APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1987, p. 1034–1041
0099-2240/87/051034-08$02.00/0
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Vol. 53, No. 5

Isolation of a Cellodextrinase from *Bacteroides succinogenes*

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Received 22 September 1986/Accepted 22 January 1987

An enzyme which released the cellobiose group from *p*-nitrophenyl cellobioside was isolated from the periplasmic space of *Bacteroides succinogenes* grown on Avicel crystalline cellulose in a continuous cultivation system and separated from endoglucanases by column chromatography. The molecular weight of the enzyme was approximately 40,000, as estimated by gel filtration. The enzyme has an isoelectric point of 4.9. The enzyme exhibited low hydrolytic activity on acid-swollen cellulose and practically no activity on carboxymethyl cellulose, Avicel cellulose, and cellobiose, but it hydrolyzed *p*-nitrophenyl lactoside and released cellobiose from cellotriose and from higher cello-oligosaccharides. These data demonstrate that the enzyme is a cellodextrinase with an exotype of function.

Bacteroides succinogenes, a strict anaerobe, is considered to be a predominant cellulolytic bacterium present in the rumen of many ruminants (6, 21). This bacterium possesses the ability to hydrolyze recalcitrant cellulosic materials such as cotton fibers and straw (19, 34). However, cell-free culture fluid from *B. succinogenes* exhibits little activity on crystalline cellulose (15, 19). Examination of cellulose fibers from a growing culture by electron microscopy revealed the close proximity of cells to cellulose, suggesting that cell-associated enzymes or other factors may be required for cellulolysis (11, 15).

According to the currently accepted model, which was developed from studies on fungal cellulolytic enzymes, extensive hydrolysis of crystalline cellulose requires synergistic action of at least three cellulase components: endo-1,4-β-glucanase (carboxymethyl cellulase [CMCase]), exo-1,4-β-glucanase (often a cellobiohydrolase), and β-glucosidase (cellobiase) (10, 36). All cellulolytic bacteria synthesize endoglucanase and either β-glucosidase (14, 29) or cellobiose phosphorylase (2) or a combination of the two (31). It was found that, during growth on cellulose, *B. succinogenes* produced multiple endoglucanase components, mostly present in the extracellular fluid, and cellobiase, mainly associated with cells (15). However, the existence of exoglucanase in bacteria has been questioned for a long time (9, 14, 28). Recently exoglucanases have been purified from a number of bacterial species, including *Streptomyces flavogriseus* (24), *Cellulomonas uda* (26), *Clostridium stercorarium* (7), and *Ruminococcus albus* (30). However, the presence of an exoglucanase has not been demonstrated in *B. succinogenes*.

In the past few years, a sensitive assay technique for the determination of an exo-type enzyme has been developed and involves the use of the substrate p-nitrophenyl-β-D-cellobioside (pNPC), an analog of cellotriose (8, 30). The exoglucanases were found to split off the cellobiose units from pNPC, producing *p*-nitrophenol, easily measurable at 405 nm (8).

In the present study, the cellular location of the cellulase activities was examined. With the use of pNPC, a cellobiosidase activity was identified and separated from other cellulase components. The properties of this new enzyme activity were investigated.

MATERIALS AND METHODS

Organism and growth conditions. *B. succinogenes* S85 (previously obtained from M. P. Bryant, University of Illinois, Urbana) was maintained as described by Groleau and Forsberg (15). For inoculum preparation, the organism was grown, as described by Schellhorn and Forsberg (32), in a medium identical to that used by Scott and Dehority (33) except that ammonium sulfate was the sole nitrogen source, with 0.2% Avicel microcrystalline cellulose PH105 (FMC Corp., Marcus Hook, Pa.) as the sole source of carbohydrate. After 4 to 6 days of incubation at 37°C, the growing culture served as an inoculum. A Multigen benchtop fermentor equipped with a 2-liter C-30 chemostat vessel and automatic pH control (New Brunswick Scientific Co., Inc., Edison, N.J.) was used to grow *B. succinogenes*. All connections of the system were made with butyl rubber tubing. The whole system was sterilized by autoclaving. Anaerobiosis was achieved and maintained by flushing the system with sterile O_2-free CO_2 at a flow rate of 8 ml/min. The chemostat medium was the same as that used by Groleau and Forsberg (15) for large-scale batch cultivation, except that ammonium sulfate (1.8 g/liter) was the sole nitrogen source, Avicel cellulose (1%) was the only readily available carbon source, and sodium carbonate was added to the medium to a final concentration of 0.16% (wt/vol). All medium components, except the vitamins, Na_2CO_3, and cysteine, were added to an 8-liter aspiratory bottle, which was then sterilized at 121°C for 45 min. After autoclaving, the medium was gassed with sterile O_2-free CO_2. After cooling, separately sterilized vitamin and Na_2CO_3 stock solutions were added. Two hours later, a sterile O_2-free cysteine solution was added. Complete reduction of the medium was indicated by the loss in color of resazurin. The outlet of the medium bottle was then aseptically connected to an Accu-rated peristaltic pump tubing (inner diameter, 1.52 mm) (Gilson Minipuls 2 peristaltic pump; Mandel Scientific Co., Rockwood, Ontario, Canada) leading into the medium inlet assembly of the chemostat culture vessel. Homogeneous suspension of the Avicel cellulose in the medium bottle was maintained by continuously stirring with a magnetic stirrer and added to the culture vessel as described by Lee et al. (22). The vessel was filled to one-third

