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PURIFICATION AND PARTIAL CHARACTERIZATION OF TWO EXTRACELLULAR ENDOGLUCANASES FROM CELLULOMONAS FERMENTANS

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SUMMARY. Avicelase assay of gel slices after non-denaturing polyacrylamide gel electrophoresis of concentrated supernatants from Cellulomonas fermentans revealed four active bands. One of them corresponded to the principal active band on CM-cellulose. Among the three others, at least one did not correspond to any active band on CM-cellulose and might reflect the presence of an exoglucanase (EC 3.2.1.91). The active band on CM-cellulose was composed of two endoglucanases (EC 3.2.1.4), called CFA and CFB, which we purified by the means of DEAE-Trisacryl chromatography and high performance liquid chromatography (anion exchange chromatography and gel chromatography). These two monomeric enzymes differ in their molecular weights (40 000 and 57 000 for CFA and CFB, respectively) and in their catalytic constants in the reaction with CM-cellulose (Km were 1.5 g/l and 59 g/l for CFA and CFB, respectively), but have similar modes of action on this substrate and similar substrate specificities.

The enzymatic degradation of cellulose is a problem which has still not yet been completely solved even though it has been investigated for a long time (1-8). Cellulose has been hydrolysed by several enzymes differing in substrate specificity, but which interact synergistically (2,3,8). Some, called endo-β-1,4-glucanases (EC 3.2.1.4), hydrolyse the cellulose molecule randomly in the region of low crystallinity of the cellulose fibre. The randomly acting enzymes increase the number of cellulose chain ends available to the enzymes liberating end groups, called exo-β-1,4-glucanases (EC 3.2.1.91). Studies on the components of the cellulase complex have indicated that there exist isoenzymes of endoglucanase and exoglucanase (9-11). The origin of these isoenzymes and especially their role in enzymatic hydrolysis have not yet been properly elucidated, however.

Cellulomonas fermentans, a cellulolytic bacteria isolated from urban waste (12) can grow on native cellulose (13). The purpose of the present study was to

Abbreviations : MOPS, 4-morpholinopropanesulfonic acid ; CM-cellulase, carboxymethyl-cellulase ; pNP-cellobiohydrolase, p-nitrophenyl cellobiohydrolase ; SDS, sodium dodecylsulfate ; HPLC, high performance liquid chromatography.

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characterize its extracellular cellulolytic system and particularly to purify the major endoglucanase components.

MATERIAL AND METHODS

Bacterial strain. The organism used in this study, Cellulomonas fermentans (strain M, DSM 3133, ATCC 43279), was isolated at our laboratory and its characterization has been reported elsewhere (12).

Growth conditions. C. fermentans was grown anaerobically at 30°C on the basal medium previously described (12) slightly modified (phosphate buffer was replaced by the mixture phosphate/MOPS at 5 mM/200 mM) to obtain a better production of CM-cellulase (13). Cultures were harvested at the beginning of the stationary phase

Cellulase assays:

CM-cellulase activity. Enzyme activity was determined by incubating 1 ml of appropriate enzyme dilution with 4 ml of 1 % CM-cellulose solution in 25 mM potassium phosphate buffer, pH 7, at 37°C. Samples were withdrawn and chilled to stop the reaction, after 0, 10 ,20 and 30 min. Reducing sugars were measured by the Park and Johnson ferricyanide method (14). One unit of activity is defined as 1 µmol of glucose equivalent liberated per min (IU).

A viscosimetric assay was also used. After incubation, samples were boiled for 10 min to inactivate enzymatic activity. The relative viscosity was determined at 20°C by comparing the flow rate of samples in a capillary pipette with that of water.

Activity on insoluble cellulosic substrates. This was determined by assaying at different times reducing sugars released from cellulose (cristalline cellulose Avicel PH 101 or amorphous cellulose) after removal of solids by centrifugation. Reaction condition and mixture were similar to those used for CM-cellulase activity, but CM-cellulose was replaced by the non-substituted cellulose substrate. One unit of activity corresponds to 1 µmol of glucose equivalent liberated per min. Amorphous cellulose was MN300 cellulose swollen as described by Walseth (15).

pNP-cellobiohydrolase activity. This was estimated by measuring the release of pNP from the pNP-β-D-cellobioside (16). Assays containing 0.9 ml of a pNP-β-D-cellobioside solution (1 g/l in a 25 mM phosphate buffer, pH 7, NaN_3 0.3 g/l) and 0.1 ml of enzyme sample were incubated at 37°C. pNP liberation was estimated at 400 nm. One unit (IU) of pNP-cellobiohydrolase activity was defined as the amount of enzyme liberating 1 µmol of pNP per min.

