

Isolation and Characterization of Endoglucanases 1 and 2 from *Bacteroides succinogenes* S85

M. MCGAVIN AND C. W. FORSBERG*

Department of Microbiology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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Two endoglucanases designated EG1 and EG2 were purified by column chromatography from the nonsedimentable extracellular culture fluid of *Bacteroides succinogenes* S85. They accounted for approximately 32 and 11%, respectively, of the total endoglucanase present in the nonsedimentable fraction. The most active enzyme (EG1) had a molecular weight of 65,000, pI of 4.8, and temperature and pH optima of 39°C and 6.4, respectively. The K_m for carboxymethyl cellulose was 3.6 mg/ml, and the V_{max} was 84 U/mg. The major products of cellulose hydrolysis catalyzed by EG1 were celotriose and cellobiose. EG2 was present as two components with molecular weights of 118,000 and 94,000. The two components had nearly identical cyanogen bromide peptide maps, thereby indicating that the 94,000-dalton component was a proteolytic degradation product of the 118,000-dalton enzyme. The larger component, which was more abundant in the culture fluid than the smaller form was, had a K_m of 12.2 mg/ml and a V_{max} of 10.4 U/mg. It was a basic protein with a pI of 9.4, a temperature optimum of 39°C, and a pH optimum of 5.8. The major product of cellulose hydrolysis was cellotetraose. EG2 exhibited specific binding to acid-swollen cellulose, whereas EG1 did not, and neither of them had affinity for crystalline cellulose. Based on the substrate specificities and the affinities of the two enzymes for cellulose, we postulated that EG2 is involved in the early stages of cellulose hydrolysis and that EG1 is active primarily on the products arising from EG2.

Bacteroides succinogenes is a predominant cellulolytic bacterium in the bovine rumen (6, 20). However, neither nongrowing cells nor cell-free culture fluid from *B. succinogenes* affects the extensive hydrolysis of crystalline cellulose (16). Therefore, our research was centered on determining the types of cellulolytic activities possessed by the bacterium, with the ultimate objective being to identify the enzymes responsible for the extensive hydrolysis of cellulose by growing cultures.

During growth of *B. succinogenes* in continuous culture with cellulose as the carbon source, endoglucanase and chloride-stimulated cellobiosidase activities have been shown to be present in both the cells and the extracellular culture fluid (18). A cellodextrinase was detected in the periplasmic space, while cellobiase activity was membrane associated (18, 19). Greater than 70% of the endoglucanase activity was present in the extracellular culture fluid of cells grown in either a chemostat culture or a batch culture after all of the cellulose was digested (11, 16, 19). Results of the batch culture study revealed that 50 to 62% of the extracellular endoglucanase was associated with sedimentable membrane fragments, 9 to 13% was associated with nonsedimentable material with a molecular weight greater than 4×10^6 , and 28 to 38% was associated with molecules with a molecular weight of approximately 45,000, as determined by exclusion chromatography (15, 16, 31). The endoglucanase activities in these various fractions were further separated by chromatography and electrophoresis, thereby suggesting that there were multiple components. Furthermore, at the genetic level, six distinct cellulase genes have been identified which code for endoglucanase activity (8); and expression of a gene from *B. succinogenes* coding for endoglucanase activity in *Escherichia coli* has been examined (34).

The objective of this study was to purify and characterize the endoglucanases that were free in the extracellular culture

fluid. Two endoglucanases with very different physical and catalytic properties were characterized, and these are described here.

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 (16). For chemostat culture, 1% (wt/vol) cellulose (Avicel PH105; FMC Corp., Marine Colloids Div., Rockland, Maine) was the only readily available carbon source. Details of medium preparation, chemostat design, and growth conditions were as described by Huang et al. (19).

Enzyme assays. Endoglucanase activity was assayed by incubating the appropriately diluted enzyme with 1.0% carboxymethyl cellulose (CMC; low viscosity; Sigma, Chemical Co., St. Louis, Mo.) at 39°C and quantifying the reducing sugars produced with the Nelson-Somogyi reagent (1) as described by Schellhorn and Forsberg (31). Similarly, xylanase, laminarinase, and lichenanase activities were assayed by determining the amount of reducing sugars released during incubation of the enzyme with 1.0% larchwood xylan (113F-0003; Sigma), 1.0% laminarin (L-9634; Sigma), and 0.5% lichenan (L-6133; Sigma), respectively. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μ mol of reducing sugars (expressed as glucose) per min. For viscometric assays, appropriately diluted enzyme was incubated with 1.0% (wt/vol) medium-viscosity CMC (Sigma) at 39°C. Samples were taken at defined time intervals over a period of 1 h, and enzyme was inactivated by heating it at 100°C for 10 min. Viscosity determinations were performed on 0.5-ml portions with a micro (cone-plate) viscometer (Wells-Brookfield) at 20°C with a 0.8-degree cone. Each sample taken was also assayed for reducing sugars. The specific viscosity was calculated by subtracting the buffer viscosity from the sample viscosity and then dividing the remainder by the buffer viscosity. Enzyme was

* Corresponding author.

assayed for cellobiosidase activity as described by Gilkes et al. (13) by using 0.5 mM *p*-nitrophenyl- β -D-cellobioside as the substrate. Similarly, *p*-nitrophenyl- β -D-glucoside was used as the substrate for β -glucosidase assays. All assays were conducted in 0.05 M potassium phosphate buffer (pH 6.5 or pH 6.0, where indicated).

Processing of culture fluid for chromatography. The effluent from the chemostat was collected in a reservoir that was maintained in a refrigerator at 4°C. After the solids were allowed to settle out, residual cells and cellulose (Avicel) were removed by centrifugation at $10,000 \times g$ for 10 min. The cell-free culture fluid was then concentrated at 4°C by using an ultrafiltration system (Pellicon; Millipore Corp., Bedford, Mass.) equipped with a 10,000-molecular-weight cutoff membrane. The concentrated culture fluid was diluted 10-fold in 20 mM potassium phosphate buffer (pH 6.5) and concentrated again to a final volume of 300 ml. Immediately prior to column chromatography, the preparation was ultra-centrifuged at $100,000 \times g$ for 2 h to sediment the membrane fragments.

