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SECOND PROGRESS REPORT

DEGRADATION OF PLANT CELL WALLS BY CELLULOSOMES OF ANAEROBIC BACTERIA

GRANT No. DPE-5544-G-SS-3061-00

PRESENTED BY ICAITI TO

AGENCY FOR INTERNATIONAL DEVELOPMENT
(A I D)

GUATEMALA, SEPTEMBER 1990

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1. INTRODUCTION

The agency for International Development (AID) of the United States of America granted ICAITI for a research program entitled - "Degradation of Plant Cell Walls by Cellulosomes of Anerobic Bacteria" under the terms and conditions of Grant No. DPE-5544-G-SS-8061-00. ICAITI subcontracted the Tel Aviv University to obtain technical services set forth in the scope of work of the contract which is enclosed in this report.

The overall aim of the research project is to understand the interaction between resting cell suspensions of cellulolytic bacteria and cell walls of plant cells in suspension culture.

A specific objectives are the characterization of the enzyme complexes ("cellulosomes") produced by Clostridium thermocellum and the study of the equilibria of their absorption to cellulosic plant cell wall degradation. Once the cellulosomes are characterized to the point that enzymatic defficiency for efficient cell wall degradation can be identified, the investigators plan to enhace the cellulosomes by adding the necessary enzymes.

In order to measure the kinetics of cellulosic cell wall degradation without interference from lignin or lignin-removing pre--treatment, the plant cell used as substrate will be grown from callus in suspension culture.

The present progress report covers the period from July 1, 1989 to August 31 1990.

2. SECOND COORDINATION MEETING (AIDE MEMOIRE)

AIDE MEMOIRE

SECOND COORDINATION MEETING OF THE CDR PROJECT
"DEGRADATION OF PLANT CELL WALLS BY CELLULOSOMES OF ANAEROBIC BACTERIA"

Participants:
Raphael Lamed, Tel Aviv University
Edward Bayer, Weizmann Institute of Science, Rehovot
Fabiola de Micheo, Carlos Morales, José Francisco Calzada
ICAITI

Place:
Central American Research Institute for Industry (ICAITI)
Guatemala City, Guatemala

Dates: August 1990

A presentation of the research results was provided by both parties. The ICAITI group showed some data obtained with several strains of anaerobic cellulolytic bacteria, (an associated grant). The complete characterization of these strains is still in progess and part of the results will be included in the present research contract. The successful cultivation of suspended plant cells from alfalfa, soybean, carrot and black bean was also discussed. The studies with untreated and treated sugar cane pith as substrates for the cellulolytic action were discussed in detail.

The Israeli party discussed some of the results dealing with the enhanced solubilization of cellulose by a combined cellulosome-beta glucosidase system, the presence of cellulosome-like entities in *Bacterioides cellulosolvens* and the identification of cellulosomal and noncellulosomal xylanases in *Clostridium thermocellum* associated with the cellulose-degrading enzymes. Part of the above information has been presented in a joint publication.

Some of the major achievements of the collaborative effort are as follows:

a. Complete solubilization of a 20% suspension of crystalline cellulose using a combined enzyme system.

b. Characterization of some of the inhibitory sources of cellulosome action.

c. Identification of stabilizing agents for cellulosome and beta-glucosidase (purified from Novozyme).

d. Identification and characterization of xylanolytic activities in the cellulosome.

e. Establishment of plant cell tissue culture and screening of potential substrates for applied work.

f. Initial results using alfalfa cell walls indicated enhanced degradation by combined cellulosome plus extraneous enzyme systems.

g. Studies involving linked enzyme systems were also reported.

Future activities were discussed and the need for facilitating communication was made evident. Due to the past difficulties in using BITNET communications it was decided to interact via FAX in the future.

The ICAITI party expressed the appreciation for the excellent collaboration and support obtained from the two Israeli institutions. It has improved the capabilities of the Institute for continuing work in related areas.

Due to the collective experience obtained during the term of the present project, many ideas and approaches for tackling natural substrates were seriously discussed. Both parties agreed to submit a new proposal to the CDR program. For our continued cooperation, we decided to initially select defined natural materials of increasing complexity in their respective composition; these will be used as model substrates for attack by the cellulosome-linked extraneous enzyme system. Wherever possible, literature values for composition (notably cellulose, hemicellulose and lignin) will be taken into account. Otherwise, other natural substrates and/or treated agricultural waste products will be analyzed for their composition. The substrates will be thus ranked according to their presumed complexity. Some of the substrates which were discussed during the present coordination meeting are the following: sugar cane bagasse and pith, lemon grass bagasse, citrus albedo, coffee pulp and/or mucilage and cotton linter. It is expected that different enzyme additives (e.g., defined cellulolytic enzymes, xylanases, etc.) to be included into the linked system will be dictated by the character of the model substrate. The eventual goal is to apply this approach to substrates of relevance regarding the agricultrual wastes which accumulate in Guatemala.

The second study research stay by Fabiola de Micheo, programmed within the project, was scheduled for the early part of 1991. The third coordination meeting will be held in Tel Aviv at that time.

Raphael Lamed

Raphael Lamed

Edward Bayer

Fabiola Micheo

Fabiola de Micheo

José Francisco Calzada

3. CHARACTERIZATION OF CELLULOLYTIC STRAINS IN DIFFERENT PURIFICATION STEPS

3.0 CHARACTERIZATION OF CELLULOLYTIC STRAINS IN DIFFERENT PURIFICATION STEPS.

Cellulase systems in the bacteria described below were studied by comparing the cellulosome complexes.

Clostridium thermocellum 0162	C. thermocellum YS donated by The University of TelAviv
Clostridium thermocellum 0010	C. thermocellum DSM 1237
Clostridium thermocellum 0245	C. thermocellum DSM 2360

For characterization studies, medium and culture conditions were followed according to Bayer et al.(1983). Cellobiose was added as carbon source and pH was ajusted to 7.3. Cells were grown by inoculation under anaerobic conditions, at 60°, of 4 ml of stock culture in serum bottles containing 100 ml of medium, using sufficient medium units as to obtain one liter. Incubation times were 24 hours for strains 0162 and 0245 and 36 hours for strain 0010. The cultures were pooled and the procedure indicated in figure 3.1 was followed.

Qualitative cellulolytic activity <B-(1-4)-D-glucan degradation> was determined by the Congo Red reaction (Teather and Wood, 1982).

In vitro determination of endoglucanase activity (carboximethylcellulase-CMCase) was performed by the method of Miller et al. (1960), with carboxymethylcellulose as the substrate. The incubation temperature was 60° C, and the time of assay was 1 hour. A unit of CMCase activity was defined as the amount of enzyme which released 1 µmol of reducing sugar (using glucose as a standard) per ml of sample per minute. Specific activity was defined as the amount of enzyme which released 1 µmol of reducing sugar per mg of protein per minute.

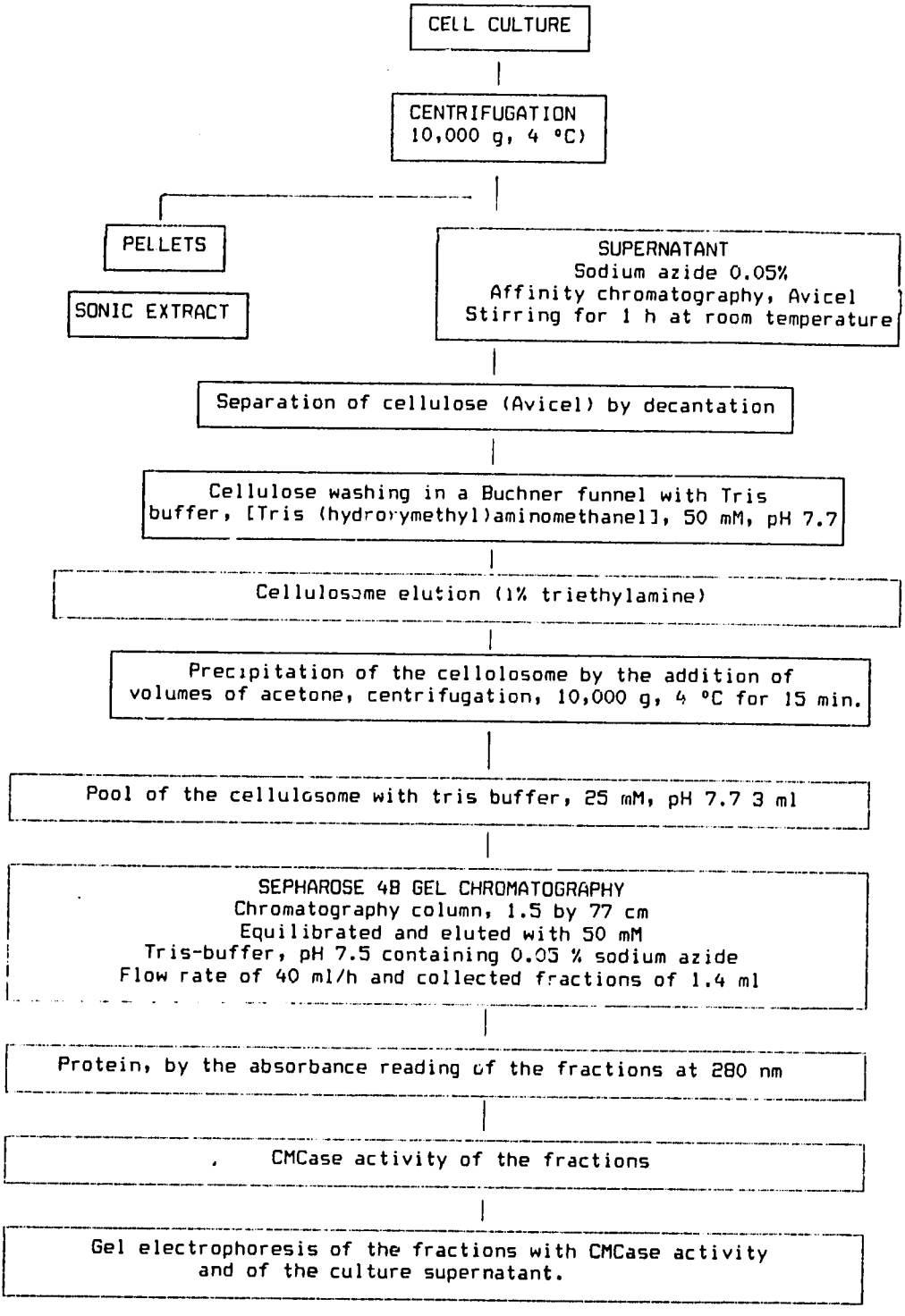
Reducing sugars were analyzed by the dinitrosalicylic acid reaction, DNS, (Miller, 1959).

Protein concentracion was determined according to Bradford (1976).

The method of Laemmli (1970), was used for slab gel electrophoresis. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was performed for 45 minutes at 120 V with 3% stacking gels and 6% separating gels. Coomassie brilliant blue R250 was used for staining the gel and a mixture of high-molecular-weight markers from Sigma Chemical Co. was used as standard.

FIGURE 3.1

SCHEMATIC DESCRIPTION, STEPS FOR CARACTERIZATION OF CELLULOSOME



The on-plate CMCase assay was carried for SDS-PAGE gels (Bayer et al. 1983), modified, a 2 % agar gel 0.8 mm thickness containing 0.1 % CMC for the agar replica was used, incubation time was 1-3 hours at 60° C.

3.2 RESULTS

Tables 1, 2, and 3 show protein content and CAMCase (endoglucanase) activity of strains 0162, 0245, and 0010, during purification steps. As can be seen from the data, the specific activity was different, strain 0245 exhibited the highest activity in all the steps; this can be correlated with the results obtained in a study on growth, enzymatic activity and adhesion of these strains (ICAITI, 1990): the specific activities estimated in the culture supernatants, 2.42, 2.53, and 0.45 U/mg protein for 0162, 0245, and 0010 strains, with approximately the same growth incubation time.

According to Bayer et al. (1985), besides triethylamine (TEA), water was used to elute the enzymatic complex from the cellulolytic matrix. Both showed similar elution abilities, a little higher with TEA for strain 0245, and with water for 0162; in strain 0010, where the CMCase activity was in general low, only TEA eluted small amount of enzymes.

Figures 1 to 6 present the absorbance at 280 nm and the endoglucanase activity (CMCase) of the fractions from sepharose 4B chromatography. All the strains showed maximum absorbance peaks near the void volume, with corresponding CMCase activity, in strains 0162 and 0245. Bayer, et al. (1985) report a turbid particulate fraction present only in cell-associated material of Clostridium thermocellum wild type that appears at the void volume. This can explain the behavior of strain 0245 (figures 5 and 6). Fractions from 20 to 32, 39 and 57 exhibited almost the same activity in strain 0162, the high absorbance in the last fraction corresponding to low activity. Strain 0245 showed maximun activity peaks were observed, in fractions 54 and 64. Although strain 0010 showed an absorbance profile like that of 0162, activity was apparent only in fractions 58 and 59. It can be assumed that after fraction 50, the activities corresponded to low molecular weight compounds.

As previously observed in the results of the purification steps, strain 0245 showed higher activity. This makes this strain attractive for further studies on cellulosome obtention. This behaviour will have to be reconfirmed by new experiments.

The electrophoretic patterns of culture supernatants are indicated in Table 4. The same fractions were present in strains 0162 and 0245, suggesting the existence of similar enzyme complexes. Strain 0010 lacked the higher molecular weight fraction. This correlates with the low molecular fractions with little CMCase activity observed in sepharose 4B chromatography.

The gel electrophoresis of the fractions which showed CMCase activity and of the sonicated supernatants presented problems, and not very good resolutions were obtained.

On-plate CMCases overlay method was not successful and clear areas due to CMCase activity were not observed for the experimental materials. For that reason, results are not presented.

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TABLE No. 1

STRAIN 0162

	Protein mg/ml	CMCase activity U/ml	Specific U/mg protein
1. Culture supernatant	0.09	0.12	1.27
2. Cellulose supernatant	0.20	0.10	0.50
3. Acetone-concentrated TEA-eluent.	0.18	0.25	1.37
4. Acetone-concentrated H2O-eluent.	0.19	0.48	2.58
5. Sonicated pellets supernatant	2.68	0.53	0.11

TABLE No. 2

STRAIN 0245

1. Culture supernatant	0.10	0.60	5.80
2. Cellulose supernatant	0.08	0.57	7.33
3. Acetone-concentrated TEA-eluent.	0.06	1.50	23.70
4. Acetone-concentrated H2O-eluent.	0.18	3.75	20.38
5. Sonicated pellets supernatant	0.42	0.26	0.62

TABLE No. 3

STRAIN 0010

1. Culture supernatant	4.50	0.45	0.10
2. Cellulose supernatant	3.87	0.20	0.05
3. Acetone-concentrated TEA-eluent.	2.38	0.15	0.23
4. Acetone-concentrated H2O-eluent.	1.05	0.00	0.00
5. Sonicated pellets supernatant	12.90	0.00	0.00

TABLE No. 4

ELECTROPHORETIC PATTERN
(MOLECULAR WEIGTH X 10-3)

Strain 0162	Strain 0245	Strain 0010
		26
28	28	28
		32
33	33	
35	35	35
39	39	39
48	48	
51	51	
		54
		57
61	61	
64	64	
		67
		70
		75
78	78	
		86
		94
		97
102	102	102
	109	
		112
114	114	
		116
120	120	120
123	123	
132	132	
137	137	
144	144	
149	149	
153	153	
160	160	

