Exploration of Two Pectate Lyases from *Caldicellulosiruptor bescii* Reveals that the CBM66 Module Has a Crucial Role in Pectic Biomass Degradation

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ABSTRACT Pectin deconstruction is the initial step in breaking the recalcitrance of plant biomass by using selected microorganisms that encode pectinolytic enzymes. Pectate lyases that cleave the α -1,4-galacturonosidic linkage of pectin are widely used in industries such as papermaking and fruit softening. However, there are few reports on pectate lyases with good thermostability. Here, two pectate lyases (CbPL3 and CbPL9) from a hyperthermophilic bacterium, *Caldicellulosiruptor bescii*, belonging to family 3 and family 9 polysaccharide lyases, respectively, were investigated. The biochemical properties of the two CbPLs were shown to be similar under optimized conditions of 80°C to 85°C and pH 8 to 9. However, the degradation products from pectin and polygalacturonic acids (pGAs) were different. A family 66 carbohydrate-binding module (CbCBM66) located in the N terminus of the two CbPLs shares 100% amino acid identity. A CbCBM66-truncated mutant of CbPL9 showed lower activities than the wild type, whereas CbPL3 with a CbCBM66 knockout portion was reported to have enhanced activities, thereby revealing the different effect of CbCBM66. Prediction by the I-TASSER server revealed that CbCBM66 is structurally close to BsCBM66 from *Bacillus subtilis*; however, the COFACTOR and COACH programs indicated that the substrate-binding sites between CbCBM66 and BsCBM66 are different. Furthermore, a substrate-binding assay indicated that the catalytic domains in the two CbPLs had strong affinities for pectate-related substrates, but CbCBM66 showed a weak interaction with a number of lignocellulosic carbohydrates. Finally, scanning electron microscopy (SEM) analysis and a total reducing sugar assay showed that the two enzymes could improve the saccharification of switchgrass. The two CbPLs are impressive sources for the degradation of plant biomass.

IMPORTANCE Thermophilic proteins could be implemented in diverse industrial applications. We sought to characterize two pectate lyases, CbPL3 and CbPL9, from a thermophilic bacterium, *Caldicellulosiruptor bescii*. The two enzymes share a high optimum temperature, a low optimum pH, and good thermostability at the evaluated temperature. A family 66 carbohydrate-binding module (CbCBM66) was identified in the two CbPLs, sharing 100% amino acid identity. The deletion of CbCBM66 dramatically decreased the activity of CbPL9 but increased the activity and thermostability of CbPL3, suggesting different roles of CbCBM66 in the two enzymes. Moreover, the degradation products of the two CbPLs were different. These results revealed that these enzymes could represent potential pectate lyases for applications in the paper and textile industries.

KEYWORDS *Caldicellulosiruptor*, pectate lyase, pectin, polysaccharide lyase, family 66 carbohydrate-binding module

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Pectin is an intercellular cement that gives plants structural rigidity; it was discovered at concentrations of 15% to 30% in fruit fibers, vegetables, legumes, and nuts (1). In terms of chemistry, it is a heterogeneous polysaccharide with a molecular weight in the range of 20 to 400 kDa (2). The simplest structure of pectin is a linear polymer of galacturonic acids (GAs) with α -1,4-galacturonosidic linkages, which is named homogalacturonan (HG). More complicated forms of pectin are linked by D-galacturonate and L-rhamnose residues, which are designated type I and II rhamnogalacturonans (RGI and RGII, respectively). The side chains of RGI and RGII are modified mostly by neutral sugars such as D-galactose, L-arabinose, D-xylose, and L-fucose (3).

Pectin depolymerization is an initiating step for the bioconversion of plant biomass. Therefore, pectate lyases, a group of enzymes that break down pectin in nature, are used as additives in generating commercial cellulase cocktails and consolidated-bioprocessing (CBP) microorganisms (4). The mechanism of action of pectate lyase is to cleave the α -1,4-galacturonosidic linkage of polygalacturonic acid (pGA) by a β -elimination reaction, thereby forming a double bond between positions C-4 and C-5 in oligogalacturonates (1, 4). The Carbohydrate-Active Enzymes Database (CAZy) (www.cazy.org) (5) classifies pectate lyases into families 1, 2, 3, 9, and 10 of the polysaccharide lyases (PLs). Most of these lyases are identified from microorganisms such as those from the genera *Bacillus*, *Clostridium*, *Erwinia*, *Fusarium*, *Pseudomonas*, *Streptomyces*, and *Thermoanaerobacter*, which are mostly alkaline (pH 8 to 11) and Ca^{2+} dependent (4).

