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Novel cellulase recycling method using a combination of *Clostridium thermocellum* cellulosomes and *Thermoanaerobacter brockii* β-glucosidase

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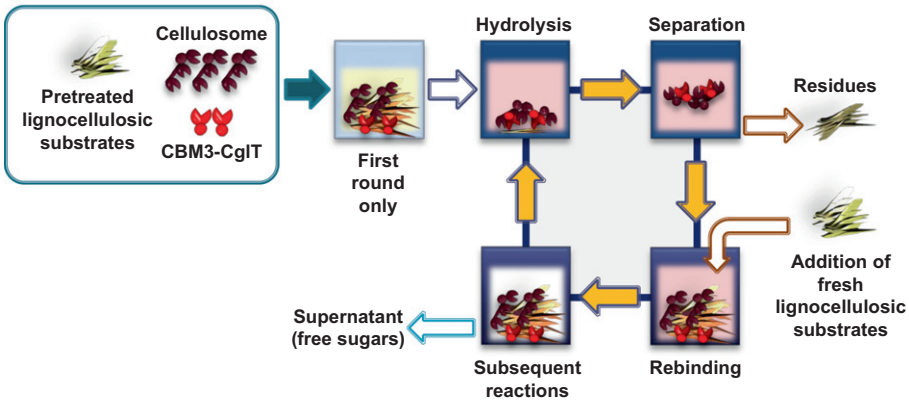
HIGHLIGHTS

- CBM3–CglT was created for enzyme recycling using *C. thermocellum* cellulosomes.
- Cellulosomes and CBM3–CglT recycled with high cellulose degradation ability.
- Cellulosomes and CBM3–CglT effectively recycled ammonia pretreated rice straw.
- Delignification of pretreated rice straw improved the recycling rounds.
- This enzyme recycling system has great potential.

GRAPHICAL ABSTRACT



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ABSTRACT

This report describes a novel recycling method utilizing a combination of *Clostridium thermocellum* cellulosomes and *Thermoanaerobacter brockii* β-glucosidase (CglT). To recover cellulosomes and CglT through re-binding to additional cellulose, a chimeric CBM3–CglT was created by fusing carbohydrate binding module (CBM3) from the scaffolding protein CipA into the N-terminal region of CglT. When a recycling test using cellulosomes and CBM3–CglT was performed on microcrystalline cellulose, the process was capable of 4 rounds of recycling (1% w/v cellulose/round). Although irreversible absorption of cellulosomes and CBM3–CglT into the residues was observed when ammonia-pretreated rice straw and delignified rice straw was used as substrates, a maximum of 2 and 4 recycling rounds (1% w/v glucan/round) were achieved, respectively, consistent with a 70% saccharification rate. This novel recycling method using cellulosomes and CBM3–CglT has great potential as an effective lignocellulose degradation system.

1. Introduction

Lignocellulose consists of three major polymers, cellulose, hemicellulose and lignin, and is expected to be utilized as an abundant renewable resource. However, the plant cell wall is difficult to hydrolyze because the cellulose is surrounded by a lignin seal that is covalently associated with hemicellulose, and cellulose can occur as a tightly packed crystalline structure (Harris and Stone, 2009). Thus, the rate-limiting step in lignocellulose conversion to useful end products, such as bioethanol, is the hydrolysis of cellulose and hemicellulose polymers to sugars.

Among cellulolytic microorganisms, *Clostridium thermocellum*, an anaerobic, thermophilic, and spore-forming bacterium, is the

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most potent cellulose-degrading bacterium known to produce multienzyme complexes, cellulosomes (Bayer et al., 2004; Demain et al., 2005). The cellulosome (2.0–3.5 MDa) of *C. thermocellum* consists of the large (197 kDa), non-catalytic, multimodular scaffolding protein CipA, which includes nine cohesins, four hydrophilic modules, and a family 3 carbohydrate-binding module (CBM3). The catalytic units are non-covalently attached to scaffolding via high-affinity type I interactions between dockerin domains of the catalytic units and cohesins on the scaffolding (Bayer et al., 2004). Recently, genome sequencing efforts have identified more than 70 dockerin-containing cellulases and hemicellulases in the genome of *C. thermocellum* ATCC27405 (Demain et al., 2005). Thus, the cellulosome of *C. thermocellum* provides for a surprisingly large variety of enzymes and obviously attractive enzymatic properties for the degradation of complex plant biomass. In a previous study (Waeonukul et al., 2012), it has been demonstrated drastic improvements in the cellulolytic activities of cellulosomes from *C. thermocellum* S14, in combination with β-glucosidase (CglT) from *Thermoanaerobacter brockii* (Breves et al., 1997). The combination of cellulosomes and β-glucosidase exhibited effective saccharification ability for ammonia-soaked rice straw, with an enzyme loading 1/10 the amount of a combination of fungal cellulases to achieve the same saccharification level, strongly indicating that this combination system has great potential as an alternative cellulolytic system to fungal cellulases.

The commercial production of chemicals and fuels from lignocellulosic biomass by enzymatic means continues to require considerable research in terms of both technical and economic aspects. Although the cellulase system of the filamentous fungus *Trichoderma reesei* is one of the most effective for the hydrolysis of cellulosic materials, cellulase enzymes continue to be one of the major costs associated with the hydrolysis process (Gnansounou and Dauriat, 2010; Nguyen and Saddler, 1991). Recycling of cellulases has been explored as an effective way of reducing the high cost of enzymes, and recycling strategies using *T. reesei* cellulases have been reported (Girard and Converse, 1993; Qi et al., 2011; Ramos et al., 1993). In *T. reesei* cellulases, endoglucanases (EG I, EG II, EG III, and EG V), cellobiohydrolases (CBH I and CBH II) and xylanases (XYN I, XYNII, XYNIII, and XYNIV) are individually secreted, and act synergistically to degrade lignocellulose. On the other hand, the *C. thermocellum* cellulosome is organized as a macromolecular machine of cellulases and hemicellulases via the scaffolding protein CipA. It appears that a recycling system using cellulosomes simplifies the enzyme recovery process required for lignocellulose degradation, compared to systems using *T. reesei* cellulases, since it may be unnecessary to account for the recovery of each cellulosome component due to the complexation of cellulases and hemicellulases.

