Enzyme and Microbial Technology 115 (2018) 52–61

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/enzmictec

The spatial proximity effect of beta-glucosidase and cellulosomes on cellulose degradation

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ARTICLE INFO

Keywords:
Clostridium thermocellum
Proximity
Beta-glucosidase
Cellulosome
Cellulose degradation

ABSTRACT

Low-cost saccharification is one of the key bottlenecks hampering the further application of lignocellulosic biomass. *Clostridium thermocellum* is a naturally ideal cellulose degrading bacterium armed with cellulosomes, which are multienzyme complexes that are capable of efficiently degrading cellulose. However, under controlled condition, the inhibition effect of hydrolysate cellobiose severely restricts the hydrolytic ability of cellulosomes. Although the addition of beta-glucosidase (Bgl) could effectively relieve this inhibition, the spatial proximity effect of Bgl and cellulosomes on cellulose degradation is still unclear. To address this issue, free Bgl from *Caldicellulosiruptor* sp. F32 (*Ca*BglA), carbohydrate-binding module (CBM) fused *Ca*BglA (*Ca*BglA-CBM) and cellulosomal type II cohesin module (CohII) fused to *Ca*BglA (*Ca*BglA-CohII) were successfully constructed, and their enzymatic activities, binding abilities and saccharification efficiencies were systematically investigated *in vitro* and *in vivo*. *In vivo*, with the adjacency of *Ca*BglA to cellulosomes, the saccharification efficiency of microcrystalline cellulose increased from 40% to 50%. For the pretreated wheat straw, the degradation rate of the combination of cells and the *Ca*BglA-CohII or the *Ca*BglA-CBM was as efficient as that of the free *Ca*BglA (approximately 60%). This study demonstrated that the proximity of *Ca*BglA to cellulosomes had a positive effect on microcrystalline cellulose but not on pretreated wheat straw, which may result from the nonproductive adsorption of lignin and the decreased thermostability of *Ca*BglA-CBM and *Ca*BglA-CohII compared to that of *Ca*BglA. The above results will contribute to the design of cost-effective Bgls for industrial cellulose degradation.

1. Introduction

Lignocellulosic biomass is the largest renewable organic resource in nature. The sustainability and bioavailability of this biomass make it a promising feedstock for producing biofuels and biochemicals. However, its natural recalcitrance is the biggest barrier hampering cost-effective lignocellulose deconstruction [1–3]. *Clostridium thermocellum* has been demonstrated to be the most efficient biomass degrading microorganism on earth and bears abundant multienzyme complexes that are designated cellulosomes [4–9]. However, the feedstock inhibition from the end-product cellobiose severely limits the hydrolytic ability of cellulosomes, and thus hampers further saccharification of the cellulose [10,11].

Noticeably, the cell-free cellulosomes bind to the insoluble substrate, where the hydrolysate cellobiose accumulates near the interface of the cellulosomes, and thus, only a small part of the soluble Bgl can be involved to immediately convert cellobiose into glucose, which indicates that the synergistic effects of the cellulosomes and the free Bgl were negligible [12]. Therefore, a suitable spatial distance between cellulosomes and Bgl seems to be an important factor that affects the efficiency of cellulose degradation. Previous studies developed efficient biological saccharification strategies, such as *in vitro* by coupling the purified cellulosomes with a chimeric CohII-fuse Bgl [12] or *in vivo* by using the wild type of *C. thermocellum* cultures supplemented with the CBM-fused Bgl [13]. Recently, we constructed a recombinant strain of *C. thermocellum* Δ*pyrF:Ca*BglA making the *Ca*BglA as a part of the

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https://doi.org/10.1016/j.enzmictec.2018.04.009
Received 12 January 2018; Received in revised form 30 March 2018; Accepted 23 April 2018
Available online 01 May 2018
0141-0229/

cellulosome [14]. The recombinant strain could produce a secretory exoglucanase Cel48S-bearing Bgl (*Ca*BglA) and could be used as an efficient whole-cell catalyst to enhance both the cellulose-degrading ability and sugar productivity without the extra adding of Bgl [14]. These strategies have been performed on the combination of Bgl and cellulosomes to enhance the efficiency of cellulose degradation and saccharification. However, it is still difficult to assess which strategy is optimal due to the usage of different Bgls and the combination system. The objective of this study was to systematically assess whether the spatial proximity effect of Bgls and cellulosomes would enhance cellulose degradation and moreover determine which type of combination system would enhance cellulose degradation the most.

