High Speed Atomic Force Microscopy Visualizes Processive Movement of *Trichoderma reesei* Cellobiohydrolase I on Crystalline Cellulose

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Fungal cellobiohydrolases act at liquid-solid interfaces. They have the ability to hydrolyze cellulose chains of a crystalline substrate because of their two-domain structure, i.e. cellulose-binding domain and catalytic domain, and unique active site architecture. However, the details of the action of the two domains on crystalline cellulose are still unclear. Here, we present real-time observations of *Trichoderma reesei* (Tr) cellobiohydrolase I (Cel7A) molecules sliding on crystalline cellulose, obtained with a high speed atomic force microscope. The average velocity of the sliding movement on crystalline cellulose was 3.5 nm/s, and interestingly, the catalytic domain without the cellulose-binding domain moved with a velocity similar to that of the intact TrCel7A enzyme. However, no sliding of a catalytically inactive enzyme (mutant E212Q) or a variant lacking tryptophan at the entrance of the active site tunnel (mutant W40A) could be detected. This indicates that, besides the hydrolysis of glycosidic bonds, the loading of a cellulose chain into the active site tunnel is also essential for the enzyme movement.

Cellulose is the most abundant organic compound on Earth and is the major structural component of plant cell walls. In nature, cellulose chains, linear polymers of β-1,4-linked D-glucose units, are packed into ordered arrays to form crystalline cellulose I (1). Because cellulose chains have stable inter- and intermolecular hydrogen bonds in the crystal (3), cellulose I is quite resistant not only to chemical hydrolysis, but also to enzymatic degradation. To decompose cellulose, many microorganisms produce a set of different enzymes. Cellobiohydrolases (CBHs) are a group of cellulases that can hydrolyze glycosidic linkages at a crystalline surface (4, 5). *Trichoderma reesei* Cel7A (formally known as CBH I) is one of the best studied cellobiohydrolases, having a two-domain structure composed of a small cellulose-binding domain (CBD) belonging to carbohydrate-binding module family 1 and a glycoside hydrolase family 7 catalytic domain (CD) (Fig. 1), which are defined in the CAZy database (6–11). A similar two-domain structure is common for other glycosidases degrading insoluble carbohydrates. The CD of TrCel7A has a 50-Å long, tunnel-shaped active site, which binds a single cellulose chain and hydrolyzes the glycosidic bond to produce cellobiose, a β-1,4-glucose dimer, from the reducing end of the chain (12–16). Loss of the CBD causes a decrease of crystalline cellulose decomposition, but has less or no effect on the hydrolysis of soluble or amorphous cellulose (17–19). TrCel7A is thought to hydrolyze the crystalline cellulose chain in a processive manner, making consecutive cuts without releasing the chain. This is attributed to the unique, tunnel-shaped active site architecture of CBHs (20).

Atomic force microscopy (AFM) has a capacity to image individual biomolecules (21) and has been used to visualize *Trichoderma* CBH I (Cel7A) on the surface of cellulose (22). However, only very slow processes can be recorded by the technique because AFMs require minutes to scan certain area, and many bioprocesses occur at a much higher rate. In the present study, therefore, we have observed TrCel7A molecules using high speed AFM (HS-AFM) and analyzed how this enzyme behaves on crystalline cellulose substrate.

**EXPERIMENTAL PROCEDURES**

**Cellulose and Enzyme Preparations**—Cellulose from green alga, *Cladophora* sp., was used in this study (23). Cel7A from *T. reesei* was purified from a commercial cellulase mixture, Celluclast® 1.5L (Novozymes; available from Sigma-Aldrich) as described previously (23), and CD was prepared by the papain treatment of purified, intact TrCel7A as described earlier (6). Hydrolysis of highly crystalline cellulose from *Cladophora*, phosphoric acid-swollen cellulose prepared from Avicel (Funacel; Funakoshi Co., Ltd., Tokyo, Japan) was performed as described previously (23, 24). The production and purification of the TrCel7A E212Q mutant (two-domain version) have been reported earlier (25). The TrCel7A W40A mutant (two-domain version) was produced in *T. reesei* ALKO3414 (Roal Ltd., Rajamäki, Finland) (*cel7A* and *cel7B*) and purified basically as described earlier (25, 26). The detailed characterization of the W40A mutant will be described elsewhere. TrCel7A and the homologous endoglucanase I, Cel7B, are difficult to separate...
during purification. For all of the Cel7A preparations used here, the possible endoglucanase contamination was carefully checked using hydroxyethyl cellulose (Sigma) and Ostazin brilliant red H-3B hydroxyethyl cellulose (Sigma) which are good substrates for endoglucanases but are not hydrolyzed by cellbiohydrolases, basically as described earlier (25). All enzyme samples were estimated to contain less than 0.1% endoglucanase contamination.