Activity on xylan. This was determined by measuring the release of reducing sugars from xylan. A suspension of commercial xylan (Fluka) was centrifuged at 12 000 g for 10 min in order to eliminate soluble reducing substance. The pellet was washed. Reaction conditions and mixture were similar to those used for CM-cellulase activity, but CM-cellulose was replaced by washed xylan. At various times of incubation, the reducing sugars released were measured after removal of solids by centrifugation, by the ferricyanide method. 1 unit (IU) of xylanase activity is defined as the amount of enzyme that released reducing power equivalent to 1 µmol of xylose per min.

Protein determination. Protein was determined by a modified Lowry method (17) using bovine serum albumin as a standard. In fractionation experiments protein was estimated by its absorbance at 280 nm (A_{280}).

Release of substrate-bound enzymes. The technique of Langsford et al. was used (18).

Polyacrylamide gel electrophoresis. Analytical disc gel electrophoresis was carried out by the method of Lugtenberg et al. (19) using 7.5 % acrylamide gel with bromophenol blue as the tracking dye. A current of 3 mA/gel was applied and protein was stained with Coomassie blue. To locate enzymatic activities, the gel was cut into 2 mm thick slices and each slice was incubated overnight in 0.2 ml 25 mM phosphate buffer, pH 7. This elution buffer was used for assaying enzymatic activities as described above. Polyacrylamide gel electrophoresis in the presence of SDS was performed according to Lugtenberg et al. (19) using 11 % acrylamide gel electrophoresis, molecular weight standards(Sigma) consisted to

bovine serum albumin (68 000), ovalbumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), trypsinogen (24 000) and trypsin inhibitor (20 100). Molecular weights of native proteins were calculated as described by Hedrick and Smith (20) with respect to protein standards (aldolase, 158 000; bovine serum albumin, 68 000; ovalbumin, 45 000; trypsin inhibitor, 20 100).

Chemicals. Cellulose MN300 was purchased from Macherey and Nagel, Avicel, xylan, acrylamide and bisacrylamide from Fluka, DEAE-Trisacryl from Pharmindustrie, CM-cellulose medium viscosity (degree of substitution 0.7, degree of polymerization 11 000), pNP, pNP-β-D-cellobioside, bovine serum albumin, MOPS, Tris and SDS from Sigma. All other reagents were of the highest purity available except for guanidine hydrochloride (Prolabo).

RESULTS

Analysis of the enzymatic components involved in cellulose hydrolysis, secreted by Cellulomonas fermentans

In the last exponential phasis of growth 85% of CM-cellulase activity was in the supernatant. In order to display and compare protein compositions of cellulolytic activities secreted by C. fermentans in culture on cellobiose and cellulose MN300 (2 g/l), extracellular fractions were analyzed by polyacrylamide disc gel electrophoresis. Cultures were harvested at the beginning of the stationary phase of growth. Supernatant of cellobiose culture was obtained by centrifugation of culture medium at 12 000 g for 20 min. Extracellular enzymatic fraction of the culture on cellulose was obtained as described by Langsford et al. (18). These protein solutions were first concentrated by ultrafiltration in an Amicon cell with a PM 10 membrane and dialyzed against Tris-HCl 5 mM pH 7.5 buffer to obtain similar CM-cellulase activity (25 IU/ml and 21 IU/ml, for cellobiose and cellulose concentrated supernatants, respectively). Gel electrophoresis of these concentrated supernates revealed a complex mixture of proteins (Fig. 1). Analysis of their enzymatic activities after elution from the gel matrix gave similar profiles with CM-cellulase and pNP-cellobiohydrolase and a fairly similar profile with Avicelase with four separate bands of cellulase activities. One of these bands corresponded to the principal CM-cellulase and pNP-cellobiohydrolase bands. Among the three other bands, at least one did not correspond to any CM-cellulase band and might reflect the presence of an exoglucanase component (Rf 0.73-0.74).

