Appl Microbiol Biotechnol (2006) 71: 654–660
DOI 10.1007/s00253-005-0249-6

Takamitsu Arai · Akihiko Kosugi · Helen Chan · Roger Koukiekolo · Hideaki Yukawa · Masayuki Inui · Roy H. Doi

Properties of cellulosomal family 9 cellulases from *Clostridium cellulovorans*

Received: 10 August 2005 / Revised: 31 October 2005 / Accepted: 5 November 2005 / Published online: 11 March 2006
© Springer-Verlag 2006

Abstract The cellulosomal family 9 cellulase genes *engH*, *engK*, *engL*, *engM*, and *engY* of *Clostridium cellulovorans* have been cloned and sequenced. We compared the enzyme activity of family 9 cellulosomal cellulases from *C. cellulovorans* and their derivatives. EngH has the highest activity toward soluble cellulose derivatives such as carboxymethylcellulose (CMC) as well as insoluble cellulose such as acid-swollen cellulose (ASC). EngK has high activity toward insoluble cellulose such as ASC and Avicel. The results of thin-layer chromatography showed that the cleavage products of family 9 cellulases were varied. These results indicated that family 9 endoglucanases possess different modes of attacking substrates and produce varied products. To investigate the functions of the carbohydrate-binding module (CBM) and the catalytic module, truncated derivatives of EngK, EngH, and EngY were constructed and characterized. EngHΔCBM and EngYΔCBM devoid of the CBM lost activity toward all substrates including CMC. EngKΔCBM and EngMΔCBM did not lose activity toward CMC but lost activity toward Avicel. These observations suggest that the CBM is extremely important not only because it mediates the binding of the enzyme to the substrates but also because it participates in the catalytic function of the enzyme or contributes to maintaining the correct tertiary structure of the family 9 catalytic module for expressing enzyme activity.

T. Arai · H. Chan · R. H. Doi (✉)
Section of Molecular and Cellular Biology,
University of California,
Davis, CA 95616, USA
e-mail: rhdoi@ucdavis.edu
Tel.: +1-530-7523191
Fax: +1-530-7523085

Present address:
A. Kosugi
Japan International Research Center for Agricultural Sciences (JIRCAS),
305-8686 Tsukuba, Ibaraki Japan

Present address:
R. Koukiekolo
SIMS Department,
100 Sussex drive,
ON K1A 0R6 Ottawa, Canada

H. Yukawa · M. Inui
Research Institute of Innovative Technology for the Earth (RITE),
Kyoto, Japan

Introduction

Many cellulolytic microorganisms produce an intricate type of multienzyme complex, termed the cellulosome (Doi and Kosugi 2004). Although all β-glucanases in the complex hydrolyze the same β-1,4-glycosidic bond, their mode of action is different, either randomly attacking the cellulose molecule internally (endoglucanase) or degrading it processively from one of the two ends (exoglucanase) (Zverlov et al. 2005).

The glycoside hydrolases such as the cellulases are composed of several functional domains. The most common arrangement is a catalytic domain connected to a carbohydrate-binding module (CBM) via a linker sequence, although many cellulases contain additional functional domains other than a CBM (Ohmiya et al. 1997; Tomme et al. 1995). Catalytic domains of cellulases are categorized into 13 groups of the 97 glycoside hydrolase families (Henrissat and Bairoch 1996; (http://afmb.cnrs-mrs.fr/~pedro/CAZY/ghf.html). CBMs, most of which bind preferentially to cellulose, are also classified into 43 families on the basis of amino acid sequence similarities (http://afmb.cnrs-mrs.fr/~pedro/CAZY/cbm.html). Now, more than 170 genes have been identified in the family 9 glycoside hydrolases. Family 9 catalytic domains are very similar, displaying an (α/α)6-barrel fold and equivalent catalytic machinery. However, enzyme activity differs for family 9 enzymes. The differences may be due to their neighboring modules, which cocrystallize with their respective family 9 catalytic domains (Bayer et al. 1998a,b).

