Enzymatic hydrolysis of cellulosic materials using synthetic mixtures of purified cellulases bioengineered at *N*-glycosylation sites

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Received: 22 May 2018 / Accepted: 1 September 2018 / Published online: 5 September 2018
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Abstract

Mutant forms of recombinant endoglucanase II (EG II, N194A), cellobiohydrolase I (CBH I, N45A) and cellobiohydrolase II (CBH II, N219A) from *Penicillium verruculosum* with enhanced cellulase activities, achieved by engineering of enzyme *N*-glycosylation sites in our previous studies, were used as components of the binary and ternary mixtures of cellulases in hydrolysis of Avicel and milled aspen wood. Using the engineered forms of the enzymes at a dosage of 10 mg/g substrate resulted in significant boosting of the glucose release from cellulose in the presence of excess β -glucosidase relative to the performance of the corresponding wild-type mixtures at the same loading. The boosting effects reached 11–40% depending on the reaction time and substrate type. In hydrolysis of both cellulosic substrates by the binary mixtures of cellulases, all the enzyme pairs exhibited synergism. The magnitude of the synergistic effects (K_s) did not depend notably upon the induced mutations in the enzymes, and they were in the range of 1.3–1.8 for the combinations of EG II with CBH I (or CBH II), and 2.3–2.9 for the CBH I–CBH II pair. The results of this study should provide a basis for the development of a more effective fungal strain capable of producing cellulase cocktails with enhanced hydrolytic performance against lignocellulosic materials.

Keywords Cellulase · Protein engineering · *N*-glycosylation · Enzymatic hydrolysis of cellulose · Boosting effect · Synergism

Introduction

The production of second-generation biofuels from plant biomass has become a reality in the last few years, and the number of industrial bioconversion processes based on renewable lignocellulosic feedstocks will continue to grow (Gusakov 2013; dos Santos et al. 2016). Cellulases (cellobiohydrolases and endo-1,4- β -glucanases) are key enzymes used in lignocellulose bioconversion, and therefore, finding new more active cellulases or improving the properties of the already known enzymes by bioengineering techniques remains an important challenge for researchers working in

this field (Bommarius et al. 2014; Trudeau et al. 2014; Payne et al. 2015).

One of the recent and unconventional approaches for enhancing the cellulase activity is based on engineering the *N*-glycosylation sites in the enzyme molecule. In the case of cellobiohydrolase I (CBH I) from *Trichoderma reesei* and *Penicillium funiculosum*, belonging to family 7 of glycoside hydrolases (GH7), removing an existing or introducing a new *N*-glycosylation motif in a certain position of the protein globule by site-directed mutagenesis boosted the sugar production in cellulose hydrolysis up to 70% (Adney et al. 2009). Using a similar approach, we have previously obtained the mutant forms of recombinant CBH I (rPvCel7A), CBH II (rPvCel6A) and EG II (rPvCel5A) from *Penicillium verruculosum* with enhanced cellulase activities (Dotsenko et al. 2016a, b; Gusakov et al. 2017). However, it should be noted that the *N*-glycans removal may sometimes cause a detrimental effect on the enzyme activity (Dotsenko et al. 2016a; Gusakov et al. 2017). Mechanistic models summarizing the influence (positive or negative) of *N*-linked oligosaccharides on the activity of cellobiohydrolases from

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the GH6 and GH7 families were presented in our previous publication (Gusakov et al. 2017).

Testing the performance of synthetic mixtures of individual cellulases to estimate the potential of multienzyme cocktails for enzymatic saccharification of lignocellulosic feedstocks proved to be a useful instrument in such kind of research (Gusakov et al. 2007; Billard et al. 2012; Trudeau et al. 2014). In this paper, we used the engineered forms of recombinant CBH I (N45A), CBH II (N219A) and EG II (N194A) from *P. verruculosum* with the highest specific activities (Dotsenko et al. 2016a, b; Gusakov et al. 2017) as components of the binary and ternary cellulase mixtures in hydrolysis of Avicel and milled aspen wood and compared the performance of the corresponding reference wild-type enzyme mixtures. The synergistic effects between the components were studied as well.

