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The cellulase of the anaerobic bacterium *Clostridium thermocellum*: isolation, dissociation, and reassociation of the cellulosome*

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ABSTRACT

The cellulosome of *Clostridium thermocellum*, isolated from the extracellular cellulase system by affinity chromatography on Whatman CC41 cellulose, contained >95% of the carboxylmethylcellulase activity applied to the column and could extensively solubilise "crystalline" cellulose when incubated in the presence of Ca^{2+} and dithiothreitol. An aqueous solution of the cellulosome could be dissociated into polypeptides by 0.5% sodium dodecyl sulphate–10mM ethylenediaminetetra-acetic acid–10mM dithiothreitol at 25° or 30° and pH 5. After dialysis, the polypeptides reassociated and there was a complete recovery of activity towards "crystalline" cellulose.

INTRODUCTION

Much of the research on the mechanism of cellulase action has been focused on the extracellular cellulase systems of fungal origin[1–3]. By comparison, little work has been published on the extracellular cellulases of bacteria, which, in the main, cannot degrade "crystalline", hydrogen-bond-ordered cellulose. An exception is the cellulase of the anaerobic bacterium *Clostridium thermocellum*[4], but little is known about the mechanism of action of this enzyme. The most significant observations have been that (*a*) the activity towards crystalline cellulose resides in a multicomponent enzyme complex (the so-called cellulosome) which has[5–7] a molecular mass of ~ 2×10^3 kDa, (*b*) the complex comprises[5] 14 subunits with masses in the range 45–120 kDa, (*c*) the activity towards crystalline cellulose is significant[4] only in the presence of Ca^{2+} and dithiothreitol (DTT), and (*d*) most of the subunits show endoglucanase activity with the notable exception of one with a molecular mass of ~ 210 kDa, which may be involved in binding the complex to the substrate[5,8].

Clarification of the mechanism of action has been frustrated by the difficulty of dissociating the cellulosome into its components without extensive loss in activity towards crystalline cellulose. Hitherto, relatively drastic conditions[7,8] have been used to try to dissociate the cellulosome. However, we now report that the dissociation can be effected under mild conditions and that the resulting polypeptides can be reassociated to

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give a cellulase that has the same activity towards crystalline cellulose as the original cellulosome.

EXPERIMENTAL

Materials. — *Clostridium thermocellum* NCIB 10682 was obtained from the National Collection of Industrial and Marine Bacteria (Torry, Aberdeen), Acrylagel and Bis-acrylagel from National Diagnostics, and CM-cellulose (low viscosity), sodium dodecyl sulphate (SDS), EDTA, and 1,4-dithio-DL-threitol (DTT) from the Sigma Chemical Company.

Preparation of the cell-free cellulase. — The bacterium was grown in GM-medium[9], using Solka Floc SW-40 (1%) as the carbon source, in a 16-L stirred-tank fermenter (SF-16, New Brunswick Scientific Company) for 48 h at 60°. Nitrogen was bubbled constantly through the suspension. The culture was filtered through a GF/A filter (1.6 μm) or centrifuged (76 000*g* for 20 min. Cell-free cellulase was then isolated by adding $(NH_4)_2SO_4$ to 80% saturation at 0°, as described[10].

Isolation of the cellulosome. — The pH of a sample (5 mL) of enzyme solution containing protein[11] (76 mg) and CM-cellulase activity (315 I.U.) was adjusted to 7.7, using aqueous 0.5% w/v NaOH, and applied to a column (10 × 1.5 cm) of Whatman CC41 cellulose, equilibrated with 50mM Tris HCl buffer (pH 7.7). The column was eluted with the buffer and then with distilled water. Activity towards crystalline cellulose was associated only with the fraction eluted with distilled water. The fraction was concentrated 10-fold using an Amicon PM-10 membrane and stored at −18°.

