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Essential 170-kDa subunit for degradation of crystalline cellulose by *Clostridium cellulovorans* cellulase

(carboxymethylcellulase/endo-1,4-β-glucanase/cellobiohydrolase/exo-1,4-β-glucanase)

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ABSTRACT **The cellulase complex from *Clostridium cellulovorans* has been purified and its subunit composition determined. The complex exhibits cellulase activity against crystalline cellulose as well as carboxymethylcellulase (CMCase) and cellobiohydrolase activities. Three major subunits are present with molecular masses of 170, 100, and 70 kDa. The 100-kDa subunit is the major CMCase, although at least four other, minor subunits show CMCase activity. The 170-kDa subunit has the highest affinity for cellulose, does not have detectable enzymatic activity, but is necessary for cellulase activity. Immunological studies indicate that the 170-kDa subunit is not required for binding of the catalytic subunits to cellulose and therefore does not function solely as an anchor protein. Thus this core subunit must have multiple functions. We propose a working hypothesis that the binding of the 170-kDa subunit converts the crystalline cellulose to a form that is capable of being hydrolyzed in a cooperative fashion by the associated catalytic subunits.**

Cellulases produced by microorganisms play a major role in the degradation of cellulosic materials and the conversion of this major organic carbon compound to utilizable forms of energy. It is important to understand the mechanism whereby cellulases can degrade crystalline cellulose, particularly since the reaction consists of an interaction between a complex soluble enzyme and a solid particulate substrate. The cellulase from *Clostridium thermocellum*, a thermophilic anaerobe, has been studied extensively (1–3) as well as that from the fungus *Trichoderma reesei* and other microorganisms (see ref. 4 for reviews).

We have approached this problem by analyzing the cellulase produced in large quantities by a mesophilic, spore-forming, anaerobic microorganism, *Clostridium cellulovorans* (5). The advantages of using this particular organism are the presence of a cellulase complex of a size and property that simplified its purification and that of its subunits, the absence of any affinity factors (3) for its activity, the production of large quantities of extracellular cellulase, and the absence of contaminating extracellular proteases.

Our studies have revealed the presence of a large protein with a molecular mass of 170 kDa (P170) that has no apparent enzymatic activity but that is essential for coordinating the activities of a number of cellulose-degrading enzymes associated with it. The role for P170 appears to be different from that reported for the anchor protein by Demain and his colleagues (6) for the *C. thermocellum* cellulase, since the *C. cellulovorans* carboxymethylcellulases (CMCases; EC 3.2.1.4) and cellobiohydrolase (CBHase; EC 3.2.1.91) can bind to crystalline cellulose even in the absence of P170. However, P170 is absolutely required as part of the cellulase enzyme complex for the degradation of crystalline cellulose and thus acts as a "coordinator" for the cellulase complex.

MATERIALS AND METHODS

Cell Culture and Medium. *C. cellulovorans* (ATCC 35296) was grown anaerobically as described (5) except for the carbon source, which was 0.1% Avicel (PH-105, FMC).

Cellulase Isolation and Purification. A 500-ml culture was grown to late stationary phase at 37°C. The culture was centrifuged for 10 min at 8000 × *g* and the supernatant was precipitated with $(NH_4)_2SO_4$ at 80% saturation. The pellet was dissolved in 4 ml of PC buffer (50 mM phosphate/12 mM citrate, pH 7.0/1 mM sodium azide), dialyzed against the same buffer, and mixed with 4 ml of Avicel suspension (1 g/ml), which resulted in binding of the enzyme to cellulose. The cellulose/enzyme slurry was incubated for 10 min at 37°C and centrifuged for 10 min at 10,000 × *g*. The pellet was suspended, incubated, and centrifuged sequentially as above with 4-ml portions of 1 M NaCl/PC buffer (twice); 4 ml of PC buffer (once); 4 ml of deionized H_2O (twice); and finally 400 ml of deionized H_2O. The protein in the last wash was precipitated with $(NH_4)_2SO_4$ at 80% saturation; the pellet was resuspended in 8 ml of PC buffer and dialyzed against PC buffer. Ten microliters of each fraction was used for SDS/PAGE and enzyme assays.

