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Molecular Cloning and Biochemical Characterization of a Family-9 Endoglucanase With an Unusual Structure From the Gliding Bacteria *Cytophaga hutchinsonii*

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Abstract

Cytophaga hutchinsonii was originally isolated from sugarcane piles. This microorganism therefore probably produces an array of enzymes allowing it to digest cellulosic substrates. *C. hutchinsonii* thus represents a rich source of potentially effective cellulase enzymes that can be harnessed for conversion of biomass to simple sugars. These sugars can then be used as feedstock for ethanol production or other chemical syntheses. In this study, we report the PCR cloning of an endoglucanase gene (Cel9A) from *C. hutchinsonii* using degenerated primers directed at the catalytic domain. Alignment of the amino acids sequence revealed that Cel9A has a gene structure totally different from the other known cellulose degraders. The most striking feature of this cloned protein is the absence of a cellulose-binding domain (CBD), which to date was believed to be imperative in cellulose hydrolysis. Consequently, the

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Cel9A gene, encoding β-1,4 endoglucanase from *C. hutchinsonii* was overexpressed in *Escherichia coli* with a His-Tag based expression vector. The resulting polypeptide, with a molecular mass of 105 KDa, was purified from cell extracts by affinity chromatography on cellulose. Mature Cel9A was optimally active at pH 5.0 and 45°C. The enzyme efficiently hydrolyzes carboxymethyl-cellulose (CMC). Analysis of CMC and filter paper hydrolysis suggests that Cel9A is a nonprocessive enzyme with endo-cellulase activities.

Index Entries: Cytophaga; cellulase; cellulose-binding domain.

Introduction

Converting plant biomass into biofuels and usable chemicals has been a long-term goal of scientists studying cellulases and related glycosyl hydrolases *(1)*. Much progress has been made in identifying, cloning, expressing, and characterizing cellulases from both aerobic and anaerobic bacteria and from fungi *(2)*. Natural biomass substrates, such as corn fiber or sugarcane bagasse, are structurally complex and many other enzymes besides cellulases are needed for efficient degradation of the polysaccharides to monosaccharides. The organisms known to degrade cellulose release a battery of enzymes with different specificities, endoglucanase, exoglucanase, and β-glucosidase *(3)*. Many of the cellulase genes that have been cloned, have been characterized by DNA sequence analysis. On the basis of these studies and other work, at the protein level, some generalizations regarding the structure and function of cellulases are emerging. In a review, Knowles et. al. *(4)* noted that, notwithstanding the lack of sequence similarity, at both the DNA and protein levels, among cellulases and their genes, there are certain general features that these cellulases share. Cellulases appear to contain three separate domains: (1) an N-terminal hydrolytic domain, (2) a serine-, proline-, and threonine-rich linker region, and (3) a C-terminal domain which is responsible for the binding of the enzymes to the substrate. As noted above, the lack of sequence similarity may be the consequence of independent gene evolution, whereas, within an organism, selection pressure may be sufficient to enable gene duplication and mutagenesis to create and maintain intraspecific gene families *(5)*. The ancestral or evolutionary source sequence for cellulase genes may have been a gene that encodes a pre-existing glycolytic enzyme(s) *(5)*. At this time, a detailed understanding of mechanism underlying some cellulase genes, such as *Cytophaga hutchinsonii*, remains to be elaborated.

Cytophaga hutchinsonii is a Gram-negative soil bacterium isolated from sugarcane piles. It grows poorly in minimal liquid medium with carbohydrate polymers such as cellulose, starch, cellobiose, or xylan as the sole carbon source. But it is thought to be an important organism in the degradation of biomass *(6)*. Consequently, this study was designed to evaluate the cellulose decomposing potential of *C. hutchinsonii* by cloning, expressing, and characterizing the endoglucanase gene from this organism. In the

present study, we show that the enzyme Cel9A, belonging to the glycoside hydrolase family 9, is an endoglucanase with a gene structure different from known cellulose degraders and investigated its properties and possible role in the degradation of plant biomass.

