Determination of Subunit Composition of *Clostridium cellulovorans*
Cellulosomes That Degrade Plant Cell Walls

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*Clostridium cellulovorans* produces a cellulase enzyme complex (cellulosome). In this study, we isolated two plant cell wall-degrading cellulosomal fractions from culture supernatant of *C. cellulovorans* and determined their subunit compositions and enzymatic activities. One of the cellulosomal fractions showed fourfold-higher plant cell wall-degrading activity than the other. Both cellulosomal fractions contained the same nine subunits (the scaffolding protein CbpA, endoglucanases EngE and EngK, celllobiohydrolase ExgS, xylanase XynA, mannanase ManA, and three unknown proteins), although the relative amounts of the subunits differed. Since only cellubiase was released from plant cell walls by the cellulosomal fractions, celllobiohydrolases were considered to be key enzymes for plant cell wall degradation.

**MATERIALS AND METHODS**

**Materials.** Avicel (crystalline cellulose) was purchased from FMC Corporation. Carboxymethyl cellulose (CMC) (low viscosity), locust bean gum, xylan from oat spelt, and pectin from apples were purchased from Sigma. Powder of the water-insoluble fraction of corn stems was supplied by Meiji Seika Kaisha, Ltd.

**Enzyme preparation.** *C. cellulovorans* ATCC 35296 was grown by the method of Sleat et al. (21). The bacteria were cultivated anaerobically at 37°C for 2 days in 250 ml of medium containing a carbon source at a concentration of 0.5%. The culture supernatant was concentrated and dialyzed as described previously (19) and was dissolved in 10 ml of phosphate-buffered saline (PBS) buffer (50 mM KH$_2$PO$_4$, 50 mM K$_2$HPO$_4$, pH 6.8).

**Purification of cellulosomes.** Seventy-five milliliters of concentrated culture supernatant of *C. cellulovorans* cells grown with pectin was precipitated with
(NH$_4$)$_2$SO$_4$, dialyzed, and purified by cellulose affinity chromatography as described previously (19). The cellulose-binding fraction was ultrafiltered with Ultra free max 100 k NMWL (Millipore) and concentrated to a volume of 1 ml. Then the 1 ml concentrated solution was applied to a High Q cartridge (1 ml; Bio-Rad) equilibrated with 50 mM Tris-HCl buffer (pH 8.0). After the column was washed with 3 ml of the same buffer, it was eluted with a stepwise gradient of NaCl (50 mM to 1 M).

Assay of enzymatic activities. The activities on corn stem powder (for cellwallase), Avicel (for cellulase), CMC (for endoglucanase), xylan (for xylanase), locust bean gum (for mannanase), and pectin (for pectate lyase) were assayed at pH 6.0 and 37°C by measuring the liberated sugars as D-glucose equivalents by the Somogyi-Nelson method (27). Each reaction mixture consisted of 250 μl of a 1% substrate solution, 100 μl of 500 mM sodium acetate buffer (MES)–NaOH buffer (pH 6.0), and 150 μl of an enzyme solution. The incubation times were 30 min for endoglucanase, xylanase, mannanase, and pectate lyase activities and 12 h for cellulase and cellwallase activities. Activity was expressed in units; 1 U was defined as the amount of enzyme that released 1 μmol of reducing sugar per min.

Protein determination. The concentration of protein was measured by the method of Bradford (1) with a protein assay kit from Bio-Rad, using bovine serum albumin as the standard.

Preparation of antibodies. Antibodies against CbpA (formerly called anti-P170) and ExgS (formerly called anti-P70) were prepared as described previously (15). Antibody against EngE was prepared as described previously (15) by using recombinant EngE expressed in Escherichia coli (22).

SDS-PAGE, zymograms, and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 10% polyacrylamide gel by the method of Laemmli (12). To determine the approximate molecular weight of the band to calculate the relative molar amount, the molecular weight of each protein was expressed relative to the amount of the P1 band, which was arbitrarily assigned a value of 1. Endoglucanase and xylanase zymograms were obtained by using 0.1% CMC or 0.1% xylan copolymerized with 10% polyacrylamide and 0.1% SDS as described previously (19). For Western blotting, proteins were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The membrane was treated with antibody (diluted 1:5,000) and stained as described previously (22).

