Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 349 (2006) 20–23
BBRC
www.elsevier.com/locate/ybbrc

Degradation of cellulosome-produced cello-oligosaccharides by an extracellular non-cellulosomal β-glucan glucohydrolase, BglA, from *Clostridium cellulovorans*

Akihiko Kosugi [a], Takamitsu Arai [b], Roy H. Doi [c,*]

[a] *Japan International Research Center for Agricultural Sciences (JIRCAS), Tsukuba, Ibaraki 305-8686, Japan*
[b] *Research Institute of Innovative Technology for the Earth (RITE), Kyoto, Japan*
[c] *Section of Molecular and Cellular Biology, University of California, Davis, CA 95616, USA*

Received 22 June 2006
Available online 17 July 2006

Abstract

Clostridium cellulovorans degrades cellulose efficiently to small oligosaccharides, which are used as an energy source. To characterize enzymes related to degrading small oligosaccharides, a gene was cloned for an extracellular non-cellulosomal β-glucan glucohydrolase (BglA) classified as a family-1 glycosyl hydrolase in *C. cellulovorans*. Recombinant BglA (rBglA) had higher activity on long glucooligomers than on cellobiose. When cellulosomes and rBglA were incubated with cellulose, the oligosaccharides produced were degraded more effectively to cellobiose and glucose, than with cellulosomes alone, indicating that BglA facilitated the degradation of accessible cello-oligosaccharides produced from cellulose by *C. cellulovorans* cellulosomes. Thus, this is an example of an extracellular non-cellulosomal enzyme working in a cooperative manner with cellulosomes to degrade cellulose to sugars.

Keywords: *Clostridium cellulovorans*; β-Glucan glucohydrolase; Non-cellulosome; Cello-oligosaccharides; Family-1 glycosyl hydrolase; Cellulosome; β-Glycosidase; Recombinant protein; Synergistic effects; Cellulose degradation

In order to utilize polysaccharides such as glucans and xylans to meet carbon and energy requirements, heterotrophic organisms depend on a catabolic pathway involving the interaction of multiple hydrolytic enzymes, transporters, and regulatory systems coordinating gene expression of pathway-specific proteins [1,2]. Enzymatic hydrolysis of homopolymer cellulose, present as the most abundant polysaccharide on earth, requires endoglucanases (EC 3.2.1.4), which randomly hydrolyze internal β-1,4 glycosidic bonds, cellobiohydrolases and exoglucanases (EC 3.2.1.91), which remove cellobiose from either the non-reducing or reducing ends of cellooligomers, and β-glucosidases (EC 3.2.1.21) and β-glucan glucohydrolase (EC 3.2.1.74), which preferentially act, respectively, on cellobiose and cellooligomers to release glucose [1]. In addition, it is also well-known that cellooligomers such as cellobiose are metabolized by cellobiose phosphorylase (EC 2.4.1.20), which cleaves cellobiose by phosphorolysis, yielding glucose-1-phosphate as one of the products [1,3].

Clostridium cellulovorans, an anaerobic, mesophilic, and spore-forming bacterium, produces a large extracellular polysaccharolytic multicomponent complex called the cellulosome, and is one of the most efficient cellulolytic organisms. *C. cellulovorans* utilizes not only cellulose but also hemicelluloses, such as xylan, and several other carbon sources for growth [4,5]. Thus, this bacterium has the capacity to catabolize a wide variety of β- and β-linked glucans, while related glucan-catabolizing enzymes are less well understood in this bacterium.

In this paper, we describe the characterization of an extracellular non-cellulosome β-glucan glucohydrolase

* Corresponding author. Fax: +1 530 752 3085.
E-mail address: rhdoi@ucdavis.edu (R.H. Doi).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.07.038

(BglA) from *C. cellulovorans*, which acts in concert with cellulosomes to degrade small oligosaccharides required for carbon catabolism.

