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Characterization of the Extracellular Cellulase from a Mesophilic Clostridium (Strain C7)

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An extracellular, 700,000-M_r multiprotein complex that catalyzed the hydrolysis of crystalline cellulose (Avicel) was isolated from cultures of *Clostridium* sp. strain C7, a mesophile from freshwater sediment. In addition to cellulose (Avicel, ball-milled filter paper), the multiprotein complex hydrolyzed carboxymethylcellulose, cellodextrins, xylan, and xylooligosaccharides. Hydrolysis of cellulose or cellotetraose by the complex yielded cellobiose as the main product. Cellopentaose or cellohexaose was hydrolyzed by the complex to cellotriose or cellotetraose, respectively, in addition to cellobiose. Xylobiose was the main product of xylan hydrolysis, and xylobiose and xylotriose were the major products of xylooligosaccharide hydrolysis. Activity (Avicelase) resulting in hydrolysis of crystalline cellulose required Ca^{2+} and a reducing agent. The multiprotein complex had temperature optima for Avicelase, carboxymethylcellulase, and xylanase activities at 45, 55, and 55°C, respectively, and pH optima at 5.6 to 5.8, 5.5, and 6.55, respectively. Electron microscopy of the 700,000-M_r enzyme complex revealed particles relatively uniform in size (12 to 15 nm wide) and apparently composed of subunit structures. Elution of strain C7 concentrated culture fluid from Sephacryl S-300 columns yielded an A_{280} peak in the 130,000-M_r region. Pooled fractions from the 130,000-M_r peak had carboxymethylcellulase activity but lacked Avicelase activity. Except for the inability to hydrolyze cellulose, the 130,000-M_r preparation had a substrate specificity identical to that of the 700,000-M_r protein complex. A comparison by immunoblotting techniques of proteins in the 130,000- and 700,000-M_r preparations, indicated that the two enzyme preparations had cross-reacting antigenic determinants.

Research carried out by various investigators has clearly established that the cellulase systems of anaerobic bacteria are different from those of white- and soft-rot fungi (7). Although much progress has been made in understanding the mode of action of fungal cellulases, very little is known about the mechanism of cellulose hydrolysis by clostridia and other anaerobic bacteria.

One of the most extensively studied cellulase systems of anaerobic bacteria is the multicomponent cellulolytic complex (cellulosome) of *Clostridium thermocellum*, a thermophile originally isolated from fermenting manure (22). Clusters of cellulosomes (polycellulosomes) present on the surface of *C. thermocellum* cells are responsible for the attachment of the cells to cellulose (7).

Recent work in our laboratory has determined that *Clostridium* sp. strain C7, a mesophile isolated from mud of a freshwater swamp, produces a multiprotein complex capable of degrading crystalline cellulose (5). The cellulase complex of strain C7 differs from that of *C. thermocellum* because it is not associated with the cells to a significant extent, it has a considerably smaller molecular weight, and it does not play a role in cell attachment to the insoluble substrate, inasmuch as the complex is extracellular and the cells do not attach themselves to cellulose (5).

In this report we describe biochemical and ultrastructural characteristics of the extracellular multienzyme complex utilized by strain C7 for the hydrolysis of crystalline cellulose.

(A preliminary report of part of this work was presented previously [Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, K40, p. 251].)

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MATERIALS AND METHODS

Bacterial strain and culture conditions. *Clostridium* sp. strain C7 (15), was cultivated in prereduced chemically defined medium MJ-CB (5). Cultures were incubated in an atmosphere of N_2 at 30°C unless specified otherwise.

Enzyme preparations. The Avicelase-active, 700,000-M_r protein complex was isolated from the supernatant fluid of strain C7 cultures grown in medium MJ-CB (5). Fractions corresponding to an A_{280} peak that eluted in the 130,000-M_r region from a Sephacryl S-300 column were obtained as described elsewhere (5). The 130,000-M_r fractions lacked Avicelase activity (i.e., ability to hydrolyze crystalline cellulose) but possessed carboxymethylcellulase (CMCase) activity. Enzyme preparations were stored in an N_2 atmosphere at 4°C.

Enzyme assays. Avicelase activity was determined by measuring the decrease in turbidity of a suspension of Avicel (type PH 105, 20-μm particles; FMC Corp., Marcus Hook, Pa.), and CMCase activity was determined by measuring the production of reducing sugars from carboxymethylcellulose (CMC; sodium salt, medium viscosity; Sigma Chemical Co., St. Louis, Mo.) (5), except where indicated otherwise. A unit of Avicelase activity was defined as the amount of enzyme resulting in the hydrolysis of 0.5 μg of Avicel per h. A unit of CMCase activity was defined as the amount of enzyme that released 1.0 μmol of reducing sugar (expressed as glucose) per min under the assay conditions. Ball-milled filter paper-hydrolyzing activity was determined by the same method used for Avicelase activity, except that ball-milled filter paper (15) was used as the substrate instead of Avicel. The procedure and reaction mixture used to determine xylanase activity were the same as those used to determine CMCase activity, except that 7 to 28 μg of protein was used and CMC was replaced by xylan (from larchwood; Sigma)

washed three times in 70% ethanol. CMCase and xylanase reaction mixtures were incubated for 10 min at 42°C.

