Insights into the roles of non-catalytic residues in the active site of a GH10 xylanase with activity on cellulose

Yindi Chu1,2, Tao Tu3, Leena Penttinen4, Xianli Xue5, Xiaoyu Wang4, Zhuolin Yi3, Li Gong3,4, Juha Rouvinen5, Huiying Luo1, Nina Hakulinen2, Bin Yao3,4, and Xiaoyun Su1

From the 1Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China, the 3Department of Chemistry, University of Eastern Finland, Joensuu Campus, Joensuu FIN-80101, Finland, the 4Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, China, and the 5Key Laboratory of Molecular Animal Nutrition and Feed Sciences, College of Animal Science, Zhejiang University, Hangzhou 310058, China

Edited by Gerald W. Hart

Bifunctional glycoside hydrolases have potential for cost-savings in enzymatic decomposition of plant cell wall polysaccharides for biofuels and bio-based chemicals. The N-terminal GH10 domain of a bifunctional multimodular enzyme CbXyn10C/Cel48B from Caldicellulosiruptor bescii is an enzyme able to degrade xylan and cellulose simultaneously. However, the molecular mechanism underlying its substrate promiscuity has not been elucidated. Herein, we discovered that the binding cleft of CbXyn10C would have at least six sugar-binding subsites by using isothermal titration calorimetry analysis of the inactive E140Q/E248Q mutant with xylo- and cello-oligosaccharides. This was confirmed by determining the catalytic efficiency of the wild-type enzyme on these oligosaccharides. This work was supported by National Natural Science Foundation of China Grant 31672458, National Key Research and Development Program of China Grant 2016YFD0501409-02, China Modern Agriculture Research System Grant CARS-42, and the Elite Youth Program of Chinese Academy of Agricultural Sciences. The work at University of Eastern Finland was conducted with financial support from the Academy of Finland (grant decisions 287241 and 292705). The authors declare that they have no conflicts of interest with the contents of this article.

This work was supported by National Natural Science Foundation of China Grant 31672458, National Key Research and Development Program of China Grant 2016YFD0501409-02, China Modern Agriculture Research System Grant CARS-42, and the Elite Youth Program of Chinese Academy of Agricultural Sciences. The work at University of Eastern Finland was conducted with financial support from the Academy of Finland (grant decisions 287241 and 292705). The authors declare that they have no conflicts of interest with the contents of this article.

This article contains supplemental Figs. S1 and S2 and Tables S1 and S2. The atomic coordinates and structure factors (codes SOFJ, SOPK, and SOFL) have been deposited in the Protein Data Bank (http://wwwpdb.org/).

1 Both authors contributed equally to this work.
2 To whom correspondence may be addressed: Dept. of Chemistry, University of Eastern Finland, P. O. Box 111, 70211, Yliopistokatu 7, Joensuu FIN-80101, Finland. E-mail: nina.hakulinen@uef.fi.
3 To whom correspondence may be addressed: Feed Research Institute, No. 12 S. Zhongguancun St., Beijing 100081, China. Tel.: 86-10-82106065; E-mail: binyao@caas.cn.
4 To whom correspondence may be addressed: Feed Research Institute, No. 12 S. Zhongguancun St., Beijing 100081, China. Tel.: 86-10-82106094; E-mail: suxiaoyun@caas.cn.

and –1 near the cleavage site, whereas residues playing moderate roles could be located at more distal regions of the binding cleft. Manipulating the residues interacting with substrates in the distal regions directly or indirectly improved the activity of CbXyn10C on xylan and cellulose. Most of the key residues for cellulase activity are conserved across GH110 xylanases. Revisiting randomly selected GH10 enzymes revealed unreported cellulase activity, indicating that the dual function may be a more common phenomenon than has been expected.

Cellulose and xylan are glucose- and xylose-configured biopolymers, respectively, and are the two major constituents of many plant cell wall polysaccharides. Glucose can be readily fermented into ethanol, and the five-carbon xylose is now also a feedstock of biofuels and bio-based chemicals, thanks to the vast progress achieved in recent years in engineered yeasts, Escherichia coli, and other alternative microbes (1, 2). Therefore, depolymerization of cellulose and xylan, more favorably in a manner of enzymatic catalysis, is required for the current wave of biofuel and biorefinery industries.

Economically viable biocatalysis of cellulose and xylan is dependent on efficient enzymes produced at low cost. Among the many endeavors to reduce the cost of the hydrolysis of plant cell wall polysaccharides by cellulases and xylanases, one is to discover or engineer an enzyme(s) with dual or even multiple functions on more than one substrate. For example, a new endoxylanase activity was engineered into a GH43 exo-acting arabinofuranosidase by site-directed mutagenesis (3), and a bifunctional cellulase/xylanase was obtained by fusion expression of the Thermotoga maritima Cel5C and XynA (4). Because cellulose and xylan collectively represent the major plant cell wall polysaccharides for many energy crops and agricultural crop residues, it is of practical interest to screen for or to engineer efficient enzymes with dual activities on cellulose and xylan. Interestingly, although members in many cellulase families have been discovered to have activity on xylan (5), only few xylanases are observed to have a cellulase activity.

The Gram-positive anaerobic bacterium Caldicellulosiruptor bescii (formerly named as Anaerocellum thermophilum (6)) is a thermophilic extremophile with excellent ability in the deg-
Insights into a bifunctional GH10 xylanase/cellulase

radation of plant cell wall polysaccharides (7) and even lignin (8). Six multimodular, bicatalytic domain-bearing glycoside hydrolases are encoded by a plant cell wall polysaccharide utilization gene cluster (9). Many of these enzymes in this gene cluster have been biochemically characterized and proven to be efficient in hydrolyzing cellulose, xylan, and mannann, which constitutes the biochemical basis for the high efficiency in plant biomass utilization by this bacterium (10–15). We have shown previously that CbXyn10C, the N-terminal GH10 catalytic domain of the multimodal bifunctional CbXyn10C/Cel48B, is one of the few GH10 xylanases to have cellulase activity (13).

Andrews et al. (16) analyzed the structural and biochemical basis of GH10 xylanases with perceivable activity on cellulose. Later, structure-based engineering of such an enzyme from Streptomyces olivaceoviridis was carried out to improve the cellulase activity (17). Despite these achievements, it is noticed that the previous studies mainly focused on the residues interacting with the sugar subsites close to where the cleavage takes place (namely −2, −1, and +1). The full scenario of the binding cleft of such a type of bifunctional enzyme is not yet available in the literature. With activities against both xylan and cellulose (and also barley β-glucan), CbXyn10C serves as a good candidate in industrial applications and therefore represents an excellent model for investigating how this type of enzyme can interact with both xylan and cellulose.

To understand the molecular mechanism underlying the catalytic promiscuity of CbXyn10C with xylan and cellulose, we first carried out biochemical and crystallographic analyses of this enzyme. Then the systematic mutational studies for the predicted residues interacting with the xylan or cellulose were performed. Molecular dynamic simulation was also used to observe behavior of a representative mutant with improved activity on cellulose. Our research provides structural and mutational insights into the roles of the amino acids in substrate catalysis for this GH10 xylanase with dual activities on xylan and cellulose.