FIGURE 1

SEPHAROSE 4B GEL CHROMATOGRAPHY, STRAIN 0162

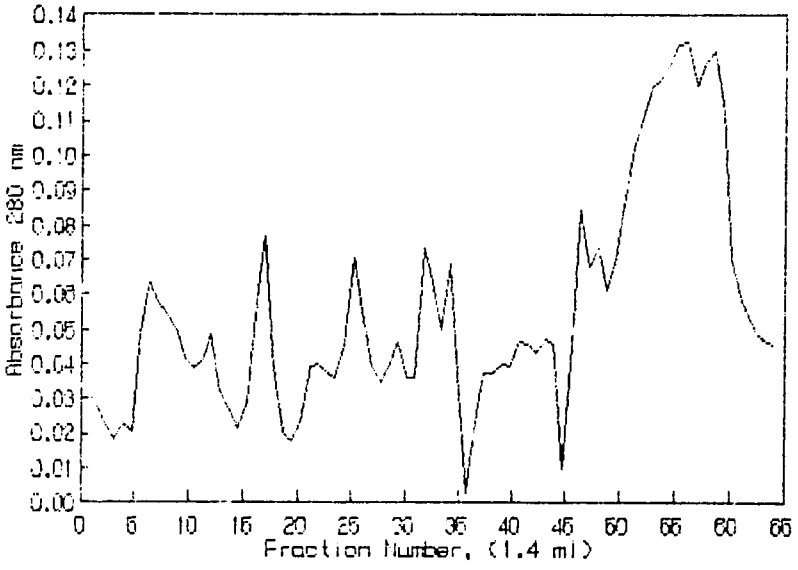


FIGURE 2

SEPHAROSE 4B GEL CHROMATOGRAPHY, STRAIN 0162

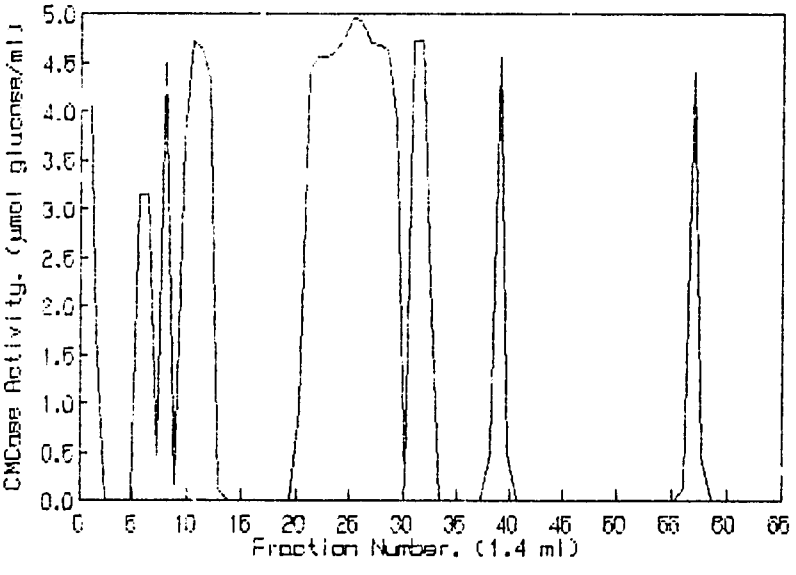


FIGURE 3

SEPHAROSE 4B GEL CHROMATOGRAPHY, STRAIN 0010

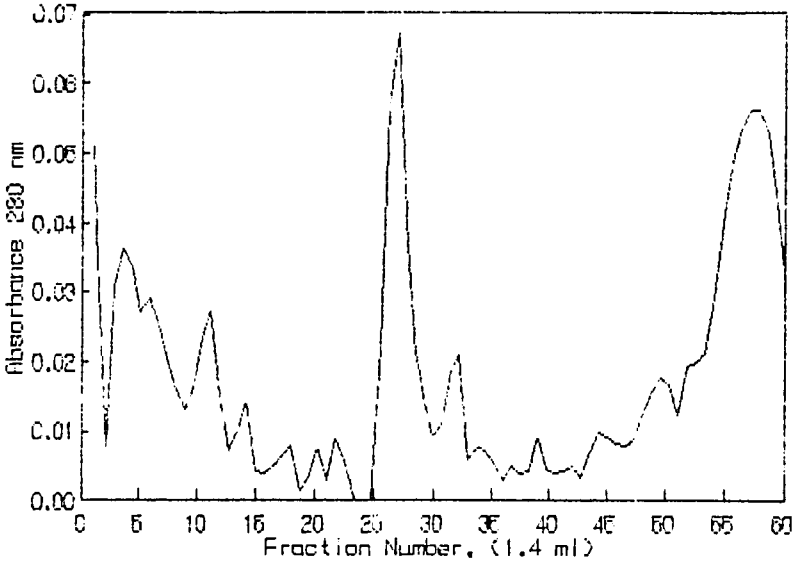


FIGURE 4

SEPHAROSE 4B GEL CHROMATOGRAPHY, STRAIN 0010

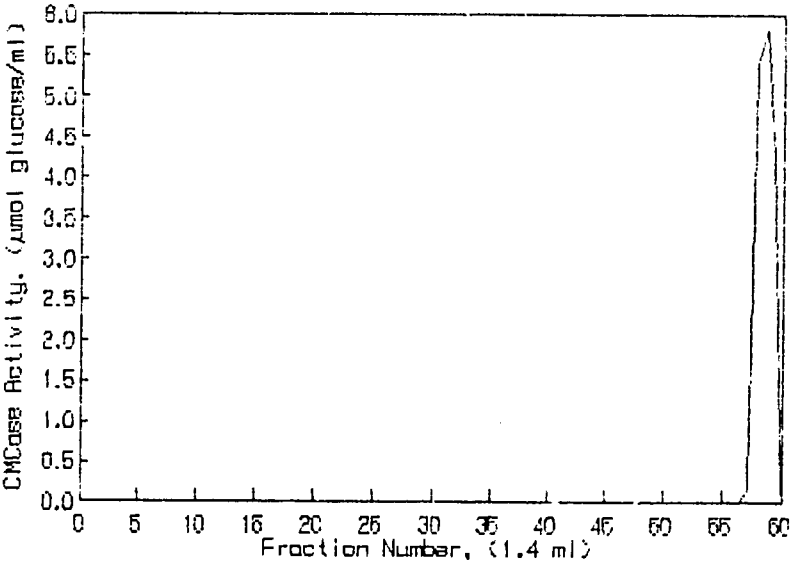


FIGURE 5

SEPHAROSE 4B GEL CHROMATOGRAPHY, STRAIN 0245

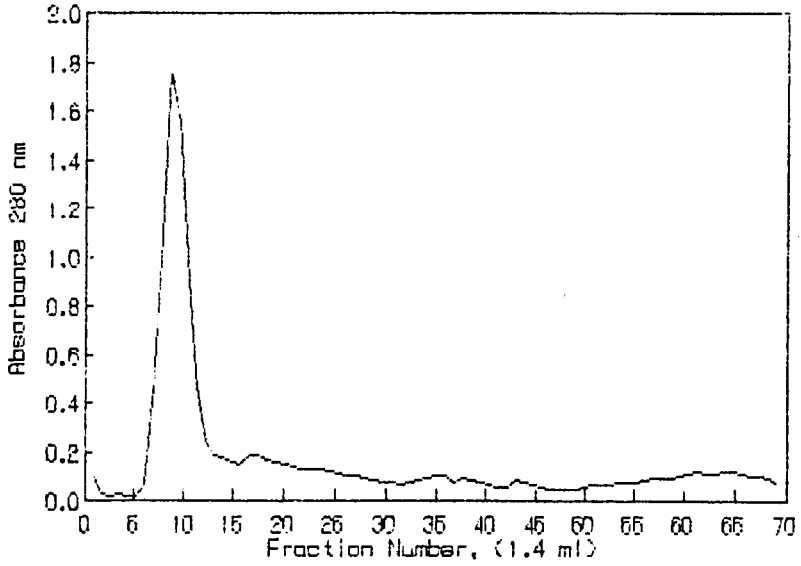
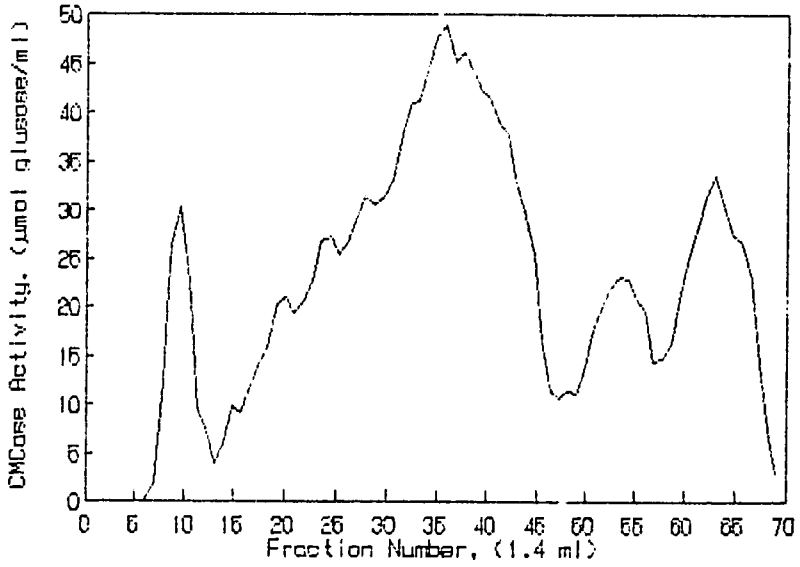


FIGURE 6

SEPHAROSE 4B GEL CHROMATOGRAPHY, STRAIN 0245



4. SUGAR CANE PITH DEGRADATION

4.0 SUGAR CANE PITH DEGRADATION.

4.1 TREATMENT OF SUGAR CANE PITH BY ORGANOSOLV PROCESS

The material was treated in a ratio of solid to liquid of 1:8 with a mixture of 60% ethanol-40% water (by weight), 3% of sodium hydroxide. The mixture was cooked in a pressurized rotating digester for 4 hours at 175° C. The chemical analysis was determined by the method described by Goering and Van Soest (1970). The composition is shown in table Nº 1.

4.2 ENZYMATIC DEGRADATION OF SUGAR CANE PITH.

4.2.1 Untreatrd (30 mesh)

4.2.2 Treated (20 and 30 mesh)

The hydrolysis of sugar cane pith was carried out as follows:

1. Addition of Cellulosome (8 µg/mg cellulose)

2. Addition of Cellulosome (8 µg/mg cellulose) and B-glucosidase (Novozym) (0.15 U/mg cellulose)

Crude B-Glucosidase (Novozym 188) was kindly donated by Novo Industries. The enzyme was dialyzed against acetate buffer 0.05 M, pH 5. The activity was determined following the method described by Berghem and Pettersson (1974), by measuring the release of p-nitrophenol from p-nitrophenyl-B-D-glucopyranoside (PNPG). One unit of activity is defined as the amount of enzyme liberating 1 µmol p-nitrophenol per minute.

The assay mixture containing substrate equivalent to 1 % cellulose, the enzymes as mentioned above, and the following compounds according to Lamed and Bayer (1986), EDTA 2 mM, $CaCl_2$ 10 mM, cysteine hidrochloride 2 mM, acetate buffer 50 mM, at pH 5, in 150x13 mm test tubes, were sealed with rubber stoppers under a stream of nitrogen gas, and incubated with intermittent shaking at 60° C for 96 hours, taking samples at 0, 4, 10, 24, 34, 48, and at 96 hours. Controls at 0 time and blanks without enzymes were carried out during the given incubation hours. The samples were centrifuged and the supernatants boiled for 10 minutes. Reducing sugars were determined by dinitrosalicylic acid method, (Miller, 1959) and glucose by Glucose-Oxidase (PGO) technique from Sigma (Kit Nº 510).

4.3 RESULTS.

TREATMENT OF SUGAR CANE PITH BY ORGANOSOLV PROCESS

Table Nº 1 shows the chemical composition of sugar cane pith. Lignin and hemicellulose losses were observed, after the treatment, 30 and 39 % respectively, consequently an increase in cellulose occurred (34 %).

ENZYMATIC DEGRADATION OF SUGAR CANE PITH.

A preliminary assay was carried out with 20 mesh untreated and treated sugar cane pith, with and without the addition of B-glucosidase (from almonds) to cellulosome and substrate. Degradation was not observed up to 96 hours of incubation.

After the Novozym enzyme was obtained, it was used in a small trial with 20 mesh treated sugar cane pith only. The values of reducing sugars (µmoles glucose/ml) and of glucose (PGO) µmoles/ml during incubation time are presented in table Nº 2. It can be observed that the hydrolysis stopped after 72 hours, glucose release was low corresponding to 3.45 % of solubilization of the cellulose.

A third group of experiments was done with 30 mesh untreated and treated material, looking for the effect of the particle size on the degradation. A similar behavior was observed with in no increase hydrolysis after approximately 68 hours. It is obvious that the treated meterial had an advantage, almost reaching three times the reducing sugars as compared with the untreated case.

When a comparison of results for different particle sizes was made, it is possible to see that both treated material (20 and 30 mesh) had better reducing sugar production. In the release of glucose both the effect of particle size and pretreatment were evident and positive. The same holds true for the solubilization of cellulose.

Since the cellulosome is capable of degrading cellulose to cellobiose, glucose release was observed only when degradation was carried with cellulosome plus B-glucosidase.

The results suggest that the organosolv treatment, besides chemical changes, enhanced the susceptibility to enzymatic hydrolysis. However, in order to increase the degradation of sugar cane pith, experiments must be considered with different concentrations of cellulosome and B-glucosidase. The study of other agroindustrial substrates and their responses to enzymatic hydrolysis according to their own structural characteristics would be also of interest.

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TABLE Nº 1

CHEMICAL ANALISIS OF SUGAR CANE PITH

	Untreated Pith	Treated Pith
	%	%
Neutral detergent fiber (NDF)	90.64	93.50
Acid detergent fiber (ADF)	62.00	76.27
Permanganate lignin	11.72	8.26
Cellulose	45.87	61.52
Hemicellulose (NDF-ADF)	28.64	17.23
Moisture	4.91	4.15

TABLE No. 2

SUGAR CANE PITH
(treated, 20 mesh)

Reducing sugars (DNS) µmoles glucose/ml

Incubation hours	0	24	48	72	96
Cs.	0.00	3.27	4.98	6.88	7.07
Cs+Nov	0.00	5.10	6.26	7.32	7.32

Glucose (PGO) µmoles/ml

Incubation hours	0	24	48	72	96
Cs.	0.00	0.00	0.00	0.00	0.00
Cs+Nov	0.00	1.22	1.79	2.13	2.09

Solubilization of Cellulose (Percentage)
(Glucose, PGO)

Incubation hours	0	24	48	72	96
	0.00	1.98	2.90	3.45	3.39

Cellulosome (Cs) : 8 µg/mg cellulose

Novozym (Nov) : 0.15 U/mg cellulose 5

TABLE No. 5

SUGAR CANE PITH
(Untreated, 30 mesh)

Reducing sugars (DNS) µmoles glucose/ml

Incubation hours	0	4	10	24	34	48	68	96
Cs.	0.00	0.17	0.572	1.12	2.13	3.00	3.51	3.51
Cs+Nov	0.00	0.94	1.27	1.86	2.75	3.50	3.77	3.77

Glucose (FGO) µmoles/ml

Incubation hours	0	4	10	24	34	48	68	96
Cs.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cs+Nov	0.00	0.67	1.14	1.40	1.90	2.25	2.39	2.39

Solubilization of Cellulose (Percentage)
(Glucose, FGO)

Incubation hours	0	4	10	24	38	48	68	96
	0.00	1.08	1.84	2.26	3.07	3.63	3.87	3.87

Cellulosome (Cs) : 8 µg/mg cellulose

Novozym (Nov) : 0.15 U/mg cellulose

TABLE No. 4

SUGAR CANE FIBR
(treated, 30 mesh)

Reducing sugars (DNS) µmoles glucose/ml

Incubation hours	0	4	10	24	34	48	68	96
Cs.	0.00	1.68	2.14	2.50	2.98	3.33	3.70	3.70
Cs+Nov	0.00	2.00	3.98	7.32	8.85	9.33	9.98	9.98

Glucose (FGO) µmoles/ml

Incubation hours	0	4	10	24	34	48	68	96
Cs.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cs+Nov	0.00	0.74	1.19	2.50	2.87	3.30	3.51	3.51

Solubilization of Cellulose (Percentage)
(Glucose, FGO)

Incubation hours	0	4	10	24	38	48	68	96
	0.00	1.20	1.93	4.05	4.65	5.35	5.80	5.80

Cellulosome (Cs) : 8 µg/mg cellulose

Novozym (Nov) : 0.15 U/mg cellulose

5. CELL SUSPENSION PREPARATION

5.0 CELL SUSPENSION PREPARATION

5.1 CALLUS CULTURES.

5.2 CELL SUSPENSION CULTURES.

5.1 CALLUS CULTURES.

Plant material: CARROT, SOYBEAN, BLACK BEANS AND ALFALFA.

Explants:

Carrot: Cambial region.

Soybean: Radicles and leaves from aseptically grown 5-day-old seedlings.

Black beans: Radicles from seeds. Radicles and leaves from aseptically grown 5-day-old seedlings.

Alfalfa: Seeds. Radicles and leaves from aseptically grown 5-day-old seedlings

5.1.1 Preparation and sterilization of explants.

1) Alcohol desinfection (70%) for 30 seconds. (Seeds).
2) Sodium hypochlorite disinfection (0.5 %) for 20 minutes. (Seeds and carrot explants).
3) Rinse in sterile distilled water (3 times). For excision of radicles from seeds, these were soaked overnight in sterile water.

5.1.2 Explant cultures.

From step 3 the work was done aseptically in a laminar air-flow cabinet. Seeds and cut explants were transferred to glass vials and glass culture vessels containing the basal medium of Murashige and Skoog (MS) (1962), suplemented per liter with 0.5 mg thiamine, 0.2 mg glycine, 0.5 mg nicotinic acid, 0.5 mg pyridoxine-HCl, 100 mg myoinositol, 30 g of sucrose as carbon source, 7 g of agar and the pH was adjusted to 5.7. In alfalfa cultures, 200 mg of casein hydrolysate and 200 mg of malt extract per liter of medium were added.

For seedlings, medium without growth regulators was used.

Growth regulator suplementation was performed as follows:

1.- 2,4-Dichlorophenoxiacetic acid, (2,4-D): 1 mg/l.
Kinetin: 0.089 mg/l.

2.- 2,4-D: 2 mg/l.
Kinetin: 0.5 mg/l.

3.- p-Chlorophenoxyacetic acid (pCAA): 2 mg/l.
2,4-D: 0.5 mg/l.
Kinetin: 0.025 mg/l.

4.- 2,4-D at three concentrations: 1, 2, and 3 mg/l.

Treatment Nº 3 was assayed only in black beans.

Cultures were incubated at 25º - 27º C, one group in darkness, another under 2000 lux illumination using a 16 hour photoperiod. After calli were formed, periodic subcultures were made to fresh medium until friable calli were obtained.

5.2 CELL SUSPENSION CULTURES

Cell suspension cultures were obtained by transfer of friable callus lumps to liquid medium of the same composition incubating the flasks in an orbital shaker with an agitation rate of 100 r.p.m. After initiation of the cell suspension and increase in cell density, subcultures involved aseptic transfer to fresh medium through a stainless steel 40 mesh sieve.

5.3 RESULTS.

Copies of some of the photographs of formed calli and of the cell suspensions are annexed.

The treatment results depended on the plant material. For carrot, better calli were obtained with treatment Nº 4 at a concentration of 2.0 mg/l of 2,4-D. The same result was obtained with cell suspension cultures.

Alfalfa and soybean callus and cell suspension cultures showed very good formation with treatment No. 2. Seeds in alfalfa and leaves in soybean were the most promising explants.

Black beans calli from radicle and from leaves were produced better using treatments Nº 2 and Nº 3, however for cell suspension, the last one was the best.

In all cases calli formation was obtained after one month, and subcultures were carried out every month. Cell suspension cultures were iniciated after 30 days by transfer of 3 months calli to liquid medium, with subcultures every 8 days.

In general illumination conditions produced better calli formation. Cell suspensions showed good growth rates in the darkness.

5.4 BIBLIOGRAPHY

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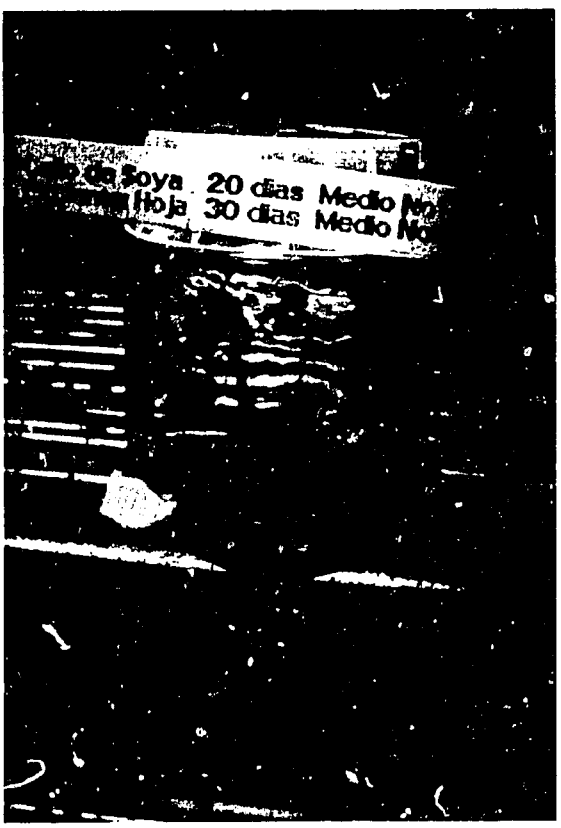
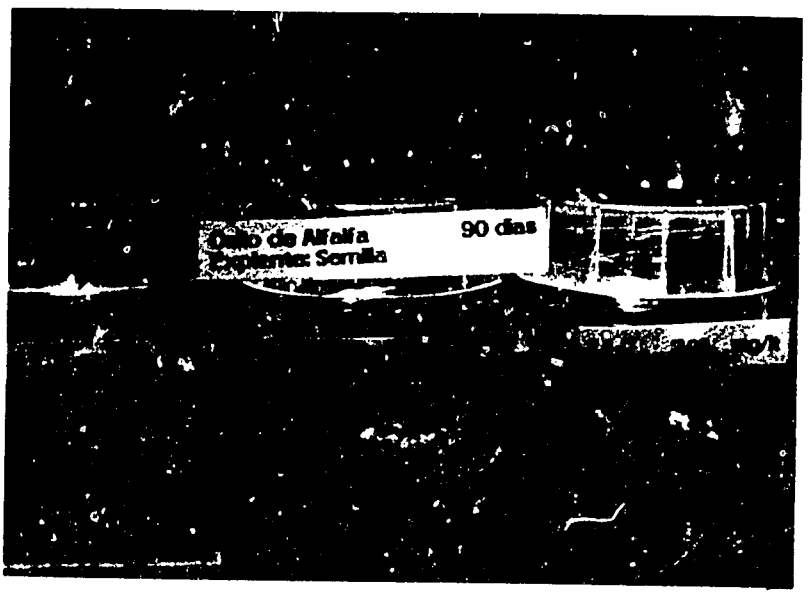
SOY BEAN CALLUS



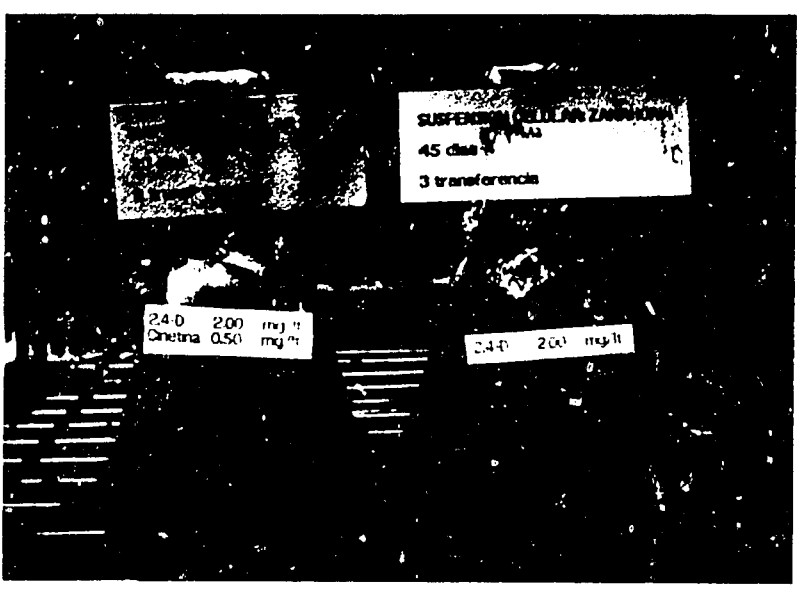
BLACK BEAN CALLUS
FROM LEAVES

BLACK BEAN CALLUS
FROM RADICLE

ALFALFA CALLUS
FROM SEEDS



SOY BEAN AND CARROT
CELL SUSPENSIONS



6. REPORT FROM TEL AVIV UNIVERSITY

TEL AVIV UNIVERSITY אוניברסיטת תל-אביב

RESEARCH AUTHORITY רשות המחקר

January 24, 1990

Jose Francisco Calzada
Head Applied Centroamericano Da
Investigaciony Tecnologia Industrial
(ICAITI)
Avenida La Reforma 4-47. Zone 10
Guatemala, C.A.

