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Studies on Cellulose Hydrolysis by *Acetivibrio cellulolyticus*†

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Acetivibrio cellulolyticus **extracellular cellulase extensively hydrolyzed crystalline celluloses such as Avicel (FMC Corp., Food and Pharmaceutical Products Div., Philadelphia, Pa.) but only if it was desalted and supplemented with Ca^{2+}. The Ca^{2+} effect was one of increased enzyme stability in the presence of the ion. Although preincubation of the cellulase complex at 40°C for 5 h without added Ca^{2+} had a negligible effect on endoglucanase activity or on the subseqent hydrolysis of amorphous cellulose, the capacity of the enzyme to hydrolyze crystalline cellulose was almost completely lost. Adsorption studies showed that 90% of the Avicel-solubilizing component of the total enzyme preparation bound to 2% Avicel at 40°C. Under these conditions, only 15% of the endoglucanase and 25% of the protein present in the enzyme preparation adsorbed to the substrate. The protein profile of the bound enzyme, as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was complex and distinctly different from the profile observed for total cellulase preparations. The specific activity of *A. cellulolyticus* cellulase with respect to Avicel hydrolysis was compared with that of commercially available *Trichoderma reesei* cellulase.**

In the search for possible commercial sources of cellulase, certain anaerobes have been the subject of considerable attention because of their highly efficient cellulose metabolism. At the same time, however, doubts have been expressed about the usefulness of these organisms in this regard because of difficulties in obtaining enzyme preparations capable of duplicating the high in vivo hydrolysis rates. There has been speculation that this discrepancy may result from disruption of a highly ordered cellulase complex located largely or entirely on the cell surface (5). However, attempts at gaining a better understanding of the factors involved have been frustrated by problems encountered in fractionation and biochemical characterization of the cellulase complexes.

Recently, progress has been made in characterizing the cellulase system of *Clostridium thermocellum*, a thermophilic anaerobe. Johnson et al. (7) reported that Ca^{2+} and a thiol-reducing agent were required for maximum cellulase activity. Under optimum conditions, the specific activity of *C. thermocellum* cellulase was much higher than that observed for cellulase produced by aerobic fungi such as *Trichoderma reesei*. It was subsequently shown by Johnson and Demain (6) that thiol-binding reagents inactivated Avicel (FMC Corp., Food and Pharamceutical Products Div., Philadelphia, Pa.)-solubilizing activity but had little effect on endoglucanase activity. A major part of the cellulolytic activity of *C. thermocellum* appears to be associated with high-molecular-weight aggregates which have been termed cellulose-binding factors (10). These aggregates contain, in addition to several endoglucanases, a 210,000-dalton protein which is thought to be responsible for adhesion of the aggregates to cellulose (1, 9, 10). Like *C. thermocellum*, *Acetivibrio cellulolyticus* is a highly efficient cellulolytic anaerobe which produces several electrophoretically distinct endoglucanases (13). We have previously demonstrated a requirement for Ca^{2+} and reducing agents by the cellulase complex of this organism. In the present communication we expand on these findings and present some results on the adsorption of the *A. cellulolyticus* enzyme to cellulosic substrates.

MATERIALS AND METHODS

Organism and growth conditions. *A. cellulolyticus* (NRCC 2248, ATCC 33288), a mesophilic anaerobe isolated by Patel et al. (15), was grown on 1% Avicel PH105 in fermentors as described by Patel and MacKenzie (16).

Enzyme preparations. *A. cellulolyticus* cells were harvested aerobically by centrifugation, and the supernatant was retained as the source of cellulase. The cellulase was then precipitated with ammonium sulfate (80% saturation). The precipitate was collected by centrifugation at 20,000 × *g* for 20 min and suspended in 10 mM phosphate buffer (pH 6.0). The material was then desalted by passage through a Bio-Gel P-6 (Bio-Rad Laboratories, Richmond, Calif.) column (2.6 by 70 cm); the eluting buffer was 10 mM phosphate (pH 6.0). The desalted enzyme preparation obtained by this step served as the total *A. cellulolyticus* enzyme complex in all experiments.

Celluclast (*T. reesei* cellulase) was obtained from Novo Laboratories, Copenhagen, Denmark. This enzyme was used in comparative studies involving *A. cellulolyticus* and *T. reesei* cellulases.