Methodology

Cloning Experiment

Stock cultures of *C. hutchinsonii* were inoculated in liquid Luria-Bertani (LB) medium and grown at 26°C overnight with shaking. Cells were harvested by centrifugation at 13,000 rpm. DNA preparation and electrophoresis of DNA fragments were completed using routine procedures *(7)*. Amplification of the DNA fragment encoding a portion of the cellulase gene of the *C. hutchinsonii* endoglucanase was performed using the polymerase chain reaction (PCR) with the following primers: 5'-AGT TGG TAT GAT GCT GGT GAT CAT-3' as the forward primer, and 5'- CTG ACT GTT ATT TGG AAC TGC TCT-3' as the reverse primer *(8)*.

Degenerate PCR primers were designed as follows: the forward/reverse primers were based on alignment of highly conserved sequences. Several bacterial endoglucanases were pooled together and aligned by CLUSTAL *(9)*. The generated blocks were pasted into the computer software CODEHOP, known to design primers from nonconsensus sequences *(10)*. PCR reactions (25 µL) with the prepared oligonucleotide primers were performed in an MJ gradient thermocycler. The amplification conditions were: denaturation for 1 min at 94°C followed by 35 amplification cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C and a final elongation step of 5 min at 72°C. Products were separated by agarose gel electrophoresis; a product of about 500 bp was purified from the gel with Qiagen gel purification kit *(11)*.

The purified PCR product was then cloned into *E. coli* XLB-10 Gold by transformation. The transformed clones were then grown on LB medium supplemented with ampicillin (100 µg/mL). The positives clones were screened on X-gal plates. White colonies were allowed to grow on LB_{amp} overnight. Plasmids were isolated and run on a gel for verification. The ones with the right size were purified as described earlier. After purification, 18 µL of the pure DNA was sent away for sequencing. The resulting sequence was analyzed with the software program SEQUENCHER *(12)* and specific primers were designed for chromosome walking to fish the full gene out.

Expression Experiment

Stock cultures of *C. hutchinsonii* were used. They were grown in LB media for inoculums and in minimal media for biochemical assays. The cultures were allowed to shake (150 rpm) at 26°C overnight in a C24 incubator shaker. The main buffers used were NEST (0.1 *M* Tris-HCL, pH 8.0),

HEPES (0.01 *M* + 10% glycerol) and 40–60 mL of 0.6 *M* $(NH_4)2SO_4$ + 0.1 *M* NaCl + 5 m*M* KPi, pH 6.0).

E. coli XLB-Gold was used as a host for recombinant plasmids. Plasmid pGEM and pET28 were used as shuttle and expression vectors, respectively *(13,14)*. *E. coli* cells were grown on LB medium supplemented with ampicillin (100 μg/mL) and Kanamycin (60 μg/mL) when needed. Synthesis of endoglucanase was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) when the optical density (OD) of the cultures reached 0.2 (1 OD corresponds to 0.33 g/L). Bacteria were harvested by centrifugation 8 h after induction (13,000 rpm for 10 min).

Cel9 under the control of its own promoter was poorly expressed in *E. coli (15)*. To increase protein expression, Cel9A was introduced into plasmid pGEM under the control of the ptac promoter. The construction was carried out as follows *(7)*. The catalytic domain of Cel9A was framed by using its own ATG at the initiation site and by adding two stop codons (TAATAA) at the termination site. Because no single restriction site was available at the beginning of the sequence, a Not1 site was introduced by site-directed mutagenesis. A Sac1 site was also introduced by the same procedure before the termination site. A strain harboring this plasmid was used to obtain high quantities of EGCCel9A after induction.