Materials and methods

Bacterial strains and media. C. cellulovorans (ATCC35296) [4,5] was used as the source of chromosomal DNA and cell wall preparations. *C. cellulovorans* was grown anaerobically at 37 °C in serum bottles containing the previously described medium [6] which included 10 g cellobiose, 10 g acid-swollen cellulose (ASC) [7], 10 g xylobiose, and 10 g xylan/L. *Escherichia coli* Novablue and BL21 (DE3) (Novagen) were used as cloning hosts for production of recombinant proteins and were grown at 18, 30, or 37 °C in Luria–Bertani medium containing ampicillin (100 μg/ml; Sigma) or kanamycin (50 μg/ml; Sigma).

Cloning and DNA sequencing for BglA. To clone genes possessing activity for β-glycosidase activity, the λZAPII *C. cellulovorans* genomic library [6] (Stratagene) was screened by overlaying with 0.7% soft agar containing 1 mM 4-methylumbelliferyl β-D-glucopyranoside (4-MUG) (Sigma). The positive plaques were isolated by detection, under UV light, of fluorescent haloes surrounding recombinant plaques. To isolate full-length genes encoding activity, positive clones were obtained by *in vivo* excision and rescued with the use of ExAssist helper phage (Stratagene). Positive clones were further screened for hydrolysis activity for 4-MUG, and two colonies with activity were isolated. DNA sequence was determined from double-stranded plasmid DNA by the dideoxy chain termination method. Sequence data were analyzed and compared using the BLAST program.

Construction of pEBGLA29. The two primers containing artificial *Xba*I or *Xho*I sites (underlined) were used to amplify full-length BglA (5′-CTA GTC TAG ATA CTA ACA AAT ATT G-3′ and 5′-CCG CTC GAG CTT ATT AGA TCT TTC TAT-3′). The amplified fragment was digested with the cognate restriction enzymes and inserted between the *Xba*I and *Xho*I sites of pET29b (Novagen) to generate pEBGLA29. This plasmid provided a six-histidine tag on the C terminus.

Purification of rBglA. rBglA was purified from *E. coli* BL21(DE3) (Novagen) strains harboring pEBGLA29. When a culture of *E. coli* BL21(DE3) harboring pEBGLA29 had reached an optical density at 600 nm of 0.5 at 30 °C in Luria–Bertani medium supplemented with kanamycin (50 μg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the cells were further cultivated at 30 °C for 4 h. The cells were collected, suspended in buffer 1 (50 mM phosphate, 300 mM NaCl, and 10 mM imidazole, pH 8.0) and disrupted by sonication. The cell extracts were applied to a Ni–nitrilotriacetatic acid agarose column (Qiagen). Recombinant BglA (rBglA) was eluted by buffer 1 with 250 mM imidazole. The eluted proteins were concentrated to 1.5–2.0 mg/ml by ultrafiltration (Ultra free biomax-30; Millipore). Protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce) with bovine serum albumin as the standard.

Enzyme assays. The glycosidase activity was measured by the absorbance of liberated *p*-nitrophenol from *p*-nitrophenyl-β-D-glucopyranoside (pNGP), *p*-nitrophenyl-β-D-cellobioside (pNCB), *p*-nitrophenyl-β-D-xylopyranoside (pNXP), *p*-nitrophenyl-α-L-arabinopyranoside (pNAP), *p*-nitrophenyl-α-L-arabinofuranoside (pNAF), *p*-nitrophenyl-β-D-galactopyranoside (pNGa), *o*-nitrophenyl-β-D-galactopyranoside (oNGa), *p*-nitrophenyl-β-D-fucopyranoside (pNFP), *p*-nitrophenyl-β-D-lactopyranoside (pNLP), *p*-nitrophenyl-β-D-glucoronide (pNGlo), and *p*-nitrophenyl-α-D-glucopyranoside (pNaGP), at 410 nm. Assay mixtures containing each substrate at 1 mM in 50 mM sodium phosphate buffer (pH 6.0) were incubated for 10 min at 37 °C, and the reactions were stopped by addition of Na_2CO_3 [1,6]. One unit of the activity toward *p*-nitrophenol derivatives was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenyl/min. The activity of the enzyme for ASC was determined by measuring the release of reducing sugars with the Somogyi-Nelson method, with D-glucose as the standard. Hydrolysis of cellobiose, cellotriose, and cellotetraose was measured using the glucose hexokinase kit following the manufacturer's instructions (Sigma) [1].