Dissociation of the cellulosome. — Samples (500 μL) of aqueous enzyme solution (500 μL) containing CM-cellulase activity (3 I.U.) and protein[12] were heated severally at 25, 30, 45, or 60° for 25 min in a reaction mixture (725 μL)[12] which was variously 0.5, 0.2, 0.1, or 0.05% with respect to SDS, 70 or 10mM with respect to EDTA, and 10mM with respect to DTT. Some mixtures (950 μL) were buffered at pH 3.5 or 5 with sodium acetate (final concentration 0.05M). Activity of the dissociated cellulosome towards crystalline cellulose (cotton fibre) was measured using an aliquot containing 0.3 I.U. of CM-cellulase (see below).

Enzyme assays. — Cellulase activity was assayed using soluble carboxymethylcellulose (CM-cellulose) and crystalline cellulose (cotton fibre).

(*a*) *CM-cellulase activity.* — A mixture (2 mL) containing aqueous 1% (w/v) CM-cellulose (1 mL), 0.2M 4-morpholine-ethanesulphonic acid (MES) buffer (0.5 mL, pH 6.0), and diluted enzyme was incubated at 60° for 15 min. The reaction was stopped by adding 2 mL of Somogyi reagent, and the reducing sugar was determined by the Somogyi–Nelson[12] method using D-glucose as standard. Neither SDS nor DTT interfered significantly, at the dilution used, with either the CM-cellulase activity or the reducing sugar assay.

(*b*) *Activity towards cotton.* — A mixture (5 mL) consisting of dewaxed[13] cotton fibre (2 mg), 0.2M MES buffer (1.25 mL, pH 6.0), 70mM $CaCl_2$ (0.5 mL, 7mM final concentration), 100mM DTT (0.5 mL, 10mM final concentration), 0.05M NaN_3 (0.05

mL), enzyme (0.3 I.U. of CM-cellulase), and water was incubated at 60° for 72 h. The residual cellulose was then determined using the potassium dichromate–sulphuric acid reagent[14].

Determination of protein. — Protein was determined using either the bicinchonic acid (BCA) reagent, as described in the Pierce catalogue (No. 23220/2325) or, where appropriate, the method of Bradford[11]. Each reagent was calibrated using bovine serum albumin as standard.

SDS-polyacylamide gel electrophoresis (SDS-PAGE). — An LKB 2050 midget or an LKB 2001 vertical electrophoresis apparatus[19] was used with 0.2% of SDS in both the stacking (4%) and separating gels (6%), with a constant current of either 40 or 100 mA, respectively. The buffer consisted of 0.1% of SDS in 25mM Tris–glycine (pH 8.9).

The dissociated samples were prepared for electrophoresis by diluting (1:1) the solution containing the cellulosome with "sample treatment buffer", which contained 0.2% of SDS, 10% of glycerol, and 0.02% of Bromophenol Blue in 125mM Tris–HCl buffer (pH 6.8). The sample treatment buffer did not dissociate the cellulosome unless the mixture was boiled or unless the pH was reduced to 5.5. Similarly, the SDS in the gel did not cause any dissociation.

Some samples were denatured before electrophoresis by boiling for 5 min in a solution which contained 2% of SDS, 10% of glycerol, 5% of 2-mercaptoethanol, and 0.02% of Bromophenol Blue in 125mM Tris–HCl buffer (pH 6.8). This heat-treatment step was omitted for samples in which the cellulosome was dissociated under conditions designed to maximise the recovery of activity towards crystalline cellulose.

After electrophoresis, the gel was fixed in aqueous 10% trichloroacetic acid for 5 min and stained with 0.25% of Coomassie Blue in acetic acid–methanol–water (1:3.5:8). The mixture of protein standards contained myosin (205 kDa), β-D-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine plasma albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa).

RESULTS

Isolation of the cellulosome. — The cellulosome was obtained by affinity chromatography on Whatman CC41 cellulose (see Experimental). The fractions eluted with buffer and water contained almost equal amounts of protein, but the former was not active towards crystalline cellulose and contained only 5% of the total CM-cellulase activity eluted from the column. In contrast, the latter fraction (the cellulosome[6]) contained 95% of the CM-cellulase activity and effected 82% solubilisation of cotton in the presence of Ca^{2+} and DTT. Chromatography of the cellulosome fraction on a column (100 × 1.5 cm) of Sepharose 6B equilibrated with 20mM Tris–HCl buffer (pH 7.7), which had been calibrated with Blue Dextran and high molecular weight standards (Pharmacia), separated a large protein (~ 2×10^3 kDa) and 4.5% of a smaller component (~ 2.5×10^5 kDa).