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of its working volume. The chemostat was maintained at 39°C with agitation set at 60 rpm. The pH was regulated at 6.5 by the addition of 5 N NaOH. Growth of the culture was initiated with a 40% (vol/vol) inoculum of the growing cells. After 24 h, the medium was pumped into the culture vessel at a dilution rate of 0.025 h^{-1}. To prevent extensive sedimentation of Avicel cellulose inside the tubing during its passage to the vessel, the connection between the medium reservoir and the culture vessel was made as short as possible and the medium was pause pumped at a high pumping rate for 40 s at intervals of approximately 15 min. Outflow from the culture vessel was collected in a reservoir placed in a refrigerator (4°C). Culture purity was checked by phase-contrast microscopy. Under these growth conditions, the biomass in the chemostat culture was approximately 1.2 g of protein per liter.

Localization of cellulase components. The cell suspension was carefully removed from the chemostat effluent reservoir without disturbing the residual Avicel cellulose settled at the bottom and was centrifuged at 10,000 × *g* for 10 min at 4°C. The cells were then fractionated by using an osmotic shock technique essentially the same as that described by Neu and Heppel (27). The cells were washed twice in half the previous sample volume with cold 0.05 M sodium phosphate buffer (pH 6.5) supplemented with 0.8% (wt/vol) NaCl (phosphate-buffered saline) and suspended in the same volume of 25% sucrose–1 mM EDTA. The suspension was shaken for 10 min at 22°C. After centrifugation at 20,000 × *g* for 20 min at 22°C, the cells were quickly and vigorously suspended in the same volume of ice-cold water. The suspension was shaken gently for 10 min at 4°C and then centrifuged at 30,000 × *g* for 20 min at 4°C. The supernatant (periplasmic fraction) was removed, and the pellet was suspended in the same volume of phosphate-buffered saline and sonicated for nine 20-s bursts with 1-min intervals on ice. After centrifugation at 35,000 × *g* for 1 h at 4°C, the supernatant (cytoplasmic fraction) and the sediment (membrane fragment) were obtained. To calculate the protein and enzyme activities recovered during osmotic shock, a sample of the cell suspension from the chemostat reservoir was sonicated in the same manner and centrifuged at 35,000 × *g* for 1 h at 4°C to yield the membrane fragments and the total nonsedimentable preparations. Two enzymes, *p*-nitrophenyl galactosidase (25) and phosphoglucose isomerase (12), were used as cytoplasmic and periplasmic markers, respectively (3).

The accessibility of the cell-associated cellobiosidase activity to its substrate in the extracellular fluid was examined by comparing the cellobiosidase activities displayed by the intact cells and by the suspension of disrupted cells. *B. succinogenes* cells grown in the chemostat were harvested, washed with 0.05 M sodium phosphate buffer, pH 6.5, supplemented with 0.2 M NaCl and suspended in half the previous sample volume of the same buffer. Samples of the cell suspension were treated as follows: one sample was mixed with an equal volume of the suspending buffer to serve as a washed cell preparation; the second sample was mixed with an equal volume of the solubilizing solutions (2% [wt/vol] Triton X-100, 50 mM EDTA, 0.2 M NaCl, 0.05 M sodium phosphate buffer, pH 6.5); the third sample was sonicated for eight 30-s bursts with 1-min cooling intervals on ice and then mixed with an equal volume of the suspending buffer; and the fourth sample was sonicated and mixed with an equal volume of the solubilizing solution. All four samples were incubated at 0°C with shaking for 30 min and centrifuged at 30,000 × *g* for 30 min at 4°C. The cellobiosidase activity in the supernatant and the sediment of each sample was measured.

Enzyme assays. Cellobiosidase activity was determined according to Gilkes et al. (13). Properly diluted enzyme (0.05 ml) in 0.10 M sodium phosphate buffer, pH 6.5, was incubated with 0.05 ml of 1 mM *p*-nitrophenyl-β-D-cellobioside (Sigma Chemical Co., St. Louis, Mo.) in a 96-well microtiter plate at 39°C. The reaction was stopped by addition of 0.05 ml of 1 M Na_2CO_3. The absorbances were measured with a microtiter plate reader (model 307; Bioteck Instruments, Burlington, Vt.) equipped with a 405-nm filter. Other aryl glycosidase activities were determined in the same way except that pNPC was replaced with appropriate *p*-nitrophenyl glycosides. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per min.

Endoglucanase activity was assayed by incubating the enzyme with 1.0% carboxymethyl cellulose (Sigma C-8758; low viscosity; degree of substitution, approximately 0.7; degree of polymerization, approximately 400) at 30°C, and the reducing sugars produced were quantified with the Nelson-Somogyi reagent (1) as described by Schellhorn and Forsberg (32). The assays were conducted in 0.025 M sodium phosphate buffer, pH 6.5. Similarily, xylanase, laminarinase, and lichenanase activities were assayed by determining the amount of the reducing sugars released during incubation of the enzyme with, respectively, 1.0% (wt/vol) xylan (lot 113F-0003; Sigma), 1.0% (wt/vol) laminarin (lot L-9634; Sigma), and 0.5% (wt/vol) lichenan (lot L-6133; Sigma) for 60 min at 39°C. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugars (expressed as glucose) per min.