Column chromatography. All purification steps were performed at 4°C, and all buffers used in the purification contained 0.01% sodium azide as a preservative. DEAE-Sepharose CL-6B, S-Sepharose, CM-Sepharose, phenyl-Sepharose, Polybuffer exchanger 94, Polybuffer 74, and Sephadex G-100 were purchased from Pharmacia (Uppsala, Sweden). Hydroxylapatite HTP and Bio-Gel P-150 were obtained from Bio-Rad Laboratories (Richmond, Calif.). All chromatographic columns were prepared according to the instructions of the manufacturers.

Purification of endoglucanase 1. Concentrated culture fluid containing 327 mg of protein was applied to a column (2.6 by 30 cm) of DEAE-Sepharose CL-6B that was equilibrated in starting buffer (20 mM potassium phosphate buffer [pH 6.5], 0.01% sodium azide). After the sample was applied, the column was washed with 1 bed volume of starting buffer, and then 500 ml of a linear buffer gradient from 20 to 500 mM was applied. The initial flow rate was 50 ml/h, and 5-ml fractions were collected. The endoglucanase peak fractions eluting in the buffer gradient were desalted and concentrated by ultrafiltration through a membrane (PM-10; Amicon Corp., Lexington, Mass.). The concentrated protein was subsequently applied to a column (1.0 by 28 cm) of hydroxylapatite. After the column was washed with 1 bed volume of starting buffer, the bound proteins were eluted with a linear potassium phosphate buffer gradient (20 to 500 mM, pH 6.5). The fractions containing endoglucanase activity were pooled, concentrated, and then equilibrated with 25 mM piperazine hydrochloride (pH 5.5). The concentrated protein was applied to a column (0.9 by 15 cm) of Polybuffer exchanger 94 that was equilibrated with 25 mM piperazine hydrochloride (pH 5.5). Proteins were eluted with a pH gradient developed by elution of the column with 12 bed volumes of 10-fold diluted Polybuffer 74 adjusted to pH 4.0 with HCl. Fractions of 1.2 ml were collected. Fractions from the two peaks of endoglucanase activity were pooled separately and applied to a column (2.6 by 68 cm) of Sephadex G-100 equilibrated with 50 mM potassium phosphate buffer (pH 6.5). The column was eluted with equilibration buffer at a flow rate of 16 to 18 ml/h, and 5-ml fractions were collected. Active fractions from Sephadex G-100 were made to 4 M in NaCl and applied to a column (1.5 by 25 cm) of phenyl-Sepharose equilibrated with 4 M NaCl in 50 mM potassium phosphate buffer (pH 6.5). Bound proteins were eluted with a descending linear gradient of NaCl from 4.0 to 0.0 M, which was applied over a volume of 300 ml at a flow rate of 37 ml/h.

Active fractions were concentrated and desalted by ultrafiltration with a membrane (PM-10; Amicon).

Purification of endoglucanase 2. Concentrated culture fluid containing 2.5 g of protein was diluted 10-fold in 20 mM potassium phosphate buffer (pH 6.5) containing 5% (wt/vol) betaine (Sigma), concentrated to a volume of 300 ml, and applied to DEAE-Sepharose as described above for endoglucanase 1 (EG1). The nonbinding fraction was applied to a column (2.5 by 38.5 cm) of S-Sepharose equilibrated with the buffer described above containing betaine. Following sample application, the column was washed with 270 ml of buffer and then was eluted with a linear gradient (0 to 600 mM) of KCl that was made up in equilibration buffer and applied over a volume of 800 ml. The initial flow rate was 40 ml/h, and 5-ml fractions were collected. The major peak of endoglucanase activity that eluted in the gradient was concentrated by ultrafiltration through a membrane (PM-10; Amicon), diluted 10-fold in equilibration buffer, and concentrated again. The concentrated protein was applied to a column (1.7 by 25 cm) of CM-Sepharose, and the column was washed with 100 ml of equilibration buffer. Protein was eluted with a linear gradient of KCl (0 to 400 mM, made up in equilibration buffer) applied over a volume of 400 ml. The initial flow rate was 15 ml/h, and 4.5 ml-fractions were collected. The two peaks of endoglucanase activity (A and B) which were eluted in the gradient were pooled separately and concentrated in the absence of betaine. Both fractions were applied separately to a column (1.5 by 28 cm) of hydroxylapatite equilibrated in 300 mM potassium phosphate buffer (pH 6.5). Each column was washed with 50 ml of equilibration buffer, followed by application of a linear buffer gradient from 300 to 800 mM applied over a volume of 400 ml. The initial flow rate was 30 ml/h, and 5-ml fractions were collected. After hydroxylapatite chromatography, fraction A was subject to rechromatography on CM-Sepharose to achieve a more complete separation from fraction B, while fraction B was subjected to gel filtration on Bio-Gel P-150, followed by rechromatography on CM-Sepharose to remove contaminating protein from fraction A. The purified proteins were dialyzed against 20 mM potassium phosphate buffer (pH 6.5) containing 0.01% sodium azide and stored at 0 to 4°C.

A summary of the purifications of EG1 and EG2 is given in Table 1.

SDS-PAGE, gradient gel electrophoresis, and IEF. The sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) system used in this study was that of Laemmli (22). The protein standards used were a low-molecular-weight kit for EG1 from Pharmacia or a high-molecular-weight kit for endoglucanase 2 (EG2) from Bio-Rad. Staining was with Coomassie brilliant blue R-250 (0.1% in 40% methanol-10% acetic acid; Sigma), and gels were destained in 12% ethanol-7% acetic acid. Molecular weights were also estimated by nondenaturing polyacrylamide gradient gel electrophoresis (4 to 30% acrylamide). The gels were prepared according to the instructions of Pharmacia, with the exception that 4% sucrose was included in the gel. Gradient gels were stained with a silver stain kit (Bio-Rad) according to the instructions of the manufacturer.