Enzyme assays. (i) Turbidimetric assay. Hydrolysis of Avicel PH105 and acid-swollen cellulose prepared by the method of Ferchak et al. (3) was followed by a decrease in turbidity (measured at 660 nm, 1.5-cm light path), as described by Johnson et al. (7). Assays contained 0.1% Avicel or 0.25% acid-swollen cellulose, 0.02% NaN_3, and 50 mM MOPS (morpholinepropanesulfonic acid; pH 6.0) in a final volume of 5 ml. Assays containing Avicel were typically supplemented with 10 mM $CaCl_2$. All assays were performed at 40°C without shaking. True cellulolytic activity resulting in extensive hydrolysis (solubilization) of unmodified forms of cellulose, such as Avicel, could be readily monitored by this assay method.

(ii) Carboxymethylcellulase. Carboxymethylcellulase or endoglucanase assays were performed at 45°C as described previously (11). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar (expressed as glucose) per min.

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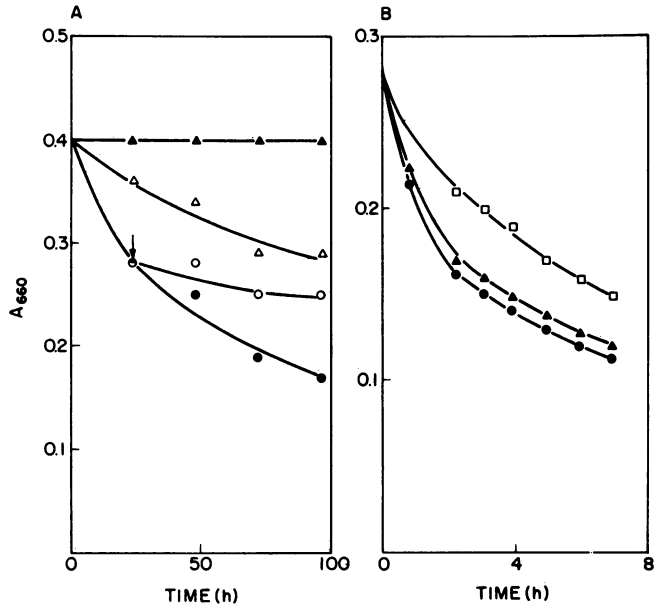

FIG. 1. Effect of Ca^{2+} and Hg^{2+} on the hydrolysis of Avicel (A) and acid-swollen cellulose (B) by *A. cellulolyticus* cellulase. Symbols: ▲, no additions; ●, 10 mM Ca^{2+}; □, 10 μM Hg^{2+}; △, 10 mM Ca^{2+} and 10 μM Hg^{2+}. The inhibitory effect of 10 μM Hg^{2+} on Avicel hydrolysis was also tested by its addition (↓) at 24 h (○) to a hydrolysis mixture containing 10 mM Ca^{2+}. Hydrolysis mixtures contained 0.4 U of endoglucanase per ml.

(iii) Avicelase. Assays contained various concentrations of Avicel PH105, 10 mM Ca^{2+}, 0.02% NaN_3, and 50 mM MOPS (pH 6.0) in a final volume of 2 ml. After 24 h of incubation at 40°C without shaking, reducing sugar release was estimated as described earlier (11) by the Somogyi modification (19) of the Nelson method (14) with glucose as the standard.

Adsorption studies. The adsorption of different components of the cellulase preparation to increasing concentrations of Avicel PH105 was studied at 40°C. In addition to enzyme and substrate, adsorption mixtures contained 10 mM Ca^{2+}, 0.02% NaN_3, and 50 mM MOPS (pH 6.0) in a total volume of 6 ml. After 1 h of incubation, the cellulose was removed by centrifugation at 20,000 × *g* for 10 min, and the nonadsorbed protein, endoglucanase, and total cellulase (determined turbidimetrically with Avicel PH105 as the substrate) were measured. Protein concentrations were estimated by the Bradford method (2) with a Bio-Rad reagent and with bovine gamma globulin as the standard.

Electrophoresis. The protein profiles of enzyme-substrate complexes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Enzyme-substrate complexes were prepared as described above with Avicel and acid-swollen cellulose at concentrations of 0.1% and 0.25%, respectively. The enzyme-substrate complexes were subjected to SDS-PAGE with the buffer system described by Laemmli (8). Bands were visualized as described previously (12).

