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Two Components of an Extracellular Protein Aggregate of *Clostridium thermocellum* Together Degrade Crystalline Cellulose†

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ABSTRACT: The extracellular cellulase system of *Clostridium thermocellum* ATCC 27405 was fractionated on a Sepharose 2B gel filtration column by using assays based on the hydrolysis of Avicel and (carboxymethyl)cellulose (CMC). Four A_{280} peaks were eluted, with most of the Avicelase (cellulase active on crystalline cellulose such as Avicel) and (carboxymethyl)cellulase (CMCase) activities coinciding with the second peak (approximate M_r 6.5×10^6). A lower molecular weight CMCase was also detected. Avicelase-containing fractions displayed six major protein bands (M_r 60000–250000) and many other minor bands on a sodium dodecyl sulfate (SDS) gel, indicating that Avicelase exists as a multisubunit protein aggregate. The Avicelase-containing protein aggregate was dissociated by mild SDS treatment, and the resulting subunits or subcomplexes were resolved on an Ultrogel AcA 34 gel filtration column in the presence of 0.1% SDS. Although none of the individual fractions obtained displayed significant Avicelase activity, Avicel was degraded by a combination of two fractions after the removal of SDS. One of the fractions showed only a single major protein species (M_r 82000, designated S_S) on an SDS gel. The other fraction was a mixture of at least six protein species of higher molecular weights. The major protein species (M_r 250000, designated S_L) of the latter fraction was purified by elution from an SDS gel and displayed no Avicelase activity. However, S_L degraded Avicel when combined with S_S. These results indicate that the degradation of crystalline cellulose by *C. thermocellum* can be accomplished by combining two protein components (S_S and S_L) existing in the extracellular protein aggregate. The roles, if any, of the other peptides present in the protein aggregate are not known, but we can say that they are not required for activity on the substrate Avicel under the conditions of our assay.

Clostridium thermocellum produces an extracellular cellulase system capable of degrading crystalline cellulose such as cotton and Avicel (Johnson et al., 1982b). Studies of the *C. thermocellum* cellulase system are important due to the potential use of this bacterium in the direct conversion of cellulosics to liquid fuel or chemicals (Avgerinos & Wang, 1980). Two endo-β-glucanases (1,4-β-D-glucan glucanohydrolase; EC 3.2.1.4) with molecular weights of 56000 (endoglucanase A; Petre et al., 1981) and 83000–94000 (Ng & Zeikus, 1981) have been purified and characterized from this organism. A gene (*celA*) coding for endoglucanase A has been cloned and expressed in *Escherichia coli* (Cornet et al. 1983) and in *Saccharomyces cerevisiae* (Sacco et al. 1984); its nucleotide sequence has been determined (Beguin et al., 1985). Another gene (*celB*) coding for another endoglucanase (endoglucanase B) has also been cloned in *E. coli* (Cornet et al., 1983), and its gene product with a molecular weight of 66000 has been purified from *E. coli* (Beguin et al., 1983). Five additional genes coding for endoglucanases have also been cloned into *E. coli* (Millet et al., 1985). These five genes differ from one another and from *celA* and CelB in their restriction maps. Recently, endoglucanase C (M_r 39000; Petre et al., 1986) and endoglucanase D (M_r 65000; Joliff et al., 1986) were purified from the *E. coli* clones that harbor *cel* genes. The endoglucanases that have been studied degrade (carboxymethyl)cellulose (CMC),[1] a soluble cellulose derivative.

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CMC, (carboxymethyl)cellulose; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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So far, no exoglucanase (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91) has been reported in *C. thermocellum*.

It is generally accepted that "true" cellulase activity (degradation of crystalline cellulose) of fungal origin (such as that of *Trichoderma reesei*, *Trichoderma koningii*, and *Sporotrichum pulverulentum*) results from the synergistic action of endoglucanase and exoglucanase (Bisaria & Ghose, 1981; Ljungdahl & Eriksson, 1985; Mandels, 1981). We have shown, in the past, that the true cellulase activity of *C. thermocellum* is unique since it is dependent on the presence of Ca^{2+} and a thiol-reducing agent (Johnson et al., 1982b) and a metal ion is probably involved (Johnson & Demain, 1984). Its specific activity appears to be much higher than that of *T. reesi* cellulase (Johnson et al., 1982b). Although the formation of (carboxymethyl)cellulase (CMCase) in *C. thermocellum* is constitutive and not subject to carbon source repression (Garcia-Martinez et al., 1980), we found that the formation of the true cellulase is subject to regulation by carbon source in a manner different from other cellulolytic microorganisms (Johnson et al., 1985). Despite these studies, the mechanism by which crystalline cellulose is degraded by this bacterium remains a mystery. Purification of the cellulase component(s) that degrades crystalline cellulose has been hindered by the extensive aggregation of the extracellular proteins produced by this bacterium (Ait et al., 1979; Lamed et al., 1983a; Petre et al., 1981).