Materials and methods

Enzymes

Recombinant wild-type and mutant forms of *P. verruculosum* CBH I (rPvCel7A), CBH II (rPvCel6A) and EG II (EG IIa, rPvCel5A) were expressed in *Penicillium canescens* PCA10 (*niaD*[−]) strain and purified as described elsewhere (Dotsenko et al. 2016a, b; Gusakov et al. 2017). The engineered forms of the enzymes showing the highest specific activities against cellulosic substrates (Avicel, CMC) were used as components of binary and ternary cellulase mixtures; they were: N45A mutant of CBH I, N219A mutant of CBH II, N194A mutant of EG II. Purified β -glucosidase (BGL) from *Aspergillus niger* was isolated as described elsewhere (Korotkova et al. 2009).

Enzymatic hydrolysis of cellulosic substrates

Microcrystalline cellulose (Avicel PH-101 from Sigma, St. Louis, MO, USA) and milled aspen wood pretreated as described elsewhere (Volkov et al. 2014) were used as substrate in the hydrolysis experiments by binary and ternary mixtures of cellulases.

Hydrolysis of the cellulosic materials was carried out in 2-mL test tubes at 40 °C and pH 4.5 (0.1 M Na-acetate buffer) with constant stirring (1000 rpm) on a thermostatic shaker TS-100 (Biosan, Latvia). The substrate concentration in the reaction mixture was 5 g/L. In all cases, the dosage of binary and ternary mixtures of cellulases was 10 mg of total protein per 1 g of substrate. Protein was measured using the Lowry assay (Peterson 1979). To prevent the inhibition of cellobiohydrolases by cellobiose and to convert all the oligosaccharides formed into glucose, excess of purified BGL from *A. niger* was added to the reaction mixture at

the concentration of 0.015 mg/mL. To prevent microbial contamination in the reaction system, 1 mM sodium azide was added. After 24, 48 and 72 h of hydrolysis, 0.1 mL samples of the reaction mixture were taken and centrifuged for 2 min at 13,400g, and then the concentration of glucose was determined in the supernatant by the glucose oxidase—peroxidase assay using a Fotoglucose kit (Impact Ltd., Russia); calibration was carried out using D-glucose (Reakhim, Russia). The experiments were performed in triplicate.

Calculation of the coefficient of synergism

The coefficient of synergism (K_s) for the binary mixtures of cellulases was calculated using the following formula:

$$K_s = [\text{Glc}]_{1+2} / (X \times [\text{Glc}]_1 + (1 - X) \times [\text{Glc}]_2),$$

where $[\text{Glc}]_{1+2}$ is the concentration of glucose after a certain time of cellulose hydrolysis (24, 48 or 72 h) by the binary combination of enzyme 1 and enzyme 2 at the total dosage of 10 mg/g substrate; $[\text{Glc}]_1$ is the concentration of glucose after the same hydrolysis time by the enzyme 1 at the dosage of 10 mg/g substrate; $[\text{Glc}]_2$ is the concentration of glucose after the same hydrolysis time by the enzyme 2 at the dosage of 10 mg/g substrate; X is the mass fraction of the enzyme 1 in the binary mixture (in all cases the cellulose hydrolysis was carried out with added excess of BGL).

Results

Cellulose hydrolysis by binary mixtures of recombinant cellulases

First, the hydrolysis of Avicel and milled aspen wood was carried out with binary mixtures of the engineered forms of recombinant *P. verruculosum* cellulases (N194A mutant of EG II, N45A mutant of CBH I, N219A mutant of CBH II) and those of wild-type. The enzyme mass fraction in the mixtures varied from 0 to 100% with a stepwise increment of 20% while maintaining the total loading of cellulases at the level of 10 mg protein per gram of substrate. The experiments were carried out for 72 h, every 24 h a sample was taken and the glucose concentration was determined.

