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Mini-scaffoldin enhanced mini-cellulosome hydrolysis performance on low-accessibility cellulose (Avicel) more than on high-accessibility amorphous cellulose

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ABSTRACT

A glycoside hydrolase family 5 *Bacillus subtilis* endoglucanase, a family 9 *Clostridium thermocellum* processive endoglucanase, and a family 48 *Clostridium phytofermentans* ISDg cellobiohydrolase were assembled together by the high-affinity interaction between three cohesins in a mini-scaffoldin (mini-CipA) and dockerins in three cellulases, forming the mini-cellulosome. This mini-cellulosome exhibited enhanced hydrolytic activity on low-accessibility cellulose (microcrystalline cellulose, Avicel) and high accessibility cellulose (regenerated amorphous cellulose, RAC) as compared to the non-complexed cellulase mixture at the same enzyme amount. The stimulation factors (SF, i.e., activity ratios of the mini-cellulosome to the non-complexed cellulase mixture) were larger on Avicel than on RAC regardless of substrate/enzyme ratios. Also, SF increased when substrate/enzyme ratio increased. The different hydrolysis patterns of the mini-cellulosome and cellulase mixture on Avicel and RAC suggested that the construction of synthetic cellulosomes would be an efficient way to significantly enhance cellulose hydrolysis rate and digestibility, especially in the case of low-accessibility recalcitrant cellulose at low enzyme usage.

1. Introduction

Lignocellulosic biomass is the most abundant renewable bioresource [1]. The utilization of a small fraction of collectable low-cost cellulosic materials, including crop residues (e.g., corn stover) as well as dedicated bioenergy grass and wood, would produce a significant fraction of sustainable transportation biofuel so that it would decrease reliance on crude oil, enhance energy security, and decrease net greenhouse gas emissions [2]. Cost-effective sugar release from recalcitrant lignocellulose, however, remains challenging [3,4]. During biomass saccharification, low mass-specific activity cellulase results in a large use of cellulase [5]. The weight ratio of substrate to enzyme (i.e., [S]/[E]) for cellulose hydrolysis is at least one order of magnitude higher than that for starch hydrolysis, resulting in higher enzyme cost [5]. Therefore, increasing specific activity of cellulase would reduce enzyme usage so to enhance overall economy of biomass-based biorefineries. Mass-specific activity of cellulase can be enhanced by several approaches: improvement in individual components by directed evolution [6,7] and rational design [8], reconstitution of non-complexed cellulase cocktails [1,9,10], and construction of complexed cellulases (called synthetic cellulosomes) [11–18].

Enzymatic hydrolysis of cellulose requires synergetic action among endoglucanase (EG, EC 3.2.1.4), cellobiohydrolase (CBH) (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). In the *Trichoderma* fungal cellulase system, the dominant components are EG I (Cel7B) and III (Cel5A), cellobiohydrolase I (Cel7A) and cellobiohydrolase II (Cel6A), suggesting that these components play a central role in hydrolyzing cellulose. EG cuts accessible β-1,4-glucosidic bond of cellulose chains randomly. CBH I and CBH II act on reducing end and non-reducing end, respectively. It is thought that the respective cellulase component in bacterial cellulase system are glycoside hydrolase family 5 endoglucanase Cel5, family 48 cellobiohydrolase (Cel48) acting on reducing end, and family 9 processive endoglucanase (Cel9) acting on non-reducing end [10].

Anaerobic bacteria and fungi often produce complexed cellulases – cellulosomes, whose catalytic units are linked by non-hydrolytic scaffoldins [19–22]. Inspired by natural cellulosomes, Bayer and his coworkers have proposed to construct designer cellulosomes with tailored subunit components through the high-affinity interaction between cohesins and dockerins [23].

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A number of building blocks, including cohesins, dockerins, catalytic modules (e.g., cellulases and hemicellulases), carbohydrate binding modules (CBMs), and linkers, have been reassembled into various designer cellulosomes in vitro [11–15]. However, most of these designer cellulosome studies focused on the assembly of different cellulosomes and demonstrated enhanced synergy due to scaffoldins, few studies attempted to investigate the ratios of substrate to enzyme on the stimulation effects of cellulosomes on different substrates in that decreasing cellulase usage (i.e., mg cellulase per g of cellulose) is vital to cost-effective sugar release from pretreated cellulosic materials [5]. Only Fierobe et al. reported that the stimulation effects of mini-cellulosomes on Avicel and bacterial cellulose, and found that stimulation effects were inversely related to substrate concentration at a fixed cellulase concentration [15]. It was vital and interesting to study the in-depth relationship between different action mode cellulase components linked by scaffoldins or not, and cellulose characteristics.

Substrate accessibility to cellulase may be the most important substrate characteristic impacting enzymatic cellulose hydrolysis, as compared to degree of polymerization (DP), crystallinity, pore volume, particle size, and so on [4,10,24]. Microcrystalline cellulose (Avicel) is made from wood pulp by acid hydrolysis that can remove most amorphous cellulose and all hemicellulose, but it still contains a significant fraction of amorphous cellulose [10,25]. Regenerated amorphous cellulose (RAC) is prepared from Avicel through a series of steps: cellulose slurrying in water, cellulose dissolution in concentrated phosphoric acid, and regeneration in water [26]. As a result, RAC, a completely disordered insoluble substrate, has approximately 20 times cellulose accessibility of that of Avicel [10,27], while it has the same DP when ice-cold concentrated phosphoric acid is used [28]. Avicel and RAC represent two extreme model cellulosic materials featuring very low and very high substrate accessibility, respectively [10]. Most pretreated cellulosic materials produced by dilute acid pretreatment, steam explosion, hot water, cellulose solvent-based lignocellulose fractionation, soaking in aqueous ammonia have substrate accessibility falling between those of Avicel and RAC [4,10]. Therefore, the study of cellulosome and non-complexed cellulase hydrolysis on these two model cellulosic materials at different [S]/[E] ratios would help to develop advanced enzyme systems so as to decrease cellulase usage.