To match the culture conditions of *C. thermocellum*, the thermotolerant Bgl from *Caldicellulosiruptor* sp. F32 (*Ca*BglA), which has been demonstrated to have an optimum temperature of 75 °C and a half-life of up to 48 h at 75 °C [15], was selected for this study. A CBM3a domain and a type II cohesin domain (cellulosomal type II dockerin-binding module) were fused to *Ca*BglA to generate new domain-added enzymes with the ability to bind cellulose (via *Ca*BglA-CBM) or the cellulosomes (via *Ca*BglA-CohII) directly, respectively. Then, the enzyme activity and binding ability of *Ca*BglA, *Ca*BglA-CBM and *Ca*BglA-CohII were investigated. Moreover, to evaluate the spatial proximity of *Ca*BglA to the cellulosomes during cellulose degradation, the hydrolysis efficiency of microcrystalline cellulose and pretreated wheat straw were also investigated. This study will contribute to the design of cost-effective Bgls for industrial cellulose degradation.

2. Materials and methods

2.1. Bacterial strains and cultivation

Caldicellulosiruptor sp. strain F32 (CGMCC 1.5183; China General Microbiological Collection Center, Beijing, China) was cultured at 75 °C anaerobically in GS-2 medium. *C. thermocellum* strain DSM1313 was cultured at 55 °C anaerobically in modified GS-2 medium (KH_2PO_4 1.5 g, $K_2HPO_4{\cdot}3H_2O$ 2.1 g, urea 2.1 g, $MgCl_2{\cdot}6H_2O$ 1.0 g, $CaCl_2{\cdot}2H_2O$ 150 mg, $FeSO_4{\cdot}6H_2O$ 1.25 mg, cysteine-HCl 1 g, MOPS-Na 10 g, yeast extract 6.0 g, trisodium citrate·$2H_2O$ 3.0 g, resazurin 0.1 mg/L, and pH 7.4) [16]. Avicel (5 g/L) was used as the sole carbon source. *Escherichia coli* strains were grown in Luria-Bertani medium (LB) with shaking (200 rpm) at 37 °C. Antibiotics were added to the medium when required: kanamycin, 50 μg/mL. *E. coli* DH5α (Takamorra Bio, Shiga, Japan), *E. coli* BL21 (DE3), and plasmid pET-28a (+) (Merck KGaA, Darmstadt, Germany) served as the cloning host, expression host, and vector, respectively.

2.2. Cellulosomes preparation

Cells of *C. thermocellum* DSM 1313 were cultured for 2 days using GS-2 media, and then the cellulosomes were purified via an affinity purification method [16,17]. In short, the cells of *C. thermocellum* DSM1313 were cultured in GS-2 medium with 5 g/L of Avicel as the sole carbon resource for 2 days at 55 °C. The supernatants were collected and then incubated overnight with 17 mg of phosphoric acid swollen amorphous cellulose at 4 °C. Then, the pellets were collected and resuspended with 10 mL of 50 mM of Tris-HCl buffer (pH 7.0) containing 10 mM of $CaCl_2$ and 5 mM of dithiothreitol, and they were then dialyzed against 1 L of sterile distilled water for at least 8 h. After the phosphoric acid swollen amorphous cellulose was dissolved, the supernatants were concentrated to 1 mL by ultrafiltration (100 kD, Sartorius Stedim Biotech) and analyzed by SDS-PAGE.