Results

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC)5 can be used to characterize the number of substrate-binding subsites (18). As reported earlier (13), Glu-140 and Glu-248 are the two catalytic residues, respectively. A catalytic-inactive mutant, i.e. E140Q/E248Q, was therefore constructed, expressed, purified, and used in the ITC experiments. Commercially available ligands, including five xylose-configured oligosaccharides (xylobiose, X2; xylotriose, X3; xylotetraose, X4; xylopentaose, X5; and xylohexaose, X6) and two glucose-configured oligosaccharides (cellopentaose, G5; and cellohexaose, G6) were used in the ITC experiments. The representative data for ITC-binding reactions were given in Fig. 1. The binding of E140Q/E248Q to both xylo-oligosaccharides and cello-oligosaccharides was basically driven by a large loss of enthalpy with slight acquisition of entropy (Table 1), similar to the Geobacillus stearothermophilus GH10 intracellular xylanase IXT6 but distinct to extracellular XT6 in terms of the entropy change (18). The binding constants (Ks) of E140Q/E248Q to xylotriose to xylohexaose were 500–700 × 103 M−1, ~10-fold higher than that for xylobiose but ~2-fold lower than that for xylotriose. This suggests that CbXyn10C has at least six sugar-binding subsites for xylene-configured substrates.

The binding constant of E140Q/E248Q to cellohexaose was about 18 × 103 M−1, 27-fold higher than that for xylohexaose but about 70-fold lower than for xylotriose. The ITC data together for both the xylo- and cello-oligosaccharide ligands, it was clear that the binding cleft of CbXyn10C harbors at least six sugar-binding subsites. It is noteworthy that the binding of cello-oligosaccharides was much weaker than that of xylo-oligosaccharides. This is consistent with the finding that CbXyn10C is a robust xylanase with much less cellulase activity (13).

Next, the catalytic efficiency of CbXyn10C with xylo- and cello-oligosaccharides was determined. This could not be determined for xylobiose, cellobiose, cellotriose, and celtetraose (Table 2). Among the other xylo-oligosaccharides, the catalytic efficiency was lowest for xylotriose (3.3 ± 0.4 mm−1 s−1). This value increased by 15-fold for xylotetraose (49 ± 3 mm−1 s−1), 25-fold for xylopentaose (81 ± 10 mm−1 s−1), and 99-fold (326 ± 20 mm−1 s−1) for xylotriose. The significant trend of ascending further confirmed the notion that there are at least six substrate-binding subsites for CbXyn10C. Low catalytic efficiency was observed for cellopentaose (0.009 mm−1 s−1), which increased by 4.4-fold for xylotriose (Table 2). Although both the binding constants (as determined in ITC) and catalytic efficiencies increased with higher degrees of polymerization for either configuration of the oligosaccharides, we noticed that the catalytic efficiencies from X3 to X6 steadily increased, whereas the binding constants for X3 to X5 appeared to be at the same magnitude. This may be due to the difference in the binding of shorter oligomers to subsites. Only the binding of oligosaccharides to the center of the active site leads to catalysis.

Structure of the free form of CbXyn10C

The crystal structure of the CbXyn10C WT (supplemental Fig. S1) was determined at the resolution of 1.34 Å. The data collection and refinement statistics are listed in Table 3. One polypeptide chain was found in the asymmetric unit. Analysis of the electron density maps indicated that polypeptide chain was ordered from Pro-1 to Ser-336. The continuous electron density was observed even before Pro-1 suggesting that amino acids from expression vector at N terminus are well-ordered. The overall structure of CbXyn10C displayed a typical β/αs-TIM barrel fold and “salad-bowl” shape of GH10 family enzymes. The TIM barrel consisted of a parallel eight-stranded β-sheet and seven α-helices and was surrounded by two short β-strands. In addition, one 310 helix at the N terminus was located at the bottom of the barrel. The overall structure of CbXyn10C was similar to those of other GH10 family enzymes.
such as the *G. stearothermophilus* xylanase IXT6 (PDB code 2Q8X; 44% identity) (19). The root mean square deviation (r.m.s.d.) between IXT6 and CbXyn10C is 0.72 Å over 256 Cα atoms. The catalytic residues Glu-140 and Glu-248 as well as Glu-46, Lys-50, His-87, Trp-91, Asn-139, His-218, Trp-297, and Trp-305 are strictly conserved and positioned similarly in the active site compared with those in other GH10 family enzymes.

In addition, 1.5-Å resolution structure of the inactive mutant in its free form was solved (data not shown). The conformations of CbXyn10C and the inactive mutant E140Q/E248Q were almost identical because superposition of the two molecules gave an r.m.s.d. of 0.17 Å.

Table 1

| Ligand      | *K*′ | *K*′ × 10^3 | Δ*H*′ | ΔS′ | ΔG′ |
|-------------|------|-------------|-------|-----|-----|
| Xylobiose   | 1.13 | 60 ± 12     | -4.50 | 0.30 | 0.17 | 4.67 |
| Xylotriose  | 0.98 | 584 ± 100   | -5.52 | 0.19 | -5.71 |
| Xylotetraose| 1.06 | 524 ± 114   | -5.71 | 0.24 | -5.21 |
| Xylopentaose| 1.06 | 701 ± 98    | -3.44 | 0.21 | -5.65 |
| Xylohexaose | 0.80 | 1220 ± 111  | -7.31 | 0.08 | -5.39 |
| Cellopentaose| 1.00 | 0.65 ± 0.07 | -1.93 | 0.11 | 2.09 |
| Cellohexaose| 1.00 | 17.5 ± 1.4  | -3.04 | 0.23 | -3.27 |

Values are represented as means ± S.D. calculated from three independent experiments.

Table 2

| Substrate       | Catalytic efficiency (kcat/Km, mM s^-1) |
|-----------------|----------------------------------------|
| Xylobiose       | ND*                                   |
| Xylotriose      | 3.3 ± 0.4                             |
| Xylotetraose    | 49 ± 3                                |
| Xylopentaose    | 81 ± 10                               |
| Xylohexaose     | 326 ± 20                              |
| Cellobiose      | ND*                                   |
| Cellotriose     | ND*                                   |
| Cellotetraose   | ND*                                   |
| Cellopentaose   | 0.009                                 |
| Cellohexaose    | 0.04                                  |

*ND means too low to be determined.

Although xylohexaose was used in crystallography analysis with E140Q/E248Q, the continuous electron density for seven xylose residues was found from subsites −3 to +4 (Fig. 2A). This could be caused by impurity from xylohexaose. However, we did not find supporting evidence from mass spectroscopy analysis (data not shown). Therefore, the observed seven xylose units were more likely from a superimposed structure of two binding patterns of CbXyn10C E140Q/E248Q having xylohexaose occupying −3 to +3 and −2 to +4, respectively. For
simplicity, xyloheptaose will be used in the subsequent description. The crystal structure of E140Q/E248Q-xyloheptaose (Fig. 2A) was determined at 1.16-Å resolution. The data processing and refinement statistics are also summarized in Table 3. The structure of E140Q/E248Q complexed with xyloheptaose closely resembles (71% amino acid identity) that of XT6 from G. stearothermophilus complexed with xylohexaose. The coordinates (PDB 4PRW) have been submitted to the Protein Data Bank, but there is no publication date available.

