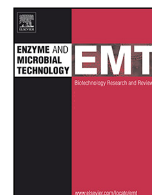
Enzyme and Microbial Technology 93 (2016) 113–121

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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



Characterization of a *Cellulomonas fimi* exoglucanase/xylanase-endoglucanase gene fusion which improves microbial degradation of cellulosic biomass

Kwabena O. Duedu [a,b,*], Christopher E. French [a]

[a] Institute of Quantitative Biology, Biochemistry and Biotechnology, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3FF, UK
[b] Department of Biomedical Sciences, School of Basic & Biomedical Sciences, University of Health & Allied Sciences, Ho, Ghana

ARTICLE INFO

Article history:
Received 24 May 2016
Received in revised form 4 August 2016
Accepted 5 August 2016
Available online 8 August 2016

Keywords:
Biomass conversion
Multicatalytic cellulases
Cellulose
Citrobacter freundii
Cellulomonas fimi

ABSTRACT

Effective degradation of cellulose requires multiple classes of enzyme working together. However, naturally occurring cellulases with multiple catalytic domains seem to be rather rare in known cellulose-degrading organisms. A fusion protein made from *Cellulomonas fimi exo-* and *endo-* glucanases, Cex and CenA which improves breakdown of cellulose is described. A homologous carbohydrate binding module (CBM-2) present in both glucanases was fused to give a fusion protein CxnA. CxnA or unfused constructs (Cex + CenA, Cex, or CenA) were expressed in *Escherichia coli* and *Citrobacter freundii*. The latter recombinant strains were cultured at the expense of cellulose filter paper. The expressed CxnA had both *exo-* and *endo-* glucanase activities. It was also exported to the supernatant as were the non-fused proteins. In addition, the hybrid CBM from the fusion could bind to microcrystalline cellulose. Growth of *C. freundii* expressing CxnA was superior to that of cells expressing the unfused proteins. Physical degradation of filter paper was also faster with the cells expressing fusion protein than the other constructs. Our results show that fusion proteins with multiple catalytic domains can improve the efficiency of cellulose degradation. Such fusion proteins could potentially substitute cloning of multiple enzymes as well as improving product yields.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

About 75% of the total biomass of lignocellulosic materials is made of cellulose and hemicellulose, making cellulose the most suitable feedstock for production of biofuels and renewable feedstock chemicals [1–3]. Cellulose can be degraded naturally and used as a source of energy by various bacteria and fungi. Such organisms produce *endo*-β-1,4-glucanases (EC 3.2.1.4), *exo*-β-1,4-glucanases or cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) [4,5]. The combined action of these enzymes results in saccharification of cellulose. Endoglucanases and cellobiohydrolases break the long cellulose into short-chain units (cellobiose and other short oligonucleotidesaccharides) which are hydrolysed by β-glucosidases into glucose [4,6]. The endoglucanases and cellobiohydrolases usually consist of two functional units, the catalytic domain (CD) which hydrolyses the β-1,4-glycosidic bonds in cellulose and a non-catalytic CBM which binds to cellulose. In addition to their substrate binding function, CBMs have been shown to improve the enzymatic activity of their cellulases. CBMs may loosen individual cellulose chains from the cellulose surface prior to hydrolysis or enhance the solubilisation of individual glucan chains of the cellulose surface. In addition, some CBMs also serve as thermostabilizing domains [7–10].

The cellulases of *Cellulomonas fimi* are well studied, and the genome has been sequenced [11]. Two of the best studied cellulases of *C. fimi* are the endoglucanase CenA and the bifunctional exoglucanase/xylanase Cex. The N-terminal family-2 CBM of CenA, unlike those of other endoglucanases, has been shown to disrupt the structure of cellulose fibres, resulting in the release of fine cellulose particles without any detectable hydrolytic activity [12]. Cex possesses a homologous CBM at its C-terminus. The N-terminal GH10 catalytic domain of Cex hydrolyses cellulose and cellotetraose to release cellobiose from non-reducing chain ends [13,14], and has also been shown to possess endoglucanase activity as well as activity against xylan [15–17]. In both CenA and Cex, the CBM is linked to the respective catalytic domain by a proline-threonine (PT) linker. Whist the PT linker acts as a flexible tether, the CBM anchors the catalytic domains onto amorphous, semi-crystalline and crystalline cellulose chains enabling the catalytic domains to hydrolyse nearby

