Bioresource Technology 101 (2010) 5534–5538

Contents lists available at ScienceDirect

Bioresource Technology

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

Glycoside hydrolase family 9 processive endoglucanase from *Clostridium phytofermentans*: Heterologous expression, characterization, and synergy with family 48 cellobiohydrolase

Xiao-Zhou Zhang [a], Noppadon Sathitsuksanoh [a,b], Y.-H.P. Zhang [a,b,c,*]

[a] Department of Biological Systems Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA
[b] Institute for Critical Technology and Applied Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA
[c] BioEnergy Science Center of Department of Energy, Oak Ridge, TN 37831, USA

ARTICLE INFO

Article history:
Received 2 December 2009
Received in revised form 26 January 2010
Accepted 28 January 2010
Available online 4 March 2010

Keywords:
Clostridium phytofermentans
Cellulase
Family 9 glycoside hydrolase
Processive endoglucanase
Synergy

ABSTRACT

The glycoside hydrolase family 9 cellulase (Cel9) from *Clostridium phytofermentans* has a multi-modular structure and is essential for cellulose hydrolysis. In order to facilitate production and purification of Cel9, recombinant Cel9 was functionally expressed in *Escherichia coli*. Cel9 exhibited maximum activity at pH 6.5 and 65 °C on carboxymethyl cellulose in a 10-min reaction period. The hydrolysis products on regenerated amorphous cellulose (RAC) were cellotetraose (a major product), cellotriose, cellobiose and glucose, and 71–80% of the reducing sugars produced by Cel9 were in soluble form, suggesting that Cel9 was a processive endoglucanase. The highest synergy between *C. phytofermentans* Cel9 and *C. phytofermentans* cellobiohydrolase Cel48 on Avicel was about 1.8 at a ratio of about 1:5. Cel9 alone was sufficient to solublize filter paper while Cel48 was not; however, it enhanced the solublization process along with Cel9 synergistically. This study provided useful information for understanding of the cellulose hydrolysis mechanism of this cellulolytic bacterium with potential industrial importance.

1. Introduction

Cellulose is the most abundant renewable biological resource and a low-cost energy source (Lynd et al., 2008; Zhang, 2009). The production of biobased products and biofuels from renewable lignocellulose is important for the sustainable development of human beings. High costs of cellulases, which are responsible for hydrolyzing pretreated cellulosic material, are one of the largest obstacles to commercialization of biomass biorefineries (Taylor et al., 2008; Zhang et al., 2006b; Zhu et al., 2009).

Clostridium phytofermentans ISDg is an obligately anaerobic, mesophilic Gram-positive bacterium. It can utilize a broad range of carbon sources for generation of ethanol, acetate, CO_2 and hydrogen in one step (Warnick et al., 2002). *C. phytofermentans* has been regarded as a model microorganism for consolidated bioprocessing (CBP) (Warnick et al., 2002; Zhang et al., 2010). *C. phytofermentans* appears not to produce complexed cellulases due to the lack of scaffolding, cohesin, and dockerin sequences (Zhang et al., 2010). The genomic sequence suggests that a gene cluster contains two open reading frames – Cphy3367 encoding a putative family 9 glycoside hydrolase (Cel9) and Cphy3368 encoding a confirmed cellobiohydrolase Cel48 (Zhang et al., 2010). Different from other *Clostridium* spp. that contain 5–16 different *cel9* genes, *C. phytofermentans* has a sole *cel9* gene (Tolonen et al., 2009). A *cel9*-knockout study showed that Cel9 was the essential component of the cellulases responsible for microbial cellulose utilization (Tolonen et al., 2009; Wilson, 2009). However, the basic properties of Cel9 have not been characterized yet.

In this study, in order to obtain high-purity bacterial cellulase Cel9 and facilitate its production, cel9 gene was functionally expressed in *Escherichia coli*, and the recombinant protein was purified and characterized. Also, the synergy between Cel9 and Cel48 was studied.

2. Methods

2.1. Chemicals and materials

All chemicals were reagent grade or higher, purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Regenerated amorphous cellulose (RAC) was prepared from Avicel through cellulose dissolution in concentrated phosphoric acid followed by regeneration (Zhang et al., 2006a). Cellodextrin standards were prepared by hydrolysis of Avicel by

* Corresponding author. Address: Department of Biological Systems Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA. Tel.: +1 540 231 7414; fax: +1 540 231 3199.
E-mail address: ypzhang@vt.edu (Y.-H.P. Zhang).