The Avicel hydrolysis by both engineered and wild-type EG II–CBH I or EG II–CBH II combinations showed maximum conversion yields to glucose when the CBH mass fraction was 100%, although the product yields after different reaction times in the mixture containing 80% of CBH I were rather close to those maximum values (Fig. 1). This may be explained by the fact that Avicel, a highly crystalline substrate, is a specific substrate for cellobiohydrolases, while being much more resistant to the action of EG II (Morozova et al. 2010). However, since Avicel contains

Fig. 1 Glucose release from Avicel (5 g/L) as a result of the synergistic action of the wild-type (a) and engineered (b) cellulases from *P. verruculosum* used in binary mixtures at the total protein loading

some amorphous cellulose in its structure, up to 30–35% (Sinityn et al. 1991), susceptible to the action of endoglucanases, a synergism between EG II and CBH I (or CBH II) was observed (Fig. 1), as it is evidenced by the difference between experimental curves and theoretical straight lines. This difference is the result of generating new cellulose chain ends, which are catalytic target sites for CBHs. All EG II–CBH I (CBH II) combinations tested showed maximum synergistic effects when the CBH mass fraction in the binary mixture was 20%: the K_s for engineered and wild-type EG II–CBH I mixtures was 1.8 ± 0.1 , while for EG II–CBH II mixtures it was 1.3 ± 0.1 .

In the case of the engineered and wild-type CBH I–CBH II mixtures, the highest glucose yields from Avicel were observed when the mass ratio between the enzymes was 6:4 (Fig. 1), that is, both cellobiohydrolases had similar importance in the hydrolysis of this substrate. The maximum synergism was observed when the CBH II mass fraction in the binary mixture was 80%. The K_s decreased with increasing the reaction time, and it was 2.85–2.86, 2.77–2.80 and 2.55–2.56 for 24, 48 and 72 h, respectively.

Using the engineered forms of cellulases in the binary mixtures provided 29–38% increase in the concentration of glucose obtained in Avicel hydrolysis after a different reaction time relative to the compositions based on the

of 10 mg/g substrate, 40 °C, pH 4.5. Purified BGL from *A. niger* was added in excess to the reaction system at 0.015 mg/mL concentration

wild-type enzymes (Table 1). It should be noted that the boosting effects shown in the table are given for the enzyme pairs exhibiting not the highest synergism but the highest glucose yields. These effects did not depend significantly upon the time of hydrolysis, and they were the greatest for EG II–CBH I and CBH I–CBH II combinations (35–38%).

In hydrolysis of pretreated aspen wood by engineered and wild-type EG II–CBH I mixtures, the maximum yields of glucose were obtained when the CBH I mass fraction was 60%, while in the case of EG II–CBH II combinations the optimum CBH II content was 80% (Fig. 2). For binary CBH I–CBH II compositions, the highest glucose concentrations were achieved when the mass fraction of CBH II was 60%.

The maximum synergistic effects during the hydrolysis of aspen wood by engineered and wild-type EG II–CBH I combinations were observed at the CBH I content of 60% ($K_s = 1.7 \pm 0.2$), while for the EG II–CBH II mixtures, the maximum synergism was observed at the CBH II content of 40–60% ($K_s = 1.4 \pm 0.1$). The highest synergistic effects for mixtures based on cellobiohydrolases were observed when the mass ratio between CBH I and CBH II was 6:4, that is, at a lower content of CBH II compared to Avicel hydrolysis; the K_s varied in the range of 2.27–2.53. However, in general, the synergistic effects for enzyme pairs in the hydrolysis of both substrates were rather similar.

Table 1 Boosting effects achieved with binary mixtures of engineered *P. verruculosum* cellulases in hydrolysis of cellulosic substrates

Substrate	Enzyme mass fraction (%)			Boosting effect (%) ^a		
	EG II	CBH I	CBH II	24 h	48 h	72 h
Avicel	20	80	—	38 ± 2	37 ± 3	35 ± 2
	20	—	80	29 ± 2	30 ± 2	32 ± 2
	—	60	40	38 ± 3	37 ± 3	36 ± 2
Aspen wood	40	60	—	35 ± 3	38 ± 4	40 ± 3
	20	—	80	25 ± 2	28 ± 3	33 ± 3
	—	40	60	18 ± 2	26 ± 3	35 ± 3

^aIncrease in the glucose concentration for a mixture of engineered cellulases relative to the glucose concentration obtained with a mixture based on wild-type enzymes