Pectate lyases are widely applied in industries such as papermaking, fruit softening, juice and wine clarification, plant fiber processing, and oil extraction (6). Biotechnological usage is highly dependent on the biochemical properties of these enzymes, such as temperature, pH, and salt concentration (1). Commercial pectate lyases have been isolated mostly from mesophilic bacteria of the genus *Bacillus*, including *B. subtilis*, *B. licheniformis*, *B. cereus*, *B. circulans*, *B. pasteurii*, *B. amyloliquefaciens*, and *B. pumilus* (1, 4). Among them, the recombinant pectate lyase BacPelA from *B. clausii* had high cleavage activity on methylated pectin. Its maximum activities were observed at pH 10.5 and 70°C, and it showed the highest degumming efficiency reported to date (7). A pectate lyase, PelS6, from the *B. amyloliquefaciens* S6 strain displayed highly thermostability and survival at a wide range of pHs, which could be suitable for juice clarity (8). To identify thermostable and highly active enzymes, the number of studies investigating thermophilic microorganisms such as those from the genera *Thermotoga* and *Caldicellulosiruptor* has been growing recently (9–14).

A previous study indicated that a pectin deconstruction gene cluster is vital for the growth of thermophilic *Caldicellulosiruptor bescii* on plant biomass. However, the biochemical characteristics were unclear (15). Figure 1A shows three adjacent genes, *Cbes_1853*, *Cbes_1854*, and *Cbes_1855*, that encode a rhamnogalacturonan lyase, CbPL11; a pectate lyase, CbPL3; and a pectate disaccharide lyase, CbPL9, respectively (16). Alahuhta et al. presented a crystal structure of the catalytic domain (CD) of CbPL3 and described CbPL3 as a unique pectate lyase with a low optimum pH (11, 17). However, detailed properties of these pectate lyases remained unclear. Meanwhile, CbPL3 and CbPL9 share the same N-terminal domain, which is predicted to be a family 66 carbohydrate-binding module (CBM66) with 100% protein sequence similarity (18). The function of this CbCBM66 is still unknown. In this study, the pectate lyases CbPL3 and CbPL9 and their truncated mutants were heterologously expressed in *Escherichia coli*. The properties of the different domains in the two enzymes were biochemically and functionally analyzed.

RESULTS AND DISCUSSION

Cloning and expression of pectate lyases and their truncated mutants from *C. bescii*. A rhamnogalacturonan lyase (CbPL11), a pectate lyase (CbPL3), and a pectate disaccharide lyase (CbPL9) were identified in a pectin depolymerization gene cluster of *C. bescii* (Fig. 1A) (15–17). However, their biochemical characteristics were unclear. To determine their roles in pectin deconstruction, we expressed these three lyases and their truncated mutants in an *E. coli* protein expression system. Unfortunately, the

FIG 1 (A) Gene organization of three pectate lyase genes in a gene cluster of *Caldicellulosiruptor bescii*. (B) Schematic structures of *CbPL3*, *CbPL9*, and their truncated mutants *CbPL3-CD*, *CbPL9-CD*, and *CbCBM66*. (C) Analysis of purified proteins by 12% SDS-PAGE. The molecular masses of *CbPL3*, *CbPL3-CD*, *CbCBM66*, *CbPL9*, and *CbPL9-CD* are 45.6, 26.9, 22.3, 67.8, and 49 kDa, respectively. Lane M is a protein marker with a range from 25 to 116 kDa.

recombinant forms of *CbPL11* and its catalytic module were hardly overexpressed in BL21(DE3) and C41(DE3) due to unknown proteolysis degradation (data not shown). This enzyme contains a catalytic domain that belongs to a family 11 polysaccharide lyase (PL) and a family 3 carbohydrate-binding module (CBM3). A previous study demonstrated the lack of glycosylation between two linker modules, which may cause unexpected proteolysis after the heterologous expression of *C. bescii* CelA in *B. subtilis* (19, 20). Two other lyases, *CbPL3* and *CbPL9*, contained the same region, with 100% protein similarity; this region was previously identified as a concanavalin A-like lectin/glucanase (CALG) domain by the NCBI database and the InterPro server (21). Recently, research classified this domain as a family 66 CBM (CBM66) (Fig. 1B) (18). Phylogenetic analysis suggested that *CbPL3* and *CbPL9* are more closely related to pectate lyases from the genus of *Bacillus* than to the ones from other bacteria (see Fig. S1 in the supplemental material). To understand their functions, full-length *CbPL3* and *CbPL9* and their truncated mutants *CbPL3-CD* (i.e., containing only the catalytic domain), *CbPL9-CD*, and *CbCBM66* were expressed in *E. coli*, as confirmed by SDS-PAGE (Fig. 1C).

