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Published Ahead of Print 25 October 2013.

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A Distinct Model of Synergism between a Processive Endocellulase (TfCel9A) and an Exocellulase (TfCel48A) from Thermobifida fusca

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Lignocellulosic biomass is digested in nature by the synergistic activities of enzymes with complementary properties, and understanding synergistic interactions will improve the efficiency of industrial biomass use for sustainable fuels and chemicals. Cel9A and Cel48A from a model bacterium, Thermobifida fusca (TfCel9A and TfCel48A, respectively), are two cellulases with different properties and have previously been shown to synergize well with each other. TfCel9A is a processive endocellulase with relatively high activity on crystalline cellulose. TfCel48A is a reducing end-directed exocellulase with very low activity on crystalline cellulose. Neither enzyme fits its respective role in the classical synergism model of enzymatic cellulose digestion. Using the results of time course, endpoint, and sequential addition activity assays, we propose a model of synergistic cooperation between the two cellulases. TfCel9A is most effective on fresh bacterial cellulose with a presumably uniform surface at the molecular level. Its processive activity likely erodes the surface and thus reduces its own activity. TfCel48A is able to hydrolyze the TfCel9A-modified substrate efficiently and replenish the uniform surface required by TfCel9A, creating a feedback mechanism. The model of synergistic interactions is comparable to an earlier proposed model for Trichoderma reesei Cel7A and Cel7B, but the roles of endo- and exocellulases are reversed, a finding which suggests that bacteria and fungi may have evolved different approaches to efficient biomass degradation.

Fuels and chemicals derived from lignocellulosic biomass have the potential to displace a significant fraction of petroleum-based products in the near future. One of the key challenges in the utilization of biomass for such applications is the cost associated with enzymatic hydrolysis of cellulose to fermentable sugars. Consequently, mechanistic studies of cellulases and other biomass-degrading enzymes will play an important role in the advancement of biomass use for the production of renewable low-carbon-footprint fuels and chemicals.

In nature, most biomass is degraded by the complementary action of multiple enzymes produced by cellulolytic microorganisms. Cellulose constitutes a major component of plant cell walls and is composed of anhydrous glucose units covalently linked via β-1,4 glycosidic bonds to form long polymer chains. Hydrogen bonding between individual chains forms a recalcitrant heterogeneous material of variable crystallinity that is relatively resistant to enzymatic hydrolysis. Cellulases belong to different glycoside hydrolase (GH) families and are commonly divided into two major classes. Exocellulases typically contain an active site inside a tunnel and thus initiate hydrolysis at cellulose chain ends. Endocellulases usually contain an active site in an open cleft and are able to initiate hydrolysis anywhere along the cellulose chain. The processivity of cellulases is defined as the average number of consecutive cleavages that a cellulase will carry out before its dissociation from the chain. Due to technical limitations, the processivity of cellulases acting on bulk cellulose is usually reported as a relative value. The processivity of cellulases is defined as the average number of consecutive cleavages that a cellulase will carry out before its dissociation from the chain. Due to technical limitations, the processivity of cellulases acting on bulk cellulose is usually reported as a relative value.

For exocellulases, it is usually the ratio of soluble to insoluble reducing ends after digestion. Exocellulases are believed to be more processive than endocellulases, in particular, as judged by the soluble-to-insoluble-reducing-end postdigestion ratios. There is a subclass of processive endocellulases, the most studied of which is Cel9A from Thermobifida fusca (TfCel9A).