N-terminal amino acid sequencing. The proteins blotted onto an Immobilon-P polyvinylidene difluoride membrane were sequenced by using a model 477 protein sequencer (Applied Biosystems).

RESULTS

Effects of carbon sources in the media on plant cell wall-degrading activity (cellwallase activity). To determine the effects of carbon sources in the media on the production of plant cell wall-degrading activity, C. cellulovorans was cultured with several different carbon sources.

Since corn stover is a potential substrate for biomass conversion to obtain fermentable sugars (9), we used corn stem powder as the substrate for the assay of the plant cell wall-degrading activity of C. cellulovorans enzymes. The amount of reducing sugar liberated from the powdered corn stems was designated the cellwallase activity. As shown in Table 1, the culture grown with cellobiose produced the most cellwallase activity, and the culture supernatant from cells grown with pectin exhibited the highest cellwallase specific activity.

Partial purification of cellulosomes that degrade plant cell walls. Since the enzyme from cells grown with pectin exhibited the highest cellwallase specific activity, cellulosomes were partially purified from a culture supernatant from cells grown with pectin. To collect the cellulose-binding proteins, which are mainly cellulosomes (19), the culture supernatant was purified by cellulose affinity chromatography. Then, in order to remove small proteins (mainly noncellulosomal enzymes), the cellulosomal fraction was ultrafiltered with a membrane that eliminated molecules smaller than 100 kDa. The concentrated solution was subjected to anion-exchange chromatography. As shown in Fig. 1, cellwallase activity eluted in two peaks (designated cellulosome 1 and cellulosome 2). Although the two cellulosomal fractions contained the same level of cellwallase activity, cellulosome 1 had much higher cellwallase activity than cellulosome 2.

Comparison of cellulosome 1 and cellulosome 2. To compare the properties of cellulosome 1 and cellulosome 2, SDS-PAGE patterns and enzymatic activities of both cellulosomes were determined. A silver-stained SDS-PAGE gel containing cellulosome 1 and cellulosome 2 is shown in Fig. 2A. The results indicated that the two cellulosomal fractions contained the same nine main components (designated P1 to P9). To roughly determine the relative amount of each subunit, the gel was stained with Coomassie brilliant blue R-250 (data not shown) and analyzed by densitometry on the assumption that the density of a band was proportional to the actual mass of protein. The molar amount of each protein was expressed relative to the amount of the P1 band, which was arbitrarily assigned a value of 1. (Fig. 2B). Cellulosome 1 contained larger amounts of P6, P7, P8, and P9 than cellulosome 2, while cellulosome 2 contained three times more P2 than cellulosome 1 contained. The enzymatic activities of both cellulosomes are shown in Table 2. The cellwallase specific activity of cellulosome 1 was four times greater than that of cellulosome 2. Also, cellulosome 1 had higher Avicelase, xylanase, and mannanase specific activities and lower endoglucanase specific activity than cellulosome 2. Neither cellulosome 1 nor cellulosome 2 showed pectate lyase activity. It was recently found that a culture supernatant of pectin-grown C. cellulovorans had pectate lyase activity (Tamaru et al., unpublished). Thus, the present results indicate that pectin-grown cells produce either a noncellulosomal pectate lyase or a cellulosomal pectate lyase that dissociates from the cellulosome during growth.

High-performance liquid chromatography analysis of hydrolytic products showed that only cellobiose was liberated from both Avicel and corn stem powder by both cellulosomal fractions (data not shown).