0960-8524/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2010.01.152

using a mixture of concentrated HCl/H_2SO_4 and separated by 2-column chromatography (Zhang and Lynd, 2003). The purity of the isolated cellodextrins was much higher than that of the Sigma products (Zhang and Lynd, 2003). The oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA).

2.2. Bacterial strains and growth conditions

E. coli DH5α (Invitrogen, Carlsbad, CA) was used as a host cell for DNA manipulation, and *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA) was used as the host for recombinant protein production. The Luria–Bertani (LB) medium with 100 μg/mL ampicillin was used for *E. coli* cell growth and recombinant protein production.

2.3. Construction of plasmid

The DNA sequence encoding the mature Cel9 (25-985 amino acids) (GenBank accession number: ABX43720) was amplified from the genomic DNA of *C. phytofermentans* ISDg (ATCC Accession number 700394) by a primer pair of P1 (5′-GGAAT GGATC CATAA AGGAG GTATA ATATG GCGGA AACCA ATTAT AATTA CGGA-3′, *Bam*HI site underlined) and P2 (5′-CCTGA TCTCG AGTTT ATGGT TCGAC TCCCC AAACC A-3′, *Xho*I site underlined). The PCR product was digested with *Bam*HI/*Xho*I and ligated into the corresponding sites of the vector pET20b (Novagen, Madison, WI) to generate pET20b-cel9 for producing Cel9.

2.4. Enzyme expression and purification

The strain *E. coli* BL21 (pET20b-cel9) was cultivated in 250 mL of the LB medium supplemented with 1.2% glycerol at 37 °C for the production of Cel9. When A_{600} reached ~1.6, 50 μmol/l isopropyl-beta-D-thiogalactopyranoside (IPTG) was added and the temperature was decreased to 18 °C for ~16 h. The cell pellets were resuspended with 35 mL of 50 mmol/l Tris–HCl buffer (pH 8.0). After ultrasonication and centrifugation, 70 mg of RAC was added in the supernatant for adsorbing the target protein at room temperature. After washing once with de-ionized water, the Cel9 was desorbed by using 80% ethylene glycol (Hong et al., 2008). The purified Cel9 in ethylene glycol was stored at −20 °C. The production and purification of Cel48 of *C. phytofermentans* were performed as previously described (Zhang et al., 2010).

2.5. Enzyme assays

Enzyme activity was assayed at 50 °C based on initial reaction rates in a 10-min reaction period (Zhang et al., 2009). The reaction mixtures (0.5 mL) contained 1% (wt/vol) of the substrate (e.g., Avicel PH-105, RAC, carboxymethyl cellulose) in a 50 mmol/L 2-N-morpholino-ethanesulfonic acid (MES) buffer (pH 6.0) containing 1 mmol/L $CaCl_2$. Enzyme concentration in the reactions was 2 μg/mL (~20 nmol/L), unless otherwise noted. The reactions were terminated by boiling for 5 min. After centrifugation, aliquots of the supernatants were assayed for the release of the reducing sugars. Concentration of reducing sugars was determined by the 2,2′-bicinchoninate method (Waffenschmidt and Janeicke, 1987) with modifications described by Zhang et al. (Zhang and Lynd, 2005) and with glucose as the standard, where the reduced reaction temperature (75 °C) can generate more accurate results for the reducing sugar ends for mixed cellodextrins. One unit of activity was defined as the amount of enzyme that released 1 μmol of reducing end per min.

2.6. Chromatographic assays

The enzymatic hydrolysis products of soluble cellodextrins were analyzed by thin-layer chromatography. The samples were separated on a Whatman 60 Å silica gel plate with a developing solution of ethyl acetate–H_2O–methanol (8:3:4, v/v) (Zhang and Lynd, 2006a). The sugars were detected by charring with an acid/alcohol solution (90 mL methanol, 5 mL sulfuric acid, and 5 mL acetic acid) plus 0.5 mL *p*-anisaldehyde. After the hydrolysis of RAC by 20 μg/mL Cel9 for 6 h, the soluble cellodextrins were analyzed by using a Beckman high-performance liquid chromatography (HPLC) equipped with a Bio-Rad HPX-42A column and a refractive index detector at a flow rate of 0.4 mL/min (Zhang and Lynd, 2003).

2.7. Assessment of synergy

Synergy between Cel9 and Cel48 was determined in 1 mL of the 0.4% (wt/vol) Avicel solution containing either Cel9 only, or Cel48 only or both of these two enzymes at different molar ratios. The total amount of the enzymes in the 1-mL reaction solution was fixed at 10 μg. After 24-h incubation at 50 °C, the concentrations of soluble sugars were measured by using the phenol–sulfuric acid method with glucose as the standard (DuBois et al., 1956; Zhang et al., 2009). The degree of synergistic effect was defined as the ratio of the observed activity of the combined enzymes to the sum of the individual activities (Beldman et al., 1988).

