| H168A             | 5.0 ± 0.1 | 1389.9 ± 1.1 | 7.9 ± 0.4 | 53.6 ± 3.9 |
| H169A             | 10.1 ± 1.0 | 571.2 ± 27.1 | 2.0 ± 0.2 | 37.1 ± 1.9 |
| N208A             | ND | ND |     | ND | ND |
| Y270A             | ND | ND |     | ND | ND |
| Y270F             | ND | ND |     | ND | ND |
| H277A             | 9.5 ± 0.3 | 1157.4 ± 3.8 | 12.5 ± 0.2 | 664.9 ± 5.0 |
| W347A             | ND | ND |     | ND | ND |
| V299A             | 2.4 ± 0.3 | 1408.7 ± 28.0 | 3.9 ± 0.4 | 321.4 ± 23.3 |
| W302A             | 8.4 ± 0.4 | 2165.2 ± 53.0 | 8.3 ± 0.4 | 465.5 ± 3.7 |
| V299A/W302A       | 3.7 ± 0.2 | 1077.6 ± 34.4 | ND | ND |

*ND, the activity was nondetectable.

Table 3

|           | CtCel5E-apo | CtCel5E_E314A-xylobiose | CtCel5E_E314A-cellobiose |
|-----------|-------------|-------------------------|-------------------------|
| PDB code  | 4U3A        | 4U5I                    | 4U5K                    |
| Data collection |      |                         |                         |
| Resolution (Å) | 25.2-4.0 (2.49-2.40) | 25.2-5.0 (2.59-2.50) | 25.2-6.5 (2.74-2.65) |
| Space group  | P4_2_2      | P4_2_2                  | P4_2_2                  |
| Unit cell: a/b/c (Å) | 74.93/74.93/254.20 | 75.02/75.02/256.18 | 74.94/74.94/254.89 |
| No. of reflections measured | 283,909 (19,844) | 26,220 (2588) | 129,124 (12,681) |
| Complete (%) | 97.0 (70.6) | 98.8 (100.0) | 99.8 (100.0) |
| Rmerge (%)  | 7.9 (47.7)  | 8.2 (51.3)  | 11.9 (48.8)  |
| Mean I/σ(I) | 23.7 (4.3)  | 23.1 (3.3)  | 11.6 (3.0)  |
| Multiplicity | 10.3 (9.5)  | 4.9 (5.2)   | 5.1 (5.1)   |
| Refinement  |             |                         |                         |
| Rwork (%)  | 20.3 (27.0) | 20.3 (26.7) | 18.3 (28.1) |
| Rfree (%)  | 24.8 (35.0) | 24.7 (31.8) | 25.4 (34.6) |
| Geometry deviations |       |                         |                         |
| Bond lengths (Å) | 0.006 | 0.007 | 0.007 |
| Bond angles (degrees) | 1.01 | 1.29 | 1.30 |
| No. of atoms/mean B-values (Å²) | 4929/41.1 | 5214/51.5 | 5576/40.3 |
| Ramachandran plot (%) |       |                         |                         |
| Most favored | 95.5 | 93.5 | 92.1 |
| Allowed     | 4.5  | 6.2  | 7.4  |
| Disallowed  | 0.0  | 0.3  | 0.5  |

* Rmerge = \sum_i \sum_{ij} \sum_{hkl} |I_{hkl}(i) - \langle I_{hkl}(i) \rangle| / \sum_i \sum_{ij} \sum_{hkl} |I_{hkl}(i)|.
Characterization and Structures of CtCel5E

FIGURE 3. Apo-form structure of CtCel5E and structural comparison with CelCCA and TmCel5A. A, dimeric structure of the apo-form CtCel5E shown in cyan. B, the apo-CtCel5E structure is depicted as a ribbon diagram. The α helices and β strands of the TIM barrel are colored in cyan and purple, respectively. The additional short α helix, two β short strands, and a 3_10 helix are colored in blue, orange and green, respectively. The side chains of catalytic Glu-209 and Glu-314 are depicted as sticks. The two missing regions are shown as green dots. C, superimposition of the active-site residues in the CtCel5E structure (yellow) onto those in the structures of TmCel5A (purple) and CelCCA (green). D, CtCel5E structure (colored as in B) superimposed onto the TmCel5A structure (PDB entry 3AMC; purple) focusing on the region of two short β strands. E, CtCel5E structure (colored as in B) superimposed onto the C. cellulosolyticum CelCCA structure (PDB entry 1EDG; green), focusing on the region of two short β strands.