The procedure for purification of EGCCEL9A was conducted based on a modified method from Wilson's laboratory at Cornell University and Sambrook *(15)*. Cells of *C. hutchinsonii* were grown from a frozen stock in LB broth, and used to inoculate 250 mL of the same medium. This culture was grown overnight and used to inoculate a 6-L fermenter. Fermentation was maintained at 30°C, pH 7.2, with shaking at 150 rpm. The supernatant was clarified by centrifugation and loaded onto a 90-mL phenyl-Sepharose column, 5 cm in diameter. The column was washed with 250 mL of 1 *M* ammonium sulfate, 10 m*M* NaCl, 5 m*M* KPi pH 6, followed by 350 mL of 0.3 *M* ammonium sulfate, 5 m*M* NaCl, 5 m*M* KPi pH 6. Thirty milliliters of this buffer were left on the column and 5 m*M* KPi, pH 6.0 was added slowly to elute the Cel9A protein. The resulting fractions were tested for CM-cellulose activity and also visualized on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels (10% acrylamide–4X separating buffer: 0.4% SDS, 1.5 *M* Tris-HCl, pH 8.8). The cleanest fractions were combined. The protein was further purified using a 25-mL Q Sepharose column in 10 m*M* Bis-Tris/Hcl pH 5.8 + 10% glycerol buffer. Cel9a was eluted using a 0–0.5 *M* NaCl gradient in the same buffer. The best fractions as judged by SDS-PAGE gels were combined to yield 36 mg (6.0 mg/L culture) of Cel9A. The preparations visually appeared to be pure on SDS gels and no contaminating CM-cellulase bands were seen on native gels which had been overlaid with CM-cellulose.

For cloning into the pET vector, pET28 was used as the expression vector. Cel9A was successfully cloned into pET28 *(14)*. pGEM generated high quantities of cells, whose plasmids have been isolated, and sequence to avoid any unexpected mutations.

Characterization Experiment

After analysis of the sequence chromatograph picture, the DNA sequence was transferred into National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) for homology search *(16)*. A specific percentage of identity was revealed for each of the sequence in the database (GENEBANK) showing homology with our input sequence. The software program PROSITE was also used to analyze the amino acid sequence. The ScanProsite tool allows the scanning of protein sequences for the occurrence of patterns, profiles and rules (motifs) stored in the PROSITE database, or to search protein database(s) for hits by specific motif(s) *(17)*.

CM-cellulose filter paper assays were run as described previously for CMC assay in 50 m*M* Kpi, pH 5.5 buffer at 30°C. Reducing sugar was determined using dinitrosaliccylic acid (DNS) Procedure *(18)*. pH studies were carried out using buffers made with 50 m*M* NaH_2PO_4, 50 m*M* boric acid, and 40 m*M* acetic acid, and adjusted to the desired pH with NaOH. Protein concentrations were measured using the Bradford Protein Determination Method *(19)*. Low protein concentrations in the supernatant have to be acid concentrated in order to avoid false reading by the Lowry method *(20)*.

Effects of pH on Activity

Enzyme activity was determined in the following buffer systems (50 m*M*): acetate, pH 4.0 to 8.0, and phosphate, pH 3.0 to 9.0. Samples were incubated overnight at 30°C and the resulting activity was measured spectrophotometrically at a wavelength of 600 nm *(18)*.

Effects of Temperature on Stability

Temperature stability was determined by incubating concentration protein (0.3 mg/mL) at the indicated temperatures (4, 16, 25, 50, and 65°C) in buffer containing 10 m*M* $CaCl_2$ or 2 m*M* EDTA or no supplements. After an overnight incubation of 14 h, cellulase activities were measured as previously described *(18)*.

Viscosity

Decrease in viscosity was determined by measuring the decrease in the time necessary for 1 ml of reaction mixture (0.5% CMC in buffer B, 55 µg/mL Cel9A) to travel 20 cm down a vertical glass pipet after incubation with enzyme at 30°C for 1 h. Known endo- (E4) and exo-cellulases (E3) were used as standard *(21)*. Water with zero viscosity was used as reference sample or blank.

Proces.sivity

Cellulase enzymes attack the cellulose chain by either doing one or several successive cleavages, releasing thereby non-reducing or reducing ends *(1)*. Such behavior is termed enzyme processivity and it is measured by the ratio between reducing and nonreducing ends released. In this study,

enzyme processivity was performed with filter paper (FP) as the substrate. Buffer used in this experiment was sodium acetate, pH 4.0 and 5.0. It was incubated with the substrate (FP) and Cel9A overnight at optimum temperature (30°C). Of the seven incubations, three were used to determine the combined reducing ends (CRE) and four were pooled together for the super reducing ends (supernatant where the soluble sugars are to be found). The insoluble reducing ends (IRE) were determined after washing the filter paper three times with buffer.