CAMERICANO DE INVESTIGACION
Y TECNOLOGIA INDUSTRIAL, GUATEMALA
FEB. 2 1990

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Dear Sir,

Re: Prof. Raphael Lamed
Dr. E.A. Bayer
Grant # DPE-5544-G-SS-8061
our No. 451-341

Enclosed please find Interim Scientific Report for the period January - December 1989 for the above research project.

We thank you for your kind assistance and cooperation.

Sincerely yours,

Sofia Ninio
Accounting Official

encl.

cc:Prof. R. Lamed
Mr. Meir Yaskil
US Embassy, Tel Aviv

11948

PARA ACCION
A LA Campo
PARA INFORMACION
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RAMAT AVIV, 69978 TEL AVIV, ISRAEL, TEL. 410405 ,429624 .טל ,69978 קריית האוניברסיטת, רמת-אביב, תל-אביב

PROGRESS REPORT JANUARY-DECEMBER 1989

DEGRADATION OF PLANT CELL WALLS BY CELLULOSOMES OF ANAEROBIC BACTERIA

7 2 -11- 1990

Presented by Professor Raphael Lamed and Dr. Edward A. Bayer,
Tel-Aviv University, to ICAITI, Grant No. DPE-5544-G-SS-8061
of Agency for International Development (AID)

OVERVIEW

The efforts during the first year of the research initially involved the coordination of research activities during the inaugural meeting with Guatemalian scientists, followed by a several weeks' training period of Fabiola De-Micheo (7). The research during this period centered on three topics: a. Characterization of cellulosome-like complexes in Bacteroides cellulosolvens b. A survery on xylanases of Clostridium thermocellum, in particular cellulosome associated with a potential to assist in plant cell wall degradation and c. Initial supercellulosome evaluation and construction.

The first topic yielded very interesting results suggesting several lines of similarities in cellulosome-like complexes in the completely unrelated bacterium B. cellulosolvens and the previously characterized C. thermocellum. In particular, an S1-like glycosylated subunit was found in the new cellulosome without evident enzymatic activity, but displaying anomalous SDS-PAGE electrophoretic behaviour, immunochemical reactiviy, lectin-binding and sugar composition, similar to those of the S1 in the cellulosome of C. thermocellum. Defined xylanase and endoglucanase activities and protein components which exhibit these activities were also found in the new cellulosome-like complexes. A tendency to form insoluble precipitates somewhat complicates the study of these new complexes.

In the second topic we have made significant progress and are ready to submit a paper entitled "Cellulosomal and non-cellulosomal xylanases of Clostridium thermocellum relationship to cellulose degrading enzymes", and are quite convinced, on the basis of the study, that the cellulosome contains natural enzymes dedicated to the

degradation of the major hemicellulose component xylan, even without the ability to use this polymer as a carbon source.

In the third topic we have made some very encouraging advances. In particular, we are confident that provided the right conditions and ancillary enzyme/s to be incorporated into the cellulosomes are available, we will be able to achieve the complete hydrolysis of very dense cellulose suspensions. The key enzyme to this discovery is B-glucosidase. The enzyme from A. niger was found most suitable for the task and was studied both as a crude enzyme and after purification. Activation in rate and degree of hydrolysis were detected, the latter phenomenom was found to depend mainly on initial celluloose concentration. Initial studies in chemical combination of this enzyme into the cellulosome indicate that it would be possible to mimic the effect and perhaps to even enhance it further. Study in search of a suitable pectinase to serve for complementary purposes are currently underway.

Comparison of high molecular cellulolytic complex in Bacteroides cellulosolvens to the cellulosome of C. thermocellum

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Bacteroides cellulosolvens is an anaerobic mesophilic gram negative bacterium. It was first isolated by Murray et al. (1984) from sewage sludge. It ferments cellulose and cellobiose but not xylan, xylose, glucose or other sugars. It was isolated from a stable co-culture with C. saccharolyticum which appears to remove toxic metabolites and to feed on the cellulolytic degradation products.

The culture obtained from American Type culture collection (ATCC 35603) apparently contains the latter organism as a contaminent technique before initiating the study of its putative high molecular

weight celluloytic system.

Adsorption of B. cellulosolvens to cellulose and other polymers

Table 1 describes the adsorption of B. cellulosolvens (A=0.85) to various polymers (1.6%). It was also compared to that of C. thermocellum under the same conditions. Cells of both strains were adsorbed to Avicel, alfafa, hay, wheat, straw and several cationic polymers. The latter ionic based adsorption was usually inhibited by increasing the salt concentration. The two organisms differed mainly in that the Bacteroides strain was adsorbed to insoluble xylan, while Clostridium did not. In addition, the adsorption to cellulose was inhibited by soluble methyl cellulose only in the former organism. Other soluble polymers and sugars, including cellubiose, did not interfere with the adherence in both organisms.

Table 1: Percent adsorption of B. cellulosolvens and C. thermocellum to 'insoluble' polymers.

polymer	B. cellulosolvens	C. thermocellum
alfalfa hay	76	53
wheat straw	95	84
xylan	84	0
starch	12	0
Avicel	46	38
agarose	0	0
Carboxyl methyl cellulose	28	0
PEI-cellulose	14	-
DEAE-cellulose	14	-
QAE-Sephadex	100	-

Surface labeling of B. cellulosolvens with 'cellulosome recognizing' lectin.

Labeling cells grown on both cellulobiose and cellulose by the lectin from Griffonia simplicicolia was previously observed in several cullolytic bacteria suggesting a similarity to C. thermocellum (Lamed et al., 1987). Since the reaction of the GSI lectin was traced to the cellulosome, and in particular to one glycosylated subunit termed S1, it was assumed to be part of a putative cellulolytic complex also in these other bacteria. This assumption was found to be correct for B. cellulosolvens and the lectin-binding proteins served as a target for both purification studies and as a marker. I[illegible] GSI binding was characterized only in high molecular weight fractions in cell extracts and cell-free growth media for both cellobiose and cellulose as carbon source.

Initial biochemical characterization of bacterial proteins with regard to involvement in large celluloytic lectin-binding complexes

---

Fractionation of the spent medium of cellobiose-gram cells was attempted by Sepharose 4B gel filtration and by immobilized GSI lectin. This procedure led to the isolation of two high molecular weight fractions (10 million and 0.7 million daltons) reactive with the lectin and containing several major peptides. About 70% of the endoglucanase and xylanase activities were bound to the Sepharose-bound GS1 lectin. Fig. 1 shows the analysis of the cellulosome-like complex of this bacterium. From the figure it is clear that a major glycosylated subunit (apparent MW = 230 kDal) is responsible for the binding to Sepharose-GS1. Other proteins which forma complex with

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this glycosylated subunit are also bound. The activity binding data and direct Zymogram staining of SDS-PAGE gels suggest strongly that both xylanase and CMCase active proteins are included in the complex. In particular, a major 84 kDal peptide appears to be a xylanase and a 50 kDal band is a CMCase (not shown).

Initial studies directed to construction of a supercellulosome

In addition to the cellulases of Clostridium thermocellum (Lamed and Bayer, 1988), xylanase activity has also been detected on the extracellular growth medium despite the reported inability of this organism to grow on xylan as the sole source of energy. It was therefore of interest to determine whether such activity is associated with the cellulosome. In addition, we were interested in understanding the interaction of these proteins with xylan and the effect of xylan on their interaction with the insoluble cellulose substrate.

Fractionation of extracellular xylanase activity

In order to determine the distribution of the observed xylanase activity of C. thermocellum, the extracellular constituents of cellulose-grown cells were fractionated according to size and interaction with cellulose or xylan. The results showed that most of the activity is associated with the high-molecular-weight fraction. More than half of the xynalase activity is bound to cellulose but none adhered to xylan. The association of xylanase activity with high-molecular-weight cellulose-bound fractions suggests that a major portion of this activity in C.thermocellum is contained in the cellulosome. It was thus decided to further test this possibility.

Characteristics of xylanase activity in the cellulosome

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The degradation of xylan by the purified cellulosome was analyzed and a specific activity of 100 umoles xylose-$min^{-1} \cdot mg^{-1}$ was obtained. The activity reached a maximum within the pH range of 6 and 7.5 and the reaction exhibited a broad temperature optimum with a maximal activity of about 70°C. The major products of the reaction included xylobiose (as the main reaction product), xylotriose, as well as various unidentified oligosaccharides. Very low levels of xylose were also detected. After long periods of reaction (days) most of the xylotriose was converted to xylobiose which formed the major reaction product. The activity was sensitive to inhibition by certain divalent metal ions. The order of sensitivity of cellulosome action to metal ions was $Cu^{+2} > Pb^{+2} >> Zn^{+2} > Co^{+2}$, consistent with earlier studies on the action of crude xylanase activity using trinitrophenyl xylan as a substrate. Xylose, glucose or cellobiose failed to inhibit the xylanase activity. The cellulosome failed to convert pure B-xylobiose to xylose.

Interaction of the cellulosome with xylan

Despite the high levels of xylanase activity in the cellulosome, C. thermocellum strain YS failed to grow on either xylan or xylose as the major carbon and energy source. We examined whether the cellulosome binds also to xylan using a biotinylated preparation of the cellulosome. We found that essentially no cellulosome bound to xylan. In contrast, the cellulosome was completely adsorbed to relatively low amounts of cellulose. Moreover, the addition of large amounts of xylan to the cellulosome-containing medium failed to interfere with the adsorption of the cellulosome to cellulose.

Non-cellulosomal xylanases

In many respects the characteristics of the non-cellulosomal xylanase activity are very similar to those exhibited by the cellulosome. The temperature and pH maximum of the non-cellulosomal xylanases were found to be identical to that of the cellulosome. Xylobiose was the predominant product. The inhibitory effect of divalent metal ions on the non-cellulosomal xylanase activity is essentially identical to that on the cellulosomal activity; i.e. $Cu^{+2} > Pb^{+2} >> Zn^{+2} > Co^{+2}$.

Comparative polypeptide pattern of cellulosomal and non-cellulosomal xylanases

Despite the striking similarities in the above-described characteristics of the cellulosomal and non-cellulosomal xylanase activities, their respective polypeptide patterns are markedly different (Fig. 1). The non-cellulosomal polypeptides range from M_r 27,000 to 70,000, far below the average molecular mass of, and migrating in positions inconsistent with those of the cellulosomal subunits which range from M_r 48,000 to 210,000. Even more pronounced, however, is the difference in the regenerated xylanase activities following SDS-PAGE separation. In the cellulosome-associated fraction, several major xylanase activities can be detected corresponding to S2, S7, S9 and/or S10 and S13. Two to three minor activities, e.g. S3, S5 and S11 could also be discerned. In the non-cellulosomal fraction none of the major xylanase activities are coincident with those of the cellulosome. The major non-cellulosomal xylanase activities exhibit M_r values of 65,000, 47,000, 45,000, 40,000 and 30,000.

Xylanase and endoglucanase activities of cellulosomal subunits

When parallel samples of the cellulosome (treated at 100_oC in sample buffer) were subjected to SDS-PAGE and tested for xylanase and CMCase activities, some of the separated subunits appeared to exhibit similar levels of both specificities (Fig. 2); notably the S2 and S13 subuinits showed very high activities. On the other hand, the S7 and S9, $S10_1$ subunits appeared to contain xylanase activity only. Similarly, at relatively high sample concentrations the S11 subunit appeared to exhibit only xylanase activity. At such concentration levels a series of additional activity bands were also visible (mol. mass between 120,000 and 150,000), most of which appeared to reflect both xylanase and CMCase activities.

Cellobiohydrolase-like activity of cellulosomal subunits

Using 4-methylumbelliferyl B-D-cellobioside as a substrate, the highest activity levels were observed associated with the S3 and S9(S10) subunits. Lower levels of activity were detected in the S4 and S7 subunits.

Disposition of B-glucosidase and B-xylosidase activities in C. thermocellum

B-Glucosidase and B-xylosidase activities were found exclusively in cell-associated extracts; both activities were essentially absent from cell-free (cellulosomal and noncellulosomal) fractions. FLPC of the whole cell extract of C. thermocellum revealed that the respective activities were confined to separate peaks, activity (M_r ~ 54,000) and a second which exhibited B-xylosidase activity (M_r ~ 200,000).

Inhibition of cellulosome Avicelase activity by cellobiose

B-glucosidase was potentially a good candidate to improve

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cellulosome action since previous work demonstrated inhibition of cellulosome action by high concentrations of cellobiose (Lamed et al. 1985)

It was first essential to demonstrate that low concentrations of cellobiose are also severely inhibitory. The kinetics of Avicel (0.6 mg/ml) degradation was followed turbidometrically in the presence of different concentrations of cellobiose for 26 hts, employing 8 micrograms cellulosome per mg of cellulose in 50 mM acetate buffer, pH 5, containing 10 mM CaC12, 2mM EDTA and 5 mM cysteine under anaerobic nitrogen atmosphere. Half-maximal inhibition was reached at concentrations as low as 0.2% of cellobiose (Fig. 3). Our previous studies have also shown that the inhibition by 2% cellobiose did not result from interference of the disaccharide with adsorption of the cellulosome to Avicel.

The next obvious step was to seek a B-glucosidase which would be sufficiently stable to operate at a temperature of 60_oC and at the appropriate pH range of 5.0 - 6.5, which would allow optimal operation of the cellulosome. Other conditions, such as the presence of $CaC1_2$ and cysteine, which optimize cellulosome action, must also conform with the chosen B-glucosidase.

After screening experiments, the Novo enzyme (cellobiase, Novozyme) was chosen since it was active and stable under the above conditions. For more details on stability of the purified enzyme, see below.

Demonstration of B-glucosidase enhancement of cellulosome Avicelase

The stage was now set to test the effect of B-glucosidase on the rate and extent of decomposition of Avicel by the cellulosome. This was done by employing 20, 50 and 200 mg Avicel per ml reaction

mixture. The amount of cellulosome added depended on the Avicel concentration and was chosen to be 8 microgram/mg Avicel corresponding to a 3-fold excess of the amount initially adsorbed on the cellulose surface. The concentration of B-glucosidase was in all experiments also proportional to the concentration of cellulose and cellulosome added, and was 0.06 units per mg Avicel (units correspond to micromole glucose/min obtained under the reaction conditions from cellobiose). The reaction was assayed by analysis of reducing sugars by the DNS method calibrated by either glucose (in the presence of B-glucosidase) and/or cellobiose, which is the sole end product of the cellulosome, in the absence of B-glucosidase. In most cases, all assays gave similar results of solubilization, which were also confirmed by turbidometric analysis. The time-course of 5% Avicel solubilization, in the absence or presence of B-glucosidase, is shown in Fig. 4. About 3-fold enhancement of both the initial rates and degree of solubilization could be observed. Complete solubilization was evident after 96 hrs in the presence of B-glucosidase and 50% solubilization after about 15 hrs, while less than 35% was dissolved after 96 hrs in the absence of B-glucosidase. The solubilization of 20% cellulose suspension also proceeded readily, although a second addition of cellulosome/B-glucosidase after 72 hrs was necessary to achieve the near complete solubilization of Avicel (Fig. 5).

Purification of *A. niger* B-glucosidase from Novozyme

In order to (a) ensure that the stimulatory effect was indeed due to the action of B-glucosidase and (b) to prepare a pure protein for chemical crosslinking into the cellulosome, we have developed a simple purification method for the enzyme. The method consisted of DEAE-

cellulose (Whatman) ion exchange chromatography, followed by Sephacryl 200 (Biorad) gel chromatography. The purification steps are summarized briefly in Table 2. As shown in Fig. 6 the enzyme was obtained as a practically pure polypeptide corresponding to a molecular weight of 116 kDal (C) from the rather crude commercial enzyme (A) via the partly enriched ion exchange fraction (B).

Table 2.

Purification of the Cellobiase from Novozyme

Fraction	Total Activity (PNPG units)	Total Protein (mg)	Specific Activity (u/mg)
Novozyme	3486	300	12
DEAE-cellul.	2019	39	53
Sephacryl-200	519	5	103

* 1 βNPG unit = ~~4.7~~ 0.493 cellobiase units (micromole glucose/min, 60c)

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Stability of purified B-glucosidase

The stability of the purified cellobiase (Fig. 7) was assessed after incubation for different times, up to 72 hrs at pH 5.0 and at a temperature of 60_oC in the absence of presence of 1% glucose. The activity was assayed employing the standard PNPG assay. More than 60% of the activity was retained after 72 hrs, provided glucose was also present. In the absence of glucose only 30% of the activity was retained after 24 hrs.

Chemical modification/crosslinking

Biotinylation of cellulosome and B-glucosidase:

The standard procedure employing biotin N-hydroxy-succinimide ester was employed (Wilchek and Bayer, 1990). The success of modification, namely the incorporation of significant amount of biotin to the enzyme was assessed by a dot blot procedure employing biotinyl-alkaline phosphatase and avidin. The effect of the chemical modification on the activities was assayed by the PNPG assay for B-glucosidase and by the effects on endoglucanase (CMCase) and Avicel solubilizing activity of the cellulosome. Under the conditions employed no damage of activities was observed in any of the asdsays of either B-glucosidase or cellulosome. The activity to solubilize Avicel was in fact slightly increased by the chemical modification of the cellulosome.

Various approches to the crosslinking reaction have been initiated with promising results, including coupling of sugar (periodate)-oxidized cellulosome with adipic dihydrazide (WSC-coupled)-derivatized B-glucosidase. Initial studies are also underway to identify an appropriate pectinase for super-cellulosome studies..pa

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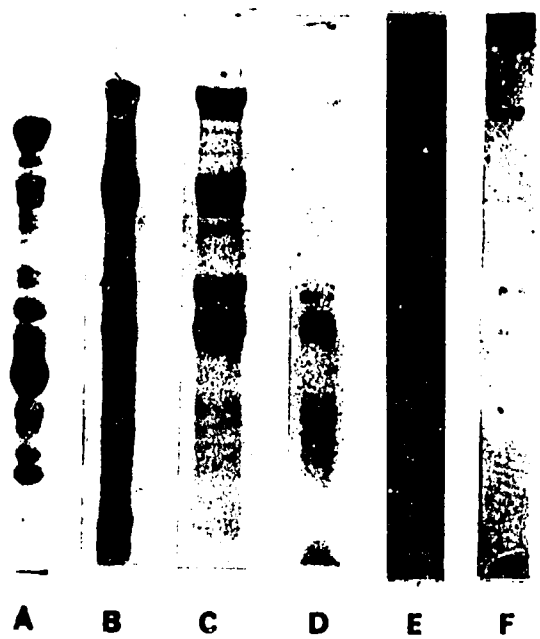

Fig. 1.

Characterization of the putative cellosome of B. cellulosolvens.

A. Coomassie blue pattern of purified cellulosome from C. thermocellum (serving as protein markers).
B. Coomassie blue staining pattern of total secreted protein bands from B. cellulosolvens grown on cellobiose.
C. Protein bands in the 700,000 dalton fraction eluted from gel filtration column (Sepharose 4B).
D. Proteins eluted from GSI-lectin column.
E. GSI-lectin-based staining of material from C.
F. Carbohydrate staining pattern (PAS) of C.

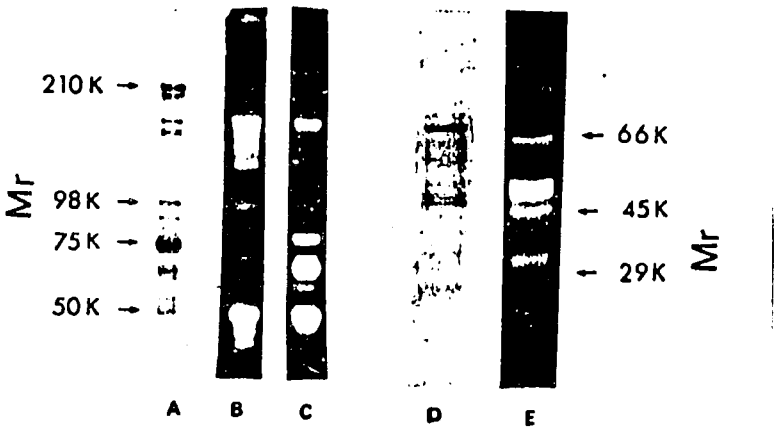

Fig. 2.

