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Supporting Online Material for

## **Traffic Jams Reduce Hydrolytic Efficiency of Cellulase on Cellulose Surface**

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Materials and Methods

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**Other Supporting Online Material for this manuscript includes the following:**  
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Movies S1 to S9

1 **Supporting Online Materials**

2

3 **Traffic Jams Reduce Hydrolytic Efficiency of Cellulase on Cellulose Surface**

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7 **Materials and Methods**

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## 1 **Materials and Methods**

2 *Cellulose and enzyme preparations*- Cellulose from green alga *Cladophora* sp., having more  
3 than 95% crystallinity, was used in this study (14, 26). Cel7A from *T. reesei* was purified from  
4 a commercial cellulase mixture, Celluclast<sup>®</sup> 1.5L (Novozyme, available from Sigma-Aldrich)  
5 as described previously (26), and hydrolysis of highly crystalline celluloses from *Cladophora*  
6 was performed as described previously (26).

7 *T. reesei* Cel6A wild-type was purified from a *T. reesei* strain lacking the endogenous genes  
8 for the major endoglucanases Cel5A and Cel7B. The presence of contaminating cellulolytic  
9 activities was ruled out by measuring the activities towards MeUmb(Glc)<sub>1</sub>, MeUmb(Glc)<sub>2</sub>,  
10 and hydroxyethyl cellulose (HEC), as described earlier (27). Concentrations of purified  
11 wild-type protein were determined from UV absorbance at 280 nm using the molar extinction  
12 coefficient  $\epsilon = 104,000 \text{ M}^{-1} \text{ cm}^{-1}$ , based on the result of total amino acid analysis (28).

13  
14 *High-speed AFM observations*- High-speed atomic force microscopic observations were  
15 carried out using a laboratory-built HS-AFM apparatus and a BL-AC10EGS-A2 small  
16 cantilever (Olympus Corporation), based on previous reports (12, 29-31). Two microliters of  
17 crystalline cellulose suspension in water (0.2 %) was dropped on a graphite sample disc,  
18 which was rinsed 3 times with 18  $\mu\text{l}$  of 20 mM sodium acetate buffer, pH 5.0, after incubation  
19 for 5 minutes at 25°C. Crystalline cellulose on the sample disc was initially observed without  
20 enzyme in 70  $\mu\text{l}$  of the same buffer, followed by the addition of 20  $\mu\text{M}$  enzyme solution with  
21 final concentration of 2.0  $\mu\text{M}$ . The AFM images were taken at 1-4 frames  $\text{sec}^{-1}$  at 25°C.  
22 Digital movies were constructed using Adobe ImageReady CS2 (Version 9.0, Adobe Systems  
23 Inc.). The linear movement of individual molecule was tracked and analyzed by the use of a  
24 newly developed routine in Igor Pro (Version 6.4, WaveMetrics, Inc.), which was basically  
25 designed to follow the center of the moving particle. AFM data analysis software Gwyddion

1 (Version 2.2, <http://gwyddion.net/>) was used for making the 3-dimensional movies in Fig. 4  
2 and movies S8 and S9 as described previously (32).

3  
4 *Adsorption of TrCel7A on crystalline celluloses- Cladophora cellulose* (either cellulose I<sub>α</sub> or  
5 cellulose III<sub>I</sub>, 0.1% w/v) was incubated with various concentrations of enzymes (final  
6 concentration, Abs<sub>280</sub>=0.04-0.8) in 1 ml of 50 mM sodium acetate buffer, pH 5.0, at 30 °C  
7 with inversion for 120 min. The free protein concentration [F] (μM) at equilibrium was  
8 measured after centrifugation (15,000g x 30 sec) by measuring the absorbance at 280 nm of  
9 the supernatant (900 μl). An absorption coefficient at 280 nm of 88,250 M<sup>-1</sup>cm<sup>-1</sup> was used for  
10 *TrCel7A* as described previously (26). The amount of adsorbed enzyme (A,  
11 nmol/mg-cellulose) was calculated by subtraction of the amount of free enzyme from the  
12 amount of added enzyme. The amount of adsorbed enzyme was plotted versus free enzyme  
13 concentration, based on a two-binding-site model for *TrCel7A* analysis, using the following  
14 equation:

$$15 \quad A = A_1 \cdot [F] / (1/K_{ad1} + [F]) + A_2 \cdot [F] / (1/K_{ad2} + [F])$$

16 where A<sub>1</sub> and A<sub>2</sub> are the adsorption maxima of high- and low-affinity binding  
17 (nmol/mg-cellulose); K<sub>ad1</sub> and K<sub>ad2</sub> are the adsorption constants of the high- and low-affinity  
18 binding sites (μM<sup>-1</sup>).