SDS–PAGE and zymogram analysis. SDS–PAGE was performed in a 10% polyacrylamide gel. Gels were stained for 4-MUG hydrolysis activity. The activity staining was performed using PAGE with 10% (wt/vol) nondenaturing polyacrylamide gel. After electrophoresis, the gel was incubated for 30 min with 50 mM phosphate buffer (pH 6.0) containing 25% isopropanol at 4 °C and washed twice with the same buffer without isopropanol. After being washed, the gel was incubated for 60 min at 37 °C in 50 mM phosphate buffer (pH 6.0) containing 1 mM 4-MUG. Positive bands were detected by fluorescence under UV illumination [6].

Preparation of intracellular and extracellular materials from C. cellulovorans grown on cellobiose, acid swollen cellulose (ASC), xylobiose, and xylan medium. Extracellular materials (cellulosomal and non-cellulosomal fractions) were prepared from supernatants of cultures grown on cellobiose, ASC, xylobiose, and xylan and purified according to their size and interaction with cellulose as described previously [8]. Intracellular fraction of *C. cellulovorans* was prepared by sonication of cellobiose grown cells [8]. The sonicated cells were separated into cell debris and supernatant by centrifugation (22,000*g*, 30 min, 4 °C). The supernatant was used as the intracellular fraction for the active staining.

Results and discussions

Nucleotide and amino acid sequences of bglA gene

To clone and characterize the glucan-hydrolyzing enzymes related to oligosaccharides, we carried out screening of a previously constructed *C. cellulovorans* genomic library for β-glycosidase activity by overlaying with soft agar containing 4-MUG. Two positive clones were isolated, and they had a 3.62-kbp insert in common (pG1) (GenBank Accession No. AY268940). The pG1 encoded two genes consisting of *bglA* and *orf1*, which predicted proteins with β-glycosidase and Fe–S oxidoreductase activities, respectively. The *bglA* gene encoded a protein of 445 amino acids with a molecular weight of 51,565. The alignment of the predicted amino acid sequence revealed that mature BglA was classified as a family-1 glycosyl hydrolase, and had high homology for β-glycosidase from several thermophilic bacteria such as *Thermoanaerobacter brockii*, *Caldicellulosiruptor saccharolyticus*, and *Thermotoga maritime* (55–56% identities). Although BglA was found in the extracellular fraction, a signal peptide was not obvious in the derived amino acid sequence.

Characterization and identification of BglA

To characterize the properties of BglA, we designed a fusion protein rBglA with a six-histidine tag at its C-terminus and purified rBglA from *E. coli* BL21 (DE3) harboring pEBGLA29. rBglA had high specific activities on pNGP, pNCB, pNGa, oNGa, and pNFP (177, 130, 133, 130, and 129 U/mg protein, respectively). rBglA also showed weak hydrolytic activity for pNXP, pNAP, and pNLP without pNGlo and pNaGP.

The pH for optimum rBglA activity was 6.0, and the enzyme was stable over a pH range of 5–8 when incubated at 40 °C for 12 h with no substrate. The temperature for maximum activity was found to be 50 °C at pH 6.0.