*HS-AFM Observations*—HS-AFM observations were carried out using a NanoLiveVision (Olympus Corporation) equipped with a BL-AC10EGS-A2 small cantilever (Olympus Corporation), based on a previous report (27). Two microliters of crystalline cellulose suspension in water (0.2%) were dropped on a graphite sample disc, which was rinsed three times with 18 ml of 20 mM sodium acetate buffer, pH 5.0, after incubation for 5 min. Crystalline cellulose on the sample disc was initially observed without enzyme in 70 °C and the products were analyzed by HPLC (LC-2000 series; Jasco, Tokyo, Japan), using a Corona™ Charged Aerosol Detector™ (ESA Biosciences, Chelmsford, MA) as described elsewhere (28). Soluble cellulosiglassurides with degree of polymerization 1–6 were used as standards.

*Image Analysis*—Digital images were analyzed using Adobe ImageReady CS2 (version 9.0, Adobe Systems, Inc.). The rate of enzyme movement was determined by measuring the distance relative to a reference point on one of the frames in pixels, and this was converted to length using the size of the observation area.

**RESULTS AND DISCUSSION**

Highly crystalline cellulose prepared from *Cladophora* sp. was hydrophobically immobilized on graphite grid and were observed by HS-AFM after the addition of Cel7A. A schematic presentation is shown in Fig. 1. As shown in Fig. 2 and supplementary Video S1, we have obtained HS-AFM images of small particles sliding on the surface of highly crystalline cellulose. These images showed that the particles slide in a single direction without becoming detached from the substrate. Analysis of the images showed that the size of the particles was 3.1 nm (Fig. 2, B and C), which is consistent with the height estimated from the crystal structure of the catalytic module of *TrCel7A* (Fig. 2D). Because these moving particles were not seen in the absence of the enzyme, we conclude that they are *TrCel7A* molecules sliding on crystalline cellulose. *TrCel7A* is known to hydrolyze cellulose from the reducing end of the chain (14, 16), and the previous transmission electron microscopy studies also showed unidirectional hydrolysis, narrowing of the microcrystals at the reducing end side when highly crystalline cellulose was treated with *TrCel7A* (15). In the present study, cellulose crystals showed similar narrowing after incubation with the *TrCel7A*, which was confirmed by transmission electron microscopy observations and accompanied by cellbiose production as the only soluble product, detected by HPLC (data not shown). Therefore, we conclude that the *TrCel7A* molecules are moving in the direction from the reducing end to the non-reducing end. We have also tested several CBH II (Cel6) types of enzyme, which have been known to hydrolyze non-reducing end of crystalline cellulose (29), but we have not been able to observe any movement on the cellulose surface (data not shown), suggesting less processive action of CBH II.

The adsorption of the family 1 CBD on crystalline cellulose requires three aromatic amino acids (three tyrosines on the CBD*TrCel7A* surface) (30), and the binding sites involve the hydrophobic (110) planes of cellulose crystals (31). During the HS-AFM observations, many molecules were seen not only on crystalline cellulose, but also on the background, hydrophobic graphite surface. However, the enzyme molecules on the background only vibrated, possibly because of Brownian motion, but did not slide in any direction. The fact that the sliding movement was observed only on cellulose indicates that the enzyme movement requires the combination of the enzyme and the substrate and does not arise only as a result of simple enzyme adsorption via hydrophobic interactions.

The individual *TrCel7A* molecules were all sliding with a similar velocity on the crystalline cellulose surface, and the average rate of movement on cellulose I for intact *TrCel7A* was estimated to be 3.5 ± 1.1 nm s⁻¹ (n = 69), as shown in Fig. 3. In addition, HS-AFM observations using the *TrCel7A* CD, isolated after proteolytic cleavage of the intact enzyme (Fig. 4), were also performed. No particles were observed on the substrate when the isolated CD was added at a concentration similar to that of the intact wild-type enzyme (data not shown); however, a 10-fold increase in the protein concentration (to 20 μM) resulted in the appearance of sliding particles, as shown in...
Interestingly, the average velocity of the sliding CD was similar to that of the intact TrCel7A. This indicates that adsorption on crystalline cellulose through the CBD is not essential for the movement of TrCel7A, and CD alone seems to be responsible for the sliding on the substrate.