Xylanolytic and endoglucanase activity profiles of SDS-PAGE-separated cellulosomal and non cellulosomal components.

A. Coomassie blue staining of the purified cellulosome.
B. Detection of endoglucanase activity in the cellulosome.
C. Detection of xylanase activity in the cellulosome.
D. Coomassie blue staining of the noncellulosomal extract.
E. Detection of xylanase activity in the noncellulosomal fraction.

Lanes A - C, 6% gels.
Lanes D - E, 10% gels.

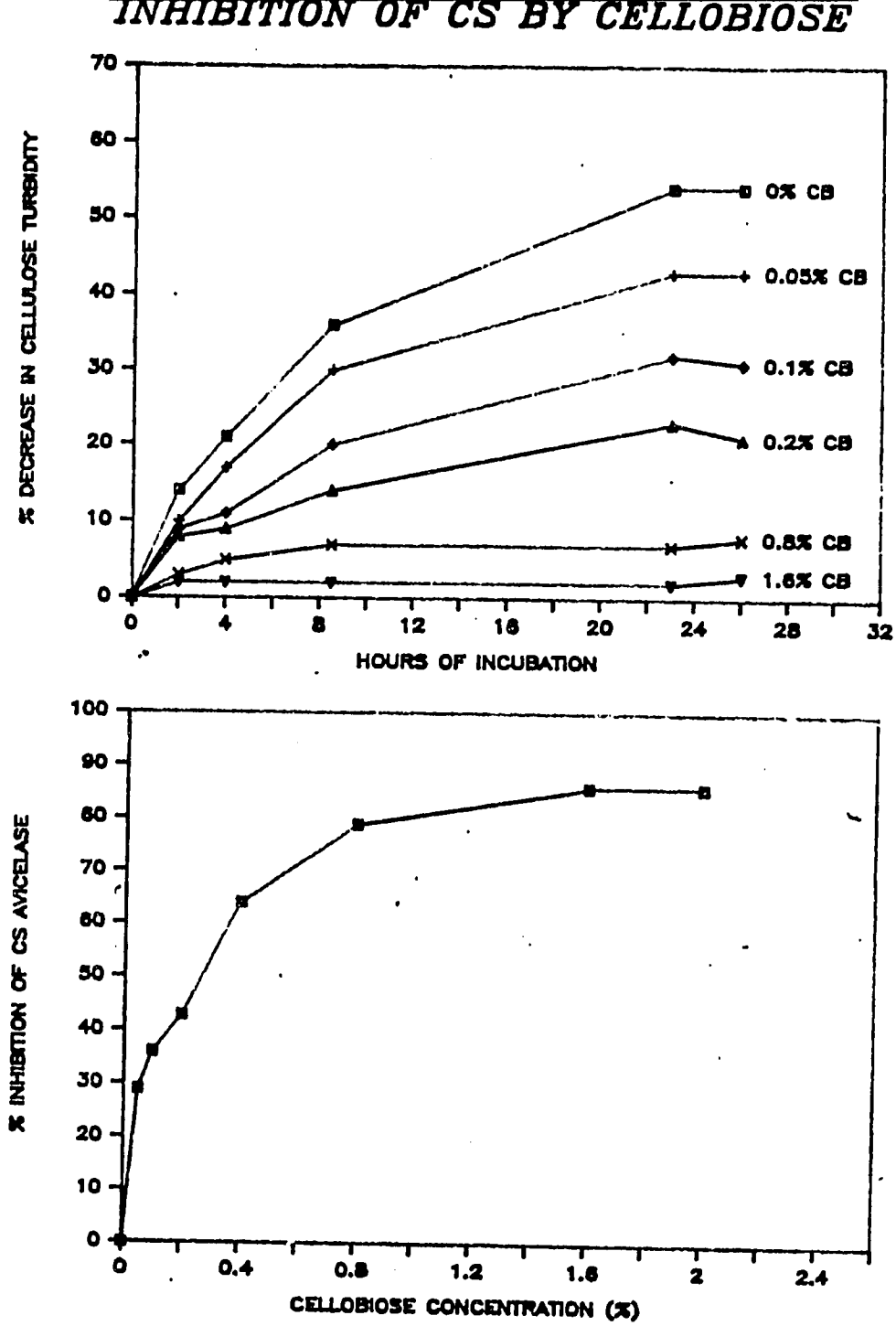

Fig. 3.

Inhibition of 5% Avicelase activity of the cellulosome by cellobiose.

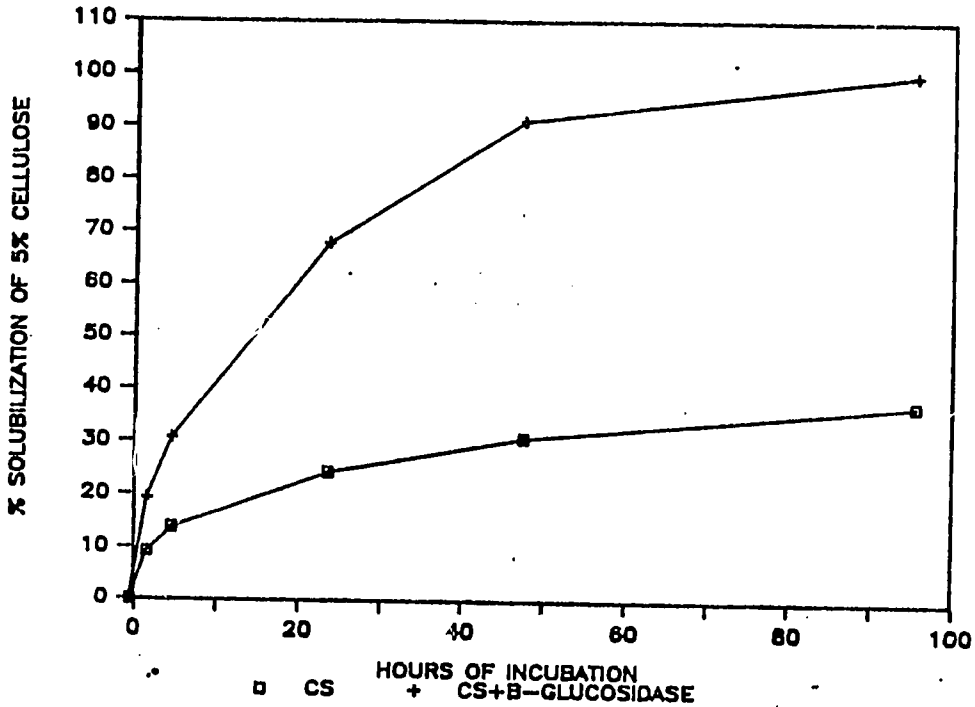

Fig. 4

B-Glucosidase-induced enhancement of solubilization of microcrystalline cellulose by the cellulosome of C. thermocellum.

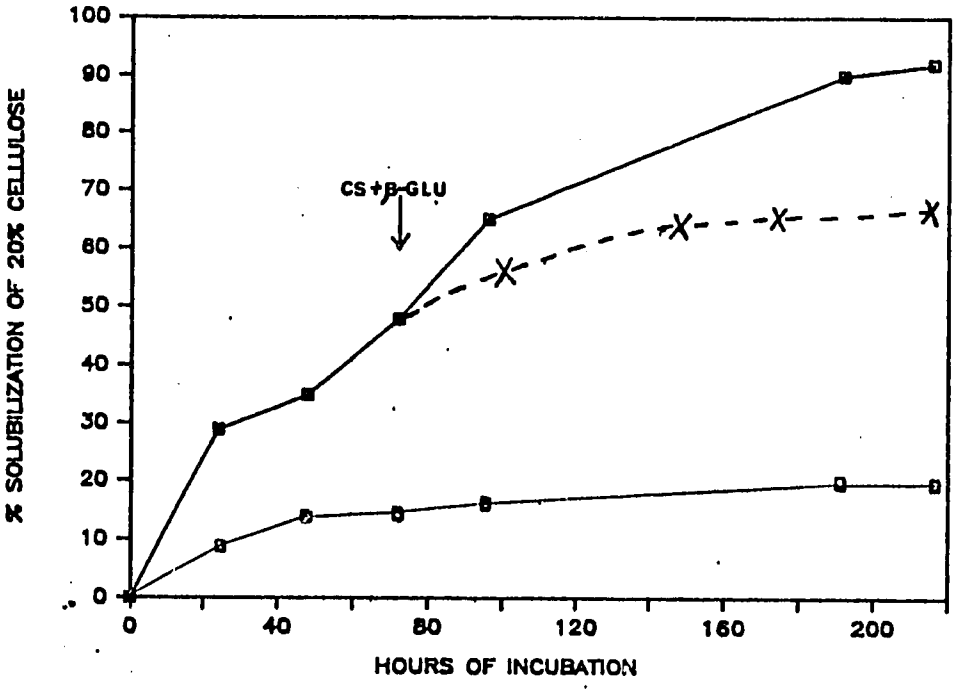

Fig. 5

Time course of solubilization of 20% Avicel by the combined cellulosome-B-glucosidase system. After 72 hrs, a second portion of B-glucosidase was added which further enhanced the degradation of Avicel to near completion.

Fig. 6

Purification of cellobiase from Novozyme

A. Commercial preparation.
B. DEAE - cellulose chromatography.
C. Sephacryl 200 chromatography.

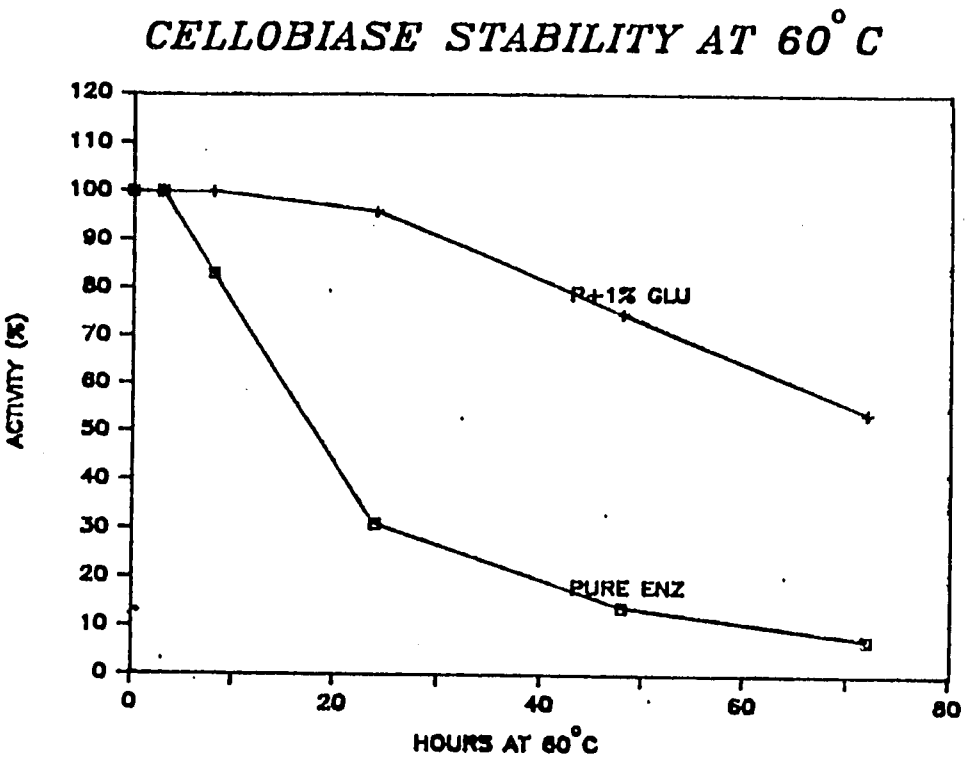

Fig. 7

Stability of the purified B-glucosidase to incubation at 60_oC in the presence or absence of glucose.

7. JOINT PUBLICATION

Efficient cellulose solubilization by a combined cellulosome-β-glucosidase system

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Running title: Enhanced solubilization of cellulose

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*Abbreviation used: PNPG, p-nitrophenyl β-D-glucopyranoside

ABSTRACT

The cellulosome, the multienzyme complex of the cellulase system of Clostridium thermocellum which mediates the solubilization of insoluble cellulose, is strongly inhibited by the major end product, cellobiose. By combining a purified β-glucosidase from Aspergillus niger with the cellulosome, accumulated cellobiose was hydrolyzed thereby resulting in a dramatic enhancement (up to 10-fold) of cellulose degradation. The observed enhancement was expressed both in the rate and degree of solubilization of microcrystalline cellulose, compared with that observed for the unsupplemented cellulosome. Near-complete conversion of cellulose to glucose could be obtained from dense substrate suspensions (up to at least 200 g/liter).

INDEX ENTRIES

Clostridium thermocellum; cellulosome; cellulase; cellulose, solubilization of; Aspergillus niger; Novozym; β-glucosidase; cellobiase

INTRODUCTION

For many years, the cellulase system of Clostridium thermocellum has been promoted for the industrial conversion of cellulose to glucose or other potentially useful products (e.g., ethanol). As the most prevalent example of an anaerobic thermophilic cellulolytic strain, this organism is considered particularly attractive for industrial application (1). Several approaches have been attempted. Some groups have tried to employ intact cell systems for this purpose. Others have utilized crude cell-free extracts or partially purified cellulase preparations. Both strategies have essentially failed. On the one hand, cells of C. thermocellum are sensitive to relatively low concentrations of ethanol (one of the major and the preferred product). On the other hand, both crude and purified enzyme preparations are subject to potent inhibition which leads to ineffectual solubilization of cellulosic substrates.

An important measure of the efficacy of cellulose degradation has been described and termed "true" cellulase activity, or the capacity to completely solubilize microcrystalline cellulose (2). In C. thermocellum, the major portion of true cellulase activity resides in the cellulosome, a high-molecular-weight, multienzyme protein complex (3-5). However, the capacity of this complex to degrade high concentrations of cellulose is limited; in the past, only the solubilization of relatively dilute suspensions of cellulose have been reported (2,6,7).

In earlier works, Demain and colleagues have shown that a crude cellulase preparation can be inhibited by cellobiose (8). In a similar vein, we demonstrated (6) that the true cellulase activity of the purified cellulosome is also inhibited by cellobiose (the major end product). In

this communication, we show that a purified fraction of a commercially available β-glucosidase from A. niger counteracts the observed cellobiose-induced inhibition of cellulose degradation by the cellulosome of C. thermocellum. This phenomenon results in the near complete conversion of microcrystalline cellulose to glucose at concentrations of at least 200 g per liter employing 5-10 mg of the combined enzyme preparation per gram of cellulose.

EXPERIMENTAL

Materials

Avicel PH 105 (microcrystalline cellulose) was a product of FMC (Philadelphia, PA). Crude β-glucosidase (Novozym 188) was kindly provided by Mrs. Eilat Reisner, the local agent of Novo Industries a/s (Bagsvared, Denmark).

Purified cellulosome fractions were obtained from the cell-free broth of cellulose-grown cells of C. thermocellum strain YS as described previously (9). The 2-step procedure consisted of affinity chromatography on cellulose and gel filtration on a Sepharose 4B column.

DEAE-cellulose (DE 52) was purchased from Whatman Ltd., (Kent, UK). Cysteine-HCl, cellobiose, glucose, p-nitrophenyl β-glucopyranoside, and the glucose determination kit (No.510) were obtained from the Sigma Chemical Co. (St. Louis, Mo).

Enzymatic Activities

The PNPG assay. This assay was used to rapidly assess the presence of β-glucosidase activity during the purification procedure for this enzyme. Samples were incubated for 30 min with a 1-ml solution containing p-nitrophenyl-β-D-glucopyranoside in 50 mM sodium acetate buffer (pH 5) at

60°C. The reaction was terminated upon addition of 30 μl of 2 M Na_2CO_3. The absorbance at 400 nm was measured. A unit of PNPGase activity is defined as the number of micromoles of p-nitrophenol (ϵ_{400} = 13901 $M^{-1}cm^{-1}$) formed per min.

Cellobiase activity. The assay was performed under conditions coincident with those required for efficient solubilization of cellulose by the cellulosome. The assay was carried out at 60°C under nitrogen for 60 min. The assay mixture consisted of 2 mM EDTA, 10 mM $CaCl_2$, 5 mM cysteine and 2% cellobiose in 50 mM sodium acetate buffer, pH 5.0. The amount of glucose formed from cellobiose was determined using the combined glucose oxidase/peroxidase system. A unit of activity is defined as the number of micromoles of cellobiose converted to glucose per min.

Cellobiose inhibition assay. The assay is based on the capacity of cellobiose to inhibit "true cellulase" activity in the cellulosome (6). The solubilization of cellulose was determined turbidometrically by a procedure modified from Johnson et al. (2). The reaction was conducted with continuous shaking in horizontally oriented rubber bung-sealed 13-mm test tubes under nitrogen atmosphere. A suspension (3 ml) of Avicel (0.06%) in 50 mM sodium acetate buffer (pH 5.0), containing 2 mM EDTA, 10 mM $CaCl_2$, and 5 mM cysteine-HCl, was incubated for 26 h at 60°C. The suspension was brought to a concentration of 5 μg/ml of cellulosome. Turbidity was measured at the indicated times at 660 nm by inserting the incubation tube into a Spectronic 20 spectrophotometer (Milton Roy Analytical Products Div., Rochester, NY). The degree of solubilization in a given sample was calculated as the ratio of turbidity decrease to the initial turbidity of the Avicel suspension. In inhibition experiments, cellobiose was added at the indicated concentrations.

Purification of β-Glucosidase

DEAE-Cellulose chromatography. Novozym (600 mg) was diluted with 1 liter of 10 mM potassium phosphate buffer, pH 6.4 (phosphate buffer) and DEAE-cellulose (50 ml resin preswollen in the same buffer) was added. The suspension was stirred for 1 h at 4°C. The supernatant fluids were decanted, and the DEAE-cellulose was packed in a column (1.5 x 28 cm). The column was then washed with 200 ml of the phosphate buffer. A linear gradient of increasing NaCl concentration was applied by means of two reservoirs, one containing 150 ml of phosphate buffer and the other containing 150 ml of 0.5 M NaCl in the same buffer. Fractions of 4.0 ml were collected at a rate of 24 ml/h. The peak of β-glucosidase (determined by the PNPG assay to elute at about 0.2 M NaCl) was pooled and concentrated to 1 ml by ultrafiltration (Omegacell® unit membrane, 10 K nominal mol. wt. limit; Filtron Technology Corp., Clinton, MA).

Gel filtration chromatography. The DEAE-cellulose fraction was applied to a Sephacryl S200 column (1.5 x 82 cm), equilibrated in phosphate buffer, containing 0.05% sodium azide. Fractions (2 ml) were collected at a rate of 40 ml/h. The β-glucosidase-containing peak was pooled and dialyzed against 0.1 M sodium acetate buffer, pH 5.0. The specific cellobiase activity of the purified enzyme was determined to be 173 units per mg enzyme.

Miscellaneous Methods

Protein was determined by the Bradford method (10). Reducing sugars were determined by the DNS method (11) and calibrated using either glucose or cellobiose as indicated below. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed in 6% or 10% gels essentially as described previously (12).

Bulk Solubilization of Cellulose

A 6-ml suspension of microcrystalline cellulose at the desired concentration was prepared in 11-ml rubber bung-sealed serum bottles using 50 mM sodium acetate buffer (pH 5.0), containing 2 mM EDTA, 10 mM $CaCl_2$ and 5 mM cysteine-HCl. A stock solution of the cellulosome was introduced to give a ratio of 8 mg per g substrate. This ratio of cellulosome-to-substrate was previously determined to provide maximal levels of activity (6). When required, purified β-glucosidase was added at 86 PNPG units per g substrate (equivalent to 42 cellobiase units per g substrate). For experiments using 20% cellulose, a second dose of the combined enzyme system was applied at the desired time interval. The bottles were flushed with nitrogen gas, and the incubation was carried out with shaking at 60°C. The reaction bottles were sampled anaerobically using a syringe equipped with an 18-gauge needle. Assay of reducing sugars was performed after removal of residual cellulose by centrifugation. For reaction mixtures containing β-glucosidase, calibration of the assay was performed with a glucose standard, since the final (and only) detectable product was glucose; for reaction mixtures lacking β-glucosidase, cellobiose (by far the predominant product of cellulosome action) was used for calibration. The above-described assessment of cellulose degradation was complemented by turbidometric measurements of appropriate dilutions (see section on cellobiose inhibition assay) of the respective reaction mixtures.