Cellobiosidase (EC 3.2.1.91) activity was determined by measuring colorimetrically the amount of *p*-nitrophenol released from *p*-nitrophenol-β-D-cellobioside (Sigma). Reaction mixtures contained 8 μmol of *p*-nitrophenol-β-D-cellobioside, 0.2 ml of enzyme preparation (approximately 0.02 mg of protein, dialyzed against distilled water for 4 h at 4°C), and distilled water, in a total volume of 1 ml and were incubated at 42°C. Reactions were terminated by the addition of 0.4 ml of 1 M Na_2CO_3, and the A_{410} was measured. β-Glucosidase (EC 3.2.1.21) and β-xylosidase (EC 3.2.1.37) activities were determined in the same manner, except that *p*-nitrophenol-β-D-cellobioside was replaced with 10 μmol of *p*-nitrophenol-β-D-glucopyranoside (Sigma) and 10 μmol of *p*-nitrophenyl-β-D-xylopyranoside (Sigma), respectively.

Hydrolysis of cellobiose or cellodextrin was determined by detecting hydrolysis products as follows. Each reaction mixture contained the following, in a total volume of 1.0 ml: cellobiose (Sigma) or a cellodextrin (cellotriose, cellotetraose, cellopentaose, or cellohexaose; V-Labs, Inc., Covington, La.), 0.3 mg; enzyme preparation (dialyzed against distilled water for 4 h at 4°C), approximately 0.02 mg of protein; and distilled water. Reaction mixtures were incubated at 42°C for 20 h. Products were separated by thin-layer chromatography (TLC) as described below. Xylobiose or xylooligosaccharide hydrolysis was determined by the same method used for cellodextrin hydrolysis, except that xylobiose (V-Labs) or a xylooligosaccharide preparation (0.48 mg, prepared by the method of Lee et al. [14] and donated by K. E. Chambers) was used as the substrate instead of cellodextrins, and the reaction mixtures were incubated for 17 h.

The pH optima for Avicelase, CMCase, and xylanase activities were determined by substituting the buffer in standard reaction mixtures (5) (see above) with 0.1 M acetate buffer (pH 5.1 to 6.0), 0.1 M succinate-NaOH buffer (pH 5.1 to 6.0), or citrate-phosphate buffer (pH 6.1 to 7.0). To determine the stability of the cellulase complex at different temperatures, enzyme preparations (19.2 μg of protein) were preincubated at 40 to 60°C for 20 min in the absence of substrate and with or without $CaCl_2$ (5 mM) before activities were determined. Stability in the presence of O_2 was determined by incubating enzyme samples (3 ml, 174 μg of protein) in air at 4°C with occasional shaking for aeration before Avicelase activity was determined in 0.3-ml samples (17.4 μg of protein).

Protein was measured by the method of Bradford (4) with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the protein standard, unless specified otherwise.

Products of enzymatic action. Reaction mixtures used to determine hydrolysis products were the same as those described above except that (i) buffer was omitted, (ii) the enzyme preparations were dialyzed against distilled water for 4 h at 4°C, and (iii) the substrates listed below were used. Reactions were stopped by heating at 60°C for 5 to 10 min. Hydrolysis products formed from Avicel, ball-milled filter paper, CMC, cellobiose, and cellodextrins (cellotriose, cellotetraose, cellopentaose, cellohexaose; V-Labs) were separated by TLC by using Baker flex silica gel plates (Fisher Scientific Co., Pittsburgh, Pa.). Samples (80 to 120 μl of reaction mixture) and sugar standards (50 to 100 μg in 5 to 10 μl) were applied to plates by spotting 5-μl samples. Plates were developed with butanol-ethanol-water (7:4:3, vol/vol/vol). Plates to which cellopentaose and cellohexaose reaction mixtures had been applied were developed twice in the same solvent. To detect carbohydrates, the plates were sprayed with a 5% (vol/vol) solution of concentrated sulfuric acid in absolute ethanol and then air dried and heated at 110°C for 15 min. Hydrolysis products formed from xylobiose, xylan (washed as described above), and a xylooligosaccharide preparation (described above) were separated by TLC with Whatman LK5 silica gel plates (Baxter Healthcare Corp., McGaw Park, Ill.). After the samples (10 μl of reaction mixture) and sugar standards (xylobiose [V-Labs], xylotriose, xylotetraose, and xylopentaose [a gift from R. B. Hespell]; 15 to 80 μg in 2 to 7 μl) were applied, the plates were developed with nitroethane-ethanol-water (1:3:1, vol/vol/vol). To detect carbohydrates, the plates were sprayed with an *N*-(1-naphthyl)ethylenediamine dihydrochloride solution (3) and then air dried and heated at 110°C for 15 min.