Clostridium cellulovorans produces a cellulase enzyme complex (cellulosome) containing a variety of cellulolytic subunits attached to the nonenzymatic scaffolding compo-

nent termed CbpA (Shoseyov et al. 1992). So far, the following eight cellulosomal cellulase genes of *C. cellulovorans* have been cloned and sequenced: *engB*, *engE*, *engH*, *engK*, *engL*, *engM*, *engY*, and *exgS* (Doi et al. 2003). In addition, the cellulosomal mannanase gene *manA* (Tamaru et al. 2000), the pectate lyase gene *pelA* (Tamaru and Doi 2001), and the xylanase genes *xynA* (Kosugi et al. 2002) and *xynB* (Han et al. 2004) have been cloned and sequenced. Since plant cell walls contain hemicellulose and pectin as well as cellulose, *C. cellulovorans* cellulosomes contain enzymatic activities required to hydrolyze each of the major components of plant cell walls.

The presence of at least five family 9 cellulosomal enzymes has suggested that each of them may have different functions. This paper compares the five family 9 enzymes to determine whether they have different properties. The result indicate that they indeed have different specificities toward different substrates and that this may play a role in more efficient degradation of plant cell wall celluloses.

Materials and methods

Strains and growth conditions *C. cellulovorans* strain (ATCC 35296) (Sleat et al. 1984) was used for the isolation of the genomic DNA. The *Escherichia coli* strains used in this study were NovaBlue and BL21(DE3) (both from Novagen). Recombinant *E. coli* strains were cultured in Luria broth supplemented with kanamycin (50 μg/ml) at 37°C.

Plasmids and plasmid constructions The plasmid vectors used in this study were pET22b, pET29b, pET41 Ek/LIC, and pET42b (Novagen). The plasmids used to produce rEngH, rEngK, rEngL, rEngM, rEngY, and the truncated derivatives were constructed as follows: DNA fragments encoding each gene were amplified by PCR from the *C. cellulovorans* genomic DNA with LA Taq DNA polymerase (Takara), and an appropriate combination of primers containing multicloning sites for inserting the PCR fragments into plasmid vectors was synthesized. After sequencing the inserted DNA fragments for confirmation of the absence of mutations, the inserted fragments were transferred to BL21(DE3) (Table 1).

Purification of EngH, EngL, EngM, EngY, and truncated derivatives To produce recombinant proteins, 1.5 l of cultures of *E. coli* recombinants was grown to mid-log phase (A_{600}=0.6), and after incubation for 1 h at 10°C, isopropyl-β-thiogalactopyranoside was added to the cultures to give a final concentration of 0.5 mM. After an additional incubation of 5 h at 20°C, cells were harvested, washed, and disrupted by sonication. When cells, except for EngH- and EngK-containing cells, were disrupted by sonic oscillation, protease inhibitor tablets (Roche) were added. Cell debris was removed by centrifugation, and the cell-free extracts

Table 1 Oligonucleotides used for cloning

Primer	Sequence	Plasmid
EngHF	5'GTTGGATCCGTTATCAGGAATCTTGG GTGCAACTTC3'	pET42EngH
EngHR	5'TTACTCGAGCTGATAAAAGTAG3'	pET42EngH
EngKF	GACGACGACAAGATGGCCACAGTACT AAGTGTTTCAACAGTTGC3'	pET41EngK
EngKR	5'GAGGAGAAGCCCG GTTTAGTGGTGGTGGTGGTGGTGGTC GACAGAAAGAAGTTTCTTCTT3'	pET41EngK
EngLF	5'CGAATTCGGCACCTAAATTTGA3'	pET22EngL
EngLR	CCGTCGAGACCAAGAAGTAAC3'	pET22EngL
EngMF	5'TCGAGCTCCTGCAACGAATTTTGAT3'	pET29EngM
EngMR	5'CCGCTCGAGTGCAAGCAGTTGTTTCTT3'	pET29EngM
EngYF	5'TCGGATCCGGACACTACTGTCTCA3'	pET22EngY
EngYR	5'TTTTAGTCGACGAAGCTATTAATTTG3'	pET22EngY
EngHCATF	5'GACGACGACAAGATGTTATCGGAAT CTTGGGTGCAAC3'	pET41EngHΔCBM
EngHCATR	5'GAGGAGAAGCCCGGT TTAGTGGTGGTGGTGGTGGTGAAATTCAC TTACATTTATGAAGTATTTGAATGA3'	pET41EngHΔCBM
EngKIgF	5'GTTGGATCCGGGATATGTAGCTGAAACTCCAGAA3'	pET22EngKΔCBM
EngKR	TTACTCGAGAGAAAGAAGTTTCTT3'	pET22EngKΔCBM
EngMCATF	5'GTTGGATCCGCCACCAGAAGAGCCAACAAATGCGG3'	pET22EngMΔCBM
EngYLICIgF	5'GACGACGACAAGATGAACGAAAAATCCT ATGCTCCAATAAAGGTTAAT3'	pET41EngYΔCBM
EngYLICR	5'GAGGAGAAGCCCGGTTTAGTGGTGGTGG TGGTGGTGGTCGACGAAGCTATTAATTTGGCC3'	pET41EngYΔCBM