...equivalent to Trp-210 of TmCel5A in CtCel5E, explaining the lack of mannanase activity for CtCel5E. In contrast, xylose, a 5-carbon sugar, does not have C6-OH. As such, the xylanase activity of CtCel5E is not affected by these missing interactions. Moreover, His-168 and Asn-208 of CtCel5E only form hydrogen bonds with the equatorial C2-OH of (−1)-xylose, further weakening the binding of mannanose that has an axial C2-OH.

However, the loss of the interactions with mannnose C1-, C2-, and C6-OH in CtCel5E, as mentioned above, apparently only abolishes its mannanase activity and not its cellulase activity, perhaps because there are more interactions for cellobiose binding. Structural comparison (Fig. 5C) suggests that His-95, His-96, Asn-135, and Glu-136 of TmCel5A form hydrogen bonds with the C1-C3-OHs of the (−1)-glucose, and the corresponding residues His-168, His-169, Asn-208, and Glu-209 of CtCel5E make similar interactions. However, His-169 of Cel5E provides hydrogen bonds with C3-OH of (−1)-xylose, C3-OH of (−1)-glucose, and C6-OH of (−2)-glucose, whereas the corresponding His-96 in TmCel5A only forms hydrogen bonds with C3-OH of either (−1)-glucose or (−1)-mannose. Therefore, CtCel5E has xylan binding capability without mannan binding affinity while retaining its cellulose binding activity.

Particularly, unlike Tyr-198 of TmCel5A, which forms a hydrogen-bonding interaction with (−1)-glucose, the corresponding Tyr-270 of CtCel5E is 14.4 Å away (Fig. 5D). Based on its position, Tyr-270 might be responsible for binding with the (+2)-sugar moiety of substrate or can be mobile during catalysis because it is located near the flexible loop.

Roles of Active-site Residues Probed by Site-directed Mutagenesis—To verify the roles of these structure-predicted important residues for either activity, we performed site-directed mutagenesis studies, and the data are summarized in Table 2. His-168 forms two hydrogen bonds with C2- and C3-OH of the (−1)-glucose and one hydrogen bond with the C2-OH of (−1)-xylose. The H166A mutant showed 2-fold increased $K_m$ and 11-fold decreased $k_{cat}$ for cellulase activity but abolished xylanase activity. Similarly, His-169 also forms hydrogen bonds with both ligands, and thus its Ala mutant also displayed a $K_m$ increased by 5-fold and a $k_{cat}$ of cellulase activity decreased by 3-fold but a $k_{cat}$ of BW significantly decreased by 24-fold. Moreover, based on the structures (10), the corresponding residue of His-277 in TmCel5A is only important for mannanase activity. Accordingly, the H277A CtCel5E mutant retained most of its cellulase and xylanase activities.

In contrast, N93A, N208A, Y270A, Y270F, and W347A were inactive, suggesting that these residues are catalytically required. From the structures, Asn-93 forms a hydrogen bond with the (−2)-sugar of both ligands. Asn-208 is responsible for binding with C2-OH of the (−1)-sugar of both ligands, and Trp-347 may provide hydrophobic and hydrogen bond interactions. Tyr-270 is essential, although it is not in direct contact with the bound ligands. Additionally, Y270F was inactive, suggesting the essential role of OH in the side chain. Asn-349 also forms hydrogen bonds with the C2-OH of (−2)-xylose and (−2)-glucose.

Improvement of Activities by F267A—As shown above, Tyr-270 is an essential residue and may need to undergo relocation for catalysis. Phe-267 close to Tyr-270 has hydrophobic interactions with Val-299 and Trp-302 in the α6 helix, which may prevent the flexible loop from relocation. By replacing Phe-267 with Ala, the F267A mutant CtCel5E actually displayed 4-fold higher $k_{cat}$ for cellulase activity and 2-fold higher $k_{cat}$ for xyla-
nase activity compared with CtCel5E (Table 2), and it produced the same products as the wild-type form (data not shown). The pH and temperature profiles indicate that the mutant enzyme has higher activities than the wild-type enzyme, and the optimal pH for cellulase activity is down-shifted to 4 (Fig. 5E). This finding supports our hypothesis that the flexible loop needs to undergo relocation for catalysis. To further confirm the role of the hydrophobic interactions between Val-299, Trp-302, and Phe-267 in activity, we mutated Phe-267 to either positively charged Lys or negatively charged Glu or replaced Val-299 or Trp-302 with Ala. The mutant enzymes were expressed and purified in a manner similar to that for the wild type. As expected, F267E and F267K mutant enzymes showed significantly impaired activities, supporting our hypothesis. However, V299A and W302A retained or slightly increased their cellulase activity but had lower xylanase activity (Table 2), indicating that Val-299 and Trp-302 may play some role in xylanase activity. Accordingly, the V299A/W302A double mutant showed abolished xylanase activity.