Synergistic interactions between different cellulases can strongly enhance the rate and extent of enzymatic cellulose hydrolysis. Since Reese et al. first documented synergism between different cellulase fractions in 1950 (5), a lot of work has been carried out to understand the mechanisms by which these enzymes act on their substrates. In 1979, Wood and McCrae proposed what became the classical endocellulase-exocellulase model of enzymatic cellulose hydrolysis (6). According to the model, endocellulases attack the amorphous fractions of cellulose, creating more chain ends for exocellulase attack. In turn, exocellulase activity exposes new amorphous regions within the bulk substrate and thus stimulates additional endocellulase activity. Support for this model has been extensively demonstrated in the literature, but it is also likely that the model is incomplete (7–9). Synergistic interactions have also been demonstrated for different types of exocellulases that preferentially attack either the reducing or the nonreducing end of the cellulose chain (10, 11). More recently, synergism between cellulases and auxiliary activity (AA) proteins of the AA10 (formerly CBM33) and AA9 (formerly GH61) families has been demonstrated (12–14). In addition, removal of obstacles and the release of unproductively bound exocellulases appear to be important components of effective synergistic cellulase mixtures. Continued studies of synergistic interactions in cellulose hydrolysis can therefore contribute to the development of models more complete than those that are presently available.

Here we report data for synergistic interactions between Cel9A and...
and Cel48A, two cellulases secreted by the model cellulolytic bacteria *Thermobifida fusca* (TfCel9A and TfCel48A, respectively). TfCel9A is a processive endocellulase which contains two cellulose binding modules, CBM2 and CBM3c (17), and is relatively effective as a single cellulase in the digestion of crystalline cellulose (4). CBM2 is attached via a flexible linker (which includes a fibronectin III domain) to CBM3c. CBM3c is, in turn, connected almost rigidly to the GH9 catalytic domain (18) and is believed to be responsible for the processivity of TfCel9A and ability of TfCel9A to digest crystalline cellulose effectively (17, 18). TfCel48A is a reducing end-directed exocellulase with very poor activity on crystalline cellulose (19). Despite its low activity, TfCel48A makes up about a third of total secreted cellulases when *T. fusca* is grown on cellulose (20), and it has been shown to interact synergistically with other *T. fusca* cellulases, including TfCel9A (4, 19). Given that TfCel9A is processive and is the most effective *T. fusca* cellulase on crystalline substrates (4), it is unlikely to serve the typical role assigned to endocellulases in the endosyneryxsis—exosyneryxsis model. In addition, the very low activity of TfCel48A on crystalline cellulose also makes it incompatible with the presumed role of exocellulases in the classical model of enzymatic cellulose digestion. Hence, it is likely that TfCel48A and TfCel9A employ a distinct mechanism of synergism, and here we propose a model for their cooperation.

**MATERIALS AND METHODS**

**Substrates and enzymes.** Bacterial cellulose (BC) was produced by *Acetobacter aceti subsp. xylinum* ("Acetobacter xylinum") and was obtained as a gift from Monsanto. BC cake was washed three times with deionized (DI) water by centrifugation and resuspended in DI water with 0.04% sodium azide (Sigma-Aldrich). The concentration was determined as dry weight per volume. Recombinant TfCel9A and TfCel48A were expressed in *Escherichia coli* BL21 cells and purified as previously described in references 17 and 21, respectively.

**Time course activity assays.** All reactions were conducted in triplicate in Eppendorf 2-ml Protein LoBind plastic tubes. One milligram substrate was combined with 83 nM TfCel9A and/or 750 nM TfCel48A in 0.6 ml 50 mM sodium acetate buffer, pH 5.5. Only buffer and substrate were combined for use as negative controls. Upon mixing, the BC reaction mixtures were immediately placed in a 50°C water bath. Samples were removed in triplicate at the given time points and placed on dry ice to stop the reaction. Frozen samples were later placed in a boiling bath for 10 min in order to denature the enzyme. It was verified experimentally that boiling does not alter the soluble sugar profiles detected by high-performance liquid chromatography (HPLC). The remaining reaction was removed and the soluble sugar concentrations were measured using a Shimadzu HPLC system fitted with a Bio-Rad Aminex HPX-87P analytical column and a refractive index detector. The mobile phase was Milli-Q water at a flow rate of 0.6 ml/min. Sample injection was performed by an autosampler installed on the instrument.