Affinity-purified antibodies to the 700,000-M_r protein complex. One ml of the dialyzed 700,000-M_r enzyme preparation (140 μg) was mixed with 1 ml of Maalox (William H. Rorer, Inc., Fort Washington, Pa.) as an adjuvant, and the mixture was injected intramuscularly into a rabbit. The 700,000-M_r enzyme preparation did not contain contaminating proteins that eluted at the 130,000-M_r position, as determined by reapplication of the 700,000-M_r preparation to a Sephacryl S-300 gel filtration column. A booster injection (1 ml, 100 μg of protein) was administered after 21 days. Bleeding was performed on day 33. Ouchterlony (19) immunodiffusion analysis verified that antibodies to the 700,000-M_r protein complex were present in the antiserum.

Immunoglobulin G (IgG) was isolated from the rabbit antiserum by affinity chromatography by applying the antiserum to columns of protein A (Genzyme Corp., Boston, Mass.) coupled to Affi-Gel 10 (Bio-Rad), prepared according to the instructions of the manufacturer. In this procedure, the buffer system of Goding (8), which included binding buffer (0.02 M potassium phosphate [pH 7.4], 0.13 M NaCl) and eluant (0.58% [vol/vol] glacial acetic acid in 0.15 M NaCl), was used. Specific antibodies to the 700,000-M_r protein complex were affinity purified from the isolated IgG preparation by applying the latter to columns of 700,000-M_r protein complex coupled to Affi-Gel 15 (Bio-Rad), prepared according to the instructions of the manufacturer, with phosphate-buffered saline (PBS; 0.2 M potassium phosphate [pH 7.6], 0.25 M NaCl) as the binding buffer and 5 M potassium thiocyanate as the eluant.

Enzyme-linked immunosorbent assay. Wells of microdilution plates (Immulon II; Dynatech Laboratories, Inc., Chantilly, Va.) were coated overnight at 4°C with fractions (50 μl each, 0.5 to 5 μg of protein) from gel filtration chromatography of concentrated strain C7 culture supernatant fluid (5). The wells were washed three times with PBS containing 5% (vol/vol) Tween 80, blocked with PBS containing 1% (wt/vol) bovine serum albumin (200 μl/well) for 1 h at room temperature, and then washed three times as described above. Affinity-purified antibodies to the 700,000-M_r protein complex were diluted in PBS to 0.047 μg/ml. Diluted antibodies (100 μl) were added to each well, incubated for 1 h at room temperature, and then washed three times as described above. Goat anti-rabbit IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) was added (1:5,000 dilution in PBS, 100 μl) to each well. After incubation for 1 h at room temperature, the wells were washed three times, and finally 100 μl of a mixture (1:1 by volume) of 3,3′,5,5′-tetramethylbenzidine and H_2O_2 (commercially prepared solutions; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was added to

each well. The optical density at 650 nm of the wells was determined by means of a Vmax Kinetic Microplate Reader (Molecular Devices Corp., Palo Alto, Calif.).

Antibody inhibition of Avicelase activity. Avicelase activity was inhibited by using affinity-purified antibodies to the 700,000-M_r protein complex. Various volumes of an antibody solution containing 60 μg of protein per ml were incubated with 100-μl portions of an enzyme preparation containing 20 U of Avicelase activity (6 μg of protein). Preimmune IgG (control IgG; 60 μg of protein per ml) was used instead of affinity-purified antibodies in control mixtures. Incubation was in an N_2 atmosphere at room temperature for 1 h and then at 4°C for 5 h before Avicelase activity was determined.

Immunoblotting. Proteins present in enzyme preparations were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (5). Polyacrylamide gels were washed two times for 30 min each by immersing them in transfer buffer (a mixture of 1,200 ml of methanol and 4,800 ml of an aqueous solution containing 18.7 g of Tris hydrochloride and 86.5 g of glycine). The electrophoretic transfer of proteins from polyacrylamide gels to 0.45-μm-pore-size nitrocellulose membranes (Schleicher & Schuell Co., Keene, N.H.) was accomplished with a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) operated at 55 V (constant voltage) for 2.5 h, by the procedure of Towbin et al. (21). Upon completion of the transfer, the membranes were immersed in a blocking solution consisting of 5% nonfat dry milk (Carnation Co., Los Angeles, Calif.) in Tris-buffered saline (TBS; 20 mM Tris hydrochloride [pH 7.5], 0.85% NaCl) for 1 h at room temperature on a rocking platform shaker. Then the membranes were washed two times, 5 min each, in TBS containing 1% (wt/vol) bovine serum albumin. The control serum (preimmune), affinity-purified antibodies, or cellulosome-specific (*C. thermocellum* mutant AD2-adsorbed [2]) antibodies (a gift from R. Lamed and E. Morgenstern), all diluted with TBS to 0.3 μg/ml, were incubated individually with each membrane overnight at room temperature. The membranes were washed two times in TBS and incubated for 2 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Finally, the membranes were washed three times in TBS and developed for 5 to 15 min in a mixture (1:1 by volume) of H_2O_2 and 4-chloro-naphthol (commercially prepared solutions; Kirkegaard & Perry Laboratories). Color development was stopped by rinsing membranes in distilled water.