* Corresponding author at: Department of Biomedical Sciences, School of Basic & Biomedical Sciences, University of Health and Allued Sciences, PMB 31, Ho, Ghana.
E-mail addresses: koduedu@gmail.com, kduedu@uhas.edu.gh (K.O. Duedu), c.french@ed.ac.uk (C.E. French).

http://dx.doi.org/10.1016/j.enzmictec.2016.08.005
0141-0229/© 2016 Elsevier Inc. All rights reserved.

cellulose chains [18–22]. In addition the CBM initiates mobility and facilitates the diffusion of the catalytic domain [23].

Gene fusion is an essential tool in systems and synthetic biology, and has been widely used in the production of synthetic bifunctional enzymes [24–27]. In cellulose hydrolysis, gene fusion has been used in studying functions and effectiveness of various CBMs [28–30] as well as to create enzymes with multiple activities [31–33]. Warren and colleagues described a fusion of Cex and CenA made by the use of restriction enzymes. Although the resulting fusion had both endoglucanase and exoglucanase activities, it lacked the flexible PT linkers of both enzymes and all of the CBM of Cex, and most of the CBM of CenA. It did not bind to cellulose, but, like native CenA and Cex, was translocated to the periplasm when expressed *in E. coli* [34]. A xylanase-endoglucanase gene fusion of *Clostridium thermocellum xynX* gene and an enhanced *Erwinia chrysanthemi PY35 cel5Z*:Ω gene has been constructed using restriction independent overlapping [32].

Although various fusions of cellulases have been reported, their effectiveness for cellulose degradation *in vivo* has not been examined. It has been previously demonstrated that recombinant *Citrobacter freundii* (a close relative of *E. coli* possessing the native ability to assimilate cellobiose) expressing CenA and Cex is able to grow at the expense of crystalline cellulose [35,36]. Here, the preparation of a Cex-CenA fusion protein retaining all the essential properties for effective cellulose degradation is described.

2. Methods

2.1. Bacteria strains and growth conditions

Escherichia coli JM109 was used as host for initial cloning, activity testing and secretion experiments. *Citrobacter freundii* NCIMB 11490 (ATCC 8090) was used as host strain for cellulose utilization experiments. We chose *C. freundii* because it is a close relative of *E. coli* and has natural ability to utilize cellobiose and hence eliminating the requirement for the supply of β-glucosidase to cultures. Luria agar (LA) [37] supplemented with chloramphenicol was used for all plate cultures unless otherwise stated. Our initial experiments showed that 40 μg/ml chloramphenicol slowed down the growth of *C. freundii* constructs. A lower concentration of 15 μg/ml was found to be optimal and was used for *C. freundii* cultures whereas 40 μg/ml was used for *E. coli* cultures. For liquid cultures, Luria broth (LB) [37] or M9 minimal medium [38] supplemented with 0.1% w/v yeast extract was used. All organisms were grown at 37 °C. In addition, liquid cultures were incubated with shaking at 200 rpm. Cellulose degradation experiments were conducted using M9 minimal medium supplemented with Ford's Gold medal blotting paper (2 cm squares, approx. 50 mg each) or Whatman GB003 pure cellulose blotting paper (1 cm squares, approx 30 mg each). Cultures containing filter paper were agitated vigorously for 1 min every 24 h on a vortex mixer. Aliquots of the culture were taken from time to time, serially diluted with phosphate buffered saline (PBS) and plated on LA without antibiotics. Colonies on the dilution plates were counted, averaged and plotted as growth curves. The total growth of cells was estimated by calculating the area under the growth curves.

