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The anaerobic fungus *Neocallimastix frontalis*: isolation and properties of a cellulosome-type enzyme fraction with the capacity to solubilize hydrogen-bond-ordered cellulose

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Summary. A minor component isolated from the extracellular cellulase of the anaerobic rumen fungus *Neocallimastix frontalis* by adsorption on cellulose had a remarkable capacity to degrade crystalline hydrogen-bond-ordered cellulose. When produced in a semi-defined medium the component comprised normally less than 4% of the total protein and only 0.3% of the protein in cultures containing rumen fluid. The minor component showed endoglucanase (carboxymethylcellulase) and β-glucosidase activity and effected the extensive hydrolysis of "crystalline" cellulose in the form of the cotton fibre when acting alone. Glucose was the sole product of hydrolysis. The specific activity of the crystalline cellulose solubilizing factor (CCSF) in degrading cotton fibre was much higher than any other cellulase or cellulase component reported so far. The activity of the CSSF to crystalline hydrogen-bond-ordered cellulose resides in a high molecular mass complex of 670 kDa, that comprised a number of subunits ranging in size from 68 to 135 kDa.

Introduction

Microbial cellulose is potentially commercially important for the generation of fermentable glucose from lignocellulosic wastes (Eriksson and Wood 1985). The extracellular cellulase that has been studied most extensively in this context is that from the aerobic fungus *Trichoderma reesei* (Mandels 1982). Unfortunately, from a commercial point of view the specific activity of this cellulase is low when crystalline cellulose is the substrate, and this has prevented its industrial use for the production of glucose. Recently, it was demonstrated that the extracellular cellulase from the anaerobic rumen fungus *Neocallimastix frontalis* was remarkable in that it was much more active on cellulose in one of its most recalcitrant forms, namely, the cotton fibre, than the extracellular cellulase of *T. reesei* (Wood et al. 1986, 1988).

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Clearly, an understanding of the reasons for the high activity shown by *N. frontalis* cellulase may provide information essential to the effective commercial use of this enzyme. In this report we demonstrate that the extracellular cellulase of *N. frontalis* contains a single multicomponent cellulase complex that is responsible for the activity towards crystalline cellulose, hereafter called the crystalline cellulose-solubilising factor (CCSF). In this respect at least it is similar to the extracellular cellulase of the bacterium *Clostridium thermocellum*, which degrades crystalline cellulose with a multicomponent enzyme complex called the cellulosome (Lamed et al. 1983; Lamed and Bayer 1988).

Materials and methods

Chemicals. Acrylagel and bis-acrylagel for polyacrylamide gel electrophoresis (PAGE) were purchased from National Diagnostics (Highland Park, N. J., USA), Ultrogel AcA 22 was from Pharmacia (carboxymethylcellulose, Milton Keynes, UK); (CMC; low viscosity grade) and was bought from Sigma (Poole, Dorset, UK); low molecular mass standards were purchased from BDH (Poole, Dorset, UK). Blue dextran (2×10^6), thyroglobulin (660 kDa), ferritin (440 kDa) and catalase (232 kDa) were all from the calibration kit for gel filtration sold by Pharmacia, Milton Keynes, UK. All other chemicals were bought from Sigma.

Preparation of enzymes. N. frontalis cellulase: *N. frontalis* RK21 was isolated from sheep digesta (Wood et al. 1986) and cultured exactly as described by Wood et al. (1986) or by using strips of Whatman cellulose filter paper suspended in semi-defined salts medium defined by Lowe et al. (1985). The concentration of the carbon source was 0.5% (w/w). Fermentation was allowed to proceed for 9 days. The cell-free enzyme was concentrated (Wood et al. 1986) by ultrafiltration using a hollow fibre cartridge (Amicon, Danvers, Mass., USA; H1P10-8) followed by equilibration in an Amicon cell (PM 10 membrane) with 0.1 M KH_2PO_4-NaOH buffer, pH 6.7.