Biochemical characterization. Based on the crystal structures of *CbPL3-CD* and the trigalacturonic acid complex reported by Alahuhta et al., three Ca^{2+} ions were directly involved in substrate binding (11). To determine the relationship between enzyme activity and Ca^{2+} , the purified proteins, namely, *CbPL3*, *CbPL3-CD*, *CbPL9*, and *CbPL9-CD*, were subjected to an *in situ* assay in the presence of Ca^{2+} and the chelating agent

FIG 2 Enzyme characterization. (A) Interaction of calcium and the chelating agent EDTA with CbPL3. A total of 2 nM purified CbPL3 was incubated with 1% polygalacturonic acid (pGA) at 65°C for 20 s. Next, 0.5 mM Ca^{2+} was added to this mixture for another 20 s. Subsequently, 1 mM EDTA inhibited the activity, and this activity was recovered with 5 mM Ca^{2+} . The absorbance at 235 nm was measured in a quartz cuvette. (B) Optimum calcium concentrations. (C) Optimum pHs. (D) Optimum temperatures. (E) Thermostabilities of CbPL3, CbPL9, CbPL3-CD, and CbPL9-CD at 65°C. (F) Specific activities (units per micromole) of pectate lyases and their truncated mutants with a synthetic substrate (pGA) and natural substrates (citrus pectin and apple pectin). The detailed procedure is described in Materials and Methods.

EDTA. As shown in Fig. 2A and Fig. S2, the absorption lines at 235 nm were flat (0 to 30 s) in the absence of Ca^{2+} . The absorption value increased (70 to 100 s) after the addition of Ca^{2+} (0.1 mM) and was quenched by the addition of 0.5 mM EDTA (130 to 160 s). Finally, the absorption value was increased by the addition of Ca^{2+} (1 mM) (190 to 220 s). Furthermore, relative activity was examined in the presence of different concentrations of Ca^{2+} (Fig. 2B). Two full-length pectate lyases and their catalytic modules exhibited their highest lyase activities at 0.25 mM Ca^{2+} , except for CbPL9, at 0.125 mM Ca^{2+} (Fig. 2B). Many reports indicated that polysaccharide lyase activities

TABLE 1 Specific activities of two CbPLs and their catalytic domains on different carbohydrate substrates

Enzyme	Sp act (U/ μ mol) ^a										
	pGA (mean $\pm$ SD)	Citrus pectin	Apple pectin	Rhamno-GA	CMC-Na	Avicel	Beechwood xylan	Alginate	β -1,3(-1,4)-Glucan	Levan	Inulin
CbPL3	457.7 $\pm$ 4.9	231.3	15.7	7.9	ND	ND	ND	ND	ND	ND	ND
CbPL3-CD	439.2 $\pm$ 10.7	241.5	12.9	2.6	ND	ND	ND	ND	ND	ND	ND
CbPL9	21.1 $\pm$ 1.1	10.6	9.9	0.7	ND	ND	ND	ND	ND	ND	ND
CbPL9-CD	5.2 $\pm$ 0.2	1.8	1.7	0.1	ND	ND	ND	ND	ND	ND	ND

^aND, not determined.

are mostly Ca²⁺ dependent; for example, recombinant pectate lyase (*recPEL* S6) from *Bacillus amyloliquefaciens* S6 showed maximal activity at 0.5 mM CaCl₂ (8). In this study, after CbPLs and their catalytic domains were purified from *E. coli*, the detection of lyase activities was not possible in Ca²⁺-free buffer. The enzyme activity considerably increased after the addition of Ca²⁺ (Fig. 2A and B). This phenomenon indirectly indicates a weak binding affinity of Ca²⁺ for the catalytic regions of the two pectate lyases in the absence of substrates. We thus speculated that Ca²⁺, to some extent, is involved in the catalytic step by establishing a bridge between CbPLs and carbohydrate substrates; however, Ca²⁺ does not bind to the CbPL protein alone.