Results and Discussion

DNA sequence analysis of the cloned DNA fragment coding for the cellulase gene identified an open reading frame (ORF) of 2934 bases, starting at nucleotide (nt) 30 with an ATG codon (Fig. 1). This sequence has 31% homology with endB gene coding for the endoglucanase EGB from *Pseudomonas sp*. *(23)*. Comparison of nucleotide sequence of Cel9A with sequences from the DATABANK using the FASTA program revealed that Cel9A belongs to family 9 of Glycosyl-Hydrolases and particularly to subfamily E1. Alignments of all the E1 sequences and determination of the percentages of similarity were done using the CLUSTAL program. This alignment suggest that Cel9A consists of a catalytic Domain of 480aa and a C-terminal adjoining immunoglobulin (Ig)-like domain of unknown function, comprising 102aa, that is present in all family E1 enzymes. An additional sequence of 31aa was present at the N-terminal part of Cel9A. This sequence turned out to be a signal sequence based on hydrophobicity analysis by the software program SIGNAL-P *(22)*.

A search for homologous proteins revealed a glycosyl hydrolase family-9 catalytic domain located from amino acid 30 to 590 and an Ig-like domain from amino acid 600 to 890. An alignment of Cel9ACD and other family 9 showed a 99.3% homology between these proteins. A CLUSTAL alignment of 2 family 9 catalytic domains performed with the DNA STAR MEGALIGN Program, revealed 30% similarity between them. It is remarkable that 30% of the residues are identical in all members of the family. There is an Ig-like domain module in Cel9A from Amino acid 600 to 890 that is homologous to domains found in four other endoglucanases. This Ig-like domain is only weakly homologous to the three Ig-like repeats found in the family 9 enzyme *Thermobifida fusca.* Thus far, the functions of these domains, which consist of repeating units of approx 120 amino acids, remain unknown for microbial carbohydrates.

Cel9A showed a pH optimum at 6.0–6.5 and retained >50% of its activity from pH 5.0 to pH 9.0 (Fig. 2). Thermostability, as tested by preincubation without substrate at various temperatures, decreased markedly above 50°C. When SC assays were run overnight at different temperatures, activity was the same at 40°C and 50°C and declined to approx 30% at 60°C (Fig. 3). The isoelectric point (pI) of the cloned protein was calculated to be 6.5 from the number of basic and acidic residues. Cel9A does not cleave xylan, although an operon homologous to xylan operon in other organisms

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Fig. 1. Nucleotide sequences of the cloned endoglucanase gene. The putative catalytic region is indicated in italics. The signal peptide (N-Terminal) and the immunoglobulin-like domain (C-Terminal) are shown in bold.

has been found within the genome (unpublished data). These data infer the possibility that the xylan operon might be involved either in cellulose or other polysaccharides hydrolysis; however, this hypothesis remains to be