With this complex structure, the interaction of CbXyn10C with the xylose-configured substrate could be clearly demonstrated. Xyloheptaose makes numerous interactions with amino acid residues in the substrate-binding cleft. The xylosidic oxygen atom connecting the xylose subunits at positions $-1$ and $+1$ is hydrogen-bonded to the Gln-140 NH$_2$ group with an N$\cdots$O distance of 2.9 Å. The corresponding Glu-140 could thus reach to protonate xylosidic oxygen atom. Gln-248 is close (3.2 Å) to the C1 carbon of the xylose residue at subsite $-1$. In a wild-type enzyme, a corresponding nucleophilic Glu would be poised to directly attack C1 to produce the covalent intermediate. This xylose ring at the reaction site has a distorted boat conformation $B_3$, O(20). Hydrogen bonds between the O2 and the side chain of Asn-139, O3 and the side chains of Lys-50 and His-87, and O5 and the side chain of His-218 further enhanced the binding of this xylose residue. Also, the xylose residue at subsite $-2$ forms hydrogen bonds with Asn-47, Lys-50, and Trp-297. All remaining xylose subunits ($-3$, $+1$, $+2$, and $+4$), but not $+3$ (hydrogen bonding with Asn-186), do not form

| Table 3: X-ray data collection and refinement statistics |
|----------------------------------------------------------|
| **Data collection**                                       |
| Beamline        | WT       | E140Q/E248Q_xyloheptaose | E140Q/E248Q_cellohexaose |
| Space group     | $I422$   | $I422$                  | $I422$                   |
| Unit cell; $a$, $b$, $c$ (Å)                             | 183.84,183.84,56.13 | 183.61,183.61,56.24      | 185.83,185.83,56.32      |
| Resolution (Å)                                          | 92–1.34 | 54–1.16                 | 65–1.87                  |
| Unique reflections                                      | 108,971 (7803)    | 162,835 (7361)          | 40,841 (2968)            |
| Redundancy                                               | 12.7 (10.7)       | 7.7 (4.5)               | 7.1 (7.4)                |
| $R_{merge}$ (%)                                          | 11.7 (205.1)      | 14.6 (114.3)            | 11.7 (122.8)             |
| $I/\sigma(I)$                                           | 12.7 (1.7)        | 6.2 (1.0)               | 11.5 (1.6)               |
| Completeness (%)                                         | 0.99 (0.58)       | 0.97 (0.55)             | 1.0 (0.68)               |
| **Refinement**                                           |
| Resolution (Å)                                          | 1.34               | 1.16                     | 1.87                     |
| $R_{work}/R_{free}$ (%)                                  | 16.3/18.3          | 17.2/18.6                | 15.3/18.5                |
| No. of non-H atoms                                       | 2976               | 3005                     | 2840                     |
| Protein                                                 | 1421               | 1121                     | 1116                     |
| Ligand                                                  | 350                | 569                      | 332                      |
| Solvent                                                 | 16                 | 14                       | 28                       |
| Mean $B$ factors (Å$^2$)                                 | 14                 | 18                       | 65                       |
| r.m.s.d. from ideal geometry                             | 31                 | 32                       | 40                       |
| Bond lengths (Å)                                         | 0.008              | 0.009                    | 0.011                    |
| Bond angles (°)                                          | 1.007              | 1.088                    | 1.53                     |
| Ramachandran plot (%)                                    | 98                 | 98                       | 97                       |
| Favored                                                 | 0                  | 0                        | 0                        |

Figure 2. Crystal structures of the double mutant E140Q/E248Q complexed with xyloheptaose (A) and cellohexaose (B). The $F_o - F_c$ electron density maps for the omitted substrates are represented in blue at 3σ contour level.
direct hydrogen bonds with the residues in the substrate-binding cleft. It is common for GH10 xylanases that most hydrogen-bonding interactions are located at subsites −2 and −1. There is no significant difference for the number of hydrogen bondings with xylan between CbXyn10C and other xylanases (supplemental Table S1). However, we can note that the xylose ring packs against hydrophobic flat face of aromatic residues at subsite +1 (with Tyr-185), at +3 (with Tyr-256), and at +4 (with Trp-223). The xylose subunits at subsites +1, +2, and +3 have the lowest average B factor, indicating a tighter binding of the xylose subunits to the inner subsites than to the peripheral subsites. Our observation of strong electron density for these xylose rings indicated that their conformational freedom is clearly reduced by intermolecular interactions with the enzyme.

Complex structure of E140Q/E248Q with cello-oligosaccharides

E140Q/E248Q exhibited weak binding to cello-oligosaccharides in ITC analyses. Using cellohexaose as the longest commercially available cello-oligosaccharide with the strongest binding (Table 1), we were able to obtain the crystal of E140Q/E248Q complexed with cellohexaose. The omit electron density map showed fairly substantial density at subsites +4, +3, +2, +1, −1, and −2 (Fig. 2B). The electron density is quite continuous, but there is a break between subsites +1 and +2. In addition, electron density is clearly partial at subsites −1 and −2. Generally, the electron density for the cello-oligomer ligand does not correspond to the single molecular structure, which suggests that the observed density is the result of different binding modes of the oligomer, characteristic of the promiscuous activity toward cello-oligomers for this enzyme. However, we were able to refine cellohexaose in the active site occupying approximately the same space as xylohexaose. The glucose residue at subsite −1 has a similar boat-like conformation to the xylose residue at the same site. The distance between the C1 atom and Gln-248 is 3.4 Å suggesting that Glu-248 would be able to act as a nucleophile also toward the glucose residue bound at this position. Also, Glu-140 could act as an acid/base catalyst because the distance between the corresponding Glu-140 and glycosidic oxygen would be short (2.9 Å). Glucose ring has an additional C6–O6 group compared with xylose. In the complex model, it is packed against a flat ring of Trp-305, whose position is slightly moved. In addition, the positions of Trp-91 and the following loop have changed as well as the conformation of the side chain of Tyr-256, which all indicate plasticity in the active site (Fig. 3). The overall changes between the complex structures of cellohexaose and xylohexaose are small, suggesting that this enzyme is quite able to bind to cello-oligosaccharides and catalyze their hydrolysis.

Molecular dynamics simulation of cellohexaose binding to CbXyn10C

Although the crystal structure of E140Q/E248Q complexed with cellohexaose was obtained, the electron density map is not complete for all the binding subsites. Analysis of CbXyn10C with cellohexaose through molecular dynamics simulation could provide more detailed information of how the enzyme interacts with cellulose. Herein, we docked cellohexaose to the CbXyn10C catalytic site and carried out molecular dynamics simulations employing the program Amber. After docking and subsequent equilibration, stable positions for the ligands were established (Fig. 3). In general, both xylo- and cellohexaosecarriers were found to be slightly twisted into a conformation that could favor hydrolysis. In the docked complex structure, His-87, Trp-91, Tyr-185, Ile-189, Trp-223, and Trp-305 could interact with cellohexaose via hydrophobic stacking. Residues such as Glu-46, Asn-47, Lys-50, Gln-94, Asn-139, Asn-186, Gln-216, His-218, Asn-220, Tyr-256, and Trp-297 could be involved in hydrogen bonding with cellohexaose. These residues created a narrow, deep cleft at the subsite side.