Analytical isoelectric focusing (IEF) was performed in a horizontal gel electrophoresis cell (Bio-Phoresis; Bio-Rad). The gel composition was that described by Huang and Forsberg (18), with the exception that carrier ampholytes (2% vol/vol; LKB, Bromma, Sweden) covered a pH range from 3.5 to 10, and 1.0 N NaOH was used as the cathode buffer. The gel was run at 8 W of constant power until 1,500

TABLE 1. Purification of EG1 and EG2

Enzyme and purification step	Total protein (mg)	Total units of enzyme	Sp act (U/mg)	Yield (%)
EG1				
1. Crude culture fluid	326.8	686.3	2.1	100.0
2. DEAE-Sepharose	150.0	120.0	0.8	17.5
3. Hydroxylapatite	10.6	85.9	8.1	12.5
4. Chromatofocusing	1.1	26.6	24.2	3.9
5. Sephadex G-100	0.5	24.7	49.4	3.6
6. Phenyl Sepharose	0.07	5.8	82.3	0.9
EG2				
1. Crude culture fluid	2,494	2,020	0.81	100
2. DEAE-Sepharose nonbinding	1,597	2,140	1.34	106
3. S-Sepharose	84.9	220	2.59	10.9
4. Fraction A from CM-Sepharose				
Hydroxylapatite	3.2	23.9	7.40	1.2
Rechromatography on CM-Sepharose	3.1	19.0	6.1	0.9
5. Fraction B from CM-Sepharose				
Hydroxylapatite	15.8	85.1	5.4	4.2
Bio-Gel P150	11.9	61.8	5.2	3.1
CM-Sepharose rechromatography	8.2	42.0	5.1	2.1

V was achieved, at which point samples were applied to plastic wells that were placed on the surface of the gel. Subsequent electrophoresis was at 1,500 V for 1.5 h. Fixing, staining with Coomassie brilliant blue, and destaining were done as described by Huang and Forsberg (18). The isoelectric pH of the proteins were determined by reference to the position of protein IEF standards (Pharmacia) that were focused in a separate lane of the same gel.

Determination of products of cellooligosaccharide and acid-swollen cellulose hydrolysis. Hydrolysis products of cellotriase, cellotetraose, and cellopentaose (Pfanstiehl Laboratories, Inc., Waukegan, Ill.) and cellohexaose (provided by R. B. Hespell) by the purified enzymes were determined as described by Huang and Forsberg (18). Assay mixtures contained 100 μ l of 1 mg of cellooligosaccharide per ml and 5.2×10^{-3} U of EG1 or 7.9×10^{-3} U of EG2 in a final volume of 200 μ l of 25 mM potassium phosphate buffer (pH 6.5 for EG1 or pH 6.0 for EG2). Incubation was for 20 h at 39°C, and the reaction mixture was terminated by heating it in a boiling water bath for 10 min. For acid-swollen cellulose hydrolysis, conditions were the same as described above, with the exception that 100 μ l of 1% acid-swollen cellulose, prepared as described by Schellhorn and Forsberg (31), was used as the substrate.

Binding of enzyme to substrate. Purified enzymes were diluted in 20 mM KPO₄ buffer (pH 6.0) and mixed with an equal volume of either 2% cellulose (Avicel) or 0.56% acid-swollen cellulose (as determined by a phenol-sulfuric acid assay [9]) that was made up in the same buffer. Identical samples containing a 200-fold excess of bovine serum albumin were also prepared. The mixtures were incubated for 30 min at 39°C with gentle vortexing every 7.5 min. After 30 min, the cellulose-bound enzyme was removed by centrifugation in a microfuge and the supernatants were assayed for activity, with 1% CMC used as the substrate.

Other analytical procedures. Protein was determined by the method of Bradford (4), with bovine serum albumin used as a standard. Glycoprotein stains were performed as described by Fairbanks et al. (10) by using the periodic acid-Schiff base reagent. The carbohydrate content of acid-swollen cellulose preparations was determined by the phenol-sulfuric acid assay of Dubois et al. (9). The pH optima of the enzymes were studied at 39°C, with CMC as the sub-

strate. For EG1, buffers were disodium citrate-NaOH from pH 5.0 to 6.7, while sodium phosphate buffer was used between pH 6.4 and 8.0. For EG2, sodium acetate was used between pH 4.0 and 5.6, while sodium phosphate was used between pH 5.4 and 7.8. All buffers were at concentrations of 50 mM. The dependence of the endoglucanase activity on substrate concentration was examined at 39°C in 50 mM potassium phosphate buffer (pH 6.4) for EG1 or pH 6.0 for EG2. Appropriately diluted enzyme was incubated with CMC at concentrations that varied between 1 and 17 mg/ml, and the liberated reducing sugars were quantified. K_m and V_{max} values were calculated by the double-reciprocal method of Lineweaver and Burk (25). For cyanogen bromide peptide mapping, the protocol was exactly as described by Goding (14). EG2A and EG2B were electrophoresed on a 0.75-mm-thick SDS-polyacrylamide gel. Coomassie brilliant blue-stained protein bands containing approximately 9 μ g of protein were excised and equilibrated in 70% formic acid (ACS grade; Fisher Scientific Co., Pittsburgh, Pa.). Cyanogen bromide (Eastman Kodak Co., Rochester, N.Y.) dissolved in dimethylformamide (BDH, Poole, England) was added to give a final concentration of 40 mg of CNBr per ml. After gentle shaking for 1.5 h at room temperature, CNBr was removed by two washes of 20-min duration in 10 ml of 10% acetic acid. The acid was neutralized with 1.0 M Tris hydrochloride (pH 8.0). The gel slices were then equilibrated with SDS-PAGE reducing buffer and loaded onto a 1.5-mm-thick gel with a 5% stacking gel and a 12% resolving gel. After electrophoresis, the gel was stained with a silver reagent (Bio-Rad).