Identification of cellulosomal subunits. Since cellulosome 1 exhibited higher cellwallase specific activity, the cellulosomal subunits of cellulosome 1 were identified. To identify the subunits, Western blotting, N-terminal amino acid sequencing, and a zymogram analysis of endoglucanase and xylanase were performed. The results are summarized in Table 3.
The Western blot analysis was performed by using anti-CbpA, anti-EngE, and anti-ExgS. As shown in Fig. 3, anti-CbpA reacted only with P1 and anti-EngE reacted only with P2. These results indicated that P1 was CbpA and that P2 was EngE. The apparent molecular weights of P1 and P2 were in good agreement with the calculated molecular weights of CbpA (20) and EngE (22). Anti-ExgS reacted mainly with P4, and there were weak cross-reactions with P1, P3, and other...
proteins (Fig. 3). Since the ExgS used for preparation of anti-ExgS was purified from culture supernatants of *C. cellulovorans* (15), the purified ExgS used for antibody preparation probably contained P1 and P3 as contaminants. Thus, Western blotting revealed the presence of CbpA, EngE, and ExgS.

To confirm that P4 is ExgS, the N-terminal amino acid sequence of P4 was determined. The results (Table 3) showed that the N-terminal amino acid sequence of P4 was identical to that of ExgS. The apparent molecular weight of P4 was in good agreement with the theoretical molecular weight of ExgS (13). The N-terminal amino acid sequences of P3, P5, P6, P7, P8, and P9 were also determined. The N-terminal sequences of P5 and P6 did not match the N-terminal sequences of any known proteins. Thus, P5 and P6 appear to be unidentified cellulosomal proteins. The N-terminal sequence of P8 could not be read, and its N terminus is probably blocked. The N-terminal sequences of P3, P7, and P9 were in good agreement with the derived molecular weights of EngK, XynA, and ManA. The N-terminal sequence analyses, therefore, showed that EngK, ExgS, XynA, and ManA were present.

To produce cellulosomes with high cellwallase activity, the effects of carbon sources in the medium on cellwallase production were determined. Pectin was found to be the best carbon source for *C. cellulovorans* to produce high cellwallase specific activity. Recently, it was found that the enzyme from cells grown with pectin also had the highest protoplast-forming specific activity (Tamaru et al., unpublished). Thus, pectin is considered a good carbon source for *C. cellulovorans* to produce plant cell wall-degrading enzymes.

Two different cellulosomal fractions were isolated from the supernatant of cells grown with pectin by anion-exchange chromatography of the cellulose-binding fraction. Both cellulosome 1 and cellulosome 2 contained nine subunits, although the relative amounts of the subunits differed in the two cellulosomal fractions. Moreover, the enzymatic activities in the fractions were also different. These results indicated that *C. cellulovorans* grown with pectin produced heterogeneous cellulosomes, as has been shown with *C. papyrosolvens* C7 cellulosomes (17, 18).

Since scaffolding protein CbpA has nine cohesin domains that bind enzymatic subunits, CbpA could bind at most nine enzymatic subunits (3). Therefore, if the isolated cellulosomal fractions were completely purified, the molar amounts of enzymatic subunits P2 to P9 should satisfy two requirements: (i) each molar amount of enzyme should be an integer of CbpA and (ii) the sum of the molar amounts of enzymes should be nine. To make the molar amounts of the enzyme shown in Fig. 2B integers, we rounded the data to obtain the following molar amounts: for cellulosome 1 P1 to P9, 1, 1, 1, 3, 1, 2, 1, 1, and 2, respectively; and for cellulosome 2 P1 to P9, 1, 3, 2, 3, 1, 1, 0, 0, and 1, respectively. Consequently, the values resulted in sums for the relative molar amounts of P2 to P9 that were greater than nine for both cellulosomal fractions. Thus, these

**DISCUSSION**

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![Western blot analysis of cellulosome 1. Lane 1, gel stained with Coomassie brilliant blue R-250; lane 2, gel immuno-stained with anti-CbpA; lane 3, gel immuno-stained with anti-EngE; lane 4; gel immuno-stained with anti-ExgS. The numbers on the left are the molecular masses (in kilodaltons) of the markers.](image-url)
results indicate that both isolated cellulosomal fractions were impure; i.e., they probably contained mixtures of cellulosomes. Moreover, even if the compositions of cellulosomes in the isolated cellulosomal fractions were similar to each other, there were still large numbers of possible permutations for enzymatic subunits to bind to CbpA. Thus, we assumed that the two isolated cellulosomal fractions contained heterogeneous mixtures of cellulosomes.