SDS-PAGE, under non-denaturing conditions (without boiling), of the fraction eluted with buffer and the cellulosome revealed that the former comprised some low

molecular weight proteins, whereas the latter was composed mainly of high molecular weight material that did not enter the gel in SDS-PAGE. After the cellulosome fraction had been boiled in SDS–2-mercaptoethanol, SDS-PAGE revealed that the complex had dissociated into five major subunits (210–215, 95, 75, 68, and 51 kDa) and eight minor components (205, 195, 166, 150, 145, 134, 107, and 87 kDa) that were only observed when the chromatograms were heavily loaded. Several cellulosome preparations, isolated from different cultures of the same strain of bacterium, gave similar patterns of dissociation.

Dissociation of the cellulosome. — The aqueous solution of the cellulosome (containing 3 I.U. of CM-cellulase activity) was treated with a solution containing 0.2% of SDS, 10mM EDTA, and 10mM DTT severally at 30, 45, and 60°, and at pH 5.5. As shown in Fig. 1, the cellulosome was dissociated extensively by each treatment to give patterns of polypeptides which were similar to each other and to that resulting from boiling the cellulosome in aqueous 2% SDS containing 5% of 2-mercaptoethanol. A notable difference was that the major polypeptide in the region 205–215 kDa seemed to vary in mass according to the temperature used for dissociation (Fig. 1, lanes 4–6). Dissociation at 30° additionally yielded a small amount of a component with a mass of ~48 kDa. Only traces of the undissociated cellulosome (which was unable to enter the gel in SDS-PAGE) were detected. Thus, the pattern of dissociation of the cellulosome varies according to the temperature used.

When the dissociating agents were removed from the mixture of polypeptides by dialysis in a collodion membrane against 20mM Tris–HCl buffer (pH 7.7) containing 2.5mM DTT, SDS-PAGE showed that reassociation had occurred to give a high molecular weight complex which did not enter the gel (Fig. 1, lanes 7 and 8) to a significant extent; only traces of the dissociated polypeptides (48–215 kDa) remained.

The activity of the reassociated polypeptides was tested towards cotton fibre. As shown by the results in Table I, 65–70% of the activity of the undissociated cellulosome

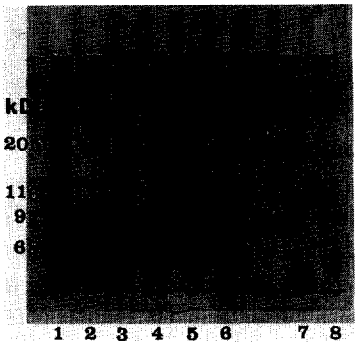

Fig. 1. Composition of *C. thermocellum* cellulosome dissociated by SDS–EDTA–DTT at 30, 45, and 60°, and reassociated by dialysis (see Experimental). Lanes: 1, molecular weight markers; 2, cellulosome; 3, cellulosome dissociated by boiling with 2% SDS–2-mercaptoethanol for 5 min (control); 4, cellulosome dissociated at 30°; 5, cellulosome dissociated at 45°; 6, cellulosome dissociated at 60°; 7, cellulosome reassociated after dissociation at 30°; 8, cellulosome reassociated after dissociation at 45°; 15 μg of protein were applied to each lane.