Fig. 2 Glucose release from milled aspen wood (5 g/L) as a result of the synergistic action of the wild-type (a) and engineered (b) cellulases from *P. verruculosum* used in binary mixtures at the total pro-

tein loading of 10 mg/g substrate, 40 °C, pH 4.5. Purified BGL from *A. niger* was added in excess to the reaction system at 0.015 mg/mL concentration

The boosting effects achieved for the binary mixtures of engineered cellulases in the hydrolysis of aspen wood varied in the range of 18–40% depending on the enzyme pair and hydrolysis time (Table 1). In general, the boosting effects became more pronounced with an increase in the reaction time, and they were the highest for combinations of EG II–CBH I (40%) and CBH I–CBH II (35%) after 72 h of hydrolysis.

Cellulose hydrolysis by ternary mixtures of recombinant cellulases

Using an approach similar to that for the binary mixtures of cellulases (see above), the hydrolysis of Avicel and milled

aspen wood was carried out under the action of ternary compositions of engineered and wild-type EG II, CBH I and CBH II (in the presence of excess *A. niger* BGL). The results are shown in Figs. 3 and 4, where points inside the triangles correspond to the ternary mixtures.

In hydrolysis of Avicel (Fig. 3), two of the most efficient ternary mixtures based on both wild-type and engineered enzymes had the mass ratios between EG II, CBH I and CBH II of 2:4:4 or 2:6:2, and this was valid for all reaction times (24–72 h). Although it should be noted that the glucose yields for the mentioned ternary mixtures were rather similar to the product yields demonstrated by the best binary compositions of CBH I and CBH II. This was

Fig. 3 Ternary diagrams showing the glucose release from Avicel (5 g/L) under the action of the wild-type (**a**) and engineered (**b**) cellulases from *P. verruculosum* used in mixtures at the total protein load-

ing of 10 mg/g substrate in the presence of excess BGL (0.015 mg/mL), 40 °C, pH 4.5. Different colors correspond to glucose concentration levels (in g/L) shown on the left

due to the already mentioned fact that the contribution of an endoglucanase (EG II) to hydrolysis of Avicel was much lower in comparison to that for cellobiohydrolases.

In hydrolysis of milled aspen wood (Fig. 4), one of the best ternary mixtures had the same mass ratio between EG II, CBH I and CBH II (2:4:4) like in the case of Avicel. The second optimal composition had a ratio between the components of 2:2:6, that is, the lignocellulosic substrate basically demanded the higher content of CBH II in the mixture, relative to Avicel. Unlike the Avicel case, in the hydrolysis of aspen wood, the best binary mixtures based on only cellobiohydrolases could not compete with ternary mixtures of the optimal composition, containing 20% of EG II.

When compared with compositions based on the wild-type cellulases, mixtures of the engineered enzymes, having the same ratios between the components, yielded 31–38% and 11–28% increase in the glucose concentration obtained from Avicel and aspen wood, respectively (Table 2). In hydrolysis of Avicel, the magnitude of the boosting effect slightly decreased with increase in the reaction time from 24 to 72 h, while in the case of the lignocellulosic substrate, an opposite tendency took place and the highest boosting effect for two engineered mixtures (27–28%) was observed after 72 h of hydrolysis.

Discussion

Elimination of N-linked glycans in certain positions of the recombinant CBH I, CBH II and EG II of *P. verruculosum* by applying Asn to Ala mutations at the respective N-glycosylation sites has previously been shown to be an effective technique for enhancing their activity against cellulosic substrates (Dotsenko et al. 2016a, b; Gusakov et al. 2017). Using binary and ternary mixtures of the engineered forms of the mentioned cellulases in hydrolysis of Avicel cellulose and a lignocellulosic material (pretreated aspen wood) resulted in significant boosting of the glucose formation in the presence of extra added BGL compared to the mixtures of the wild-type enzymes of the same composition (Tables 1, 2). Unlike in the case of Avicel, in hydrolysis of aspen wood the magnitude of the boosting effect became greater with an increase in the reaction time from 24 to 72 h, and the maximum (40%) effect was observed for a binary mixture containing 40% of EG II and 60% of CBH I. Two best ternary mixtures of the engineered cellulases provided 27–28% increase in the glucose concentration obtained from the lignocellulosic substrate after 72 h of hydrolysis. The higher boosting effect of engineered enzymes is more important at longer hydrolysis times in the case of aspen wood, but not Avicel, because the first one, unlike the second model