PAGE and Zymogram. SDS/PAGE was performed in 7.5% polyacrylamide gels (7). Native PAGE was performed in 4% polyacrylamide gels (8). CMCase zymograms (9) were prepared by using 0.1% CM-cellulose that was copolymerized with 7.5% polyacrylamide. CMCase activities were visualized with Congo red after a 2-hr incubation at 37°C.

IgG Production and Purification. One milligram of isolated cellulase was resolved by preparative SDS/PAGE. The gel was then soaked in ice-cold 0.25 M KCl and the 170-kDa (P170) band was cut out. The protein was electroeluted overnight at 4°C in 25 mM Tris/192 mM glycine (pH 8.3), at 100 V (constant voltage), using the Elutrap system (Schleicher & Schuell). The P170 (100 μg) was emulsified with an equal volume of complete Freund adjuvant (Difco) and used to inject subcutaneously two white New Zealand female rabbits at multiple injection sites. After 1 week, the rabbits were given a booster injection with 100 μg of protein emulsified with incomplete adjuvant. Two weeks later 50 ml of blood was taken and allowed to clot for 1 hr at 37°C. Serum was recovered after overnight incubation at 4°C by centrifugation at 10,000 × *g*, and IgG was purified by using DEAE Affi-Gel Blue (Bio-Rad) according to the manufacturer's instructions. Western blot analysis was performed to confirm specific interaction with P170 at 1:1000 dilution. Anti-rabbit IgG alkaline phosphatase-conjugated antibodies (Bio-Rad) were used as secondary antibodies according to the manufacturer's protocol.

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Abbreviations: CMCase, carboxymethylcellulase; CBHase, cellobiohydrolase.

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FPLC Analysis. A 100-μl (1-mg) aliquot of cellulase was injected into an FPLC system (Pharmacia) equipped with a Superose 12 sizing column. Proteins were eluted with 0.15 M NaCl/PC buffer at 0.5 ml/min and monitored at 280 nm. Fractions containing 250 μl were collected.

Enzyme Assays. Cellulase activity was measured by spotting 10 μl of enzyme solution on an agar plate containing 0.1%, 24-hr pebbled–milled cellulose (Whatman no. 1 filter paper) in PC buffer. Plates were incubated for 16 hr at 37°C and cellulase activity was estimated by the ability of the enzyme to form a clear halo on an opaque background. CMCase activity was tested on PC/agar plates containing 0.1% CM-cellulose. Ten microliters of enzyme solution was spotted and incubated for 16 hr at 37°C; then the plates were stained with 1% Congo red solution for 5 min and destained several times by washing with 1 M NaCl. Orange halos on a red background indicated CMCase activity (10). CBHase activity was measured on PC/agar plates containing 0.5 mM 4-methylumbeliferyl β-cellobioside. Ten microliters of enzyme solution was spotted and incubated for 2 hr at 37°C. CBHase activity was estimated by the fluorescence intensity resulting from UV illumination (11).

RESULTS

Purification of the Cellulase Complex. The extracellular cellulase complex was isolated from the crude protein mixture by taking advantage of the extremely high affinity of the complex for cellulose. The cellulase could not be washed off the cellulose either by 1 M NaCl or by PC buffer. Repeated washing of the cellulose with a large volume of deionized H_2O eluted the cellulase complex, which was resolved by SDS/PAGE to reveal three major protein bands having molecular masses of 170 kDa (P170), 100 kDa (P100), and 70 kDa (P70) and at least six minor proteins (Fig. 1, lane 7; Fig. 2, lane 1). The proteins in the last elution fraction (i.e., the last deionized H_2O wash; Fig. 1, lane 7) from cellulose migrated as a single band in native PAGE (Fig. 1*B*), which indicated that the *C. cellulovorans* cellulase was a multiprotein complex composed of different subunits having a heterogeneous stoichiometry. Among the three major proteins, P170 was eluted only at the last deionized H_2O wash, whereas P100 and P70 were continuously "leaked" during all the washings (Fig. 1*A*).