2.3. Cloning, expression and purification

The *Caldicellulosiruptor* sp. F32 genome sequence and annotation data were obtained from GenBank (accession number is APGP00000000) DNA encoding Bgl (the GenBank accession number is JX030398) was amplified from *Caldicellulosiruptor* sp. F32 genomic DNA by PCR using 5′ **CTAGCT AGCATGAGTTTCCCAAAAGGATTTTTG**, 5′GGAATTC**CATATG**ATGAGTT TCCCAAAAGGATTTTTG, 3′CCG**CTCGAG**TTACGAATTTTCCTTTATATAC TGCTG and 3′CGG**ACTAGT**CGAATTTTCCTTTATATACTGCTG (*Nhe*I, *Nde*I, *Xho*I and *Spe*I sites in boldface type, respectively). A DNA encoding linker from CipA was amplified from *C. thermocellum* DSM 1313 genomic DNA by PCR using 5′GGAATTC**CATATG**GGAGATACAACAGAACCTG and 3′CGG**ACTAGT**CCTTACTGCATCCAGATCAT (*Nde*I and *Spe*I sites in boldface type). DNA encoding CBM3a from the CipA anchoring protein was amplified from *C. thermocellum* DSM 1313 genomic DNA by PCR using 5′ GGAATTC**CATATG**ACGCCCACCAGGCCATC and 3′CGG**ACTAGT**CTGTGT TGATGGTACTAC AC (*Nde*I and *Spe*I sites in boldface type). The DNA encoding type −II cohesin module from SdbA anchoring protein was amplified from *C. thermocellum* DSM 1313 genomic DNA by PCR using 5′ CTA**GCTAGC**GATAAAGCCTCGAGC and 3′ AAGGAAAAAA**GCGGCCGC**A TCCGGCTGTATT AC (*Nhe*I and *Not*I sites in boldface type). The PCR amplified *Bgl* gene was digested by *Nhe*I/*Xho*I and ligated into pET28a, which resulted in the final vector pBgl. The PCR amplified Bgl and linker gene were digested by *Nde*I/*Spe*I and ligated into pET28aNS [18] and resulted in the two vectors pBglN and pLinker; these two vectors were then digested (using *Spe*I/*Bam*HI and *Nhe*I/*Bam*HI, respectively) and ligated into pET28aNS, which resulted in the vector pBgl-linker. The PCR amplified CBM3a, CohII, the vectors of pBglN and pBgl-linker were digested (using *Nhe*I/*Xho*I, *Nhe*I/*Not*I, *Spe*I/*Xho*I, and *Spe*I/*Not*I, respectively) and ligated, which resulted in the final vectors pBglN-CBM3a and pBgl-linker-CohII. Expression of the proteins was achieved by adding isopropyl-β-D-thiogalactopyranoside (IPTG, 0.3 mM of final concentration) to mid-exponential phase cultures of *E. coli* BL21 (DE3) that harbored target plasmids with incubation for an additional 4 h at 37 °C. Then, the cells were harvested by centrifugation at 5000*g*, 4 °C for 30 min, and the cell pellets were resuspended in 10 mL of buffer (containing 20 mM of phosphate sodium, pH 8.0, 500 mM of NaCl, and 30 mM of imidazole) and disrupted by a high-pressure cell disruptor and sonication. The supernatant was purified on a His trap™ HP (GE healthcare) column and eluted with a linear gradient to 500 mM of imidazole. The fractions were collected and concentrated for later use as purified enzymes. The yields of purified Bgl (defined as *Ca*BglA), Bgl-CBM3a (defined as *Ca*BglA-CBM) and L-Bgl-linker-CohII (defined as *Ca*BglA-CohII) were 34.5, 35.5 and 29.5 mg per L of culture medium, respectively.

2.4. Enzymatic activity assay

The optimal growth temperature of *C. thermocellum* DSM 1313 is 55 °C. In order to consistent with this temperature, the activity of Bgls was tested under the same temperature rather than its optimal temperature (75 °C).

The enzymatic activity of *Ca*BglA, *Ca*BglA-CBM and *Ca*BglA-CohII was measured using 4-nitrophenyl-β-D- galactopyranoside (*p*NPG, Solarbio) as a substrate. The enzymatic activity was performed at 55 °C, and 0.01 μg samples were incubated for 5 min within 100 μL of a solution containing 4 mM of *pNPG* and 50 mM of citrate buffer (pH 5.5). The reaction was terminated by adding 150 μL 1 M Na_2CO_3 and the absorbance at 405 nm was measured. One unit of enzyme activity was defined as the amount of enzyme to hydrolyze 1 μmol *p*NPG and to liberate 1 μmol *p*NP per min. The thermostability was measured as the ratio between the retaining activity and the initial activity was measured using *p*NPG after 4, 8, 12 and 16 days of treatment at 55 °C. The initial activity was defined as 100%. SDS-PAGE was also used to visualize the enzymatic thermostability. The glucose inhibition was determined by adding 0–1000 mM of glucose to the standard reaction mixture at 55 °C for 5 min with *p*NPG as the substrate. The glucose inhibition was defined as the glucose concentration required to inhibit 50% of the initial enzymatic activity. The optimal pH value was measured at 55 °C for 5 min in 50 mM citrate buffer from pH 4.0–6.8. The kinetic constant was determined by measuring the initial rates at