19  
20 *Synergistic hydrolysis of crystalline cellulose-TrCel7A and TrCel6A* (the *TrCel6A/TrCel7A*  
21 mixing ratio was 0/2.0, 0.5/1.5, 1.0/1.0, 1.5/0.5, or 2.0/0 μM) were incubated in 50 mM  
22 sodium acetate buffer, pH 5.0, with 0.1 % cellulose III<sub>I</sub> (14) at 30°C for 30 min, then  
23 centrifuged (x 15,000 g). The soluble products thus obtained were analyzed by HPLC  
24 (LC-2000 series; Jasco, Tokyo, Japan), using a Corona<sup>TM</sup> Charged Aerosol Detector<sup>TM</sup> (ESA  
25 Biosciences, Chelmsford, MA) as described previously (33). The supernatant was separated

1 on Shodex<sup>®</sup> Asahipak NH<sub>2</sub>P-50 (Showa Denko K.K., Kanagawa, Japan) with a linear gradient  
2 of acetonitrile/H<sub>2</sub>O (60/40 to 50/50, v/v). The amount of products was quantified using  
3 cellooligosaccharides with degree of polymerization (DP) = 2-7 (Seikagaku Corporation,  
4 Tokyo, Japan) as standards.

5

1 **Captions for Supporting Online Movies**

2 **Movie S1**. HS-AFM images of *TrCel7A* (final conc. 2.0  $\mu\text{M}$ ) sliding on crystalline cellulose  
3  $\text{I}_\alpha$ .  $x/y=150/90$  nm, 10x playback, total time 153 s. Scale bar and height lookup table (LUT) of  
4 the images are shown in Fig. 1A.

5

6 **Movie S2**. HS-AFM images of *TrCel7A* (final conc. 2.0  $\mu\text{M}$ ) on cellulose  $\text{III}_1$ .  $x/y=180/100$   
7 nm, 10x playback, total time 466 s. Scale bar and height LUT of the images are shown in Fig.  
8 2C.

9

10 **Movie S3**. HS-AFM images of *TrCel7A* (final conc. 2.0  $\mu\text{M}$ ) on cellulose  $\text{III}_1$ .  $x/y=72/40$  nm,  
11 5x playback, total time 154 s. Scale bar and height LUT of the images are included in the  
12 images.

13

14 **Movie S4**. HS-AFM images of *TrCel7A* (final conc. 2.0  $\mu\text{M}$ ) on cellulose  $\text{III}_1$ .  $x/y=150/75$  nm,  
15 10x playback, total time 90 seconds. Scale bar and height LUT of the images are shown in Fig.  
16 3B.

17

18 **Movie S5**. HS-AFM images of *TrCel7A* (final conc. 2.0  $\mu\text{M}$ ) on cellulose  $\text{III}_1$  peeling the  
19 surface of the same cellulose fiber of movie S4.  $x/y=150/75$  nm, 10x playback, total time 372  
20 seconds. Scale bar and height LUT of the images are shown in Fig. 3B.

21

22 **Movie S6**. HS-AFM images of cellulose  $\text{III}_1$  before the enzyme addition.  $x/y=250/125$  nm,  
23 10x playback, total time 18 seconds

24

25 **Movie S7**. HS-AFM images of crystalline cellulose  $\text{III}_1$  with the addition of *TrCel6A* (10 s)

1 and *TrCel7A* (505 s) with final concentrations of 2.0  $\mu\text{M}$  each.  $x/y=250/125$  nm, 10x  
2 playback, total time 832 seconds.

3

4 **Movie S8**. Three-dimensional HS-AFM images of cellulose III<sub>I</sub> incubated with *TrCel6A*. This  
5 movie was created from the first 480 s of movie S7. *TrCel6A* was added (10 s) at a final  
6 concentration of 2.0  $\mu\text{M}$ . Height LUT of these images is shown in Fig. 4.

7

8 **Movie S9**. Three-dimensional HS-AFM images of cellulose III<sub>I</sub> incubated with  
9 *TrCel6A+TrCel7A*. This movie was created from the latter part of movie S7. After 495 s from  
10 the addition of *TrCel6A* (5 s from the initial frame in this movie), *TrCel7A* was added at a  
11 final concentration of 2.0  $\mu\text{M}$ .