We now describe the isolation of a cellulase-containing protein aggregate by gel filtration chromatography, the dissociation of this aggregate by a mild sodium dodecyl sulfate (SDS) treatment, and the reconstitution of Avicelase activity after the removal of detergents. The results indicate that the degradation of crystalline cellulose by *C. thermocellum* can be accomplished by combining two protein components.

Preliminary reports of these results have been presented (Wu & Demain, 1985, 1986).

Experimental Procedures

Organism and Medium. C. thermocellum ATCC 27405 was used throughout this study. The chemically defined medium (MJ) and the anaerobic cultivation techniques were those described by Johnson et al. (1981).

Cultivation Conditions. Seed cultures were grown in Hungate tubes by using cellobiose as carbon source. Fermentations were carried out in 2.8-L Fernbach flasks by using cotton as carbon source. Into 2 L of medium was inoculated 20 mL of exponentially growing seed culture. Prior to and during inoculation, the flasks were bubbled with nitrogen. After 48 h of incubation at 60 °C, the whole broth was filtered through several layers of cheesecloth to remove residual cotton and then centrifuged at 18000*g* for 20 min. The supernatant fluid containing the enzyme(s) was kept frozen until used. When cellobiose was used as carbon source, the culture broth was harvested by centrifugation after 14 h of fermentation at 60 °C.

Concentration of the Enzyme. To 1 volume of the enzyme preparation was added 2 volumes of cold (4 °C) acetone. The resulting mixture was centrifuged at 18000*g* for 10 min. The precipitate was resuspended in a small amount of 50 mM Tris-HCl buffer (pH 7.1), and insoluble materials were removed by centrifugation.

Gel Chromatography. Gel chromatography was carried out at room temperature on 1.6 × 90 cm columns.

(*a*) *Sepharose 2B Gel Chromatography.* The acetone-concentrated crude enzyme (15 mg of protein in 2.5 mL of Tris-HCl buffer, pH 7.1) was applied to a Sepharose 2B (Pharmacia Fine Chemicals) column equilibrated with 50 mM Tris-HCl buffer (pH 7.1) and eluted with the same buffer at a flow rate of 7 mL/h. Four-milliliter fractions were collected.

(*b*) *Ultrogel AcA 34 Gel Chromatography.* The Avicelase-containing fractions eluted from the Sepharose 2B column were pooled and the proteins concentrated by acetone precipitation. Such concentrate (1.2 mL containing 2.8 mg of protein) was incubated with 80 μL of SDS (10%), 160 μL of DTT (100 mM), 240 μL of EDTA (500 mM), and 6 mg of cellobiose (a cellulase inhibitor; Johnson et al., 1982a) at 60 °C for 25 min. The protein sample thus treated was fractionated on an Ultrogel AcA 34 (LKB) column equilibrated with buffer A (50 mM Tris-HCl buffer, pH 7.1, 1 mM DTT, 1 mM cellobiose, and 0.1% SDS) and eluted with the same buffer at a flow rate of 16 mL/h. Four-milliliter fractions were collected into tubes that contained both 0.6 mL of 75% glycerol and 50 μL of 10% cholic acid. Fractions were stored at 4 °C and assayed for cellulase activities within 3 weeks.

Reconstitution of Avicelase Activity. To tubes containing aliquots of chromatography fractions from the Ultrogel AcA 34 column (Figure 2), alone or in combination, was added cold acetone (final concentration 67%). The resulting mixture was placed at −20 °C for 30 min before it was centrifuged in a microcentrifuge (Model 5414, Brinkmann, Westbury, NY). The precipitate was resuspended in a small volume of 50 mM Tris-HCl buffer (pH 7.1). To each tube was added 5 μL each of 1 mM $FeSO_4$ and 1 mM $FeCl_3$ before Avicelase assay; however, this was later found to have no effect on Avicelase activity.

Gel Electrophoresis. (*a*) *Nondenaturing Polyacrylamide Gel Electrophoresis.* Gradient polyacrylamide gels (3–12% or 3–27%) were purchased from Integrated Separation Sciences (Newton, MA). The running buffer contained 90 mM Tris-HCl and 80 mM boric acid, pH 8.4. Electrophoresis was carried out at 100 V for 24 h.

(*b*) *SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE).* Polyacrylamide gel electrophoresis in the presence of SDS was performed as described by Laemmli (1970).