To determine the affinity of rBglA for oligosaccharides, the kinetics properties with cellobiose, cellotriose, and

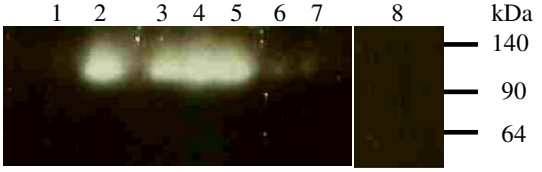

Fig. 1. Identification of BglA in *C. cellulovorans* and expression of rBglA. Lane 1: cellulosomal fraction of *C. cellulovorans*; lane 2: non-cellulosomal extracellular fraction of *C. cellulovorans*; lane 3: purified rBglA; lane 4: extracellular fraction of *C. cellulovorans* culture grown with cellobiose; lane 5: extracellular fraction of *C. cellulovorans* culture grown with ASC; lane 6: extracellular fraction of *C. cellulovorans* culture grown with xylobiose; lane 7: extracellular fraction of *C. cellulovorans* culture grown with xylan; lane 8: intracellular fraction of cellobiose grown *C. cellulovorans* cells. A 10 μl aliquot of the each fraction was applied to the polyacrylamide gel.

cellotetraose were determined. As a result, the K_m and V_{max} for each substrate were shown to be 2.9, 1.7, and 1.1 mM, and 7.1, 28.6, and 31.3 μmol/min/mg protein, respectively. These results showed that rBglA has higher affinity for oligosaccharides with longer chain length than that with a short chain such as cellobiose. Inhibition of enzyme activity by end products was observed at a glucose concentration of 20 mM, and competitive inhibition was determined by Lineweaver–Burk plots (data not shown).

To determine whether BglA in *C. cellulovorans* is influenced by growth on carbon sources and to confirm its extracellular location, we carried out active staining analyses with 4-MUG using several carbon sources. When cellobiose and cellulose were used as sole carbon sources, high activity of BglA was detected exclusively in the extracellular non-cellulosomal fraction (Fig. 1, lanes 2, 4, and 5) and not in the intracellular fraction prepared from cells by sonication (Fig. 1, lane 8). Furthermore BglA activity could not be detected in any fractions when xylobiose and xylan were the growth substrates (Fig. 1, lanes 6 and 7). These results suggest that BglA activity is regulated by the carbon source in the growth medium, and that BglA functions extracellularly. The active form of BglA was also revealed to be a homo-dimeric structure from the active staining results.

Contribution of BglA to cellulose degradation by C. cellulovorans cellulosomes

To observe whether BglA played any role on cellulose degradation by *C. cellulovorans*, we measured synergistic activity for cellulose hydrolysis between rBglA and the native cellulosome and non-cellulosome fractions prepared from *C. cellulovorans* grown on cellobiose as the carbon source. It was observed that the mixture of cellulosomal and non-cellulosomal fractions showed the highest hydrolysis activity, compared with incubations with each fraction alone. Thus it was clearly shown that it was necessary for cooperation between the cellulosome and the non-cellulosomal fraction to obtain maximum cellulose degradation activity in *C. cellulovorans*. Similar synergistic effects were observed when rBglA and the cellulosomal fraction were incubated with ASC; however synergism was not observed between rBglA and the non-cellulosomal fraction.

Table 1
Synergistic effects between cellulosome and non-cellulosomal fractions for cellulose degradation

Enzyme fraction	Amount of released sugar (μg/ml)[a]
No enzyme	0.0
Cellulosomal fraction (CS)	506.9 ± 16.6
Non-cellulosomal fraction (nCS)	209.5 ± 17.27
CS and nCS	1555.3 ± 15.59
CS and rBglA	695.4 ± 22.35
nCS and rBgaA	238.5 ± 10.82
rBglA	24.1 ± 5.9

[a] Activities are expressed in amounts of released sugar (μg/ml of reaction mixture). CS and nCS was shown cellulosomal and non-cellulosomal fractions of *C. cellulovorans*, respectively. The reaction mixtures contained 0.5% (wt/vol) ASC, 50 mM phosphate buffer (pH 6.0). Each reaction was incubated with and 0.50.9 mg (rBglA), 0.6 mg (CS), and 0.7 mg (nCS) of protein, respectively. The incubation was carried out at 50 °C for 16 h. Each value is the mean of three determinations ± the standard deviation.