In a preliminary study, it was observed that the major CM-cellulase band (Rf 0.54-0.55) could appear in the form of two very close bands (data not shown).

Purification of the major extracellular endoglucanases

Electrophoretic analysis did not reveal any difference in the number of CM-cellulase components produced in culture on cellulose and cellobiose. Consequently, it was decided to produce endoglucanase activity in a cellobiose culture. Culture supernatant therefore contained the totality of the extracellular enzymatic activity and it was not necessary to desorb enzymes from residual cellulose with possibly denaturing treatments. Fermentation was stopped after 5

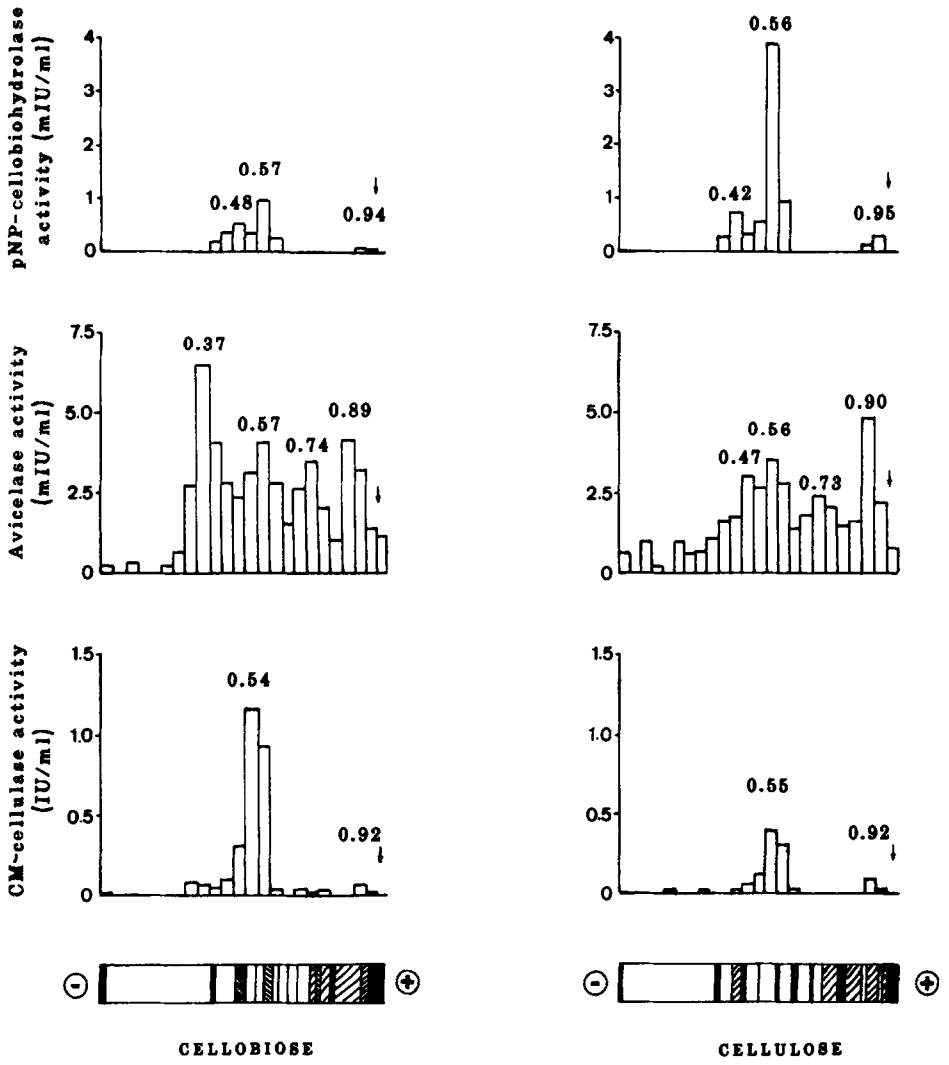

Fig. 1 . Distribution of extracellular proteins and main cellulolytic activities after electrophoresis on 7.5% polyacrylamide gel (cultures on cellobiose and cellulose). CM-cellulase, Avicelase and pNP-cellobiohydrolase activities were measured on gel slices of 2 mm width. Arrows mark the extent of migration of the bromophenol blue tracking dye.

