Effect of carbon source on the cellulosomal subpopulations of *Clostridium cellulovorans*

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*Clostridium cellulovorans* produces a cellulase enzyme complex called the cellulosome. When cells were grown on different carbon substrates such as Avicel, pectin, xylan, or a mixture of all three, the subunit composition of the cellulosomal subpopulations and their enzymic activities varied significantly. Fractionation of the cellulosomes (7–11 fractions) indicated that the cellulosome population was heterogeneous, although the composition of the scaffolding protein CbpA, endoglucanase EngE and cellobiohydrolase ExgS was relatively constant. One of the cellulosomal fractions with the greatest endoglucanase activity also showed the highest or second highest cellulase activity under all growth conditions tested. The cellulosomal fractions produced from cells grown on a mixture of carbon substrates showed the greatest cellulase activity and contained CbpA, EngE/EngK, ExgS/EngH and EngL. High xylanase activity in cellulose, pectin and mixed carbon-grown cells was detected with a specific cellulosomal fraction which had relatively larger amounts of XynB, XynA and unknown proteins (35–45 kDa). These results in toto indicate that the assembly of cellulosomes occurs in a non-random fashion.

**INTRODUCTION**

Cellulosomes are multi-enzyme complexes produced by anaerobic cellulolytic bacteria that efficiently degrade various plant cell-wall polysaccharides (Bayer et al., 2004; Doi & Kosugi, 2004; Doi et al., 2003; Ohara et al., 2000; Schwarz, 2001). The *Clostridium cellulovorans* cellulosome complex contains a nonenzymic scaffolding component that incorporates, at most, nine enzyme subunits into the complex (Shoseyov et al., 1992). The assembly of cellulosomal subunits into the complex is dictated by tenacious interactions between cohesins and dockerins (Tokatlidis et al., 1993; Yaron et al., 1996). *C. cellulovorans* produces cellulosomes containing a variety of cellulolytic and hemicellulolytic subunits attached to the nonenzymic scaffolding component termed CbpA (Doi et al., 2003).

Naturally occurring plant cell wall compounds are structurally heterogeneous polymers and are composed primarily of cellulose, hemicellulose, pectin and lignin (Taiz & Zeiger, 1991). The existence of various carbon-containing polymers in plant cell walls may require the *C. cellulovorans* cellulosomes to have different subunit compositions and subpopulations to degrade plant cell walls efficiently. Recent transcriptional and proteomic studies show that different carbon sources in the *C. cellulovorans* culture medium have a significant effect on cellulosomal enzymic composition and activity (Han et al., 2003a, b, 2004b). Previously, Pohlschroder et al. (1994, 1995) found that the extracellular cellulase system of *Clostridium papyrosolvens* consisted of at least seven distinct high-molecular-mass protein complexes (500–660 kDa). All the complexes had endoglucanase-active protein subunits, but only two had xylanase-active subunits.

In this study, we fractionated partially purified cellulosomes from *C. cellulovorans* into subpopulations and determined their subunit compositions and enzymic activities. The data showed the presence of heterogeneous populations of cellulosomes isolated from cells grown on different carbon sources. What are the prerequisites for the formation of functional subpopulations of cellulosomes? A partial answer may involve enzyme induction and heterogeneous populations of cohesins and dockerins.

**METHODS**

**Bacterial strain and growth conditions.** *C. cellulovorans* ATCC 35296 was grown under strictly anaerobic conditions at 37°C in 1 or 10 l of a previously described medium (Shoseyov & Doi, 1990; Sleat et al., 1984), which included 1% (w/v) Avicel (microcrystalline cellulose, FMC), 1% xylan (birch wood, Sigma), 1% pectin (apples, Sigma) and 1% mixed polysaccharides [Avicel/xylan/pectin (3:1:1, by wt)].