thus obtained were used for enzyme purification. EngL, EngM, and EngY were purified by a HiTrap chelating column (Amersham Pharmacia Biotech). EngH and EngK were applied to S-protein agarose (Novagen), and the proteins bound to resin were purified and pooled according to the product manual. The glutathione S-transferase (GST) tag was removed from the recombinant proteins by recombinant enterokinase (Novagen) digestion after binding to S-protein agarose. The pooled solution was desalted and concentrated into sodium phosphate buffer (pH 7.0) by use of an Ultrafree 10-kDa membrane (Millipore). After the HiTrap chelating column, the EngMΔCBM was applied to a RESOURCE Q (Amersham Pharmacia Biotech) column. The purity of each fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The protein concentration was determined with bovine serum albumin as the standard using the Micro BCA Protein Assay Reagent Kit (Pierce).

Enzyme assays Enzyme activity was assayed in the presence of 0.5% (wt/vol) concentration of each polysaccharide at 37°C in 50 mM acetate buffer (pH 6.0). Enzyme concentration was 0.5 mM. Samples (500 μl) were collected at appropriate times and immediately mixed with 500 μl chilled 0.38 M sodium carbonate containing 1.8 mM cupric sulfate and 0.2 M glycine. The reducing sugars were determined by reductometry with the Dygert et al.'s (1965) method. One unit of activity was defined as the amount of enzyme releasing 1 μmol of glucose equivalent per minute from carboxymethylcellulose (CMC). All assays were repeated three times.

Analysis of hydrolysis products Cellooligosaccharides (SEIKAGAKU) (cellobiose to cellohexaose, 5 mg each) were incubated with 0.1 U of the purified enzyme in 1 ml of 50 mM sodium succinate buffer (pH 6.5) at 37°C. Thin-layer chromatography (TLC) of the hydrolysis products was performed on a DC-Fertigplatten SIL G-25 plate (Macherey-Nagel, Dorne) developed with a solvent of 1-propanol/water (85:15, vol/vol). Cellooligosaccharides were visualized by spraying the plate with aniline–diphenylamine reagent (Gasparic and Churacek 1978).

Results

Domain structure and identity of family 9 cellulosomal cellulases

Five family 9 cellulosomal glycosidases were expressed in *E. coli* and characterized. EngK and EngM had the same domain composition, whereas EngH, EngL, and EngY had different domain compositions. Each family 9 glycosidase had an accessory domain (CBM or Ig-like domain) at the N or C terminus except for EngL. EngL was composed of only a catalytic and a dockerin domain. Figure 1 shows the domains of family 9 cellulosomal enzymes from *C. cellulovorans*. The family 9 catalytic domain of EngK extended from 310 to 819 amino acids and exhibited extensive sequence homology with the catalytic domains

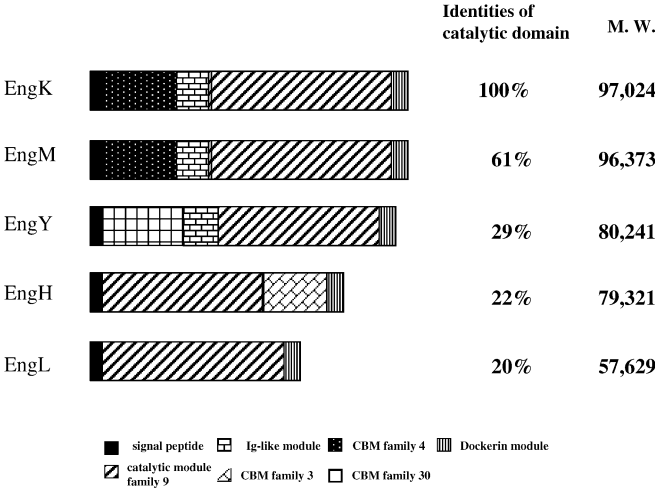

Fig. 1 Molecular architecture and molecular weight of family 9 cellulases from *Clostridium cellulovorans*. Amino acid sequences were obtained from NCBI: EngH, U34793; EngK, AF132735; EngL, AF132735; EngM, AF132735; EngY, AF105330

of the other family 9 cellulases, e.g., 69% identity with EngM, 32% identity with EngY, 23% identity with EngH, and 22% identity with EngL (Fig. 1).