2.2. Molecular biology procedures

Design and construction of genetic parts were done in accordance with the BioBrick assembly standard [39]. The strains, plasmids and oligonucleotides used in this study are described in Table 1. PCR was performed using Kod Polymerase (Novogen) according to manufacturer's protocol. 'For amplification of GC rich genes, glycerol (10% v/v) was included in reaction mixtures, and the initial denaturation step in each cycle was extended to 60 s. The PCRs were performed in 50 μl of reaction with 1 μl of the supercoiled plasmid DNA and 1x reaction buffer with 1 mM $MgCl_2$, 0.2 mM each of dNTPs, 1 U of KOD DNA polymerase and 0.4 μM each of forward and reverse primers. Thermal cycling was performed at 95 °C for 1 min; followed by 30 cycles of 95 °C for 20 s, 65 °C for 10 s, 72 °C for 20 s/kb; followed by 72 °C for 1 min. Expected product sizes and specific extension times have been provided in the Supplementary material (Sup. Table 1). DNA sequencing was performed by the Sanger sequencing method using the BigDye® Terminator v3.1 cycle sequencing kit (Life Technologies) according to the manufacturer's protocol.

2.3. Construction of the fusion protein

The gene encoding the fusion protein was constructed from previously prepared pSB1C3 plasmids containing BBa_K523016 (P_{lac} + *lacZ'α* + RBS + *cex*) and BBa_K523015 (P_{lac} + *lacZ'α* + RBS + *cenA*). The *lac* promoter was BBa_J33207 and ribosome binding site was BBa_J15001 [40]. An NcoI restriction site was introduced into the CBM of the two constructs by PCR such that the amino acid sequences were not changed. The *cenA* construct was amplified using the forward primer cenAfNcoI and reverse primer pSBNX3insr2. Likewise the *cex* construct was amplified using the forward primer pSBNX3insf2 and reverse primer cexrNcoI. Primer sequences are provided in Table 1. PCR amplifications were performed using KOD hot start DNA polymerase (Novagen) using the MJ Research PTC-200 DNA Engine Thermal Cycler. The amplified PCR products were each purified using the QIAquick PCR purification kit (QIAGEN GmbH, Germany). The purified DNA products were then digested with NcoI (New England Biolabs, Inc.) for one hour and purified. The purified products were ligated overnight at 16 °C in a 30 μl reaction volume containing 10 μl of each digested product, 3 μl T4 DNA ligase buffer (with ATP), 1 μl T4 ligase, 1 μl T4 polynucleotide kinase (PNK) and 5 μl nuclease free water. The ligation product was used as a template for PCR with primers pSBNX3insf2 and pSBNX3insr2 using KOD polymerase. PCR products were electrophoresed on 0.8% agarose gels in 1X TAE buffer. Electrophoresis was performed at 100 V, 50 mA for 40 min using the Bio-Rad mini-sub cell horizontal electrophoresis system. The gel was post-stained with SYBR-Safe nucleic acid stain (1 μl 10,000× stock in 50 ml deionized water) for 20 min and visualized on the Life Technologies Safe Imager 2.0. Bands were excised and purified using the QIAquick gel extraction kit (QIAGEN GmbH, Germany). The purified products were digested with EcoRI and PstI at 37 °C for one hour. The plasmid pSB1C3 was also digested with EcoRI/PstI. Plasmid digests and PCR product digests were ligated together as described above and cloned into *E. coli* JM109. White clones were tested for Cex and CenA activities as described below. Positive clones were grown overnight in 5 ml Luria broth supplemented with 40 μg/ml chloramphenicol. Plasmid DNA was isolated using the QIAprep spin miniprep kit (QIAGEN GmbH, Germany) and tested for insert size. Constructs with the right sizes were confirmed by Sanger sequencing performed by Edinburgh Genomics, University of Edinburgh, UK. All procedures were performed according the respective manufacturers' protocols.

2.4. Enzyme activity assays

Enzyme activity within cells harbouring cloned genes were tested qualitatively using 4-methylumbelliferyl β-D-cellobioside (MUC) [20] and carboxymethyl cellulose (CMC) [41] substrates. To detect endoglucanase activity in *E. coli* or *C. freundii* harbouring *cenA* or *cxnA* genes, bacterial colonies were grown on LA with appropriate antibiotics, 0.38 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Melford) and 0.2% w/v Carboxymethyl cellulose (Sigma

Download English Version:

https://daneshyari.com/en/article/6488216

Download Persian Version:

https://daneshyari.com/article/6488216

Daneshyari.com