Insight into the Underlying Synergy between Exo–Lytic Cellulases

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Abstract

Lignocellulosic biomass can be converted into biofuels and biochemicals, through a synergetic degradation by cellulases. The underlying cooperative mechanism is of paramount importance for the development of efficient enzyme cocktails. The synergy between exo–lytic cellulases, however, still remains poorly understood. In the present contribution, we decipher the synergism by investigation of the enzyme–enzyme interactions (EEIs) between two exo–lytic cellulases—*Talaromyces emersonii* Cel7A (*Te*Cel7A) and *Trichoderma reesei* Cel6A (*Tr*Cel6A), and between *Te*Cel7A and other main components in the cocktails, and propose a novel synergistic way. The enzymes are found to be apt to bind around the eight substrate–enclosing loops (SEls) of *Te*Cel7A, of which the *Tr*Cel6A possesses the strongest binding energy with *Te*Cel7A. The combination between *Te*Cel7A and *Tr*Cel6A is further investigated experimentally by Microscale Thermophoresis, confirming the existence of their interactions. Due to the EEIs, the flexibility of the SEls, which mediate dissociation of *Te*Cel7A from cellulose, is increased. We further found that the improved flexibility of loop B3 is pivotal to accelerate dethreading process, helping improving enzymatic hydrolytic efficiency. In view of our theoretical and experimental results here and previous experimental phenomena, the carbohydrate binding modules of the exo–lytic enzymes, which enable them to absorb on the same plane of the substrate, are conjectured to enhance the degree of the EEIs. This work brings to light an underlying synergy between exo–lytic cellulases, and is conducive to a systematic understanding of the synergetic actions in cellulase cocktails.

Introduction

Lignocellulosic biomass can be converted into many value–added products, providing prominent economic and environmental benefits [1–3]. Enzymatic hydrolysis has emerged as one of the most significant technologies for the conversion of the biomass into glucose [4]. To complete hydrolysis of the complex natural substrate, a variety of enzymes with different specificities, such as endo–beta–1,4–glucanases (EGs, EC 3.2.1.4), cellobiohydrolases (CBHs, EC 3.2.1.91) and beta–glucosidases (BGs, EC 3.2.1.21), acting in synergy, are required. How to degrade efficiently the biomass by cellulase cocktails was, therefore, investigated widely to design optimal enzymatic combinations and ratios [5–7].

Cellulose, the most important component of lignocellulose can be degraded gradually into glucose by three steps, i.e., (i) EGs cleave randomly beta–1,4 linkages of the cellulosic chains, producing reducing or non–reducing ends, (ii) CBHs assault the cellulose chain from these ends, yielding cellobiose, and (iii) BGs eventually convert cellobiose to glucoses [8–12]. Furthermore, EG can remove amorphous–unordered substrate areas, hence helping cellobiohydrolase I (CBHI or Cel7A) interact well with cellulosic substrate [13–15]. In the work of Ng et al., BG was found to alleviate the inhibition of cellobiose on CBHI [16]. This sort of synergism, resulting from the positive effect of EG or BG on the degradation of cellulose by CBHI was widely recognized [17].

According to the study of Trudeau et al., substantial cooperation between exo–lytic enzymes, i.e., CBHI and cellobiohydrolase II (CBHII or Cel6A) also exists [14], whereas the mechanism is poorly understood.
The rate of glycoside hydrolase family 7 (GH7) CBHI leaving cellulose after catalysis, namely dissociation rate is known to be the rate–limiting step when employed alone [22–29]. The high flexibility of the substrate–enclosing loops (SELS, Fig. 1) has been proved to accelerate enzymatic dissociation, improving the yield of cellobiose [30, 31]. Furthermore, it has been reported that the activity of cellulase can be increased or decreased by the structural changes due to the association with other proteins or ligands [32–34]. It is, therefore, reasonable to believe that the cooperation between the two exo–lytic cellulases may arise from the influence of CBHII on the flexibility of SELs in CBHI by enzyme–enzyme interactions (EEIs).