Cellobiase activity was determined with cellobiose as the substrate at a final concentration of 1.0% (wt/vol). Glucose liberated was measured with glucose oxidase as described by Groleau and Forsberg (15). Phosphoglucose isomerase activity was measured by the method of Bergmeyer (4).

Estimation of protein. Protein was determined by the method of Bradford (5), with bovine serum as a standard.

Preparation of samples for column chromatography. Cellulose-grown cells were harvested from the chemostat maintained under the conditions specified earlier and subjected to osmotic shock as described above. The osmotic shockate was concentrated approximately 25-fold by ultrafiltration at 0°C through an Amicon PM10 membrane (Amicon Canada Ltd., Oakville, Ontario). The concentrated material was then diluted with 5 volumes of ice-cold 0.02 M potassium phosphate buffer, pH 6.5, and reconcentrated. This process was repeated twice.

Column chromatography. DEAE-Sepharose CL-6B was obtained from Pharmacia, Uppsala, Sweden. Hydroxylapatite HTP and Bio-Gel P-150 were obtained from Bio-Rad Laboratories Ltd., Richmond, Calif. Isoelectric focusing (IEF) carrier ampholytes were from LKB, Bromma, Sweden. All chromatographic columns were prepared according to the manufacturer's instruction. The experiments involving column chromatography were performed at 4°C.

A DEAE-Sepharose CL-6B column (2.6 by 31 cm) was preequilibrated with 0.02 M potassium phosphate buffer, pH 6.5. After sample application, the column was washed with 1 bed volume of the equilibration buffer and then eluted with a linear gradient of 0.02 to 0.5 M potassium phosphate buffer, pH 6.5, over a volume of 500 ml. Finally, the column was eluted with 1.0 M potassium phosphate buffer, pH 6.5. The buffer gradient profile was determined by measuring the conductivity of fractions and relating the values to a calibra-

TABLE 1. Localization of the cellulase enzymes in *B. succinogenes*[a]

Fraction	Enzyme activity (nmol/min per mg of protein)					Protein (mg/ml)
	Cellobiosidase	CMCase	Cellobiase	pNPGase	PGIase	
S_1 (cell-free culture fluid)[b]	12 (39)	386 (69)	25 (10)	12 (71)	48 (32)	0.296 (28)
S_2 (wash)	1	20	5	1	5	0.023
S_3 (wash)	0.5	8	1.5	0.5	—[c]	0.013
S_4 (sucrose supernatant)	2.5	30	0.05	0.5	13.5	0.068
S_5 (periplasmic fraction)[d]	12 (62)	20 (21)	0.05 (0)	0 (0)	47.5 (61)	0.096 (15)
S_6 (cytoplasm)	6.5 (33)	31.5 (33)	47 (33)	2.5 (63)	26 (34)	0.168 (26)
S_7 (membranes)	1 (5)	43 (46)	96 (67)	1.5 (37)	4 (5)	0.372 (58)
Recovery (%)[e]	115	96	72	106	97	97

[a] Cells were grown in a chemostat with 1% (wt/vol) cellulose as the carbon source. pNPGase, *p*-nitrophenyl galactosidase; pGIase, phosphoglucose isomerase.
[b] Values in parentheses for S_1 are the percentages of the total enzyme activity or protein of the culture found in the extracellular cell-free culture fluid.
[c] —, Not determined.
[d] Values in parentheses for S_5, S_6, and S_7 are the percentages of the total cell-associated activity or protein found in each fraction.
[e] Recovery was calculated based on the total activity or the protein content of a sonicated culture.

tion curve. The fractions containing cellobiosidase activity were pooled and concentrated by ultrafiltration through an Amicon PM10 membrane. The concentrated sample was diluted with 0.02 M potassium phosphate buffer, pH 6.5, and reconcentrated. A sample of the cellobiosidase preparation obtained from the DEAE-Sepharose column was loaded onto a hydroxylapatite column (1.0 by 28 cm), which was equilibrated with 0.2 M potassium phosphate buffer, pH 6.5. The column was washed with the equilibration buffer, and the proteins were eluted with a linear potassium phosphate buffer gradient (0.02 to 0.5 M; pH 6.5). The buffer gradient was measured as described above. The fractions containing cellobiosidase activity were again pooled and concentrated. The sample from the hydroxylapatite column was applied to a Bio-Gel P-150 column (2.5 by 75 cm), which was equilibrated with 0.05 M sodium phosphate buffer, pH 6.5, and proteins were eluted with the same buffer. The void volume of the column was determined with a ferritin (horse spleen) solution. For molecular weight determination, the column was calibrated with protein standards including RNase A, chymotrypsinogen A, ovalbumin, and albumin.