2.8. Degradation of filter paper

The 2-mL reaction mixtures contained 20 mg of filter paper disk. Cel9 and Cel48 were added in the reactions alone or together at a concentration of 20 μg/mL for each. The tubes were incubated at 50 °C. The concentrations of soluble sugars were measured by using the phenol–sulfuric acid method with glucose as the standard (DuBois et al., 1956; Zhang et al., 2009).

2.9. Other assays

Protein concentration was measured by the Bradford assay (Bradford, 1976). Protein samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

3. Results and discussion

3.1. Sequence analysis

The *cel9* gene of *C. phytofermentans* encodes a protein of 985 amino acids containing a signal peptide (residue 1–24) with a typical cleavage site (ADA-A). The BLAST analysis suggested that mature Cel9 (~104.9 kDa) has a modular structure (Fig. 1a). The amino acid sequence of 29-466 is a Cel9 catalytic module, which has a high similarity (87%) with the *Clostridium stercorarium* CelZ (Jauris et al., 1990). The Cel9 catalytic module also has a similarity of 81% with the *Clostridium cellulolyticum* Cel9G (Mandelman et al., 2003). The molecular structure of Cel9 was predicted by SWISS-MODEL workspace (Bordoli et al., 2009) based on the template of the crystal structure of *C. cellulolyticum* Cel9G (PDB: 1G87) (Mandelman et al., 2003). The predicted three-dimensional structure model of Cel9 catalytic module resembles the template structures very well (data not shown). The putative catalytic base (D80), the proton donor (E446), and aromatic residues (H150, W280, D335, D337 and Y442) involved in substrate stacking are also highly conserved, suggesting that the *C. phytofermentans* Cel9 may share the same mechanism for cellulose hydrolysis by attacking cellulose

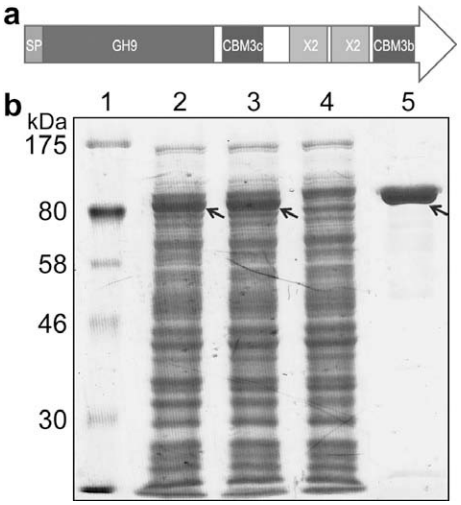

Fig. 1. The modular architecture of Cel9 and its production in *E. coli*. (a) The modular structure of Cel9. SP, signal peptide; GH9, glycoside hydrolase family 9 catalytic module; CBM3c, family 3c carbohydrate-binding module; X2, X2 domain; CBM3b, family 3b carbohydrate-binding module. (b) The expression and purification of Cel9 from *E. coli* BL21. Lanes: 1, protein molecular mass markers; 2, total protein extract; 3, soluble protein extract; 4, soluble protein extract after Cel9 was adsorbed by RAC; 5, 1.3 μg purified Cel9. The position of Cel9 is indicated by an arrow.

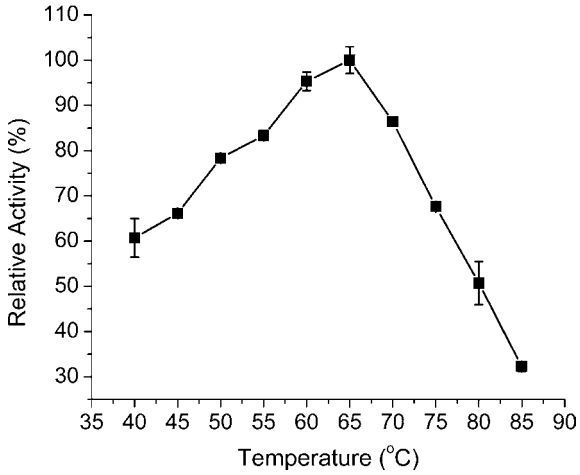

Fig. 2. Activity as function of temperature of Cel9 on CMC in a 50 mM MES buffer. The error bars represent the standard deviation of the triplicate measurements.

chain from the non-reducing end (Mandelman et al., 2003). There are a family 3 carbohydrate-binding module (CBM3) (residue 485–636), which belong to type c CBM3 based on the sequence identity (Jindou et al., 2006), two X2 domains (Mosbah et al., 2000) (residue 648–731 and 741–824) in tandem, and a C-terminal type b CBM3 (residue 838–981) (Jindou et al., 2006).