In this study, we assembled the designer mini-cellulosome containing three bacterial cellulases – an endoglucanase Cel5, a processive endoglucanase Cel9, and a cellobiohydrolase Cel48. Family 5 endoglucanase (BsCel5), family 9 processive endoglucanase (CtCel9) and family 48 cellobiohydrolase (CpCel48) were obtained from *Bacillus subtilis* [7], *Clostridium thermocellum* [10] and *Clostridium phytofermentans* ISDg [29], respectively. A mini-scaffoldin (mini-CipA) containing three type I cohesins and one family 3 CBM was truncated from *C. thermocellum* CipA [19,30,31]. The stimulation effects of this designer mini-cellulosome were investigated on two model substrates at different [S]/[E] ratios compared to the non-complexed mixture.

2. Materials and methods

2.1. Chemicals

All chemicals were reagent grade or higher, purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Microcrystalline cellulose – Avicel PH105 (20 μm) – was purchased from FMC (Philadelphia, PA). RAC was prepared from Avicel as previously described [26]. The oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA).

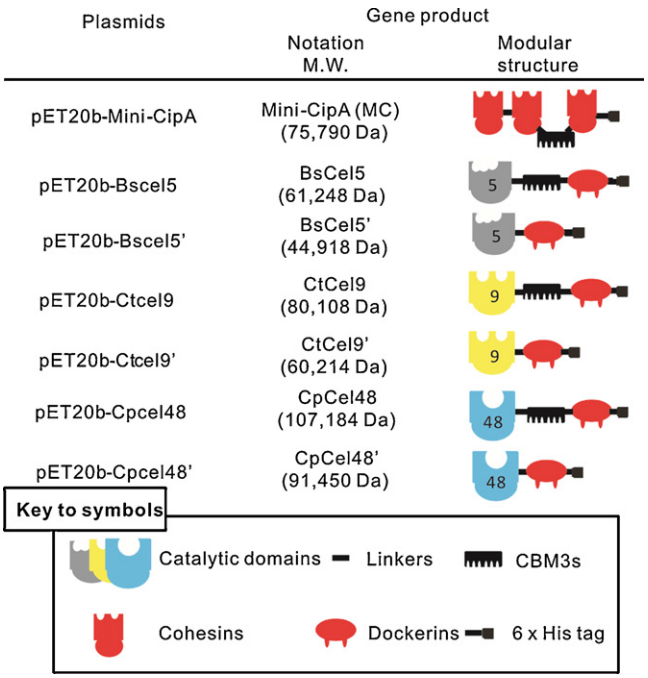

Fig. 1. Schematic representation of the recombinant proteins used in this study.

2.2. Strains and medium

Escherichia coli JM109 was used as a host cell for DNA manipulation, and *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA) was used as a host cell for recombinant protein expression. The Luria–Bertani (LB) medium was used for *E. coli* cell culture and recombinant protein expression. Ampicillin (100 μg/mL) was added in the LB medium.

2.3. Construction of plasmids

The sequences of all PCR primers used are listed in Table 1. Plasmids and recombinant proteins are summarized in Fig. 1. All plasmid sequences were verified by DNA sequencing. The DNA sequence encoding the truncated mini-CipA (26–723 amino acids, GenBank Accession number: L08665) was amplified based on the genomic DNA of *C. thermocellum* ATCC 27405 by a primer pair of mini-CipA_For and mini-CipA_Rev. The PCR product was digested with NdeI/XhoI and ligated into NdeI/XhoI-digested pET20b (Novagen, Madison, WI), yielding pET20b-mini-CipA. The dockerin module was added to the C-terminus of Cpcel48 by overlap-extension PCR. The DNA encoding the catalytic domains of CpCel48 (GenBank Accession number: ABX43721) with or without CBM were amplified from pP43N-Cpcel48 [29] by two primer pairs of CpCel48_For and CpCel48_Rev as well as CpCel48_For and CpCel48′_Rev, respectively. The DNA fragment encoding a dockerin domain (DocS, 673–741 amino acids) from CtCelS (GenBank Accession number: L06942) was amplified from the genomic DNA of *C. thermocellum* by a primer pair of DocS_For and DocS_Rev. The two resultant overlapping fragments were mixed as the template for the next round PCR by a primer pair of CpCel48_For/DocS_Rev. These resultant fragments were cloned into NdeI/XhoI-digested pET20b, thereby generating pET20b-Cpcel48 and pET20b-Cpcel48′. pET20b-Ctcel9 and pET20b-Ctcel9′ were also obtained using PCR amplification and overlap-extension polymerase chain reaction method. The DNA encoding the mature CtCel9 (28–739 amino acids, GenBank Accession number: CAA43035) was amplified from the genomic DNA