Fig. 4 Ternary diagrams showing the glucose release from milled aspen wood (5 g/L) under the action of the wild-type (a) and engineered (b) cellulases from *P. verruculosum* used in mixtures at the

total protein loading of 10 mg/g substrate in the presence of excess BGL (0.015 mg/mL), 40 °C, pH 4.5. Different colors correspond to glucose concentration levels (in g/L) shown on the left

Table 2 Boosting effects achieved with ternary mixtures of engineered *P. verruculosum* cellulases in hydrolysis of cellulosic substrates

Substrate	Enzyme mass fraction (%)			Boosting effect (%) ^a		
	EG II	CBH I	CBH II	24 h	48 h	72 h
Avicel	20	40	40	38 ± 3	33 ± 3	31 ± 2
	20	60	20	38 ± 3	34 ± 2	33 ± 2
Aspen wood	20	40	40	14 ± 2	22 ± 3	28 ± 3
	20	20	60	11 ± 2	21 ± 2	27 ± 3

^aIncrease in the glucose concentration for a mixture of engineered cellulases relative to the glucose concentration obtained with a mixture based on wild-type enzymes

substrate, is an example of a real renewable lignocellulosic feedstock that may be used in bioconversion processes, in which the longer reaction time provides the higher concentration of glucose.

In hydrolysis of both cellulosic substrates by the binary mixtures of cellulases, all the enzyme pairs exhibited synergism. The magnitude of the synergistic effects (K_s) did not depend notably upon the induced mutations in the enzymes. For engineered and wild-type EG II–CBH II combinations, the K_s varied in the range of 1.3–1.4, while for the mixtures based on EG II–CBH I the K_s was slightly higher (1.7–1.8). This kind of synergism was the result of a well-known mechanism when new cellulose chain ends formed by EG II represented the starting points for

the processive action of cellobiohydrolases (van Dyk and Pletschke 2012; Payne et al. 2015). The higher synergistic effects have also been reported for the mixture of EG V (Cel45A) with CBH I (Cel7A) in comparison with the combination of EG V–CBH II (Cel6A) from *Humicola insolens*, and the lower synergism in the second case was explained by the occasional endo-character of the CBH II (Boisset et al. 2001). The same explanation may also be applied for our studies, since the *P. verruculosum* CBH II, unlike the CBH I, exhibits some minor activity against barley β -glucan and carboxymethylcellulose (CMC) (Morozova et al. 2010), including the activity assay by viscometry that reflects the endo-depolymerase action of cellulases.

The binary mixtures based on *P. verruculosum* CBH I–CBH II displayed the highest degree of synergism in the hydrolysis of both substrates ($K_s = 2.3\text{--}2.9$). Such kind of exo–exo synergism has also been reported for pairs of CBH I–CBH II from *T. reesei* acting on crystalline cellulose III (more than twofold effect) (Igarashi et al. 2011), *Myceliophthora thermophila* (formerly *Chrysosporium lucknowense*) acting on cotton ($K_s = 2.75$) (Gusakov et al. 2007) and *H. insolens* acting on bacterial cellulose (a synergy higher than fourfold was observed at the Cel7A/Cel6A ratio of 1:2) (Boisset et al. 2001). Although the exo–exo synergism may also partially be explained by the occasional endo-character of the CBH II, more likely it was caused by the ultrastructural heterogeneity of the substrates (Boisset et al. 2001). Using a metric to quantify binding-target arrangements determined by photoactivated localization microscopy, Fox et al. (2013) showed that combinations of cellulases binding within similar but nonidentical morphologies can have synergistic activity. CBH I and CBH II initiating their attack on cellulose from different ends of a polysaccharide molecule can also mutually enhance the enzyme mobility during its processive movement along the cellulose surface by removing structural obstacles for this movement, analogous to relieving a traffic jam (Igarashi et al. 2011).