Protein expression of family 9 enzymes and their derivatives

Family 9 enzymes and their derivatives were constructed as described in "Materials and methods." Each of the purified preparations gave a major band upon SDS-PAGE analysis, and their molecular sizes were in good agreement with those deduced from the nucleotide sequences (Fig. 2). EngKΔCBM, EngMΔCBM, and GST-EngYΔCBM, with deleted CBMs, consisted of an Ig-like domain and a family 9 catalytic domain; EngHΔCBM, with deleted CBM, consisted of only a family 9 catalytic domain (Fig. 3).

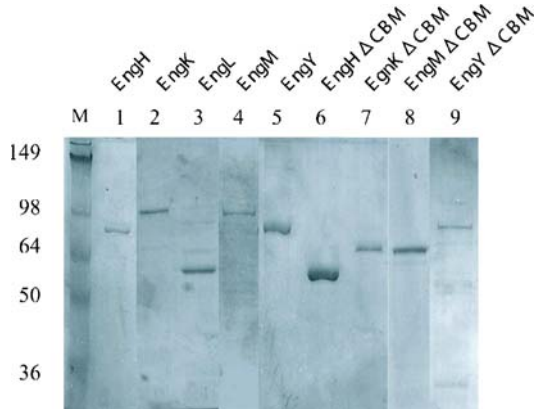

Fig. 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified derivatives of family 9 cellulase. The gel was stained with Coomassie brilliant blue. *Lane M* protein molecular mass standard (molecular masses shown at the *left*), *lane 1* EngH, *lane 2* EngK, *lane 3* EngM, *lane 4* EngL, *lane 5* EngY, *lane 6* EngHΔCBM, *lane 7* EngKΔCBM, *lane 8* EngMΔCBM, *lane 9* EngYΔCBM

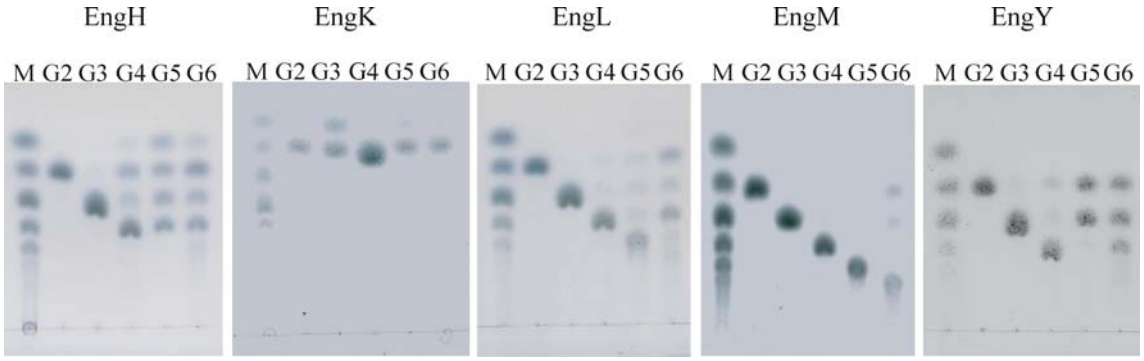

Fig. 3 Molecular architecture and molecular weight of recombinant enzymes lacking a CBM used in this study

These truncated proteins were purified from whole-cell lysates of *E. coli* recombinants by column chromatography.