3.2. Production and purification of Cel9

As shown in Fig. 1b, the recombinant Cel9 was expressed as the major protein (~43 mg/L) in *E. coli*, and most was soluble (Fig. 1b, lane 3). Cel9 was purified based on its ability to strongly bind RAC. After an affinity adsorption on RAC, followed by desorption by ethylene glycol (Hong et al., 2008), Cel9 was purified to homogeneity (Fig. 1b, lane 5) with a yield of ~28%, which was estimated based on the total enzyme activity recovered after the purification. The apparent molecular mass of the purified protein was estimated to be about 90 kDa by SDS–PAGE analysis. This value was slightly lower than the theoretical value of 104.9 kDa based on its deduced amino acid sequence, and this phenomenon of faster immigration rate was also observed for the *C. phytofermentans* Cel48 (Zhang et al., 2010).

3.3. Characterization of Cel9

The effect of pH on the activity of Cel9 was examined on carboxymethyl cellulose (CMC) in 50 mM disodium hydrogen phosphate–citric acid buffers (pH 5–7) in a 10-min reaction period. The pH optimum was 6.5 (data not shown). The effect of temperature on the activity of Cel9 was examined with the same substrate under the standard conditions; the optimal temperature was 65 °C (Fig. 2). The high optimal temperature for Cel9 and Cel48 (60 °C) (Zhang et al., 2010) from a mesophilic organism is somewhat surprising, but can be explained by possible acquisition of the *cel9–cel48* gene cluster from a thermophilic microorganism through horizontal gene transfer. Cel9 exhibited the highest activity of 1747 ± 9 U/μmol on CMC. It showed relatively low activities of 482 ± 9 and 261 ± 23 U/μmol on RAC and Avicel, respectively. As shown in Fig. 3a, Cel9 had no detectable activity on cellobiose (G2); only a small fraction of cellotriose (G3) was hydrolyzed into G2 and glucose; cellotetraose (G4) was hydrolysed into G3, G2, and G1; cellopentaose (G5) was mainly hydrolyzed into G3 + G2 and slightly into G4 + G1; and cellohexaose (G6) was preferentially

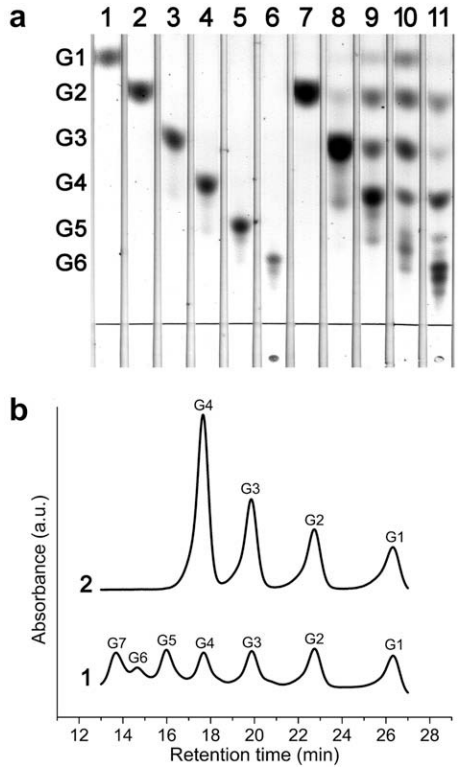

Fig. 3. Chromatography analysis of hydrolysis products. (a) Thin-layer chromatography analysis of hydrolysis products of soluble cellodextrins. G1–G6 (lanes 1–6) refer to the positions of standards (4 μg of each): glucose (G1), cellobiose (G2), cellotriose (G3), cellotetraose (G4), cellopentaose (G5), and cellohexaose (G6), respectively. Reactions containing G2–G6 (0.4%) were incubated at 37 °C for 16 h with 20 μg/mL Cel9. Samples loaded in lanes 7–11 were the hydrolysis products of G2 (2 μL), G3 (4 μL), G4 (3 μL), G5 (3 μL), and G6 (4 μL), respectively. The trails for lanes 8–11 were due to high loading of long chain cello-oligosaccharides and their low solubility in the developing solution. (b) HPLC analysis of hydrolysis products on RAC. Curve 1, the standards; G7 refers to celloheptaose. The reactions (1 mL) containing 1% RAC were incubated at 50 °C with (curve 2) 2 μg/mL Cel9.