TABLE I

The effect of temperature on the cellulase activity of the cellulosome in the dissociated and reassociated states

Enzyme/dissociating solution (DS)	*Dissociating temperature (degrees)*	*Hydrolysis of cotton (%)*[a]		
		A	*B*	*C*
Original cellulosome	–	–	–	71
Cellulosome	30	–	71	–
Cellulosome + DS	30	48	–	–
Cellulosome	45	–	64	–
Cellulosome + DS	45	39	–	–
Cellulosome	60	–	63	–
Cellulosome + DS	60	26	–	–

[a] Effected by an enzyme solution containing 0.3 I.U of CM-cellulase activity. Columns: A, the cellulosome was dissociated using a solution of 0.2% SDS, 10mM EDTA, and 10mM DTT (see Experimental), then reassociated by dialysis at room temperature in a collodion membrane against 20mM Tris–HCl buffer (pH 7.7) containing 2.5mM DTT; B, control (the untreated cellulosome was incubated at the temperatures indicated); C, untreated cellulosome.

towards cotton fibre was recovered (*cf.* columns B and C in Table I). The enzyme dissociated at 25° and then reassociated gave results (not shown in Table I) similar to those obtained at 30°. The pH of the dialysis buffer used to reassociate the subunits was critical. At pH values <5, the recovery of activity towards crystalline cellulose was poor; buffers of pH ≥ 6.5 were the best.

There was no marked dissociation of the cellulosome at 25–30° and pH 7 or 6, as judged by SDS-PAGE. Slight dissociation was apparent in the absence of SDS in sodium acetate buffer of pH ≤ 5.0, but dissociation increased markedly in the presence of a mixture of SDS (0.05%), EDTA (10mM), and DTT (10mM).

The patterns of polypeptides produced on dissociation at pH 3.5 in the presence of 10mM EDTA, 10mM DTT, and SDS at 0.05 or 0.1% at 25° were shown by SDS-PAGE (Fig. 2, lanes 3 and 4) to be similar to that observed on boiling in SDS (Fig. 2, lane 1) in that it contained a 215-kDa polypeptide. When dissociation was effected at pH 5, the 215-kDa polypeptide was absent, but new polypeptides in the region 180–190 kDa were generated (Fig. 2, lanes 8 and 9).

SDS-PAGE showed that the components which were dissociated under different conditions (Fig. 2, lanes 3, 4, 8, and 9) were associated into high molecular weight components when SDS and other reagents were removed by dialysis (Fig. 2, lanes 6, 11, and 12) and these components could not enter the gel to a significant extent. In one instance, much of the complexed material was unable to penetrate even the 4% stacking gel (Fig. 2, lane 6). When the reassociated complexes were boiled in SDS–2-mercaptoethanol, they showed the same subunit composition as the original cellulosome on SDS-PAGE (not shown in Fig. 2).

The results in Table II show that a large proportion of the activity of the undissociated cellulosome towards cotton was recovered when the cellulosome which

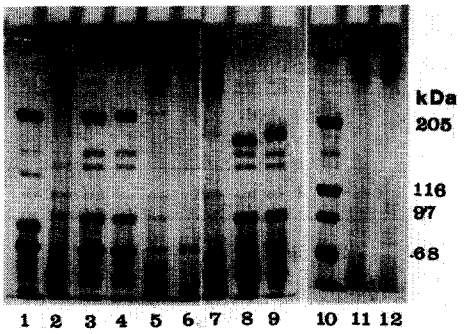

Fig. 2. Composition of *C. thermocellum* cellulosome dissociated by a mixture of SDS–EDTA–DTT at pH 3.5 and 5, and 25° then reassociated (see Experimental). Lanes: 1, cellulosome dissociated by boiling in SDS–2-mercaptoethanol; 2, cellulosome treated with buffer at pH 3.5; 3, cellulosome dissociated with a solution containing 0.05% SDS at pH 3.5; 4, as in 3 but with 0.1% SDS; 5, as in 2 but dialysed; 6 as in 3 but dialysed; 7, cellulosome treated at pH 5.0; 8 and 9, cellulosome dissociated by solutions at pH 5 containing 0.05 and 0.1% SDS, respectively; 10, marker proteins; 11, as in 8 but dialysed; 12, as in 9 but dialysed; 15 μg of protein were applied to each lane.