Enzymatic property and activity of EngH, EngK, EngL, EngM, and EngY

Each of the recombinant enzymes was tested for substrate specificity and activity. The purified rEngH had relatively the highest specific activity toward CMC (5.875 U/mg) and β-glucan (1.018 U/mg). rEngK had the highest activity toward Avicel (0.018 U/mg) and the lowest activity toward CMC. rEngL had activity only toward CMC (0.632 U/mg), Avicel (0.002 U/mg), and acid-swollen cellulose (ASC; 0.009 U/mg). rEngM had activity toward CMC (0.096 U/mg), Avicel (0.006 U/mg), ASC (0.001 U/mg), and lichenan (0.104 U/mg). rEngY had relatively high specific activity as EngH toward CMC (5.423 U/mg). All five family 9 enzymes had no activity toward Birchwood xylan and oat spelts xylan (Table 2).

rEngH had the highest activity toward ASC and CMC. rEngK had the highest activity toward Avicel. These results suggested that these two enzymes played a key role in degradation of insoluble cellulose.

EngH, EngM, and EngY were maximally active around pH 8.0, 6.0, and 6.5, respectively, and EngK, EngL, EngKΔCBM, and EngMΔCBM had an optimum pH of 7.0 when the enzyme activity was assayed by 10-min incubation at 42°C in Britton and Robinson universal buffer solutions (50 mM phosphoric acid–50 mM boric acid–50 mM acetic acid; the pH was adjusted to 3–9 with NaOH). EngH, EngK, and EngM were stable in the pH range of 5–9, and EngL and EngY were stable at the pH range of 5–8 when incubated at 37°C for 30 min in the same buffer solutions without any substrates. The optimum temperature for activity was found to be 42°C for EngH and EngM and 37°C for EngK and EngY. All enzymes were stable at 40°C for 10 min at pH 7.0 in the absence of substrates (Table 3), except for EngMΔCBM, which was stable at 35°C.

Analysis of hydrolysis products

The action of the enzyme on cellooligosaccharides was qualitatively analyzed by TLC. When cellotetraose, cellopentaose, and cellohexaose were treated with rEngH, glucose, cellobiose, cellotetraose, cellopentaose, and cellohexaose were produced. rEngH cleaved cellulose randomly. When cellotetraose, cellopentaose, and cellohexaose were treated with rEngK, cellobiose was the main product (Fig. 4). The enzyme was not active toward cellotriose and cellobiose. These results indicated that rEngK was an exoglucanase that hydrolyzed substrates processively.

When cellotetraose (G3), cellopentaose (G5), and cellohexaose (G6) were treated with rEngH, glucose, cellobiose (G2), cellotetraose (G4), G5, and G6 were produced. rEngH cleaved cellulose randomly. rEngL, rEngM, and rEngY also degraded cellooligosaccharides randomly. rEngL and rEngM exhibited slow reaction rates for cellooligosaccha-

Table 2 Activities of rEngH, EngK, rEngL, rEngM, EngHΔCBM, EngKΔCBM, EngMΔCBM, and EngYΔCBM toward various substances

Substrate	Specific activity (U/mg)								
	rEngH	rEngK	rEngL	rEngM	rEngY	EngHΔCBM	EngKΔCBM	EngMΔCBM	EngYΔCBM
CMC	5.875	0.021	0.632	0.096	5.423	ND	0.136	0.003	ND
Avicel	0.014	0.018	0.002	0.006	0.003	ND	ND	ND	ND
ASC	0.065	0.057	0.009	0.001	0.016	ND	0.003	ND	ND
β-Glucan	1.018	0.038	ND	NT	0.197	ND	ND	ND	ND
Lichenan	0.025	ND	ND	0.104	0.031	ND	ND	ND	ND
Laminarin	ND	ND	ND	ND	ND	ND	ND	ND	ND
Oat spelts xylan	ND	ND	ND	ND	ND	ND	ND	ND	ND
Birchwood xylan	ND	ND	ND	ND	ND	ND	ND	ND	ND

CMC Carboxymethylcellulose, *ASC* acid-swollen cellulose, *ND* no activity detected, *NT* no test

Table 3 Properties of purified rEngH, rEngK, rEngL, EngM, rEngY, EngKΔCBM, and EngMΔCBM

	rEngH	rEngK	rEngL	rEngM	rEngY	EngKΔCBM	EngMΔCBM
Optimum pH	8.0	7.0	7.0	6.0	6.5	7	7
pH stability	5–9	5–9	5–8	5–9	5–8	NT	NT
Optimum temperature (°C)	42	37	42	42	37	NT	NT
Thermostability (°C)	<40	<40	<40	<40	<40	<40	<35

NT No test

rides smaller than G5. These results indicated that family 9 enzymes indeed had different substrate specificities and that this fact may play a role in the efficient degradation of plant cell wall celluloses.