hydrolyzed into G4 + G2, and to a less extent into G5 + G1 or two G3. Fig. 3b showed that G4 is the dominant product and G3, G2 and G1 are the minor products. No soluble product was detected without adding the enzyme in the reaction. The cleavage patterns of Cel9 on soluble cellodextrins and insoluble cellulose were quite the same as those of CelZ from *C. stercorarium* (Bronnenmeier and Staudenbauer, 1990). The other members of the GH9 family have different substrate specificities and end-products (Arai et al., 2006). For example, CelI from *C. thermocellum* released G2 as the major product, and G3 and G1 as the minor products from Avicel (Gilad et al., 2003). CelG from *C. cellulolyticum* produced G5 and G4 as the initial products on amorphous cellulose and Avicel (Gal et al., 1997).

The processivity of processive endoglucanse usually is determined by the ratio of the generated soluble reducing ends to insoluble reducing ends (Irwin et al., 1993; Reverbel-Leroy et al., 1997). The distribution of reducing sugars generated by Cel9 on RAC was monitored. As shown in Fig. 4, the ratios of the soluble to insoluble fraction increased from 2.4 to 3.9 when the incubation time was prolonged from 0.5 to 3.5 h. GH9 proteins, including Cel9, belong to the subfamily theme B (Gilad et al., 2003), such as *C. stercorarium* CelZ (Jauris et al., 1990), *C. thermocellum* CelI (Gilad et al., 2003), *C. cellulolyticum* CelG (Gal et al., 1997), and *Thermobifida fusca* E4 (Irwin et al., 1998). They act both as endoglucanases and exoglucanases from non-reducing ends, which are termed as processive endoglucanase.

3.4. Synergy between Cel9 and Cel48

Since (i) the genes of Cel48 and Cel9 form a gene cluster, (ii) Cel48 (molecular mass ~99.5 kDa) is a cellobiohydrolase and Cel9 is a processive endoglucanase, and (iii) Cel48 works on the reducing ends and Cel9 acts on non-reducing ends based on its structure homology modeling, the synergism between Cel48 and Cel9 was studied in a 0.4% (wt/vol) Avicel suspension (Fig. 5). Since specific activity of Cel9 (261 U/μmol) was much higher than that of Cel48 on Avicel (15.1 U/μmol) (Zhang et al., 2010), total amount of soluble sugars released was proportional to the amount of Cel9 (Fig. 5). Under all tested conditions regardless of the ratios of the two enzymes, the combination of Cel48 and Cel9 always released more soluble sugars than that of the sum of individual enzymes. The maximum degree of synergy effect was approximately 1.55 when Cel48/Cel9 was ca. 5.

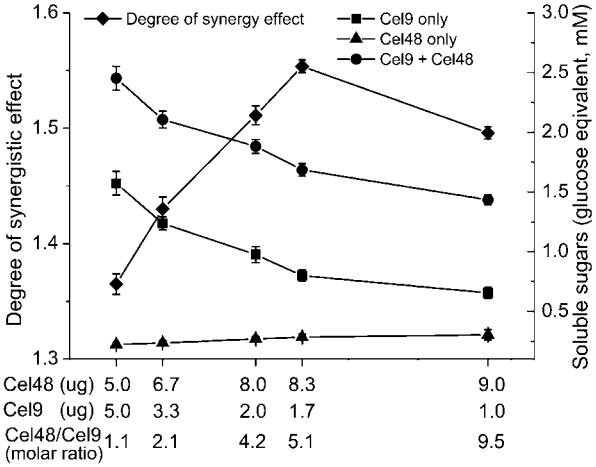

Fig. 5. Hydrolysis of Avicel by Cel9 and Cel48, alone and in combination. Avicel was incubated with indicated amount of Cel48 or/and Cel9 in 1 mL of reaction mixture. The molar ratios of Cel48 to Cel9 were indicated when they were added in combination as described in Section 2.7. The concentrations of soluble sugars were determined by phenol–sulfuric acid method and the degrees of synergy effect were calculated as described in Section 2.7. The error bars represent the standard deviation of the triplicate measurements.