C. thermocellum cellulase: *C. thermocellum* (NCIB 10682) was grown in a mineral salts medium (Garcia-Martinez et al. 1984) containing 1% (w/v) Solka Floc SW 400 for 48 h as previously described (Johnson et al. 1982). The cell-free crude extract was obtained by centrifuging the culture fluid for 20 min at 75000 *g*: the enzyme was precipitated by the addition of solid $(NH_4)SO_4$ to

85% saturation. The enzyme was dissolved in 50 mM TRIS-HCl buffer, pH 7.7, and an aliquot (5 ml) containing 75 mg protein was applied to a column (1.0 cm × 14.7 cm) of Whatman CC41 equilibrated with 50 mM TRIS-HCl buffer, pH 7.0. The column was washed first with buffer and then the cellulosome was eluted with water.

T. reesei cellulase: *T. reesei* was cultured in a fermentor as previously described (Mandels 1982). The cell-free enzyme was precipitated with $(NH_4)_2SO_4$ (20–80% saturation), redissolved in 0.1 M acetate buffer, pH 5.0, and desalted on a column of Sephadex G-25.

Assay methods. Enzyme assays were carried out in 0.1 M KH_2PO_4-NaOH buffer, pH 6.7, at 40° C. Cellulase activity was determined by measuring the residual cellulose left after incubating the enzyme with dewaxed cotton fibre (Corbett 1963; Wood 1969) or by measuring the release of reducing sugars (Nelson 1952) from a solution of CMC (Wood and McCrae 1977) using glucose as standard. β-Glucosidase activity was determined using 1 mM 4-methylumbelliferylglucoside as substrate. One international unit of carboxymethylcellulase (CMCase) and β-glucosidase is defined as the release of 1 μmol reducing sugar (glucose equivalent) or 4-methylumbelliferone per minute, respectively. Activity towards cotton is expressed as the percentage hydrolysis effected in 42–72 h under the standard conditions (Wood et al. 1986). Protein was quantified by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Sodium dodecyl sulphate (SDS)-PAGE. The subunit composition of the fungal enzymes was determined using 11% (w/v) gels according to the method of Laemmli (1970). BDH low-molecular-mass standards were used to calibrate the gels. Protein bands were detected using the Coomassie Blue stain (Wood and McCrae 1977).

Hydrolysis of various cellulosic substrates. The reaction mixture consisted of 5 mg of either cotton fibre, Whatman cellulose powder CC41, barley straw, oat straw, birchwood α-cellulose (Green 1963) or Avicel (microcrystalline cellulose), 2.5 ml of 0.1 M KH_2PO_4-NaOH buffer, pH 6.0, 0.1 ml NaN_3 (0.05 M) and enzyme solution to give a final volume of 5.0 ml. The enzyme solution contained 100 μg protein. After incubation for 140 h (90 h in the case of Avicel) at 37° C, the tubes were centrifuged (3000*g* for 10 min) and the reducing sugar in the supernatant was determined. The H_3PO_4-swollen cellulose (Wood 1971) was added to the tube as a suspension (0.5 ml containing 10 mg/ml).

Comparison of activities of N. frontalis, C. thermocellum and T. reesei cellulase on cotton fibre. A 2-mg sample of dewaxed cotton fibre was incubated in a 12-ml centrifuge tube with buffer (4 ml) and an aqueous solution of either *N. frontalis*, *C. thermocellum* or *T. reesei* cellulase in a total volume of 5.0 ml for 54 h. In each case the enzyme solution contained 25 μg of protein. For *N. frontalis* and *C. thermocellum* cellulases 0.1 M KH_2PO_4-NaOH buffer, pH 6.0, containing 0.05 M NaN_3 (bacteriostat) was used: activity of *T. reesei* cellulase was measured in 0.1 M acetate buffer, pH 5.0, containing 0.05 M NaN_3. The enzymes were incubated at their temperature optimum: 40° C for *N. frontalis*; 60° C for *C. thermocellum*; 50° C for *T. reesei*. The residual cotton fibre was measured, as previously described (Wood 1969).

