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Purification and Characterization of an Exo-β-1,4-Glucanase from *Ruminococcus flavefaciens* FD-1

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An exo-β-1,4-glucanase (Exo A) from *Ruminococcus flavefaciens* FD-1 was purified to homogeneity and characterized. Enzyme activity was monitored during purification by using the substrate *p*-nitrophenyl-β-D-cellobioside (NPC). Over 85% of the NPC activity was found to be extracellular once the filter paper was degraded (7 days). Culture supernatant was harvested, and the protein was concentrated by ultrafiltration. The retentate (≥300,000 M_r), containing most of the activity against NPC, was then fractionated with a TSK DEAE-5PW column. This yielded a sharp major peak of NPC enzyme activity, followed by a broader, less active area that appeared to contain at least six minor peaks of lower enzymatic activity. Further purification was achieved by chromatography with a hydroxylapatite column. Finally, gel filtration chromatography yielded a homogeneous enzyme (Exo A) as determined by silver stains of both sodium dodecyl sulfate- and nondenaturing electrophoresis gels. Substrate specificity experiments and the products of cellulose digestion indicate that the enzyme was an exo-β-1,4-glucanase. Exo A required Ca^{2+} for maximal activity and had an apparent K_m of 3.08 mM for NPC, with a V_{max} of 0.298 μmol/min per mg of protein. The enzyme had an M_r of 230,000, as determined by gel filtration chromatography, and was a dimer of 118,000-M_r subunits. The N-terminal amino acid sequence of the enzyme is presented.

Cellulolytic organisms produce several cellulase enzymes which have different specificities and modes of action. At least three types of cellulase enzymes are involved in the degradation of cellulose by fungi (25). Endo-β-1,4-glucanase (EC 3.2.1.4) is produced by all cellulolytic microorganisms and is more commonly referred to as carboxymethylcellulase (CMCase) or C_x cellulase. Exo-β-1,4-glucanase (EC 3.2.1.91) is also referred to as C_1 cellulase or cellobiohydrolase. β-1,4-Glucosidase (EC 3.2.1.21) is widely distributed among organisms and is commonly known as cellobiase or β-glucosidase. The most efficient and complete hydrolysis of cellulose is thought to be the result of the combined synergistic action of both endoglucanase and exoglucanase.

Two of the most important cellulolytic bacteria in the rumen are *Ruminococcus flavefaciens* and *Ruminococcus albus* (2, 11). Typically, these two species ferment cellulose, cellobiose, and xylan. The cellulase systems of both *R. flavefaciens* and *R. albus* appear to have many similarities. However, in general, *R. flavefaciens* degrades crystalline cellulose more efficiently than *R. albus* (2, 11). This observation suggests that *R. flavefaciens* possesses exoglucanase activity.

Pettipher and Latham (29) investigated the cellulase complex from *R. flavefaciens* as a crude preparation and concluded that the most active enzymes present in the complex were of the exo-β-1,4-glucanase type. However, the authors did not purify any of the enzymes present or investigate the number and types of cellulolytic enzymes in the different molecular weight complexes. The authors concluded that the major enzyme type was exo-β-1,4-glucanase, based on the products of cellulolytic activity (cellobiose, cellotriose, and a small amount of glucose) and the changes in viscosity versus reducing sugar. However, valid interpretations of cellulase enzyme type cannot be made from data generated by a mixture of cellulolytic enzyme activities. Cellulase enzymes must be purified to homogeneity before conclusive characterization and categorization of enzyme type can be made. Therefore, the presence or absence of a true exoglucanase in *R. flavefaciens* has not been demonstrated.

Recently, there have been preliminary reports of the purification of a β-glucosidase and a cellobiosidase from *R. albus* (26, 27). The aim of this study was to demonstrate the existance of an exoglucanase in *R. flavefaciens*; this required purification, identification, and characterization of exoglucanase activity in a strain of *R. flavefaciens*. This paper describes the purification and characterization of an exo-β-1,4-glucanase (Exo A) from *R. flavefaciens* FD-1.