RESULTS

Turbidimetric assays demonstrated that *A. cellulolyticus* cellulase preparations obtained by the desalting of ammonium sulfate precipitates were capable of extensive hydrolysis of Avicel PH105 (Fig. 1A). Assuming that there is a direct relationship between turbidity and cellulose concentration at various points in the assay, this represents a weight loss of 60% over the assay period. The previous observation (11) that the hydrolysis of cellulose-azure by *A. cellulolyticus* cellulase was greatly enhanced by Ca^{2+} and strongly inhibited by Hg^{2+} was further investigated with turbidimetric assays. In the absence of Ca^{2+}, the enzyme preparation was completely inactive against Avicel. Although the results presented in Fig. 1 were obtained at a Ca^{2+} concentration of 10 mM, levels as low as 1 mM gave similar hydrolysis rates. An initial rapid rate gave way to a slower, but linear, one. The addition of Hg^{2+} at a concentration of 10 μM resulted in a 50% reduction in the Avicel hydrolysis rate. The addition of Hg^{2+} 24 h after the initial rapid hydrolysis almost completely inhibited further hydrolysis.

The effects of Ca^{2+} and Hg^{2+} on the hydrolysis of acid-swollen cellulose were quite different from those observed for Avicel. Although Hg^{2+} reduced the hydrolysis rate, Ca^{2+} was not observed to significantly increase the hydrolysis rate (Fig. 1B).

The effect of Ca^{2+} on Avicel solubilization by *A. cellulolyticus* cellulase was apparently one of increased enzyme stability in the presence of the ion (Fig. 2A). Enzyme preparations that were preincubated at 40°C before being added to assays containing Avicel and 10 mM Ca^{2+} were found to have drastically reduced activity levels. A 1-h preincubation resulted in a 50% drop in activity, and a 5-h preincubation caused almost total inactivation of Avicel-solubilizing activity. It seems probable that only the more readily hydrolyzed regions of the substrate were attacked in the latter instance. When the same preincubations were performed in the presence of 10 mM Ca^{2+}, there was only a slight loss of Avicel-solubilizing activity (Fig. 2B).

The effect of Ca^{2+} on the solubilization of Avicel and

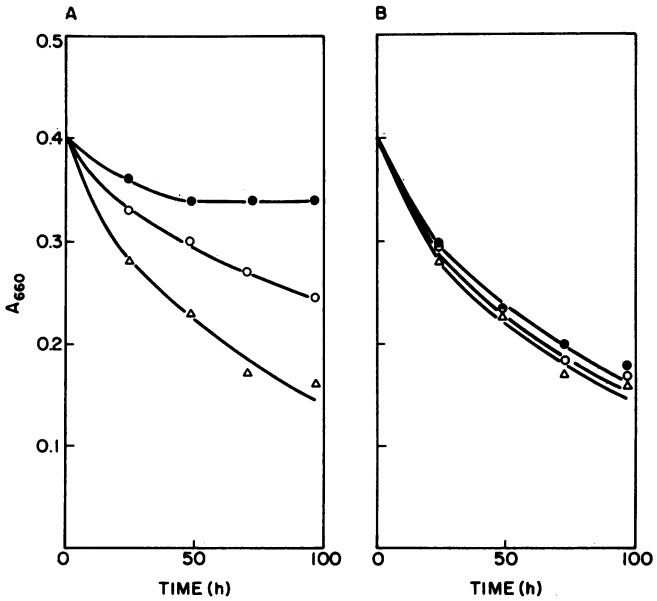

FIG. 2. Stability of the Avicel-solubilizing activity of *A. cellulolyticus* at 40°C in 50 mM MOPS buffer (pH 6.0) (A) and in 50 mM MOPS buffer (pH 6.0) supplemented with 10 mM Ca^{2+} (B). Symbols: △, controls; ○, 1-h incubation before substrate addition; ●, 5-h incubation before substrate addition. Hydrolysis mixtures contained 0.4 U of endoglucanase per ml and were supplemented with 10 mM Ca^{2+}.