Electron microscopy. Preparations of the 700,000-M_r protein complex (45 μl, 6.5 μg) were mixed with 1 μl of a suspension of bacteriophage T4 (5×10^8 PFU per ml; a gift from C. B. Thorne), which was included as an internal standard for calibrating measurements of protein dimensions (1). One drop of the mixture was placed onto a glow-discharged, carbon-coated 400-mesh copper grid. After 30 s, excess sample was removed by touching to filter paper, and then samples on the grids were washed with droplets of water and stained with 2% uranyl acetate (pH 7.0) for approximately 30 s. Samples were examined with a Siemens 102 transmission electron microscope operating at an accelerating potential of 80 kV.

RESULTS

Effect of DTT and Ca^{2+} on enzyme activity. Avicelase activity of the 700,000-M_r protein complex required a reducing agent, dithiothreitol (DTT), for the hydrolysis of crystalline cellulose (Fig. 1). In the absence of DTT, the rate of solubilization of Avicel was greatly reduced and activity ceased after approximately 48 h (Fig. 1). When 10 mM (final concentration) DTT was present in the reaction mixture, Avicel was completely solubilized after approximately 168 h.

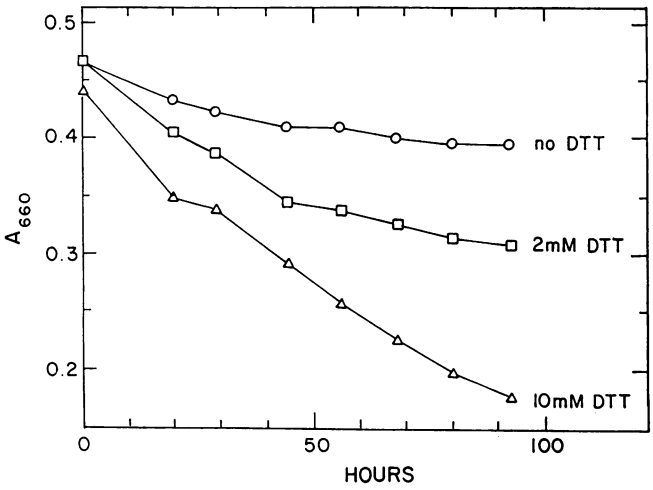

FIG. 1. Influence of DTT concentration on Avicel hydrolysis by the 700,000-M_r protein complex. The protein complex (0.01 μg) was incubated with Avicel, and activity was determined as described in Materials and Methods.

Ca^{2+} appeared to be specifically required for Avicelase activity, and the addition of EDTA to reaction mixtures resulted in complete loss of activity (Fig. 2). Activity was not stimulated by the addition of 5 mM (final concentration) $MgCl_2$ (Fig. 2).

Enzyme substrates and products. The 700,000-M_r protein complex hydrolyzed Avicel and CMC (5). In addition, the protein complex hydrolyzed xylan and a mixture of xylooligosaccharides but not xylobiose (Table 1), indicating that the cellulase-active complex had xylanase as well as Avicelase activity but lacked β-xylosidase activity. The latter activity is more than 80% cell associated in log-phase cultures of strain C7 grown with cellobiose or xylan as the growth

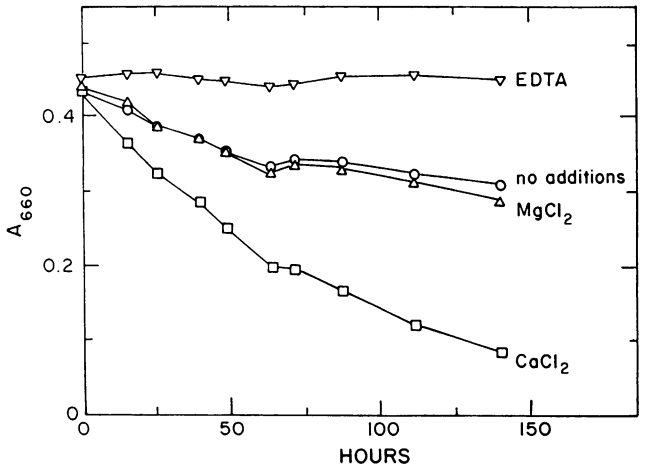

FIG. 2. Influence of Ca^{2+} on Avicel hydrolysis by the 700,000-M_r protein complex. Reaction conditions were the same as in Fig. 1, except in some reaction mixtures $CaCl_2$ was replaced with 5 mM EDTA or 5 mM $MgCl_2$ (final concentrations) or omitted (no additions).