The effect of metal ions was examined using pectate lyases and their catalytic modules in the presence of 0.25 mM Ca²⁺. The enzyme activity was strongly inhibited by most of the divalent cations (Tables S1 and S2). The optimum-pH assay revealed that CbPL3-CD, CbPL9, and CbPL9-CD exhibited their best activities at pH 9.0 (Fig. 2C). Wild-type CbPL3 exhibited optimal activity at pH 8.5 and retained over 10% of its activity at pH 7 (11). As shown in Fig. 2C, CbPL3 had a broad pH range, and more than 75% of its activity was retained at pH 7 to 10. This phenomenon could be due to the difference in the two buffer recipes. At the optimum temperature, CbPL3, CbPL3-CD, and CbPL9 exhibited their highest activities at 85°C, whereas CbPL9-CD showed activity at 80°C (Fig. 2D). The half-lives of these enzymes were more than 15 h at 65°C, suggesting their thermal stability at elevated temperatures (Fig. 2E and Fig. S3). The pectate lyase PelN from *Paenibacillus* sp. strain 0602 has half-lives of approximately 9 and 42 h at 50°C and 45°C, respectively (22). Without a rationally designed mutation, the activities of wild-type *A. luchuensis* mut. *kawachii* PelA (AkPelA) decreased to 16%, 10%, and 8.5% of the maximal activity after 60 min of incubation at 60°C, 70°C, and 80°C, respectively (37). To our knowledge, the pectate lyases CbPL3 and CbPL9 from *C. bescii* have higher optimum temperatures and better thermostabilities than their derivatives from other species. Hence, these lyases are good candidates for applications in the food, paper, and textile industries.

Specific enzyme activity (units per micromole) was examined under the optimum conditions of 85°C, pH 9, and 0.25 mM Ca²⁺ by using various substrates (Fig. 2F and Table 1). CbPL3-CD showed higher activities than CbPL3. However, the activity of CbPL9-CD was lower than that of CbPL9. Based on the gel image in Fig. 1C, purified CbPL9 and CbPL9-CD did not appear to be very stable, and multiple protein bands appeared. Therefore, the low specific activity of CbPL9-CD may be a result of the heterogeneity of CbPL9-CD fragments in the protein sample because a fraction of the enzyme may not be active. Moreover, kinetic analysis of the enzymes demonstrated that the deletion of CbCBM66 reduced the catalytic efficiencies of the two CbPLs mainly by decreasing their turnover rates (k_{cat}) (Table 2).

Binding and degradation by CbPLs against different carbohydrate substrates.

The in-tube polysaccharide adsorption (ITPA) method was used to detect the interactions of proteins and insoluble substrates and determine substrate-binding properties (23, 24). As shown in Fig. 3, two full-length CbPLs and their CbCBM66-truncated mutants, namely, CbPL3-CD and CbPL9-CD, clearly bound to pectin-derived substrates such as pGA, pectin from apple, pectin from citrus, switchgrass (SG), and poplar leaves but bound very weakly (or undetectably) to pectin-free carbohydrate polymers such as

TABLE 2 Kinetic analysis of two *CbPLs* and their truncated mutant on the substrate polygalacturonic acid

Enzyme	Mean sp act (U/ μ mol) $\pm$ SD	Mean $V_{\max}$ (U/mg) $\pm$ SD	Mean K_m (mg/ml) $\pm$ SD	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \text{ ml mg}^{-1}$)
<i>CbPL3</i>	10.0 $\pm$ 0.1	1,446 $\pm$ 162.4	1.6 $\pm$ 0.3	2,132.7	1,314.9
<i>CbPL3-CD</i>	16.3 $\pm$ 0.4	1,135 $\pm$ 92.3	1.33 $\pm$ 0.2	919.2	691.1
<i>CbPL9</i>	0.3 $\pm$ 0.02	57.3 $\pm$ 4	0.79 $\pm$ 0.1	127	132.5
<i>CbPL9-CD</i>	0.1 $\pm$ 0.004	26.4 $\pm$ 1.7	0.96 $\pm$ 0.1	6.49	6.8

arabinoxylan from wheat, xylan from beechwood, and Avicel. *CbCBM66* showed no binding affinity for these substrates, except for pectin from citrus (Fig. 3 and Fig. S4). Thus, the catalytic modules, not CBM66, in the two *CbPLs* could be involved in substrate binding.

