

Pectate lyase A, an enzymatic subunit of the *Clostridium cellulovorans* cellulosome

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Clostridium cellulovorans uses not only cellulose but also xylan, mannan, pectin, and several other carbon sources for its growth and produces an extracellular multienzyme complex called the cellulosome, which is involved in plant cell wall degradation. Here we report a gene for a cellulosomal subunit, pectate lyase A (PelA), lying downstream of the *engY* gene, which codes for cellulosomal enzyme EngY. *pelA* is composed of an ORF of 2,742 bp and encodes a protein of 914 aa with a molecular weight of 94,458. The amino acid sequence derived from *pelA* revealed a multidomain structure, i.e., an N-terminal domain partially homologous to the C terminus of PelB of *Erwinia chrysanthemi* belonging to family 1 of pectate lyases, a putative cellulose-binding domain, a catalytic domain homologous to PelL and PelX of *E. chrysanthemi* that belongs to family 4 of pectate lyases, and a duplicated sequence (or dockerin) at the C terminus that is highly conserved in enzymatic subunits of the *C. cellulovorans* cellulosome. The recombinant truncated enzyme cleaved polygalacturonic acid to digalacturonic acid (G2) and trigalacturonic acid (G3) but did not act on G2 and G3. There have been no reports available to date on pectate lyase genes from *Clostridia*.

The plant cell wall is organized as a complex network composed of polysaccharides such as cellulose, hemicellulose, and pectin. In particular, pectin is a heteropolysaccharide composed of α -1,4-linked galacturonate chains with a high percentage of methyl esterification. Pectin degradation requires the combined action of several enzymes that can be classified into two groups, i.e., methylesterases, which remove methoxyl groups from pectin, and depolymerases (hydrolases and lyases), which cleave the bonds between galacturonate units. Pectate lyases (pectate transeliminase, EC 4.2.2.2) are known to play a critical role in the depolymerization process during soft rotting and catalyze random cleavage of internal α -1,4-linkages of polygalacturonate or methylesterified pectins through a transeliminative mechanism, thereby generating 4,5-unsaturated oligogalacturonates (1, 2). The breakdown of pectin located in the middle lamella and primary cell wall leads to the maceration of plant tissues (3, 4).

Clostridium cellulovorans, an anaerobic, mesophilic, and spore-forming bacterium, is able to use polysaccharides such as cellulose, pectin, xylan, and several other carbon sources for its growth (5). When *C. cellulovorans* grows in media containing these carbon sources, the bacterium produces an extracellular multienzyme complex called the cellulosome (6) with a total molecular weight of about 1×10^6 and capable of hydrolyzing crystalline cellulose (7). To elucidate the relationships between the structure and function and the regulatory systems of a variety of enzymes involved in the cellulosome system of *C. cellulovorans*, we have cloned, sequenced, and characterized several cellulosomal genes (8, 9). The *C. cellulovorans* cellulosome comprises three major subunits (P170, P100, and P70) (7, 10), i.e., the scaffolding protein CbpA (11), the endoglucanase EngE (12), and the exoglucanase ExgS (13). In addition to these major subunits, we have cloned and sequenced the cellulosomal subunits, including the endoglucanases EngB (14), EngH, EngK, EngL, EngM, and EngY (10, 13, 15), and a mannanase, ManA (15), each containing a duplicated sequence (DS or dockerin)

consisting of highly conserved 22-aa repeats. Therefore the *C. cellulovorans* cellulosomal enzymes identified to date are capable of degrading cellulose, xylan, lichenan, and mannan. However, because we have recently succeeded in converting *Arabidopsis* and tobacco cells to protoplasts with the *C. cellulovorans* cellulosomes (Y.T., S. Ui, H. Chan, R.H.D., and B. Liu, unpublished data), and it has been reported that pectin could serve as a carbon source for growth (5), we believed that the *C. cellulovorans* cellulosome must also have pectinase activity. The present paper provides data that indicate that a cellulosomal gene *pelA* and the enzyme encoded by this gene can degrade pectin and that pectate lyase A (PelA) contains a DS at its C terminus. Thus the versatility of the *C. cellulovorans* cellulosome in degrading plant cell wall materials is further established. Furthermore, as far as we are aware, there have been no reports about pectate lyase genes in *Clostridium* species.