RESULTS

Purification of EG1. When the endoglucanases present in the extracellular culture fluid from *B. succinogenes* were separated on DEAE-Sepharose, 13.6% of the activity applied to the column was eluted in the application buffer, while 32% was eluted as a major single sharp peak of activity in the buffer gradient; this was followed by a series of minor peaks (Fig. 1). The major peak of endoglucanase activity was closely associated with a peak of cellobiosidase activity, most of which was removed by subsequent chromatography on hydroxylapatite. Chromatofocusing resolved the endo-

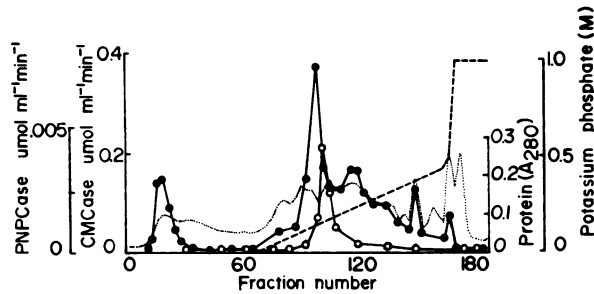


FIG. 1. DEAE-Sepharose chromatography of culture fluid proteins from cellulose-grown *B. succinogenes*. Fractions 88 to 109 were pooled. Symbols: ●, endoglucanase (carboxymethyl cellulase; CMCCase); ○, cellobiosidase (*p*-nitrophenyl-β-D-cellobiosidase; PNPCase); ···, protein; ----, potassium phosphate buffer gradient.

glucanase activity into two peaks (Fig. 2), both of which yielded a single protein of 65 kilodaltons (kDa) after further purification (Fig. 3A). In subsequent purifications on DEAE-Sepharose, the distribution of the endoglucanase changed; and 45% of the enzyme was then eluted in the application buffer before the gradient was applied, and only 17.5% was found in the EG1 peak. Low recovery became a problem, and it was discovered that the addition of 5% (wt/vol) betaine to the column buffers improved the overall recovery of EG1 from the culture fluid. This compound reportedly minimizes protein-protein interactions (Fast Protein Liquid Chromatography Ion Exchange and Chromatography; Laboratory Separation Division, Pharmacia). With betaine in the first ion-exchange column buffer, the final phenyl-Sepharose chromatography step could be omitted while the same degree of purification was maintained. This resulted in a twofold increase in the amount of EG1 protein which could be recovered from the crude culture fluid.

However, in the subsequent purifications, only one endoglucanase peak was observed on chromatofocusing, which yielded a purified protein of 65 kDa, which was identical in size to both endoglucanases purified from the two peaks of activity observed in Fig. 2.

Purification of EG2. For purification of EG2, 2.5 g of culture fluid protein containing 5% (wt/vol) betaine was applied to a column of DEAE-Sepharose. EG1 was purified

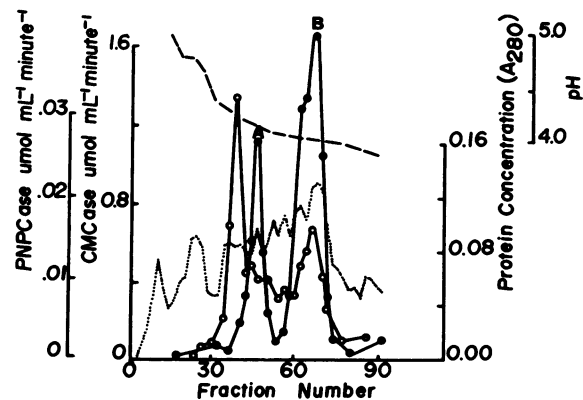


FIG. 2. Chromatofocusing of endoglucanase activity eluted from hydroxylapatite. Fractions 43-49 (peak A) and 56-74 (peak B) were pooled separately. Symbols: ○, cellobiosidase (PNPCase); ●, carboxymethyl cellulase (CMCCase); ···, protein; ----, pH.

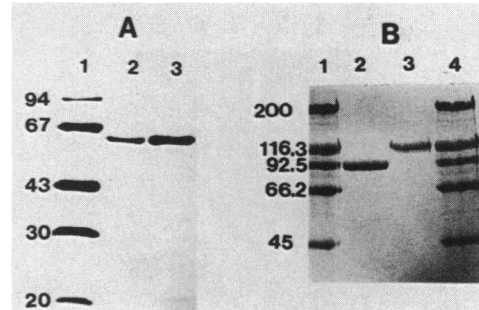


FIG. 3. SDS-PAGE of the purified endoglucanases. (A) A 10% polyacrylamide gel; lane 1, low-molecular-weight standards (Pharmacia), in kilodaltons; lane 2, 1.0 μg of endoglucanase purified from peak A in Fig. 2; lane 3, 1.0 μg of EG1 purified from peak B in Fig. 2. (B) A 7.5% polyacrylamide gel; lanes 1 and 4, high-molecular weight-standards (Bio-Rad), in kilodaltons; lane 2, 1.5 μg of purified EG2A; lane 3, 1.5 μg of purified EG2B.

from the protein which bound to the column, while the nonbinding material which contained 64% of the total protein was applied to the cation exchanger S-Sepharose. The major endoglucanase peak that eluted in the salt gradient contained 5% of the applied protein and 10.9% of the activity originally applied to DEAE-Sepharose. When this was subsequently applied to a column of CM-Sepharose and eluted with a shallow KCl gradient, two peaks of activity were observed (Fig. 4). In order to achieve complete separation of these two peaks, peak A (fractions 78 to 88) was subjected to chromatography on hydroxylapatite and then applied a second time to CM-Sepharose, while peak B (fractions 91 to 96) was subjected to chromatography on hydroxylapatite, followed by gel filtration on Bio-Gel P-150 and a second application to CM-Sepharose. In an initial purification, Sephadex G-100 chromatography was employed after hydroxylapatite chromatography. However, the enzyme bound to the Sephadex and could not be eluted. The additional chromatographic steps after hydroxylapatite chromatography resulted in a loss of protein and enzyme activity, but they were necessary to accomplish the effective separation of the enzymes in peaks A and B observed on CM-Sepharose. These enzymes are referred to as EG2A and EG2B.