TABLE 1. Substrate specificity[a] of the 700,000- and 130,000-M_r enzyme preparations

Substrate	Substrate specificity of the following prepns:	
	700,000 M_r	130,000 M_r
Cellobiose	−	−
Cellotriose	−	−
Cellotetraose	+ (G_2)	+
Cellopentaose	+ (G_2, G_3, trace G_1)	+
Cellohexaose	+ (G_2, G_4, trace G_1 and G_3)	+
CMC[b]	+	+
Ball-milled filter paper	+ (G_2, trace G_1 and G_3)	−
Avicel	+ (G_2, trace G_1 and G_3)	−
Xylobiose	−	−
Xylan	+ (X_2, trace X_1 and X_3)	+
Xylooligosaccharides	+ (X_2, X_3)	+
p-Nitrophenyl-β-D-glucopyranoside	−	−
p-Nitrophenyl-β-D-cellobioside	+	+
p-Nitrophenyl-β-D-xylopyranoside	+	+

[a] Substrates hydrolyzed (+) or not hydrolyzed (−) by the enzyme preparations were determined as described in the Materials and Methods. Products of the 700,000-M_r complex are indicated within parentheses as follows: G_1, glucose; G_2, cellobiose; G_3, cellotriose; G_4, cellotetraose; X_1, xylose; X_2, xylobiose; X_3, xylotriose.

[b] The reaction yielded sugars that were probably methylated and migrated at rates comparable to those of G_1 to G_4 on TLC plates.

substrate (K. Chambers, personal communication). Cellodextrins larger than cellotriose were hydrolyzed by the complex (Table 1). The 700,000-M_r complex did not have β-glucosidase activity, inasmuch as cellobiose and the cellobiose analog *p*-nitrophenol-β-D-glucopyranoside were not substrates. Although the natural sugars cellotriose and xylobiose were not hydrolyzed, the analogs of these sugars, *p*-nitrophenol-β-D-cellobioside and *p*-nitrophenol-β-D-xylopyranoside, respectively, were hydrolyzed (Table 1). These results suggest that hydrolysis of sugar analogs does not necessarily imply that the sugars corresponding to the analogs serve as substrates for cellulase systems. Avicel and ball-milled filter paper were not substrates for the 130,000-M_r enzyme preparation (5). Otherwise, the substrate specificity of the 130,000-M_r enzyme preparation was identical to that of the 700,000-M_r complex (Table 1). The occurrence of xylanase activity in both the 700,000- and 130,000-M_r preparations and the lack of Avicelase activity in the 130,000-M_r preparation suggest that distinct active sites for these activities are present in the 700,000-M_r complex.

Complete or partial hydrolysis of Avicel or ball-milled filter paper by the 700,000-M_r complex resulted in the production of cellobiose and of trace amounts of glucose and cellotriose (Fig. 3, Table 1). Xylobiose was the main product of hydrolysis of xylan, with trace amounts of xylose and xylotriose formed (Table 1), and xylobiose and xylotriose were the main products of xylooligosaccharide hydrolysis (Table 1). Larger oligosaccharides were never detected when xylan or an insoluble form of cellulose served as the substrate. CMC hydrolysis yielded products that migrated on TLC plates at rates comparable to those of glucose (trace), cellobiose, cellotriose, cellotetraose, and larger oligosaccharides (Table 1). The results indicated that the 700,000-M_r protein complex has multiple enzyme activities. Hydrolysis products formed by the action of the 130,000-M_r enzyme preparation on its substrates were identical to those of the 700,000-M_r complex.

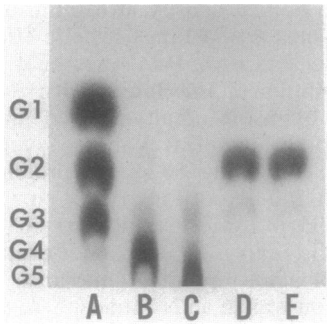

FIG. 3. TLC analysis of the hydrolysis products from the complete degradation of Avicel (lane D; 100 μl) and ball-milled filter paper (lane E; 100 μl) by the 700,000-M_r protein complex. Glucose and cellotriose spots in lanes D and E are very faint. The experimental details were described in Materials and Methods. Lanes: A, mixture of glucose (G1), cellobiose (G2), and cellotriose (G3), 50 μg each ; B, cellotetraose (G4), 50 μg; C, cellopentaose (G5), 100 μg.