To confirm whether the cellulose hydrolysis products were influenced by the addition of rBglA and the non-cellulosomal fraction to the cellulosomal fraction, we analyzed the hydrolysis products by thin-layer chromatography (data not shown). The hydrolysis products by only the cellulosome fraction were mainly glucose, cellobiose and several cello-oligosaccharides. When cellulosomal and non-cellulosomal fractions were incubated with ASC, the hydrolysates contained increasing amounts of smaller saccharides such as glucose, cellobiose and short chain cello-oligosaccharide, compared with the cellulosomal fraction alone. In addition, when rBglA was incubated with the cellulosomal fraction, the depolymerization of cellulose was accelerated slightly, compared with cellulosomal fraction alone. These results were in good agreement with that of the measurements of the amount of released sugars (Table 1).

Therefore, these results indicated that BglA facilitated the degradation of accessible cello-oligosaccharides produced from cellulose by *C. cellulovorans* cellulosomes. Thus, this is an example of a non-cellulosomal enzyme working in a cooperative manner with cellulosomes to degrade cellulose to sugars.

Conclusion

An extracellular non-cellulosomal β-glucan glucohydrolase (BglA), which was classified as a family-1 glycosyl hydrolase, was isolated from *C. cellulovorans*. BglA was located and functioned extracellularly according to zymogram analysis using cellulosomes and non-cellulosome fractions. rBglA showed higher activity on long glucooligomers than on cellobiose. Synergistic effects were detected on analysis of ASC degradation with the cellulosome fraction and rBglA, indicating that BglA plays a cooperative

role in the degradation of accessible cello-oligosaccharides produced from cellulose by *C. cellulovorans* cellulosomes.

Acknowledgments

We are grateful to Helen Chan for skillful technical assistance. This research was supported in part by Grant DE-FG02-04ER15553 from the US Department of Energy.

References

[1] D.A. Yernool, J.K. McCarthy, D.E. Eveleigh, J.-D. Bok, Cloning and characterization of the glucooligosaccharide catabolic pathway β-glucan glucohydrolase and cellobiose phosphorylase in the marine hyperthermophile *Thermotoga neapolitana*, J. Bacteriol. 182 (2000) 5172–5179.

[2] R.A. Warren, Microbial hydrolysis of polysaccharides, Annu. Rev. Microbiol. 50 (1996) 183–212.

[3] L.R. Lynd, P.J. Weimer, W.H. van Zyl, I.S. Pretorius, Microbial cellulose utilization: fundamentals and biotechnology, Microbiol. Mol. Biol. Rev. 66 (2002) 506–577.

[4] R.H. Doi, A. Kosugi, K. Murashima, Y. Tamaru, S.O. Han, Cellulosomes from mesophilic bacteria, J. Bacteriol. 185 (2003) 5907–5914.

[5] R.H. Doi, A. Kosugi, Cellulosomes: plant-cell-wall-degrading enzyme complexes, Nat. Rev. Microbiol. 2 (2004) 541–551.

[6] A. Kosugi, K. Murashima, R.H. Doi, Characterization of two noncellulosomal subunits, ArfA and BgaA, from *Clostridium cellulovorans* that cooperate with the cellulosome in plant cell wall degradation, J. Bacteriol. 184 (2002) 6859–6865.

[7] T.M. Wood, Preparation of crystalline, amorphous, and dyed cellulase substrates, Methods Enzymol. 160 (1988) 19–25.

[8] A. Kosugi, K. Murashima, Y. Tamaru, R.H. Doi, Cell-surface-anchoring role of N-terminal surface layer homology domains of *Clostridium cellulovorans* EngE, J. Bacteriol. 184 (2002) 884–888.