**Preparation of cellulosomal (sub)populations.** Cellulosomes were purified from culture supernatants of *C. cellulovorans*, as described previously (Shoseyov & Doi, 1990). The culture supernatants...
were obtained during the stationary phase (4–7 days) by centrifuga-
tion, precipitated by 80% (w/v) ammonium sulfate and dialysed. The extracellular material was then mixed with Avicel, which resulted in binding of the cellulosome complex to Avicel. After incubation for 1 h at 4 °C, the suspension was poured into a column which was washed with three volumes 50 mM Tris/HCl buffer (pH 7-5) to elute the unattached fractions. The bound frac-
tion was eluted from the cellulose column with deionized water and concentrated with Ultrafree Biomax (10 kDa cut-off, Millipore) before being subjected to gel filtration on a HiLoad 26/60 Superdex 200 prep grade column (320 ml, Amersham Biosciences) equili-
brated with 50 mM Tris/HCl buffer (pH 7-5). The high-molecular-
mass fractions were collected as the cellulosomal fraction by using FPLC (Amersham Biosciences), and concentrated with Ultrafree Biomax (10 kDa cut-off, Millipore). The concentrated solution was applied to a RESOURCE Q (1 ml, Amersham Biosciences) equili-
trated in an equilibration buffer [6 M urea, 2% (w/v) SDS, 0-375 M Tris/HCl (pH 8-8), 20% (w/v) glycerol] with 2% (w/v) dithiothreitol, followed by equilibration buffer with 2-5% (w/v) iodoacetamide. The second-dimension gels were cast using a linear gradient of 4–15% polyacrylamide. The equilibrated tube gels were secured to the 2-D gels using agarose, and SDS-PAGE was carried out as described previously (Gorg et al., 2000; Laemmli, 1970). Proteins were fixed in the gels by soaking in a solution containing 40% (v/v) methanol and 10% (w/v) acetic acid for approximately 1 h and subsequently visualized by Coomassie blue staining (Genomic Solutions). For Western blot analysis, proteins (1 μg) were separated by SDS-PAGE and blotted onto a PVDF membrane (Immobilon-P, Millipore). The membrane was treated with antibody (diluted 1:5000) and stained as described previously (Tamaru & Doi, 1999).

Zymography. Zymography for xylanase and carboxymethylcellulase (CMCase) was performed by using 0-1% (w/v) of each substrate incorporated into the polyacrylamide. After proteins (1 μg) were separated by SDS-PAGE, the gels were renatured and incubated in a renaturation buffer (100 mM succinic acid, 10 mM CaCl₂, 1 mM dithiothreitol, pH 6-3) for 1 h at 37 °C with gentle shaking. The clearing zones corresponding to enzyme activities were visualized with 0-3% (w/v) Congo red (stained for 10 min and destained with 1 M NaCl solution) (Béguin, 1983).

Enzyme assays. The activities on Avicel (for cellulase), carboxy-
methylcellulose (CMC) (for endoglucanase), pectin (for pectate lyase) and xylan (for xylanase) were assayed at pH 6-0 and 37 °C by measuring the liberated reducing sugars as D-glucose equivalents by the Somogyi–Nelson method (Somogyi, 1952; Wood & Bhat, 1988). Each reaction mixture consisted of 250 μl 1% substrate solution, 100 μl 250 mM sodium acetate buffer (pH 6-0), and 150 μl enzyme solution. The incubation times were 30 min for endoglucanase, pectate lyase and xylanase activities and 18 h for cellulase activity. One unit of each enzyme activity was defined as the amount of enzyme which released 1 μmol reducing sugar min⁻¹ (μmol⁻¹) for Avicelase assays) under the condition indicated.

RESULTS

Effects of carbon source in the medium on subpopulations of cellulosomes and their enzyme activity

To collect cellulose-binding proteins, which are mainly cellulosomes, the culture supernatant was initially partially purified by cellulose affinity chromatography. In order to remove the noncellulosomal enzymes, the cellulose-binding fraction was subjected to gel-filtration chromatography that allowed the collection of molecules of 400–900 kDa. The mean molecular mass was approximately 750 kDa. The partially purified cellulosomes were fractionated by anion-exchange chromatography. The elution profiles showed that approximately three to five major peaks, including approximately four to six small fractions, were eluted between 0 and 1 M NaCl (data not shown). Fractionation indicated that the cellulosome population was hetero-
geneous. Only small amounts of proteins eluted between 0 and 0-05 M or 0-3 and 1 M NaCl, and exhibited no CbpA subunit; therefore the fractions from these regions were not considered as cellulosomes (data not shown).

To determine the effects of carbon sources in the medium on the subpopulations of cellulosomes, C. cellulovorans was cultured with four different carbon substrates. Each sample was subjected to anion-exchange chromatography in duplicate to control for variations of elution profiles (data not shown). The elution profiles of the cellulosomal subpopula-
tions after anion-exchange chromatography were dramati-
cally different depending on the culture conditions used (Fig. 1).