GH7 CBHIs are one of the most abundant enzymes in native fungal secretions [35, 36]. The structure and function of these enzymes, especially Trichoderma reesei (T. reesei) CBHI and Talaromyces emersonii CBHI (TeCel7A) have been deciphered clearly by crystallography and molecular dynamics (MD) simulations [37–40], making them excellent model systems. In the present work, the relevant molecular–level synergetic mechanism of exo–lytic cellulases–TeCel7A and T. reesei CBHII (TrCel6A) was deciphered using docking, MD simulations and Microscale Thermophoresis (MST) experiment. In brief, this work provides useful insight into the synergetic actions between cellulases.

Results And Discussion

Enzyme–Enzyme Docking and Interactional Energy

HADDOCK [41] was employed to investigate the EEIs of TeCel7A with TrCel6A, EG, BG and TeCel7A itself, respectively. Table 1 shows the docking results for these assemblies, wherein TeCel7A–EG processes the best Z–score. Binding affinity (ΔG) and the dissociation constant (Kd) were then measured by PRODIGY [42]. TeCel7A–TrCel6A features the strongest affinity of −17.2 kcal/mol. These results suggest that the two enzymes can interact powerfully, mainly owing to their flexible SELs (see Fig. 1). However, TeCel7A is not apt to bind with itself in view of the lowest HADDOCK score, Z–Score and binding affinity of TeCel7A–TeCel7A assembly, compared with the other enzyme–enzyme complexes.

|                        | TeCel7A–TrCel6A | TeCel7A–EG | TeCel7A–BG | TeCel7A–TeCel7A |
|------------------------|-----------------|------------|------------|----------------|
| HADDOCK score          | −57.3 ± 11.5    | −92.9 ± 3.8| −59.3 ± 7.8| −40.7 ± 3.9    |
| Z–Score                | −2.1            | −2.7       | −1.9       | −0.8           |
| ΔG (kcal/mol)          | −17.2           | −12.7      | −15.3      | −10.5          |

On the basis of protein–protein docking, multi–microsecond MD simulations were then employed to decipher their underlying mechanism in detail. The four initial structures of TeCel7A, TrCel6A, EG, BG and the four protein–protein complexes of TeCel7A–TrCel6A, TeCel7A–EG, TeCel7A–BG and TeCel7A–TeCel7A were sufficiently relaxed in an explicit water environment and each followed by a 500 ns MD
simulation, except for *Te*Cel7A–*Tr*Cel6A, which followed by 1.5 µs. The evolution of the center-of-mass (COM) distance between the two proteins in each complex structure (Fig. S1) suggests that the well-equilibrated states have been reached. As reported in Table 2, the binding free energies calculated by the MM-PBSA method indicate that the two exo–lytic cellulases possess the strongest binding free energy among the four enzyme-enzyme assemblies. In view of results of docking and MD simulations, we therefore suggest that *Tr*Cel6A is the most promising one to influence the hydrolytic function of *Te*Cel7A.

### Table 2

| System          | ΔG   | ΔE<sub>MM</sub><sup>b</sup> | ΔG<sub>PB</sub> | ΔG<sub>SA</sub> |
|-----------------|------|-----------------------------|----------------|----------------|
| *Te*Cel7A-*Tr*Cel6A | -23.4 | -1232.2                   | -3207.8         | -2.1           |
| *Te*Cel7A-EG    | -7.8 | -519.6                    | -996.1          | -48.4          |
| *Te*Cel7A-BG    | -16.2 | -158.0                    | -3076.4         | -14.8          |
| *Te*Cel7A-*Te*Cel7A | -14.6 | -284.1                    | -2562.7         | -69.1          |

*All quantities are in kcal mol<sup>-1</sup>.*  
<sup>b</sup> ΔE<sub>MM</sub> energy includes the intermolecular noncovalent interactions and the change of the conformational energies.

### Binding Modes and Interaction Interface of *Te*Cel7A–*Tr*Cel6A

As depicted in Fig. 2a, the optimal docking pose for *Te*Cel7A–*Tr*Cel6A indicates that the two exo–lytic cellulases bind with each other with their SELs. Furthermore, the other enzymes were found to combine to the region around SELs of *Te*Cel7A as well (Fig. S2). Eight hydrophobic residues in *Te*Cel7A (Ala375, Ala376, Met378, Leu379, Ala389, Ile396, Ala397 and Val407) were found to locate at the interactional interfaces of the complexes (Fig. 2b). It is well known that the unproductive adsorption caused by the binding of hydrophobic sites between cellulases and lignin is one of the main limiting factors in the enzymatic hydrolysis [23, 43]. EEIs can properly prevent cellulases from the adsorption of lignin residues, thereby contributing to increasing the efficiency of enzymatic hydrolysis.