Temperature and pH optima. The temperature optima for Avicelase, CMCase, and xylanase activity by the 700,000-M_r protein complex were 45, 55, and 55°C, respectively, and the pH optima were 5.6 to 5.8, 5.5, and 6.55, respectively. The dissimilarities in temperature and pH optima for these activities support the idea that the hydrolysis of each substrate involves different active sites within the protein complex.

Effect of temperature, $CaCl_2$, and aeration on stability of the 700,000-M_r protein complex. Preincubation of the 700,000-M_r protein complex for 20 min at 55°C resulted in complete loss of Avicelase (Fig. 4A) and CMCase (Fig. 4B) activities. Temperature stability of Avicelase activity was enhanced by the addition of $CaCl_2$ (Fig. 4A). Ca^{2+} has been shown to stabilize Avicelase activity in a cellulase preparation from *Acetivibrio cellulolyticus* (17). $CaCl_2$ had no effect on the temperature stability of CMCase (Fig. 4B).

Although enzyme preparations were routinely stored in an O_2-free atmosphere (see Materials and Methods), enzyme preparations stored in air exhibited Avicelase activity when assayed in standard reaction mixtures (under anaerobic conditions). Aerated samples (see Materials and Methods) retained essentially all of the initial Avicelase activity for at least 6 months.

Immunological analysis. A comparison of proteins in 130,000- and 700,000-M_r enzyme preparations was carried out by using the Western immunoblotting technique with affinity-purified antibodies to the 700,000-M_r protein complex. By means of these procedures we detected more than 15 proteins in the 700,000-M_r cellulase preparation and approximately 11 proteins in the 130,000-M_r preparation (Fig. 5). One band in the 130,000-M_r enzyme preparations, which migrated as a 116,000-M_r protein, was stained most intensely and comigrated with a protein band present in the 700,000-M_r protein complex. This suggested that the 116,000-M_r protein occurred in both the 130,000- and 700,000-M_r enzyme preparations. In general, our results indicated that proteins in the 130,000- and 700,000-M_r enzyme preparations had one or more major antigenic determinants in common. Several protein bands detected in 130,000-M_r enzyme preparations did not correspond to bands in 700,000-M_r preparations. Possibly, protein processing resulted in altered migration rates.

Specific antibodies to the *C. thermocellum* YS multiprotein cellulase complex termed the cellulosome (2, 12) reacted with two proteins (approximately 72,000 and 132,000 M_r) in

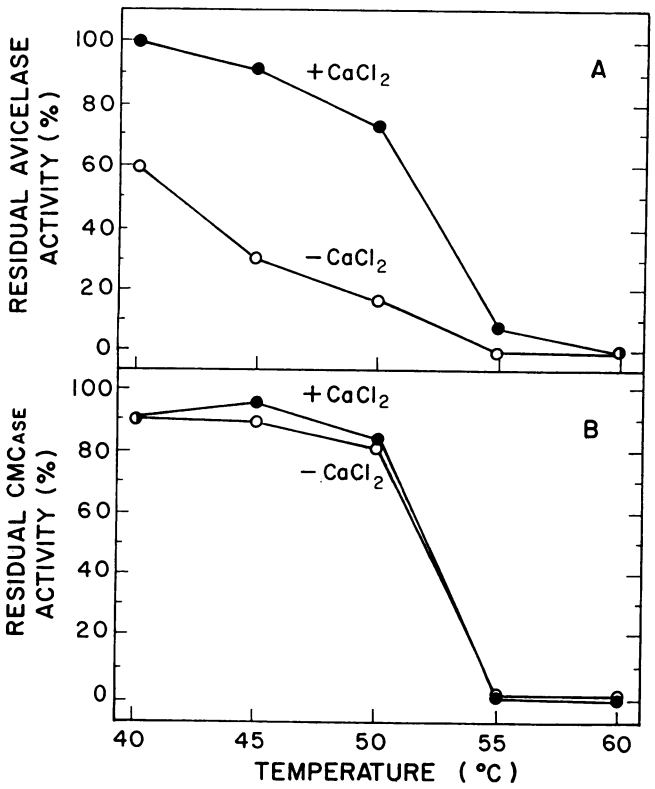

FIG. 4. Temperature stability of Avicelase (A) and CMCase (B) activities by the 700,000-M_r protein complex in the presence and absence of $CaCl_2$. The protein complex (approximately 30 or 0.16 U of Avicelase or CMCase, respectively) was preincubated without any addition or in the presence of $CaCl_2$ (5 mM, final concentration) for 20 min at the appropriate temperature in an N_2 atmosphere for Avicelase activity or in air for CMCase activity.