CMCase Activity. A test for CMCase activity was performed on the proteins separated by SDS/PAGE (Fig. 2, lane 2). The major CMCase activity was associated with P100. Among the three major proteins, P70 was less active based on the intensity of the Coomassie blue band compared to its activity band. CMCase activity was also found with P73, P72, P65, and P58, but not with P170, P43, and P40. The significance of these results with respect to P170 is that this major protein, although it had no detectable hydrolytic activity, possessed the highest affinity for cellulose (see Fig. 1*A*). A similar protein was found recently in *C. thermocellum* (6) and is believed to play a role as an "anchor" protein, an essential component that is required for the degradation of crystalline cellulose fibers.

Molecular Mass of the Native Cellulase Complex. To determine the molecular mass of the native cellulase complex, the enzyme was injected into an FPLC system equipped with a molecular sizing column. Total protein, CMCase, and cellulase activities were monitored (Fig. 3). Two major CMCase activities were detected and coincided with two protein peaks at 900 and 100 kDa (Fig. 3 *A* and *B*). The major cellulase activity appeared at the 900-kDa peak, but there was considerable cellulase activity even in the fractions with molecular mass lower than that of the 12-kDa protein standard, cytochrome *c* (Fig. 3 *A* and *C*). SDS/PAGE followed by silver staining (Fig. 4) of the 900- and 100-kDa peaks and fractions 58 and 66 (arrows at bottom of Fig. 3*A*) revealed that all fractions containing cellulase activity contained P170, P100, and P70. The most surprising observation was the detection of relatively high amounts of P170 in fractions 58 and 66, indicating a strong affinity of the lower molecular

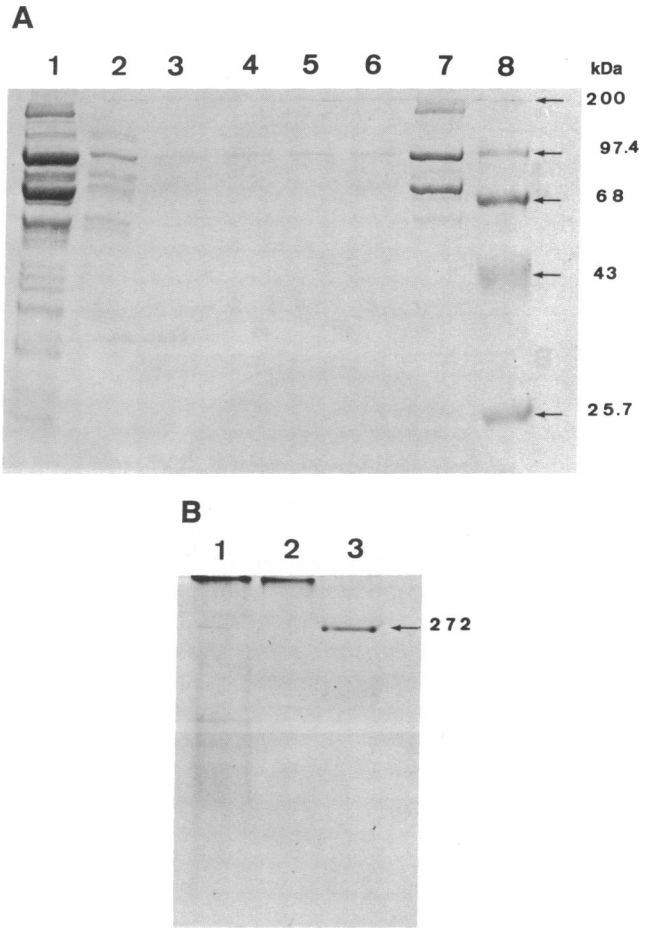

FIG. 1. PAGE analysis of the affinity-purified cellulase complex. (*A*) SDS/PAGE. Lane 1, 0–80% $(NH_4)_2SO_4$ fraction (10 μl); lane 2, proteins that did not bind to the cellulose (10 μl); lane 3, 4-ml 1 M NaCl wash (10 μl); lane 4, 4-ml PC buffer wash (10 μl); lanes 5 and 6, 4-ml deionized-H_2O washes (10 μl); lane 7, 400-ml deionized-H_2O wash concentrated to 8 ml (10 μl); lane 8, molecular mass markers. (*B*) Native PAGE. Lane 1, 0–80% $(NH_4)_2SO_4$ fraction (10 μl); lane 2, the purified cellulase (see *A*, lane 7); lane 3, 272-kDa urease marker.