Elution of Proteins from SDS–Polyacrylamide Gels. Proteins were eluted from SDS–polyacrylamide gels as described by Hager and Burgess (1980) with the following modifications. Concentrated, dialyzed crude cellulase (containing 1.2 mg of protein) was subjected to electrophoresis on a 6% SDS–polyacrylamide gel. The elution buffer was prepared by mixing 10 mL of 100 mM glycine buffer (pH 10), 1 mL of glycerol, 130 μL of 10% SDS, 8 mg of DTT, 17 mg of cellobiose, and 100 μL of 2% sodium azide. Following elution overnight in 1.5 mL of elution buffer at room temperature, the crumbled gel was removed by filtering the mixture through glass wool. Tubes containing 20 μg of bovine serum albumin plus gel elution filtrate alone or in combination with a chromatography fraction were precipitated by acetone. The resulting protein precipitate was resuspended in 200 μL of 100 mM glycine buffer (pH 10.0) containing 5 mM DTT. Five microliters each of 1 mM $FeSO_4$ and 1 mM $FeCl_3$ was added to each tube, and they were placed at room temperature overnight before they were assayed for Avicelase activity.

Measurement of Cellulase Activity. (*a*) *Assay of Avicelase Activity.* Avicelase was determined by the decrease in turbidity (660 nm) of an Avicel suspension as described by Johnson et al. (1985). In experiments shown in Figures 4–7, the assay mixture contained a lower amount (1 mg) of Avicel. When $FeSO_4$ and $FeCl_3$ were added to the enzyme, the air in the head space of the assay tubes was replaced by nitrogen.

(*b*) *Assay of CMCase Activity.* CMCase was assayed as described by Sakajoh (1983). The reaction mixture contained

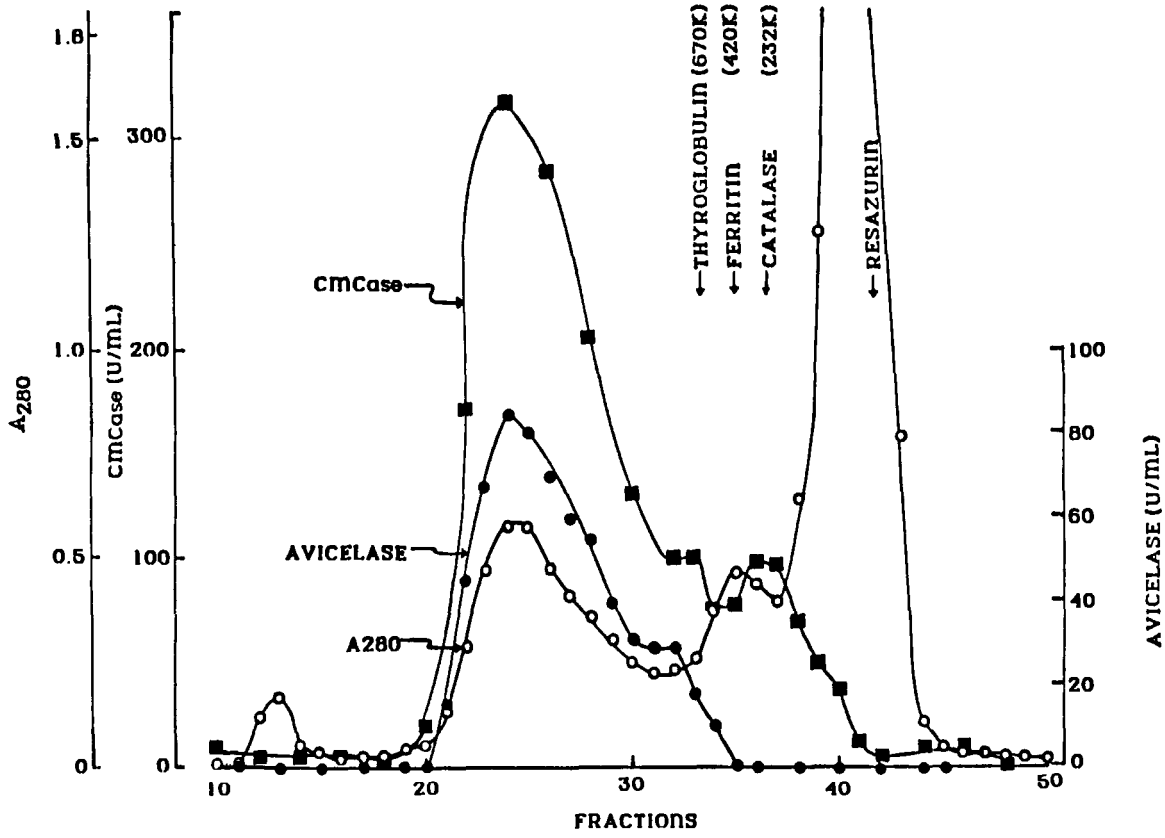

FIGURE 1: Sepharose 2B chromatography of the acetone-concentrated crude enzyme of *C. thermocellum*. The elution points of molecular weight markers are designated by arrows.