RESULTS

Inhibition of Cellulosome by Cellobiose

In previous work (6), we have shown that the solubilization of cellulose by the purified cellulosome of *C. thermocellum* strain YS undergoes near-complete inhibition by 2% cellobiose. This study had been prompted by an earlier finding by Johnson *et al*. (8) who showed a similar inhibitory effect of cellobiose on the crude cellulase system in a different strain of *C. thermocellum*.

The phenomenon of cellulase inhibition by end products of cellulolysis was examined by analyzing the decrease in cellulose turbidity (Fig. 1A). This particular assay system is considered to be a measure of the "true cellulase activity" of the given enzyme preparation. In these experiments, the measurement of reducing sugar by the DNS procedure would be inapplicable due to the cellobiose or glucose (as potential inhibitors) added exogenously to the reaction mixture.

Using this system, half-maximal inhibition was reached at exogenous cellobiose concentrations of about 0.1% (Fig. 1B). Using the same assay system, only nominal levels of inhibition were observed upon addition of equivalent levels of glucose, similar to the results reported for the crude cellulose system of strain ATCC 27405 (8).

Purification of *A. niger* β-Glucosidase

Since cellobiose (the major product of cellulosome action on cellulose) appeared to be the major cause for inhibition, we considered converting the cellobiose produced to glucose using an appropriate enzyme. This would especially be important for industrial application where hydrolysis of high concentrations of substrate would be required. We therefore looked for a suitable source of enzyme.

Novozym, a crude preparation from culture filtrates of the fungus A. niger, is commercially available from Novo Industries, and a simple 2-step procedure was developed in this work for the purification of the β-glucosidase. A similar procedure was recently described by McCleary and Harrington (13).

The protocol consisted of DEAE-cellulose ion-exchange chromatography followed by gel filtration on a Sephacryl S-200 column (Table 1). The active fractions from each step and the crude enzyme were subjected to SDS-PAGE. As shown in Figure 2, the purified enzyme displayed a single polypeptide band. The molecular weight, as estimated by SDS-PAGE, was 116,000.

In order to further assess the suitability of the purified enzyme to the thermophilic reaction conditions consistent with the cellulosome action on cellulose, various stability properties of the β-glucosidase were examined. Thus, the activity of the purified enzyme was determined after incubation for different time periods (up to 72 hr) at 60°C (pH 5) in the presence or absence of 1% glucose (Fig. 3). More than 60% of the activity was retained after 72 hr when the enzyme was incubated in the presence of glucose. In the absence of the sugar, a significant decrease in the stability of the enzyme was observed under the above-described conditions; after 24 hr, only about 30% of the original activity remained.

Enhancement of Avicelase Activity by the Combined Enzyme System

The effect of β-glucosidase addition to the reaction mixture was tested by measuring reducing sugar either turbidometrically or using the DNS method (11). This was accomplished employing up to 200 mg Avicel per ml reaction mixture, the amount of cellulosome and β-glucosidase applied was proportional to the concentration of Avicel.

The addition of β-glucosidase to the reaction mixture increased the level of cellulose solubilization (Fig. 4). At 20 mg of substrate per ml, the addition of β-glucosidase enhanced the level of solubilization about 2-fold. A 3-fold enhancement was observed at 50 mg of substrate per ml. Complete solubilization in this case was evident after 96 hr in the presence of β-glucosidase, compared to only about 30% in the absence of the enzyme. The most striking result was observed at 200 mg of substrate per ml reaction mixture; in this case, a 10-fold enhancement was observed. At such high concentrations of substrate, however, a fresh addition of the combined enzyme system was required at an intermediate stage of the process to achieve complete solubilization (see arrow, Fig. 4C). In most cases, the β-glucosidase caused the enhancement in both the initial rates and degree of solubilization. It is interesting to note that independent of the initial concentration of C. thermocellum substrate in the reaction mixture, the final level of solubilization effected by the cellulosome alone (without added β-glucosidase) was surprisingly constant (about 15 mg/ml). Similar results were obtained employing crude β-glucosidase (Novozym) to give equivalent levels of activity as described above for the combined enzyme system containing the purified β-glucosidase preparation.

DISCUSSION

Recent advances in the understanding of the cellulolytic system of C. thermocellum have led to the renewed interest in its cellulases, and in particular to the cellulosome, as a practical means for the conversion of cellulose to soluble sugars. The theoretical cellulolytic potential of this bacterial system has been estimated to be about 50-fold better than that of fungal systems (2), although their utilization for industrial processes has yet to be realized.

In order to achieve optimal levels of product formation, it is imperative to understand the key requirements for this system. In this regard, it has previously been demonstrated that the solubilization of cellulose, either by the crude cellulase system of C. thermocellum (2,8) or by the purified cellulosome (6), is highly dependent upon the conditions of its medium. The presence of Ca^{2+} and reducing conditions (e.g., thiols) markedly enhances the degradation of the insoluble substrate. Even more critical is the status of the major enzymatic end product, cellobiose, the accumulation of which results in the potent inhibition of the solubilizing action. Despite such modulating effects on the degradation of microcrystalline cellulose, the latter substances do not affect the enzymatic activity on a variety of modified substrates, e.g., CMC, dyed cellulosics or amorphous cellulose.

In designing a process for efficient enzymatic degradation of cellulose, the major consideration is to rid the system of the resultant cellobiose so as to prevent feedback inhibition of the cellulase apparatus. The cell apparently does so in nature by a combination of mechanisms (1). Firstly, the substrate is degraded extracellularly by means of polycellulosomal protuberances; part of the product is converted to glucose by the resident membrane-associated β-glucosidase, the glucose and the residual cellobiose are then incorporated intracellularly via the appropriate transport system. Moreover, additional amounts of cellobiose may be utilized in nature by other saccharolytic (but non-depolymerizing) satellite bacterial strains which occupy the same ecosystem. In this way, cellobiose is effectively removed from the environment and the cellulase system (specifically the cellulosome) continues to degrade the substrate

efficiently. In the cell-free state, the cellulosome produces saturating levels of cellulose, and cellulolysis is thus inhibited.

Conceptually, the combination of a cellulase system with β-glucosidase is not new. This approach has been tried in the past using fungal systems with varying degrees of success (15-18). Different strategies have been examined, including the batchwise mixture of the enzymes, the recycling of cellulase reaction products through immobilized β-glucosidase, and co-immobilization of both cellulase and β-glucosidase components. Nevertheless, these processes were economically prohibitive, since catalytic efficiencies were extremely low; very high enzyme-to-substrate ratios were required to effect high levels of solubilization. In another recent example where a bacterial system was employed, Kadam and Demain (7) combined a cloned β-glucosidase from C. thermocellum ATCC 27405 with the crude cellulase system from the same strain and demonstrated the enhanced degradation of relatively low concentrations of cellulose.

The major contribution of the present study is that a purified cellulolytic entity, the cellulosome of C. thermocellum, can be combined with a readily available β-glucosidase (Novozym) to effect efficient solubilization of very high substrate concentrations. In doing so, we employed relatively low concentrations of the respective enzymes compared with the fungal system. In applying the cellulosome system for naturally occurring cellulosics, it is noteworthy that the cellulosome already comprises numerous xylanases among its components which complement the inherent cellulolytic activity thus effecting concomitant solubilization of hemicellulose (19,20). Indeed, preliminary experiments led to significant

solubilization of dried alfalfa using the combined cellulosome-β-glucosidase system. In future applications, we plan to extend these studies by combining other enzymes with the cellulosome system for the improved solubilization of dense concentrations of natural cellulosic substrates.

ACKNOWLEDGMENT

Parts of this work were performed with the support of the U.S.-Israel Cooperative Development Research Program funded by the U.S. Agency for International Development (Washington, D.C.).

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Table 1. Purification of the β-glucosidase from Novozym

Fraction	Total Activity (PNPG units)[a]	Total Protein (mg)	Specific Activity (units/mg)
Novozym	37650	600	65
DEAE-Cellulose	21800	78	286
Sephacryl S200	5600	10	554

[a]One PNPG unit is equivalent to 0.493 cellobiase units (micromole glucose per min, 60°C)

FIGURE LEGENDS

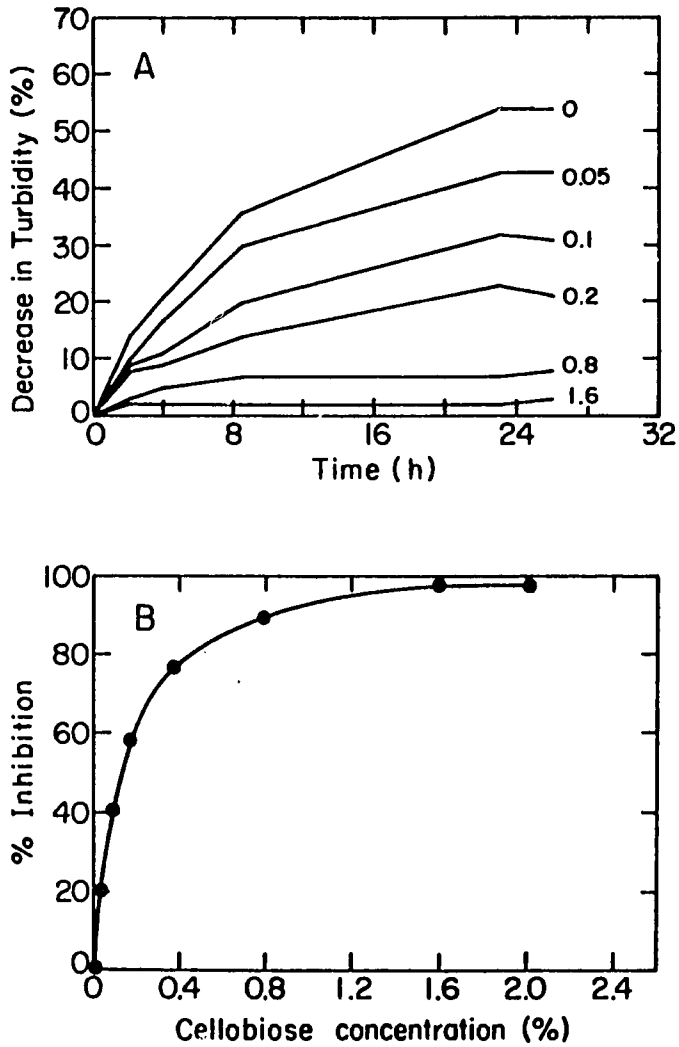
Figure 1. Cellobiose-induced inhibition of true cellulase activity in the cellulosome. Panel A shows the decrease in turbidity generated by the action of the cellulosome either alone (0) or in the presence of the indicated concentrations (in %, wt/vol) of cellobiose. Note that both the initial rate and the degree of solubilization of the substrate are severely affected by the addition of cellobiose. Panel B shows the extent of inhibition of cellulose degradation after incubation at 24 h as a function of the concentration of cellobiose.

Figure 2. Polypeptide profile of β-glucosidase preparations following the major steps of the purification procedure. Lane A, crude commercially obtained enzyme (Novozym); Lane B, DEAE-cellulose fraction; Lane C, purified enzyme.

Figure 3. Stability of purified β-glucosidase under conditions compatible with those required for efficient cellulosome action. The purified enzyme was incubated in the presence (+Glc) or absence (-Glc) of 1% glucose at 60°C (pH 5.0) in 0.1 M sodium acetate buffer, containing 2 mM EDTA, 10 mM $CaCl_2$ and 5 mM cysteine.

Figure 4. Solubilization of various concentrations of cellulose by the combined cellulosome-β-glucosidase system versus that of the cellulosome alone. The figures show the time course of solubilization of the indicated concentrations of cellulose either by a combination (•) of the cellulosome (8 μg per mg cellulose) and the β-glucosidase (0.04 cellobiase units per mg substrate) or by an equivalent amount of

the cellulosome alone (o). At the highest level of cellulose concentration (Panel C), the extent of solubilization reached a plateau at about 60%. The reaction could be increased to near-completion (X) by the introduction (arrow) of a second sample of the combined enzyme system.



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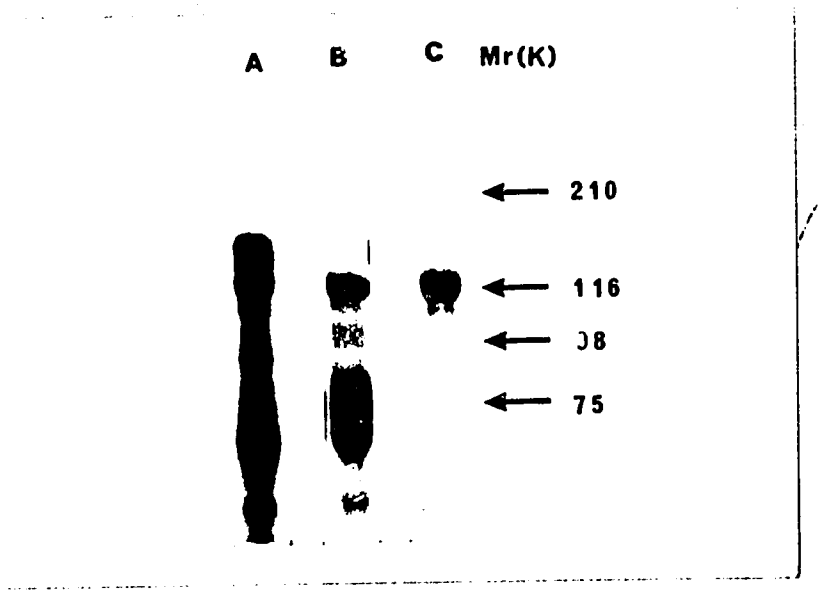
(Fig. 1)



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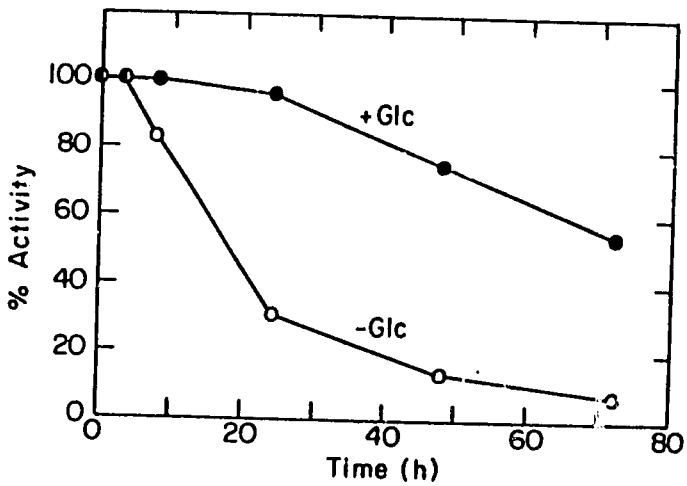
(Fig. 2)



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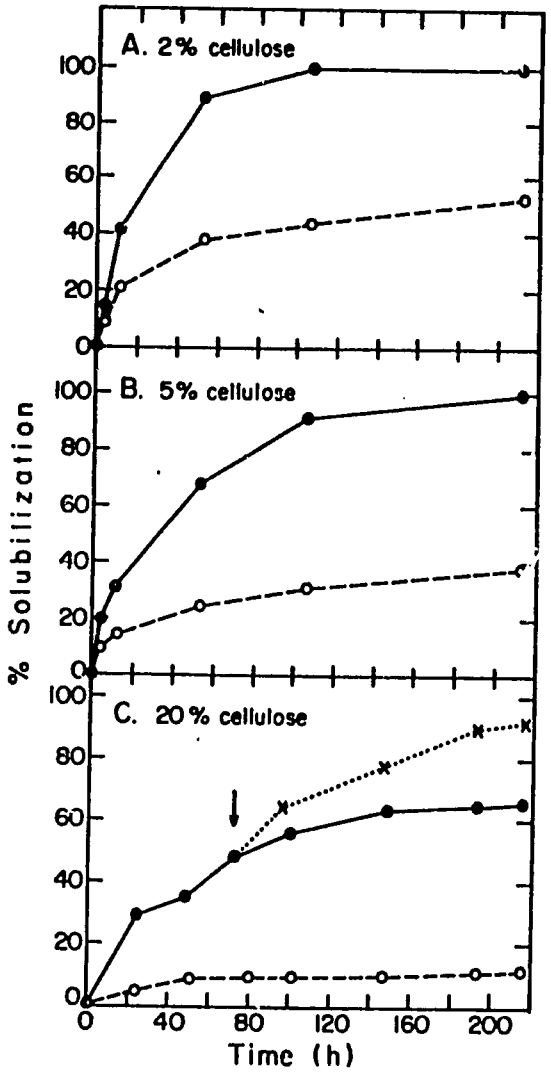
(Fig. 3)



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(Fig. 4)

8. OTHER PUBLICATIONS

Current Microbiol. in press

Cellulosome-like entities in Bacteroides cellulosolvens

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ABSTRACT

Cellulosome-like complexes were identified in the broth and sonic extracts of cellobiose- and cellulose-grown cells of Bacteroides cellulosolvens. The extracellular fractions contained 3 to 4 major polypeptides and several minor polypeptide bands which were localized in two major gel filtration peaks indicating average molecular weights of about 700 kDa and >10 MDa. A relatively large molecular weight component (M_r 230 kDa) was found to contain carbohydrate, but no apparent enzymatic activity of its own could be detected. The cell sonicate displayed a more complicated polypeptide profile, and glycosylated polypeptides were larger (ca. 310 and 290 kDa) than that of the extracellular fraction. The 230-kDa extracellular component interacted strongly with the GSI isolectin from Griffonia simplicifolia, exhibited immunochemical cross-reactivity with the S1 subunit of the cellulosome from Clostridium thermocellum, and displayed anomalous pH- and salt-dependent migratory behavior in SDS-PAGE. Taken together, this evidence strongly suggests a structural similarity between the glycoconjugates of these two distinct cellulolytic bacteria. A major 84-kDa polypeptide was identified as a xylanase, and a 50-kDa polypeptide displayed endoglucanase activity. Additional biochemical and cytochemical evidence indicated that cellulosome-like cellulolytic complexes are associated with the cell surface in this bacterium.

INTRODUCTION

Aggregative forms of cellulases have been identified in several cellulolytic bacteria either as cell-bound complexes and/or in the cell-free form (10). In the case of Clostridium thermocellum, the most extensively studied cellulolytic bacterium, adherence to cellulose is directly mediated by a multicellulase complex termed the cellulosome (1,12). The cellulosome of C. thermocellum was found to be exquisitely organized for highly efficient degradation of its insoluble cellulosic substrate. The cellulosome complex

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Abbreviations used: CMC, carboxy methyl cellulose; FITC, fluorescein isothiocyanate; GSI, isolectin I from Griffonia simplicifolia; PBS, phosphate-buffered saline, pH 7.4; PAGE, polyacrylamide gel electrophoresis

contains at least 14 different subunits, many of which exhibit endoglucanase, xylanase and exoglucanase-like activities (17). One of its most interesting components, the S1 subunit, has no known enzymatic role, but may contribute indirectly to the observed synergism among the other components of the cellulosome (11,16). The S1 is highly glycosylated and antigenic (4,11).

We have recently reported that a variety of cellulolytic bacteria exhibit a range of structural properties which are remarkably similar to those of C. thermocellum (13). These include the presence of cell surface protuberance-like structures, the interaction with an αGal-specific lectin which selectively recognizes the S1 subunit of the cellulosome, and the cross-reactivity with an anti-cellulosome-specific antibody preparation. One of these strains, Bacteroides cellulosolvens, a Gram-negative mesophilic bacterium, was previously found to contain a very active cell-associated cellulase (6,21) and to exhibit the above-mentioned properties reminiscent of the cellulosome from C. thermocellum (5). The purpose of this work was to determine whether such a multienzyme complex exists in B. cellulosolvens and to characterize its properties on the molecular level, using the cellulosome from C. thermocellum as a reference.