Isolation of the CCSF by adsorption on cellulose. This was done by adding 5 ml dry Avicel PH 101 to 5.8 ml of concentrated enzyme solution at 4° C. Phosphate buffer (0.1 M K_2HPO_4-NaOH, pH 6.7) was added to bring the volume to 20 ml and the suspension was stirred at 4° C for 10 min. The suspension was centrifuged (100 000*g* for 20 min) and the pellet resuspended in the phosphate buffer (final volume 20 ml). The reaction mixture was stirred for 10 min at 4° C and then centrifuged as before. The washing procedure on the pellet was carried out once with 20 ml buffer and then three times with 20 ml water.

Kinetics of hydrolysis of crystalline cellulose by cellulase components from N. frontalis, C. thermocellum and T. reesei. The CCSF used was that isolated from the crude cellulase (semi-defined medium) preparation of *N. frontalis* using affinity chromatography on Avicel (above). The cellulosome of *C. thermocellum* was isolated in a similar way to the CCSF of *N. frontalis* but using a column of Whatman cellulose powder instead of Avicel (Bhat and Wood 1992). Affinity chromatography on cellulose was found to be unsuitable for the purification of the cellulase of *T. reesei*, which is known to comprise individual cellobiohydrolases, endoglucanases and β-glucosidases that interact synergistically in solubilizing crystalline cellulose (Eriksson and Wood 1985). The *T. reesei* cellulase was used without fractionation. This would seem acceptable as nearly all of the extracellular protein in the culture fluid is cellulase (Mandels 1982).

Results and discussion

Isolation of the CCSF by adsorption on cellulose

Attempts to isolate the CCSF by affinity chromatography on a column of Avicel PH 101 was not successful because the activity of the enzyme was such that even at 4° C the Avicel was rapidly hydrolysed to colloidal particles that clogged the column. After a time, the fine particles appeared in the column effluent. The problem of clogging was successfully circumvented by stirring the culture supernatant and the Avicel in a beaker, isolating the Avicel by centrifugation, and then washing the Avicel with buffer and water, as described in Materials and methods. Table 1 shows the results of a typical purification experiment. The various washes of the Avicel adsorbent were assayed for endoglucanase (CMCase), β-glucosidase, protein, and activity to cotton fibre.

As can be seen from Table 1, the CCSF was not desorbed from the Avicel until it had been washed three times with water, at which stage all the CCSF was removed in a single wash. The third water wash was iden-

Table 1. Distribution of the various enzyme activities during fractionation of *Neocallimastix frontalis* crude cellulase (rumen fluid-containing medium) by affinity on cellulose

Fraction	CMCase (%)	β-Glucosidase (%)	Protein (%)	Cotton solubilisation (%)[a]
Non-adsorbed enzyme	67.9	91.5	91.8	0.0
1st buffer wash of cellulose	8.6	6.0	5.7	0.0
2nd buffer wash	2.1	0.7	1.2	0.0
1st water wash	2.0	0.4	0.6	0.0
2nd water wash	3.9	0.4	1.0	1.0
3rd water wash	1.8	0.1	0.3	80.7
4th water wash	0.0	0.0	0.3	0.0

The percentages quoted for the amount of enzyme found in the various washes are based on the amount of activity fractionated: CMCase, carboxymethylcellulase

[a] 25 μg of protein was used for the assay of activity towards cotton fibre

Table 2. Protein and enzyme activities in crude culture filtrate[a] of *N. frontalis* and in the fraction adsorbed on cellulose (CCSF)

Enzyme	CMCase			β-Glucosidase			Protein	
	IU total	% total	Specific activity	IU total	% total	Specific activity	mg	% total
Crude culture filtrate[a]	137.2	100.0	1.1	71.8	100.0	0.6	125	100.0
CCSF	10.4	7.6	2.6	0.6	0.8	0.2	3.9	3.2

Specific activity = activity per milligram of protein: CCSF, crystalline cellulose-solubilizing factor; IU, international units of activity
[a] prepared in semi-defined medium

tified as containing CCSF when it was found to be the only sample with the capacity to degrade cotton fibre extensively. Of the enzyme eluted from the Avicel adsorbent, only 0.1% of the β-glucosidase and 1.8% of the endoglucanase was associated with the CCSF. Fractionation carried out on enzyme isolated from cultures containing semi-defined medium were similar, but 7.6% of the endoglucanase (CMC ase), 0.8% of the β-glucosidase and 3.2% of the protein in the enzyme preparation was associated with the CCSF (Table 2).