days when maximum enzymatic activity was attained. The various steps in the purification were performed at 4°C. They are summarized in Table 1. The supernatant was obtained by centrifugation of the culture at 12 000 g for 20 min. Then it was dialyzed against Tris-HCl 5 mM, pH 7.7 buffer (buffer A) until the conductivity matched that of the buffer. This dialyzed supernatant was gently stirred overnight with 5 ml/liter of DEAE-Trisacryl previously equilibrated using the same buffer. The DEAE-Trisacryl was then allowed to settle . The supernatant was decanted and the gel poured into a column with a diameter of 2.1 cm and washed with 4 vol. of buffer A. CM-cellulase activity was then eluted

Table 1 . Purification of endoglucanases from Cellulomonas fermentans

Procedure	Fraction	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg protein)	Purification factor[a] (-fold)	Yield (%)
Culture supernatant		104.3	139.8	1.3	-	-
DEAE-Trisacryl M chromatography	0.25 M	28.8	123.7	4.3	3.3	88
TSK-DEAE-5PW HPLC	A	2.9	53.1	18.3	14.1	
	B	7.5	25.0	3.3	2.5	56
G 2000 SW HPLC of fraction A	AF	1.56	30.8	19.7	15.1	
G 2000 SW HPLC of fraction B	BF	0.464	11.4	24.6	18.9	30
TSK-DEAE-5PW HPLC of fraction AF	AEF	0.068[b]	1.17	17.2	13.2	9

(a) cumulative yield of the two endoglucanases calculated as : total CM-cellulase activity of purified fractions/CM-cellulase activity of initial supernatant
(b) proteins were determined by the spectrophotometric method described by Warburg and Christian (21).

with NaCl 0.25 M in buffer A. This fraction was dialyzed extensively against water before being freeze-dried. It was dissolved in the minimal volume of buffer A, and was then again dialyzed against the same buffer before being loaded onto a TSK-DEAE-5PW HPLC column, previously equilibrated with buffer A (Fig. 2). After washing, adsorbed proteins were eluted with a linearly increasing NaCl-gradient so that two major well-defined fractions exhibiting CM-cellulase activity (A and B) could be separeted. These fractions containing each an endoglucanase component were separately pooled and dialyzed extensively against water before being freeze-dried. After being dissolved in buffer B (sodium phosphate 0.1 M, NaCl 0.1 M, pH 7), they were individually chromatographed on a G 2000 SW HPLC column (gel filtration) equilibrated with buffer B at the flow rate 6 ml/h. The active fractions were pooled. Fraction AF containing an endoglucanase called CFA and fraction BF containing an endoglucanase called CFB were analysed by non-denaturing polyacrylamide gel electrophoresis (7.5 %). The photographic reproduction of gels shown in Fig. 3 indicated that fraction BF contained a single protein component. This was confirmed by electrophoresis in 6-11 % polyacrylamide gels. On the other hand, fraction AF contained a major protein component the endoglucanse CFA with minor amounts of contamination proteins. Further purification of this endoglucanase was achieved by anion-exchange HPLC on the TSK-DEAE-5PW column (flow rate 60 ml/h) after dialysis of the AF fraction against water and then buffer A. A sodium chloride gradient was applied from 0 to 0.25 M with several phases having various slopes . Especially, a long stationary phase at 0.14 M in NaCl was programmed and permitted a better separation of the active fraction, named AFE. It migrated as