### Influence of *Tr*Cel6A on Flexibility and Catalytic Region of *Te*Cel7A

As depicted in Fig. 3a, the interactional interface of *Te*Cel7A–*Tr*Cel6A is changed after 1.5 µs MD simulation, like that of other enzyme-enzyme complexes (Fig. S3). The phenomena indicate that this nonspecific association between enzymes cannot always sustain. Since the enzymes should work on their exclusive substrates by traditional synergetic forms, we, therefore, suggest that the separation of the enzyme-enzyme complexes after EEIs is beneficial to hydrolysis cycle. Figure 3b shows the root mean square fluctuation (RMSF) values of the residues in *Te*Cel7A with and without combining *Tr*Cel6A. It is apparent that flexibility of the loops A1, B1, B2 and B3 is improved significantly by EEIs. In the enzymatic hydrolysis, improved flexibility of the SELs will accelerate dissociation – the rate–limiting step of GH7 CBHI degrading cellulose alone, increasing the yield of cellobiose.
It is acknowledged that the initial binding of TeCel7A to cellulose is certainly influenced by the opening of SELs [44, 45]. As shown in Fig. 4a, the cross-sectional area (CSA) formed by the center of mass (COM) distances of loops B2, B3 and A3 were measured. Figure 4b indicates that the CSA of TeCel7A is expanded notably by association with TrCel6A. Before the degradation of lignocellulose by TeCel7A, the EEIs between the two exo-lytic cellulases in hydrolytic system will help the initial association of TeCel7A with cellulose. Andersen et al. have reported that the highest synergy was observed at the beginning of hydrolysis [46], consistent with our theoretical analysis.

**Dethreading Process**

Vermaas et al. have reported that dethreading is the predominant mechanism for dissociation of GH7 CBHI from cellulose [30]. We, therefore, speculate that highly extent of synergy between the two exo-lytic cellulases occurs in dethreading process – the longest time-consuming action of GH7 CBHI degrading cellulose. As shown in Fig. 5a, loops A1, B1 and B2 absorb on the surface of cellulose before dissociation. The notably improved flexibility of these three loops after EEIs (see Fig. 3b) can enhance motility of CBHI, helping the enzyme leave the surface of substrate. The results of Vermaas et al. further indicate that −1 to −3 transition is the rate-limiting step for dethreading, especially −1 to −2 [30]. As illustrated in Fig. 5b, many hydrogen bonds are formed between −1 glucosyl ring and the enzyme, influencing the rate of dethreading significantly. Acceleration of dethreading was deemed to depend on modulating interactions around the −1 state [30]. Loop B3, closest to the −1 site is, therefore, the most promising SEL to influence dethreading. In view of the improved flexibility of loop B3 (see Fig. 3b) after EEIs, it will interact with −1 glucosyl ring frequently, facilitating motion of the substrate. We, therefore, suggest that the key to the synergy of exo-lytic cellulases is to improve the flexibility of loop B3, thus accelerating dethreading.

**Existence of EEIs and the Role of Carbohydrate Binding Module (CBM)**

To verify the existence of combination between exo-lytic cellulases experimentally, MST experiment were performed, characterizing the binding of TeCel7A to TrCel6A. The relevant result indicates that the interactions of TeCel7A with TrCel6A exist, but weak (Kd = 140 ± 60 µM, see Fig. 6). In view of the result of Fig. 3a and the corresponding theoretical analysis, the weak combination between exo-lytic cellulases is conjectured to be reasonable. The degree of cooperation between them is, therefore, suggested to depend on the frequency of EEIs. In the study of Badino et al., the synergetic extent of the two exo-lytic cellulases achieved the highest level when both the two enzymes possess CBMs [21]. In addition, their synergy was decreased step by step if one or both of them do not have CBMs [21]. We conjecture that CBMs can enable the two exo-lytic cellulases to locate at the surface of cellulose to interact adequately, enhancing the degree of EEIs. Overall, the synergy between the exo-lytic cellulases can arise from increased flexibility of the key loops in TeCel7A by EEIs, degree of which is improved by their CBMs.
Conclusions