700,000-M_r protein complex preparations from strain C7 (Fig. 6). Apparently, the cellulosome of *C. thermocellum* and the 700,000-M_r protein complex of strain C7 have proteins with cross-reacting antigenic determinants. No proteins in 130,000-M_r enzyme preparations were detected by the cellulosome-specific antibodies (Fig. 6). Cellulosome-specific antibodies did not cross-react with nondenatured 700,000-M_r enzyme complex preparations from strain C7, as determined by an enzyme-linked immunosorbent assay. Possibly, proteins in the enzyme complex that were separated electrophoretically and detected by cellulosome-specific antibodies by the immunoblotting technique were sterically hindered from antibody binding when in the native form.

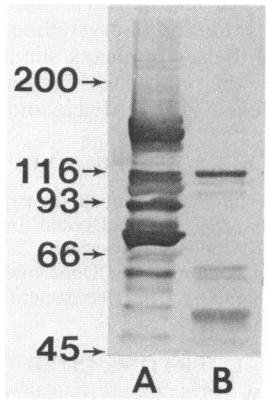

FIG. 5. Immunoblot analysis of the 700,000-M_r (lane A) and 130,000-M_r (lane B) enzyme preparations of strain C7 after sodium dodecyl sulfate-polyacrylamide gel electrophoresis with affinity-purified antibodies to the 700,000-M_r protein complex. Samples contained approximately 3.7 μg of protein. Preimmune serum controls (see Materials and Methods) were negative. The numbers indicate M_rs in thousands.

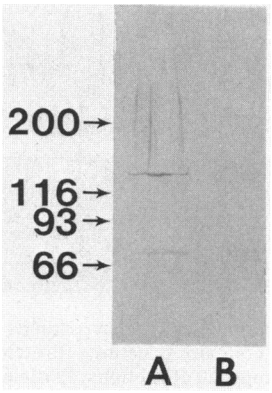

FIG. 6. Immunoblot analysis of the 700,000-M_r (lane A) and 130,000-M_r (lane B) enzyme preparations of strain C7 after sodium dodecyl sulfate-polyacrylamide gel electrophoresis with cellulosome-specific antibodies. Samples contained approximately 3.7 μg of protein. Preimmune serum controls (see Materials and Methods) were negative. The numbers indicate M_rs in thousands.

Avicelase activity was inhibited (approximately 55% inhibition in the presence of 200 μl of antibodies) by affinity-purified antibodies to the 700,000-M_r protein complex. This observation suggested that the antibodies may bind to the active site(s) on the protein complex or may bind to cellulose-binding site(s) and prevent binding of the enzyme to the substrate. When the enzyme preparation was preincubated with various amounts of a preparation of affinity-purified antibodies, a typical antibody inhibition curve was obtained. Increases in antibody concentration resulted in increases in enzyme inhibition.

Ultrastructure. Electron microscopy of negatively stained preparations of the 700,000-M_r protein complex revealed particles that were relatively uniform in size and apparently composed of subunit structures (Fig. 7). Several morphological types of particles were present. Circular-type particles had a diameter of 12 to 14 nm and usually a central hole or depression and symmetrical projections (Fig. 7a). Other particles were more or less rectangular in shape, 13 to 15 nm long and 6.5 to 8.5 nm wide (Fig. 7b to d), often with a notch in the center of the long axis (Fig. 7b, indicated by arrows). A central groove or cleft, parallel to the long axis of the rectangular-type particles, was always present (Fig. 7b to d). Possibly, these two types of particles represent two orientations of the quaternary structure of the 700,000-M_r protein complex. A plausible simple structure consistent with the measured dimensions of these two types of particles is a cylinder approximately 7.5 nm in height composed of two rings of subunits, approximately 13 nm in diameter. By this interpretation, the cleft seen in the rectangular forms would be the juncture of two superimposed rings, and the circular forms would be the circular faces of the cylinders. Based on the average dimensions of 16 different particles, the average

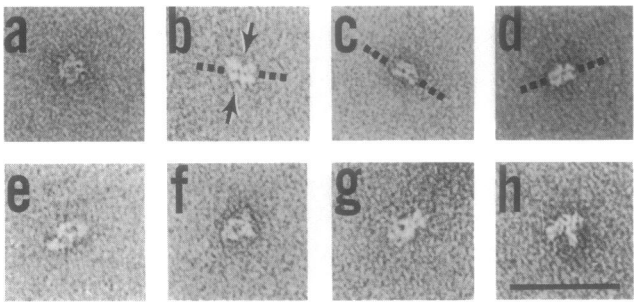

FIG. 7. Transmission electron micrographs of negatively stained 700,000-M_r protein complex structures. Protein complex structures apparently were composed of subunits. Particle morphological types included the following: the circular type (a); rectangular type (b to d), often seen with a notch in the center of the long axis (b, indicated by arrows) and a central groove or cleft parallel to the long axis (b to d, indicated by dotted lines); and irregular shapes (e to h). Bar, 50 nm.

molecular mass of these cylindrical particles was estimated to be about 800,000 daltons.