1.1 mL of 1% (carboxymethyl)cellulose (CMC, Type 9M31; Hercules Inc., Wilmington, DE), 0.4 mL of 100 mM succinate buffer (pH 5.7), and various amounts of enzyme in a total volume of 1.8 mL. The reaction was carried out at 60 °C for 15 min and then terminated by the addition of 0.2 mL of 2% sodium carbonate and 1 mL of cyanide/carbonate solution (10 mM KCN; 49 mM Na_2CO_3). Two milliliters of 0.05% potassium ferricyanide was added, and the solution was vortexed and boiled in a water bath for 30 min. The tubes were cooled and the residual ferricyanide was measured at 420 nm (Halliwell, 1961; Park & Johnson, 1949). One unit of CMCase activity was defined as the amount of enzyme necessary to cause a decrease of absorbance at 420 nm by 1 optical density unit (1-cm path length; Turner 330 spectrophotometer; 1 CMCase unit thus defined equals 0.03 IU). The experimental values were corrected for the reducing group(s) present in the enzyme preparation and the substrate (CMC).

Protein Assay. Protein was assayed by the method of Bradford (1976) using the Bio-Rad protein assay kit (Bio-Rad Laboratories). When the sample contained SDS, protein was determined by the modified Lowry method (Peterson, 1977). Bovine serum albumin was used as standard.

Results

Nondenaturing Gel Electrophoresis of the Crude Enzyme. Aggregation of the extracellular proteins produced by *C. thermocellum* ATCC 27405 in chemically defined MJ medium was examined by nondenaturing gel electrophoresis. Cells were grown on cotton, and the culture filtrate, concentrated by acetone precipitation, was subjected to electrophoresis on a 3–27% gradient polyacrylamide gel. After 24 h of electrophoresis at 100 V, a significant amount of protein migrated only slightly into the gel (data not shown). The same phenomenon was observed when the culture filtrate was concentrated by ultrafiltration using an Amicon PM-10 membrane to avoid a decrease in the dielectric constant and subsequent aggregation caused by acetone. Although the mobility of a protein species on a nondenaturing gel is determined by its size as well as by its charge, the molecular weight estimation based on a Sepharose 2B column (as shown in the next section) indicated that the failure of the protein to migrate into the gel is most likely due to the large size of the aggregate. Since the size-exclusion limit of a 3% polyacrylamide gel is at least 3 million daltons (Margolis & Kendrick, 1968), the molecular weight of the aggregate was thought to exceed this value. To determine whether the aggregation was caused by growth on an insoluble carbon source, we replaced cotton with cellobiose. Again, we found that most of the protein remained at the top of the gel after electrophoresis.

Isolation of an Avicelase- and CMCase-Containing Protein Aggregate. The cotton-grown culture filtrate was concentrated by acetone precipitation, and the concentrate containing 15 mg of protein was fractionated on a Sepharose 2B column. Four absorbance peaks (A_{280}) were observed (Figure 1); most of the Avicelase and CMCase activities coincided with the second peak. A minor peak of a low molecular weight CMCase was also detected (fractions 36–37, Figure 1). The Avicelase-containing fractions (fractions 22–32) eluted before thyroglobulin (M_r 670 000). On an SDS gel, these fractions showed multiple protein bands, including six major bands with molecular weights ranging from 60 000 to 250 000 (data not shown). When examined by nondenaturing gel electrophoresis on a 3–12% polyacrylamide gel, protein samples in these fractions only barely migrated into the gel. These results indicate that Avicelase exists as a huge protein aggregate that also displays CMCase activity.

To determine whether nucleic acid is involved in the aggregation, we examined the absorption spectrum of the A_{280} peak fraction (fraction 24 of Figure 1). Although the concentrated crude enzyme showed an absorption maximum at

Table I: Partial Purification of Clostridial Cellulase

step	total vol (mL)	total act. (units) Avicelase	total act. (units) CMCase	total protein (mg)	yield (%) Avicelase	yield (%) CMCase	sp act. (units/mg of protein) Avicelase	sp act. (units/mg of protein) CMCase
(1) crude broth	130	3900	1360	15.6			250	90
(2) acetone concentrate	2.5	3750		15.6	96		240	
(3) Sepharose 2B (peak II, fractions 21–28)	32	2300	580	4.5	60	40	510	130

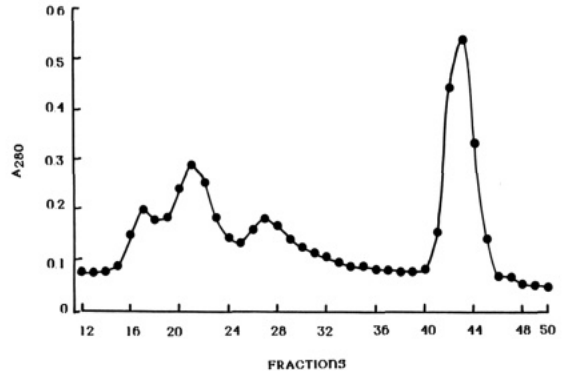

FIGURE 2: Ultrogel AcA 34 chromatography of peak II from Sepharose 2B column after treatment with SDS.