**BC hydrolysis assays of different concentrations of TfCel48A with a constant concentration of TfCel9A.** Reaction mixtures were prepared in the same manner as described above for the time course assays. TfCel9A (8.3 nM) was combined with 0 to 158 nM TfCel48A, and the mixture was incubated with 1 mg BC for 19 h. Samples were then processed for HPLC in the same manner as described above.

**Sequential BC addition assays.** Sequential BC addition assays were conducted under the same conditions used for the time course assays. For endpoint sequential assays, enzymes and substrate were incubated for 20 h and boiled for 5 min. The corresponding synergistic partner was then added, and the reaction mixtures were incubated for 2.25 h. A comparison between the total amount of soluble sugar produced by enzymes on fresh and pretreated substrates was made. In the case of substrate pretreatment, the total amount of sugar produced before the addition of the synergistic partner was determined experimentally and subtracted from the total amount of soluble sugar present after digestion by the freshly added enzyme. For time course sequential assays, reaction mixtures were initially prepared by mixing 83 nM TfCel9A with 1 mg BC. Triplicate samples were collected at the intervals indicated below and frozen on dry ice to stop the reaction. After overnight incubation, all remaining reaction mixtures were boiled for 5 min and divided into two groups. TfCel9A (83 nM) was added to one group, and TfCel48A (750 nM) was added to the other samples. Samples from both groups were removed in triplicate at the indicated intervals. After overnight incubation, the remaining reaction mixtures containing TfCel48A were boiled for 5 min and 83 nM TfCel9A was added. Samples were removed in triplicate at the time intervals indicated below. Controls were run to ensure that boiling of the reaction mixtures was sufficient to stop all additional activity when they were incubated at 50°C. It was also verified experimentally that boiling does not alter the HPLC profiles of the soluble sugars or affect the reactivity of BC.

**Data analysis.** HPLC data were processed with the OriginPro (version 8) program (OriginLab Corporation). Product identities and concentrations were determined by Gaussian peak fitting, using standard solutions with known concentrations of soluble cellobioseGlucosacharides for reference. Soluble sugar concentrations at time zero were subtracted from all of the subsequently obtained concentrations. The soluble sugar produced upon initial mixing of the enzyme and the substrate is primarily due to the burst activity, as described previously (22, 23), whereas the model used here is concerned with the digestion of the more recalcitrant portions of cellulose. The A- and b-parameter values of the time course profiles (where A is the total activity of the added enzyme, and b is the hydrolysis power factor that quantifies the dependence of activity on time) were determined using the nonlinear least-squares fit of equation 1.

**Kinetic model of cellulose hydrolysis.** We recently developed a kinetic model for mechanistic studies of enzymatic cellulose hydrolysis (21). The model relies on two parameters to quantify cellulose digestion by individual cellulases and their mixtures over time. It is based on a pseudo-zero-order Michaelis-Menten kinetic scheme but replaces an activity constant with a time (digestion)-dependent activity coefficient:

\[ X = A \cdot t^b \]