MATERIALS AND METHODS

Organism and growth conditions. Bacteroides cellulosolvens ATCC 35603 was cultured anaerobically at 37°C according to Murray et al. (20) using either cellobiose (Sigma Chemical Co., St. Louis, MO) or microcrystalline cellulose (Avicel, E. Merck AG, Darmstadt, FRG) as substrate.

Electron microscopy. Cationized ferritin staining and processing for scanning electron microscopy was performed as reported earlier (14).

Lectin-induced agglutination and labeling of cells. Cell suspensions were washed once by centrifugation (17,000 g, 10 min) with PBS, and the cells were suspended to one absorbance unit (400 nm). A 50-μl sample was combined with 10 μl of FITC-GSI (0.4 mg/ml, Sigma). After 10 min at 25°C, the cells were washed once, suspended to the original volume with PBS, and mounted on a glass microscope slide for fluorescence analysis under an Olympus BH2 fluorescence microscope using a blue exciter filter.

Adherence assay. The assay was performed essentially according to Bayer et al. (2). Briefly, a 1-ml aliquot of a washed cell suspension (A_{400} = 2.5) was brought to a total volume of 3 ml with 1 ml of 20% (wt/vol in PBS) microcrystalline cellulose and 1 ml of PBS (containing the various additives when indicated). The suspension was vortexed for 30 s and the cellulose and bacterial cells were allowed to settle at room temperature for 60 min. The turbidity (A_{400}) of the supernatant fluids was measured and compared with control tubes wherein PBS was substituted for the cellulose suspension.

Determination of cellulolytic and xylanolytic activities. Cellulase and xylanase activities were performed by determining reducing sugars released from the respective polymer. CMCase activity was determined essentially according to Miller et al. (15); briefly, a solution containing the enzyme sample, CMC (low viscosity, 1% wt/vol) and citrate-phosphate buffer (50 mM K_2HPO_4, 12.5 mM citric acid, pH 6.3), was brought to a total volume of 2 ml with distilled water. The sampes were incubated for 2 hr at 38°C. A 3-ml aliquot of DNS reagent was added and the tubes were heated at 90°C for 10 min. The extent of reaction was measured spectrophotometrically at 640 nm. Xylanase activity was determined in a similar manner by substituting 0.5% (wt/vol) xylan for the CMC. A cellobiose standard was used for cellulase and xylose for xylanase activity.

Gel chromatography. Samples (5-25 mg, 5 ml) were applied to a Sepharose 4B column (1.4 x 63 cm), and gel filtration was carried out at room temperature. The column was equilibrated and eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 0.05% sodium azide. The flow rate was 0.25 ml/min, and fractions of 1.5 ml were collected.

Lectin labeling of cell-derived material. The desired extracellular or cell-associated sample was examined for lectin binding by a dot blot assay. Dot blots were dried and quenched with 2% bovine serum albumin in PBS. Blots were incubated for 1 hr with GSI-peroxidase (3 μg/ml, Sigma). After incubation, the blots were rinsed several times with PBS, and substrate solution [0.013% (wt/vol) H_2O_2 in a Tris-saline buffer (pH 7.4), containing 0.5 mg of chloro-1-naphthol per ml (initially prepared from a 3-mg/ml methanolic stock solution)] was added. Color development occurred within 5-10 min, and the reaction was terminated by washing the blots with tap water.

Cellulose-based affinity chromatography. The cell-free supernatant fluids from a 1-liter culture were brought to pH 7.7 with 1 N NaOH, and 10 g of microcrystalline cellulose were added. The suspension was then stirred for 1 hr at room temperature. The supernatant fluids were removed through a sintered glass funnel, and the residue was washed with 50 mM Tris-HCl buffer (pH 7.4). The adsorbed proteins were eluted from the cellulosic matrix with 100 ml of a 1% solution of triethylamine. The eluent solution was immediately neutralized using 10% acetic acid.

Lectin-based affinity chromatography. A 0.1-ml sample of GSI-agarose (EY Labs, Inc., San Mateo, CA) was washed with Tris-HCl buffer and added to a 1-ml solution containing 20-fold concentrated spent growth medium from B. cellulosolvens. The suspension was mixed for 60 min at room temperature, centrifuged and the supernatant fluids were saved for subsequent studies. The adsorbed proteins were eluted from the affinity column by 1 M galactose.

Slab-gel electrophoresis. The conditions for SDS-PAGE (6% gels) were those described previously (7). Native PAGE was carried out under similar conditions with the omission of SDS and mercaptoethanol. Gels were stained with Coomassie brilliant blue R250, destained and photographed. In some cases, gels were stained for the presence of carbohydrates using the PAS method (23).

Blot transfer of separated proteins was carried out electrophoretically onto nitrocellulose sheets. GSI-conjugated peroxidase was employed to detect the relevant carbohydrate-containing polypeptide component. Staining with anticellulosomal antibodies was carried out as described previously (13).

Zymograms. In order to detect enzymatic activity associated with a given polypeptide band, the gels were stained by a modification of the Congo Red procedure (3). Gels were run as above with the addition of the desired substrate, i.e., xylan (0.1% soluble fraction) for xylanase activity or 0.1% CMC for endoglucanase activity. Following the electrophoresis step, the gels were washed twice for 30 min with citrate-phosphate buffer containing 25% (vol/vol) 2-propanol and twice again with the alcohol-free buffer alone. The washed gels were incubated for 16 hr at 37°C and stained with 0.1% Congo Red. Finally, the gels were rinsed with 1 M NaCl.

RESULTS

Reisolation and characterization of B. cellulosolvens. In the early stages of this work, we encountered difficulties in maintaining the strain used (ATCC 35603). During growth in a simplified synthetic medium (18), which contained 0.5% cellobiose as carbon source, an unidentified mesophilic sporulating rod was consistently found to overtake the original nonsporulating rod. Since the bacterium was originally isolated from a stable coculture with Clostridium saccharolyticum (19,20), we suspected that the latter was the contaminant strain. After reisolation of the bacterium employing a roll bottle modification of the roll tube technique, the nonsporulating strain (B. cellulosolvens) could be transferred repeatedly in cellobiose-containing medium and exhibited a doubling time of 13 hr. In accordance with the original publication on this bacterium (20), the reisolate grew on cellulose and cellobiose as growth substrates, but not on starch, xylan, xylose, glycerol, glucose, fructose, galactose and arabinose. The culture was insensitive to 25 μg/ml penicillin G, but sensitive to 2.5% yeast extract, results which are in line with the original publication.

Adherence of B. cellulosolvens to insoluble polymers. Cells of B. cellulosolvens have been reported earlier to adhere to cellulose (8). This observation was confirmed here by the strong adherence of the cells to cellulose during growth, even upon vigorous shaking of the culture. In order to characterize the specificity of the adherence, washed cells were added to various charged and uncharged cellulosic and noncellulosic polymers, either in the presence or in the absence of potentially inhibitory sugar derivatives and salts. Adherence of B. cellulosolvens to microcrystalline cellulose was very similar to that of C. thermocellum in terms of the capacity of the polymer to adsorb the cells, and the lack of effect of various additives to interfere with the adsorption (including 5% concentrations of glucose, galactose or cellobiose, and 1 M concentratios of NaCl, CMC, hydroxyethyl cellulose or polyethylene glycol).

Methyl cellulose inhibited adherence in B. cellulosolvens but not in C. thermocellum. Like C. thermocellum, alfalfa and wheat straw adsorbed the bacterium. Significantly, in contrast to C. thermocellum, cells of B. cellulosolvens readily bound to the insoluble fraction of birch xylan. Starch and agarose particles adsorbed cells of B. cellulosolvens only nominally. Positively charged forms of cellulose such as DEAE- and PEI-cellulose adsorbed the bacterium, and the adsorption was partially inhibited by salt.

Scanning electron microscopic delineation of the cell surface. Protuberant structures on the cell surface were visualized employing the cationized ferritin procedure (14) in line with our previously published observations, obtained before reisolation of the bacterium (13). Extended protuberances linking the bacteria to cellulose particles could be observed in a cellulose-grown culture (not shown). Cellobiose-grown cells exhibited smaller and less abundant protuberances compared to cellulose-grown cells.

Cell surface labeling and agglutination by GSI isolectin. Several different cellulolytic bacteria were previously reported to undergo labeling by an isolectin from Griffonia simplicifolia which is specific for terminally linked α-galactosyl moieties, suggesting the general occurence of αGal on the surface of this bacterium. The reisolate was extensively labeled and agglutinated by an FITC derivative of this lectin (Fig. 1).

Fractionation and analysis of cell-derived material. The growth supernatant of cellobiose-grown B. cellulosolvens was concentrated and applied to 6% native PAGE gels The results (Fig. 2) indicated the presence of very high-molecular mass entities which barely penetrated the interface of the stacking and separating gels. Upon gel filtration on Sephacryl S200, the concentrate eluted in the void volume fraction.

Whole-cell sonicates and cell-free supernatant fluids, derived from cellobiose- and cellulose-grown B. cellulosolvens, were fractionated by gel filtration on Sepharose 4B. The fractions obtained were analyzed for protein and for their interaction with the αGal-specific lectin in a semiquantitative dot blot assay. In all cases (Fig. 3), two regions of lectin-binding activity were observed which apparently corresponded to aggregate forms eluting at about 700 kDa and in the void volume (>10 MDa).

SDS-PAGE of the carbohydrate-containing fractions from extracellular material derived from cellobiose-grown cells (Fig. 3A, 700-kDa region) showed four major bands (M_r 230, 140, 100 and 84 kDa) and a number of minor bands (Fig. 4). The very high-molecular-weight region (>10 MDa) showed a similar SDS-PAGE pattern (data not shown). Lectin-based staining of the blotted material indicated that the 230-kDa band was mainly labeled. PAS staining (not shown) verified the latter as the major carbohydrate-containing component. An essentially identical picture emerged for the analogous material from cellulose-grown cells (Fig. 3C). Anti-cellulosome antibody (from C. thermocellum) also crossreacted mainly with the 230-kDa band (Fig. 4).

Regarding cell-associated material, the labeling pattern was differnt than that of the extracellular polypeptide pattern. Coomassie staining of SDS-PAGE gels showed a relatively complex set of polypeptides. Lectin staining revealed a strong signal which coincided with a comparatively weak Coomassie-stained doublet

at about 310 kDa and 290 kDa. The latter two bands could be segregated by gel filtration (data not shown), both were localized in separate outermost fractions of the 700-kDa region. Interestingly, the 290-kDa band eluted in the larger molecular-mass fraction and the 310-kDa band followed in the lower-sized fraction, again suggesting that these glycopolypeptides occur in solution as complexes.

Affinity chromatography of extracellular material. Samples of the supernatant fluids (20-times concentrated) from cellobiose-grown cells (containing 3.4 and 9.6 units/ml endoglucanase and xylanase activity, respectively), were adsorbed to either microcrystalline cellulose or lectin-Sepharose (GSI) resin. In either case, the majority of enzymatic activity was adsorbed to the respective matrix; cellulose adsorbed 96% of the endoglucanase activity and 94% of the xylanase activity, the lectin-Sepharose adsorbed 68% and 70% of the corresponding activity. Free galactose (0.1 M) completely inhibited the binding to the lectin-Sepharose matrix.

Elution of the material was less than quantitative for both processes. Partial elution from the lectin-Sepharose could be effected by 1 M galactose, Triethylamine (pH 11) succeeded in releasing about 20% of the adsorbed material from cellulose. In either instance, three major polypeptide bands were reproducibly eluted (the 230-kDa glycosylated component, the 100-kDa and 84-kDa components), and one major component (140 kDa) failed to be adsorbed by both affinity resins. The data thus indicate that at least three major components comprise a cellulose-binding, αGal-containing, enzyme complex in B. cellulosolvens.

Localization of enzyme activity. Since both xylanase and endoglucanase activities are associated with the protein complex which is adsorbed and eluted from both cellulose and lectin affinity resins, it was of interest to identify which proteins were responsible for these activity. Using the Congo Red procedure together with the appropriate substrate (CMC or xylan), it was found that none of the major components expressed endoglucanase activity (Fig. 5). Rather, a relatively minor band (M_r ~50 kDa) appeared to be an endoglucanase. In the case of xylanase activity, one of the major bands (84 kDa) displayed a strong signal, and a variety of other minor and smaller polypeptide components exhibited lower levels of xylanase activity. It should be noted that the incubation conditions used for these zymograms were not ideal. It has been reported that $CaCl_2$ and ascorbic acid significantly enhance the endoglucanase activity in this bacterium, but it was not feasible to include these additives in the zymogram assay.

Immunochemical crossreactivity of cell-derived components with anti-cellulosome antibody. We have described previously (11) an antibody preparation which reacts with high specificity toward the S1 subunit of the cellulosome from C. thermocellum. Using this antibody on SDS-PAGE-separated samples of cellobiose-grown supernatant fractions of B. cellulosolvens, it was shown that the 230-kDa component was preferentially labeled (Fig. 4). Identical results were obtained for the affinity-purified complex. As would be expected, the signals obtained were somewhat weaker than that of the equivalent immunochemical interaction with the S1 subunit of C. thermocellum.

Anomalous behavior of the glycosylated component. We have recently found that pretreatment of the C. thermocellum cellulosome with acidic or low ionic strength conditions leads to an alteration in the mobility of the S1 subunit in SDS-PAGE (17). It was therefore of interest to determine whether a similar effect could be observed for components of the B. cellulosolvens system. Indeed, dialysis of the cell-free supernatant fluids against distilled water or pH 4.5 acetate buffer caused a similar anomalous electrophoretic effect in the glycosylated 230-kDa component. As for the C. thermocellum system, the usual SDS-PAGE profile of the latter component could be rectified by simple adjustment of the pH or ionic strength.

DISCUSSION

The plant cell wall, the natural target of attack by cellulolytic bacteria, is degraded by the concerted action of several enzymes. These enzymes must bind initially to their target substrate and are effective only through the combined synergistic operation. A unique advantage for the enzyme-bearing microorganism must exist in such a highly competitive ecological environment. In many cases, the primary selective advantage is gained by the adhesion of the bacterial cell to the substrate, a process which is accompanied by the localization of critical hydrolytic enzymes at the cell-substrate interface.

B. cellulosolvens is an efficient crystalline cellulose degrading microorganism which binds tightly to cellulose derivatives and to xylan. Most of its cellulolytic enzymes appear to be maintained in a cell-associated form (21). The specificity of substrate adherence for this bacterium was broader than that of the well-characterized anaerobic thermophilic strain, Clostridium thermocellum, which exhibits a rather strict preference for underivatized cellulose. The inhibition of the adherence by methyl cellulose, a potent inhibitor of endoglucanases (22) suggests that cell-bound enzymes may indeed be involved in cell attachment as proposed earlier for C. thermocellum (9,12).

Other properties of the B. cellulosolvens system were found to be strongly reminiscent of the C. thermocellum system. In spite of the inability of the bacterium to grow on xylan and xylose alone as carbon sources, high xylanase activity was found both in secreted and cell-associated forms. At least one major polypeptide band was found to represent a true xylanase without detectable endoglucanase activity. The major products of xylan hydrolysis were xylobiose and xylotriose, but the cells also appear to contain an independent β-xylosidase which eventually converts these products to xylose (unpublished results). The xylanolytic activities appear to be physically associated into the cellulosome-like complex analogous to the situation in C. thermocellum (17).

Also noteworthy is the presence in the complex of a high-molecular-weight αGal-containing glycoconjugate(s) whose properties are strikingly similar to those of the S1 subunit of C. thermocellum. In this regard, neither glycoconjugate appears to exhibit enzymatic activity alone, but both exhibit an unusual pH- or salt-dependent anomalous effect in their electrophoretic mobility properties in SDS-PAGE (16). At least one endoglucanase was also detected in gels, although the complex itself appeared to account for at least 70% of the total endoglucanase activity of the extracellular material, indicating, perhaps, the presence of other undetected cellulases in the complex.

The results presented in this communication strongly support previous reports concerning the nature of anaerobic degradation of cellulose mediated by exocellular complexes of hydrolytic enzymes. Additional work on the molecular level is required in order to better understand the intra and intermolecular

interactions among the cellulosome components, the cell wall and the cellulosic substrate. It is also of particular interest to further examine the generality of these findings for other types of cellulolytic organisms.

ACKNOWLEDGMENT

Parts of this work were performed with the support of the U.S.-Israel Cooperative Development Research Program funded by the U.S. Agency for International Development (Washington, D.C.).

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FIGURE LEGENDS

Fig. 1. Agglutination and cell surface labeling of B. cellulosolvens cells by fluorescein-derivatized lectin. Cells were grown on cellobiose-containing medium, washed twice with PBS and labeled with FITC-GSI for 5 min at room temperature. Panel A, phase micrograph of untreated cells; panel B, phase micrograph of lectin-treated cells; panel C, fluorscence micrograph of lectin-treated cells.

Fig. 2. Native polyacrylamide gel electrophoretogram of concentrated growth medium from C. thermocellum (panel A) and B. cellulosolvens (panel B). Arrow indicates the interface between the stacking (3%) and separation (6%) gels.

Fig. 3. Gel filtration analysis of cell-associated and cell-free material of B. cellulosolvens. Whole-cell sonic extracts and cell-free growth fluids derived from B. cellulosolvens grown on either cellulose- or cellobiose-containing medium were fractionated by gel filtration on Sepharose 4B. The cross-hatched areas designate fractions which were reactive with the GSI lectin. The elution positions of the void volume (V_0) and the 670-kDa protein thyroglobulin (TG) are shown. Panel A, extracellular material from cellulose-grown cells; Panel B, extracellular material from cellobiose-grown cells; Panel C, cell-associated material from cellulose-grown cells; Panel D, cell-associated material from cellobiose-grown cells.

Fig. 4. Interaction of B. cellulosolvens protein components with lectin and with anticellulosome antibodies. Cells were grown on cellobiose-containing medium and the lectin-binding fraction of cell free material was prepared by gel filtration chromatography (see Fig. 3B, 700-kDa region of shaded area). The cell-associated preparation consisted of the total sonic extract obtained from washed cells grown on the same medium. Protein fractions were separated by SDS-PAGE, blotted onto nitrocellulose paper prior to labeling with either the GSI lectin or anticellulosomal antibody (Ab). Coomassie-stained samples (CBB) are provided for reference.

Fig. 5. Detection of endoglucanase and xylanase activities in extracellular protein complexes of B. cellulosolvens. The 700-kDa region of the cell-free growth culture derived from cellobiose-grown cells (Fig. 3B) was pooled, concentrated and separated by SDS-PAGE. The endoglucanase- and xylanase-containing bands were detected in gels which included the corresponding substrate. The gels were stained subsequently using the Congo Red procedure. Panel A, Coomassie brilliant blue staining; Panel B, CMCase activity; Panel C, xylanase activity.