The degradation products were examined by thin-layer chromatography (TLC) after the incubation of *CbPLs* and their mutants with pGA and pectin. In the beginning phase, the main products of *CbPL3* on pGA were unsaturated digalacturonic acid (uD), unsaturated trigalacturonic acid (uT), and other oligogalacturonic acids (Fig. 4A). The dominant end products after 12 h were uD and uT. The unsaturated oligogalacturonic acids migrated faster than the saturated ones due to the double bond formed between positions C-4 and C-5 in oligogalacturonates (25). For *CbPL9*, the end products were mainly uT and unsaturated tetragalacturonates (uTetra), with a small amount of uD (Fig. 4B). Therefore, the two *CbPLs* were endo-type pectate lyases with different end products. As shown in Fig. 4C, *CbCBM66*-truncated mutants, namely, *CbPL3-CD* and *CbPL9-CD*, showed end products similar to those of their full-length enzymes against either pGA or pectin from citrus. The same result was identified in *CbPL9* and *CbPL9-CD*. Hence, the deletion of *CbCBM66* did not influence the degradation step of *CbPLs*. Furthermore, a mixture of *CbPL3* and *CbPL9* released the products of uD and uT in the presence of pGA and pectin from citrus. Overall, *CbCBM66* was not involved in the binding and degradation of pectin-derived substrates.

Characterization of *CbCBM66*. The protein sequence of *CbCBM66* was annotated initially as a domain from the family of concanavalin A-like lectins/glucanases (CALG), which contain 12 to 14 antiparallel β -strands arranged in two curved sheets (26). The members of the CALG superfamily are classified according to their diverse functions into six families, namely, endoglucanases, xylanases, cellobiohydrolases, β -1,3(-1,4)-glucanases, alginate lyases, and peptidases (26, 27). Recently, this *CbCBM66* sequence was annotated as belonging to the Pfam06439 family (or DUF1080). Based on Pfam

FIG 3 Interaction of *CbPLs* and their truncated mutants with substrates. Purified proteins (20 μ g) were mixed with predetermined amounts of the given substrates. The suspension was centrifuged, and the washed pellets (bound fraction ["+"]) and supernatant fluids (unbound fraction ["-"]) were collected and subjected to SDS-PAGE after mixing with sample buffer.

FIG 4 (A and B) Time course of degradation products of pGA by CbPL3 (A) and CbPL9 (B) determined by using TLC analysis. The marker contains standard chemicals such as monogalacturonic acid (M), digalacturonic acid (D), and trigalacturonic acid (T). For lane C, pGA was incubated in the absence of the enzyme for 12 h, and the following dots show products liberated after 0.0, 0.25, 0.5, 1.0, 2.0, 3.0, and 12.0 h of enzymatic action. (C) Degradation products of pGA and pectin by one or a combination of different enzymes.

analysis, the protein members from Pfam06439 and family 16 glycoside hydrolase (β -1,3-1,4-glucanase) were structurally similar; however, their active sites were different. The results of DNS (3,5-dinitrosalicylic acid) and ITPA assays showed that the hydrolysis activities and binding affinities of CbPL3, CbPL9, and CbCBM66 were undetectable (or very low) for carbohydrate substrates such as sodium carboxymethylcellulose (CMC-Na), xylan from beechwood, β -1,3(-1,4)-glucan from barley, alginate, and Avicel (Table 1 and Fig. 3 and 5A). Hence, CbCBM66 has neither a CALG- nor a Pfam06439-derived function.

CbCBM66 was classified as belonging to the family 66 carbohydrate-binding modules (CBM66) (18). At present, CBM66 members are predominantly located in GH32 enzymes (18). Sequence analysis of CbCBM66 using BLAST identified 11 protein modules that exhibited high sequence similarity among *Caldicellulosiruptor* spp. (protein identity of >85%) but low similarities to other pectate lyases (protein identity of <50%) and GH32 enzymes (<25%). This finding reveals the unique ecological niche of *Caldicellulosiruptor* CBM66. The only resolved structure in this family is BsCBM66 (PDB accession no. 4AZZ), which is located at the C terminus of the GH32 β -fructosidase from *Bacillus subtilis* (18). The Iterative Threading Assembly Refinement (I-TASSER) server predicted that CbCBM66 is structurally close to BsCBM66 from *B. subtilis* (28); however, COFACTOR and COACH (29) indicated that the substrate-binding sites in CbCBM66 are different from those in BsCBM66 (Fig. 5B and C). Furthermore, neither β -fructosidase activities nor binding affinities CbCBM66 and CbPL3 were detected on levan and inulin (Table 1 and Fig. 5A). This finding reveals that CbCBM66 does not exhibit the function of glycoside hydrolases or polysaccharide lyases.