Analytical IEF. Analytical IEF was performed in a Biophoresis horizontal gel electrophoresis cell (Bio-Rad), which was connected to a refrigerated water-circulating bath maintained at 4°C. Polyacrylamide solutions containing 5.76% (wt/vol) acrylamide, 0.24% (wt/vol) *N,N'*-methylene bisacrylamide, 10% (vol/vol) glycerol, 1% (wt/vol) glycine, 2% (wt/vol) carrier ampholytes, 0.1% (vol/vol) *N,N,N',N'*-tetramethylenediamine, and 0.025% (wt/vol) ammonium persulfate were cast between two glass plates separated by 1.5-mm-thick spacers. To obtain a pH range of 2.5 to 6.0, 1% each of the ampholytes pH 2 to 5 and pH 4 to 6 was used. Ampholyte (pH 5 to 7, 2%) and H_3PO_4 (1 N) were used as the cathode and anode solutions, respectively. The gel was prefocused for 20 min each at 100, 200, and 400 V. Up to 50 μl of the sample was applied in a plastic well placed on the gel surface. Subsequent electrofocusing was for 10 to 12 h at 400 V. Sample application wells were removed 1 h after electrophoresis was started. For detection of cellobiosidase activity, the agarose solution containing 0.03% (wt/vol) methylumbelliferyl-β-D-cellobioside, 2% (wt/vol) agarose, and 0.25 M sodium phosphate buffer, pH 6.5, was heated to 100°C and poured onto a gel support film for agarose (Bio-Rad). The solidified agarose gel was overlaid on the IEF gel. The sandwiched gels were incubated at 37°C and periodically checked under a long-wave UV light. Cellobiosidase releases from methylumbelliferyl cellobioside the methylumbelliferone group, which fluoresces blue under long-wave UV. The fluorescent bands on the gel were photographed under a long-wave UV light. For the protein stain, the gel was fixed for at least 1 h in a fixing solution (3.46% [wt/vol] sulfosalicylic acid, 11.5% [wt/vol] trichloroacetic acid in water) and then washed for 30 min or longer with a destaining solution (20% [vol/vol] ethanol, 8% [vol/vol] acetic acid in water), stained for approximately 40 min at 40°C in a staining solution (0.115% [wt/vol] Coomassie brilliant blue in the destaining solutions), and finally washed with several changes of the destaining solution. Pharmacia IEF standard was run according to the manufacturer's instructions.

Hydrolysis of cello-oligosaccharides and acid-swollen cellulose. Hydrolysis products of cellobiose and the higher cello-oligosaccharides, cellotriose, cellotetraose, cellopentaose (Pfanstiehl Laboratories Inc., Waukegan, Ill.), and cellohexaose (provided by R. B. Hespell) by the cellobiosidase preparation, were examined. The enzyme was incubated at 39°C for 10 h with 100 μg of each of the oligosaccharides in 0.05 M sodium phosphate buffer, pH 6.5, in a final volume of 0.2 ml. The reactions were stopped by heating the assay mixtures in a boiling-water bath for 10 min. For control, the reaction was terminated immediately after the enzyme was mixed with the substrate. The hydrolysates were freed from ions by mixing with the ion-exchange resins Amberlite IR-120 (hydrogen form) and Rexyn 201 (carbonate form), filtered through 0.45-μm-pore-size Metricel filters, and analyzed by using a high-pressure liquid chromatography (HPLC) system (Waters Associates, Milford, Mass.), which was equipped with a model ERC-7510 refractive index detector (Erma Optical Works, Ltd., Tokyo, Japan) and a model 730 data module (Waters Associates). Separation was achieved on a Bio-Rad cello-oligosaccharide column. The column temperature was maintained at 80°C. Degassed water was used as an eluant at a flow rate of 0.4 ml/min.

For examination of hydrolysis of acid-swollen cellulose by the cellobiosidase, the enzyme was incubated at 39°C for 16 h with 390 μg of acid-swollen cellulose in 0.05 M sodium phosphate buffer, pH 6.5, in a final volume of 0.2 ml. The hydrolysate was pretreated and analyzed by HPLC as described above.

RESULTS

Localization of cellulase components. The cellulose-grown cells were fractionated as described in Materials and Methods, and the cellulase activities in each fraction were as-

TABLE 2. Cellobiosidase activity of intact and treated *B. succinogenes* cells[a]

Treatment	Cellobiosidase activity (nmol/min per mg of protein)	Relative activity (%)	Activity solubilized (%)[b]
Untreated intact cells	6.9	40	4
Triton-EDTA	19.6	112	101
Sonication	16.9	100	100
Sonication-Triton-EDTA	17.9	104	100

[a] Cells were grown in a chemostat with 1% (wt/vol) cellulose as the carbon source.

[b] Percentage of total activity recovered in the nonsedimentable fraction (30,000 × *g* for 30 min).

sayed. It can be seen in Table 1 that three tested cellulase activities had different cellular distributions. Over half of the cellobiosidase activity was cell associated, whereas approximately 70% of the endoglucanase (CMCase) activity was present in the cell-free culture supernatant. Most of the cellobiase was found to be cell-bound. Comparison of the cellobiosidase activity in the three cellular fractions seems to suggest that this enzyme is mainly located in the periplasmic space of the cells. Incomplete release of the periplasmic enzymes during osmotic shock may be responsible for the cellobiosidase activity detected in the cytoplasmic fraction. In a similar experiment, almost 80% of the cell-associated cellobiosidase activity was released by osmotic shock. In contrast, no cellobiase activity was detected in the periplasmic fraction. Thus the cell-bound cellobiase activity appears to be present in the cytoplasm and associated with the membrane. The cell-associated endoglucanase activity was found in all cellular fractions.