Fig. 1

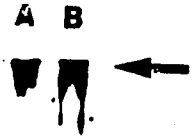

Fig. 2

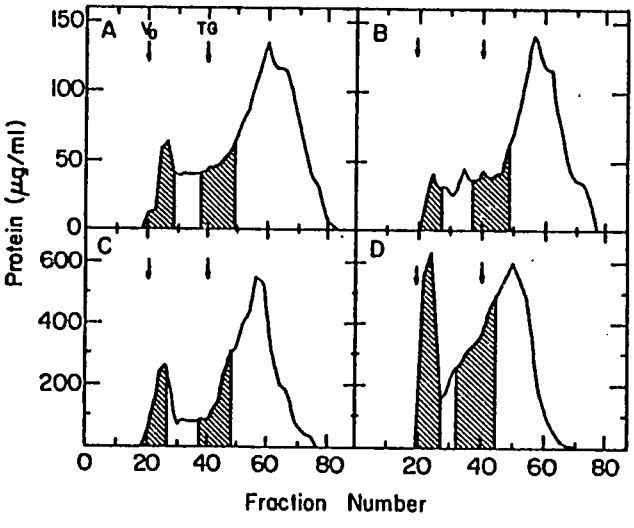

Fig. 3

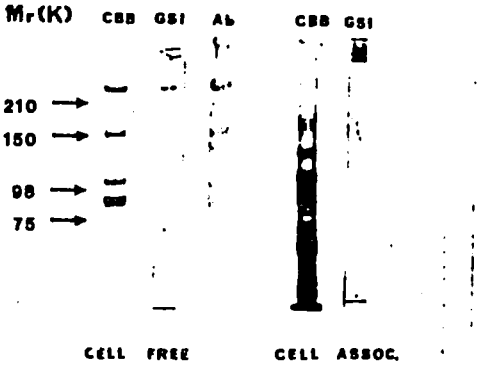

Fig. 4

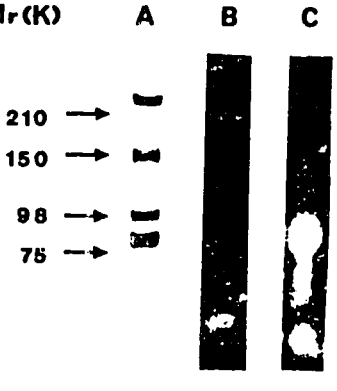

Fig. 5

J. Bacteriol. in press

Cellulosomal and noncellulosomal xylanases of *Clostridium thermocellum*: Relationship to cellulose-degrading enzymes

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Xylanase activity of *Clostridium thermocellum*, an anaerobic thermophilic cellulolytic bacterium, was characterized. The activity was localized both in the cellulosome (the principal multienzyme, cellulose-solubilizing protein complex) and in noncellulosomal fractions. Each of these fractions contained at least four major polypeptide bands which contributed to the xylanolytic activity. In both cases, pH and temperature optima, product pattern as well as other features of the xylanase activity were almost identical. The main difference was in the average molecular weights of the respective polypeptides which appeared responsible for the activity. In the noncellulosomal fraction, xylanases of M_r ranging from 30,000 to 65,000 were detected. Distinct from these were the cellulosomal xylanases which exhibited much larger M_r values (up to 170,000). The cellulosome-associated xylanases corresponded to known cellulosomal subunits, some of which also exhibited endoglucanase activity, others coincided with subunits which expressed cellobiohydrolase-like (exoglucanase) activity. In contrast, the noncellulosomal xylanases hydrolyzed xylan exclusively. β-Glucosidase and β-xylosidase activities were shown to be the action of different enzymes; both were associated exclusively with the cell and were not components of the cellulosome. Despite the lack of growth on and utilization of xylan or its degradation products, *C. thermocellum* produces a highly developed xylanolytic apparatus which is interlinked with its cellulase system.

In nature, potentially useful biomaterials often exist as complex multicomponent polymers. For example, the plant cell wall comprises an intricate structural array consisting mainly of cellulose, hemicellulose and lignin. Although a variety of different bacteria and fungi can successfully degrade these substrates, only little is known about the exact mechanism(s) of microbial degradation of such biomaterials.

Recent work in our laboratory (16,17) has contributed to a better understanding of the efficient degradation of crystalline cellulose by the bacterium *Clostridium thermocellum*. The cellulosome, a high-molecular-weight, multicomponent protein complex which exists in cell-surface bound and cell-free forms, has been shown to be responsible both for cellular adherence to cellulose and for the degradation of cellulose to cellobiose by the intact organism (5,22). The strong adsorption to cellulose and the molecular organization to form a defined complex has been proposed to generate efficient degradation of cellulose by the intact organism (4,21). Indeed, the cellulosome was found to be responsible for the "true"

Abbreviations used: CMC, carboxymethyl cellulose; MeUmb-cellobiose, 4-methylumbelliferyl β-*D*-cellobiopyranoside; MeUmb-Glc, 4-methylumbelliferyl β-*D*-glucopyranoside; MeUmb-Xyl, 4-methylumbelliferyl β-*D*-xylopyranoside; PNP-Glc, *p*-nitrophenyl β-*D*-glucopyranoside; PNP-Xyl, *p*-nitrophenyl β-*D*-xylopyranoside; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline, pH 7.4; SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; TLC, thin layer chromatography

(11

cellulase activity (i.e., the capacity to fully solubilize cellulose) in *C. thermocellum* (20).

Previous publications (11,26,28) have also suggested the presence of xylanases in the extracellular growth medium of *C. thermocellum* despite the reported (10,11) inability of this organism to grow on xylan as the sole source of carbon and energy. The definition of an enzyme as a xylanase is complicated by the commonly observed cross-specificity of xylanases and cellulases (13,32). In one report (29), two different enzymes derived from *C. thermocellum* were described wherein one was shown to exhibit only carboxymethyl cellulase (CMCase) activity whereas the other consisted of a combination of CMCase and xylanase activities. These particular proteins, when purified, were found not to be associated into the large-molecular-weight complex (the cellulosome). It was therefore of interest to characterize in general terms the xylanase activities associated with the cellulosome and to compare their properties with those of noncellulosomal xylanases. In addition, we were interested in understanding the interaction of these proteins with xylan and the effect of xylan on their interaction with the preferred substrate, i.e., insoluble cellulose.

MATERIALS AND METHODS

Materials. Cellobiose, carboxymethyl cellulose (CMC, sodium salt, low viscosity), 4-methylumbelliferyl *β-D*-cellobiopyranoside (MeUmb-cellobiose), 4-methylumbelliferyl *β-D*-glucopyranoside (MeUmb-Glc), 4-methylumbelliferyl *β-D*-xylopyranoside (MeUmb-Xyl), *p*-nitrophenyl *β-D*-glucopyranoside (PNP-Glc), and *p*-nitrophenyl *β-D*-xylopyranoside (PNP-Xyl) were obtained from Sigma Chemical Co., St. Louis, Mo. Microcrystalline cellulose (Avicel) was purchased from E. Merck AG (Darmstadt, FRG); xylose was a product of Fluka AG, Buchs, Switzerland. Xylobiose, xylotriose and birch xylan (Roth, FRG) were kindly provided by Prof. G. Zeikus, Dept. of Biochemistry, Michigan State University.

Organism, substrates and culture conditions. Cultures of *C. thermocellum* strain YS were grown anaerobically at 60°C in serum bottles containing the previously described (18) medium which included the desired carbon source. Cells were grown on cellobiose for 8 to 9 h, according to predetermined growth curves. Cellulose-grown cells were cultivated for about 24 h (until the cellulose substrate was no longer visible in the cultures); attempts to grow *C. thermocellum* on either xylan or xylose were carried out for at least one week.

Preparation of extracellular and cell-associated material. Extracellular (cell-free) material was obtained from the growth cultures by centrifuging the cells at 12,000 x *g* for 30 min. The supernatant fluids were collected and concentrated 50 fold by ultrafiltration using an Amicon PM10 membrane. Cell-associated material was prepared from whole-cell sonic extracts as follows. Cells, cultured in 200 ml of medium, were washed once with Tris buffer (pH 7.5) and resuspended in about 3 ml of the same buffer. The suspension was sonicated for 10 min in ice with a 70% duty cycle using a tapered disruptor horn in a sonicator cell disruptor (model W-225 R; Heat Systems-Ultrasonics, Inc. Plainview, NY). Particulate matter was removed by centrifugation (30,000 x *g*) for 20 min.

Fractionation of extracellular xylanase. Extracellular constituents of cellulose-grown cells were fractionated either according to size or according to their interaction with cellulose or xylan. In the former case, 50-fold concentrated extracellular material was subjected to gel filtration on a Sepharose 4B column (0.8 x 100 cm). The column was equilibrated and eluted with 50 m*M* Tris-HCl buffer, pH 7.5, containing 0.05% sodium azide. Fractionation on either cellulose or xylan was performed by bringing the unprocessed cell-free medium to

1.2% with the desired polymer. After a 1-h incubation period at room temperature, the stirred suspension was centrifuged using a clinical centrifuge, and the clarified supernatant was collected.

Purification of the cellulosome. The cellulosome was purified from the spent cell-free growth medium of cellulose-grown cells of *C. thermocellum*. The procedure is based on a two-step process which includes affinity chromatography on cellulose and gel filtration on Sepharose 4B as described previously in detail (18).

Fractionation of xylan substrate. In order to prepare soluble and insoluble fractions of xylan, a suspension (0.5%) of commercial xylan was prepared and stirred for 1 hr at room temperature. The mixture was centrifuged for 10 min at 17,300 x *g*; the supernatant, comprising the soluble fraction, was removed and saved; the pellet, comprising the insoluble fraction, was washed twice with 20 volumes of PBS, collected and saved.

Xylanase activity. Xylanase activity was assayed by incubating the desired enzyme preparation in the presence of 0.5% (wt/vol) xylan (total fraction) in citrate-phosphate buffer (50 m*M* K_2HPO_4, 12.5 m*M* citric acid, pH 6.3) at 60°C (unless otherwise stated in the text). In cases where a pH range was desired, citrate buffers were used between pH 2.9 to 7.4 and phospate buffers were used between pH 6.3 and 8.3. There was essentially no difference in the results noted for the overlapping buffer region (pH 6.3 to 7.4). Appearance of reducing sugars was assayed by the dinitrosalicylic acid procedure (24).

Stability assay. Cellulosomal or noncellulosomal xylanases were treated with either 0.2% SDS, sample buffer or sample buffer without mercaptoethanol. When desired, samples were heated to 70°C or 100°C for 10 min. Xylanase activity was then determined as described above.

Analysis of reaction products. Xylan degradation products were qualitatively determined by thin layer chromatography (TLC) on DC-Plastikfolien silica gel 60 F_{254} (Merck) using acetone-ethyl acetate-acetic acid (2:1:1). Plates were visualized by spraying with a 1:1 (vol/vol) mixture of 0.2% methanolic orcinol and 20% sulfuric acid; plates were then heated.

CMCase activity. In vitro determination of endoglucanase activity was performed according to Miller *et al.* (24) using CMC as a substrate. The incubation was performed for 30 min at 60°C. A unit of CMCase activity was defined as the amount of enzyme which released 1 μmol of reducing sugar (using glucose as a standard) per ml of sample per min under the conditions indicated.

β-Glucanase activity and β-xylosidase activity. β-Glucanase activity and β-xylosidase activities were estimated by measuring the release of *p*-nitrophenol from the appropriate substrate (PNP-Glc, PNP-Xyl or MeUmb-Xyl). Samples (5 μl) were dissolved in 1 ml of citrate-phosphate buffer, containing 10 m*M* of the corresponding substrate, and the reaction mixture was incubated for 30 min at 60°C. The reaction was terminated by adding 30 μl of 2 *M* Na_2CO_3, and the optical density of the liberated *p*-nitrophenol was measured at 405 nm. The release of 4-methylumbelliferone was measured at 347 nm by difference absorption spectrophotometry or by fluorometry (pH 10) at emission wavelengths >435 nm (excitation 335-400 nm).

Bacterial adhesion assay. A washed cell suspension was brought to A_{400} = 2 with PBS. Increasing volumes of the cell suspension were brought to a total volume of 1 ml and either 10 mg xylan (insoluble fraction) or 10 mg Avicel was added. The suspension was vortexed for 20 s and rocked gently at room temperature. After 20 min, the suspension was centrifuged using a clinical centrifuge (1500 rpm) for 4 min. The supernatant fluids were collected and the turbidity (A_{400}) was measured and compared to that of control tubes wherein PBS was substituted for the cell suspension.

In some experiments, the effect of xylan on the adhesion of the bacterial cells to cellulose was evaluated by the addition of 2.5 mg/ml xylan (soluble fraction).

Cellulosome adhesion assay. The purified cellulosome was biotinylated according to Bayer and Wilchek (6). A solution of the biotinylated cellulosome was treated with increasing amounts of either microcrystalline cellulose or an insoluble xylan preparation. The suspension was rocked for 20 min at room temperature, centrifuged at 4300 x *g*, and 1-μl samples containing serial dilutions of the supernatant fluids were loaded onto nitrocellulose strips. The strips were quenched for 1 h with 2% BSA in PBS, washed 3 times with PBS and incubated for 30 min with solutions containing streptavidin complexes with biotinylated alkaline phosphatase (7). The strips were then rinsed again with PBS, and substrate solution [10 mg naphthol AS-MX phosphate (sodium salt) and 30 mg Fast Red TR salt dissolved in 100 ml of 0.1 *M* Tris-HCl buffer, pH 8.4] was added. The reaction was terminated by washing the blots with distilled water. The amount of biotinylated enzyme remaining in each sample was determined either visually or by densitometric readings of the dot blots. The data were compared to a serially diluted biotinylated cellulosome standard.

Electrophoresis and in situ *visualization of enzyme activities.* SDS-PAGE was performed in 6% or 10% gels essentially as described previously (15). The gels were stained for endoglucanase or xylanase activity in a modification of the cellulase assay described by Beguin (8). A suspension (0.1% final concentration) of either CMC or soluble xylan was incorporated into the separating gel prior to addition of ammonium persulfate and polymerization. Protein samples were applied to the gel after heating for 10 min at the desired temperature (either 70°C or 100°C) in the presence of sample buffer (3% SDS, 10% glycerol, 5% β-mercaptoethanol in 31 m*M* Tris-HCl, pH 6.8).

Upon completion of electrophoresis, the gel was washed four times for 30 min in citrate-phosphate buffer (the first two washes contained 25% isopropanol), and the gel was then incubated in this buffer for 5 min at 60°C. The gel was introduced into a 0.1% solution of Congo Red and gently rocked for several minutes. Excess dye was decanted, and the gel was washed with 1 *M* NaCl until excess stain was totally removed from the active band. After a final rinse in 5% acetic acid, the background turned dark blue, thus facilitating photographic documentation. In duplicate lanes, proteins were stained with Coomassie Brilliant Blue R250, destained and photographed. In some cases, the gel was stained twice – once with Congo Red and once with Coomassie Blue. This enabled precise identification of activity with a given band.

Zymograms for exoglucanase activities. Due to the sensitivity of cellobiohydrolase-like activity to boiling in SDS (see Results Section), protein samples were applied to gels after heating for 5 min at 70°C in sample buffer. Upon completion of electrophoresis, the gels were washed twice for 30 min with citrate-phosphate buffer (pH 6.3) containing 25% isopropanol and twice for 30 min with the same buffer containing 1 m*M* MeUmb-cellobiose (without isopropanol). After washing, the gels were incubated in the substrate-containing buffer for 20 min at 60°C. Positive bands were detected by fluorescence under ultraviolet illumination at 340 nm.

β-Glucosidase activity was similarly detected on gel overlays, except that samples were boiled prior to application to gels and MeUmb-Glc was used as the substrate instead of MeUmb-cellobiose.

High performance liquid chromatography (HPLC). Gel permeation HPLC was performed using a Gilson model 303 HPLC system (Villiers le Bel, France) equipped with a Perkin Elmer model LC-75 spectrophotometer detector (Norwalk, CT). Cell-associated (sonic extract) material (200 μl, ca. 2 mg) was applied to a prepacked Superose 12 gel filtration column (Pharmacia, Uppsala, Sweden). The column was developed at 0.4 ml/hr using 0.1 *M* Tris-HCl (pH 7.5). Additional conditions conformed to the manufacturer's instructions.

RESULTS

Xylanase activity in C. thermocellum

C. thermocellum was grown on either cellulose- or cellobiose-containing medium. The cultures were harvested in the late-exponential phase of growth, and the respective fractions were examined for degradative activity on xylan. The cell-free xylanase activity derived from cellobiose-grown cells was about double that of the cell-associated activity (6.5 versus 3.1 μmol per ml broth per min). Similar but lower levels of xylanase activity were achieved for cellulose-grown cells (4.7 versus 1.4 μmol per ml broth per min for cell-free and cell-associated activity, respectively).

Fractionation of extracellular xylanase activity

It was of interest to determine the distribution of the observed xylanase activity of *C. thermocellum*. For this purpose, the extracellular constituents of cellulose-grown cells were fractionated either according to size or according to their interaction with cellulose or xylan. The cell-free growth medium was therefore subjected to gel filtration on Sepharose 4B, the high- and low-molecular-weight fractions were pooled separately, concentrated and their respective xylanase activities were determined. The results showed that about 60% of the activity was associated with the high-molecular-weight fraction. In parallel, cellulose or xylan was introduced to the extracellular material, and the amount of xylanase activity adsorbed onto the corresponding matrix was determined. In the case of cellulose, more than half of the xylanase activity was bound to the insoluble polymer. In contrast, in the case of xylan, all of the activity remained in the unbound state.

The association of xylanase activity with high-molecular-weight cellulose-bound fractions suggested that a major portion of this activity in *C. thermocellum* is contained in the cellulosome. It was thus decided to further test this possibility.

Characteristics of xylanase activity in the cellulosome

The degradation of xylan by the purified cellulosome was analyzed, and a specific activity of 100 μmoles xylose·min^{-1}·mg^{-1} was obtained. The activity reached a maximum within the pH range of 6 and 7.5, and the reaction exhibited a broad temperature optimum with a maximal activity of about 70°C (Fig. 1). The major products of the reaction included xylobiose (as the main reaction product), xylotriose, as well as various unidentified oligosaccharides (Fig. 2). Very low levels of xylose were also detected. After long periods of reaction (days) most of the xylotriose was converted to xylobiose which formed the major reaction product (data not shown).

The stability of the xylanase activity of the cellulosome to treatment with various agents was also investigated. Heating the cellulosome to 70°C for 10 min had little effect by itself on xylanase activity. Boiling the cellulosome, however, served to reduce the observed activity to about 15% of the original value. On the other hand, the combination of heating at 70°C and the presence of SDS resulted in a significant reduction (70-80%) of the xylanase activity of the cellulosome.

In addition, the activity was sensitive to inhibition by certain divalent metal ions. Interaction with 10 m*M* concentrations of Cu^{+2} and Pb^{+2} inhibited most of the activity. Zn^{+2} and Co^{+2} were less effective inhibitors; Ca^{+2} and Mg^{+2} had no effect on the xylanase activity of the cellulosome. The order of sensitivity of cellulosome action to metal ions was $Cu^{+2} > Pb^{+2} \gg Zn^{+2} > Co^{+2}$. The results are consistent with those of Tang *et al.* (29) who studied the action of a crude xylanase preparation using trinitrophenyl xylan as a substrate.

Since cellobiose, the end product of the cellulolytic process in *C. thermocellum*, is known to inhibit "true" cellulase activity in the cellulosome (14,20), we also examined whether xylose, glucose or cellobiose inhibited the xylanase activity with negative results. Soluble xylan degradation products (0.25 mg/ml) also failed to significantly inhibit the activity.

Minute levels of a β-xylosidase-like activity were observed using either PNP-Xyl or MeUmb-Xyl as substrates (data not shown). However, the cellulosome failed to convert pure β-xylobiopyranoside to xylose. This tends to corroborate the very low levels of xylose observed as a degradation product using xylan as a substrate (Fig. 2) and indicates that the cellulosome essentially lacks strict β-xylosidase activity.

Interaction of the cellulosome with xylan

Despite the high levels of xylanase activity in the cellulosome, *C. thermocellum* strain YS failed to grow on either xylan or xylose as the major carbon and energy source. Since the cellulosome is an exocellular complex which, in addition to its enzyme apparatus, is responsible for the adhesion of the bacterium to the insoluble cellulose substrate, it was interesting to determine whether the cellulosome binds also to xylan. For this purpose, a biotinylated preparation of the cellulosome was treated with incremented amounts of an insoluble xylan preparation, the polymer was pelleted, and the amount of biotinylated enzyme remaining in the supernatant fluids was determined. The results were compared to a positive control experiment in which cellulose was used as the adsorbent. In the case of xylan, essentially all of the cellulosome was found in the supernatant fraction, indicating a lack of binding to the substrate (Fig. 3). In contrast, the cellulosome was completely adsorbed to relatively low amounts of cellulose. Moreover, the addition of large amounts (10-fold) of xylan (soluble fraction) to the cellulosome-containing medium failed to interfere with the adsorption of the cellulosome to cellulose.

Noncellulosomal xylanases

In many respects, the characteristics of the noncellulosomal xylanase activity are very similar to those exhibited by the cellulosome. The pH maximum of the noncellulosomal xylanases was found to be identical to that of the cellulosome, although a much broader pH optimum was evident in the former (Fig. 1a). Likewise, the temperature maximum was identical in the two xylanase forms in *C. thermocellum*, except that a somewhat sharper temperature optimum was observed for the noncellulosomal xylanases (Fig. 1b).

The reaction products were indistinguishable for cellulosomal and noncellulosomal activities; xylobiose is the predominant product (Fig. 2). In both cases, complete depolymerization was evident from TLC analysis following long incubation periods. Interestingly, the inhibitory effect of divalent metal ions on the noncellulosomal xylanase activity is essentially identical to that on the cellulosomal activity; i.e., $Cu^{+2} > Pb^{+2} \gg Zn^{+2} > Co^{+2}$.