Further purification of the CCSF by gel filtration

The CCSF isolated by adsorption on Avicel (3rd water wash - Table 1) was concentrated in an Amicon cell using a PM 10 membrane (cut-off 10 kDa) to 0.5 ml and equilibrated by repeated concentration and dilution with 0.1 M KH_2PO_4-NaOH buffer, pH 6.7, containing 0.05 M NaN_3. The concentrate (0.25 ml) was applied to a column of Ultrogel AcA 22 (1.6 cm × 84 cm) equilibrated with phosphate buffer, pH 6.7, and eluted with the same buffer. The CCSF chromatographed as a component of molecular mass 670 kDa: a small amount of contaminating protein, which had a molecular mass of less than 200 kDa, was separated. Activity towards cotton was found only in the high-molecular-mass component, which was also rich in CMCase and β-glucosidase activity.

SDS-PAGE gel electrophoresis of the CCSF

The high-molecular-mass component dissociated on treatment with SDS/mercaptoethanol, as indicated Materials and methods, into several components that could be separated on SDS-PAGE (Fig. 1). The major polypeptide bands had molecular masses of 86, 81, 76 and 63 kDa: minor components of molecular mass 56 and 126 kDa were also present.

Comparison of the kinetics of hydrolysis of crystalline cellulose by cellulase components from N. frontalis, C. thermocellum and T. reesei

Figure 2 shows the degree of hydrolysis of crystalline cellulose, in the form of cotton fibre, effected by 2.4–15 μg of enzyme protein, using conditions established to be optimal for the different enzymes (see legend to

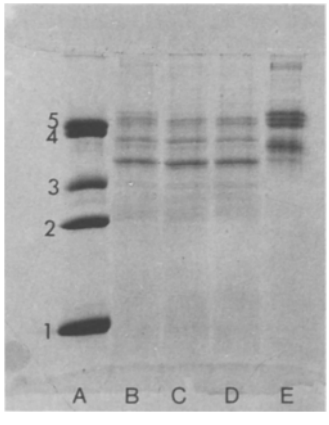

Fig. 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of *Neocallimastix frontalis* cellulase components: *lane A*, molecular weight standards (*1*, cytochrome C, 12 kDa; *2*, carbonic anhydrase, 30 kDa; *3*, ovalbumin, 45 kDa; *4*, albumin (bovine serum), 66 kDa; *5*, ovotransferrin, 77 kDa); *lane B*, concentrated unfractionated culture filtrate; *lanes C* and *D*, fraction not adsorbed on Avicel; *lane E*, crystalline cellulose-solubilizing factor (CCSF) found in 3rd water wash (Table 1)

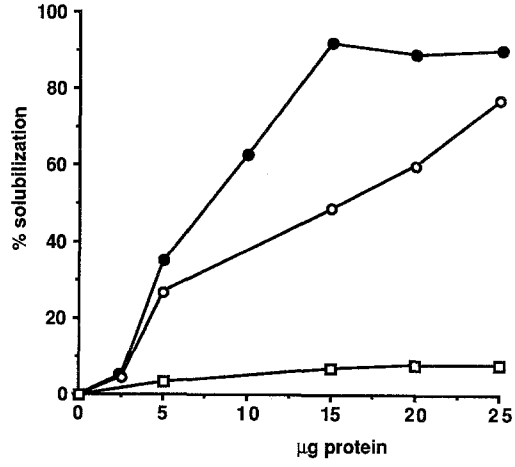

Fig. 2. Comparison of kinetics of hydrolysis of crystalline cellulose by the CCSF of *N. frontalis* (●), the cellulosome of *Clostridium thermocellum* (○), and the cellulase of *Trichoderma reesei* (□). The details of the preparation of the enzymes and the assays are given in Materials and methods. Incubation was carried out at the temperature optimum for each cellulase: 40° C, pH 6.0, for *N. frontalis*; 60° C, pH 6.0, for *C. thermocellum*; 50° C, pH 5.0, for *T. reesei* (Mandels 1982; Johnson et al. 1982)

