SHORT COMMUNICATION

Partial Purification of Cellulase from *Clostridium thermocellum*

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The cellulolytic system from culture filtrates of *Clostridium thermocellum*, when partially purified by preparative electrophoresis, showed a single protein band on analytical polyacrylamide gel electrophoresis; however, sodium dodecyl sulphate–polyacrylamide gel electrophoresis indicated that at least five proteins were present. Carbohydrate was present in the purified fraction and it is suggested that the cellulase was present as a complex.

INTRODUCTION

Many studies have dealt with cellulose-degrading mesophilic fungi and bacteria, but little is known about the formation and properties of the cellulase of thermophilic anaerobic bacteria. *Clostridium thermocellum* produces large amounts of a cellulase (Weimer & Ziekus, 1977) which is extracellular, thermally stable at 70 °C and adsorbed strongly by cellulose. We have studied the production of this cellulase during cell growth and partial purification of the enzyme indicates that it is present as a complex.

METHODS

Culture. *Clostridium thermocellum* NCIB 10682 was grown anaerobically at 60 °C on CM3 medium as described by Weimer & Zeikus (1977) with cellulose at 10 g l⁻¹. After 6 d, the culture was centrifuged and the sedimented cells and cellulose were discarded. The supernatant was concentrated 180-fold by ultrafiltration through a PM10 membrane (Amicon).

Assay of cellulolytic activity. Samples (10 ml) were withdrawn from a 1 l culture at various times: 5 ml was centrifuged at 12000 g for 10 min and the supernatant was assayed for soluble cellulase; the remaining 5 ml was assayed without centrifuging to determine the total activity.

Carboxymethylcellulase activity was determined colorimetrically by assaying reducing sugar equivalents released from carboxymethylcellulose (Sigma) at 60 °C. Assays contained an appropriate amount of enzyme, substrate (0.8 %, w/v) and 20 mM-KH₂PO₄/K₂HPO₄ buffer, pH 7.0. Reducing sugar was estimated by the ferricyanide method of Park & Johnson (1949). One unit of carboxymethylcellulase was defined as the amount releasing 1 µmol of reducing sugar min⁻¹.

Cellulase activity was determined by the release of reducing equivalents from Whatman CC41 cellulose. Assays contained an appropriate amount of enzyme, cellulose (1.25 %, w/v) and 250 mM-KH₂PO₄/K₂HPO₄ buffer, pH 7.0, and they were incubated at 60 °C for 1 h. Reducing sugar was estimated as described above. One unit of cellulase was defined as the amount releasing 1 µmol reducing sugar equivalents h⁻¹.

Analyses. Total protein was measured by the method of Lowry using bovine serum albumin as a standard. Total carbohydrate was measured by the phenol/H₂SO₄ method of Dubois *et al.* (1956) with glucose as a standard.

Determination of molecular weight. The molecular weight of the cellulase was determined by the method of Andrews (1964) from its elution volume on a Bio-Gel P-200 column (1 × 80 cm). The column was equilibrated with 0.05 M-Tris/HCl buffer, pH 7.0, containing 0.2 M-NaCl, and calibrated with the following standard proteins (all from Sigma): bovine serum albumin (mol. wt 68000), rabbit alkaline phosphatase (78000) and rabbit aldolase (145000).

Table 1. *Variation of carboxymethylcellulase and cellulase activities during growth*

The total activity was measured in untreated culture samples and the soluble activity was measured in samples from which organisms and cellulose had been removed by centrifuging, as described in Methods. One unit of carboxymethylcellulase released 1 μmol reducing sugar min^{-1} and one unit of cellulase released 1 μmol reducing sugar h^{-1} .

Incubation time (h)	Carboxymethylcellulase			Cellulase		
	Total activity (units)	Soluble activity		Total activity (units)	Soluble activity	
		(units ml^{-1})	(% of total)		(units ml^{-1})	(% of total)
13	12.4	1.3	10.0	1.0	—	—
39	40.6	14.8	36.0	2.7	1.0	37.0
63	109.0	40.6	37.0	4.3	1.6	37.0
144	88.2	37.1	42.0	3.6	1.4	38.9

Electrophoresis. Preparative acrylamide gel electrophoresis was carried out using a 7900 Uniphor column electrophoresis system (LKB). The sample (39 mg protein) was layered on top of a 5 % (w/v) gel. Electrophoresis was carried out at 5 °C at a constant current of 20 mA; the upper buffer was 0.05 M-Tris/glycine (pH 8.9) and the lower and elution buffer was 0.1 M-Tris/HCl (pH 8.1). Fractions of 3 ml were collected and the active fractions were combined and concentrated.