Materials and Methods

Bacterial Strains and Vectors. *C. cellulovorans* (ATCC 35296) (5) was used as the source of chromosomal DNA. *Escherichia coli* XL1-Blue MRF' and SOLR, λ ZAPII pBluescript SK (-), and pBluescriptII KS (+) served as cloning hosts and vectors (Stratagene). Plasmids pYT-1 and pYT-2 were derived by using *E. coli* plasmids XL1-BLUE MRF' and SOLR. λ plaques were formed after *E. coli* cells were transfected with λ phages in NZY medium containing 1 mM isopropyl- β -D-thiogalactopyranoside. *E. coli* was grown at 37°C in LB medium supplemented with ampicillin (100 μ g/ml) when required. For agar medium, LB was solidified with 1.5% (wt/vol) agar.

Recombinant DNA Techniques. The *C. cellulovorans* genomic library was previously constructed in λ ZAPII (12) and was immunoscreened with an anti-P70 (cellulase) antiserum (diluted 1:500) previously prepared from *C. cellulovorans* and alkaline phosphatase-conjugated goat anti-rabbit IgG second antibody (diluted 1:3,000) (Bio-Rad). Immunodetection was carried out with a Western Detection Kit (Bio-Rad), following the manufacturer's instructions. Positive immunocrossreacting plaques were isolated, and clones were obtained by *in vivo* excision and rescued with the use of ExAssist helper phage (Stratagene). Positive clones were further screened for endoglucanase activity by overlaying with 0.7% soft agar containing 0.3% carboxymethyl cellulose (low viscosity; Sigma). Colonies with carboxymethyl cellulase activity were recognized by the formation of clear halos on a red background after staining with 0.1% Congo red and destaining with 1 M NaCl (16). Plasmids from *E. coli*

Abbreviations: PelA, pectate lyase A; rPelA, recombinant PelA; CBD, cellulose-binding domain; DS, duplicated sequence.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF105331).

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were purified with a Qiagen kit (Qiagen, Chatsworth, CA). Agarose gel electrophoresis, transformation of *E. coli*, and ligation have been described by Sambrook *et al.* (17).

Nucleotide Sequence Analysis. The nucleotide sequence was determined from both strands by the dideoxy chain termination method (18). The 6.7-kb *EcoRI* fragment (pYT-1) was subcloned with the use of restriction enzymes, and a series of nested deletion mutants from each fragment was constructed with an *ExoIII*/mung bean nuclease kit (Stratagene). Double-stranded DNA templates were sequenced with SEQUENASE, version 2.0 (United States Biochemical). Both strands were sequenced. Homology searches in the GenBank were carried out with the BLAST program.

PelA Activity and Protein Assays. The reaction mixture consisted of 0.1 ml of PelA solution, 1 ml of 0.4% sodium polygalacturonic acid (Sigma), 0.5 ml of 200 mM Tris·HCl buffer (pH 8.0), and 0.4 ml of 0.25 mM CaCl₂ in a total volume of 2 ml. After reaction at 40°C for 10 min, 2 ml of 50 mM HCl was added to terminate the reaction, and the absorbance in the solution was measured at 235 nm (19). One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of unsaturated oligogalacturonides, equivalent to 1 μmol digalacturonide/min under the above conditions. The molar extinction coefficient value was assumed to be 4,600 M⁻¹·cm⁻¹ for unsaturated digalacturonide (19). The influence of Ca²⁺ and EDTA was investigated by the addition of CaCl₂ concentrations ranging from 0 to 1 mM and 1 mM EDTA. The optimum pH was determined by using piperazine-*N,N'*-bis(2-ethanesulfonic acid); Pipes buffer (pH 6.0 to 8.0), and Tris·HCl buffer (pH 7.5–10), each at 50 mM. The activity on substrates with various degrees of methylation was determined by substituting 26%, 67%, and 89% esterified citrus fruit pectins (Sigma), an apple pectin (Sigma), or polygalacturonate.

Protein was measured by the method of Bradford (20) with a protein assay kit from Bio-Rad and BSA as a standard.

SDS/PAGE and Zymogram Analysis. SDS/PAGE was performed in a 10% polyacrylamide gel by the method of Laemmli (21). After electrophoresis, the gel was stained with Coomassie brilliant blue R. Zymogram analysis was prepared by using 0.2% pectin from apple (Sigma) that was copolymerized with polyacrylamide. Pectinase activity was visualized with 0.05% ruthenium red (Sigma) (22).