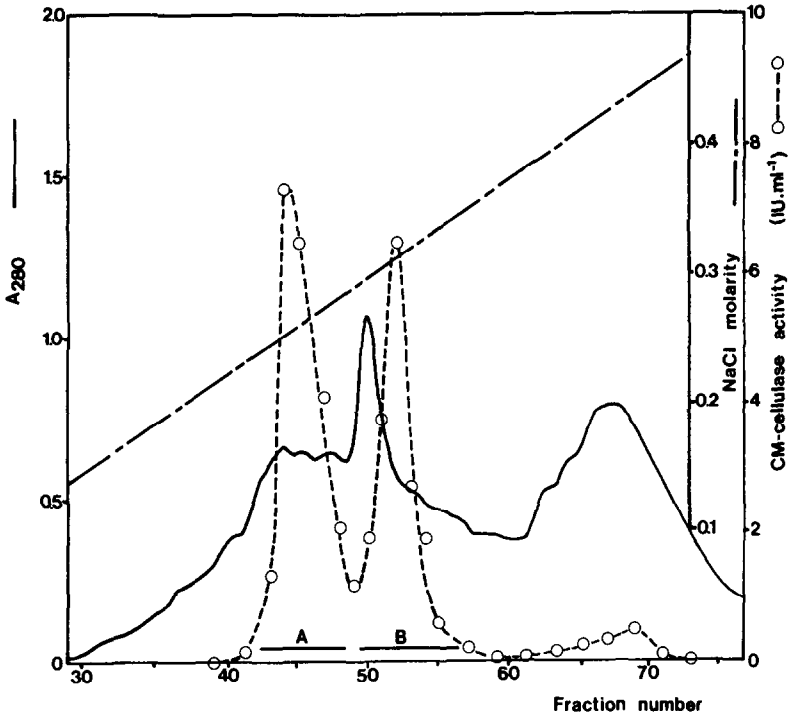

Fig. 2 . Separation of the two major endoglucanases on a TSK-DEAE-5PW column in HPLC. The column (7.5 x 75 mm) was eluted with 5 mM Tris-HCl buffer (pH 7.7) with an increasing NaCl gradient (0-0.5 M NaCl). 0.45 ml fractions were collected ; flow rate : 60 ml/h.

a single protein band in polyacrylamide gel electrophoresis (Fig. 3) indicating that the protein was homogenous .

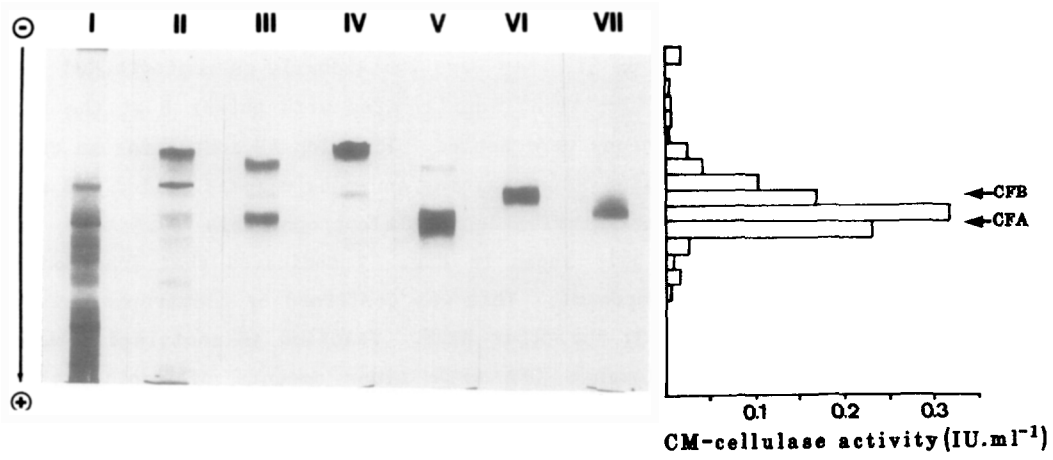

Fig. 3 . Disc polyacrylamide gel electrophoresis of pooled fractions containing CM-cellulase activity after each purification step.
a : the gels (7.5 %) were stained by Coomassie blue. I : culture supernatant (100 µg protein) ; II : 0.25 M fraction (30 µg protein) ; III : A fraction (18 µg protein) ; IV : B fraction (25 µg protein) ; V : AF fraction (18 µg protein) ; VI : BF fraction (8 µg protein) ; VII : AFE fraction (8 µg protein).
b : CM-cellulase activity (0.25 M fraction).