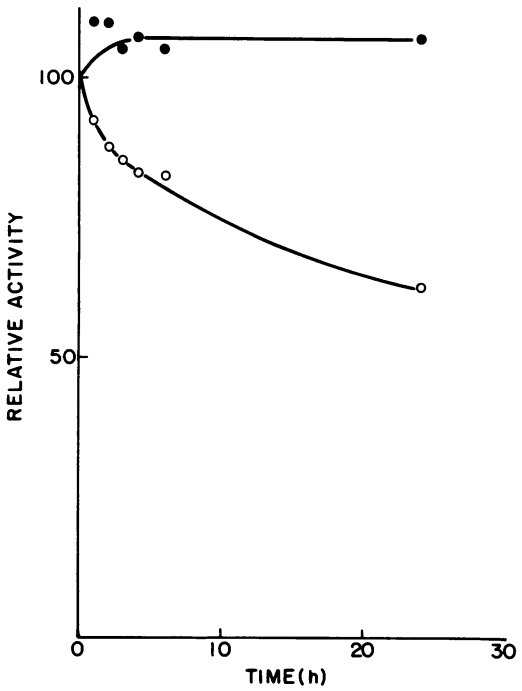

FIG. 3. Stability of *A. cellulolyticus* endoglucanase at 40°C in 50 mM MOPS buffer (pH 6.0) (○) and in 50 mM MOPS buffer (pH 6.0) containing 10 mM Ca^{2+} (●). Incubation mixtures contained 0.4 U of endoglucanase per ml and 0.02% NaN_3. Assays were performed as described in the text.

acid-swollen cellulose by *A. cellulolyticus* cellulase demonstrated that the enzymes involved in the hydrolysis of these two substrates were distinctly different. It seems likely that carboxymethylcellulase or endoglucanase is largely responsible for the hydrolysis of acid-swollen cellulose. Although Ca^{2+} did have a stabilizing effect on endoglucanase activity (Fig. 3), the half-life in the absence of this ion was much higher than that observed for Avicel-solubilizing activity. Approximately 60% of the endoglucanase remained after 24 h at 40°C in the absence of Ca^{2+}. There was no detectable loss of activity when the preincubation mixture contained 10 mM Ca^{2+}.

The susceptibility of the *A. cellulolyticus* cellulase complex to inhibition by glucose and cellobiose was tested by the turbidimetric assay procedure. Distinctly different inhibition profiles were obtained for Avicel and acid-swollen cellulose hydrolysis (Fig. 4). This provides further evidence for the involvement of different enzymes in the hydrolysis of these two substrates. Both sugars inhibited Avicel hydrolysis more strongly than acid-swollen cellulose hydrolysis. In each instance cellobiose was much more inhibitory than glucose, resulting in the complete inhibition of Avicel hydrolysis at a concentration of 100 mM.

The effect of increasing Avicel PH105 concentration on the adsorption of components of *A. cellulolyticus* cellulase to this substrate is shown in Fig. 5. Approximately 15% of the endoglucanase adsorbed throughout the range of concentrations tested (0.1 to 2.0%). Protein adsorption increased linearly from 10% at a substrate concentration of 0.1% to 25% at a substrate concentration of 2%. The degree of adsorption of Avicel-solubilizing activity was determined by performing turbidimetric assays on the nonadsorbed fractions. This activity was observed to bind strongly to the substrate. There was ca. 50% adsorption at the lowest substrate concentration and 90% at the highest. Also, there was a direct relationship between the extent of adsorption of this activity and the amount of reducing sugar released at various Avicel concentrations.

The protein profiles, as revealed by SDS-PAGE, of the total cellulase preparation, the enzyme-substrate complexes obtained with Avicel PH105 and acid-swollen cellulose, and the sedimentable portion of the cellulase preparation are shown in Fig. 6. In total cellulase preparations, a protein