257 nm, fraction 24 showed a single peak at 280 nm, indicating that no or little nucleic acid is involved in the aggregation. Fractionation on the Sepharose 2B column is summarized in Table I.

Dissociation of the Protein Aggregate into CMCase-Containing Subunits. The Avicelase-containing protein aggregate is very stable. It was not dissociated by 8 M urea (room temperature), 10 mM dithiothreitol (60 °C, 3 h) chelating agents (EDTA, 100 mM; *o*-phenanthroline, 15 mM; 2,2′-dipyridyl, 25 mM; 60 °C, 1 h), or sulfhydryl reagents (iodoacetic acid, 25 mM; *p*-(chloromercuri)benzoic acid, 80 μM; 5,5′-dithiobis(2-nitrobenzoic acid), 15 mM; room temperature). Tween 80, Tween 20, Triton X-100, or CHAPS [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate] at a concentration of 1% only partially dissociated the protein aggregate.

Among the reagents tested, only SDS effectively dissociated the aggregate into smaller units. The Avicelase-containing fractions from the Sepharose 2B column were pooled and concentrated by acetone precipitation. Such a concentrate (1.2 mL containing 2.8 mg of protein) was incubated with SDS and fractionated on an Ultrogel AcA 34 column in the presence of 0.1% SDS, and the eluted fractions were collected in tubes containing cholic acid (final concentration 0.2%) and glycerol (final concentration 10%) as described under Experimental Procedures. Four A_{280} peaks were obtained (Figure 2), and these were examined by SDS gel electrophoresis (Figure 3). Fractions 16–18 (peak I) contained a mixture of high molecular weight proteins (M_r 80 000–250 000), and fractions 20–22 (peak II) contained a mixture of low molecular weight proteins. Only one predominant protein species was detected in fractions 26–27 (peak III); this protein species also appeared in peaks I and II. Peak IV had little or no protein.

Reconstitution of Avicelase Activity. Individual fractions were examined for Avicelase activity without removal of detergents (SDS and cholic acid), and no such activity was detected. Four peak fractions (i.e., fractions 17, 21, 27, and 43) were then combined and tested for Avicelase activity. Upon incubation of the combined fractions with an Avicel suspension, only a slight drop in the turbidity of the suspension was observed. However, extensive hydrolysis of Avicel was observed after the removal of detergents by acetone precipitation as described under Experimental Procedures (data not shown). The degradation of Avicel was only observed when fractions were combined, individual fractions having no activity even after the detergents were removed by acetone precipitation (Figure 4).

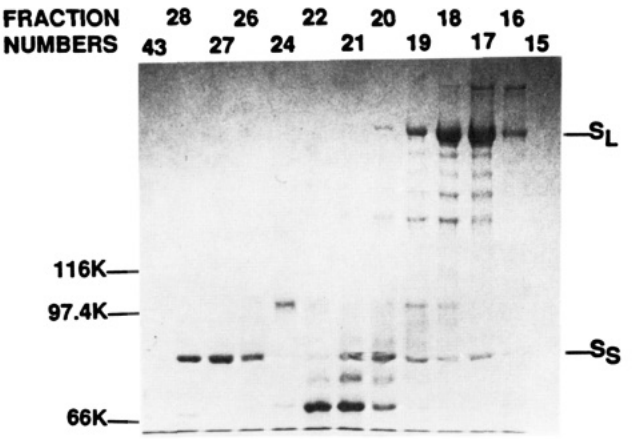

FIGURE 3: SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of the chromatography fractions from the Ultrogel AcA 34 column (Figure 2). Aliquots (80 μL) were applied to a 6% polyacrylamide gel. Proteins were stained with Coomassie brilliant blue. Molecular weight standards: β-galactosidase (116 000 daltons), phosphorylase B (97 400 daltons), bovine albumin (66 000 daltons).