SEM and reducing sugar analysis on plant biomass. Morphological changes in SGs caused by the two CbPLs were investigated by scanning electron microscopy (SEM) analysis. As shown in Fig. 6A, the surfaces of the untreated SG leaves were flat and smooth, and no fiber bundles were observed. Similar surfaces were observed on

FIG 5 (A) Binding assay of *CbCBM66* with different carbohydrate substrates. (B) Predicted structure and substrate-binding sites of *CALG* using the I-TASSER server. (C) Protein alignment of *BzCBM66* (PDB accession no. 4AZZ) from *Bacillus subtilis* and *CbCBM66*.

CbCBM66-treated SG leaves (Fig. 6B), revealing that *CbCBM66* had no degradation role. However, the surface morphologies of *CbPL3*- and *CbPL9*-treated SG leaves were significantly changed (Fig. 6C and E). The fiber bundles of the treated leaves were exposed with many deep longitudinal cracks from the inside (30). Furthermore, mixing *CbPLs* with the cellulase cocktail resulted in more splits and fractures in the morphology and higher reducing sugar concentrations than with treatment with the cellulase

FIG 6 Scanning electron microscopy analysis of treated switchgrass at magnifications of $\times 200$ and $\times 10,000$. (A) Negative control (with buffer only); (B) after *CbCBM66* treatment; (C) after pectate lyase *CbPL3* treatment; (D) after *CbPL3* and cellulase cocktail treatment; (E) after pectate lyase *CbPL9* treatment; (F) after *CbPL9* and cellulase cocktail treatment; (G) after cellulase cocktail treatment.

cocktail alone (Fig. 6C to G and Fig. 7). This finding indicates the enhanced effect of two *Cb*PLs on the hydrolysis of natural biomass.

Conclusion. In this study, two pectate lyases from *C. bescii* displayed novel enzymatic characteristics, such as high optimum temperature (80°C to 85°C), low optimum

FIG 7 Reducing ends of polysaccharides on switchgrass. Control, 0.1 g of switchgrass in 1 ml of 100 mM Tris-glycine buffer (pH 8.5); CbPL3, 0.1 g of switchgrass incubated with 10 μ g of purified CbPL3; CbPL3+Cellulase, 10 μ g of purified CbPL3 and 30 μ g of the cellulase cocktail; CbPL9, 10 μ g of purified CbPL9; CbPL9+Cellulase, 10 μ g of purified CbPL9 and 30 μ g of the cellulase cocktail; Cellulase, 30 μ g of the cellulase cocktail. All samples were incubated at 65°C for 12 h.

pH (pH 8 to 9), and thermostability (>15 h at 65°C). The catalytic activities of the two CbPLs are Ca^{2+} dependent, although Ca^{2+} does not bind to the CbPL protein alone. The two CbPLs share the same CbCBM66 domain and have 100% amino acid identity. However, the effects of CbCBM66 on the enzyme kinetics and thermostability of the two CbPLs are different. A carbohydrate-binding assay illustrated that catalytic modules, not CbCBM66, have substrate-binding affinities for most pectin-derived carbohydrate substrates. Moreover, CbCBM66 does not exhibit a CALG- or Pfam06439-related function. Thus, CbCBM66 does not act as a substrate-binding module in the two CbPLs. Furthermore, SEM and reducing sugar analyses demonstrated that the two enzymes can improve the saccharification of switchgrass. Hence, the two CbPLs exhibit potential for the degradation of plant biomass and can be applied in the paper and textile industries.

MATERIALS AND METHODS

Chemicals and strains. Monogalacturonic acid, digalacturonic acid, trigalacturonic acid, polygalacturonic acid, citrus pectin, apple pectin, rhamnogalacturonic acid, CMC-Na, Avicel, beechwood xylan, alginate, and cellobiose were purchased from Solarbio Science & Technology (Beijing, China). The vector pEASY blunt-E2, *E. coli* BL21(DE3)/pLysS competent cells, and a plasmid miniprep kit were purchased from TransGen Biotech (Beijing, China). Isopropyl- β -D-1-thiogalactopyranoside (IPTG) and ampicillin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The cellulase cocktail was a gift from Vland Biotech (Qingdao, China).

