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A kinetic study of *Trichoderma reesei* Cel7B catalyzed cellulose hydrolysis

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ABSTRACT

One prominent feature of *Trichoderma reesei* (*Tr*) endoglucanases catalyzed cellulose hydrolysis is that the reaction slows down quickly after it starts (within minutes). But the mechanism of the slowdown is not well understood. A structural model of *Tr*- Cel7B catalytic domain bound to cellulose was built computationally and the potentially important binding residues were identified and tested experimentally. The 13 tested mutants show different binding properties in the adsorption to phosphoric acid swollen cellulose and filter paper. Though the partitioning parameter to filter paper is about 10 times smaller than that to phosphoric acid swollen cellulose, a positive correlation is shown for two substrates. The kinetic studies show that the reactions slow down quickly for both substrates. This slowdown is not correlated to the binding constant but anticorrelated to the enzyme initial activity. The amount of reducing sugars released after 24 h by Cel7B in phosphoric acid swollen cellulose, Avicel and filter paper cellulose hydrolysis is correlated with the enzyme activity against a soluble substrate *p*-nitrophenyl lactoside. Six of the 13 tested mutants, including N47A, N52D, S99A, N323D, S324A, and S346A, yield ~15–35% more reducing sugars than the wild type (WT) Cel7B in phosphoric acid swollen cellulose and filter paper hydrolysis. This study reveals that the slowdown of the reaction is not due to the binding of the enzyme to cellulose. The activity of *Tr*- Cel7B against the insoluble substrate cellulose is determined by the enzyme's capability in hydrolyzing the soluble substrate.

1. Introduction

Enzymatic cellulose hydrolysis is carried out mainly by cellulases. The filamentous fungus *Trichoderma reesei* (*Tr*) can secrete a large amount of cellulases [1], including cellobiohydrolases cleaving cellobiose from cellulose chain ends, endoglucanases cleaving strands randomly, and β-glucosidases converting soluble cellodextrins and cellobiose to glucose. These cellulases except *Tr.* Cel12A [2] have two domains, a large catalytic domain (CD) and a small carbohydrate-binding module (CBM) which shares ~70% sequence identity [3], connected by an *O*-glycosylated linker peptide. The main role of CBM is to bind to cellulose and increase the enzyme concentration whereas the CD performs the glycosidic bond cleavage. However, recent studies show that CDs alone can have comparable hydrolysis efficiency as the intact cellulases provided that the substrate cellulose loading is high, e.g., 20% [4–6]. The advantage of not having CBM is that much better enzyme recycling can be achieved.

Tr. Cel7B is one of the major endoglucanases, and accounts for 5–10% of the total cellulases [7]. The X-ray structure of *Tr.* Cel7B-CD is composed of two β-sheets that pack face to face to form a β-sandwich [8]. *Tr.* Cel7B is a retaining glucosidase [9,10], and hydrolyzes glucan chains through a two-step mechanism, i.e., the glycosylation step and the deglycosylation step. Our previous computational study [11] of the *Tr.* Cel7B-CD catalyzed *p*-nitrophenyl lactoside (PNPL) hydrolysis showed that the glycosylation step involves a proton transfer from the general acid (E201) to the glycosidic oxygen of the leaving group and a nucleophile (E196) attack to the anomeric carbon, and the deglycosylation involves a proton transfer from a water molecule to E201 and a hydroxide nucleophilic attack to the anomeric carbon. Several active site non-catalytic residues form a h-bond network which is critical for the PNPL hydrolysis, and conservative mutations of these residues result in an activity loss by 100–10,000 fold [11].

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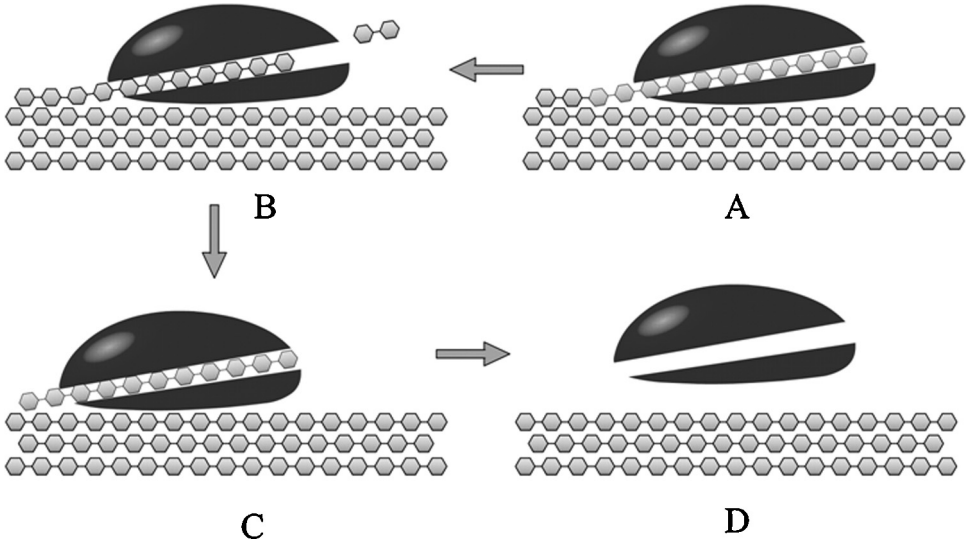

Scheme 1. Hydrolysis of cellulose by cellulose. (A) Adsorption of the cellulase to the substrate and threading of a cellulose chain into the enzyme active site; (B) cleavage of the glycosidic bond; (C) processive move of the cellulase; (D) dissociation of the cellulose.