Regularly structured particles similar to those shown in Fig. 7a to d comprised approximately 1% of the particles observed in most preparations. Most of the particles in the preparations appeared irregular in shape (Fig. 7e to h) and seemed to consist of loose aggregates of subunits. These irregularly shaped structures may derive from cylindrical particles that have become partly disrupted, perhaps as a result of the electron microscopy procedures used.

DISCUSSION

As reported elsewhere (5), the activity (Avicelase) responsible for the hydrolysis of crystalline cellulose by strain C7 resides in a multiprotein complex that differs from the cellulosome of *C. thermocellum* because it has a considerably lower molecular weight, is not associated with the cell surface, and does not function in the attachment of the cells to cellulose (5). However, the Avicelase-active complex of strain C7 has several properties in common with the cellulase systems of *C. thermocellum* and of other anaerobic bacteria. One of these common properties is the Ca^{2+} requirement for the hydrolysis of crystalline cellulose. According to MacKenzie and co-workers (17), who studied the cellulase system of *A. cellulolyticus*, Ca^{2+} does not play a direct role in the catalysis of Avicel, but it exerts its effect by stabilizing the enzyme. Possibly this conclusion applies also to strain C7, inasmuch as the temperature stability of the Avicelase activity associated with the protein complex of this bacterium was markedly enhanced by the addition of $CaCl_2$. Ca^{2+} may serve to maintain a specific structure or arrangement of the protein(s) that function in the degradation of crystalline cellulose. The CMCase activity of the multiprotein complex of strain C7, like the CMCase activities of *A. cellulolyticus* and of the cellulosome of *C. thermocellum*, was not dependent on Ca^{2+}.

Another property that the strain C7 Avicelase-active complex shares with the cellulase systems of *C. thermocellum* and other anaerobes is a requirement for a reducing agent such as DTT. Apparently, DTT provides reducing conditions that are essential for the functioning of cellulase systems (10, 11, 16).

The particles observed by electron microscopy in negatively stained preparations of the 700,000-M_r enzyme complex of strain C7 are similar in ultrastructure to cellulosomes of *C. thermocellum* (6, 13, 18). Both the strain C7 particles and the cellulosomes are composed of subunits. The *C. thermocellum* cellulosome has a larger diameter (16 to 30 nm, depending on the strain) than the particles of strain C7 (13 nm) and has a spherical or flattened-sphere shape, whereas strain C7 particles appear to be cylindrical. The strain C7 cylindrical particles seem to be composed of two rings of subunits with a cleft or groove visible at the juncture of the two rings. Similar flattened cellulosomes, consisting of subunits arranged so that they formed two halves of the cellulosome separated by a groove, were observed by Mayer and co-workers (18) in cultures of *C. thermocellum* JW20. The existence of similar ultrastructural features in multiprotein complexes from different cellulolytic bacteria suggests that a particular arrangement of the proteins within the complexes is required for crystalline cellulose hydrolysis.

The 700,000-M_r complex of strain C7 is multifunctional with respect to its enzymatic activities, inasmuch as it possesses Avicelase, CMCase, and xylanase activities. This finding is in agreement with results of previous studies, which indicated that, in other cellulolytic bacteria, the above-mentioned activities are present within a single protein complex (9, 20, 23). The different temperature and pH optima for Avicelase, CMCase, and xylanase activities in the 700,000-M_r protein complex of strain C7 suggest that different enzymatically active components within the complex are responsible for the hydrolysis of these substrates.

The results of experiments involving purified antibodies to the 700,000-M_r protein complex indicated that the 130,000- and 700,000-M_r enzyme preparations from strain C7 had proteins in common. This observation supports our previous suggestion (5) that proteins of the 130,000-M_r preparation serve as precursors for the larger Avicelase-active complex or that they may be derived from the partial dissociation of the larger complex. Antibodies to *C. thermocellum* cellulosomes cross-reacted with antigenic determinants on two proteins from the 700,000-M_r protein complex of strain C7, indicating that these proteins contained conserved regions.

The hydrolysis of crystalline cellulose by anaerobic bacteria as diverse as *C. thermocellum*, *A. cellulolyticus*, *Ruminococcus albus*, *Bacteriodes succinogenes*, mesophilic *Clostridium* sp. strain C7, and others requires the participation of large, multiprotein enzyme complexes. These enzyme complexes have features in common which may be essential for the degradation of crystalline cellulose. Further characterization of these complexes should lead to a better understanding of the intricate enzymatic mechanisms through which cellulose is degraded in anoxic environments.

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