This paper describes a novel cellulase recycling method using a combination of *C. thermocellum* cellulosomes and *T. brockii* β-glucosidase (CglT). To recover cellulosomes and CglT through re-binding to cellulose input, it constructed CBM–CglT, through the fusion of CBM3 (from CipA) to the N-terminal regions of CglT, thereby allowing the synchronization of cellulosomes and Cg1T. Here, the combination of cellulosomes and CBM3–CglT was recovered, with high hydrolysis ability, from the hydrolysate of microcrystalline cellulose and pretreated rice straw. This recycling method, using a combination of cellulosomes and CBM3–CglT, will contribute to establishing a cost-effective lignocellulose saccharification process, as a result of reduced enzyme loading.

2. Methods

2.1. Organisms, media, and growth conditions

The hyper-cellulolytic strain *C. thermocellum* S14 has been deposited with the National Institute of Technology and Evaluation Patent Microorganisms Depositary (NPMD; Chiba, Japan) as NITEP-627. *T. brockii* ATCC33075 was obtained from the American Type Culture Collection (Manassas, VA, USA). *C. thermocellum* S14 was grown on BM7CO medium (Tachaapaikoon et al., 2012). The medium was supplemented with 10 g/L microcrystalline cellulose powder (Sigmacell type-20; Sigma–Aldrich, St. Louis, MO, USA). *T. brockii* was grown in modified DSMZ 122 medium (Waeonukul et al., 2012). The medium was adjusted to pH 7.0 and supplemented with 5 g/L cellobiose as the carbon source. All BM7CO and DSMZ 122 media were degassed in boiling water and bubbled with high purity carbon dioxide gas. *Escherichia coli* DH5α (Takara Bio, Shiga, Japan), BL21 (DE3), and plasmid pET19b (Merck KGaA, Darmstadt, Germany) served as the cloning host, expression host, and vector, respectively. *E. coli* cells were grown at 37 °C in Luria–Bertani (LB) medium containing ampicillin (50 μg/mL).

2.2. Preparation of cellulosomes, recombinant CglT, and CBM3 fused CglT (CBM–CglT)

Cellulosomes were prepared from cell-free broths using *C. thermocellum* S14 culture grown in BM7CO medium supplemented with 1% (w/v) microcrystalline cellulose for 4 days (late stationary phase) at 60 °C and recovered by the affinity digestion method, as described previously (Morag et al., 1992; Waeonukul et al., 2012). Preparation of chromosomal and plasmid DNA and transformation were carried out by standard procedures or according to supplier protocols (Qiagen, Frederick, MD, USA). The previously constructed plasmid pET19CglT was used to prepare recombinant CglT (Waeonukul et al., 2012). The carbohydrate binding module (CBM3) from *C. thermocellum* scaffolding protein CipA was combined with the N-terminal region of the β-glucosidase gene (*cglT*) of *T. brockii* using the overlap extension PCR technique (Nelson and Fitch, 2011). Four primers (sense primer-1; 5′-CGCGGATCCGGTTGGCAATGCAACACCG-3′, antisense primer-1; 5′-ATTCGGATCATCTGACGGCGGTATTGTTGT-3′, sense primer-2; 5′-AACGAAATCTCTTGGAAATTTTGCATTCGGATCATCTGACGGCGG-3′, antisense primer-2; 5′-ATTGCTCAGCATCTTCGATACCATCATC-3′) containing artificial restriction enzyme recognition sites (BamHI and Bpu1102), and overlapping sites between CBM3 of CipA and cglT were designed to amplify CBM3 (sense primer-1 and antisense primer-1) and cglT (sense primer-2 and antisense primer-2) gene fragments by PCR using *C. thermocellum* and *T. brockii* genomic DNA as a template, respectively. PCR was performed with Ex Taq polymerase (Takara Bio) under standard conditions according to the manufacturer's instructions. The amplified CBM3 and cglT gene fragments were purified using a PCR purification kit (Qiagen). Overlap extension PCR was performed to create the CBM3 fused cglT gene fragment with sense primer-1 and antisense primer-2 using each fragment as the DNA template. The amplified chimeric fragment CBM3–cglT was inserted between the BamHI and Bpu1102 sites of pET19b to generate pET19CBM3–CglT. The CBM3–CglT protein was purified using a nickel affinity column chromatography (Ni-NTA agarose resins) and desalted using a desalting column. The molecular mass of purified CBM3–CglT (75 kDa) was confirmed on SDS–PAGE, demonstrating the increased size expected for the fusion of CBM3 (20 kDa) and CglT (55 kDa).

2.3. Enzyme and protein assays

The assay for recombinant β-glucosidase was performed at 60 °C in 0.1 M sodium acetate buffer (pH 6.0) containing 5 mM $CaCl_2$ under static conditions for 10 min (Waeonukul et al., 2012). Determination of β-glucosidase activity was based on measurement of the release of *p*-nitrophenol from *p*-nitrophenyl β-D-glucoside (pNPG) (Sigma–Aldrich). One unit of enzyme releases