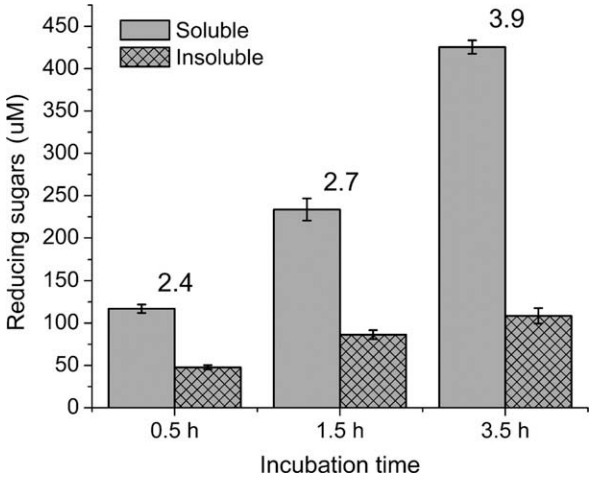

Fig. 4. Distribution of reducing sugars generated by CpCel9 on RAC. The concentrations of reducing sugars were determined by the modified 2,2′-bicinchoninate method. The ratios of the soluble fraction to insoluble fraction were given above the column. The error bars represent the standard deviation of the triplicate measurements.

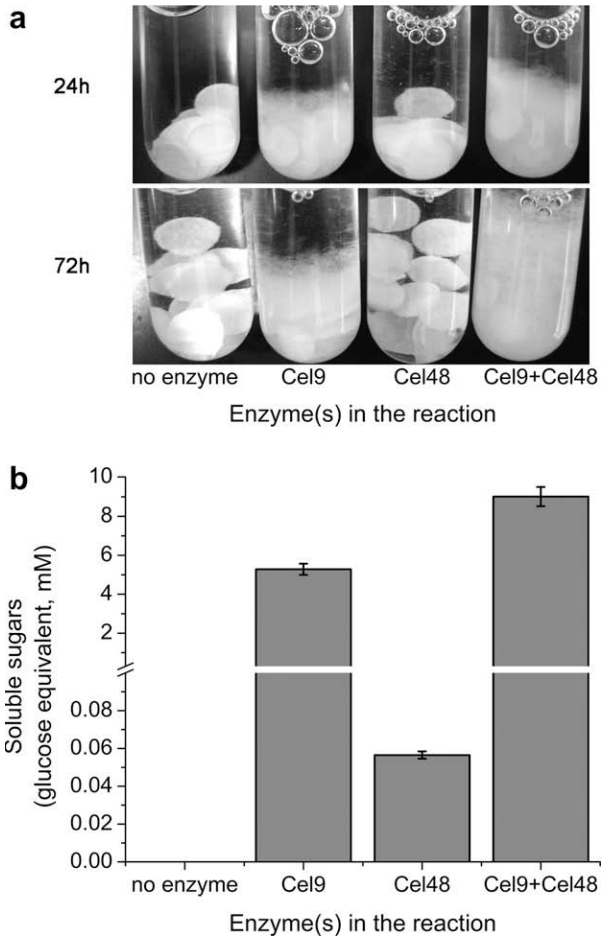

Fig. 6. Solubilization of filter paper by Cel9. (a) Degradation of filter paper by Cel9 or/and Cel48 after 24 or 72 h of incubation. (b) Soluble sugars on hydrolysis of filter paper by Cel9 or/and Cel48 after 72 h of incubation. The concentrations of soluble sugars were determined by the phenol–sulfuric acid method. The error bars represent the standard deviation of the triplicate measurements.

But the maximum solubilization occurred when Cel48/Cel9 was ca. 1 due to higher specific activity of Cel9. The synergy between endoglucanase and exoglucanase is complicated, depending on such factors as their ratio, enzyme concentration, reaction time, substrate characteristics (e.g., degree of polymerization and substrate accessibility) (Zhang and Lynd, 2004, 2006b). For example, we also found that the degree of synergy effect increased to about 1.8 when Cel48/Cel9 was 5 and the total enzymes loading was increased to 12 μg/mL (data not shown).

3.5. Solubilization of filter paper by Cel9

Furthermore, we tested the solubilization ability of Cel9, Cel48, and their combination on Whatman No. 1 filter paper (20 mg filter paper in a 2-mL reaction plus 20 μg/mL of each enzyme). As shown in Fig. 6a, Cel9 efficiently solubilized filter paper after 24 h of incubation; the efficiency was higher when Cel48 was supplemented. In contrast, the filter paper in the presence of Cel48 alone appeared nearly intact, suggesting that Cel48 did not solubilize filter paper efficiently. After a prolonged time (72 h), it was found that the combination of Cel48 and Cel9 broke filter paper more efficiently and produced many more fine fibers (Fig. 6a). The combination of Cel48 and Cel9 broke down filter paper more efficiently and produced many more fine fibers (Fig. 6a). Cel48 alone released 0.1% of the filter paper as soluble sugars; Cel9 alone released *ca.*10%; and the combination released *ca.* 17.3% with a degree of synergy effect of 1.7 (Fig. 6b). The above results showed that, although Cel48 alone did not effectively solubilize filter paper, it enhanced overall cellulose degradation.