DISCUSSION

C. thermocellum, one of the most efficient cellulolytic microbes, produces an extracellular cellulose that contains an endoglucanase/xylanase bifunctional enzyme. Our recomb-
nant CtCel5E is active for degrading CMC, PASC, BW, birch-wood, and arabinoxylan. The major products are cellobiose and cellotriose from cello-polysaccharides and xylobiose and xylotriose from xylo-polysaccharide, indicating that CtCel5E is a bifunctional /H9252-1,4-endoglucanase and /H9252-1,4-xylanase.

Although the CtCel5E we crystallized contains extra N- and C-terminal linkers, only the catalytic domain was ordered in the crystal structure. The catalytic domain adopted a canonical (α/β)8-TIM barrel architecture, which is also observed in other GH5 protein structures. Based on the previous studies of GH5 enzymes (8), Glu-209 and Glu-314 are likely to be the proton donor and nucleophile for CtCel5E activities. Accordingly, we demonstrated the loss of both activities by their replacement with Ala. Furthermore, the distance of 5 Å between Glu-209 and Glu-314 is comparable with the average distance of 5.5 Å in GH5 enzymes (24).

Although both CtCel5E and TmCel5A bind cellulose, their molecular recognition mechanisms are different. CtCel5E adopts a similar manner of binding xylobiose to binding cellobiose, mainly via His-168, His-169, Asn-208, Trp-347, and Asn-349. The site-directed mutagenesis studies (Table 2) showed that His-168 is essential for xylanase activity but that His-169 is more important for xylanase activity. Conversely, Asn-93, Asn-208, Trp-347, and Asn-349 are required for both activities.

Some Cel5 endoglucanases of GH5 are also active on mannan and galactomannan. These enzymes do not need the vital

**FIGURE 5.** The sequence and structural comparison between CtCel5E and TmCel5A. A, sequence alignment between CtCel5E constructed in this study and TmCel5A. The secondary structures that are shown at the top of sequences are for CtCel5E. B, superimposition of the mannotriose-bound TmCel5A_E253A active-site structure (PDB entry 3AZS; colored gray) with that of xylose-bound CtCel5E_E314A (yellow). Dashed lines, hydrogen-bonding interactions between the residues and the ligands. C, superimposition of the cellobiose (CBI)- and glucose (GLC)-TmCel5A_E253A active-site structure (PDB entry 3AZR; orange) with the cellobiose-CtCel5E structure (green). D, the superimposition of apo-CtCel5E (cyan) and cellobiose-TmCel5A (light orange) revealed a significant disposition of Tyr-270 in CtCel5E. The black dashed line indicates the hydrogen-bonding interactions between the residues and the substrate. 14.4 Å is the distance between Tyr-270 of CtCel5E and the equivalent residue Tyr-198 of TmCel5A that forms the hydrogen bond with (−1)-pyranose. E, pH and temperature profiles of F267A mutant CtCel5E, demonstrating its higher activities than the wild type (Fig. 2A), which is consistent with our hypothesis. Error bars, S.D.
hydrogen bonds to C2-OH of the bound cellodextrins (26–28) or can better tolerate an equatorial or axial 2-OH because of the relatively flexible structures (6, 29). In contrast, the Asn-208 side chain of CtCel5E cannot form a hydrogen bond with the axial 2-OH. Perhaps more importantly, the residues corresponding to Tyr-198, His-205, and Trp-210, which interact with (−1)-mannose in TnCel5A, are distant (Tyr-270), flexible (His-277), and absent, respectively. As a result, CtCel5E does not have mannanase activity. Whereas His-277 is less important for catalysis, as is also supported by the improvement of activity against (−1)-mannose in Stepwise fashion, the relatively flexible structures (6, 29). In contrast, the Asn-208 side chain of CtCel5E cannot form a hydrogen bond with the axial 2-OH. Perhaps more importantly, the residues corresponding to Tyr-198, His-205, and Trp-210, which interact with (−1)-mannose in TnCel5A, are distant (Tyr-270), flexible (His-277), and absent, respectively. As a result, CtCel5E does not have mannanase activity. Whereas His-277 is less important for catalysis, as is also supported by the improvement of activity against (−1)-mannose in TnCel5A, etc.

In summary, our structure and function studies not only reveal the substrate recognition mechanisms of CtCel5E but also provide a blueprint to enhance CtCel5E function. These multifunctional enzymes may be useful for biomass degradation and biofuel production.

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