Table 2 . Activity of the CFA and CFB endoglucanases towards various substrates

Substrate	Substrate concentration (g/l)	Activity ($\mu mol.min^{-1}.mg\ protein^{-1}$)	
		CFA	CFB
CM-cellulose	25	15.8	17.6
Amorphous cellulose	11.4	9.5	9.4
pNP-β-D-cellobioside	25	4.6	1.5
Cellulose Avicel PH101	25	0.075	0.1
Xylan	25	0.5	0.3

Assay mixtures contained the substrates at the indicated concentration and an appropriate amount of enzyme to give a linear rate of reaction for each substrate.

Properties of the two enzymes

Molecular weights. Native molecular weights were determined using the electrophoresis method with various acrylamide gel concentrations as described by Hedrick and Smith (22) and were found to be 40 000 in the case of CFA and 58 000 in that of CFB. SDS-polyacrylamide gel electrophoresis of these enzymes also yielded a single protein band in each case corresponding to molecular weights of 40 000 and 56 000 for CFA and CFB, respectively. These results indicate that these two enzymes are monomeric.

Activities on various substrates. Substrate specificity of CFA and CFB was studied (Table 2). CM-cellulose and at a lesser extent, amorphous cellulose were the most rapidly hydrolyzed substrates for both enzymes. On the other hand, CFA and CFB displayed a very low activity towards microcrystalline cellulose (Avicel). They showed some activity on pNP-β-D-cellobioside but no real xylanase activity, since reducing sugars liberated from the commercial xylan by the purified enzymes certainly resuled from contaminating cellulose hydrolysis.

To better define the mode of action of both purified enzymes, the relative change in CM-cellulose viscosity was plotted against the amount of reducing sugar produced. It was estimated that only 0.3 % of CM-cellulose glycosidic bonds were cleaved when the viscosity was reduced by 50 % (data not shown). No difference between the two enzymes was observed. They reduce the viscosity of CM-cellulose faster than they release reducing sugar, they also act like typical endocellulases.

Kinetics of CMC hydrolysis. Under the experimental conditions described in "Material and Methods", the rate of liberation of reducing sugars from CM-cellulose was linear with time for 40 min and 30 min respectively with CFA and CFB. Both purified enzymes showed a linear relationship between the initial rate and protein concentrations up to 0.4 μg/ml.

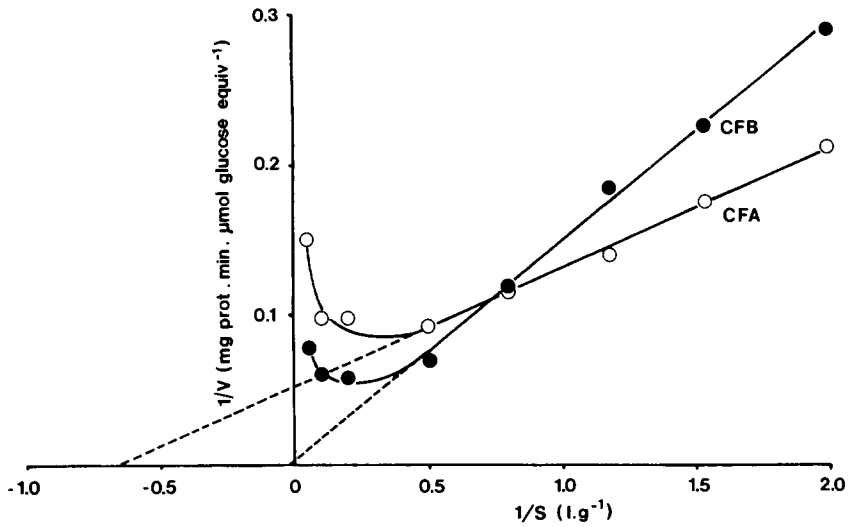

Fig. 4 . Determination of kinetic constants of endoglucanases CFA and CFB from Cellulomonas fermentans with CM-celllulose (Sigma, medium viscosity) as substrate. Linear curves were plotted using data obtained in the concentration range of 0.5 to 2.0 g/l (1/S of 0.5 to 2.0 l/g). Correlation coefficient of 0.998 and 0.997 were calculated with CFA and CFB curves, respectively.

Kinetic constants for CM-cellulase hydrolysis were obtained from Lineweaver-Burk plots. The linearity of the reciprocal plots observed in the main part of both curves (for the CM-cellulose concentration range 0.5 to 2 g/l) indicated that CM-cellulose hydrolysis by both endoglucanases followed Michaelis-Menten law (Fig. 4).