Purification of Recombinant PelA. *E. coli* harboring pYT-2 was cultured to early stationary phase at 37°C with vigorous shaking. The cells were collected by centrifugation, and the periplasmic fraction was prepared by the osmotic shock method (23). The periplasmic fraction was applied to a Q Sepharose Fast Flow column (2.6 × 22 cm; Amersham Pharmacia) equilibrated with 50 mM Tris·HCl buffer (pH 7.5). After being washed with 3 bed volumes of the same buffer, the column was eluted with a linear gradient of NaCl (0 to 0.5 M) at a flow rate of 60 ml/h. The active fractions eluted around 0.2 M NaCl were collected and concentrated by an Amicon concentrator. The concentrated enzyme solution was applied to a Sephacryl S-200 column (2.6 × 75 cm; Amersham Pharmacia) equilibrated with 50 mM Tris·HCl buffer (pH 7.5). The active fractions were used as purified enzyme.

TLC. Oligogalacturonates [galacturonic acid (G1), digalacturonic acid (G2), and trigalacturonic acid (G3)] were purchased from Sigma. Hydrolysis products were separated by TLC on a silica gel 60-plastic sheet (Merck) with a solvent of 1-butanol/water/acetic acid (5:3:2), and the oligosaccharides were visualized by spraying the plate with phosphomolybdic acid (24).

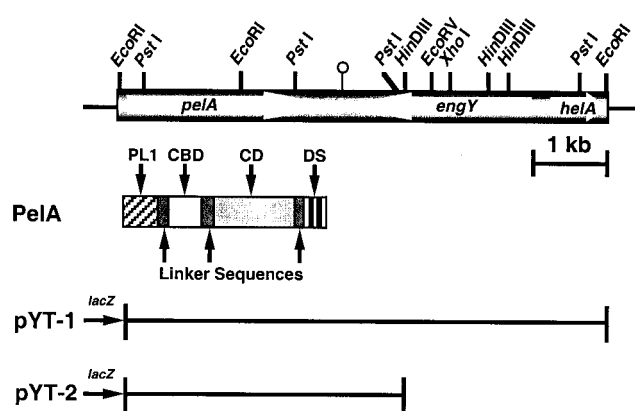


Fig. 1. Restriction enzyme maps of pYT-1 and domain structure of the PelA polypeptide. Thin lines correspond to vector plasmid DNA. pYT-1 contains the entire 6.7-kb *EcoRI* fragment. The pectate lyase gene, *pelA*, was located on a 3.7-kb fragment designated as pYT-2. The white arrows indicate the coding sequences for the PelA, EngY, and HelA polypeptides, respectively. The different domains of PelA are named and filled with different patterns. PL1, pectate lyase family 1; CD, catalytic domain.

Results

Cloning and Nucleotide Sequence of the *pelA* Gene. A *C. cellulovorans* λZAPII library was immunoscreened with anti-P70 (cellulase) antibody and further screened for endoglucanase activity. From ≈2,000 transformants, two positive clones were isolated and further characterized. Both clones contained a common 6.7-kb *EcoRI* insert. A restriction enzyme map of the cloned gene is shown in Fig. 1. As a result of DNA sequencing, the pYT-1 fragment was found to consist of three ORFs coding for a pectate lyase *pelA* gene, an endoglucanase *engY* gene, and a DNA helicase *helA* gene. As a result of determination of the coding regions, the *pelA* gene was located on a 3.7-kb *EcoRI*–*HindIII* (pYT-2) fragment (Fig. 1).

Fig. 2 shows the complete nucleotide sequence of the *pelA* structural gene along with its flanking regions. The ORF of *pelA* consists of 2,742 nt encoding a protein of 914 aa with a predicted molecular weight of 94,458. A putative ribosome-binding site, AGAATTGTA, is present at a spacing 8 bp upstream of the translational start codon ATG (25). Upstream of the coding region, possible promoter sequences, TTAACA for the –35 region and TATGAT for the –10 region with a 15-bp spacing between them, were found and showed high homologies to consensus promoter sequences for σ⁷⁰ or σ^A factor, TTGACA and TATAAT with a 17-bp spacing, found in *E. coli* or *Bacillus subtilis* (26). A possible transcription terminator consisting of a 15-bp palindrome, corresponding to an mRNA hairpin loop with a Δ*G* of –26.0 kcal/mol (*ca.* –109 kJ/mol), followed by four Ts after one nucleotide gap, was found downstream of the TAA termination codon. This structure is similar to the ρ factor-independent terminator of *E. coli* (27). Sequence of the DNA beyond the *pelA* terminator revealed a convergently oriented ORF that was identical to *engY* (Fig. 2).