Besides the two wild-type forms of the TrCel7A, two mutants were examined under similar experimental conditions. As seen in Fig. 6A, the substrate-binding tunnel of TrCel7A holds seven glucose units at subsites \(-7\) to \(-1\) and two additional glucose units in the product-binding site \(+1\) and \(+2\) (13). Glu\(^{212}\), which acts as a catalytic nucleophile (25), is located between \(-1\) and \(+1\), and there is a conserved Trp\(^{40}\) at the entrance of the active site tunnel, where its role is apparently to fix the substrate by hydrophobic interaction at subsite \(-7\). The mutation of Glu\(^{212}\) to Gln (E212Q) causes significant loss in activity, as shown in Fig. 4. In the HS-AFM images of E212Q, the mutant Cel7A appeared to be bound tightly to crystalline cellulose, and no sliding molecules could be observed under any conditions. The molecules were observed to be bound for various time periods, but always longer than 10 time frames (supplemental Video S3), indicating that they stay on the crystal surface for quite a long time without sliding movement. In addition to the inactive mutant, the mutant W40A was tested as the control of the CD and E212Q mutant. As shown in Fig. 4, this mutant has similar activity against an amorphous substrate as the wild-type, but less than 20% of the wild-type activity toward highly crystalline cellulose. This would suggest that the W40A mutant and the CD are similar in their biochemical behavior. However, in contrast to the CD, no obvious movement of W40A was observed in HS-AFM studies. Moreover, almost all of the W40A mutant molecules were observed to be bound for only a limited time period compared with the E212Q mutant (supplemental Video S4). The action of the TrCel7A W40A mutant was also examined at a much faster scan rate (four frames/s), which revealed that many molecules became attached to and detached from the crystalline cellulose surface (see supplemental Video S5). These results suggest that mutant E212Q can grasp the cellulose chain but is unable to proceed along the chain because of its inactivity, whereas mutant W40A can only rarely lift a substrate chain and undergoes repeated attachment to and detachment from the surface (see also summary in Fig. 7).

It has been suggested that the driving force of the processive movement of cellulobiohydrolases is provided by the energy from hydrolysis of glycosidic bonds (32). Molecular dynamics studies...
have further suggested that the enzyme-substrate interactions produce the force required to pull cellulose chains from the surface and also to move the enzyme along the cellulose chain, and the CBD could penetrate between cellulose chains and serve to feed the free chain into the active site tunnel of the CD (33). Based on our comparison of the two mutant and wild-type enzymes, however, both the hydrolysis of the glycosidic bond and the loading of substrate are essential for the sliding movement. Moreover, our results demonstrate that the average velocity of the CD and the intact Cel7A enzyme on cellulose surface is similar. This would clearly argue against suggestions of the family 1 CBD having an active role in penetration to the crystalline cellulose, lifting and feeding of the chain to the active site tunnel of Cel7A. Based on our studies, the role of CBD would be merely to increase the enzyme concentration on the crystalline substrate.

Motor proteins utilize high energy phosphate bonds as the energy source for movement on their substrate. Kinecin, dynein, and certain myosins are known as processive motor proteins, which show linear motion on microtubules and actin filaments, utilizing ATP as an energy source (34). Our results here indicate that TrCel7A wild-type enzyme moves linearly along the crystalline cellulose similarly to many motor proteins. The major difference is, however, the driving energy, i.e. hydrolysis of a glycosidic bond of cellulose (equal to or less than $-12.5 \text{kJ/mol}$), which has less energy than a diphosphate bond (approximately $-30 \text{kJ/mol}$) (33, 35, 36). This feature of TrCel7A is quite similar to exonucleases, which are categorized into molecular motors.

CONCLUSIONS

The present study demonstrates direct observations of TrCel7A molecules sliding on crystalline celluloses. Based on CD having sliding velocity similar to that of the wild-type and immobility of the inactive mutant (E212Q), we conclude that the movement is accompanied by a catalytic activity, and CBD enhances the concentration of enzyme molecules on the substrate but does not have more active role in cellulose hydrolysis. Moreover, our results on the W40A mutant suggest importance of hydrophobic interaction with cellulose chain for the enzyme movement. TrCel7A is the first example of a molecular motor within polysaccharide-degrading enzymes, and we suspect that other processive polysaccharide hydrolases may employ similar molecular machinery to slide on their substrate.
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