4. Conclusions

Cel9, the essential component of the *C. phytofermentans* cellulolytic system is a processive endoglucanase that shows activities on both soluble CMC and cellodextrins, and insoluble amorphous cellulose or crystalline cellulose. Cel9 and Cel48 worked together synergistically. Cel9 alone can solubilize crystalline cellulose-filter paper but Cel48 cannot. This work further demonstrated the important role of Cel9 in the *C. phytofermentans* cellulase components for the degradation of crystalline cellulose.

Acknowledgements

This work was supported mainly by the DOE BioEnergy Science Center. The BioEnergy Science Center is a US Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science. This work was also partially supported by the USDA Bioprocessing and Biodesign Center and the DuPont Young Professor Award. Noppadon Sathitsuksanoh was partially supported by the ICTAS scholar program.

References

Arai, T., Kosugi, A., Chan, H., Koukiekolo, R., Yukawa, H., Inui, M., Doi, R., 2006. Properties of cellulosomal family 9 cellulases from *Clostridium cellulovorans*. Appl. Microbiol. Biotechnol. 71, 654–660.

Beldman, G., Voragen, A.G.J., Rombouts, F.M., Pilnik, W., 1988. Synergism in cellulose hydrolysis by endoglucanases and exoglucanase purified from *Trichoderma viride*. Biotechnol. Bioeng. 31, 173–178.

Bordoli, L., Kiefer, F., Arnold, K., Benkert, P., Battey, J., Schwede, T., 2009. Protein structure homology modeling using SWISS-MODEL workspace. Nat. Protoc. 4, 1–13.

Bradford, M.M., 1976. A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Bronnenmeier, K., Staudenbauer, W.L., 1990. Cellulose hydrolysis by a highly thermostable endo-1, 4-β-glucanase (Avicelase I) from *Clostridium stercorarium*. Enzyme Microb. Technol. 12, 431–436.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.

Gal, L., Gaudin, C., Belaich, A., Pages, S., Tardif, C., Belaich, J.P., 1997. CelG from *Clostridium cellulolyticum*: a multidomain endoglucanase acting efficiently on crystalline cellulose. J. Bacteriol. 179, 6595–6601.

Gilad, R., Rabinovich, L., Yaron, S., Bayer, E.A., Lamed, R., Gilbert, H.J., Shoham, Y., 2003. CelI, a noncellulosomal family 9 enzyme from *Clostridium thermocellum*, is a processive endoglucanase that degrades crystalline cellulose. J. Bacteriol. 185, 391–398.

Hong, J., Ye, X., Wang, Y., Zhang, Y.-H.P., 2008. Bioseparation of recombinant cellulose binding module-protein by affinity adsorption on an ultra-high-capacity cellulosic adsorbent. Anal. Chem. Acta 621, 193–199.

Irwin, D., Spezio, M., Walker, L.P., Wilson, D.B., 1993. Activity studies of eight purified cellulases: specificity, synergism and binding domain effects. Biotechnol. Bioeng. 42, 1002–1013.

Irwin, D., Shin, D.H., Zhang, S., Barr, B.K., Sakon, J., Karplus, P.A., Wilson, D.B., 1998. Roles of the catalytic domain and two cellulose binding domains of *Thermomonospora fusca* E4 in cellulose hydrolysis. J. Bacteriol. 180, 1709–1714.

Jauris, S., Rucknagel, K.P., Schwarz, W.H., Kratzsch, P., Bronnenmeier, K., Staudenbauer, W.L., 1990. Sequence analysis of the *Clostridium stercorarium* celZ gene encoding a thermoactive cellulase (Avicelase I): identification of catalytic and cellulose-binding domains. Mol. Gen. Genet. 223, 258–267.

Jindou, S., Xu, Q., Kenig, R., Shulman, M., Shoham, Y., Bayer, E.A., Lamed, R., 2006. Novel architecture of family-9 glycoside hydrolases identified in cellulosomal enzymes of Acetivibrio cellulolyticus and *Clostridium thermocellum*. FEMS Microbiol Lett. 254, 308–316.

Lynd, L.R., Laser, M.S., Bransby, D., Dale, B.E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J.D., Sheehan, J., Wyman, C.E., 2008. How biotech can transform biofuels. Nat. Biotechnol. 26, 169–172.