Intact cells exhibited only 40% of their associated cellobiosidase activity toward the substrate pNPC in the suspending buffer (Table 2). However, the cell-bound cellobiosidase activity was accessible to the substrate after either sonication or treatment with 1% Triton X-100–25 mM EDTA. Complete solubilization of the cell-bound cellobiosidase activity by sonication suggests that this enzyme is not tightly associated with the membrane.

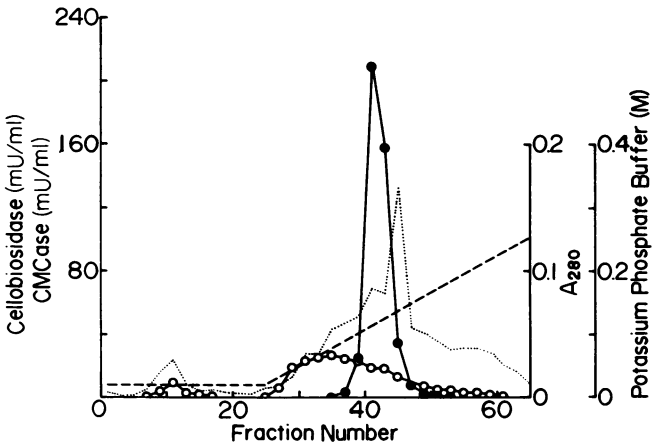

FIG. 2. Hydroxylapatite chromatography of the periplasmic cellobiosidase: column, 1.0 by 28 cm; flow rate, 15 ml/h; fraction volume, 3 ml; temperature, 4°C. Fractions 39 to 45 were pooled. Symbols: ●, cellobiosidase; ○, CMCase; ·····, protein; - - - - -, potassium phosphate buffer gradient.

Separation of cellobiosidase from endoglucanase. Since most cellobiosidase activity was associated with cells, and the extracellular culture fluid exhibited little crystalline cellulose-degrading cellulase activity, the periplasmic cellobiosidase was studied.

The osmotic shock procedure described in Materials and Methods was used to prepare the periplasmic fraction from the cellulose-grown *B. succinogenes* cells. The concentrated periplasmic enzyme preparation, which possessed both endoglucanase and cellobiosidase activities, was fractionated on DEAE–Sepharose CL-6B (Fig. 1). The cellobiosidase activity measured by the hydrolysis of pNPC was eluted as a single sharp peak after the potassium phosphate

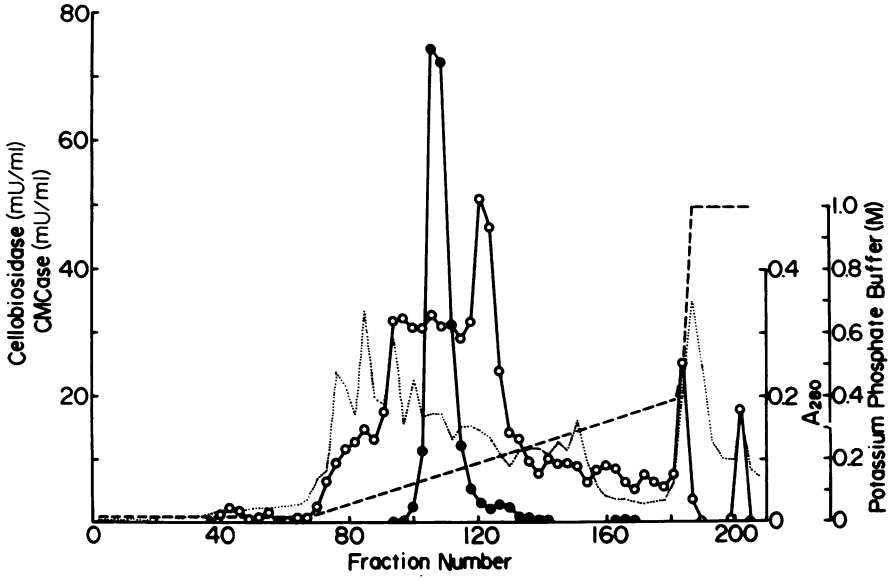

FIG. 1. Ion-exchange chromatography of the periplasmic cellobiosidase on DEAE–Sepharose CL-6B: column, 2.6 by 30 cm; flow rate, 50 ml/h; fraction volume, 5 ml; temperature, 4°C. Fractions 103 to 115 were pooled. Symbols: ●, cellobiosidase; ○, CMCase; ·····, protein; - - - - -, potassium phosphate buffer gradient.

gradient was applied. By contrast, endoglucanase activity spread over a large number of fractions with a significant proportion overlapping with the cellobiosidase activity peak. When the fractions containing cellobiosidase activity were pooled and chromatographed on hydroxylapatite, most endoglucanase activity was removed from the sharp cellobiosidase peak (Fig. 2). Final separation of the two activities was achieved on Bio-Gel P-150 (Fig. 3). The presence of a cellobiosidase, which was devoid of endoglucanase activity, was therefore demonstrated.