## References and Notes

1. M. E. Himmel *et al.*, Biomass recalcitrance: Engineering plants and enzymes for biofuels production. *Science* **315**, 804 (2007). [doi:10.1126/science.1137016](https://doi.org/10.1126/science.1137016) [Medline](#)
2. J. Jalak, P. Väljamäe, Mechanism of initial rapid rate retardation in cellobiohydrolase catalyzed cellulose hydrolysis. *Biotechnol. Bioeng.* **106**, 871 (2010). [doi:10.1002/bit.22779](https://doi.org/10.1002/bit.22779) [Medline](#)
3. B. Yang, D. M. Willies, C. E. Wyman, Changes in the enzymatic hydrolysis rate of Avicel cellulose with conversion. *Biotechnol. Bioeng.* **94**, 1122 (2006). [doi:10.1002/bit.20942](https://doi.org/10.1002/bit.20942) [Medline](#)
4. D. N. S. Hon, Cellulose: A random walk along its historical path. *Cellulose* **1**, 1 (1994). [doi:10.1007/BF00818796](https://doi.org/10.1007/BF00818796)
5. R. Wolfenden, Y. Yuan, Rates of spontaneous cleavage of glucose, fructose, sucrose, and trehalose in water, and the catalytic proficiencies of invertase and trehalase. *J. Am. Chem. Soc.* **130**, 7548 (2008). [doi:10.1021/ja802206s](https://doi.org/10.1021/ja802206s) [Medline](#)
6. T. T. Teeri, Crystalline cellulose degradation: New insight into the function of cellobiohydrolases. *Trends Biotechnol.* **15**, 160 (1997). [doi:10.1016/S0167-7799\(97\)01032-9](https://doi.org/10.1016/S0167-7799(97)01032-9)
7. T. T. Teeri *et al.*, *Trichoderma reesei* cellobiohydrolases: Why so efficient on crystalline cellulose? *Biochem. Soc. Trans.* **26**, 173 (1998). [Medline](#)
8. B. Henrissat, A. Bairoch, New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **293**, 781 (1993). [Medline](#)
9. C. Divne *et al.*, The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei*. *Science* **265**, 524 (1994). [doi:10.1126/science.8036495](https://doi.org/10.1126/science.8036495) [Medline](#)
10. J. Rouvinen, T. Bergfors, T. Teeri, J. K. Knowles, T. A. Jones, Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. *Science* **249**, 380 (1990). [doi:10.1126/science.2377893](https://doi.org/10.1126/science.2377893) [Medline](#)
11. K. Igarashi *et al.*, High speed atomic force microscopy visualizes processive movement of *Trichoderma reesei* cellobiohydrolase I on crystalline cellulose. *J. Biol. Chem.* **284**, 36186 (2009). [doi:10.1074/jbc.M109.034611](https://doi.org/10.1074/jbc.M109.034611) [Medline](#)
12. T. Ando *et al.*, A high-speed atomic force microscope for studying biological macromolecules. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 12468 (2001). [doi:10.1073/pnas.211400898](https://doi.org/10.1073/pnas.211400898) [Medline](#)
13. J. Ståhlberg, G. Johansson, G. Pettersson, A new model for enzymatic hydrolysis of cellulose based on the two-domain structure of cellobiohydrolase I. *Biotechnology (N. Y.)* **9**, 286 (1991). [doi:10.1038/nbt0391-286](https://doi.org/10.1038/nbt0391-286)
14. K. Igarashi, M. Wada, M. Samejima, Activation of crystalline cellulose to cellulose III(I) results in efficient hydrolysis by cellobiohydrolase. *FEBS J.* **274**, 1785 (2007). [doi:10.1111/j.1742-4658.2007.05727.x](https://doi.org/10.1111/j.1742-4658.2007.05727.x) [Medline](#)