Six of the nine subunits of the isolated cellulosomal fractions could be assigned to known proteins, including CbpA (20), EngE (22), EngK (25), ExgS (13), XynA (11), and ManA (23), and three subunits were unknown proteins. It has been suggested that the endoglucanase, xylanase, and mannannase activities of the cellulosomes come mainly from EngE (P2), XynA (P7), and ManA (P9), respectively. The differences in endoglucanase, xylanase, and mannannase activities between cellulosome 1 and cellulosome 2 were in quite good agreement with the differences in the relative amounts of P2, P7, and P9 found for the two types of cellulosomes.

Since the isolated cellulosomal fractions released only cellobiose from corn stem powder, degradation of cellulose to cellobiose was considered one of the key reactions for plant cell wall degradation by the cellulosomes. In general, crystalline cellulose is degraded by synergistic activity of endoglucanases and cellobiohydrolases (5–7). Cellobiohydrolase is an enzyme that releases cellobiose from crystalline cellulose. Among the enzymatic subunits on the purified cellulosome, ExgS has been characterized as a cellobiohydrolase, and its major hydrolytic product is cellobiose (13). Moreover, P4 (assigned to ExgS) is one of the major subunits of the purified cellulosomes. Thus, ExgS is expected to play an important role in degrading cellulose in plant cell walls.

The main components of the plant cell walls in corn stems are cellulose (about 40%), hemicellulose (mainly glucuronarabinoxylan; about 20%), and lignin (about 20%) (2, 9). Each cellulose microfibril in plant cell walls is cross-linked by hemicelluloses (2). Thus, it is necessary for cellulosomes to degrade hemicellulose connections to gain access to cellulose microfibrils in the plant cell walls. Although the levels of the cellulase activities of cellulosome 1 and cellulosome 2 were the same, the cellwallase activity of cellulosome 2 was much less than that of cellulosome 1. One possible explanation of this result is that the ability of cellulosome 2 to gain access to cellulose microfibrils in plant cell walls might be less than that of cellulosome 1. Since cellulosome 1 contained more XynA (P7), ManA (P9), P6, and P8 than cellulosome 2, these subunits may help the cellulosomes gain access to cellulose microfibrils more readily.

In this study, we isolated two cellulosomal fractions that degrade plant cell walls and determined the relative amounts of their enzymatic subunits. The composition of cellulosome 1, which exhibited higher cellwallase activity than cellulosome 2, could be a good starting model for designing recombinant cellulosomes that degrade plant cell walls effectively. We plan to prepare recombinant cellulosomes with the various enzymatic subunits that were identified in this study, as well as additional subunits. Analysis of these recombinant cellulosomes is expected to provide new insights not only for understanding the mechanism of plant cell wall degradation but also for preparing designer cellulosomes that degrade plant cell walls effectively.

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REFERENCES


| Table 3. Identification of the subunits of cellulosome 1 |
|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| Protein | Apparent mol wt | Zymogram* | Reacted antibody | N-terminal sequence | Assigned protein |
| P1 | 140.3 | | Anti-CbpA | cbpA | 189.1 |
| P2 | 106.7 | + + + | Anti-EngE | engE | 111.8 |
| P3 | 101.0 | + | ATMSVSGELI (EngK) | engK | 97.0 |
| P4 | 78.5 | | APVVPNPEYV (ExgP) | exgP | 80.5 |
| P5 | 75.6 | | XTSELAIK | NA | |
| P6 | 65.3 | | XDALLIS | NA | |
| P7 | 58.6 | + | ATK (XynA) | xynA | 57.0 |
| P8 | 56.5 | | NR | NA | |
| P9 | 41.5 | | ATTLGDNVVD (ManA) | manA | 44.5 |

* + + +, a clear band appeared after 2 h of incubation; +, clear bands appeared after 6 h of incubation.

† The N-terminal sequence is identical to the N-terminal sequence of the endoglucanase in parentheses.

‡ NR, N-terminal amino acid sequence could not be read.

§ NA, protein could not be assigned to known proteins.