Properties of cellobiosidase. The characteristics of the periplasmic cellobiosidase, which had been freed from endoglucanase activity, were studied.

The pH optimum of the cellobiosidase was determined by conducting the activity assays on pNPC at 39°C in 0.05 M sodium phosphate buffers at various pH values (Fig. 4). The maximum enzyme activity was found between pH 5.9 and 6.2. Approximately 50% loss of the activity occurred at either pH 5.2 or 7.0. The enzyme was almost inactive under slightly alkaline conditions. The effect of temperature on cellobiosidase activity was investigated by measuring the activity in 0.05 M sodium phosphate buffer, pH 6.0, at various temperatures. The enzyme activity was maximum at a temperature between 45 and 50°C. At 23 or 60°C, only 10% of the maximum activity was detected.

The dependence of the cellobiosidase reaction rate on substrate concentration was examined. The enzyme was incubated with various amounts of pNPC (0.2 to 2.0 mM) for 15 min at 39°C in 0.05 M sodium phosphate buffer, pH 6.0. The K_m and V_{max}, calculated by the method of Lineweaver and Burk (23), were 0.40 mM and 11.6 μmol of *p*-nitrophenol/min per mg of protein, respectively.

The molecular weight of the periplasmic cellobiosidase, as determined by Bio-Gel P-150 gel filtration, was approximately 40,000.

Analytical IEF was performed to determine the pI of the periplasmic cellobiosidase. An activity band was focused at pH 4.9, as judged by using an IEF standard calibration kit.

The action of the periplasmic cellobiosidase on various carbohydrates and arylglycosides is shown in Table 3. Of the tested substrates, only pNPC and *p*-nitrophenyl-β-D-lactoside were hydrolyzed by the cellobiosidase. Analysis of the pNPC hydrolysate on HPLC indicated that cellobiose, and not glucose, was the hydrolysis product, thus eliminating the possibility that production of *p*-nitrophenol was the result of successive removal of the glucose residues from the pNPC by a noncellobiosidase, endwise functioning enzyme.

Since pNPC is a synthetic substrate, it is therefore valuable to study the action of the cellobiosidase on some naturally occurring substrates. In this investigation, the enzyme was incubated with the oligosaccharides and acid-swollen cellulose. The hydrolysates were analyzed by HPLC. The cellobiosidase had no activity on cellobiose (Fig. 5). It degraded cellotriose into cellobiose and glucose. With cellotetraose as the substrate, only cellobiose was produced. Cellobiose was also released from the hydrolysis of cellopentaose. When cellohexaose was hydrolyzed by the enzyme, cellobiose and cellotetraose were major degradation products and cellotriose was present in a relatively small amount. The enzyme showed a very low activity on acid-swollen cellulose, and only traces of cellobiose released during hydrolysis were detected.

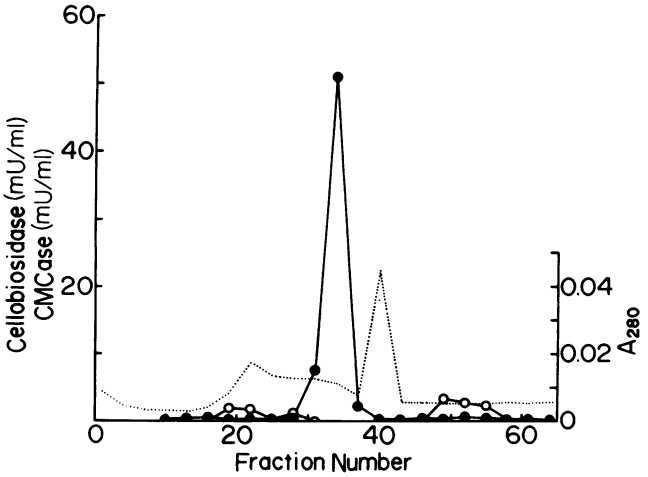

FIG. 3. Gel filtration of the periplasmic cellobiosidase on Bio-Gel P-150: column, 2.5 by 75 cm; flow rate, 7 ml/h; fraction volume, 5 ml; temperature, 4°C. Fractions 31 to 37 were pooled. Symbols: ●, cellobiosidase; ○, CMCase; ·····, protein.

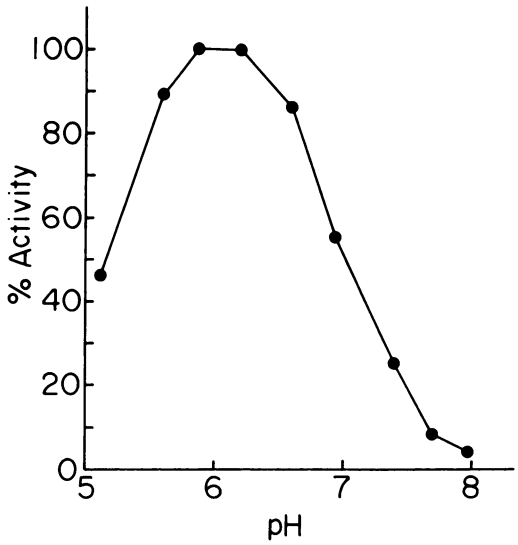

FIG. 4. Influence of pH on cellobiosidase activity from *B. succinogenes*. Sodium-potassium phosphate buffer was used at all pH values tested.