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MATERIALS AND METHODS

Organism. *R. flavefaciens* FD-1 was obtained from the stock culture collection of M. P. Bryant and B. A. White, University of Illinois, Urbana. Stock cultures were maintained on 0.1% (wt/vol) cellobiose basal medium agar (1.0%, wt/vol) slants. Prior to the 4-liter batch production of cellulase, cultures were transferred three times in the basal broth medium supplemented with 0.1% (wt/vol) acid-swollen cellulose.

Media. *R. flavefaciens* FD-1 was grown on a defined basal medium containing (per liter) 50 ml of both mineral solution no. 1 and mineral solution no. 2 (4), 0.0001% (wt/vol) resazurin, 30 ml of volatile fatty acid solution (20), 5.0 ml of vitamin solution (20) supplemented with pyridoxine (20 mg/100 ml) and pyridoxamine (20 mg/100 ml), 1 ml of Pfennig trace metal solution (22) as modified by Genthner et al. (10), 10 ml of hemin and naphthoquinone solution (12), 0.4% (wt/vol) Na_2CO_3, 42.5 ml of hydrocinnamate solution (2 mM), 37.5 ml of phenylacetate solution (2 mM), 10 ml of sodium acetate solution (0.3 M), 0.1% (wt/vol) acid-swollen

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cellulose (Whatman no. 1 filter paper), and 0.05% (wt/vol) cysteine hydrochloride. The vitamin solution, which was filter sterilized, and the cysteine hydrochloride solution were added after autoclaving. All medium preparation and manipulations were performed anaerobically under a CO_2 atmosphere as described by Hungate (13) and modified by Bryant (1).

Crude enzyme preparation. A 5-day, 200-ml culture of *R. flavefaciens* was used to inoculate 4 liters of the basal broth with 0.1% (wt/vol) acid-swollen cellulose. The culture was incubated at 39°C under constant CO_2 bubbling. At the end of 7 days of incubation, little visible cellulose remained and the culture solids were removed by centrifugation (10,000 × *g*, 20 min) at 4°C. All remaining manipulations were also conducted at this temperature. Due to the carbonate buffer used, all initial processing had to be performed under CO_2 to prevent pH fluctuations. The culture supernatant was concentrated 50-fold by ultrafiltration on an XM-300 membrane (Amicon Corp.). The retentate, containing the majority of activity against *p*-nitrophenyl-β-D-cellobioside (NPC), was washed with 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.0) and stored at 4°C until further purification could be performed.

Enzyme purification. The crude enzyme preparation was initially fractionated by high-pressure liquid chromatography (HPLC) on a Spherogel TSK DEAE-5PW column (7.5 by 75 mm; Beckman Instruments, Inc.). The column was equilibrated with 0.05 M sodium phosphate (pH 8.0) at a flow rate of 1.0 ml/min. The protein was eluted with a linear gradient of 0 to 0.5 M NaCl in 55 min. Repeated runs were made with 2.0 ml applied to the column. One sharp major peak and a broader, poorly resolved second area of activity against NPC were detected, and the major one (peak A) was chosen for further purification. Peak A was collected, pooled, and concentrated by ultrafiltration with an XM-50 membrane (Amicon Corp.). This preparation was then desalted by washing with 0.05 M HEPES (pH 7.0) and stored at 4°C. The second, minor region of lesser activity (region B) was processed in the manner just described and frozen at −20°C for study at a later time.

Peak A was further fractionated with a column (1.5 by 51 cm) of hydroxylapatite (HPT; Bio-Rad Laboratories) equilibrated with 20 mM potassium phosphate (pH 7.0). A 2-liter linear gradient of 20 to 200 mM potassium phosphate (pH 7.0) was used to elute peak A. Again two peaks of activity against NPC were detected; however, the smaller region of activity was somewhat resolved from peak A, which allowed collection of enzyme relatively free of contaminating activity. Peak A fractions, which contained activity against NPC, were pooled and concentrated with an XM-50 membrane in preparation for the final chromatographic step.