In the present work, we deciphered the molecular-level mechanism of synergy between exo-lytic cellulases by docking, microsecond-time scale MD simulations and MST experiment. Flexibility of SELs in CBHI mediates its dissociation from cellulose, and highly flexible SELs, especially loop B3, induced by EEIs is the crucial cause for the synergy between exo-lytic cellulases. Furthermore, EEIs can also decrease unproductive adsorption and open catalytic domain, help improve the efficiency of CBHI. CBMs which enable the exo-lytic cellulases to interact frequently, enhance the degree of their synergy. Put together, this work represents a step forward in the understanding of the synergistic actions in enzymatic hydrolysis, and is expected to contribute to the more efficient degradation of lignocellulose by cellulase cocktails.

Experimental

Docking and Simulation Details

The crystal structures of EG (PDB code 1KS5), TeCel7A (PDB code 3PFX) and TrCel6A (PDB code 1HGW, in which A175 was mutated back to Asp) were obtained from protein data bank (PDB). BG, secreted by Penicillium piceum, was homologically modeled by SWISS-MODEL (swissmodel.expasy.org) [47–51]. The templates, PDB codes 3ZYZ and 4IIB, have the highest homology with the target BG. The protein–protein dockings of TeCel7A–TrCel6A, TeCel7A–EG, TeCel7A–BG and TeCel7A–TeCel7A were carried out by HADDOCK to obtain the optimal binding poses.

MD simulations were then carried out for the four individual enzymes and the four protein–protein complexes. All the assemblies were solvated in an equilibrated box of water separately. The overall charge neutrality was achieved by adding Na\(^+\) ions to the solution. Each molecular system before production simulations underwent i) 5000 steps minimization and 100 ps MD simulation with protein restrained, and ii) 5 ns of water equilibration without restrained. The interactions of the four assemblies were investigated by performing a total of 6 \(\mu\)s MD simulations. The details of the molecular assemblies are provided in Table S1. The scalable program NAMD 2.13 [52] with the CHARMM36 force field [53–55], and the TIP3P water model [56] were used to perform the MD simulations. Visualization and analysis of all the MD trajectories were carried out with the VMD program [57]. Binding free energies of these complexes using MM-PBSA method were calculated as described in our previous work [33].

MST Experiment

TeCel7A and TrCel6A were expressed and purified as described in our previous work [40]. TrCel6A was labeled with NT-647-NHS (NanoTemper Technologies GmbH, Munich, Germany) at a dye-to-protein ratio of 3:1 and purified with a 1 mL Pierce® Desalting Column (Thermo Scientific) using citrate buffer (10 mM Sodium Citrate, 100 mM NaCl, 0.05% Tween 20, pH 5.0) as the buffer. Capillaries were loaded with 20 nM NT-647-NHS labeled TrCel6A and 16 concentrations of TeCel7A from 12.4 nM to 407 \(\mu\)M in citrate buffer (10 mM Sodium Citrate, 100 mM NaCl, 0.05% Tween 20, pH 5.0). MST measurements were taken using
Monolith NT.115 (NanoTemper Technologies GmbH, Munich, Germany) at room temperature. The data was handled using Software Manual MO. Affinity Analysis and MO. Control.

Declarations

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Conflict of Interest

The authors declare that they have no conflict of interest.

Availability of data and materials

Simulation details, center-of-mass distance of two proteins in each complex, optimal docking poses and final states after MD simulations of TeCel7A–EG, TeCel7A–BG and TeCel7A–TeCel7A are available in Supplementary Information.

Code availability

Not applicable.

Author’s Contributions

WSC, XGS ZYH and ZYZ designed the research. ZYZ and QYL did the simulations and experiment, respectively. WSC and ZYZ wrote the manuscript and analyzed the data.

Ethical Approval

Not applicable.
Consent to participate

The manuscript has not been published previously by any of the authors and is not under consideration for publication in another journal at the time of submission.

Consent for publication

All authors have read the manuscript and approved to submit to this journal.

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