where X is percent digestion, and t is time. The values of A and b were determined by fitting equation 1 to the time course data for cellulose digestion. Parameter A is a product of specific activity and the productively bound cellulose concentration, neither of which is directly measurable for cellulases acting on bulk substrates. At low enzyme loads, the value of A is strongly dependent on the total added enzyme concentration and follows the Arrhenius relationship with respect to temperature. Parameter b is an intrinsic constant for a given cellulase on a given substrate and is independent of the total amount of added enzyme at low enzyme-to-substrate ratios. The theoretical limits of b are 0, in which case no products are formed, and 1, in which case the activity is constant over time, as is the case in classical kinetics. Thus, the value of b is indicative of the cellulase’s ability to overcome substrate recalcitrance, and it can be used to better understand synergistic interactions between biomass-active enzymes. For example, we showed that the addition of a *T. fusca* AA10 enzyme E7 to TfCel48A acting on BC increased the b value from 0.34 to 0.65 (21), which is consistent with the presumed ability of AA10 enzymes to disrupt crystalline regions of cellulose and chitin (14, 24).
which can vary strongly for different enzymes (21). It is likely that
condition 1 is typically applicable only for the initial range of digestion,
the observed
sum of the
alone, and the
b
combined together at the same loads used in individual assays, the
reflect its ability to hydrolyze BC well. When the two enzymes are
values: 0.77 and 0.34, respectively. On the other hand, the rela-
crystalline cellulose is reflected by its low A and b
values: 0.77 and 0.34, respectively. On the other hand, the relative-
high TfCel9A A and b values of 3.35 and 0.56, respectively,
reflect its ability to hydrolyze BC well. When the two enzymes are
combined together at the same loads used in individual assays, the
b value of the mixture is 0.55, which is the same as that of TfCel9A
alone, and the A value is increased by ca. 260% compared to the
sum of the A values measured for the two cellulases alone. Equation
1 is typically applicable only for the initial range of digestion,
which can vary strongly for different enzymes (21). It is likely that
the observed drop-off is due to the contribution of additional
factors other than substrate recalcitrance to the decline of the
cellulose digestion rate (e.g., surface erosion and obstacle formation
[9, 16, 25]). Importantly, while the drop-off point is below 2%
digestion for TfCel48A and ca. 12% for TfCel9A, it increases to ca.
35% when the two enzymes are combined.

The fact that the b-parameter value of the TfCel48A-TfCel9A
mixture is the same as that of TfCel9A while the A-parameter
value is significantly increased suggests that the two enzymes acting


together are able to digest the BC much faster than either one
acting alone, but they are not more effective at overcoming sub-
cstrate recalcitrance per se. The BC hydrolysis rate increase for the
mixture may be the result of the higher activity of either or both
enzymes. As a processive enzyme, TfCel9A initially produces cel-
lotetraose (G₄), which is later cleaved in solution (by TfCel9A and
other cellulases that may be present) to cellotriose (G₃), cellobiose
(G₂), and glucose (G₁) (Fig. 2a). On the other hand, TfCel48A
produces mostly G₂ and G₃ and only a trace amount of G₄ (Fig.
2b), which is able to hydrolyze only slowly. Therefore, most of
the G₄ present during the initial stages of BC digestion by the
TfCel48A-TfCel9A mixture is produced by TfCel9A and can be
used to compare the activity of TfCel9A alone and in the mixture.
As shown in Fig. 3a, the G₄ concentration profile during the initial
14 h of BC digestion is roughly the same for TfCel9A alone and
TfCel9A in combination with TfCel48A. This strongly suggests


that the rate of BC hydrolysis by TfCel9A is not significantly in-
creased in the presence of TfCel48A and implies that most of the
rate increase observed for the two enzymes acting together is due
to additional TfCel48A activity. Further support for this proposi-
tion is shown in Fig. 3b, which demonstrates a strong dependence
of the total soluble sugar produced on an increasing amount of
TfCel48A added to a constant amount of TfCel9A in endpoint
assays of BC hydrolysis. Interestingly, even though the total
amount of soluble sugar produced is strongly dependent on
TfCel48A concentrations even at high TfCel48A/TfCel9A ratios,
the specific activity of the total added enzyme is the highest at a
ratio of 0.5. This suggests that the productively bound fraction of
TfCel48A decreases as its ratio to TfCel9A increases.

The main factors that may contribute to the decline in the rate
of enzymatic cellulose digestion at low enzyme loads are inherent
substrate recalcitrance, morphological substrate changes, and
enzyme deactivation (due to poor thermostability and/or unproduc-
tive irreversible binding). To determine whether the decline in the
rate of BC digestion by TfCel9A and TfCel48A was caused primar-
ily by substrate or enzyme-related changes, we carried out sequen-
tial BC addition endpoint assays, the results of which are shown in
Fig. 4. After BC hydrolysis assay mixtures with TfCel48A or
TfCel9A alone were incubated overnight, the same amount of
fresh BC or buffer was added to the reaction mixtures and the
mixtures were incubated for the same amount of time. For both


FIG 1 Time course BC hydrolysis assays of 750 nM TfCel48A, 83 nM TfCel9A,
and their mixture. (Inset) Magnification of the TfCel48A time course. All data
points were obtained in triplicate, and the error bars represent 1 standard
deviation. The table lists the A- and b-parameter values obtained by fitting the
time course data to equation 1.