The final purification step involved an HPLC Spherogel TSK 4000-SW column (7.5 by 300 mm; Beckman Instruments). This column was equilibrated with 0.05 M HEPES (pH 7.0) containing 0.1 M NaCl at a flow rate of 0.8 ml/min. Repeated runs were made with 0.1 ml applied to the column. This technique resolved peak A to greater than 98% homogeneity.

M_r determination. The M_r of the purified enzyme was determined by HPLC gel filtration with the TSK 4000-SW column and the conditions described above. Standards used were: bovine thyroglobulin (M_r, 670,000), bovine γ-globulin (M_r, 158,000), chicken ovalbumin (M_r, 44,000), horse myoglobulin (M_r, 17,000), and vitamin B_{12} (M_r, 1,350). The column was calibrated with the standards in separate runs before and after the enzyme was run.

Electrophoresis. After each stage of the purification process, the purity of the enzyme was checked by polyacrylamide gel electrophoresis (PAGE). Nondenaturing gels were run as described by Calza et al. (5) with a 12.5% polyacrylamide resolving gel. Sodium dodecyl sulfate (SDS)-PAGE was performed as described by Laemmli (17) with a Bio-Rad Mini-Protean II with a 7.5% polyacrylamide resolving gel. Proteins were visualized by silver staining (23). The presence of carbohydrate in proteins was tested by staining with both the alcian blue (35) and the Schiff-periodate (9) procedures. Ovalbumin was the positive control for glycoprotein staining.

Enzyme assays. During the purification, NPC assays (7) were conducted at 39°C in PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)] buffer (pH 6.8). *R. flavefaciens* FD-1 does not have a β-glucosidase (3), and therefore during purification assays with NPC we were detecting exoglucanase and some endoglucanase activity (7). Characterization of the purified NPC activity was performed by using the determined temperature optimum (39°C) and the pH optimum (0.1 M sodium phosphate buffer, pH 5.0). Incubation mixtures consisted of 100 μl of 20 mM NPC (final concentration, 8 mM), 100 μl of 10 mM $CaCl_2 \cdot 2H_2O$ (4 mM final concentration, in buffer), and 50 μl of enzyme preparation (4.2 μg per assay). Samples were incubated for 20 min, and then the reaction was terminated by the addition of 100 μl of a 1 M Na_2CO_3 solution. Activity was determined by reading A_{410} on a response spectrophotometer (Gilford Instruments).

Assays for the characterization of cellulase enzyme type with various cellulose substrates contained 1% (wt/vol) substrate. Reducing sugars were detected by a modification of the potassium ferricyanide procedure (28). The reaction was stopped by adding 1% (wt/vol) $Na_2HPO_4 \cdot 2H_2O$ containing 1% (wt/vol) NaOH. All enzyme assays were determined to be linear with respect to time and protein concentration.

Divalent cation assays. The enzyme preparation was treated with 10 mM EDTA and 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid] (pH 7.0) for 1 h prior to the assay. These chelating agents were removed by filtration on a Centricon 30 microconcentrator (Amicon). This preparation was washed three times with 0.1 M sodium phosphate buffer (pH 7.0) before testing the effects of the divalent cations. All divalent cations were added as chloride salts and tested at a final concentration of 4 mM.

Inhibitor studies. Inhibitors were incubated in the presence of the enzyme for 15 min (4°C) prior to the addition of substrate (NPC). Activity was determined as described above.

Protein determinations. Concentrations of soluble protein were determined by the difference in absorbance at 230 and 260 nm as described by Kalb and Bernlohr (14).

N-terminal amino acid sequence. The N-terminal amino acid sequence of Exo A was determined by the Biotechnology Center, University of Illinois at Urbana-Champaign. The sequence was obtained from 100 pmol of Exo A with an Applied Biosystems liquid-phase protein/peptide sequencer.