In the Avicel hydrolysis experiments by mixtures containing EG II and either CBH I or CBH II, the EG II was not essential if taken into consideration only the glucose yields at varying reaction times (Fig. 1). In most cases, the highest glucose yields were observed when the CBH I (or CBH II) was present alone (with extra added BGL) in the reaction system. However, in the hydrolysis of milled aspen wood the role of EG II has increased, and the maximum concentrations of glucose were achieved when the EG II content in the binary mixture was 20% (in combination with CBH II) or 40% (in combination with CBH I). Compared to Avicel, the pretreated lignocellulosic material was more susceptible to the action of an endoglucanase and so the contribution of EG II to the hydrolysis of this substrate increased. The lower need for EG II in the mixture with CBH II may again be explained by the occasional endo-action and the higher activity of the latter enzyme against aspen wood relative to CBH I.

In hydrolysis of Avicel and milled aspen wood, one of the most efficient ternary mixtures, based on both wild-type and engineered EG II, CBH I and CBH II, had the ratio between the components 2:4:4 (Figs. 3, 4; Table 2). This ratio is rather close to the proportion of total content of endoglucanases from GH5, GH7 and GH12 families (14% by protein) to the CBH I (35%) and CBH II (33%) content in the culture filtrate of the reference high-cellulase *P. verruculosum* B221-151 strain (the rest 18% of protein represented BGL and other non-cellulolytic enzymes) (Morozova et al. 2010). These data indicate that the fungus *P. verruculosum*

secretes a multienzyme cocktail with a ratio between cellulase components close to optimal, although the optimal ratio may vary depending on the substrate, as data presented in Figs. 3 and 4 show. In particular, the second efficient ternary mixture in Avicel hydrolysis had the ratio between EG II, CBH I and CBH II of 2:6:2, while the respective second mixture in the hydrolysis of milled aspen wood had a higher content of the CBH II (2:2:6 ratio).

In the experiments described in this report, we used recombinant wild-type cellulases and those subjected to protein engineering at *N*-glycosylation sites, all of them being expressed in the *P. canescens* fungal host. Thus, the next task should be an assembly of the engineered EG II, CBH I and CBH II with enhanced cellulase activities back in the initial *P. verruculosum* fungus. Although the expression of a single enzyme encoded by a mutated gene in the native producing strain is not a problem, as our previous studies showed (Volkov et al. 2014; Sinitsyn et al. 2016), secreting an optimal composition of a few engineered proteins into a multienzyme cocktail may become a more sophisticated task. However, the boosting effects that can be obtained in the hydrolysis of a lignocellulosic feedstock by a composition of the engineered cellulases deserve the efforts spent on achieving this goal. Using fusion constructs containing connected in series target genes, controlled by a single inducible gene promoter (Proskurina et al. 2013), is one of the approaches that may be applied for this purpose. Even if the secretion of the optimized ternary assembly of the engineered cellulases would not be achieved by one improved fungal strain, still the construction of multiple fungal strains with a single engineered cellulase in each followed by fermentation and proportional blending of the resulting broths may become an alternative approach to developing more efficient enzyme blends.

Conclusion

The engineered forms of recombinant EG II, CBH I and CBH II from *P. verruculosum*, in which *N*-glycans at a certain position of the protein globule have been removed by site-directed mutagenesis, provided up to 40% boosting in glucose release from microcrystalline cellulose and a lignocellulosic substrate when used as binary or ternary mixtures in the presence of extra added BGL (compared to the mixtures of the wild-type enzymes of the same composition at the same total protein loading). The synergistic effects (K_s) between the enzyme pairs in hydrolysis of both substrates did not depend notably upon the induced mutations, and they were in the range of 1.3–1.8 for the combinations of EG II with CBH I (or CBH II), and 2.3–2.9 for the CBH I–CBH II pair. The ratio of cellulase components (EG II/CBH I/CBH II) in one of the most efficient ternary mixtures was

rather similar to the ratio of total endoglucanase content to the CBH I and CBH II contents in a multienzyme preparation produced by a reference high-cellulase strain of *P. verruculosum* fungus. The results of this study should provide a basis for the development of a more effective fungal strain capable of producing cellulase cocktails with the enhanced hydrolytic performance against lignocellulosic materials.

Acknowledgements This work was supported by the Russian Science Foundation (Grant No. 16-14-00163).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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