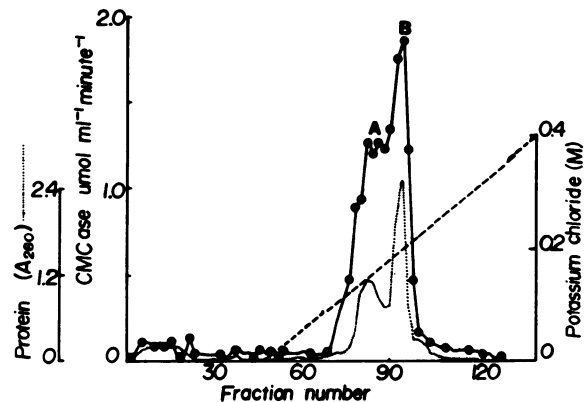


FIG. 4. CM-Sepharose chromatography of endoglucanase activity eluted from S-Sepharose. Fractions 78-88 (peak A; EG2A) and 91-96 (peak B; EG2B) were pooled separately. Symbols: ●, carboxymethyl cellulase (CMCCase); ···, protein; ----, potassium chloride gradient.

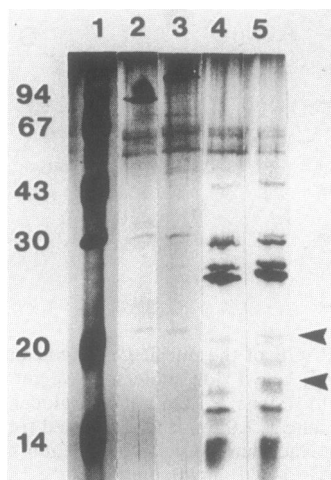


FIG. 5. Cyanogen bromide peptide mapping of EG2A and EG2B. Gel slices containing 9 μ g of EG2A (lanes 2 and 4) or EG2B (lanes 3 and 5) were treated with cyanogen bromide in 70% formic acid (lanes 4 and 5) or 70% formic acid alone (lanes 2 and 3). Arrows indicate minor cleavage products that were present only in EG2B. Lane 1 contains low-molecular-weight protein standards (Pharmacia), in kilodaltons.

Relatedness of EG2A and EG2B. The similar behaviors of these two enzymes on each of the columns used for their purification suggested that they have a high degree of physical similarity. Cyanogen bromide peptide mapping by SDS-PAGE yielded identical profiles for EG2A and EG2B, except that some minor bands were missing from the EG2A profile (Fig. 5). The results indicate that EG2A and EG2B have a common origin.

Binding of enzymes to cellulose. EG1 did not exhibit an affinity for binding to either amorphous or crystalline cellulose. EG2B had greater binding activity than EG2A, in that a greater percentage of activity bound to both acid-swollen and microcrystalline cellulose (Avicel) (Table 2). Both EG2A and EG2B exhibited a high degree of specificity for binding to amorphous cellulose. In both cases, binding to crystalline cellulose was substantially diminished in the presence of a 200-fold excess of bovine serum albumin. However, EG2B had an equally high affinity for acid-swollen cellulose both in the presence and absence of bovine serum albumin, indicating a specific interaction between enzyme and substrate.

TABLE 2. Binding of EG1 and EG2 to amorphous and crystalline cellulose

Treatment	% of activity bound to:	
	ASC ^a	Crystalline cellulose (Avicel)
EG1 without BSA ^b	28.2	17.6
EG2A		
Without BSA	68.1	24.5
With BSA	55.5	16.9
EG2B		
Without BSA	86.9	45.4
With BSA	86.4	20.5

^a ASC, Acid swollen cellulose; prepared as described by Schellhorn and Forsberg (31).

^b BSA, Bovine serum albumin; where indicated it was present in a 200-fold excess over enzyme.

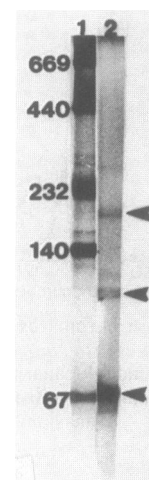


FIG. 6. Nondenaturing polyacrylamide gradient gel electrophoresis of EG1 on 4 to 30% acrylamide. Lane 1, high-molecular-weight standards (Pharmacia), in kilodaltons; lane 2, 1.5 μ g of EG1. The gel was stained with silver reagent (Bio-Rad) according to the instructions of the manufacturer. Arrows point to protein bands with molecular masses of 67, 114, and 197 kDa from the bottom to top, respectively.