Domain Structure of PelA. Analysis of the deduced amino acid sequence of PelA revealed a multidomain structure with a unique feature, i.e., a N-terminal domain homologous to pectate lyase family 1, followed by a putative cellulose-binding domain (CBD), a pectate lyase family 4 catalytic domain, and a DS (or dockerin) (Fig. 1). In addition to these domains, there were several linker sequences, i.e., a proline-threonine-rich region downstream of pectate lyase family 1, a region with eight repeats of GTTTPG between the CBD and the catalytic domain, and

| | | | | | | |
|---------------------|------|------------|----------|-------|--------|-----------------|
| PelA DS | 859 | GDLSKDGKVN | IDLAF | LKKL | LLSGDT | SGADMTV |
| EngB DS | 387 | GDVVKDGKVN | ADYAVL | KSILL | GTNTV | NVDLSV |
| EngE DS | 978 | GDVDGNDV | VNSLDFE | LKKYV | LNDT | MINKAS |
| EngH DS | 660 | GDVNNNG | IVNSMDL | AMLK | KYIL | GYEVEMNKEA |
| EngK DS | 838 | GDVNGDT | KINAIDL | AMLK | KYIL | DNSTIVIKTAN |
| EngL DS | 469 | GDVVKDGKVN | IDLAF | LKKL | LLNGIT | IGDLTV |
| EngM DS | 822 | GDINND | AKINAIDL | LAL | LKKYIL | NNDIPIVAIN |
| ExgS DS | 649 | GDVNSD | AKVN | IDLAF | LKKYIL | DSTIKINTAN |
| ManA DS | 30 | GDVNV | DGKVN | SIDMA | IVKQ | YILGTRTFSDAELKA |
| Duplicated sequence | | | | | | |
| 22 amino acids | | | | | | |
| PelA DS | 892 | ADMNKDGK | INAIDL | LALL | LKKQL | LS-COOH |
| EngB DS | 419 | SDMNKDGK | VNALDL | AVL | LKKML | LS-COOH |
| EngE DS | 1010 | ADLNKDGK | INID | LAF | LKKAI | -COOH |
| EngH DS | 692 | SDLNKDGK | INAIDF | ALL | LKKLL | LSQ-COOH |
| EngK DS | 870 | ADMNNDGK | INAIDL | LALL | LKKQL | LS-COOH |
| EngL DS | 500 | SDMNKDGK | VNAIDL | LALL | LKKLL | LG-COOH |
| EngM DS | 854 | GDINGDG | INAIDL | L | LKKQL | LA-COOH |
| ExgS DS | 681 | SDMNGDGK | VNAMDL | LALL | LKKALL | A-COOH |
| ManA DS | 64 | ADVDANGK | VNAIDL | AV | KQYIL | GSITK |

Fig. 5. Alignment of the DSs (or dockerins) of cellulosomal subunits of *C. cellulovorans*. Amino acids that are conserved in at least five of the nine sequences are highlighted. The numbers refer to amino acid residues at the start of the respective lanes; all sequences are numbered from Met-1 of the peptide.

activity to less than 5% of maximum. Among pectic substrates, maximum activity was found on polygalacturonic acid, and the purified rPelA showed less than 30% of the maximum activity on citrus and apple pectins (Fig. 7B). Moreover, as the percentage degree of methylation in pectin became higher, the purified

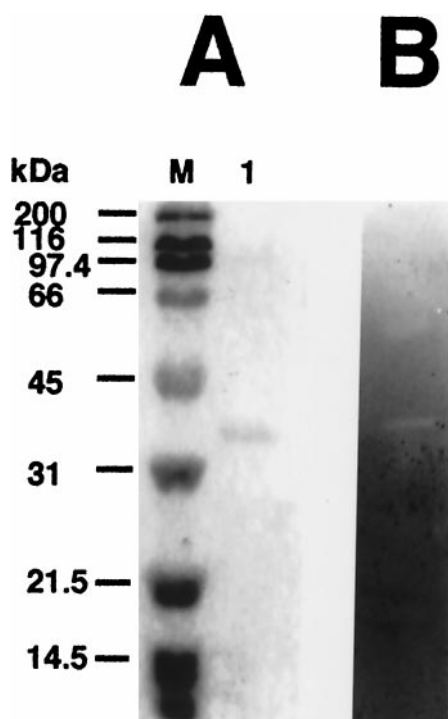


Fig. 6. Analysis of the purified rPelA by SDS/PAGE (A) and zymogram (B). Lane M, standard markers [myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase B (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.5 kDa)]; lane A 1, purified rPelA.