Contributions of amino acid residues in the binding cleft to catalysis of xylan and cellulose

The schematic diagrams showing the interaction of CbXyn10C with xylan and cellulose were shown in Fig. 4, A and B. To study the role of active-site residues in the binding of xylo- and cello-oligomers, we mutated a number of residues in the active site to alanine, and the mutants were determined for their relative activities against beechwood xylan and CMC, two model substrates for xylan and cellulose, respectively (Fig. 5, A and B). In this alanine screening, residues Lys-50, His-87, Asn-139, His-218, and Trp-297, involved in the hydrogen bonding with xylose at subsite −2 and −1, and Gln-216 interacting with xylose at subsite +1 by hydrophobic stacking were found to be critical for xylan hydrolysis because the alanine mutants of these residues retain only 9–20% of activity (Fig. 5A). These interactions are highly conserved across the GH10 xylanases (Fig. 6), and substitution of any of these residues with alanine greatly reduces the activity of the enzymes against xylo-oligosaccharides (21). The results collectively indicated that the interaction for xyloses at −2 and −1 subsites were extremely important for xylan catalysis.

Five residues (Glu-46, Asn-47, Trp-91, Tyr-185, and Trp-305) were found to be moderately important for xylan hydroly-
Figure 4. Schematic representation of the hydrogen bonding and hydrophobic stacking interactions in the binding cleft of CbXyn10C with xyloheptaose (A) and celloheptaose (B), respectively. The interaction of CbXyn10C with xyloheptaose is derived from crystallographic analysis, whereas that for celloheptaose is from molecular docking and modeling. Note the two catalytic residues (Glu-140 and Glu-248) were both replaced by glutamine here. The unit of the distance is Å.
residues, Glu-46, Lys-50, Asn-139, Gln-216, His-218, and Trp-297 may be involved in the hydrogen bonding with glucose residues at subunit $-2$, $-1$, $+1$, and $+2$, respectively. His-87, Trp-91, Tyr-185, and Trp-305 (the residue creating steric hindrance for cellulose hydrolysis (16)) formed hydrophobic stacking with the $-1$ and $+1$ glucose, respectively. Mutation of these conserved residues not only reduced their xylanase activity significantly, but also made great reduction in cellulose activity. These implied that residues particularly in the subsites $-2$ to $+1$ are important for binding to both xylo-oligosaccharides and cello-oligosaccharides. For cellulose, as few as only three residues, i.e. Asn-47, Gln-94, and Trp-223, were moderately important for cellulose hydrolysis. The ND2 atom of Asn-47 and Gln-94 may form a hydrogen bond to glucose at subsite $-2$, whereas Trp-223 may form a hydrophobic stacking with glucose at subsite $+4$. Therefore, the interactions with moderate importance in both xylan and cellulose hydrolysis could be at the subsite distal from the cleavage site. In addition, the residues Asn-186, Ile-189, and Tyr-256 were found to be slightly important for hydrolysis of cellulose. Asn-186 and Tyr-256 may form hydrogen bonds with glucose residues at subsites $+2$ and $+3$. Residue Ile-189 may form hydrophobic stacking with the glucose at subsite $+3$.

**Insights into a bifunctional GH10 xylanase/cellulase**

For the bifunctional GH10 xylanases/cellulases such as CbXyn10C, recent engineering work has been focused on the subsites close to where the cleavage takes place but not the distal regions. Therefore, in this study, we attempted to investigate whether modulating the residues interacting with the substrates, particularly those in the regions distal to the cleavage site, could enhance the enzyme’s catalytic activity or change the substrate specificity. Indeed, both the xylan- and cellulose-degrading ability of CbXyn10C could be tuned up by modulating the residues directly or indirectly interacting with the ligand distal to the cleavage site. For xylan, two alanine mutants W223A and Y256A displayed improved specific activity (Fig. 5A and Table 4). These two mutants were responsible for sandwiching the $+3$ xylose. A double mutant of W223A/Y256A did not have the additive effect. Mutation of the residues at any other subsites into alanine did not have such an improving effect. For cellulose hydrolysis, no alanine mutants for the residues directly or indirectly interacting with the cellulose ligand were found to have the additive effect. Mutation of the residues at any other subsites into alanine did not have such an improving effect. For cellulose hydrolysis, no alanine mutants for the residues directly or indirectly interacting with the cellulose ligand were found to have the additive effect. For cellulose hydrolysis, no alanine mutants for the residues directly or indirectly interacting with the cellulose ligand were found to have the additive effect. Modifying the residues indirectly interacting with the substrates has been proven to be effective in changing substrate specificity of an enzyme (22). We therefore found that mutation of Glu-52, whose OE2 atom formed a hydrogen bond with NE2 atom of Gln-94 (supplemental Fig. S2), to alanine improved the activity to 1.3-fold (Table 4). Moreover, additional mutants of Glu-52 to other residues, including E52G, E52Q, E52R, E52T, E52P, E52W, and E52V, also had increased activity on cellulose to a similar level.

**Kinetic analysis of selected mutants**

The catalytically more active mutants for xylan, which were W223A and Y256A (Fig. 5A), were selected for further kinetic analysis by modulating the residues at the subsites distal to the cleavage site

![Figure 5. Contributions of amino acid residues in the binding cleft to catalysis of xylan and cellulose](image-url)
analysis. For WT and all mutants, xylan and CMC were used as the representative substrates of xylan and cellulose, respectively. All mutants (W223A, Y256A, and W223A/Y256A) displaying improved specific activity had increased $K_m$ values for xylan, indicating that these mutants were impaired in xylan binding. However, all of them had improved turnover numbers ($k_{cat}$). Because of the comparably larger increase of $K_m$, their catalytic efficiency all decreased. At the aglycone side of the substrate, Trp-223 and Tyr-256 sandwich the xylose at subsite +3. The $K_m$ value of mutant W223A or Y256A lacking this residue only interacting with cello-oligosaccharides is marked with the open square (Fig. S2). Their $k_{cat}$ was higher than the wild type, suggesting that this sandwich interaction plays a significant role in substrate binding. Damage of this interaction might become convenient for the enzyme to release its product. For cellulose, no obvious improvement was observed for these mutants in terms of $K_m$, $k_{cat}$, and catalytic efficiency.

We did saturation mutagenesis for Glu-52. It was discovered that most, if not all, mutants had increased activity on cellulose. Except for E52D, E52P, and E52W, the other five selected mutants of Glu-52 had a very similar $K_m$ value to the wild-type but significantly increased $k_{cat}$ values (Table 4). This led to increased catalytic efficiency to ~2-fold that of the wild type. Their kinetic parameters for xylan were very similar to those of WT. Unlike these mutants, the kinetic parameters did not change much for the E52D mutant even for cellulose. The E52D mutant had an aspartate replacing the glutamate. These two residues are very like each other in character, likely accounting for the observed similar kinetic parameter for E52D and WT. With two hydrogen bonds, Glu-52 interacted with Gln-94, whose side chain formed three hydrogen bonds with atom OD1 of Asn-47, atom NZ of Lys-50 and HO–C6 of subsite −2, respectively (supplemental Fig. S2A). By replacing Glu-52 with Ala, the E52A mutant formed only one hydrogen-bonding interaction with subsite −2 (supplemental Fig. S2B). A 50-ns molecular dynamics simulation was performed for WT and E52A with cellulose, and the distance between the NE2 atom of Gln-94 and HO–C6 of subsite −2 were plotted in supplemental Fig. S2C. The simulation result suggested that the E52A mutation did not result in significant impairment in binding of the enzyme to cellulose, but it appeared to bring more flexibility to Gln-94, which could form a sterical hindrance when CbXyn10C hydrolyzes cellulose, as reported by other researchers (16) and our group (13).