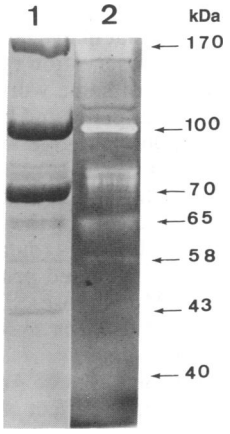

FIG. 2. CM-cellulose/SDS/PAGE analysis of the cellulase (Fig. 1, lane 7). Lane 1, Coomassie blue staining; lane 2, CMCase activity staining.

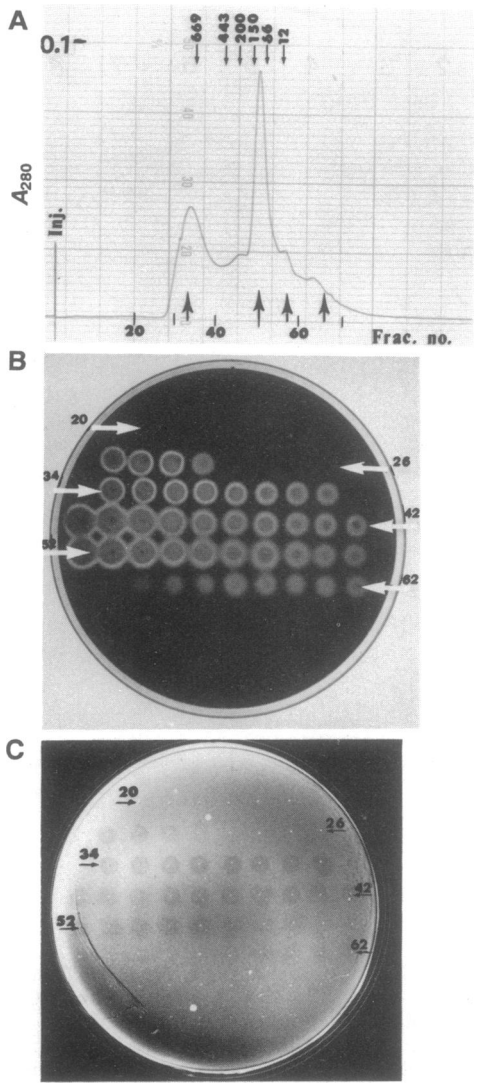

FIG. 3. FPLC analysis of the cellulase complex. (*A*) Absorbance at 280 nm of the fractions eluted from the FPLC Superose 12 column. Inj., sample injection. (*B* and *C*) CMCase and cellulase activities of fractions 20–70. Vertical arrows (*A*) at fractions 33, 51, 58, and 66 indicate sampling points for SDS/PAGE analysis (Fig. 4). The system was calibrated using molecular size markers (Sigma): bovine thyroglobulin (669 kDa), horse spleen apoferritin (443 kDa), sweet potato β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and horse heart cytochrome *c* (12 kDa).

mass components for P170 that managed to retard its passage through the column. This observation raises the question as to the validity of the claim of some workers (12, 13) for low molecular mass cellulases as estimated by gel filtration chromatography. This technique can give artifactual results, as illustrated in Figs. 3 and 4.

The nine proteins of the cellulase complex have been purified to homogeneity except for the P72 and P73, which could not be resolved satisfactorily (data not shown). CMCase activity assay of all the isolated proteins confirmed our previous findings by CM-cellulose/SDS/PAGE (Fig. 2), from which we concluded that the major CMCase activity could be related to P100.

Effect of Anti-P170 IgG on Cellulase Activity. With the anti-P170 IgG on hand, we tested the effect of this antibody on the cellulase complex to determine whether it could inhibit specific activities of the complex. The P170-specific polyclonal antibody was mixed with the isolated cellulase complex and these mixtures were tested for cellulase, CMCase, and CBHase activities (Fig. 5). Anti-P170 IgG inhibited cellulase activity but not CMCase and CBHase activities of the complex.