Mandelman, D., Belaich, A., Belaich, J.P., Aghajari, N., Driguez, H., Haser, R., 2003. X-Ray crystal structure of the multidomain endoglucanase Cel9G from *Clostridium cellulolyticum* complexed with natural and synthetic cello-oligosaccharides. J. Bacteriol. 185, 4127–4135.

Mosbah, A., Belaïch, A., Bornet, O., Belaïch, J.-P., Henrissat, B., Darbon, H., 2000. Solution structure of the module X2_1 of unknown function of the cellulosomal scaffolding protein CipC of *Clostridium cellulolyticum*. J. Mol. Biol. 304, 201–217.

Reverbel-Leroy, C., Pages, S., Belaich, A., Belaich, J.P., Tardif, C., 1997. The processive endocellulase CelF, a major component of the *Clostridium cellulolyticum* cellulosome: purification and characterization of the recombinant form. J. Bacteriol. 179, 46–52.

Taylor, L.E.I., Dai, Z., Decker, S.R., Brunecky, R., Adney, W.S., Ding, S.-Y., Himmel, M.E., 2008. Heterologous expression of glycosyl hydrolases in planta: a new departure for biofuels. Trends Biotechnol. 26, 413–424.

Tolonen, A.C., Chilaka, A.C., Church, G.M., 2009. Targeted gene inactivation in *Clostridium phytofermentans* shows that cellulose degradation requires the family 9 hydrolase Cphy3367. Mol Microbiol. 74, 1300–1313.

Waffenschmidt, S., Janeicke, L., 1987. Assay of reducing sugars in the nanomole range with 2, 2-bicinchoninate. Anal. Biochem. 165, 337–340.

Warnick, T.A., Methe, B.A., Leschine, S.B., 2002. *Clostridium phytofermentans* sp. nov., a cellulolytic mesophile from forest soil. Int. J. Syst. Evol. Microbiol. 52, 1155–1160.

Wilson, D.B., 2009. The first evidence that a single cellulase can be essential for cellulose degradation in a cellulolytic microorganism. Mol Microbiol. 74, 1287–1288.

Zhang, Y.-H.P., 2009. A sweet out-of-the-box solution to the hydrogen economy: is the sugar-powered car science fiction? Energy Environ. Sci. 2, 272–282.

Zhang, Y.-H.P., Lynd, L.R., 2003. Collodextrin production from mixed-acid hydrolysis and chromatographic separation. Anal. Biochem. 332, 225–232.

Zhang, Y.-H.P., Lynd, L.R., 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnol. Bioeng. 88, 797–824.

Zhang, Y.-H.P., Lynd, L.R., 2005. Determination of the number-average degree of polymerization of cellodextrins and cellulose with application to enzymatic hydrolysis. Biomacromolecules 6, 1510–1515.

Zhang, Y.-H.P., Lynd, L.R., 2006a. Biosynthesis of radio-labeled cellodextrins by the *Clostridium thermocellum* cellobiose and cellodextrin phosphorylases for measurement of intracellular sugars. Appl. Microbiol. Biotechnol. 70, 123–129.

Zhang, Y.-H.P., Lynd, L.R., 2006b. A functionally-based model for hydrolysis of cellulose by fungal cellulase. Biotechnol. Bioeng. 94, 888–898.

Zhang, Y.-H.P., Cui, J.-B., Lynd, L.R., Kuang, L.R., 2006a. A transition from cellulose swelling to cellulose dissolution by o-phosphoric acid: evidences from enzymatic hydrolysis and supramolecular structure. Biomacromolecules 7, 644–648.

Zhang, Y.-H.P., Himmel, M., Mielenz, J.R., 2006b. Outlook for cellulase improvement: screening and selection strategies. Biotechnol. Adv. 24, 452–481.

Zhang, Y.-H.P., Hong, J., Ye, X., 2009. Cellulase assays. Methods Mol. Biol. 581, 213–231.

Zhang, X.Z., Zhang, Z., Zhu, Z., Sathitsuksanoh, N., Yang, Y., Zhang, Y.-H.P., 2010. The noncellulosomal family 48 cellobiohydrolase from *Clostridium phytofermentans* ISDg: heterologous expression, characterization, and processivity. Appl. Microbiol. Biotechnol. doi:10.1007/s00253-009-2231-1.

Zhu, Z., Sathitsuksanoh, N., Zhang, Y.-H.P., 2009. Direct quantitative determination of adsorbed cellulase on lignocellulosic biomass with its application to study cellulase desorption for potential recycling. Analyst 134, 2267–2272.