**Discussion**

In this study, the binding cleft of CbXyn10C was found to have at least six binding substrates. The obtained CbXyn10C–cellobiohexaose structure is the only crystallized complex structure of a GH10 xylanase with a long cello-oligosaccharide. In addition, the crystallography study in combination with molecular modeling indicated that the far distal regions (~3, +3, and +4) of the binding cleft are indeed involved in interacting with the ligands via hydrogen bonding or hydrophobic interaction.

The long binding cleft has impact on its β-glucanase activity. With this knowledge, we could envision that the binding of CbXyn10C with longer glucose-configured substrates than cellobiohexaose should be even stronger and therefore more favorable for substrate degradation. This hypothesis could in turn explain why the specific activity of CbXyn10C against longer glucose-configured substrates such as the model crystalline cellulose Avicel (3.4 μmol of released sugar/min/μmol of enzyme) (13) is at a level comparable with those of “true” cellulases such as a GH9 endo-glucanase (4.5 μmol of released sugar/min/μmol of enzyme) (10) and one GH48 cellobiohydrolase (2.2 μmol of released sugar/min/μmol of enzyme) (11) from the same bacterium *C. bescii*. Therefore, although the GH10 enzymes with short binding clefts bearing as few as four substrates (such as one GH10 xylanase from *Cryptococcus albidos* (23)) can efficiently hydrolyze xylan, it appears that a long-bind-

**Table 4**

| Enzyme | CMC          | Xylan | Substrate selectivity |
|--------|--------------|-------|-----------------------|
|        | $K_m$        | $k_{cat}$ | $k_{cat}/K_m$ (xylan) | $k_{cat}/K_m$ (CMC) |
| WT     | 7.6 ± 0.8    | 9.4 ± 0.4 | 1.2 ± 0.1 | 443.0 ± 19.7 | 1278.1 ± 66.9 | 1065.1 |
| W223A  | 5.8 ± 1.1    | 8.1 ± 0.3 | 1.4 ± 0.2 | 364.9 ± 28.1 | 396.9 ± 20.4 | 283.5 |
| Y256A  | 6.6 ± 0.8    | 9.1 ± 0.4 | 1.4 ± 0.1 | 630.5 ± 100.1 | 577.8 ± 55.9 | 421.7 |
| W223A/Y256A | 5.4 ± 0.2 | 7.6 ± 0.1 | 1.4 ± 0.0 | 476.9 ± 5.6 | 303.2 ± 8.9 | 214.4 |
| E52A   | 7.8 ± 0.7    | 18.1 ± 1.2 | 23.4 ± 0.4 | 337.1 ± 13 | 1377.7 ± 140.9 | 599 |
| E52G   | 7.9 ± 0.2    | 17.8 ± 0.5 | 2.3 ± 0.1 | 439 ± 5.2 | 1284 ± 22.4 | 558.3 |
| E52D   | 6.9 ± 0.0    | 8.6 ± 0.2 | 1.2 ± 0.0 | 403.4 ± 23.7 | 1273.2 ± 129.1 | 1061 |
| E52P   | 12.1 ± 0.6   | 11.8 ± 0.7 | 1.0 ± 0 | 250 ± 30.6 | 1005.7 ± 177.5 | 1005.7 |
| E52Q   | 8.3 ± 0.8    | 19.3 ± 1.1 | 23.4 ± 0.4 | 421.2 ± 27.2 | 1355.4 ± 303.2 | 580.6 |
| E52R   | 8.5 ± 0.3    | 19.0 ± 0.3 | 2.2 ± 0.0 | 453.5 ± 13.6 | 984.1 ± 65.5 | 447.3 |
| E52T   | 7.3 ± 0.5    | 17.4 ± 1.4 | 24.4 ± 0.4 | 492.9 ± 26.6 | 1000.4 ± 112.1 | 416.8 |
| E52V   | 7.2 ± 0.3    | 17.4 ± 1.1 | 24.4 ± 0.3 | 391.7 ± 18.8 | 1335.9 ± 40.6 | 556.6 |
| E52W   | 11.0 ± 0.2   | 11.0 ± 0.2 | 1.0 ± 0 | 334 ± 9.7 | 1252.5 ± 104.2 | 1252.5 |

**Figure 6.** Multiple amino acid sequence alignment of CbXyn10C with selected GH10 xylanases. The GenBank accession numbers for the enzymes are as follows: CbXyn10C, ACM60945; Sxy110A, WP_003978188.1; DmXyn10, ACOM1799; Cjx6, AAS56792; Pxy110A, WP_012488068; CbXyn10A, ACM59335; CbXyn10B, ACM9337; Xyn10C, AHE13927; and Xyn19A, ACX30652. Although CbXyn10C/Sxy110A, CbXyn10B, Cjx6, Pxy110A, and DmXyn10 are part of the GH10 xylanases with cellulase activity, CbXyn10A, Xyn10C, and Xyn19A are enzymes previously reported without any cellulase activity. The residues interacting both with xylo-oligosaccharides and cello-oligosaccharides are marked with triangles (solid for conserved residues, and open for non-conserved residues), and the residue only interacting with cello-oligosaccharides is marked with the open square.
ing cleft is necessary for efficient hydrolysis of cellulose by CbXyn10C. Moreover, the activity of CbXyn10C on barley β-glucan (3400 μmol of released sugar/min/μmol of enzyme (13)) is only ~10-fold lower than that on beechwood xylan (39,000 μmol of released sugar/min/μmol of enzyme (13)), in sharp contrast with the low β-glucanase activity exhibited by Cellulomonas fimi Cex (1.1 × 10⁻³ of that on xylan), another GH10 bifunctional xylanase/cellulase that has been detailed for its binding pocket (21). One reason possibly accounting for the difference may be that CbXyn10C has seven binding subsites, whereas Cex has a short binding cleft with only five subsites (21), and the distal regions of Cex are not able to accommodate glucose efficiently (16).

At the first sight, there appears to be a paradox: less cellulase activity corresponds to more hydrogen bondings for CbXyn10C interacting with cellulose than with xylan (Fig. 4). Note that the binding of CbXyn10C with cellohexaose is weaker than with xylohexaose. Therefore, a well-ordered electron density map cannot be visualized for CbXyn10C-cellohexaose, particularly at subsites −2, −1, +1, and +2, where most hydrogen bonding interactions take place. As in the crystallography analysis, multiple binding states probably exist in molecular modeling for the CbXyn10C–cello-oligosaccharides complex, and the predicted hydrogen bondings are derived from a collection of these states rather than from a single state.