Physical and catalytic properties. Enzyme purified from the major chromatofocusing peak (EG1, Fig. 2) and from the major CM-Sepharose peak (EG2, Fig. 4) were chosen for further characterization. With respect to EG2A and EG2B, unless indicated otherwise data are presented only for the major form EG2B, which is referred to hereafter as EG2. The subunit molecular weights of the purified components were 65,000 for EG1, 94,000 for EG2A, and 118,000 for EG2B (Fig. 3). The native molecular mass of 67 kDa determined for EG1 by gradient gel electrophoresis (Fig. 6) differed only slightly from that determined by SDS-PAGE, indicating that the protein was a monomer. Additional protein bands of decreased intensity appeared on the gel, as indicated by the arrows of molecular masses of 114 and 197 kDa in Fig. 6. The additional protein bands corresponded roughly to the expected values for dimers (134 kDa) and trimers (201 kDa) of the monomeric form, although the possibility of a trace amount of contaminating protein cannot be excluded. This suggests a tendency of the protein to form higher-molecular-weight aggregates. The cationic nature of the EG2 enzymes necessitated the use for nondenaturing PAGE of a low-pH buffering system (β -alanine acetate [pH 4.5]) in which some of the standards did not migrate into the gel. This invalidated the electrophoretic determination of the native molecular weight.

The pIs of the purified enzymes were determined by IEF (data not shown). EG1 appeared as a doublet with pIs of 4.75 and 4.90, both of which exhibited endoglucanase activity. EG2A focused just below trypsinogen, yielding, by extrapolation, a pI of 9.18, while EG2B focused just above trypsinogen, for a pI of 9.40.

The purified enzymes EG1 (6 μ g) and EG2 (15 μ g) were tested for the presence of carbohydrate by staining SDS-polyacrylamide gels with the periodic acid-Schiff base reagent. The enzymes were not stained, while as little as 4 μ g of bovine glycoprotein (Sigma) gave a bright pink periodic acid-Schiff-positive band, indicating that the purified enzymes were not glycosylated.

The pH optimum of EG1 was observed to be 6.4. Between pH 5.9 and 7.1, the enzyme retained 90% or greater of its

maximum activity. EG2 exhibited a pH optimum of 5.8, and 90% of its maximum activity was retained at pHs of 5.4 and 6.2. The temperature optima were determined in 50 mM potassium phosphate buffer at pH 6.5 for EG1 and pH 6.0 for EG2. Both EG1 and EG2 had temperature optima at 39°C. EG1 was inactivated rapidly above this temperature, with its activity at 45°C decreasing to 25% of the maximum over a 30-min incubation, while EG2 retained 93% of its maximum activity at 45°C. EG2 was inactivated rapidly above 50°C, decreasing from 76% of maximum activity at 50°C to 4% of maximum activity at 57°C during a 20-min incubation.

The chelating agents EDTA and ethylene glycol-bis (β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and the ions mercury and copper caused severe inhibition of EG1 activity, while it was stimulated by calcium and magnesium, suggesting that there was a requirement for divalent cations (Table 3). EG2 was not stimulated by calcium or magnesium, but was inhibited 50% by 1 mM EDTA.

The K_m and V_{max} values for EG1 were 3.6 mg/ml and 84 μ mol/min per mg, respectively, while for EG2, the values were 12.2 and 10.5, respectively. The action of the enzymes on various substrates is shown in Table 4. Neither of the enzymes had activity toward either of the arylglycosides, and neither liberated reducing sugars from laminarin or xylan. EG1 had a high activity toward CMC, barley β -glucan, and lichenan; and activity on acid-swollen cellulose was higher than that of EG2 toward CMC. EG2 hydrolyzed lichenan, but slowly in relation to CMC, whereas the activity of EG1 was similar on both substrates. The products from acid-swollen cellulose hydrolysis by EG1 were composed almost entirely of cellotriose and cellobiose, with the latter being the major product (Fig. 7). Hydrolysis by EG2 resulted in a wider spectrum of products, ranging from cellopentaose to glucose, with the major product being cellotetraose. Products of oligosaccharide hydrolysis were similar to the end products of acid-swollen cellulose hydrolysis. Cellohexaose and cellopentaose were converted into cellotriose and cellobiose by EG1, whereas with EG2, cellotetraose was the major product. Cellotetraose was converted into cellobiose by EG1, but was not hydrolyzed by EG2. EG1 did not cleave cellotriose. Results of the reduction in viscosity versus the liberation of reducing sugars for EG1 and EG2 with CMC used as the substrate are presented in Fig. 8. A remarkable difference in the pattern of reducing sugar liberation over a 60-min incubation period was observed. For EG1, the re-

TABLE 4. Substrate specificity of the purified enzymes

Substrate	Concn	Sp act (μ mol/min per mg of protein) of:	
		EG1	EG2
CMC	10 mg/ml	82.3	5.4
Barley β -glucan	10 mg/ml	83.1	
Lichenan	10 mg/ml	51.5	0.5
Acid-swollen cellulose	7.8 mg/ml	7.9	0.2
Xylan	10 mg/ml	<0.01	<0.01
Laminarin	10 mg/ml	<0.01	<0.01
<i>p</i> -Nitrophenyl- β -D-cellobioside	0.5 mM	0.4	<0.01
<i>p</i> -Nitrophenyl- β -D-glucoside	0.5 mM	0.02	<0.01

lease of reducing sugar was proportionate with the time of incubation. With EG2, the release of reducing sugar was nonlinear, occurring rapidly at first and then more slowly as time progressed. This may have been due either to end product inhibition or a decreased affinity of the enzyme for CMC as the degree of polymerization was decreased. When plots of fluidity (inverse of viscosity) versus liberation of reducing sugars were constructed, identical slopes were obtained for the two enzymes (Fig. 9). Hence, both enzymes liberate equal amounts of reducing sugar per unit of viscosity decrease and therefore cleave the β (1-4) bonds of CMC with a similar, random mode of action.