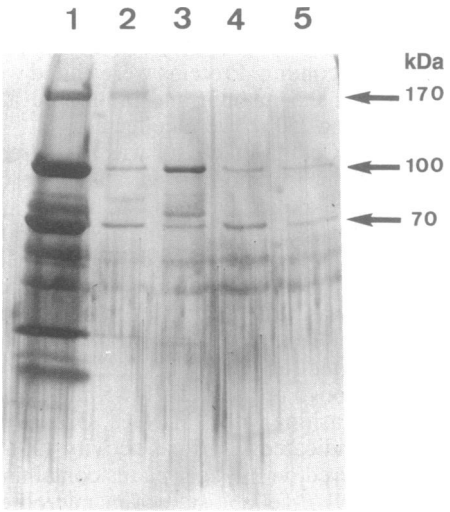

FIG. 4. SDS/PAGE analysis of the FPLC fractions. Lane 1, the cellulase complex (see Fig. 1, lane 7); lanes 2–5, fractions 33, 51, 58, and 66, respectively, from FPLC (see Fig. 3*A*).

Having the anchor protein model (6) in mind, we mixed native cellulase with cellulose and incubated the mixture for 10 min at room temperature with or without anti-P170 IgG (Fig. 6). In the absence of anti-P170 IgG the complex bound to cellulose. In the presence of anti-170 IgG the results indicated clearly that the IgG prevented P170 binding to cellulose (Fig. 6, lane 4) and no cellulase activity could be observed (Fig. 5*A*, sample 2); however, the other subunits that were released from P170 by IgG still showed CMCase and CBHase activities, and still retained the capacity to bind to the cellulose so that they could not be washed off by 1 ml of 1 M NaCl (Fig. 6, lanes 3 and 4). These results rule out the possibility that P170 is acting solely as an anchor protein for the binding of the other subunits to cellulose and indicate that it has other functions.

DISCUSSION

The *C. cellulovorans* cellulase complex shows some similarities to the *C. thermocellum* cellulase (1–3): the enzyme complex contains true cellulase activity and is composed of multiple subunits. However, its molecular mass of about 900 kDa makes it one of the smaller cellulase complexes. This smaller size, the lack of a requirement for a yellow affinity factor (3), the relative ease of purification, the stability at room temperature, the recovery of CMCase activities after SDS exposure, and the absence of contaminating proteases make the *C. cellulovorans* cellulase a favorable system for study.

These studies have revealed an explanation for the presence of "low molecular weight" cellulases as defined by molecular sizing columns, since an anomalous pattern of elution from the gel filtration column was observed. It is apparent that P170 can be restrained during its passage through a sizing column, which makes the apparent size much smaller than it actually is; all the cellulase-containing fractions contained the high molecular weight subunits necessary for cellulase activity.

The presence of multiple CMCase activities in a SDS/PAGE system can be interpreted as the result of partial degradation of P100 into smaller but still active fragments. However, this could not be the total explanation, since we have already cloned three CMCase gene-containing fragments that differ in size and restriction endonuclease cleavage patterns (unpublished results). Thus it is clear that more