Eight of 10 residues in CbXyn10C revealed to be most important for cellulose degradation in this study are well-conserved across the GH10 xylanases (Fig. 6). Glu-46 and Tyr-185 are also conserved, but with a few exceptions are replaced by Gly and Thr (for Glu-46) and isoleucine (for Tyr-185). Glu-46 and Tyr-185 are widely conserved, but with a few exceptions are replaced by Gly and Thr (for Glu-46) and isoleucine (for Tyr-185). Therefore, we randomly selected three GH10 xylanases previously reported without cellulase activity, which were the CbXyn10A from C. bescii, XynA19 from Sphingobacterium sp. TN19, and XynC01 from Achaetomium sp. Xz-8 (14, 24, 25). These enzymes were recombinantly produced and purified. We found that CbXyn10A and XynC01, but not XynA19, have perceivable activities on cellulose (8.9 and 3.9 μmol of released sugar/min/μmol of enzyme for CbXyn10A and XynC01, respectively). The cellulase activity is low, implying that the binding cleft is not optimized for cellulose for these randomly selected enzymes. Nevertheless, this still indicates the cellulase activity may not be limited to only a few enzymes, but it could be more common in members of the GH10 family than as has been expected before. This discovery will undoubtedly expand the searching area of bifunctional cellulase/xylanase, which was previously limited to the cellulase families only.

CelA from the same bacterium has an N-terminal GH9 and a C-terminal GH48 catalytic domain separated by three consecutive family 3 carbohydrate-binding modules and is highly efficient in hydrolyzing crystalline cellulose via a “digging” mechanism (15, 26). CbXyn10C is the N terminus of a multimodular enzyme CbXyn10C/Cel48B, which is the third most abundant glycoside hydrolase secreted by C. bescii grown on crystalline cellulose (27). CbXyn10C/Cel48B is extremely similar to CelA at the levels of amino acid sequence and domain organization with the main difference in the N-terminal domain (GH10 for CbXyn10C/Cel48B and GH9 for CelA, respectively). The dual activities of CbXyn10C are hypothesized to serve important roles in plant cell wall polysaccharide degradation by CbXyn10C/Cel48B, and although xylanase activity is responsible for xylan destruction, the cellulase activity of CbXyn10C may cooperate with the C-terminal GH48 domain in hydrolyzing cellulose. We are interested in engineering CbXyn10C for enhanced activities, particularly on cellulose. Previous research from other groups (16, 17) or our group (13) has shown that mutating the non-catalytic residues in the active center at subsites −2, −1, and +1 might improve the activity or change the substrate specificity of GH10 enzymes with cellulase activity. In this study, for the first time we showed that by manipulating the non-catalytic interacting residues located at the distal regions of the binding cleft either the xylanase or cellulase activity can be improved. Particularly, mutation of Glu-52 into many residues resulted in nearly doubled activity on cellulose with no large change of xylanase activity. This trait could be used for engineering the full-length CbXyn10C/Cel48B for enhanced ability to degrade lignocellulosic biomass in the future.

**Experimental procedures**

*Expression and purification of the wild-type and mutant CbXyn10C proteins*

The expression plasmid vector for wild-type (WT) CbXyn10C, denoted pET46-CbXyn10C, was reported previously (13). The expression vectors encoding CbXyn10C mutant proteins, which contained a single or double mutation, including E46A, N47A, K50A, E52A, H87A, W91A, S93A, N139A, Y185A, N186A, Q216A, H218A, N220A, W223A, Y256A, W223A/Y256A, W297A, and W305A, were constructed by site-directed mutagenesis using the Fast Mutagenesis System (TransGen Biotech, Beijing, China) (see supplemental Table S2 for primer sequences). The integrity of all the expression plasmids was verified by DNA sequencing. These plasmids were individually transformed into the E. coli BL21 (DE3) (TransGen Biotech, Beijing, China) and the recombinant E. coli strains were used for expression. Overproduction and purification of the WT and mutant CbXyn10C proteins were carried out essentially the same as described previously (13).

The CbXyn10A gene from C. bescii was cloned into pET-28a(+), and the expression plasmid was transformed into BL21(DE3), CbXyn10A was expressed and purified. XynA19 from Sphingobacterium sp. TN19 and XynC01 from Achaetomium sp. Xz-8 were expressed and purified as described previously (14, 24, 25).

**ITC**

Isothermal titration calorimetry measurements were performed at 25 °C using a MicroCal iTC200 (GE Healthcare). The double inactive mutant E140Q/E248Q of CbXyn10C was built to avoid the catalytic reaction and concurrent heat change during the titration process in the ITC measurements. Protein was extensively dialyzed against buffer, including 50 mM sodium phosphate, 150 mM NaCl (pH 7.0). All the oligosaccharides tested were also dissolved in same buffer. Each of the oligosaccharides at a desired concentration was repeatedly injected into the protein cell 18 times with the stirring speed set to 1000 rpm.

---

*Insights into a bifunctional GH10 xylanase/cellulase*
The association constant ($K_a$) was calculated accordingly using the software MicroCal Origin 7.0. The thermodynamic parameters were calculated with both the Gibbs free energy equation $\Delta G = \Delta H - T\Delta S$ and the relationship $\Delta G = -RT\ln K_a$.

**Determination of catalytic efficiencies of CbXyn10C against oligosaccharides**

All the oligosaccharides used in this study were purchased from Megazyme (Wicklow, Ireland). The catalytic efficiencies of CbXyn10C on xylo- and cello-oligosaccharides were determined by high performance anion-exchange chromatography-pulsed amperometric detector analysis according to the method described by Matsui (28). The reaction mixtures were prepared with one of the xylo- or cello-oligosaccharide substrates (200 $\mu$M) in the McIlvaine buffer, and the reaction was initiated by addition of CbXyn10C. For xylobiose (X2) and xylotriose (X3), the final concentration of CbXyn10C was 100 nM, and for xylotetraose (X4) the final concentration of the enzyme was 20 nM and that further decreased to 4 nM for xylopentaose (X5) and 2 nM for xylohexaose (X6), respectively. For cellopentaose (C5) and cellohexaose (C6), the final concentration of the enzyme was 5 and 200 nM, respectively. The substrate concentration at the beginning of the reaction ($[S_0]$) and at a specified time ($[S]$) (3 min in this study) during the reaction was fit to the equation $k = \ln([S_0]/[S])$, where $k$ is the catalytic efficiency ($k_{cat}/K_m$) (29).

**Assay of enzymatic activity**

To determine the specific activities of CbXyn10C on xylan and cellulose, 10 $\mu$L of appropriately diluted enzyme was incubated with beechwood xylan or sodium CMC in a pH 6.5 McIlvaine buffer (200 mM sodium phosphate, 100 mM sodium citrate) in a total volume of 200 $\mu$L at the optimal temperature (80 °C for cellulose and 85 °C for beechwood xylan) for certain periods of time. The released reducing sugars were determined by using the 3,5-dinitrosalicylic acid (DNS) method (30). The kinetic parameters of the wild-type CbXyn10C and its mutants were determined in the same McIlvaine buffer. The $K_m$ and $k_{cat}$ values were estimated by fitting the data to the Michaelis-Menten equation using the GraphPad Prism (version 5.01) software (GraphPad, San Diego). These assays were performed as described previously (13).