Fig. 2). It can be seen that both the CCSF of *N. frontalis* and the *C. thermocellum* cellulosome were very active on crystalline cellulose in the form of the cotton fibre: *T. reesei* cellulase was significantly less active than the other two enzymes. However, of the three enzymes tested, the CCSF of *N. frontalis* was the most active.

Comparison of activity of CCSF of N. frontalis with the non-adsorbed enzyme on various types of cellulosic material

The two types of enzyme were compared by incubating 100 μg of protein with 5 mg of a variety of cellulosic substrates. The more reactive substrates such as CMC and phosphoric acid-swollen cellulose were incubated for only 1 h and 7 h, respectively: the other celluloses were incubated for 140 h. The results (Table 3) show that those substrates that were essentially pure cellulose and that were hydrogen-bond-ordered (Whatman cellulose powder, Avicel, Birchwood α-cellulose and cotton fibre), were extensively degraded by the CCSF, but the enzymes in the fraction that was not adsorbed on Avicel (Table 1) were unable to degrade any of these substrates to a significant extent.

Barley straw and oat straw, which are highly lignified and contain hemicellulose (principally xylan) as well as cellulose (Aspinall 1983), were scarcely degraded at all by either the CCSF or the non-adsorbed fraction, and this was to be expected. However, unexpected was the observation that rye grass, which is not a heavily lignified substrate, was degraded only to a very minor degree. This suggested that the CCSF lacks activities for the degradation either of the xylan polysaccharide or for the degradation of some other polysaccharides present in small amounts in the plant cell wall, which are acting as barriers to the hydrolysis of the cellulose and xylan.

Table 3. Hydrolysis of cellulosic materials by enzyme fractions separated by affinity on cellulose

Substrate	Enzyme used	Reducing sugar (mg)	Glucose (mg)
H_3PO_4-swollen cellulose	Non-adsorbed	2.5	1.9
	CCSF	2.5	1.4
Cotton	Non-adsorbed	0.1	0.1
	CCSF	4.7	5.3
Avicel	Non-adsorbed	0.1	0.1
	CCSF	4.2	4.8
Whatman cellulose powder	Non-adsorbed	0.1	0.1
	CCSF	3.5	3.9
Birchwood α-cellulose	Non-adsorbed	0.1	0.1
	CCSF	2.3	2.5
Rye grass	Non-adsorbed	0.3	0.2
	CCSF	0.5	0.4

One hundred micrograms of the protein were used in each case. The reaction mixtures were incubated for 140 h except in the assays involving Avicel and H_3PO_4-swollen cellulose: these were incubated for 90 h and 7 h, respectively

On the basis of the evidence presented in the paper it would seem that the cellulase of the anaerobic fungus is similar to the cellulase of the bacterium *C. thermocellum* in that the component that degrades crystalline cellulose contains several components arranged in a complex. There are, however, important differences between the two complexes. For example, the CCSF of *N. frontalis* has a molecular mass of the order of 700 kDa whereas the multicomponent cellulosome complex found in *C. thermocellum* cellulase is reported to have a molecular mass of 2 million Da (Lamed et al. 1983). Other differences are to be found in the fact that:

1. The cellulosome constitutes a relatively large proportion of the extracellular enzyme (up to 95% of the endoglucanase and 50% of the total extracellular protein, depending on the culture conditions) whereas the CCSF of *N. frontalis* comprises only ≈4% of the extracellular protein and is associated with 8% of the endoglucanase.

2. The cellulosome contains no β-glucosidase and generates only cellobiose from cellulose whereas the CCSF of *N. frontalis* is rich in β-glucosidase and produces virtually pure glucose from cellulose.

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