FIG 2 Sample HPLC profiles from time courses of 750 nM TfCel48A and 83
nM TfCel9A on 1 mg BC. (a) TfCel9A initially produces mostly cellotetraose
(G₄), which is later hydrolyzed in solution to cellotriose (G₃), cellobiose (G₂),
and glucose (G₁). (b) TfCel48A produces mostly G₃ and G₂ at a constant ratio
(which is indicative of its processivity) and a trace amount of G₄. G₄ is only
slowly cleaved by TfCel48A. The data for the zero time point were collected by
mixing the samples along with all other samples and placing them in boiling
water to denature the enzymes at the same time that other samples were placed
in a 50°C bath. * indicates an artifact peak always observed in the HPLC system
employed here.

FIG 3 TfCel9A and TfCel48A relative activity in the synergistic mixture. (a)
Cellobiose (G₂) concentration profile for BC hydrolysis by TfCel9A alone and
the TfCel9A-TfCel48A (1:9 ratio) mixture; (b) extent of digestion and
specific activity as a function of the TfCel48A concentration increase with a
constant concentration of TfCel9A in BC hydrolysis 19-h endpoint assays.
TfCel48A and TfCel9A, the addition of fresh BC resulted in activity much higher than that achieved with addition of buffer only. Incubation of TfCel9A with fresh and partially digested BC resulted in the formation of the same amount of additional product as that formed in the initial digestion, while continued digestion of the original BC with fresh buffer produced only half as much additional product. This suggests that TfCel9A activity is primarily limited by the substrate-related factors and that the enzyme was not deactivated over the 2-day period during which the assays were run. Similarly, TfCel48A was able to hydrolyze fresh BC much better than the partially digested BC after overnight incubation, indicating that its activity is also primarily limited by substrate-related factors. However, the activity of TfCel48A on fresh BC resulted in only 70% additional product formation after the same incubation time, indicating that some fraction of TfCel48A was deactivated during the initial digestion. Presumably, its deactivation is primarily due to irreversible unproductive binding to the substrate, as this enzyme is generally very stable over time and multiple freeze-thaw cycles.

Sequential addition of synergistically acting cellulases can provide insight into the roles played by synergistic partners in cooperative substrate digestion (9, 26, 27). For this reason, we tested the effect of substrate pretreatment by TfCel48A and TfCel9A on each other’s activities (Fig. 5a). Substrate was incubated with either enzyme overnight, and the reaction mixtures were then boiled to denature the enzymes. The synergistic partner was then added, the reaction mixtures were incubated for 2.25 h, and the results were compared to those of assays in which the reaction mixtures were prepared with fresh BC. The results indicate that predigestion of BC with TfCel9A enhances TfCel48A overnight endpoint activity by ca. 100%. On the other hand, TfCel48A predigestion of BC did not enhance the activity of TfCel9A under the tested conditions. These observations are consistent with other published data that show enhanced exocellulase activity following substrate pretreatment with an endocellulase, but not vice versa (27).

To further understand the role of TfCel48A in its interaction with TfCel9A, we carried out more sequential enzyme addition assays such that the initial digestion with TfCel9A was followed up either with fresh addition of TfCel9A or with addition of TfCel48A followed by TfCel9A (Fig. 5b). The goal was to see whether the intermediate addition of TfCel48A would enhance the activity of the newly added TfCel9A, and this was indeed the case. After overnight incubation with TfCel9A and boiling of the reaction mixtures to denature the enzyme, addition of fresh TfCel9A led to an increase in the extent of BC digestion by less than 2.5 percentage points in 4 h. However, when fresh TfCel9A was added following intermediate overnight incubation with TfCel48A, the extent of BC digestion increased by more than 4 percentage points in 4 h, indicating that TfCel9A activity was enhanced by ca. 60% after the intermediate addition of TfCel48A.