Compared to the *Tr.* Cel7B catalyzed hydrolysis of a soluble substrate, much less is known about its hydrolysis of the insoluble substrate cellulose, especially at the molecular level. The hydrolysis of cellulose by a cellulase presumably consists of the following steps (Scheme 1): adsorption of the cellulase to the substrate, threading of a cellulose chain into the enzyme active site, cleavage of the glycosidic bond, processive movement of the cellulase, and dissociation of the cellulose [12]. Exoglucanases and endoglucanses both undergo similar steps except that endoglucanses more likely attack the amorphous regions of the cellulose without the need of the cellulose chain threading and cut the glycosidic bond randomly because endoglucanses have lower processivity. Molecular dynamics (MD) simulations suggested that the binding of *Tr.* Cel7B-CD to cellulose affects glucan clenching in the binding cleft [13] and certain loop residues facilitate the threading of the cellulose chain into the active site [14]. Kinetics studies of endoglucanases *Tr.* Cel7B, *Tr.* Cel5A, and *Tr.* Cel12A catalyzed phosphoric acid swollen cellulose (PASC) hydrolysis showed that the reaction displays an initial burst of activity followed by a rapid decline [15]. This decline has been ascribed to the enzyme being trapped by the cellulose. Compared to the intact endoglucanase, the CD alone has a weaker adsorption affinity to cellulose and its interaction with cellulose has rarely been studied experimentally. How the adsorption impacts the catalytic efficiency, whether the slowdown of the reaction depends on the form of the substrate, and what determines the reaction rate, are not clear. The detailed mechanism of the slowdown also needs further investigations.

In this work, we use *Tr.* Cel7B-CD as a model system to investigate the enzymatic cellulose hydrolysis mechanism. We first attempt to identify the binding residues by MD simulations and then introduce mutations to alter the binding property. The adsorption affinities of the WT and mutants to PASC and filter paper (FP) were determined.

2. Methods and materials

2.1. MD simulation

The apo form *Tr.* Cel7B-CD crystal structure (1EG1) [8] was used as a starting model. The protonation states of ionizable residues were determined based on a pK_a analysis by using PROPKA3 [16] where the residue is assigned as protonated (deprotonated) if the predicted pK_a is larger (equal or smaller) than 5.0. The protonation state was also examined manually. The cellulose slab was modeled based on the cellulose Iβ crystal structure presented by Nishiyama et al. [17]. The cellulose is 3 layers deep with 5 chains in each layer and each chain includes 12 glucose units. *Tr.* Cel7B-CD was docked on the cellulose surface manually using the program Hex [18], so that the active site binding groove of Cel7B-CD aligned with the middle cello-oligomer chain in the top layer (Fig. S1).

The simulations were carried out with Gromacs 4.5 [19,20]. The gromos53a6 [21,22] force field was employed to model the enzyme and the cellulose. The model was immersed in a dodecahedron box of explicit water molecules, with a 10.0 Å distance between the solvent box wall and the nearest solute atoms. There were ~60,000 atoms for the enzyme cellulose complex system. The details of the MD simulation are as follows. Initially, the system was minimized for 1000 steps. Then, with all heavy atoms restrained by the harmonic potential (k = 239 kcal/mol nm^2), the system was equilibrated in a 100 ps NVT simulation. Finally, a 100 ns NPT MD simulation was performed. In the simulation, the terminal glucose units of all cellulose chains were restrained with a force constant of 239 kcal/mol nm^2. The MD snapshots were saved every 100 ps, and the 400 snapshots from the last 40 ns MD simulation were analyzed. The hydrogen bond between *Tr.* Cel7B-CD and cellulose was assigned when the distance of the two heavy atoms (O or N) is less than 3.5 Å and the angle (hydrogen–donor–acceptor) is less than 30°.

2.2. Cloning, expression and purification of Tr. Cel7B-CD

The details about the cloning, expression and purification of *Tr.* Cel7B-CD catalytic domain were described elsewhere [11]. Briefly, the DNA encoding residues of the *Tr.* Cel7B-CD from *T. reesei* QM9414 was ligated with the vector pET-20b. The vector was transformed into an *Escherichia coli* strain DH10B. A His-tag (six histidine residues) was added to the C-terminus of the enzyme for the purification purpose. The expression vector (pET-20b-Cel7B-CD) was then transformed into *E. coli* strain Rosetta-orgami B (DE3) pLacI. The culture was grown at 37Celsius with vigorous shaking (~200 rpm). When the OD600 of the culture reached 0.8–1.2, a final concentration of 1 mM of IPTG was added to induce the expression of the protein at 16 °C and for 48 h. The lysed cells (by ultrasound sonication) were centrifuged and the resulting supernatants were purified by Ni-NTA affinity chromatography (GE, Shanghai, China). The purity was determined by sodium dodecyl sulfate polyacry-