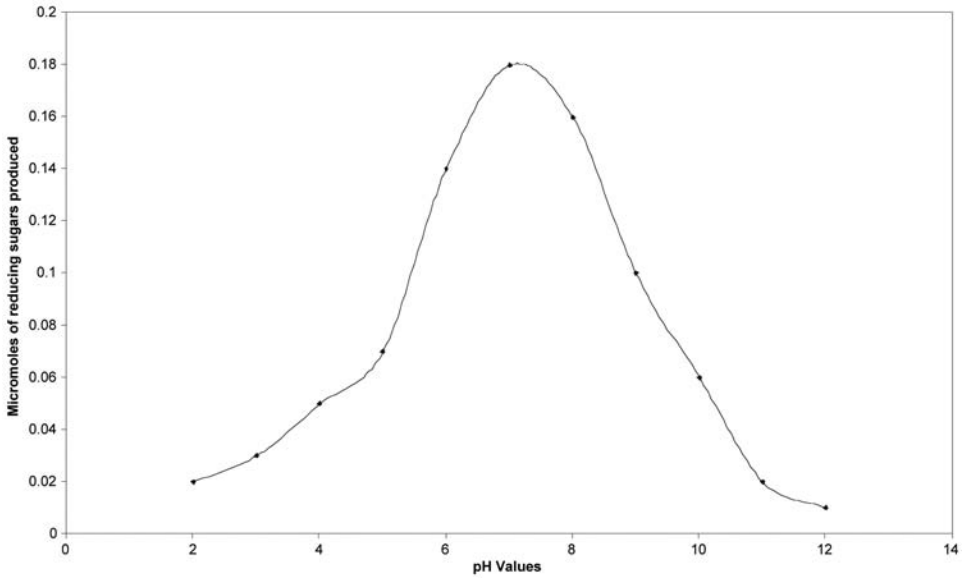

Fig. 2. Sugars assays using dinitrosaliccylic acid procedure were run overnight at 30°C in buffers with different pH and cellulase activity was measured spectrophotometrically at a wavelength of 600 nm.

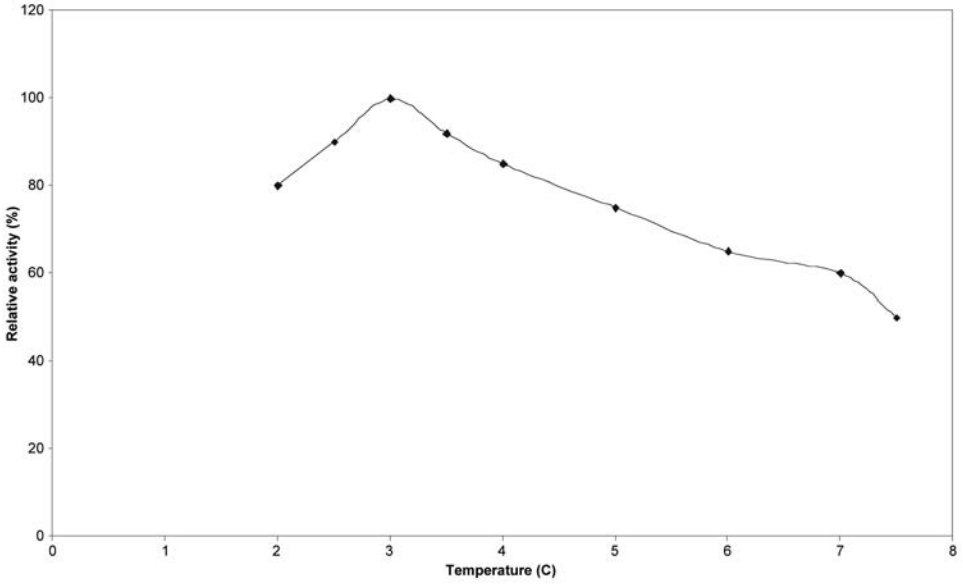

Fig. 3. Sugars assays using dinitrosaliccylic acid procedure were run for 14 h at 30°C in buffers inoculated at different temperature and cellulase activity was measured spectrophotometrically at a wavelength of 600 nm.

tested. When tested for the production of soluble and insoluble reducing sugars, Cel9A was found to produce 80% insoluble sugars (Fig. 4). Cel9A has significantly lower activity on both FP and phosphoric acid-swollen