Gene cloning, protein expression, and purification. Genomic DNA was isolated from *C. bescii* (31). Two pectate lyases, CbPL3 and CbPL9, were submitted to the NCBI database under GenBank accession no. [ACM60942](#) and [ACM60943](#), respectively (32). The genes of the two pectate lyases and their truncated mutants were amplified by PCR and then cloned into the pEASY blunt-E2 vector. The primers used in this study are shown in Table 3. The recombinant *E. coli* strains expressing the target proteins were cultivated in a 2-liter flask containing 500 ml LB medium mixed with 100 μ g/ml ampicillin at 37°C and agitated at 200 rpm. When the optical density at 600 nm (OD_{600}) of the culture medium reached 0.6 to 0.8, 0.5 mM

TABLE 3 Primers used

Clone	Primer orientation	Primer sequence (5'–3')
CbPL3-F	Forward	GCGACACTTTTAACAGATGATTTTGAAGATG
CbPL3-R	Reverse	GTATTGATGTATCTGTGATTGGGACGG
CbPL3-CD-F	Forward	ACAGCACCTACTCCGACACCAAC
CbPL3-CD-R	Reverse	GTATTGATGTATCTGTGATTGGGACG
CbCBM66-F	Forward	GCGACACTTTTAACAGATGATTTTGAAG
CbCBM66-R	Reverse	TGGTGCTACTGATGAAGTCGGTG
CbPL9-F	Forward	GCGACACTTTTAACAGATGATTTTGAAGAT
CbPL9-R	Reverse	CCTTGCTCCTATTCCTTCTAAACCACTA
CbPL9-CD-F	Forward	ACAGCACCTACTCCGACACCAAC
CbPL9-CD-R	Reverse	CCTTGCTCCTATTCCTTCTAAACCAAC

IPTG was added to the culture to induce protein expression. The cells were incubated with shaking overnight at 150 rpm at 16°C. The cell pellets were harvested by centrifugation ($8,000 \times g$ for 10 min at 4°C) and then dissolved in buffer A (20 mM Tris-HCl and 300 mM NaCl [pH 8.0]). The cell lysates were heated for 15 min at 65°C and then centrifuged ($12,000 \times g$ for 30 min at 4°C) to remove cell debris and denatured proteins after ultrasonication.

Recombinant proteins with a 6×His tag were purified using nickel-nitrilotriacetic acid (Ni-NTA)-Sefinose resin (Sangon, China) in open columns. The open column was equilibrated with 100 ml buffer A and then incubated with the supernatant for 20 min. After washing with 100 ml buffer B (buffer A with 40 mM imidazole), the target proteins were eluted with buffer C (buffer A with 300 mM imidazole). The eluted proteins were concentrated using Amicon Ultra filters (Millipore, Ireland) and confirmed by SDS-PAGE.

Activity assay of pectate lyases. The purified proteins (2 nM) were incubated in 100 mM Tris-glycine buffer (pH 8.5) containing 1% pGA at 65°C for 20 s to identify the effect of Ca^{2+} on the activities of the two CbPLs. The mixture was added with 0.5 mM Ca^{2+} for another 20 s. Enzyme activity was inhibited by treatment with 1 mM EDTA and recovered by treatment with 5 mM Ca^{2+} . The absorbance at 235 nm (A_{235}) was recorded in a quartz cuvette.

For measuring the activities of pectate lyases and their catalytic modules, purified proteins were assayed against 0.1% (wt/vol) pGA, apple pectin, and citrus pectin at the appropriate temperatures, pHs, and CaCl_2 concentrations. The A_{235} was measured due to the 4,5-unsaturated oligosaccharides. The molar extinction coefficient is $4,600 \text{ M}^{-1} \text{ cm}^{-1}$ (33). One unit of enzyme activity is the amount of enzyme that produces $1 \mu\text{mol}$ of 4,5-unsaturated oligosaccharides per min. Each experiment was performed in triplicate.

Biochemical characterization of pectate lyases. With 0.1% (wt/vol) pGA as a substrate, the effect of Ca^{2+} on pectate lyase activity was measured at pH 8.5 at 65°C by incubating the purified enzyme solutions in 100 mM Tris-glycine buffer at a Ca^{2+} concentration range of 0 to 2 mM under standard test conditions.

The effect of pH on pectate lyase activity was measured using 0.1% (wt/vol) pGA as a substrate at 65°C and 0.25 mM Ca^{2+} by incubating the purified enzyme solutions in different buffers, such as sodium citrate buffer (pH 4.0 to 6.0), Tris-HCl buffer (pH 6.0 to 9.0), and glycine-NaOH buffer (pH 9.0 to 11.0), under standard test conditions.

Furthermore, the effects of temperature on pectate lyase activity were investigated at 55°C, 65°C, 75°C, and 85°C using 0.1% (wt/vol) pGA as a substrate at pH 8.5 and 0.25 mM Ca^{2+} to determine the optimal reaction temperature. The purified enzyme solutions were incubated at the above-mentioned different temperatures, and residual activities at different times were measured under standard assay conditions to evaluate the thermal stability of the enzyme.