Enzymatic activity of EngHΔCBM, EngKΔCBM, EngMΔCBM, and EngYΔCBM

Three enzymes lacking their CBMs were synthesized and characterized. The initial rates of the reactions were measured at 37°C in various concentrations of CMC. The purified rEngKΔCBM had activity toward CMC (0.1363 U/mg) and ASC (0.003 U/mg) but showed no activity toward Avicel, lichenan, laminarin, Birchwood xylan, oat spelts xylan, and laminarin (Table 2). The enzyme was maximally active around pH 7.0 when the activity was assayed by 10-min incubation at 37°C in Britton and Robinson universal buffer solutions at various pHs. The enzyme was stable at 40°C for 10 min at pH 7.0 in the absence of substrates (Table 3).

EngMΔCBM had activity toward CMC (0.003 U/mg), in contrast with no activity toward any other substrate. The purified EngHΔCBM lacking the C-terminal CBM did not show activity toward CMC and other substrates (Table 3). The purified EngYΔCBM lacking the N-terminal CBM also did not show activity toward CMC and other substrates. These observations indicate that the hydrolytic activity of the family 9 catalytic domain of EngHΔCBM and EngYΔCBM depends completely on the presence of the CBM co-terminus to the catalytic module.

Discussion

At least eight cellulase genes have been cloned and sequenced from *C.cellulovorans*, and five of them encode enzymes classified in family 9 of the glycoside hydrolases (Henrissat and Bairoch 1996): *engH*, *engK*, *engL*, *engM*, and *engY*. There are six enzymes encoded in the cellulosome-related gene cluster (Doi et al. 2003), of which four genes encode family 9 enzymes (except for EngY).

EngK exhibited extensive sequence homology with family 9 cellulosomal enzymes of other *Clostridia*, e.g., 55% identity with CelE of *C.cellulolyticum* and 43% identity with CelK of *Clostridiumthermocellum*. CelE and CelK have properties similar to those of EngK. The final product of these three enzymes from cellulose is cellobiose, and they have similar activity patterns (Gaudin et al 2000; Kataeva et al 1999). EngM has 69% identity with EngK, but these two enzymes have different activity patterns, indicating that homology between family 9 enzymes cannot be used to predict their activity patterns.

Family 9 cellulases can be classified into three groups: enzymes consisting of a catalytic module and a family 3c CBM; enzymes consisting of an Ig-like module and a cata-

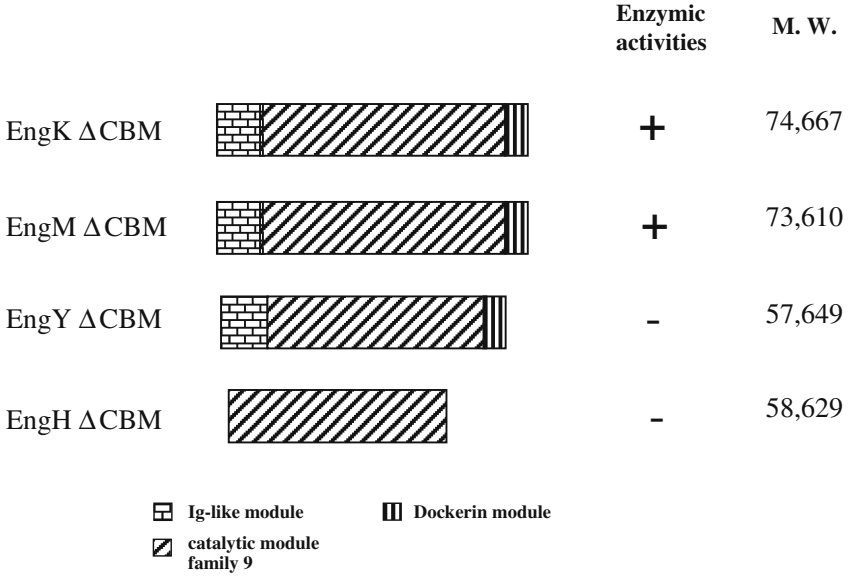

Fig. 4 Thin-layer chromatography analysis of hydrolysis products from cellooligosaccharides. Each cellooligosaccharide (5 μg, G2–G6) was incubated with purified rEngH, rEngK, EngL, EngM, and EngY for 14 h. *M* Authentic oligosaccharides, *G1* glucose, *G2* cellobiose, *G3* cellotriose, *G4* cellotetraose, *G5* cellopentaose, *G6* cellohexaose

lytic module; and enzymes consisting of only a catalytic domain. Belaich et al. (2002) divided the enzymes containing an Ig-like module into two categories depending on the presence or absence of an N-terminal family 4 CBM. For example, *C. thermocellum* CbhA and CelK contained an N-terminal family 4 CBM, but CelD did not. CelJ (CBM30-Ig-CM9)(1) and EngY, which contains a family 30 CBM as an additional module, are new types of family 9 cellulase containing an Ig-like module.