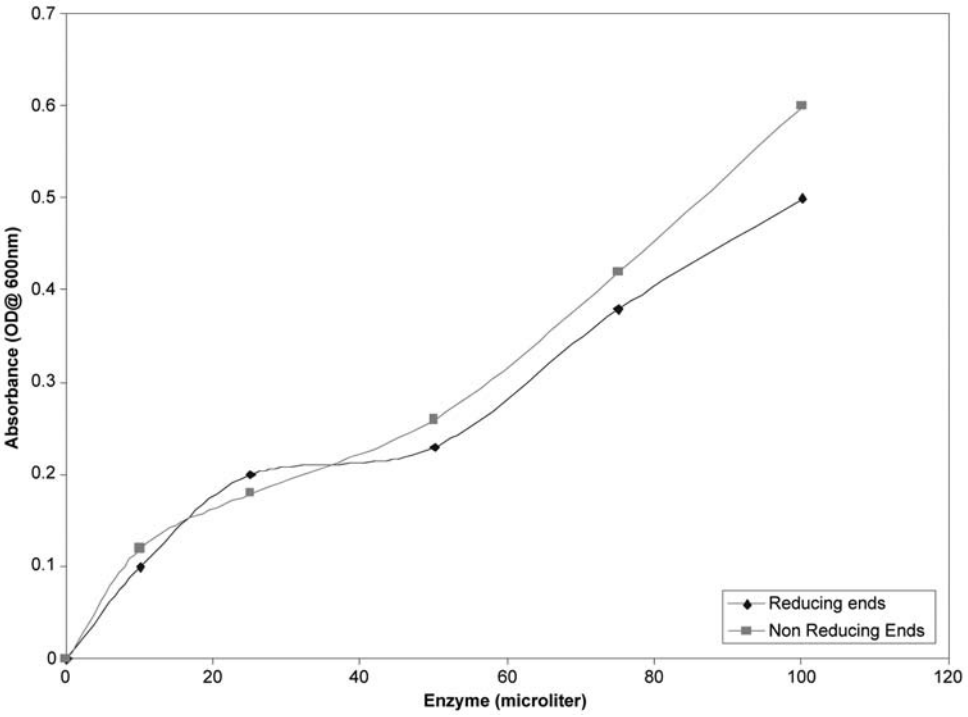

Fig. 4. Sugar assays using dinitrosaliccylic acid procedure were run overnight at 30°C, and reducing and nonreducing ends were measured spectrophotometrically at a wavelength of 600 nm.

cellulose (SC). Based on these results Cel9A appears to be a non-processive endocellulase (Fig. 5).

The amino acids that have been shown *(15)*, by site-directed mutagenesis or X-ray crystallography, to be involved in catalysis in the other family E1 enzyme, CelB from *T. fusca*, and which are conserved in all family 9 cellulases, are also present in Cel9A. But the most intriguing feature about this enzyme is the absence of a cellulose binding domain (CBD). This is not consistent with the current stand of today's literature on industrial microorganisms. All known cellulose degraders have the following structure: a catalytic domain, an adjacent CBD, a Pro/Ser/Thr rich linker and another CBD *(23)*. Cel9a differ from the other enzymes by structurally not having a linker region or any CBD domains.

The CBD is known to maintain a high concentration of the enzyme near the insoluble substrate. Other roles, such as disrupting crystalline cellulose to aid hydrolysis, have been suggested for the CBD, but such function remains unproven *(25)*. CBD has been considered as the limiting factor in hydrolysis. In the case of Cel9A, because there is no CBD present, would it be easier to achieve maximum increase in specific activity by using *C. hutchinsonii*. Specific activity is a measure of enzyme efficiency,