In addition, the effects of different metal ions and chemicals on pectate lyase activity were measured using 0.1% (wt/vol) pGA as a substrate at 65°C, at pH 8.5, and with 0.25 mM Ca^{2+} based on the standard assay method. The relative activities of the enzyme were measured using the following metal ions at 1 and 5 mM concentrations: K^+ , Mn^{2+} , Ni^{2+} , Hg^{2+} , Zn^{2+} , Fe^{2+} , Fe^{3+} , Co^{2+} , Cu^{2+} , Cd^{2+} , Mg^{2+} , and Na^+ . EDTA, SDS, urea, and guanidine hydrochloride were used at 1% and 5% concentrations.

Analysis of degradation products of CbPLs and their mutants. The degradation of polygalacturonic acid and pectin by pectate lyases was analyzed by TLC using a solvent system of ethyl acetate-acetic acid-water (4:2:3, vol/vol/vol). The reaction products were visualized by heating the TLC plate at 100°C for 10 min after spraying with 10% (vol/vol) sulfuric acid in ethanol. Unsaturated saccharides and standard samples on TLC plates were detected by using thiobarbituric acid and o-phenylenediamine staining methods, respectively. Galacturonic acid, digalacturonic acid, and trigalacturonic acid (Santa Cruz Biotechnology, Dallas, TX, USA) were used as standards (34).

Structure/function prediction of CbCBM66 by I-TASSER. The Iterative Threading Assembly Refinement (I-TASSER) system was used to predict the structure and function of CbCBM66. After inputting the sequence of CbCBM66 (amino acids [aa] 46 to 220), I-TASSER generated several structure models of the query protein by applying state-of-the-art algorithms (28). The C score (confidence score), TM score (template modeling score), value range, and root mean square deviation (RMSD) were evaluated to determine the best model. The predicted structures were refined using online tools, namely, GalaxyWeb (<http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE>) (35) and 3D refine (<http://sysbio.rnet.missouri.edu/3Drefine>) (36). Substrate-binding sites in CbCBM66 were predicted using COFACTOR and COACH (<https://zhanglab.ccmb.med.umich.edu/>) (29).

Carbohydrate-binding assay. A carbohydrate-binding assay was performed using an in-tube polysaccharide adsorption (ITPA) method as previously described (23, 24), with some modifications. The substrates were resuspended in buffer D (100 mM Tris-HCl, 10 mM Ca^{2+} [pH 8.5]) and centrifuged at $12,000 \times g$ for 10 min. Washing was conducted twice for pectin, pGA, xylan, arabinoxylan, Avicel, CMC-Na, levan, and inulin and four times for switchgrass and poplar leaves. Each substrate (2% [wt/vol]; 100 μl) was transferred into an Eppendorf tube and incubated with 20 μg of the purified proteins for 1 h at 25°C with gentle rotation. The mixture was centrifuged at $12,000 \times g$ for 10 min to sediment the polysaccharides. The supernatant (containing the unbound fraction) was transferred into an Eppendorf tube for testing, and the sediments (containing the bound fraction) were washed using the above-mentioned steps three times. The sediments were then added to 65 μl of SDS gel sample buffer and 135 μl of binding buffer. The unbound and bound fractions were boiled for 10 min and examined by SDS-PAGE.

Scanning electron microscopy and reducing sugar assays. Fresh switchgrass leaves were washed, dried in an oven at 60°C, and milled to a certain size ($\sim 5.0 \text{ mm}$). Switchgrass (0.1 g) was added to 1 ml

of 100 mM Tris-glycine buffer (pH 8.5) containing 10 μ g of the purified enzymes and/or 30 μ g of the cellulase cocktail. The reaction mixture was incubated at 65°C for 12 h and centrifuged at 12,000 $\times g$ for 5 min. The supernatant (containing the free reducing sugar) was transferred to 1.5-ml Eppendorf tubes. The precipitated part required additional dehydration prior to observation. SEM analysis was conducted to observe the ultrastructure and surface morphology of the untreated and enzyme-treated switchgrass leaves. The samples were coated with a gold layer by using a vacuum sputter coater at 200 Å. Photos were taken with a scanning electron microscope (JSM6510LV; JEOL, Japan) at a 10-kV acceleration voltage (30). The reducing sugar concentration was examined using a DNS (3,5-dinitrosalicylic acid) assay as previously described (31).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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