15. J. Lehtiö *et al.*, The binding specificity and affinity determinants of family 1 and family 3 cellulose binding modules. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 484 (2003). [doi:10.1073/pnas.212651999](https://doi.org/10.1073/pnas.212651999) [Medline](#)
16. Y. S. Liu *et al.*, Cellobiohydrolase hydrolyzes crystalline cellulose on hydrophobic faces. *J. Biol. Chem.* **286**, 11195 (2011). [doi:10.1074/jbc.M110.216556](https://doi.org/10.1074/jbc.M110.216556) [Medline](#)
17. M. Wada, H. Chanzy, Y. Nishiyama, P. Langan, Cellulose III<sub>I</sub> crystal structure and hydrogen bonding by synchrotron x-ray and neutron fiber diffraction. *Macromolecules* **37**, 8548 (2004). [doi:10.1021/ma0485585](https://doi.org/10.1021/ma0485585)
18. Y. Nishiyama, J. Sugiyama, H. Chanzy, P. Langan, Crystal structure and hydrogen bonding system in cellulose I $\alpha$  from synchrotron x-ray and neutron fiber diffraction. *J. Am. Chem. Soc.* **125**, 14300 (2003). [doi:10.1021/ja037055w](https://doi.org/10.1021/ja037055w) [Medline](#)
19. L. E. R. Berghem, L. G. Pettersson, U. B. Axiofredriksson, The mechanism of enzymatic cellulose degradation. Characterization and enzymatic properties of a  $\beta$ -1,4-glucan cellobiohydrolase from *Trichoderma viride*. *Eur. J. Biochem.* **53**, 55 (1975). [doi:10.1111/j.1432-1033.1975.tb04041.x](https://doi.org/10.1111/j.1432-1033.1975.tb04041.x)
20. B. Nidetzky, W. Steiner, M. Hayn, M. Claeysens, Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: A new model for synergistic interaction. *Biochem. J.* **298**, 705 (1994). [Medline](#)
21. C. Divne, J. Ståhlberg, T. T. Teeri, T. A. Jones, High-resolution crystal structures reveal how a cellulose chain is bound in the 50 Å long tunnel of cellobiohydrolase I from *Trichoderma reesei*. *J. Mol. Biol.* **275**, 309 (1998). [doi:10.1006/jmbi.1997.1437](https://doi.org/10.1006/jmbi.1997.1437) [Medline](#)
22. A. Koivula *et al.*, The active site of cellobiohydrolase Cel6A from *Trichoderma reesei*: The roles of aspartic acids D221 and D175. *J. Am. Chem. Soc.* **124**, 10015 (2002). [doi:10.1021/ja012659q](https://doi.org/10.1021/ja012659q) [Medline](#)
23. H. Chanzy, B. Henrissat, Unidirectional degradation of valonia cellulose microcrystals subjected to cellulase action. *FEBS Lett.* **184**, 285 (1985). [doi:10.1016/0014-5793\(85\)80623-2](https://doi.org/10.1016/0014-5793(85)80623-2)
24. T. Imai, C. Boisset, M. Samejima, K. Igarashi, J. Sugiyama, Unidirectional processive action of cellobiohydrolase Cel7A on Valonia cellulose microcrystals. *FEBS Lett.* **432**, 113 (1998). [doi:10.1016/S0014-5793\(98\)00845-X](https://doi.org/10.1016/S0014-5793(98)00845-X) [Medline](#)
25. T. M. Wood, S. I. McCrae, *Biochem. J.* **171**, 61 (1972).
26. K. Igarashi, M. Wada, R. Hori, M. Samejima, Surface density of cellobiohydrolase on crystalline celluloses. A critical parameter to evaluate enzymatic kinetics at a solid-liquid interface. *FEBS J.* **273**, 2869 (2006). [doi:10.1111/j.1742-4658.2006.05299.x](https://doi.org/10.1111/j.1742-4658.2006.05299.x) [Medline](#)
27. A. Koivula *et al.*, Immunoaffinity chromatographic purification of cellobiohydrolase II mutants from recombinant *trichoderma reesei* strains devoid of major endoglucanase genes. *Protein Expr. Purif.* **8**, 399 (1996). [doi:10.1006/prev.1996.0116](https://doi.org/10.1006/prev.1996.0116) [Medline](#)
28. H. Palonen, M. Tenkanen, M. Linder, Dynamic interaction of *Trichoderma reesei* cellobiohydrolases Cel6A and Cel7A and cellulose at equilibrium and during hydrolysis. *Appl. Environ. Microbiol.* **65**, 5229 (1999). [Medline](#)

29. M. Shibata, H. Yamashita, T. Uchihashi, H. Kandori, T. Ando, High-speed atomic force microscopy shows dynamic molecular processes in photoactivated bacteriorhodopsin. *Nat. Nanotechnol.* **5**, 208 (2010). [doi:10.1038/nnano.2010.7](https://doi.org/10.1038/nnano.2010.7) [Medline](#)
30. D. Yamamoto *et al.*, High-speed atomic force microscopy techniques for observing dynamic biomolecular processes. *Methods Enzymol.* **475**, 541 (2010). [doi:10.1016/S0076-6879\(10\)75020-5](https://doi.org/10.1016/S0076-6879(10)75020-5) [Medline](#)
31. N. Kodera, D. Yamamoto, R. Ishikawa, T. Ando, Video imaging of walking myosin V by high-speed atomic force microscopy. *Nature* **468**, 72 (2010). [doi:10.1038/nature09450](https://doi.org/10.1038/nature09450) [Medline](#)
32. K. Igarashi, M. Samejima, *Biosci. Industry* **68**, 312 (2010).
33. K. Igarashi, T. Ishida, C. Hori, M. Samejima, Characterization of an endoglucanase belonging to a new subfamily of glycoside hydrolase family 45 of the basidiomycete *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **74**, 5628 (2008). [doi:10.1128/AEM.00812-08](https://doi.org/10.1128/AEM.00812-08) [Medline](#)

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