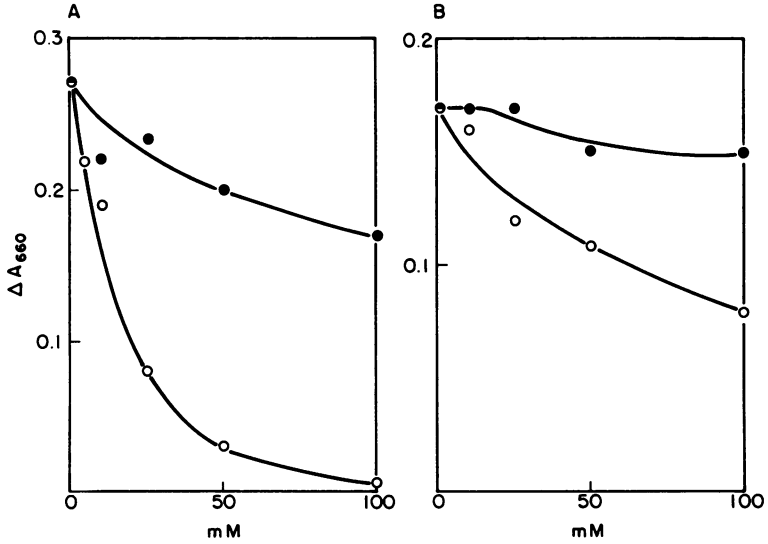

FIG. 4. Effect of glucose (●) and cellobiose (○) concentrations (millimolar) on the hydrolysis of Avicel (A) and acid-swollen cellulose (B) by *A. cellulolyticus* cellulase. Avicel hydrolysis assays were supplemented with 10 mM Ca^{2+} and performed for 96 h. Acid-swollen cellulose hydrolysis assays were performed for 24 h. The activity levels at various inhibitor concentrations are reported as the change in A_{660} from assay start to finish.

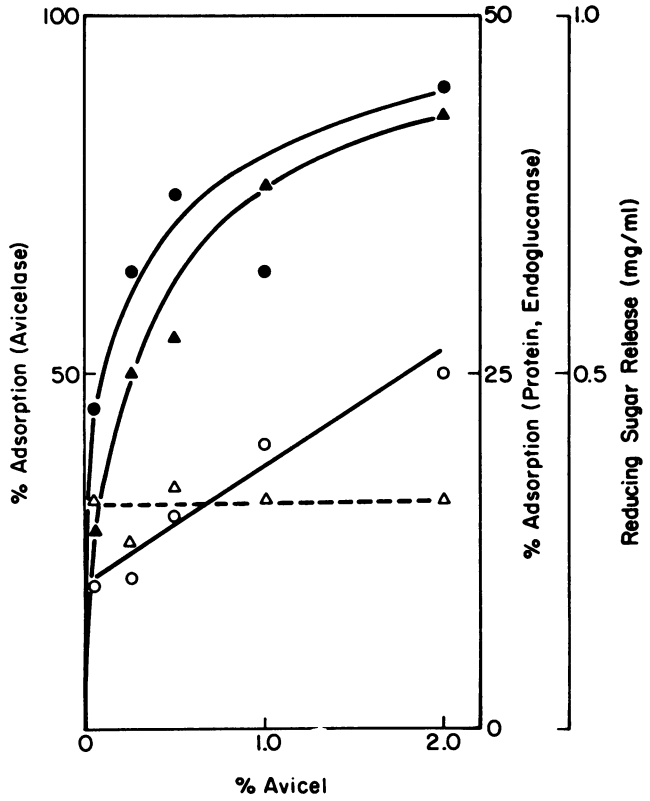

FIG. 5. Effect of Avicel concentration on reducing sugar release (▲) by *A. cellulolyticus* cellulase and on the adsorption of protein (○), endoglucanase (△), and Avicel-solubilizing activity, (Avicelase) (●) from crude *A. cellulolyticus* cellulase. In all instances, the endoglucanase concentration was 0.4 U/ml.

species with a molecular weight of 67,000 made up a large percentage of the total protein. There was, however, negligible adsorption of this protein to Avicel. The protein composition of the Avicel-enzyme complexes was distinctly different from that of the total cellulase preparations. Although many protein species were detected, two bands were predominant—one of a high but undetermined molecular weight and a second with a molecular weight of ca. 73,000. Acid-swollen cellulose–enzyme complexes also contained these two bands but differed from the Avicel-enzyme complexes in that the major protein band in the total cellulase preparations was also the major band in these complexes. High-speed centrifugation yielded a sediment with a protein composition similar to that observed in total cellulase preparations.