TABLE II

The effect of pH on the cellulase activity of the cellulosome in the intact and reassociated states

SDS concn. (%)	*pH of dissociation*	*Hydrolysis of cotton (%)*[a]	
		A	*B*
nil	–	–	71
0.05	3.5	45	–
0.1	3.5	37	–
0.05	5.0	83	–
0.1	5.0	71	–

[a] Effected by an enzyme solution containing 0.3 I.U. of CM-cellulase activity. Columns: A, the cellulosome was dissociated at 25° in the presence of 10mM EDTA, 10mM DTT, and 0.1 or 0.05% SDS for 30 min (see Experimental), then reassociated as in Table I; B, undissociated cellulosome.

had been dissociated by a solution containing 0.05% of SDS, 10mM EDTA, and 10mM DTT at pH 3.5 was dialysed against 20mM Tris–HCl buffer (pH 7.7) containing 2.5mM DTT. However, recovery of activity was even higher after dialysis when the dissociation was carried out at pH 5. Indeed, when the cellulosome was dissociated at pH 5.0 and then reassociated by dialysis, the product showed the same capacity to degrade cotton fibre as the untreated cellulosome.

DISCUSSION

The cellulosome preparation, isolated and partially purified by affinity chromatography on cellulose by the method of Lamed *et al.*[15], had a molecular weight of ~2 ×

10^6 and could be dissociated into 13–14 subunits by treatment with SDS–2-mercaptoethanol. The sizes of the subunits isolated from the cellulase produced by strain NCIB 10682 used in the present study (210–215, 205, 195, 166, 150, 145, 134, 107, 95, 87, 75, 68, and 51 kDa) are similar to, but not identical with, those generated from the cellulase produced by strain YS[5] (210, 170, 150, 115, 98, 91, 84, 75, 67, 66, 60, 57, 54, and 48 kDa).

Wu *et al.*[8] showed that a mixture of SDS, EDTA, and DTT is effective for the partial dissociation of the cellulosome. However, as noted above, unless the conditions used for the dissociation are carefully controlled, the individual polypeptides formed are denatured to some extent so that the high molecular weight complex formed on reassociation has markedly reduced activity towards crystalline cellulose. Temperature, pH, SDS concentration, and the method of disrupting the SDS–protein complexes and removing the dissociating agents are all critical if the cellulosome, after dissociation and reassociation, is to give a product with all the activity of the original undissociated cellulosome. Thus, low temperatures (25–30°) are better than higher temperatures (45–60°). Indeed, in the presence of SDS and EDTA, dissociation occurred readily at 25–30°, provided the pH was ≤ 5.5. However, cellulosome dissociated at pH ≤ 4, followed by reassociation was not very active towards cotton fibre. Reassociated cellulosome with the highest activity towards cotton fibre was obtained when the dialysis buffer had a pH of 7.7 and contained DTT. At pH ≤ 5, the reassociated product had little activity.

The degradation of crystalline cellulose by aerobic fungi involves the synergistic action of enzymes currently designated as cellobiohydrolases and endoglucanases[1]. So far, only enzymes with endoglucanase activity have been obtained in the purified state from the supernatant solutions of cultures of *C. thermocellum*[16–18]. It is possible that the mechanisms of degradation of cellulose may differ in anaerobic bacteria and aerobic fungi[16]. Electron microscopy of fragments of cellulose isolated from cultures of *C. thermocellum* indicate that the cellulose may be cleaved simply by endoglucanase-type enzymes aggregated in the cellulosome[16]. These endoglucanases are presumed to operate at every fourth glucosidic linkage in the cellulose chain by rows of polypeptide subunits which are spaced equidistantly and in a defined orientation[16]. If this hypothesis is correct, the cellulosome complex which is dissociated in part by SDS–EDTA–DTT in the present study would have to reassociate perfectly. In view of the number of components produced on dissociation, this outcome might be regarded as unlikely. However, the data presented here support such a proposition.

Elucidation of the conditions for the dissociation and reassociation of the cellulosome may help in the study of the mechanism of action of the cellulase in *C. thermocellum*, and perhaps in other cellulolytic bacteria.

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