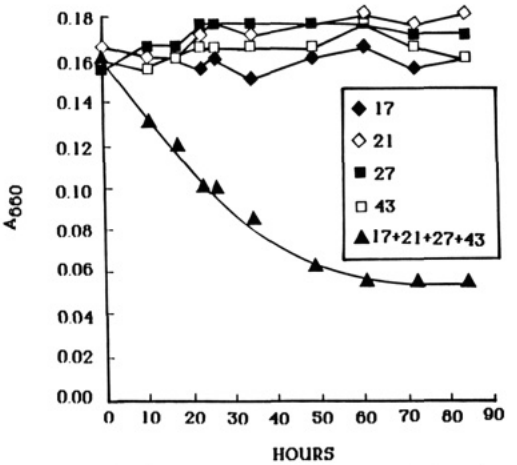

FIGURE 4: Hydrolysis of Avicel by the chromatography fractions acting alone and in combination. Aliquots (100 μL) of each fraction from the Ultrogel AcA 34 column (Figure 2) were used alone or in combination. Acetone precipitation to remove the detergents was carried out for individual fractions or after they were pooled. The resulting precipitate was resuspended in 400 μL of 50 mM Tris-HCl buffer (pH 7.1) and incubated with 1 mg of Avicel. The absorbance (660 nm) was measured with time. The protein concentrations of the individual fractions used were 50 μg/mL for 17, 150 μg/mL for 21, 70 μg/mL for 27, and 19 μg/mL for 43.

In the experiments shown in Figure 4, individual fractions had been first pooled before the removal of detergents by acetone precipitation. Alternatively, individual fractions were first acetone-precipitated to remove detergents and the resulting precipitate resuspended and then used for enzyme assay. Complete hydrolysis was again observed when fractions

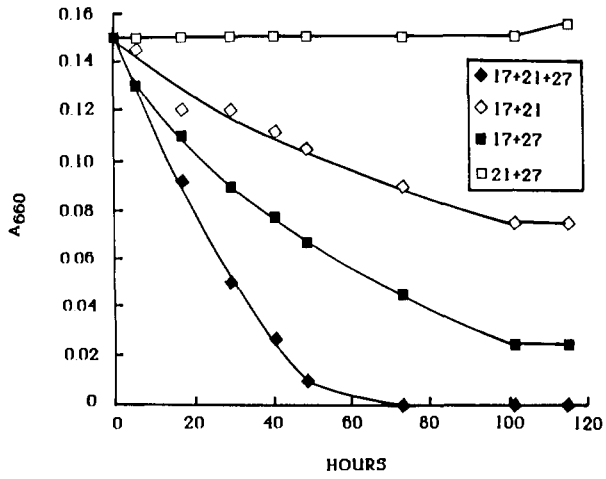

FIGURE 5: Effect of omission of the individual chromatography fractions on Avicel hydrolysis. The experimental conditions were the same as described in the legend to Figure 4 except that 150-μL aliquots of fractions were used for combination. The protein concentrations of the individual fractions used were the same as described in the legend to Figure 4.

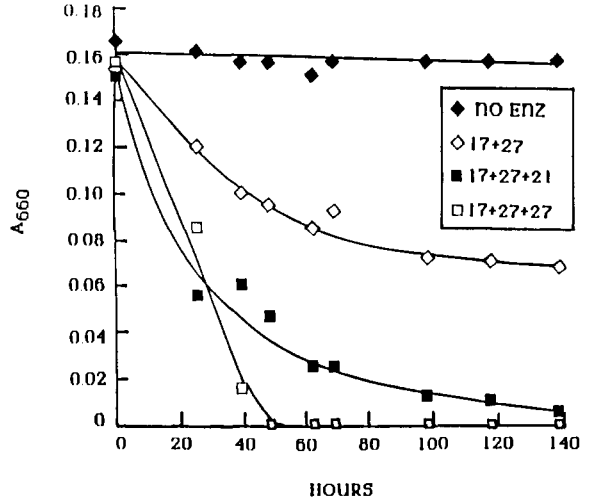

FIGURE 6: Effect of combinations of various fractions on Avicel hydrolysis. The experimental conditions were the same as described in the legend to Figure 4. In one of the samples (open squares), twice as much of fraction 27 was used. The protein concentrations of the individual fractions used were the same as described in the legend to Figure 4.

were combined. Individual fractions had no activity except fraction 17, which, when used in high amount, displayed low, but noticeable, activity toward Avicel (see Discussion).

A combination of three fractions (fractions 17, 21, and 27) was sufficient to completely solubilize Avicel (Figure 5). Although no Avicelase activity was observed if fraction 17 was omitted, omission of fraction 21 or 27 resulted in a lower hydrolysis rate. Fraction 17 alone had only very low activity as stated previously. When fraction 17 was combined with an increased amount of fraction 27, the rate of Avicel degradation was increased (Figure 6). The solubilization of Avicel was also observed when fractions 16 and 26 were combined, although individually they showed no Avicelase activity (data not shown).