The largest peak of cellulosomes from the culture grown on microcrystalline Avicel eluted between 0-10 and 0-11 M NaCl (Fig. 1A, F1). This subpopulation (Fig. 1A, F1) had the highest cellulase and CMCase specific activity (Table 1). The highest xylanase activity from the cellulose culture was present in the fourth major peak, which was found to elute around 0-2 M NaCl (Fig. 1A, F4). This subpopulation (Fig. 1A, F4) had the highest cellulase, xylanase and endoglucanase specific activities were obtained in the fourth major peak to elute at around 0-11 M NaCl (Fig. 1B, F1). However, the highest cellulase, xylanase and endoglucanase specific activities were obtained in the fourth major peak to elute at around 0-2 M NaCl (Fig. 1B, F4; Table 1). With pectin as the main carbon source, major peaks were eluted from 0-13 to 0-17 M NaCl (Fig. 1C, F2 and F3). Also, these fractions had high cellulase and endoglucanase specific activity (Table 1). The highest pectate lyase activity was found in the first major peak eluted at about 0-11 M NaCl (Fig. 1C, F1; Table 1), whereas the pectate lyase activity was not significantly different among cellulosomes obtained from cultures grown on other carbon sources (Table 1). The highest xylanase activity was found in the first major peak which eluted at around 0-11 M NaCl (Fig. 1C, F1; Table 1). The elution profile of cellulosomes from cells grown on a mixture of carbon substrates coincided with the pattern of the individual carbon substrates, especially...
with that of cellulose (Fig. 1D). Significant cellulase, xylanase and endoglucanase activities were detected in the fourth peak, which eluted around 0.19 M NaCl (Fig. 1D, F4; Table 1).

The fractions eluted from the ion exchange column showed an extremely broad elution profile and had different enzyme activities under different culture conditions. The pattern of the major peaks eluted from the column represented subpopulations of cellulosomes. These results showed that the carbon source in the growth medium dramatically affected production of cellulosomal subpopulations.

**Subunit composition of cellulosomal subpopulations from cells grown on different carbon sources**

To compare the subunit composition of cellulosomal subpopulations, SDS-PAGE and Western blot patterns were determined for cellulosomes obtained from cells grown under different culture conditions (Fig. 2). The SDS-PAGE analysis demonstrated that the cellulosomal subpopulations fractionated by anion-exchange column chromatography significantly differed quantitatively and qualitatively in enzyme subunit composition (Figs 2A, F, K and P). For example, the cellulosome subpopulation from Avicel-grown cells contained two to five major polypeptides of molecular mass 64–200 kDa (Fig. 2A). The relative amounts of individual polypeptides differed significantly, especially subunit ExgS/EngH/EngY (around 80 kDa) or XynB (around 66 kDa) (Doi et al., 2003; Han et al., 2004a). The cellulosome fraction 1 (Fig. 1, F1) which had the highest Avicelase and CMCase activity contained relatively high amounts of subunits around 80 kDa or 66 kDa (Doi et al., 2003; Han et al., 2004a). However, the subunit composition of the high-molecular-mass subunits >400 kDa was significantly dependent on the carbon source of the cellulosome fractionation. The subunit composition of the high-molecular-mass subunits >400 kDa was significantly dependent on the carbon source of the cellulosome fractionation.

**Table 1. Comparison of enzyme activities of cellulosomal fractions from different carbon sources**

<table>
<thead>
<tr>
<th>Carbon source†</th>
<th>Fraction</th>
<th>Enzyme activity (mU mg⁻¹)</th>
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<tr>
<td></td>
<td></td>
<td>CMCase (endoglucanase)</td>
</tr>
<tr>
<td>Cellulose</td>
<td>M‡</td>
<td>1·31</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>0·51</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>0·45</td>
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<td></td>
<td>F3</td>
<td>0·35</td>
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<tr>
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<td>F4</td>
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</tr>
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<tr>
<td></td>
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<td>0·44</td>
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<tr>
<td>Cellulose/pectin/xylan</td>
<td>M‡</td>
<td>1·22</td>
</tr>
<tr>
<td></td>
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<td>1·15</td>
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<tr>
<td></td>
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<td>F3</td>
<td>1·35</td>
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<td></td>
<td>F4</td>
<td>1·55</td>
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*Fractons were purified from C. cellulovorans cultivated on media containing 1% cellulose (Avicel), 1% pectin, 1% xylan or 1% cellulose/pectin/xylan mixture (3:1:1, by wt) as the sole carbon source.
†Avicelase activity is based on activity per hour (see Methods).
‡Multicomplex fraction (>400 kDa).
growth medium, the major subunits such as CbpA (189 kDa), EngE (112 kDa) and ExgS (81 kDa) were found consistently in most cellulosome subpopulations under all conditions tested (Fig. 2). However, XynA (57 kDa) was found only in certain fractions (Fig. 2, Anti-XynA).

Identification and enzyme activities of subunits of cellulosomal subpopulations

The 2-D SDS-PAGE profiles indicated that the cellulosomal fractions (F1 to F4) had different components (Fig. 3). Based on protein size and pI determined previously by MS (Han et al., 2004b), 2-D spots were determined to be CbpA, EngE, EngK, ExgS, EngH, EngL and EngB. Fraction 1 contained larger amounts of EngL than the other fractions, while fractions 2 and 3 contained larger amounts of EngE (Fig. 3, F1–F3). The subunits EngK and ExgS were clearly found in fraction 4 (Fig. 3, F4).