**DISCUSSION**

TfCel48A and TfCel9A are important components of the *T. fusca* biomass-degrading arsenal, and their properties make them incompatible with the classical endocellulase-exocellulase synergism model. To understand the potentially distinct mode of cooperation between these enzymes, we carried out endpoint, time course, and sequential addition BC hydrolysis assays, the results of which allow the following suppositions to be made. When com-

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**FIG 4** Sequential BC addition in endpoint assays. The initial 20-h BC (1 mg) digestion was followed by the addition of fresh BC (1 mg) or buffer to the reaction mixtures. The reaction mixtures were then incubated for another 20 h, and the amount of soluble sugar measured in the reaction mixtures is presented (normalized such that the value of 100 represents the amount of sugar produced after the first overnight incubation with the enzyme). Both enzymes showed enhanced activity when fresh BC was added. The double amount of soluble sugar produced by TfCel9A after the second addition of BC implies that the enzyme was largely dissociated from the original BC fraction, while in the case of TfCel48A, some enzyme may be deactivated by irreversible unproductive binding to the original substrate.

**FIG 5** Effect of sequential additions of TfCel48A and TfCel9A on each other’s activities. (a) BC was pretreated with either TfCel48A or TfCel9A for 20 h, and the reaction mixtures were boiled to denature the enzyme. The synergistic partner was then added, and the reaction mixtures were incubated for 2.25 h. Comparisons of activity on untreated and enzyme-pretreated substrate are provided. For the pretreated reaction mixtures, the provided value was adjusted by subtracting the amount of soluble sugar present in the reaction mixture measured after the pretreatment from the total amount of sugar measured at the end of the assay. (b) Initial TfCel9A (83 nM) digestion was followed by the addition of TfCel9A (83 nM) with or without an intermediate incubation with TfCel48A (750 nM). The intermediate addition of TfCel48A enhanced the activity of the freshly added TfCel9A, as evidenced by the steeper percent digestion profile following incubation with TfCel48A.
Synergistic Cooperation between TfCel9A and TfCel48A

The model of synergism between TfCel48A and TfCel9A proposed here was initiated from the time course data shown in Fig. 1 by comparing equation 1 parameter values obtained for the individual enzymes and their mixture. Additional assays were then carried out to further develop and verify the TfCel48A-TfCel9A synergism model. We believe that this study provides additional support for the utility of our recently developed kinetic model for the mechanistic studies of enzymatic cellulose digestion.

ACKNOWLEDGMENT
This work was supported by the BioEnergy Science Center, a U.S. Department of Energy (DOE) center supported by the Office of Biological and Environmental Research in the DOE Office of Science.

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A comparable model of synergism was proposed by Valjamae et al. (9) for Trichoderma reesei enzymes Cel7A (TrCel7A; CBH I, a processive exocellulase) and Cel7B (TrCel7B; EG I, a nonprocessive endocellulase). In their study of BC hydrolysis by the two cellulates, the authors concluded that the processive action of TrCel7A creates an eroded surface with obstacles that reduce the activity of the enzyme. The modified surface, however, is efficiently hydrolyzed by TrCel7B and allows sustained TrCel7A activity. TfCel9A and TfCel48A, therefore, appear to play similar roles as TrCel7A and TrCel7B, respectively, in the model proposed in the above-described study. It is important to note, however, that the roles of the endo- and exocellulases are reversed in the two models, which suggests that T. fusca and T. reesei may have evolved somewhat different approaches to biomass digestion. If this is indeed the case, it would be interesting to determine whether such differences apply more generally to cellulolytic bacteria and fungi.

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