Purification of S_L by Elution from an SDS Gel. Effective hydrolysis of a crystalline cellulose was observed when fractions 17 and 27 (or 16 and 26) were combined. Fractions 26 and 27 showed only one major protein species (M_r 82 000; designated S_S in Figure 3). However, fractions 16 and 17 contained at least six different proteins. To determine if the major protein species (M_r 250 000; designated S_L in Figure 3) in

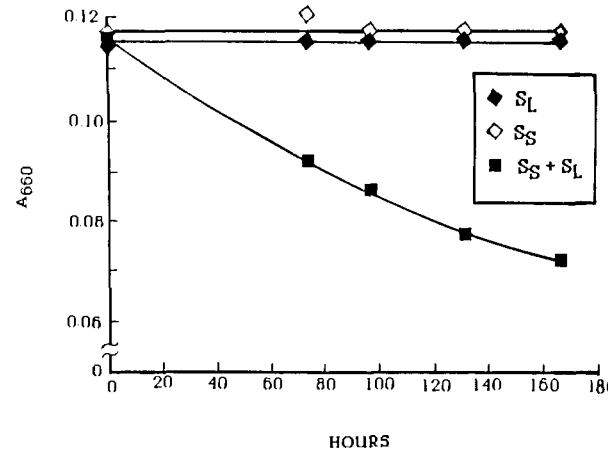

FIGURE 7: Hydrolysis of Avicel by S_L eluted from an SDS gel acting alone and in combination with S_S. S_L (M_r 250 000, Figure 3) was eluted from an SDS gel as described under Experimental Procedures. S_S (M_r 82 000, Figure 3) was equivalent to fraction 27 from the Ultrogel AcA 34 column (Figure 2). Protein in tubes included 20 μg of bovine serum albumin plus S_S (13 μg), S_L (20 μg), or both.

fraction 16 and 17 is essential for Avicelase activity, we attempted to purify it by elution from an SDS gel after electrophoresis. A crude enzyme preparation was subjected to electrophoresis on an SDS–polyacrylamide gel. The protein band corresponding to S_L was eluted from the gel as described under Experimental Procedures. S_L thus obtained had no Avicelase activity. However, when combined with S_S (equivalent to fraction 27 of the Ultrogel AcA 34 chromatography shown in Figure 2) and allowed to renature after removal of detergent as described under Experimental Procedures, it hydrolyzed Avicel (Figure 7). The specific activity obtained under these experimental conditions was approximately 1.5 units/mg, which is very low compared to that of the crude enzyme (Table I). There may be several reasons for this low activity (see Discussion).

Degradation of Avicel was also observed when S_L (after SDS was removed and the protein was allowed to renature alone) was combined with S_S from which detergents had been removed (data not shown). S_L eluted from the gel displayed a single band upon electrophoresis on an SDS gel.

S_S purified by elution from SDS gel was not active against Avicel even when combined with S_L (data not shown). This is most likely due to its inability to renature under our experimental conditions.

Activities of S_L and S_S on CMC. S_L, eluted from an SDS gel and freed of SDS by acetone precipitation, showed no activity on CMC. Neither was S_L active on other synthetic substrates such as *p*-nitrophenyl-β-glucoside (pNPG), *p*-nitrophenyl-β-cellobioside (pNPC), and methylumbelliferyl-β-cellobioside (MUC). S_S, on the other hand, released reducing sugar from CMC, indicating that it is a β-glucanase. The mode of action of S_S and the function of S_L remain to be determined.

Discussion

Aggregation of the extracellular proteins produced by *C. thermocellum* has been reported in strains NCIB 10682 (Ait et al. 1979; Petre et al. 1981), ATCC 31449 (Hon-Nami et al., 1985), YS, LQRI, and J1 (Lamed et al., 1983a) grown in complex media. Recently, the concept of a "cellulosome" was proposed which states that the extracellular cellulase components of *C. thermocellum* form a complex with an ordered structure (Bayer et al., 1983; Lamed et al., 1983a,b). This complex is thought to mediate the adherence of the cells

to the cellulose surface and the hydrolysis of cellulose. Using strain ATCC 27405, we found that the aggregation also occurred in the defined MJ medium. Although the solvent used to concentrate the enzyme may have enhanced the protein aggregation, it was not primarily induced by the solvent, since it occurred even when the crude enzyme was concentrated by ultrafiltration. Nor was the aggregate formed by the adsorption of proteins to the residual cellulose, since aggregation was found after growth in cellobiose. Molecular weight standards larger than thyroglobulin were not available, but we estimate by extrapolation that the Avicelase peak after Sepharose 2B gel filtration chromatography (fraction 24, Figure 1) has a molecular weight of 6.5 million daltons. This is the same order of magnitude as reported for the cellulosome (Lamed et al., 1983a). However, it is not possible to determine, from our data, whether the aggregate is polymorphic or has an ordered structure. Recently, aggregates with a molecular weight of 102 million daltons have been reported (Coughlan et al., 1985).