**Protein crystallization, data collection, and structure determination**

The wild-type (WT) of CbXyn10C and the inactive mutant (E140Q/E248Q) were concentrated to 10 mg/ml in a buffer containing 25 mM Tris- HCl (pH 7.5) and 150 mM NaCl. CbXyn10C WT was crystallized in a mother liquid with 0.1 M sodium cacodylate (pH 7.5). Obtained crystals were cryoprotected with 25% (v/v) 2-methyl-2,4-pentanediol. The crystals were mounted on a nylon loop and cooled immediately in liquid nitrogen prior to data collection. The diffraction data were collected at 100 K using a synchrotron radiation source (Table 3). The data were automatically processed using xia2 (31). The free enzyme structure of WT was determined by molecular replacement using the structure of xylanase from G. stearothermophilus (PDB code 2Q8X) as a template with the Phaser program. The free enzyme structure of E140Q/E248Q and its xylohexa- and cellohexaose-bound structures were solved by molecular replacement using the structure of the refined WT structure. The models were further refined using COOT (32) and Phenix (23). Hydrogen atoms were included in idealized positions and refined as a riding model. Individual anisotropic ADP for non-hydrogen atoms was refined for E140Q/E248Q complexed with xylohexaose.

**Molecular docking of CbXyn10C with cellohexaose**

The complex crystal of E140Q/E248Q with cellohexaose contained six fairly visible glucose units from subsites $-2$ to $+4$. To gain more insights into the molecular mechanism underlying the catalytic ability of CbXyn10C with cellulose, we performed a modeling study by molecular docking of CbXyn10C with cellohexaose. The coordinate files of ligand were prepared in the PRODRG server (33). The docking of the enzyme with cellohexaose was carried out by using AutoDock Vina (34). A docking grid with a size of 60 $\times$ 60 $\times$ 60 Å was used to encompass the partially bound protein–ligand complex. The docked complex was selected by the criteria of interacting energy combined with the geometrical matching quality, and the energy was minimized using YASARA software to resolve atomic clashes (35).

**Molecular dynamic (MD) simulation**

The MD simulations were carried out with the AMBER 14 simulation package and repeated twice for each system. The standard AMBER force fields ff99SB and GLYCAM_06j-1 were used to generate the topologies and parameters of the enzyme and substrate, respectively (36, 37). Each system was immersed in a dodecahedral periodic box of TIP3P water molecules that extended 10 Å from the protein atoms (38). Cl$^-$ ions and Na$^+$ ions were added to neutralize the charge. Before the MD simulations, the water molecules/ions were resolved to minimize energy via 1000 steps, followed by 20,000 steps for the side chains of the protein and then 4000 steps for the whole system to remove potentially poor contacts between the solute and solvent. After energy minimization, the systems were gradually heated from 0 to 300 K over 100 ps, followed by a 50-ns production of MD simulations with a time step of 2 fs at a temperature of 300 K and pressure of 1.0 atm that were controlled by the Langevin algorithm (39). Long-range electrostatic interactions were treated using the particle-mesh Ewald (PME) method (40). Bonds involving hydrogen atoms were constrained by the SHAKE algorithm (41).
Insights into a bifunctional GH10 xylanase/cellulase

Author contributions—Y. C. designed and performed the experiments, analyzed the data, and assisted with preparation of the figures and manuscript. T. T., L. P., J. R., and N. H. performed the crystallographic study. T. T. carried out the molecular modeling and docking. X. X., X. W., Z. Y., and L. G. provided technical assistance. H. L. gave suggestions to the modification of the manuscript. N. H., B. Y., and X. S. conceived and guided the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

References

1. Ha, S. J., Galazka, J. M., Kim, S. R., Choi, J. H., Yang, X., Seo, J. H., Glass, N. L., Cate, J. H., and Jin, Y. S. (2011) Engineered Saccharomyces cerevisiae capable of simultaneous cellobiose and xylose fermentation. Proc. Natl. Acad. Sci. U.S.A. 108, 504–509

2. Groot, J., Cepress-Mclean, S. C., Robbins-Pianka, A., Knight, R., and Gill, R. T. (2017) Multiplex growth rate phenotyping of synthetic mutants in selection to engineer glucose and xylose co-utilization in Escherichia coli. Biotechnol. Bioeng. 114, 885–893

3. McKee, L. S., Peña, M. J., Rogowski, A., Jackson, A., Lewis, R. J., York, W. S., Krogh, K. B., Viiko-Nielsen, A., Skjot, M., Gilbert, H. J., and Marles-Wright, J. (2012) Introducing endo-xylanase into an exo-acting arabinofuranosidase that targets side chains. Proc. Natl. Acad. Sci. U.S.A. 109, 6537–6542

4. Hong, S. Y., Lee, J. S., Cho, K. M., Math, R. K., Kim, Y. H., Hong, S. J., Cho, Y. U., Kim, H., and Yun, H. D. (2006) Assembling a novel bifunctional cellulase-xylanase from Thermotoga maritima by end-to-end fusion. Biotechnol. Lett. 28, 1857–1862

5. Nakazawa, H., Okada, K., Kobayashi, R., Kubota, T., Onodera, T., Ochiai, N., Omata, N., Ogasawara, W., Okada, H., and Morikawa, Y. (2008) Characterization of the catalytic domains of Trioelodera reesi endoglucanase II, and III, expressed in Escherichia coli. Appl. Microbiol. Biotechnol. 81, 681–689

6. Yang, S. J., Kataeva, I., Wiegel, J., Yin, Y., Dam, P., Xu, Y., Westpheling, J., and Adams, M. W. (2010) Classification of “Anaerocellum thermodiplod” strain DSM 6725 as Caldicellulosiruptor bescii sp. nov. Int. J. Syst. Evol. Microbiol. 60, 2011–2015

7. Kataeva, I., Foston, M. B., Yang, S. J., Pappathil, S., Biswal, A. K., Foston, M. B., Yang, S. J., Pattathil, S., Biswal, A. K., Poole, T. J., Doeppke, C., Davis, M., Westpheling, J., and Adams, M. W. (2019) Molecular and biochemical analyses of the GH44 module cellulose and biochemical analyses of CbCel9A/Cel48A, a highly secreted multidomain cellulase by Caldicellulosiruptor bescii during growth on crystalline cellulose. PloS ONE 8, e84172

8. Ye, L., Su, X., Schmitz, G. E., Moon, Y. H., Zhang, J., Mackie, R. I., and Cann, I. K. (2017) Molecular and biochemical analyses of the GH44 module of ChMan5B/Cel44A, a bifunctional enzyme from the hypertherophilic bacterium Caldicellulosiruptor bescii. Appl. Environ. Microbiol. 78, 7048–7059

9. Xue, X., Wang, R., Tu, T., Shi, P., Ma, R., Luo, H., Yao, B., and Su, X. (2015) The N-Terminal GH10 domain of a multimodular protein from Caldicellulosiruptor bescii is a versatile xylanase/β-glucanase that can degrade crystalline cellulose. Appl. Environ Microbiol 81, 3823–3833