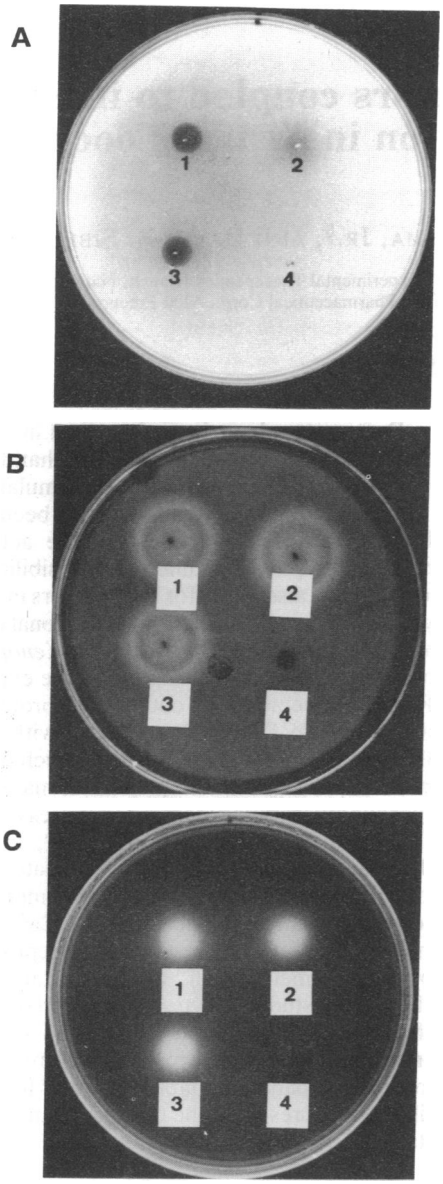

FIG. 5. Effect of anti-P170 IgG on cellulase (*A*), CMCase (*B*), and CBHase (*C*) activities. Sample 1, 10 μl of cellulase; sample 2, 10 μl of cellulase plus 1 μl of anti-P170 IgG preincubated for 10 min at room temperature, then spotted on the plate; sample 3, 10 μl of cellulase plus 1 μl of preimmune serum; sample 4, 10 μl of boiled cellulase.

than one type of CMCase is associated with the cellulase complex and in different stoichiometric relationships. However, there appear to be many more subunits and CMCases associated with the *C. thermocellum* cellulase than with the *C. cellulovorans* cellulase (1, 3).

The CBHase activity is readily detected in the native cellulase complex. However, we have not been able to detect CBHase activity in any of the protein bands after SDS/PAGE. This suggests that the CBHase activity either is sensitive to the presence of SDS and cannot be reactivated, is composed of two subunits that are separated during SDS/PAGE, or requires an association of the CBHase subunit with P170 for activity. The detection of CBHase activity and the reconstitution of cellulase activity from its subunits may require the cloning and isolation of native subunits, since all efforts at reconstitution of an active cellulase complex from subunits obtained by use of denaturing agents have been unsuccessful to date. This is in contrast to the reconstitution

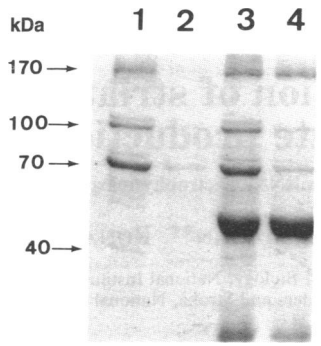

FIG. 6. Effect of anti-P170 IgG on the binding of cellulase to cellulose. Ten microliters of cellulase was incubated at room temperature for 10 min with (or without) 1 μl of anti-P170 IgG, then mixed (or not) with 10 μl of 2% cellulose (Avicel) in PC buffer at 37°C for 10 min. The cellulose was removed by centrifugation and the supernatant was analyzed by SDS/7% PAGE. Lane 1, cellulase only; lane 2, cellulase plus cellulose; lane 3, cellulase plus IgG; lane 4, cellulase plus IgG plus cellulose.

of an active cellulase from subunits of *C. thermocellum* observed by Wu *et al.* (6).

The role of P170 may be crucial to the cellulase activity. Our data indicate that its presence is necessary for cellulase activity but not for the binding of the other subunits to cellulose. We propose that P170 as a core subunit of the cellulase complex has several functions: (*i*) it serves as a core protein to which the other catalytic subunits bind, and this complex facilitates the cooperative action of the nicking enzyme (CMCase) and the subsequent attack by CBHase at the nicked sites; (*ii*) the association of the catalytic subunits to P170 is also essential and necessary for their ability to hydrolyze bonds in crystalline cellulose; and (*iii*) P170 converts the bound cellulose by an amorphogenetic action to a structure that is amenable to attack by its associated hydrolytic subunits. Thus P170 should have a number of activity domains, including binding sites for cellulose and for the hydrolytic enzymes. The size of P170 is such that it should be able to bind simultaneously to a number of hydrolytic enzymes as well as to cellulose.

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