A. cellulolyticus and *T. reesei* cellulases were compared (Fig. 7) with respect to their capacity to extensively degrade crystalline cellulose at activity levels approximating those observed in cultures actively growing on cellulose. Under the conditions used, each enzyme type should be in excess. In spite of enormous differences in protein and enzyme concentrations, the hydrolysis rates were practically identical.

DISCUSSION

Although the cellulase system of *A. cellulolyticus* is not inactivated by air, it has been shown to function optimally in a reducing environment (11). The previous observation (11) of the inhibitory effect of thiol-binding agents on dye release from cellulose-azure has now been demonstrated with a crystalline substrate, Avicel. These observations suggest

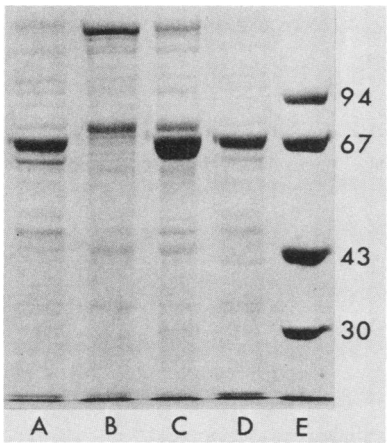

FIG. 6. SDS-PAGE of *A. cellulolyticus* total cellulase preparation (A), cellulase-Avicel complex (B), cellulase–acid-swollen cellulose complex (C), sediment obtained by centrifugation (250,000 × *g*, 18 h) of total cellulase preparation (D), and molecular weight markers (E). The numbers on the right refer to the molecular weights of the markers in thousands.

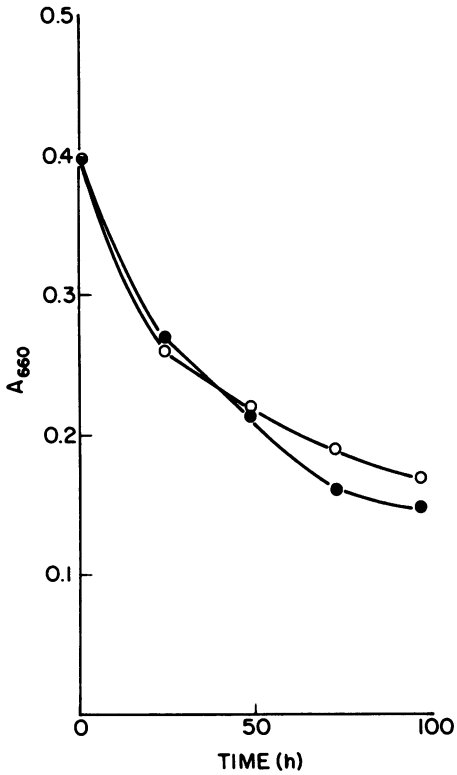

FIG. 7. Comparison of Avicel hydrolysis by *A. cellulolyticus* and *T. reesei* cellulases at activity levels similar to those obtained in culture broths, i.e., 0.4 U/ml (0.17 mg of protein per ml) and 170 U/ml (10 mg of protein per ml) for *A. cellulolyticus* (●) and *T. reesei* (○), respectively. Hydrolysis mixtures were supplemented with 10 mM Ca^{2+}.

the involvement of sulfydryl groups in the hydrolysis process. In this regard, *A. cellulolyticus* cellulase seems very similar to crude extracellular cellulase from *C. thermocellum*. This cellulase is also stimulated by the presence of a reducing agent such as dithiothreitol and is strongly inhibited by low concentrations of thiol-binding agents (6, 7). Under certain conditions, however, dithiothreitol was found to inhibit the hydrolysis of Avicel by *C. thermocellum* cellulase (6). It was proposed that the formation of oxidized products was responsible. Interestingly, dithiothreitol was found to have an inhibitory effect on the *A. cellulolyticus* enzyme in the later stages of prolonged turbidimetric assays (data not shown) and was, therefore, not generally added to these assays. The cellulase from *Ruminococcus albus*, an anaerobic rumen bacterium, also appears to require reduced conditions (18). The β-glucosidase of *Bacteroides succinogenes*, another rumen anaerobe, is inactivated by sulfydryl inhibitors, but the inhibition of cellulose-solubilizing activity by these agents has not been reported (4).