Most family 9 cellulases have noncatalytic modules such as CBM, Fg3 (fibronectin homologous module), and Ig-like modules. It is believed that a CBM in a cellulase molecule enhances the hydrolytic activity of a catalytic module adjacent to the CBM by increasing the enzyme concentration on the surface of an insoluble substrate or by supplying the catalytic module with a more easily degradable substrate, e.g., amorphous cellulose. EngY has a CBM30. CBM30 showed a wide ligand specificity including insoluble and soluble cellulosic materials, β-1,3–1,4 mixed glucan such as lichenan, and barley β-glucan (Arai et al. 2003). It seems reasonable that the family 30 CBM has an affinity for β-1,3–1,4 mixed glucan because the family 9 catalytic module of EngY has hydrolytic activity toward this substrate, and therefore, the presence of the CBM is expected to enhance its activity.

EngL has activity toward cellulose, in contrast with no activity toward hemicelluloses tested. EngL does not have a CBM. However, the absence of a CBM in EngL is not necessarily disadvantageous since EngL, a component of the cellulosome, always exists in the cellulosome, and EngL and other cellulases lacking a CBM can depend on the function of the CBM carried by the scaffolding protein and some catalytic components having their own CBMs. CelA (Béguin et al. 1985) and CelS (Wang et al. 1993), both of which are representative major catalytic components of the *C. thermocellum* cellulosome, consist of only a catalytic domain and a dockerin domain.

In general, a catalytic module and a CBM in the same polypeptide can function independently, e.g., artificial removal of the CBM from the catalytic domain does not affect the enzyme activity toward soluble substrates. Many papers show that some family 9 cellulases contain a family 3c CBM, and its presence is important for the activity of the enzyme (Arai et al 2001; Gal et al 1997; Irwin et al 1998). EngH lost activity when its CBM was removed from the catalytic module. CBM EngYΔCBM did not have any activity. The removal of a family 30 CBM from CelJ also decreased its catalytic activity (Arai et al. 2003). In doing so, it appeared to take part, in a more direct manner, in the catalytic function of the enzyme. The CBMs of this subfamily may thus be considered to serve as a cellulose-binding subsite of the catalytic domain (Bayer et al. 1998a,b).

This paper compared the five family 9 enzymes to determine whether they had different properties. The results indicated that family 9 enzymes degraded substrates differently and also yielded products that were different. Thus, it appears that having a number of family 9 enzymes in the cellulosome could facilitate the degradation of cellulose.

In addition, for several family 9 enzymes, it was shown that cellulase activity was significantly reduced by removal of the CBM from the catalytic domain. This demonstrates the importance of CBM in the function of family 9 enzymes. Site directed mutant analysis is in progress to demonstrate the importance of the CBM for some of the family 9 enzymes. The interactions between family 9 catalytic module and the other modules remain to be studied.

Acknowledgements This research was supported in part by grant DE-DDF03-92ER20069 from the U.S. Department of Energy and a grant from the RITE Institute, Kyoto, Japan.

References

Arai T, Ohara H, Karita T, Kimura T, Sakka K, Ohmiya K (2001) Sequence of *celQ* and properties of CelQ, a component of the *Clostridium thermocellum* cellulosome. Appl Microbiol Biotechnol 57:660–666

Arai T, Araki R, Tanaka A, Karita S, Kimura T, Sakka K, Ohmiya K (2003) Characterization of a cellulase containing a family 30 carbohydrate-binding module (CBM) derived from *Clostridium thermocellum* CelJ: importance of the CBM to cellulose hydrolysis. J Bacteriol 185:504–512