Lamed et al. reported that CMCase (1983a) and Avicelase (1985) activities are associated with the purified cellulosome. We also found that Avicelase and the major CMCase activities are associated with the protein aggregate (Figure 1). The association of cellulase activity with a protein aggregate is not unique in *C. thermocellum*; it has been reported [see Ljungdahl and Eriksson (1985) for review] to occur in other cellulolytic anaerobes, such as *Bacteroides succinogens* (M_r of the aggregate $>4 \times 10^6$), *Ruminococcus flavefaciens* ($M_r > 3 \times 10^6$), and *Ruminococcus albus* (M_r 1.5×10^6). Although the significance of aggregation is not clear, the quaternary structure of the aggregate in these anaerobes is probably essential for activity on crystalline cecllulose. In *T. koningii*, it has been suggested that complex formation between an exoglucanase (cellobiohydrolase) and an endoglucanase on the cellulose surface is required for effective hydrolysis (Wood & McCrae, 1979).

We found at least four different CMCases to be associated with the aggregate (data not shown). Lamed et al. (1983a) reported that at least eight components of the cellulosome display CMCase activity. The multiplicity of endoglucanases is evidenced by genetic analysis as well as biochemical studies, since seven different endoglucanase genes from *C. thermocellum* have been cloned (Cornet et al., 1983; Millet et al., 1985). An obvious question is why the organism produces so many different enzymes with seemingly the same activity. The question may be best answered after the CMCases are fully characterized.

The major impediment to the study of synergism between *C. thermocellum* cellulase components has been the aggregation of the extracellular proteins produced by this bacterium. With dissociation of the aggregate by SDS and the successful reconstitution of the Avicelase activity, we were able to demonstrate degradation of crystalline cellulose by *C. thermocellum* by a combination of two protein components. Synergism between cellulase components thus appears to be a general mechanism by which crystalline cellulose is degraded, no matter whether the cellulase system is of fungal or bacterial origin.

In reconstituting Avicelase activity, we found that Avicel was degraded by the combination of two fractions (fraction 17 with 21 or 27; Figure 5). It appeared that fractions 21 and 27 provided the same protein species (S_S, M_r 82 000) active in Avicel degradation. Fraction 17 also contained S_S in a much smaller amount (Figure 3), and this probably explains the low Avicelase activity detected in this fraction when tested alone. Although S_S is active on CMC, it has a molecular weight different from many of the CMCases identified from *C. thermocellum* (EGA, M_r 56 000, Petre et al., 1981; EGB, M_r 66 000, Cornet et al., 1983; EGC, M_r 39 000, Petre et al. 1986; EGD, M_r 65 000, Joliff et al., 1986). However, its molecular weight is close to that of the endoglucanase purified by Ng and Zeikus (M_r 83 000–94 000; 1981). Whether they are the same enzyme remains to be determined.

S_L (M_r 250 000), eluted from an SDS gel and renatured, was able to degrade Avicel when combined with S_S (Figure 7). However, S_L alone did not show any detectable activity on various synthetic substrates (pNPC, pNPG, MUC, and CMC). Interestingly, Bayer et al. (1983, 1985) reported that a major cellulosome component called S1 (M_r 210 000) seems to mediate the adherence of the bacterial cells to the cellulose surface. Unlike the wild strain, a mutant lacking cell-associated S1 failed to adhere to the solid substrate. Since S1 and S_L are similar in size, it would be interesting to determine whether S_L is a cellulolytic enzyme or an "anchoring component" (Bayer et al., 1983) that brings the cellulolytic enzyme(s) to the substrate or both.

The specific Avicelase activity obtained by combining S_S and S_L (as shown in Figure 7) is very low compared to that of the crude enzyme. Although the possibility that a cellulase component(s) other than S_S and S_L is required for the optimal activity cannot be excluded, this low specific activity is most likely due to the fact that S_L was purified by elution from an SDS gel. This purification procedure, in many cases, results in a very low recovery (0–3%) of enzyme activity (Hager & Burgess, 1980). Similarly, S_S was probably denatured to some extent due to the adverse effect of SDS. Furthermore, because of the limited amount of S_L obtainable from the gel, we were not able to determine the optimal ratio of these two components. We are currently developing methods to obtain S_L and S_S in a nondenaturing way in order to determine a more meaningful specific activity and the mechanism of the cooperative action.

In conclusion, we have demonstrated that, in *C. thermocellum*, the hydrolysis of crystalline cellulose can be accomplished by two proteins (S_S and S_L) acting synergistically. To our knowledge, the only other case of synergism reported in bacterial cellulase systems is in *Clostridium stercorarium*, whose extracellular protein is not aggregated (Creuzet et al., 1983). Cellulose degradation by *C. thermocellum* is probably a more complicated process since it involves an unusual extracellular protein aggregate with which multiple CMCases are associated. However, by focusing on S_S and S_L, it may be possible to gain a better understanding of this complex cellulolytic system. Finally, our results suggest that the molecular cloning of cellulase genes in this bacterium should be directed toward the genes coding for these two proteins which, in our opinion, are of great physiological and industrial importance.

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