The K_m and V_{max} calculated for CFA were 1.5 g/l and 19 µmol.min^{-1}.mg^{-1}, respectively. The K_m and V_{max} of CFB were found to be 59 g/l and 407 µmol.min^{-1}.mg^{-1}, respectively.

DISCUSSION

Cellulases are often very difficult enzymes to purify because of they tend to aggregate with other proteins (22,23). It emerges that the cellulolytic system of Cellulomonas fermentans does not take the form of a protein complex . This system is composed of two major endoglucanases, which were purified, and at least one exoglucanase, as shown by the electrophoretic analysis of culture supernate. This analysis revealed only one major CM-cellulase activity peak, which is composed of two enzymatic proteins which had migrated into the same zone (Fig. 3b).

These two endoglucanases were separated by high performance liquid ion exchange chromatography. With classical liquid chromatography on DEAE-Trisacryl column with a linear gradient of NaCl, this separation was not possible. The HPLC method also had the advantage of shortening the time necessary for the separation.

Previous authors have reported having difficulty in purifying cellulases by means of gel permeation methods because of the low mobility of these proteins on these matrices (24-27). The two endoglucanases of C. fermentans were found to display similar characteristics. Indeed, elution volumes of the two proteins seemed to indicate molecular weights of around 30 000 and 21 000 for CFA and CFB respectively, although the values obtained using electrophoretic methods (40 000 and 57 000, respectively) were considerably different. Some interactions between both proteins and the gel permeation matrix may thus occur.

These two purified CM-cellulase components correspond to two different proteins. SDS electrophoresis of the proteins demonstrated that they are both monomeric and cannot correspond to two different aggregated forms of a single cellulase component, as is the case with Erwinia chrysanthemi CM-cellulase (28) or Ruminococcus albus cellulase (29). Nevertheless, it cannot be ascertained wether the two endoglucanase components resulted from posttranslational modification by proteolysis or chemical substitution (e.g. glycosylation) as is the case with CM-cellulases of Cellulomonas fimi (30), or from the production of discrete, structurally unrelated enzymes. Immunological data would be of great value to solve this problem. It is noteworthy that characteristic slopes obtained by plotting fluidity against the reducing power are quite similar for both purified components (data not shown). Several authors characterizing unrelated endoglucanases, have shown differences in their mode of action on CM-cellulose (27,31). Pseudomonas fluorescens var. cellulosa produces three endoglucanases which yield similar fluidity-reducing sugar profiles, but different relative activity toward cellooligosaccharides of different chain lengths, and each had a characteristic hydrolysis pattern on amorphous and crystalline forms of cellulose (32). CFA and CFB components do not exhibit the latter difference with regard to their enzymatic properties. The sole difference we observed in the enzymatic characteristics of our two proteins was between their catalytic constants in the reaction with CM-cellulose. CFA have a higher affinity for this substrate than CFB (Km of 1.5 g/l and 59 g/l, respectively). The use of the same substrate (CM-cellulose Sigma, medium viscosity with a degree of substitution of 0.7) with both enzymes allowed comparisons of rate constants to be made. It has been shown that the Km for CM-cellulose increases with an increase in degree of substitution (33). It is consequently difficult to compare different studies conducted with different substrates. The Km values obtained for the enzymes of Clostridium stercorarium and Lenzites trabea with a Hercule CM-cellulose (DS of 0.75) were 7.14 g/l and 13,05 g/l, respectively (34,35).

It should be noted that, while the Michaelis law is obeyed at lower substrate concentrations, the velocity falls off again at high concentrations. There is no available experimental data on the basis of which to discuss the various previously described possible causes of apparent inhibition by high substrate

concentrations (36). Nevertheless, it is noteworthy that reaction mixtures containing higher CM-cellulose concentrations also showed higher viscosities. This physical parameter might influence the velocity of the reaction.

These two enzymes will be further characterized, with particular attention to their degreee of antigenic relatedness and their chemical, physico-chemical and enzyme properties with in view to elucidating their specific functions in enzymatic cellulose degradation. It would also be interesting to study the other components of the cellulolytic system of this bacteria, especially the exoglucanase component(s), of which little is known in bacteria.

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