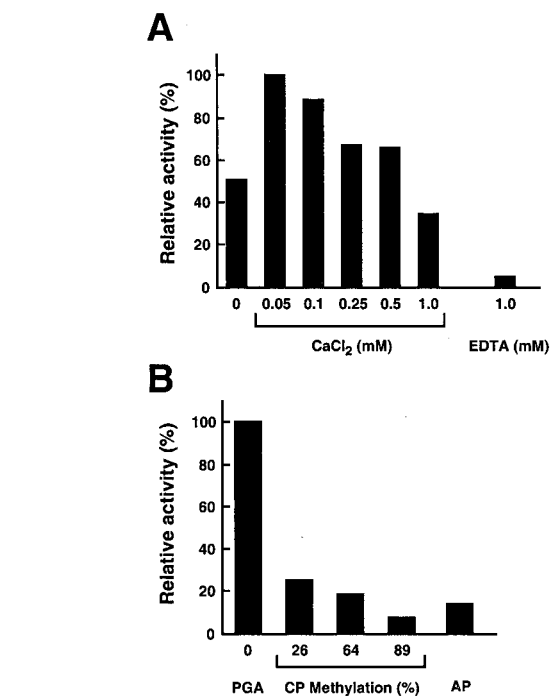


Fig. 7. Enzymatic properties of PelA. (A) The influence of Ca²⁺ was tested by using various concentrations of CaCl₂ in 50 mM Tris-HCl (pH 8) and 0.2% polygalacturonic acid. The cation requirement was confirmed by the addition of 1 mM EDTA. (B) The influence of the degree of pectin methylation on rPelA activity. Activity of rPelA on polygalacturonic acid (PGA), citrus pectins (CP) with a degree of methyl esterification from 26% to 89%, and apple pectin (AP) was determined by the standard assay condition.

rPelA revealed lower activity. As shown in Fig. 8, TLC analysis revealed that the purified enzyme cleaved polygalacturonic acid to form several oligosaccharides, such as digalacturonic acid (G2) and trigalacturonic acid (G3), but did not act on G2 and G3.

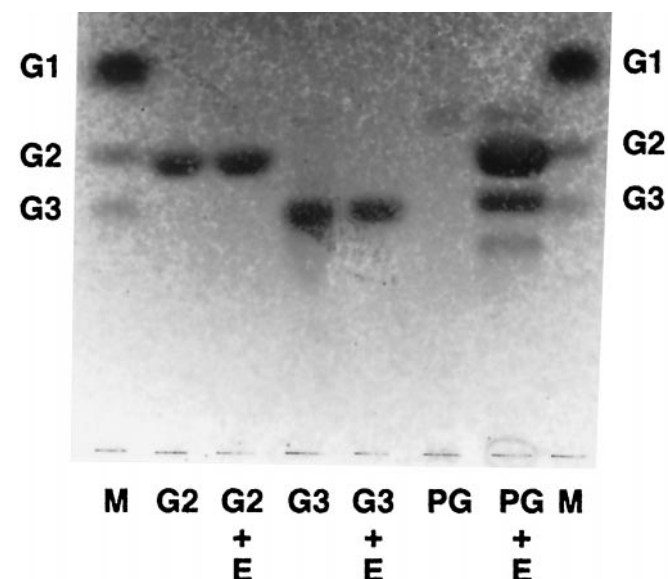


Fig. 8. TLC of the hydrolysis products of digalacturonic acid (G2), trigalacturonic acid (G3), and polygalacturonic acid (PG) with the purified rPelA. The reaction mixtures contained 50 mM Tris-HCl (pH 8), 0.05 mM CaCl₂, 5 units of enzymatic activity/ml, and 1 mg of the substrates/ml. Incubations were performed at 37°C for 12 h after the addition of rPelA or without enzyme.

One must keep in mind that these results are derived with a truncated rPelA.