Comparative polypeptide pattern of cellulosomal and noncellulosomal xylanases

Despite the striking similarities in the above-described characteristics of the cellulosomal and noncellulosomal xylanase activities, their respective polypeptide patterns are markedly different (Fig. 4). The noncellulosomal polypeptides range from M_r ~27,000 to 70,000, far below the average molecular mass of (and migrating in positions inconsistent with) those of the cellulosomal subunits which range from M_r 48,000 to 210,000.

Even more pronounced, however, is the difference in the regenerated xylanase activities following SDS-PAGE separation. In the cellulosome-associated fraction, several major xylanase activities can be detected, corresponding to molecular masses of 170,000, 84,000, 67,000 and 54,000 (i.e., S2, S7, S9 and/or S10,[1] and S13). Two to three minor activities, e.g., S3, S5 and S11 (molecular masses of 150,000, 98,000 and 60,000) can also be discerned.

In the noncellulosomal fraction, none of the major xylanase activities are coincident with those of the cellulosome. The major noncellulosomal xylanase activities exhibit M_r values of 65,000, 47,000, 45,000, 40,000 and 30,000.

Xylanase and Endoglucanase activities of cellulosomal subunits

When parallel samples of the cellulosome (treated at 100°C in sample buffer) were subjected to SDS-PAGE and tested for xylanase and CMCase activities, some of the separated subunits appeared to exhibit similar levels of both specificities (Fig. 5); notably, the S2 and S13 subunits showed very high activities. The S5 subunit expressed relatively low levels of both activities. On the other hand, the S7 and S9,10[1] subunits appeared to contain xylanase activity exclusively. Similarly, at relatively high sample concentrations, the S11 subunit appeared to exhibit only xylanase activity. At such concentration levels, a series of additional activity bands were also visible (mol. mass between 120,000 and 150,000), most of which appeared to reflect both xylanase and CMCase activities. Many of the latter bands could not be clearly discerned by Coomassie Blue or Amido Black staining, although other types of protein-specific staining (e.g., silver staining and avidin-biotin-based stain) were capable of resolving these bands (Morgenstern, Bayer and Lamed, unpublished results). If the samples were treated at 70°C[2] (instead of 100°C) prior to SDS-PAGE, the activity of the S3 subunit was markedly enhanced, indicating the sensitivity of this subunit to boiling in the presence of SDS. It should be noted that other bands may well be sensitive to treatment at 70°C, and lack of observed enzyme activity does not necessarily mean that a given band does not contribute the respective activity to the cellulosome complex *in situ*. On the other hand, using CMCase zymograms, we could not examine cellulosome samples treated at 70°C, since the subunits retained high levels of activity during electrophoresis thereby causing a smearing effect resulting in poorly resolved bands.

Cellobiohydrolase-like activity of cellulosomal subunits

In the past, MeUmb-cellobiose has been used as an indication of exoglucanase (specifically cellobiohydrolase) activity (9,27), although there has been some question as to the validity of its use for this purpose.[3] Using MeUmb-cellobiose as a substrate (Fig. 6),

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[1]The S9 and S10 subunits exhibit similar M_r values and are difficult to resolve under the SDS-PAGE conditions used in this study.

[2]These conditions (incubation at 70°C in SDS) are the mildest whereby complete denaturation of the cellulosome is effected.

[3]In this work, we do not intend to argue its legitimacy as a strict exoglucasase substrate; in this context, we refer to the activity on 4-methylumbelliferyl *β-D*-cellobioside as "cellobiohydrolase-like" activity.

the highest activity levels were observed associated with the S3, S5 and S9(S10) subunits. Lower levels of activity were detected in the S4 and S6 or S7 subunits.

Disposition of β-glucosidase and β-xylosidase activities in C. thermocellum

β-Glucosidase and β-xylosidase activities were found exclusively in cell-associated extracts. Specific activities of 0.095 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein (0.020 $\mu mol \cdot min^{-1} \cdot ml^{-1}$ broth) for β-xylosidase and 0.25 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein (0.062 $\mu mol \cdot min^{-1} \cdot ml^{-1}$ broth) for β-glucosidase were obtained. Both activities were essentially absent from cell-free (cellulosomal and noncellulosomal) fractions. It was therefore of interest to ascertain whether the activities simply reflected cross-specificities of a single enzyme or whether they represented the action of individual entities.

Gel permeation HLPC of the whole cell extract of *C. thermocellum* revealed that the respective activities were confined to separate peaks (Fig. 7), one representing β-glucosidase activity ($M_r \sim 55,000$) and a second which exhibited β-xylosidase activity ($M_r \sim 200,000$). SDS-PAGE of the partially purified β-glucosidase combined with the relevant zymogram (using MeUmb-Glc) revealed a single band ($M_r \sim 50,000$). Similar electrophoretic analysis of the β-xylosidase was inapplicable, since the enzyme activity was abolished in the presence of SDS.

DISCUSSION

The purpose of this study was to characterize the xylanase activity exhibited by the *C. thermocellum* strain YS system and to evaluate whether such activity represents simply a subsidiary activity of the cellulase system or an independent set of enzymes devoted to the degradation of hemicellulose. In addition, information was lacking regarding exocellulase activity in the cellulosome and on the occurrence and location of β-glucosidase which appears not to be an essential activity in view of the previously reported alternative phosphorylation-associated cleavage of cellobiose (3).

Although *C. thermocellum* neither utilizes xylan nor adheres to the insoluble polymer, it is quite likely that the degradation of hemicellulose would enhance the ability of the organism to reach the cellulosic component(s) of the plant cell wall substrate. Furthermore, natural degradation of plant material occurs in most cases by mixed cultures in which the cellulolytic bacterium may contribute carbon sources to the immediate environment by virtue of its hydrolytic enzymes, benefitting, at the same time, from nutrients and/or the removal of toxicants by the auxiliary bacteria (19,25). In this context, *C. thermocellum* may provide the polymer-degrading link to satellite bacterial strains which are dependent on the rapid dispersal of low-molecular-weight saccharides for growth.

It thus may not be surprising that at least eight xylanolytic polypeptides were found in this work to be secreted in *C. thermocellum* strain YS, many of which were identified to be associated with the cellulosome complex. The cellulosome, which exhibits high affinity to cellulose (21,20), appears to be a targeting tool to bring these xylanase enzymes to the surface of the insoluble hemicellulose-containing cellulosic substrate (i.e., plant cell tissue); interaction of either the intact bacterium or the purified cellulosome with soluble or insoluble xylans could not be detected. This is somewhat surprising if we consider the potential for cooperative binding properties among the many xylanases within the cellulosome complex.

While the study of the free (noncellulosomal) xylanases may be relatively simple, the study of individual cellulosome components is rendered very difficult by the extreme stability of the complex (22,16). Nevertheless, the capacity of the xylanase components (or at least a portion thereof) to undergo functional regeneration following separation by SDS-PAGE has allowed their identification in this work. In a complementary approach, Grepinet *et al.* (12) have cloned and expressed one of the xylanase genes (xyn Z) in

Escherichia coli. The xylanolytic protein did not exhibit CMCase activity, but was shown to correspond to one of the subunits (S5) of the cellulosome. In another recent work (23) three distinctly different xylanase genes from *C. thermocellum* have also been cloned and expressed in *E. coli*, two of which were considered atypical xylanases of limited activity and a third gene product which was isolated and shown to extensively hydrolyze xylan. In addition, Pape *et al.* (S. Pape, F. Mayer and J. Wiegel, Abstr. 1st Annu. Meet. Am. Soc. Microbiol. on Biotechnology, 1988, 0-15) presented evidence using a different strain (JW20) of *C. thermocellum* which suggested that the cellulase system may indeed contain other noncellulolytic activities (including xylanases). In this regard, Wiegel and Dykstra (30) reported low levels of adsorption of *C. thermocellum* to hemicelluloses extracted from steam-exploded wood. We could not detect any adhesion of the YS strain to the insoluble fraction of commercial birch xylan.

The general properties of the cellulosome-associated and noncellulosomal xylanase activities can be compared among themselves and in relation to the xylanase purified from *C. thermocellum* by Tang *et al.* (29). In doing so, we could not find significant differences in sensitivity to bivalent metal ions, pH and temperature optima, except for the relatively high molecular weight of the cellulosome-associated xylanases relative to those of the noncellulosomal system.

The multiplicity of xylanases observed in *C. thermocellum* may be caused by a variety of factors. The most important, perhaps, is the heterogeneous nature of the substrate, in particular the sugar substituents of the linear β-1,4-poly-*D*-xylopyranoside chain (e.g., arabinose, glucose, mannose, galactose etc.), which constitute about 40% of most commercial xylans (31) and up to 80% in hemicellulose (32). Additional heterogeneity is caused by significant levels of acetylation of most xylans, in particular in hard woods. The complex nature of the substrate may thus require an equally complex set of enzymes for degradation. On the other hand, post-translational modifications of the enzymes, such as glycosylation, may also contribute to the variety of individual xylanase polypeptides found in this organism.

One of the unexpected results of this study was that the purified cellulosome exhibits higher levels of xylanolytic activity than endoglucanase activity. This was also reflected in the number of distinct cellulosome subunits which express the respective activities following SDS-PAGE. In this regard, some of the cellulosomal subunits, notably S2 and S13, exhibit both xylanase and endoglucanase activities, whereas others, such as S7 and S9(10), appear to express xylanase activity alone. In the noncellulosomal fraction, all of the observed xylanases failed to exhibit detectable endoglucanase activity.

Nevertheless, the apparent predominance of xylanase over endoglucanase activity should be treated with reservation. In this context, xylan is an enzymatically more practicable substrate than cellulose; the assay methods described here for xylanase activity are reasonably reflective of the hydrolytic capacity of the relevant enzymes for their substrate. On the other hand, cellulose degradation is an elusive commodity, and endoglucanase activity is not necessarily an accurate or even representative (and certainly not the sole) measure of cellulolytic activity *per se*. For this reason, we also attempted to characterize cellobiohydrolase-like activity in the cellulosome. In this case, the pattern of labeling (using MeUmb-cellobiose as substrate) shows a distinct labeling pattern, but definitive conclusions regarding true cellobiohydrolase activity awaits more extensive end-product analysis of separated (or cloned) subunits.

The presence of a cell-associated β-glucosidase in *C. thermocellum* YS corroborates previous findings of this enzyme in a different strain (1,2). We could not trace such activity in either cellulosomal or noncellulosomal preparations, and the role of this cell-associated enzyme is not entirely clear. Even more puzzling is the presence of a distinct cell-associated β-xylosidase, the role of which is even more ambiguous than that of the β-glucosidase. It is difficult to comprehend why a bacterium, which fails to grow on (or utilize) xylose, would be energetically encumbered with a complex set of xylanolytic

enzymes capped with a seemingly superfluous β-xylosidase. There may still be a cryptic ecological advantage in retaining the xylanase system in *C. thermocellum*, and the loss of a key enzyme (e.g., xylose isomerase) may have prevented the utilization of xylose in this organism.

ACKNOWLEDGMENT

Parts of this work were performed with the support of the U.S.-Israel Cooperative Development Research Program funded by the U.S. Agency for International Development (Washington, D.C.).

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FIGURE LEGENDS

Figure 1. Effect of temperature and pH on cellulosomal and noncellulosomal xylanase activities. Samples of the purified cellulosome (•) and the noncellulosomal extract (o) were incubated with the substrate for 30 min at pH 6.3 at the indicated temperatures (Panel A) or at 60°C at the designated pH values (Panel B). In each experiment, the highest observed rate of hydrolysis was defined as 100% for the given preparation.

Figure 2. Thin layer chromatography of the major reaction products of xylanase activities in *C. thermocellum*. Cellulosomal (CS) or noncellulosomal (nCS) fractions were mixed with 0.5% xylan, incubated for two days at 60°C, and samples (0.3 and 1 μl) of each were applied to the TLC plates. Untreated xylan (Xyn) and xylose oligomers [xylose (Xyl), xylobiose (Xyl_2) and xylotriose (Xyl_3)] were included as standards.

Figure 3. Adsorption of the cellulosome to insoluble substrates. Various concentrations (as indicated) of insoluble xylan or cellulose were applied to a solution (1 mg/ml) of a biotinylated preparation of the purified cellulosome. The unadsorbed fraction was applied to dot blots and the presence of residual (biotinylated) cellulosome was determined by a streptavidin-based staining procedure. Note that the cellulosome fails to adhere to xylan but adsorbs strongly to cellulose.

Figure 4. Xylanolytic activity profile of SDS-PAGE-separated cellulosomal and noncellulosomal components. The purified cellulosome (lanes A and B) and noncellulosomal extracts (lanes C and D) were applied to 10% SDS-PAGE gels. Lanes were stained either using Coomassie blue (lanes A and C) or examined for xylanase activity (lanes B and D) by the Congo Red procedure.

Figure 5. Comparison of xylanase versus endoglucanase activities in the cellulosome. Varying amounts (μg as indicated) of the purified cellulosome were heated in sample buffer to 100°C and applied to 6% SDS-PAGE gels. Following electrophoresis, the gels were examined for xylanase or CMCase activities by the Congo Red procedure. Coomassie Blue-stained cellulosome samples (CB) are presented in the outer lanes for reference.

Figure 6. Distribution of cellobiohydrolase-like activity in the cellulosome. Varying amounts (μg as indicated) of the purified cellulosome were heated in sample buffer at 70°C and subjected to SDS-PAGE (6% separating gels). The gels were then incubated with MeUmb-cellobiose as described in the text. A Coomassie Blue-stained cellulosome sample (CB) is provided as a reference.

Figure 7. Gel permeation HPLC of cell-associated β-glucosidase and β-xylosidase activities. Whole-cell sonic extract (200 μl) was separated by HPLC on a Superose 12 column. The fractions were analyzed for protein content (-----), β-glucosidase (•) and β-xylosidase (o) activities. The positions of the void volume (V_o), glucose oxidase (GO, mol. wt. 160,000), ovalbumin (OV, mol. wt. 45,000) and the total volume (V_t) of the column are given.

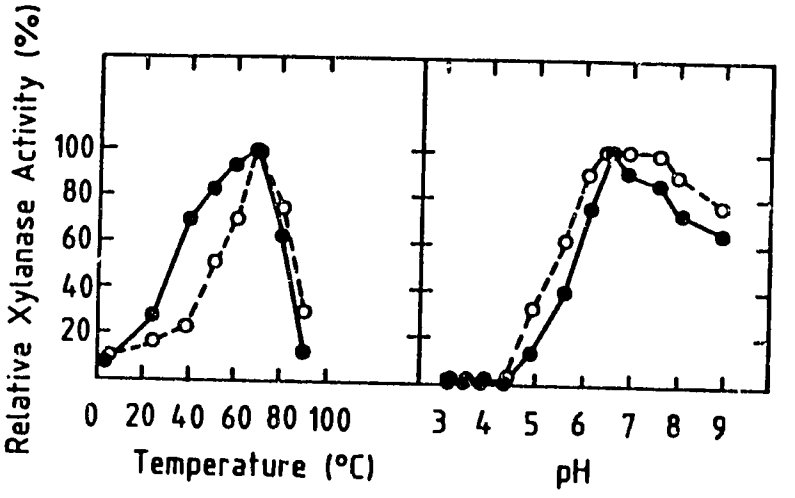

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Fig. 1

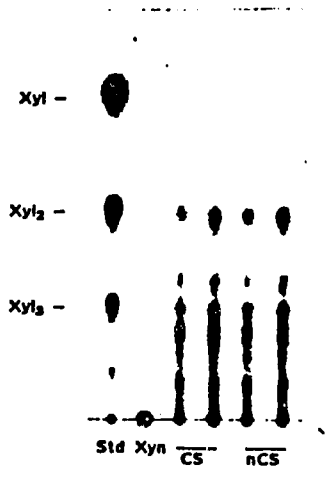

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Fig. 2

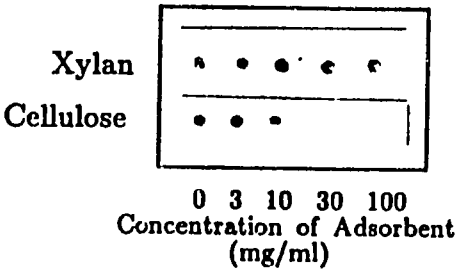

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Fig. 3

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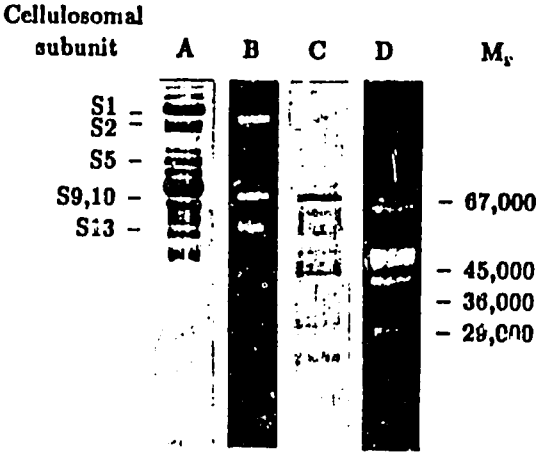

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Fig. 4

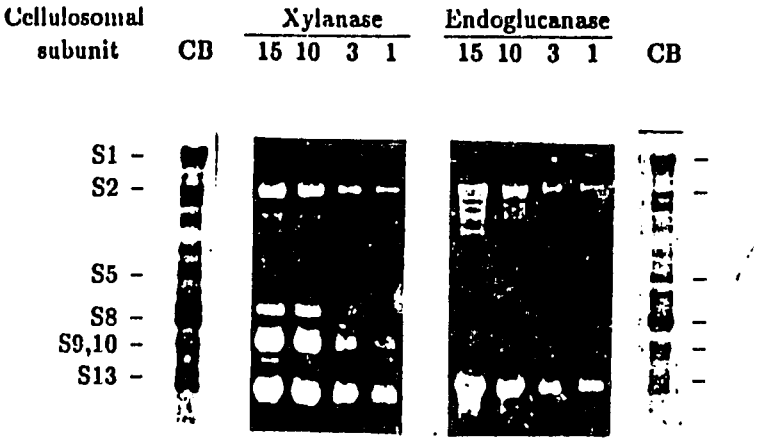

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Fig. 5

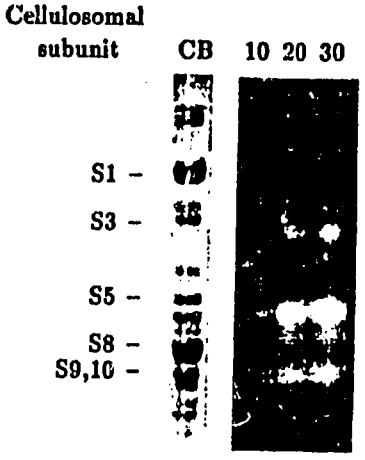

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Fig. 6

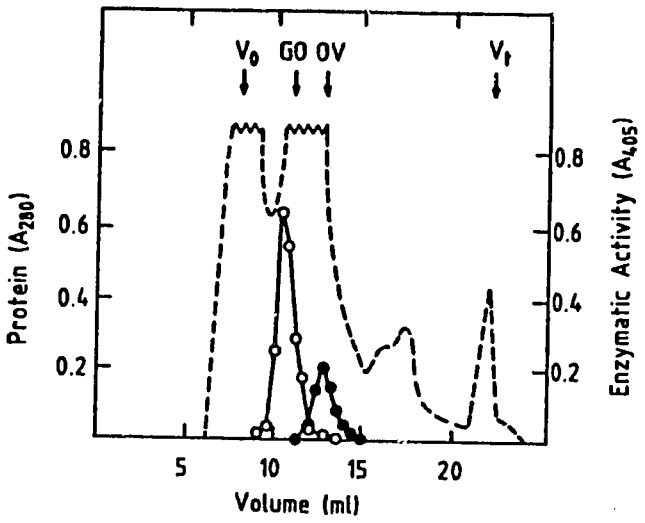

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Fig. 7

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SECOND PROGRESS REPORT

DEGRADATION OF PLANT CELL WALLS BY CELLULOSOMES OF ANAEROBIC BACTERIA

GRANT No. DPE-5544-G-SS-8061-00

ICAITI PERSONNEL:

FABIOLA DE MICHEO
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