Analytical disc gel electrophoresis was carried out at pH 8.3 by the method of Davis (1964) on 5 % (w/v) acrylamide gels with bromophenol as the tracking dye. A current of 3 mA per gel was applied for 3 to 4 h at room temperature. Protein was stained with Coomassie blue and carbohydrate was stained with the periodic acid-Schiff reagent (Zaccharius *et al.*, 1969). Reducing sugars present in the enzyme preparation were detected by the method of Gabriel & Wang (1969). To locate the enzyme activity after electrophoresis the gel was sliced into 2 mm sections and each was incubated overnight in 1 ml 5 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 7.0, before assay. Reducing sugars liberated from carboxymethylcellulose were estimated as described above.

RESULTS AND DISCUSSION

The location of carboxymethylcellulase and cellulase of *C. thermocellum* changed during growth. After 13 h incubation, only 10 % of the cellulase activity was recovered in the cellulose-free supernatant, but after 63 h, more than 30 % was recovered in the supernatant. In the young culture, most of the enzyme was bound to cellulose and as the culture aged more enzyme was released into the medium (Table 1). The total cellulase activity increased during growth and reached a constant level after 60 h growth. Consequently, for purification of the enzyme, we used the supernatant of a 6 d-old culture, harvested by centrifugation and concentrated immediately.

Attempts to purify the cellulase by gel filtration on Sephadex or by chromatography on DEAE-cellulose were unsuccessful, as were those of Lee & Blackburn (1975). The concentrated supernatant was subjected to preparative polyacrylamide gel electrophoresis as described in Methods. The yield obtained using this procedure was low, with only about 10 % of the enzyme activity being recovered. One reason for this low recovery was that a significant amount of the cellulase did not migrate and was found at the top of the gel after electrophoresis. The specific activity of the culture supernatant was 52 units (mg protein) $^{-1}$; this was increased to 105 units (mg protein) $^{-1}$ by concentration and to 140 units (mg protein) $^{-1}$ by electrophoresis.

Analytical disc polyacrylamide gel electrophoresis was carried out before and after preparative electrophoresis (Fig. 1). The concentrated supernatant showed several protein bands but only one, near the electrophoresis front, had cellulase activity. Active fractions from preparative electrophoresis showed a single protein band after staining gels with Coomassie blue, and analytical tests showed that this protein band had carboxymethylcellulase and cellulase activities and gave a positive reaction with periodic acid-Schiff reagent, indicating the presence of sugar.

Fig. 1. Disc gel electrophoresis of cellulase on 5.0 % (w/v) polyacrylamide gels at pH 8.3: (a) concentrated supernatant, stained for protein; (b) purified fraction after preparative electrophoresis, stained by Coomassie blue; (c) as b, stained for reducing sugars; (d) as b, stained with periodic acid-Schiff reagent; (e) polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, purified fraction as in b.

However, gel electrophoresis in the presence of sodium dodecyl sulphate (SDS), as described by Weber & Osborn (1969), indicated that at least five proteins were present in the purified cellulase. Activity assays on the SDS-polyacrylamide gel showed that the protein with the highest molecular weight had cellulolytic activity. The presence of several components, revealed by SDS-polyacrylamide electrophoresis, and of both carboxymethyl-cellulase and cellulase activities could indicate that the protein obtained by preparative electrophoresis is a multi-enzyme complex.

Gel filtration was used to determine the molecular weight of the complex purified by preparative electrophoresis. Since glucanases are strongly absorbed on a polysaccharide matrix and binding is of a substrate affinity type (Marshall, 1973), the molecular weight had to be determined in the absence of such interactions. A polyacrylamide gel devoid of sugar residues, Bio-Gel P-200, was therefore used and a molecular weight of 125000 ± 10000 was determined for the complex.

Total carbohydrate, estimated as glucose by the phenol/ H_2SO_4 method, represented 9.9 % (w/w) of the protein. From this determination and the positive reaction with the periodic acid-Schiff reagent, it is evident that some carbohydrates are tightly bound to the cellulase. However, the nature of the association of the protein and carbohydrates is not known. Some proteins of the multi-enzyme complex could be glycoproteins, or possibly cellodextrins resulting from incomplete hydrolysis of cellulose are bound to proteins in the complex and dissociated only in the presence of SDS. Whatever the nature of the sugar-protein binding, it appears that carboxymethylcellulase and cellulase are exoenzymes which aggregate in a soluble complex of constant molecular weight. This complex possibly represents a form of protection against proteolytic degradation.

Other papers on the purification of bacterial cellulase have not reported the presence of such a complex. Osmundsvag & Goksoyr (1975) obtained two cellulases from *Sporocytophaga myxococcoides* with a low carbohydrate content and molecular weights of 46000 and 52000, each consisting of a single polypeptide chain. However, these cells were obtained under growth conditions where cellulose was nearly exhausted, whereas in our

case, even at the end of the culture period (6 d), half of the cellulose was still present. Under these conditions, it is possible that the cellulase released into the supernatant from cellulose has bound to it cello-dextrin produced by incomplete hydrolysis of cellulose. The presence of carbohydrate residues in the purified fraction favours such a hypothesis and could account for the cellulolytic complex which accumulates in *C. thermocellum* cultures.

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