Bayer EA, Morag E, Lamed R, Yaron S, Shoham Y (1998a) Cellulosome structure; four-pronged attack using biochemistry, molecular biology, crystallography and bioinformatics. In: Claeyssens M, Nerinckx W, Piens K (eds) Carbohydrate from *Trichoderma reesei* and other microorganisms. Bookcraft, Bath, UK, pp 39–65

Bayer EA, Shimon LJW, Lamed R, Shoham Y (1998b) Cellulosomes: structure and ultrastructure. J Struct Biol 124:221–234

Béguin P, Cornet P, Aubert JP (1985) Sequence of a cellulase gene of the thermophilic bacterium *Clostridium thermocellum*. J Bacteriol 162:102–105

Belaich A, Parsiegla G, Gal L, Villard C, Haser R, Belaich JP (2002) Cel9M, a new family 9 cellulase of *Clostridium cellulolyticum* cellulosome. J Bacteriol 184:1378–1384

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

Doi RH, Kosugi A, Murashima K, Tamaru Y, Han SO (2003) Cellulosomes from mesophilic bacteria. J Bacteriol 185:5907–5914

Dygert S, Li LH, Don Florida R, Thoma JA (1965) Determination of reducing sugar with improved precision. Anal Chem 13:367–374

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

Gasparic J, Churacek J (1978) Laboratory handbook of paper and thin-layer chromatography. Ellis Horwood, Chichester, pp 153–154

Gaudin C, Belaich A, Champ S, Belaich JP (2000) CelE, a multidomain cellulase from *Clostridium cellulolyticum*: a key enzyme in the cellulosome? J Bacteriol 182:1910–1915

Han SO, Yukawa H, Inui M, Doi RH (2004) Isolation and expression of the *xynB* gene and its product, XynB, a consistent component of the *Clostridium cellulovorans* cellulosome. J Bacteriol 186:8347–8355

Henrissat B, Bairoch A (1996) Updating the sequence-based classification of glycosyl hydrolases. Biochem J 316:695–696

Irwin D, Shin DH, Zhang S, Barr BK, Sakon J, Karplus PA, Wilson DB (1998) Roles of the catalytic domain and two cellulose binding domains of *Thermomonospora fusca* E4 in cellulose hydrolysis. J Bacteriol 180:1709–1714

Kataeva I, Li XL, Chen H, Choi SK, Ljungdahl LG (1999) Cloning and sequence analysis of a new cellulase gene encoding CelK, a major cellulosome component of *Clostridium thermocellum*: evidence for gene duplication and recombination. J Bacteriol 181:5288–5295

Kosugi A, Murashima K, Tamaru Y, Doi RH (2002) Xylanase and acetyl xylan esterase activities of XynA, a key subunit of the *Clostridium cellulovorans* cellulosome for xylan degradation. Appl Environ Microbiol 68:6399–6402

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Ohmiya K, Sakka K, Karita S, Kimura T (1997) Structure of cellulases and their applications. Biotechnol Genet Eng Rev 14:365–414

Shoseyov O, Takagi M, Goldstein M, Doi, RH (1992) Primary sequence analysis of *Clostridium cellulovorans* cellulose binding protein A (CbpA). Proc Natl Acad Sci U S A 89:3483–3487

Sleat R, Mah RA, Robinson R (1984) Isolation and characterization of an anaerobic, cellulolytic bacterium, *Clostridium cellulovorans* sp. nov. Appl Environ Microbiol 48:88–93

Tamaru Y, Doi RH (2001) Pectate lyase A, an enzymatic subunit of *Clostridium cellulovorans* cellulosome. Proc Natl Acad Sci U S A 98:4125–4129

Tamaru Y, Karita S, Ibrahim A, Chan H, Doi RH (2000) A large gene cluster for the *Clostridium cellulovorans* cellulosome. J Bacteriol 182:5906–5910

Tomme P, Warren RAJ, Gilkes NR (1995) Cellulose hydrolysis by bacteria and fungi. Adv Microb Physiol 37:1–81

Wang WK, Kruus K, Wu JHD (1993) Cloning and the DNA sequence of the gene coding for the *Clostridium thermocellum* cellulase Ss (CelS), a major cellulosome component. J Bacteriol 175:1293–1302

Zverlov VV, Schantz NN, Schwarz WH (2005) A major new component in the cellulosome of *Clostridium thermocellum* is a processive endo-beta-1,4-glucanase producing cellotetraose. FEMS Microbiol Lett 15:353–358