10. Su, X., Han, Y., Dodd, D., Moon, Y. H., Yoshida, S., Mackie, R. I., and Cann, I. K. (2013) Reconstitution of a thermostable xylan-degrading enzyme mixture from the bacterium Caldicellulosiruptor bescii. Appl. Environ. Microbiol. 79, 1481–1490

11. Brunecky, R., Alahuta, M., Xu, Q., Donohoe, B. S., Crowley, M. F., Kataeva, I. A., Yang, S. J., Resch, M. G., Adams, M. W., Lunin, V. V., Himmel, M. E., and Bomble, Y. J. (2013) Revealing nature’s cellulase diversity: the digestion mechanism of Caldicellulosiruptor bescii CelA. Science 342, 1513–1516

12. Andrews, S. R., Charnock, S. J., Lacey, J. H., Davies, G. I., Claeyssens, M., Nerincx, W., Underwood, M., Sinnott, M. L., Warren, R. A., and Gilbert, H. J. (2000) Substrate specificity in glycoside hydrolase family 10—tyrosine 87 and leucine 314 play a pivotal role in discriminating between glucose and xylose binding in the proximal active site of Pseudomonas cellulosa xylanase 10A. J. Biol. Chem. 275, 23027–23033

13. Ichinose, H., Dietertavian, S., Fujimoto, Z., Kuno, A., Lo Leggio, L., and Kaneko, S. (2012) Structure-based engineering of glucose specificity in a family 10 xylanase from Streptomyces olivaceoviridis E-86. Process Biochem. 47, 358–365

14. Zolotnitsky, G., Cogan, U., Adir, N., Solomon, V., Shoham, G., and Shoham, Y. (2004) Mapping glycoside hydrolase substrate subsites by isothermal titration calorimetry. Proc. Natl. Acad. Sci. U.S.A. 101, 11275–11280

15. Solomon, V., Teplitsky, A., Shulami, S., Zolotnitsky, G., Shoham, G., and Shoham (2007) Structure—specificity relationships of an intracellular xylanase from Geobacillus steaerothermophilus. Acta Crystaloggr. D Biol. Crystallogr. 63, 845–859

16. Speciale, G., Thompson, A. J., Davies, G. J., and Williams, S. I. (2014) Dissecting conformational contributions to glycosidase catalysis and inhibition. Curr. Opin. Struct. Biol. 28, 1–13

17. Charnock, S. J., Spurway, T. D., Xie, H., Beylot, M. H., Virden, R., Warren, R. A., Hazlewood, G. P., and Gilbert, H. J. (1998) The topology of the substrate-binding clefts of glycolysis hydrolase family 10 xylasses are conserved. J. Biol. Chem. 273, 32117–32119

18. Sun, H., Yeo, W. L., Lim, Y. H., Chew, X., Smith, D. J., Xue, B., Chan, K. P., Robinson, R. C., Robins, E. G., Zhao, H., and Ang, E. L. (2016) Directed evolution of a fluorinase for improved fluorination efficiency with a non-native substrate. Angew. Chem. Int. Ed. Engl. 55, 14277–14280

19. Biely, P., Krátký, Z., and Vrsanská, M. (1981) Substrate-binding site of endo-1,4-β-xylanase of the yeast Cryptococcus albidus. Eur. J. Biochem. 119, 559–564

20. Zhao, L., Meng, K., Shi, P., Bai, Y., Luo, H., Huang, H., Wang, Y., Yang, P., and Yao, B. (2013) A novel thermophilic xylanase from Achaetomopsis sp. Xz-8 with high catalytic efficiency and application potentials in the brewing and other industries. Process Biochem. 48, 1879–1885

21. Zhou, J., Huang, H., Meng, K., Shi, P., Wang, Y., Luo, H., Yang, P., Bai, Y., Zhou, Z., and Yao, B. (2009) Molecular and biochemical characterization of a novel xylanase from the symbiotic Sphingobacterium sp. TN19. Appl. Microbiol. Biotechnol. 85, 323–333

22. Zverlov, V., Mahr, S., Riedel, K., and Bronnenmeier, K. (1998) Properties and gene structure of a bifunctional cellulolytic enzyme (CelA) from the extreme thermophile “Anaerocellum thermodiplod” with separate glycosidase and catalytic domains. Microbiology 144, 457–465

23. Locher, A., Giannone, R. J., Rodriguez, M., Jr., Shah, M. B., Mielenz, J. R., Keller, M., Antranikian, G., Graham, D. E., and Hettich, R. L. (2011) Use of label-free quantitative proteomics to distinguish the secreted cellulolytic systems of Caldicellulosiruptor bescii and Caldicellulosiruptor obsidiansis. Appl. Environ. Microbiol. 77, 4042–4054

24. Matsui, I., Ishikawa, K., Matsui, E., Miyairi, S., Fukui, S., and Honda, K. (1991) Substrate specificity of Saccharomycopsis a-amylase secreted from Saccharomycopsis cerevisiae. J. Biochem. 109, 566–569

25. Han, Y., Dodd, D., Hesper, C. W., Oheke-Adjie, S., Schroeder, C. M., Mackie, R. I., and Cann, I. K. (2010) Comparative analyses of two thermophilic enzymes exhibiting both β-1,4 mannosidase and β-1,4 glucosidic cleavage activities from Caldanaerobius polysaccharolyticus. J. Bacteriol. 192, 4111–4121
30. Miller, G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31**, 426–428
31. Winter, G., Lobley, C. M., and Prince, S. M. (2013) Decision making in xia2. *Acta Crystallogr. D Biol. Crystallogr.* **69**, 1260–1273
32. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 486–501
33. Schüttelkopf, A. W., and van Aalten, D. M. (2004) PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 1355–1363
34. Trott, O., and Olson, A. J. (2010) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **31**, 455–461
35. Krieger, E., and Vriend, G. (2014) YASARA view—molecular graphics for all devices—from smartphones to workstations. *Bioinformatics* **30**, 2981–2982
36. Kirschner, K. N., Yongye, A. B., Tschampel, S. M., González-Outeiriño, J., Daniels, C. R., Foley, B. L., and Woods, R. J. (2008) GLYCAM06: A generalizable biomolecular force field. *Carbohydrates. J. Comput. Chem.* **29**, 622–655
37. Wickstrom, L., Okur, A., and Simmerling, C. (2009) Evaluating the performance of the f99SB force field based on NMR scalar coupling data. *Biophys. J.* **97**, 853–856
38. Jorgensen, W. L., J. C., D., M. J., Impey, R. W., and Klein, M. L. (1983) Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* **79**, 926–935
39. Pastor, R. W., Brooks, B. R., and Szabo, A. (1988) An analysis of the accuracy of Langevin and molecular dynamics algorithms. *Mol. Phys.* **65**, 1409–1419
40. Essmann, U., Perera, L., Berkowitz, M. L., Darden, T., Lee, H., and Pedersen, L. G. (1995) A smooth particle mesh Ewald method. *J. Chem. Phys.* **103**, 8577–8593
41. Ryckaert, J., Ciccotti, G., and Berendsen, H. J. C. (1977) Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes. *J. Comp. Phys.* **23**, 327–341