DISCUSSION

In the purification of endoglucanase activities from the culture fluid of *B. succinogenes* grown in a chemostat with cellulose as the carbon source, it was initially observed that only 13.5% of the activity applied to DEAE-Sepharose was eluted in the application buffer (Fig. 1). However, with continued operation of the chemostat, the proportion of nonbinding activity increased dramatically, as noted in Fig. 1 of Huang et al. (19) and Table 1 of this report, in the purification of EG2. The nonbinding enzyme was associated with materials with molecular weights greater than 150,000, as indicated by its exclusion from the molecular-sieving gel Bio-Gel P-150 (unpublished data). This material may be composed of large-molecular-weight protein aggregates. In previous studies (17, 31), it was found that 9 to 13% of the nonsedimentable material was associated with protein aggregates with molecular weights of greater than 4×10^6 . These aggregates are beyond the exclusion limit of the Sepharose beads; and as the majority of the ion-exchange groups are internal to the beads, the aggregates would greatly diminish contact with the ion exchangers. Both EG1 and EG2, as well as the purified cellobiosidase (19), showed a disposition toward aggregation, as judged by nondenaturing gradient gel electrophoresis. With *Ruminococcus albus*, cellulose is degraded faster by large enzyme aggregates than by the free, low-molecular-weight species (33). Growth of *B. succinogenes* in continuous culture in the presence of excess cellulose may have selected for the formation of protein aggregates.

The molecular weight of EG1, as determined by SDS-PAGE, was identical to the molecular weight determined by Gaudet (12) by gel filtration for the crude endoglucanase activity in the culture fluid. However, in our hands, a molecular weight of 50,100 was obtained by gel filtration of purified EG1 on Bio-Gel P-150 (unpublished data). EG2 bound to Sephadex G-100, and in this respect, it was similar

TABLE 3. Influence of metal ions and chelating agents on endoglucanase activity of EG1 and EG2^a

Treatment	% of untreated activity in:	
	EG1	EG2
None	100.0	100.0
1 mM EDTA	2.1	50.9
1 mM EGTA	16.6	
1 mM HgCl ₂	0.2	5.4
1 mM CuCl ₂	38.4	2.0
1 mM CaCl ₂	138.6	96.0
10 mM CaCl ₂	172.9	
1 mM MgCl ₂	172.6	86.2
10 mM MgCl ₂	159.0	
1 mM EDTA-1 mM CaCl ₂	44.7	
1 mM EDTA-1 mM MgCl ₂	42.5	

^a Endoglucanase activity in the presence of metal ions and chelating agents was determined in 40 mM PIPES (piperazine-*N,N'*-bis(2-ethanesulfonic acid); pH 6.4) at 39°C, with CMC used as the substrate. Results are expressed as a percentage of the untreated control.

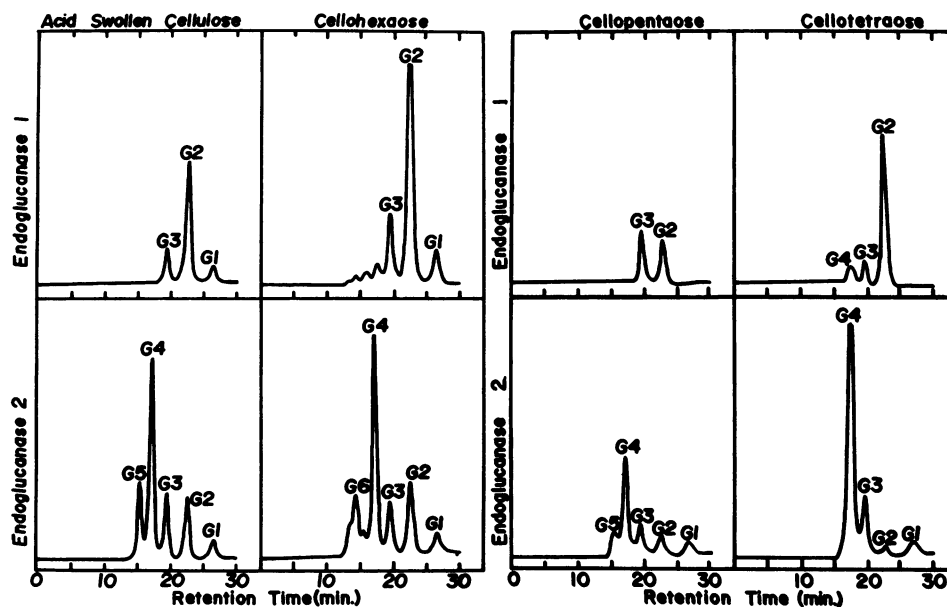


FIG. 7. Products of acid-swollen cellulose and cellooligosaccharide hydrolysis by EG1 and EG2. The enzymes were incubated with 1% acid-swollen cellulose or 0.05% cellooligosaccharide at 39°C for 22 h, and products were determined by high-pressure liquid chromatography. Abbreviations: G6, cellohexaose; G5, cellopentaose; G4, cellotetraose; G3, cellotriose; G2, cellobiose; G1, glucose.

to a 118-kDa endoglucanase from *Cellulomonas* sp. in which binding of the enzyme to Sephadex was employed in the purification process (2). EG2 was present as two components which differed in both size and charge. The similar cyanogen bromide peptide maps documented that the two proteins are related. The fact that the larger EG2B (118 kDa) was the more abundant protein and that it had a greater affinity for amorphous cellulose suggests that it is the functional form in vivo. The smaller EG2A presumably arises

from EG2B by proteolytic cleavage after its release into the extracellular culture.