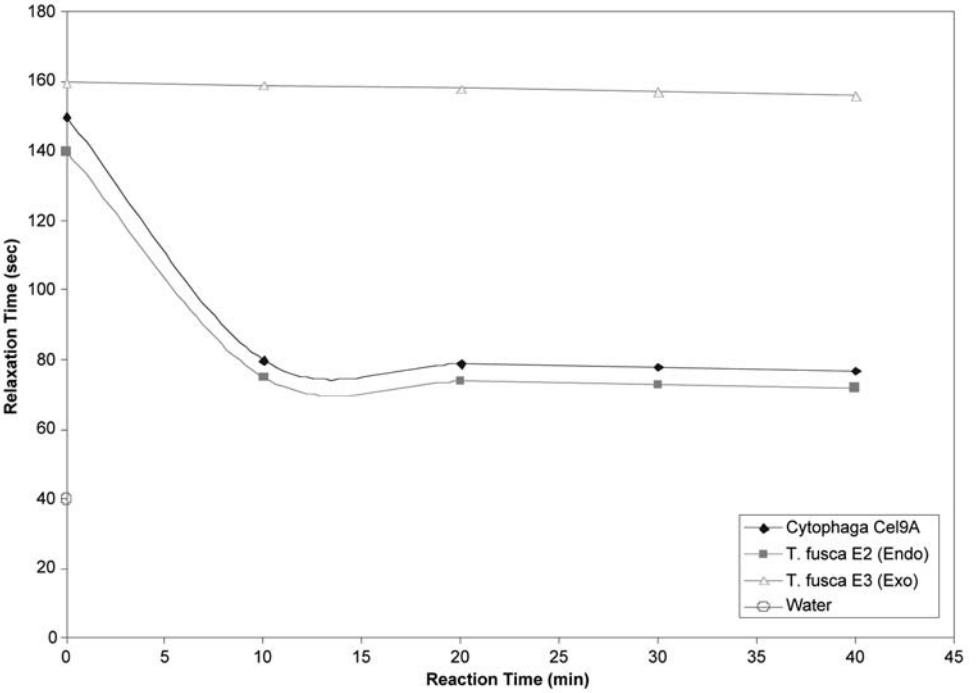

Fig. 5. Enzymes were incubated at 30°C for 40 min (reaction time) and poured down a 20-cm glass pipet, and travel time (relaxation time) was recorded.

usually constant for a pure enzyme. Generally, one unit of specific activity of CMase is defined as 1 mg glucose equivalent produced/mg protein per hour.

Conclusions

All potent cellulolytic bacteria and fungi produce a battery of cellulases, which act synergistically to solubilize crystalline cellulose. Cellulolytic systems can be associated into multienzymatic complexes (called cellulosomes) or unassociated as individual enzymes *(3)*. In both cases, enzymes have a modular structure. The unassociated enzymes consist generally of a catalytic domain responsible for the hydrolysis reaction and of a CBD mediating binding of the enzymes to the substrate. The two domains are joined by a linker peptide, which must be sufficiently long and flexible to allow efficient orientation and operation of both domains *(3)*. The cellulosomal enzymes are bound noncovalently to the cellulosome-integrating protein, which carries a CBD *(3)*.

CBDs contribute significantly to the activity of cellulases against native cellulose. This was shown in several cases by comparing the activity of bifunctional holoenzymes with core enzymes containing the catalytic domain only or by grafting a CBD onto cellulases originally consisting of a single catalytic domain *(25)*. Family-I CBDs and family-II CBDs have been

reported to be involved in the physical disruption of cellulose fibers and to release small particles from cotton fibers *(26–29)*.

In this work, we reported the cloning of a cellulase gene with no CBD from the gliding bacteria *C. hutchinsonii*. Carrard et. al. *(28)* reported that the hydrolysis of cellulose by the endoglucanase CelD is limited by the availability of substrate sites accessible to the enzyme. The same study also concluded that different CBDs increase the range of available sites to different extents. It has been claimed that the efficiency of cellulases is directly related to their affinity for the substrate *(6)*. This finding by Lynd *(6)* appears to correlate with previous observations, indicating that CBDs by themselves might have a disrupting action on native cellulose and confer enhanced activity against insoluble cellulose *(30–31)*. In this respect, it was of interest to characterize the Cel9A CD or the structure of the regions corresponding to the substrate sites whose hydrolysis is preferentially independent of a defined CBD of *C. hutchinsonii* enzyme.

Generalizing from the data published to date, family-9 enzymes appear to be reducing-end directed, processive endocellulase with some members also having very low exoglucanase activity. However, Cel9A from *C. hutchinsonii* appears to work from the non-processive ends. Therefore, relevant questions regarding cellulose hydrolysis by *C. hutchinsonii* are now being raised, such as what mechanism does this organism use to colonize its insoluble substrates? These questions remain to be answered. Wilson (Cornell University, Ithaca, NY, personal communication) suggested that this organism might cleave the cellulose chain randomly to release cellobiose and use cellobiose as energy source for metabolism, but this hypothesis remains to be experimentally tested.

Acknowledgments

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