Discussion

Much knowledge has been obtained about the cellulosomes from several *Clostridium* species (8, 28), and the previous studies have focused mainly on cellulose degradation. Although the overall features of the cellulosome are reasonably clear, there are several properties of the cellulosome that require further investigation. In particular, the versatility of substrate specificity in the cellulosome is still unknown. An understanding of the mechanism of plant cell wall degradation by the cellulosome may play an important role for biomass recycling and food and energy problems in this century. Our current strategy is to study the properties of the subunits of the *C. cellulovorans* cellulosome that include not only cellulases but also hemicellulases and pectinases. Our ultimate goal is to apply designer cellulosomes for the utilization of plant biomass as an energy source.

We have cloned and sequenced a 6.7-kb *EcoRI* fragment consisting of three ORFs, i.e., *pelA*, *engY*, and *helA* (Fig. 1). Both *pelA* and *engY* genes have a DS and interestingly possess the same transcription terminator (Fig. 2). In fact, the relationship of gene location between *pelA* and *engY* is similar to that for *pelL* and *celZ* from *E. chrysanthemi* (20, 29), although *engY* belongs to family 9 of cellulases, whereas *celZ* belongs to family 5. The deduced amino acid sequence of PelA revealed a multidomain structure. The N-terminal domain was homologous to the family 1 of pectate lyases. However, this N-terminal domain coded for a truncated enzyme without a complete catalytic domain, which was followed by a putative CBD, a complete catalytic domain belonging to family 4 of pectate lyases, and a DS. The putative CBD is one of a large group of family 2 CBDs, but the gene banks reveal only two other family II CBDs from Clostridial species. Pectate lyases are classified in five different families according to their primary amino acid sequences (30, 31). The catalytic domain of PelA belongs to family 4 of pectate lyases, which contains PelL and PelX of *E. chrysanthemi* (20, 29, 31) and PelH of *Bacillus* species PelH (GenBank accession no. AB028878). In this study, we purified and characterized the rPelA. The molecular weight of the purified rPelA was estimated to be 42,000 by SDS/PAGE (Fig. 6A), and the molecular weight as derived from the DNA sequence was 94,458. It appeared the rPelA had been cleaved by an *E. coli* protease, but its size was still sufficient to

contain the complete catalytic domain (Fig. 1). This type of partial proteolytic degradation had been shown previously for essentially all *C. cellulovorans* cellulosomal enzyme genes expressed in *E. coli*. Moreover, the purified enzyme showed pectinase activity on zymogram analysis (Fig. 6B), thus indicating that the specificity and activity of rPelA had been retained in the truncated recombinant enzyme.

Optimum pH and temperature for rPelA activity were pH 8.0 and 50°C, respectively. The purified enzyme revealed the greatest activity with polygalacturonic acid and less activity with citrus and apple pectins (Fig. 7B). Furthermore, the cleavage pattern of rPelA analyzed by TLC showed that the main products from polygalacturonic acid were digalacturonic acid (G2), trigalacturonic acid (G3), and tetragalacturonic acid (G4) (Fig. 8), and those of PelL from *E. chrysanthemi* showed G3 and larger oligomers (20). On the other hand, the main products from polygalacturonic acid for PelX from *E. chrysanthemi* were only unsaturated digalacturonic acid (uG2) (31). Furthermore, the purified rPelA did not cleave G2 and G3 (Fig. 8), whereas PelX could act on G3 to form galacturonic acid (G1) and uG2 but not on G2 (31). In addition, G4 is the best substrate for the *E. chrysanthemi* PelX, and the estimated number of binding subsites for PelX is 4, extending from subsites -2 to +2 (31). Because TLC analysis revealed that rPelA did not act on G2 and G3, the results indicate that rPelA hydrolyzes G4 or longer oligomers. Furthermore, rPelA seems to be an endo-type enzyme, whereas the *E. chrysanthemi* PelX is an exopoly-galacturonate lyase. Thus, the properties of rPelA of *C. cellulovorans* differ from those of PelL and PelX of *E. chrysanthemi* in specific activity, substrates degraded, and the products that are formed, although all of the enzymes belong to family 4 of pectate lyases.

The *C. cellulovorans* cellulosome has now been shown to be capable of hydrolyzing cellulose, lichenan, mannan, pectin, and xylan. Many of the hydrolytic properties of the whole cell can now be attributed to the enzymatic activities associated with the cellulosome. Therefore, from knowledge of the components of the plant cell wall and by combining the enzymatic subunits in designer *C. cellulovorans* cellulosomes, we should be able to degrade plant biomass efficiently and convert carbon biomass to much more utilizable forms of energy.

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