The multiplicity of endoglucanases observed in *B. succinogenes* can therefore be attributed to three factors. First, our data document the presence of at least two different endoglucanases. According to Crosby et al. (8), there are at least six genes that code for endoglucanase activity in *B. succinogenes*. A multiplicity of endoglucanases is not unusual for a cellulolytic bacterium (2, 24, 26, 27, 29, 30). Second, the endoglucanases are subject to proteolytic cleavage, giving more than one component of some of the endoglucanases in the extracellular culture fluid. Growth on cellulose has been shown to induce extracellular protease activity in *Cellulomonas fimi* (23), and culture supernatants of *Thermomonospora fusca* possess protease activity which contributes to endoglucanase multiplicity (5). Third, the endoglucanases have a tendency to aggregate, forming multimers; and presumably, they also form aggregates with

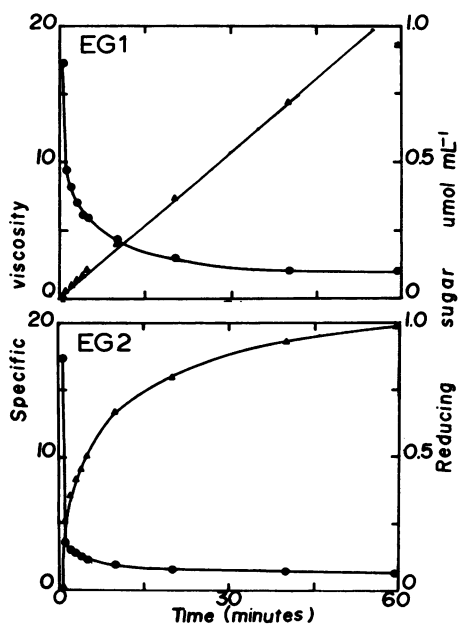


FIG. 8. Viscometric assays of EG1 and EG2, with 1% medium-viscosity CMC used as the substrate. Enzymes were diluted to give equal reducing sugar values after 1 h of incubation. Symbols: ●, viscosity; ▲, reducing sugar.

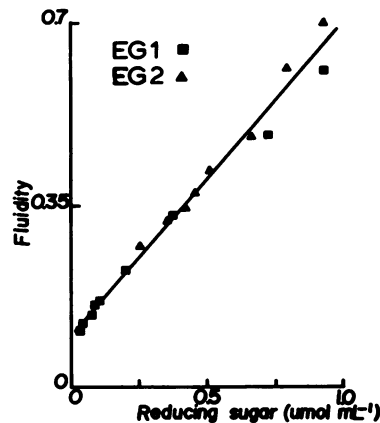


FIG. 9. Plot of fluidity versus reducing sugar for EG1 (■) and EG2 (▲). Data were derived from Fig. 8, with fluidity being defined as the inverse of specific viscosity.

other proteins. Results of studies (3) with the carboxymethyl cellulase of *Erwinia chrysanthemi* have indicated that protein aggregation can contribute to an apparent enzyme multiplicity. The activity profile of a *C. fimi* culture supernatant was shown to vary with the cellulosic substrate used for growth, culture age, and storage of the supernatant (23). Apart from the fact that endoglucanase multiplicity is caused by the factors listed above, it has also been attributed to posttranslational modification by chemical substitution, such as glycosylation. Two of three endoglucanases purified from *Cellulomonas* sp. are glycosylated (2), as is one from *Clostridium thermocellum* (29) and at least one from *T. fusca* (5). In contrast, neither of the two endoglucanases from *B. succinogenes* was a glycoprotein, although periodic acid-Schiff staining of nonsedimentable culture fluid revealed glycosylated proteins (unpublished data), and the extracellular cellobiosidase is a glycoprotein (19).

The pH and temperature optima of the purified enzymes, like those of other cellulase enzymes from *B. succinogenes*, are a reflection of the rumen environment in which the pH normally varies between 6 and 7 and the temperature varies between 37 and 40°C (18, 19, 34). The substrate specificities of EG1 and EG2 (Table 4) are typical of those for other endoglucanases. The *cel* gene product of *B. succinogenes* had a similar substrate specificity, with the exception that *p*-nitrophenyl- β -D-cellobioside was hydrolyzed (34). A cloned cellulase gene of *R. albus* expressed in *E. coli* directs the synthesis of an enzyme which could digest CMC and lichenan, but not laminarin, xylan, or cellobiose (21). The pEC2- and pEC3-encoded cloned endoglucanases of *C. fimi* have been shown to be active only on CMC and lichenan (13).

With respect to the mode of action on cellooligosaccharides, a preference for cleavage of internal glycosidic bonds appears to be a common property of both bacterial and fungal endoglucanases. With cellopentaose as the substrate, internal bond cleavage would produce cellotriose and cellobiose as the products. This was observed with purified EG1 from *B. succinogenes*, as well as with purified endoglucanases from *Cellvibrio gilvus* (7), *Myrothecium verrucaria* (35), *Clostridium thermocellum* (29), *Trichoderma viride* (32), and the purified *celC* gene product of *C. thermocellum* (28). In addition, a second endoglucanase purified from *C. thermocellum* (27) and two endoglucanases purified from *T. fusca* (5) gave glucose, cellobiose, and cellotriose as end products of cellulose hydrolysis, as did purified EG1. Hence, the pattern of cleavage of cellulose and oligosaccharides by EG1 is typical of that observed with other endoglucanases. EG2, however, displayed a feature which may be unique to this enzyme; cellotetraose was always the major end product of cellulose and cellooligosaccharide hydrolysis. The enzyme can bind to cellulose or oligosaccharides, such that five glucose residues are held within the active site; cleavage then occurs between the fourth and fifth glucose residues. With regard to the mechanism of cellulose hydrolysis, there appears to be a high probability of cooperative interaction between the two enzymes. First, EG2 possesses a high affinity for amorphous cellulose. It rapidly depolymerizes CMC, but the rate of reducing sugar production decreases as the substrate is converted into shorter polymers, with the final product being cellotetraose. Second, EG1 does not bind to amorphous cellulose but maintains a constant rate of reducing sugar production as the substrate is depolymerized; and it efficiently converts cellotetraose, the end product of hydrolysis by EG2, into cellobiose.

A widely accepted, but by no means irrefutable, model for

the enzymatic hydrolysis of cellulose by fungal cellulases envisages a mechanism whereby an endoglucanase initiates the attack on the cellulose chains and the new nonreducing chain ends that are generated are then acted upon by the endwise-acting cellobiohydrolase (36). The properties of the two enzymes reported here suggest that an additional level of cooperation between endoglucanases may be an important factor in the hydrolysis of cellulose by bacterial cellulases.

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