TABLE 3. Substrate specificity of cellobiosidase from *B. succinogenes*

Substrate	Sp act (μmol/min per mg of protein)
pNPC	5.78
p-Nitrophenyl-α-D-glucopyranoside	<0.01
p-Nitrophenyl-β-D-glucopyranoside	<0.01
p-Nitrophenyl-β-D-lactoside	2.53
p-Nitrophenyl-β-D-xylopyranoside	<0.01
Carboxymethyl cellulose	<0.01
Xylan	<0.01
Lichenan	<0.01
Laminarin	<0.01
Avicel cellulose PH105[a]	<0.01

[a] Activity on Avicel cellulose was determined by using the periplasmic fraction of the cells.

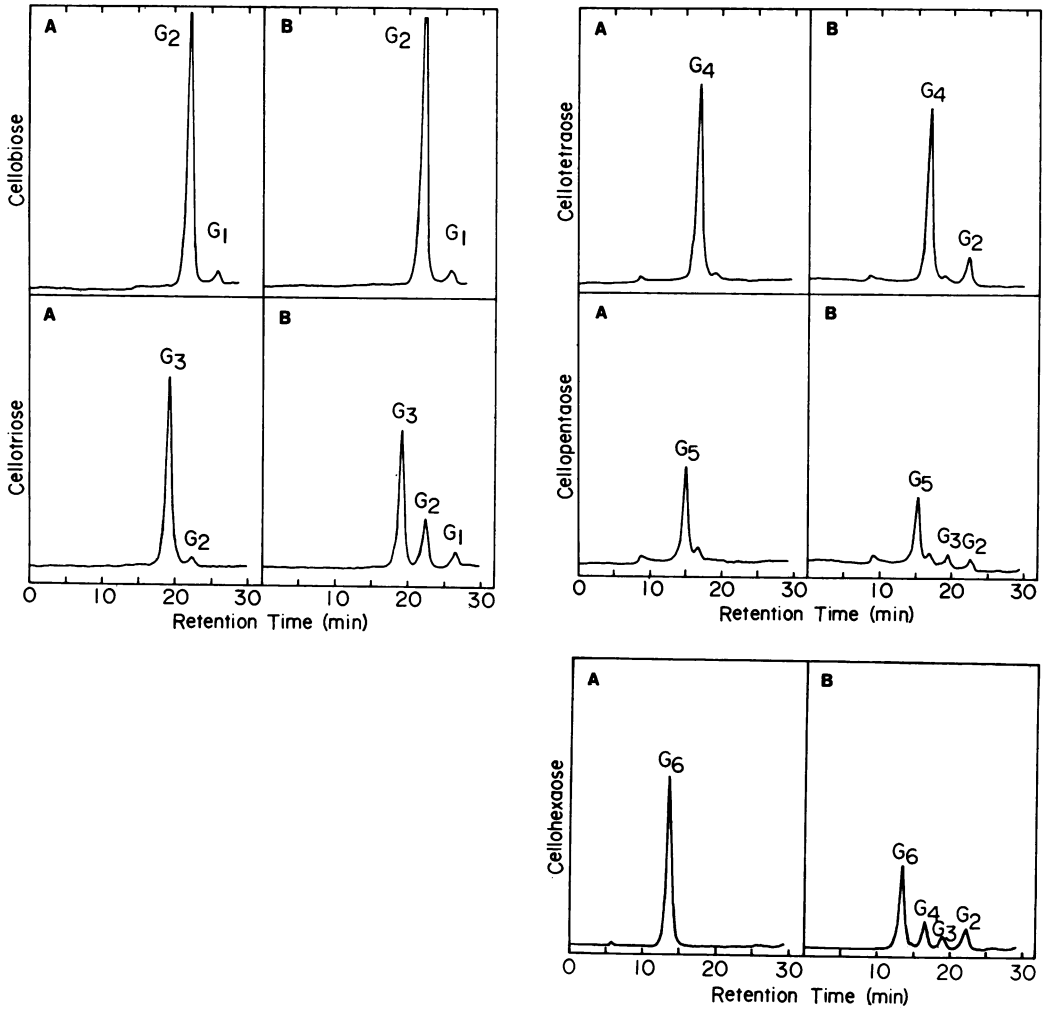

FIG. 5. HPLC analysis of the products of hydrolysis of cello-oligosaccharides by cellobiosidase. The enzyme was incubated at 39°C for 0 (A) and 10 (B) h with 100 g of cellobiose (G_2), cellotriose (G_3), cellotetraose (G_4), cellopentaose (G_5), or cellohexaose (G_6) in 0.05 M sodium phosphate buffer, pH 6.5, in a final volume of 0.2 ml. The hydrolysates were analyzed by HPLC as described in Materials and Methods.

DISCUSSION

Although endoglucanase and cellobiase produced by *B. succinogenes* have been studied for some time (11, 15–17, 32, 35), the search for an exo-type cellulase activity has been hindered by the lack of a suitable assay system. In the present study, with the use of a cellotriose analog, pNPC, we identified a periplasmic cellobiosidase.