We used SDS-PAGE combined with zymography to determine specific subunit activity in cellulosome subpopulations from cells grown on a mixture of cellulose, pectin and xylan. Fig. 4(A–C) shows the subunit composition and zymograms for CMCase and the xylan-specific components of the subpopulations. EngH (79 kDa) and EngL (58 kDa) had strong CMCase activity in cellulosomal fractions 1, 3 and 4 (Fig. 4B). EngE (112 kDa) and EngK (97 kDa) displayed moderate activity against this substrate. The hemicellulase component XynA (57 kDa) showed strong xylanase activity in cellulosomal fractions 1 and 4 (Fig. 4C). The recently sequenced and characterized XynB (66 kDa) (Han et al., 2004a) was also found in cellulosomal fractions 3 and 4 (Fig. 4C). Results obtained with gels containing xylan also showed that smaller minor components (35–45 kDa) had xylanase activity in fractions 2 and 3 (Fig. 4C).

DISCUSSION

The C. cellulovorans cellulosome consists of 7–11 distinct cellulolytic and/or xylanolytic complexes, each subpopulation with a different enzymic and polypeptide composition. These complexes were co-eluted from a gel-filtration column in a high-molecular-mass fraction (>400 kDa),
‘cellulosomal population’, and separated by subjecting the high-molecular-mass fraction to anion-exchange chromatography. The elution profiles (subpopulations) obtained from anion-exchange chromatography showed that the cellulosome population was heterogeneous, in agreement with previous studies (Murashima et al., 2002a; Pohlschroder et al., 1994).

The subunit compositions of cellulosomal subpopulations were also different when cells were grown with different carbon substrates. However, the composition of the major subunits CbpA (Shoseyov et al., 1992), EngE (Tamaru & Doi, 1999) and ExgS (Liu & Doi, 1998) was relatively constant for all the subpopulations. The cellulosomal fractions which showed higher cellulase activity contained CbpA, EngE/EngK, ExgS/EngH and EngL (Liu & Doi, 1998; Shoseyov & Doi, 1990; Shoseyov et al., 1992; Tamaru & Doi, 1999, 2000; Tamaru et al., 2000). All these enzymes are encoded in the large cbpA gene cluster cbpA-exgS-engH-engK-hbpA-engL (Tamaru et al., 2000), except for EngE. cbpA is the first gene in the large gene cluster, encoding several enzymic subunits of the cellulosomes, including the endoglucanases (EngH, EngK and EngL) and celllobiohydrolase (ExgS). These enzymes are known to synergistically degrade crystalline cellulose (Murashima et al., 2002b).

A previous genetic study in Clostridium cellulolyticum found that some of the enzymic subunits encoded by genes located in the large cluster are essential for the degradation of crystalline cellulose (Maamar et al., 2004). Thus, enzymes encoded by the large cbpA gene cluster are expected to play an important role in degrading cellulose in plant cell walls.
In order to determine whether the composition of complex plant cell walls influenced cellululosomal subpopulations, artificially mixed substrates containing cellulose, xylan, and pectin were also investigated in this study. The highest xylanase activity was detected with the specific cellulosomal fractions which had XynB (Han et al., 2004a), XynA (Kosugi et al., 2002) and four unknown xylanase proteins (35–45 kDa) (Fig. 4C, F3 and F4). This subpopulation of cellulosomes may play a major role in degrading the hemicellulose network and allowing access of other subpopulations to cellulose microfibrils in plant cell walls.

These results suggest that a regulatory system in *C. cellulovorans* controls the ratio of cellululosomal subpopulations that make up the cellululosomal population. How are specific subpopulations of cellulosomes assembled? Several factors may play a role. One factor is the induction of certain enzymes by substrates that determine the relative amounts of the various cellulosomal enzymes present for cellulosome assembly (Han et al., 2003b, 2004b; Kosugi et al., 2001; Murashima et al., 2002a). Another factor may involve the specificity of interaction between dockerins and their cognate cohesins in CbpA (Doi et al., 2003; Park et al., 2001). Some cohesins may contain a specific region that is bound by the dockerins of certain enzymes which is absent in other cohesins (Bayer et al., 2004; Doi et al., 2003; Park et al., 2001). Thus the composition of subpopulations may depend on the amount of various enzymes present and the interaction of different categories of cohesins and dockerins. Preliminary data show that different cellululosomal enzymes do interact differently with various cellulosomal cohesins (H. Y. Cho & R. H. Doi, unpublished data). The results presented indicate that cellulosome assembly occurs in a non-random fashion. The characterization of the cellulosomal subpopulations provides new insights into the construction of complex cellulase-xylanase cellulosomes that are capable of degrading plant cell walls more effectively.

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