Another distinctive characteristic of *A. cellulolyticus* and *C. thermocellum* cellulases is the Ca^{2+} requirement. Johnson et al. (7) reported that the addition of 7 mM Ca^{2+} or Mg^{2+} to crude *C. thermocellum* cellulase doubled the rate of Avicel hydrolysis and that the addition of 10 mM EDTA to the assays completely eliminated hydrolytic activity. Pettipher and Latham (17) reported that endoglucanase from *R. flavefaciens*, a rumen anaerobe, was inhibited by EDTA and that the activity could be restored by the addition of Ca^{2+} or Mg^{2+}. It was previously reported that *A. cellulolyticus* endoglucanase was relatively unaffected by Ca^{2+} or Mg^{2+} but that dye release from cellulose-azure by the enzyme was enhanced by up to 400% by 10 mM Ca^{2+} (11). In the present study we have shown that there is an absolute Ca^{2+} requirement for Avicel hydrolysis. We have also shown that Ca^{2+} does not play a direct role in Avicel hydrolysis but that it exerts its effect by preventing the inactivation of Avicel-solubilizing activity.

The importance of choosing the appropriate assay techniques in the study of cellulases cannot be overemphasized. An excellent example of this is provided by the results presented here on the role of Ca^{2+} in maintaining enzyme stability. The involvement of Ca^{2+} as an essential component of *A. cellulolyticus* cellulase was evident only when Avicel was used as the substrate. Because of low in vitro activities on crystalline cellulose, substrates such as Avicel have not been commonly used in studies on cellulases from anaerobic bacteria. The results presented here offer some explanations for these low activities and suggest that conclusions drawn from studies on amorphous and derivatized forms of cellulose may have little relevance in the hydrolysis of native forms of cellulose.

The protein profile, as revealed by SDS-PAGE, of the Avicel-cellulase complexes bears considerable similarity to that of *C. thermocellum* "cellulosomes." These are complexes with a molecular weight of 2.1×10^6. They contain at least 14 protein bands, several of which are endoglucanases and a 210,000-dalton protein which is thought to be responsible for binding the complexes to cellulose (1, 9, 10). The *A. cellulolyticus* complexes also contain a large number of protein bands and a major protein species with a molecular weight in the 200,000 range. Although the amount of *A. cellulolyticus* endoglucanase which adsorbed to 1% Avicel (ca. 15% of the total) was considerably less than the amount of *C. thermocellum* endoglucanase which adsorbed to 1% microcrystalline cellulose (65% of the total), ca. 25% of the total protein content of the preparations bound in each instance.

There is a striking difference in specific activities between the extracellular cellulase produced by anaerobic bacteria such as *A. cellulolyticus* and that produced by aerobic microorganisms such as *T. reesei*. However, this difference is obvious only when crystalline substrates such as Avicel are used. Although similar endoglucanase-specific activities were observed for these two organisms, the specific activities for Avicel hydrolysis were dramatically different. At identical Avicel hydrolysis rates, *T. reesei* assays contained 10 mg of total cellulase protein per ml (170 U of endoglucanase per ml), as compared to 170 μg of total cellulase protein per ml (0.4 U of endoglucanase per ml) for *A. cellulolyticus* assays.

It seems reasonable to speculate that further research on highly cellulolytic anaerobes such as *A. cellulolyticus* (16) may yield cellulase preparations which are superior to those from aerobic sources. Cellulolytic anaerobes offer the advantages of potentially lower enzyme production costs and higher specific activities. We have shown that, under appropriate conditions, the high in vivo activities can be duplicated in vitro. However, it should be pointed out that the benefits of high specific activities are largely eliminated by the fact that the actual amounts of enzyme produced are small compared to those produced by highly cellulolytic aerobes such as *T. reesei*. Also, many questions remain unanswered with respect to the biochemistry of cellulolysis and the application of these enzymes to cellulose saccharification processes. The roles of exoglucanases and cellulose-binding proteins are still not clear. The component(s) of the cellulase complex which require(s) Ca^{2+} and reducing conditions has yet to be identified. The nature of the inhibitor(s) present in oxidized culture supernatants is not known. The hydrolytic capacities of these enzymes at substrate concentrations that are realistic from an industrial viewpoint have not been investigated. These are the types of problems on which future work should focus.

ACKNOWLEDGMENT

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