In this investigation *B. succinogenes* cells grown on cellulose in a continuous culture system were fractionated, and the enzyme activity against pNPC, as well as endoglucanase and cellobiase in each fraction, was assayed. It was observed that approximately 70% of the total endoglucanase activity exhibited by the cells was extracellular, while most cellobiase activity remained cell-bound (Table 1). This observation supports the data from the previous study in which *B. succinogenes* was grown in batch culture on cellulose (15). Compared with endoglucanase and cellobiase, the activity against pNPC showed a distinct distribution pattern. This activity was mainly associated with cells. Fractionation of the cells further revealed that most cell-bound cellobiosidase activity was located in the periplasmic space, where little cellobiase activity and relatively low endoglucanase activity were present. As a result of its location, the cell-bound cellobiosidase displayed a reduced access to its substrate in the extracellular environment.

To examine the characteristics of the cellobiosidase, the enzyme was isolated from the periplasmic space of the cells and separated from other cellulase components by column chromatography. Some properties of the partially purified *B. succinogenes* cellobiosidase appear to be different from those of the cellobiosidase isolated from the culture supernatant of another rumen cellulolytic bacterium, *R. albus* (30). The estimated molecular weight for the *B. succinogenes* enzyme was 40,000 by gel filtration. In comparison, the *R. albus* enzyme seems to be a dimer of two polypeptide chains, each with molecular weight of 100,000. The optimal pH for the *B. succinogenes* cellobiosidase lies between 5.9 and 6.2, compared with 6.8 for the *R. albus* enzyme. The optimal temperature for the *B. succinogenes* enzyme is 45 to 50°C, compared with 37°C for the cellobiosidase from *R. albus*. The optimal pH and temperature of the *B. succinogenes* cellobiosidase, however, are similar to those of the endoglucanase isolated from the same bacterium (16). Both enzymes seem to be well adapted to the rumen environment in which the pH normally varies be-

tween 6 and 7 and the temperature between 37 and 40°C. The pI is 4.9 for the cellobiosidase of *B. succinogenes* and 5.3 for that of *R. albus*. The *B. succinogenes* cellobiosidase appears to have a high affinity for pNPC with a K_m of 0.4 mM. Deshpande et al. (8) reported that two exoglucanases from *Trichoderma reesei* had K_ms of 0.252 and 2.51 mM, respectively, for pNPC, and an exoglucanase from *Sporotichum pulverulentum* had a K_m of 1.67.

The substrate specificity of the periplasmic cellobiosidase isolated from *B. succinogenes* and its action on cello-oligosaccharides demonstrate that it has some hydrolytic properties in common with well-characterized cellobiohydrolases. The enzyme was capable of hydrolyzing *p*-nitrophenyl lactoside as well as *p*-nitrophenyl cellobioside (Table 3). This is in agreement with the finding that these two aryl glycosides were attacked by fungal exoglucanases (8). Moreover, the periplasmic cellobiosidase cleaved the agluconic bond and not the holosidic bond of pNPC. By contrast, endoglucanases may exhibit activity on both bonds (8). Like the cellobiohydrolases from many other cellulolytic bacteria and fungi, the *B. succinogenes* enzyme showed little activity on carboxymethyl cellulose, a substrate for endoglucanase, presumably because the chain ends on carboxymethyl cellulose were too highly substituted for enzyme binding to occur and exo-type enzyme action would be blocked once the available unsubstituted cellobiose residues were removed (7, 24, 26, 37). With cello-oligosaccharides as substrates, the mode of action of the enzyme was ascertained. The enzyme did not hydrolyze cellobiose, but released cellobiose from cellotriose, cellotetraose, and cellopentaose. The preference of the enzyme for cleavage of the penultimate glucosyl bond was further demonstrated in the case of cellohexaose hydrolysis. The presence of a small amount of cellotriose in the cellohexaose hydrolysate presumably was due to the nonspecific action of the enzyme. It has been reported that the cellobiohydrolases purified from *Streptomyces flavogriseus* and *T. reesei* released small amounts of glucose and cellotriose in addition to the main product cellobiose from cellulose substrates (18, 24). Previous reports on the ability of purified cellobiohydrolases to attack crystalline cellulose have revealed differences. However, with a few exceptions, most purified cellobiohydrolases seem to be unable to hydrolyze crystalline cellulose to any significant extent (14, 20, 26). The enzyme isolated from *B. succinogenes* showed no activity towards Avicel crystalline cellulose. In contrast to many purified fungal cellobiohydrolases, the *B. succinogenes* enzyme appears to be unable to effect extensive hydrolysis of acid-swollen cellulose. It seems, therefore, that the enzyme isolated from *B. succinogenes* is a cellodextrinase with an exo-type function.

The function of the periplasmic cellodextrinase presumably is to hydrolyze cellodextrins, which enter the periplasmic space, to cellobiose and glucose, which can be readily transported into the cell. This is the first report of an enzyme with the unique properties to fulfill this hydrolytic role.

ACKNOWLEDGMENTS

Appreciation is expressed to R. B. Hespell for provision of the cellohexaose and to Donna Eby for typesetting the manuscript.

L.H. was supported in part by a Graduate Visa Student Scholarship from the University of Guelph. This research was supported by contract funding from the National Research Council of Canada and by an Operating Grant from the Natural Sciences and Engineering Research Council.

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