TLC. Products of cellulose hydrolysis were analyzed by thin-layer chromatography (TLC). Samples were desalted as described by Russell (30) and spotted on silica plates (20 by 20 cm; Keiselguhr F254). The mobile phase consisted of isopropanol-ethyl acetate-H_2O (42:35:23). Products were visualized by H_2SO_4, followed by charring (33). The migration parameter R_f for each cellulodextrin was: glucose, 0.77; cellobiose, 0.68; cellotriose, 0.53; cellotetraose, 0.38; cellopentaose, 0.27; cellohexaose, 0.

TABLE 1. Purification of Exo A from *R. flavefaciens* FD-1

Step	Vol (ml)	Activity[a] (U/ml)	Total activity (U)	Protein (mg/ml)	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
Culture supernatant	4,200	1.44×10^{-2}	60.7	0.299	4.83×10^{-2}	100.00	1.00
300 XM retentate	85	5.76×10^{-1}	48.95	4.12	1.42×10^{-1}	80.64	2.94
Peak A DEAE-5PW	161.5	4.03×10^{-2}	6.5	0.167	2.41×10^{-1}	10.71	4.99
Peak A DEAE-5PW 100YM retentate	8.1	7.16×10^{-1}	5.8	1.94	3.71×10^{-1}	9.55	7.65
Peak A HPT	130	1.15×10^{-2}	1.5	0.063	1.8×10^{-1}	2.47	3.7
Peak A HPT 100YM retentate	6.8	2.00×10^{-1}	1.36	0.986	2.03×10^{-1}	2.24	4.2
TSK 4000SW	54.4	1.18×10^{-2}	0.64	0.104	1.13×10^{-1}	1.05	2.34

[a] Units are micromoles of NPC hydrolyzed per minute.

Chemicals. Sigmacell, carboxymethylcellulose (CMC, low viscosity grade), and NPC were purchased from Sigma Chemical Co. Filter paper used as the cellulose source for growth (Whatman no. 1) was purchased from Whatman Inc. Protein M_r standards for gel filtration chromatography and protein standards for SDS-PAGE were from Bio-Rad Laboratories. All other chemicals were of the highest grade commercially available.

RESULTS

Purification. *R. flavefaciens* FD-1 degrades 0.1% (wt/vol) acid-swollen cellulose in 6 to 7 days. Maximal activity against NPC (cell associated or in the culture supernatant) could be detected when little visible cellulose remained in the culture. Once the cellulose was cleared, greater than 85% of the NPC hydrolysis activity was found to be extracellular. A crude enzyme preparation was made by concentrating the culture supernatant by ultrafiltration (300,000-M_r cutoff). The retentate contained essentially all the activity against NPC. A summary of purification is presented in Table 1.

The ≥300,000-M_r retentate was fractionated by DEAE ion-exchange HPLC (Fig. 1). Two areas of activity against NPC were detected, and the major sharp peak (peak A) was chosen for further purification, as this peak was reasonably well resolved and contained a high level of NPC activity. Peak A (fractions 37 to 41) was collected, pooled, and concentrated by ultrafiltration (50,000-M_r cutoff). The second activity region (fractions 42 to 55) contained at least six other poorly resolved peaks of lower activity (region B). Peak A showed little activity against CMC, whereas region B showed a much higher level of CMC hydrolyzing activity (data not shown). Region B was processed in the manner described above and frozen at −20°C for study at a later time.

Further purification of peak A was achieved by HPT chromatography (Fig. 2). Again two NPC activity peaks were detected. The smaller peak was resolved from peak A, allowing collection of enzyme relatively free of contaminating activity from the smaller peak. Peak A (fractions 44 to 56) was pooled and concentrated by ultrafiltration (50,000-M_r cutoff) in preparation for final purification.

The final purification step was gel filtration HPLC (Fig. 3), which resolved peak A to greater than 98% homogeneity. This material (fraction 13) was used for further characterization. The final purification was approximately 2.34-fold, and the overall yield of the purified enzyme was 1.05% (Table 1). The assays during the purification process were performed at pH 6.8 and in the absence of exogenous Ca^{2+}. Therefore, the final specific activity of Exo A was underestimated. Further-

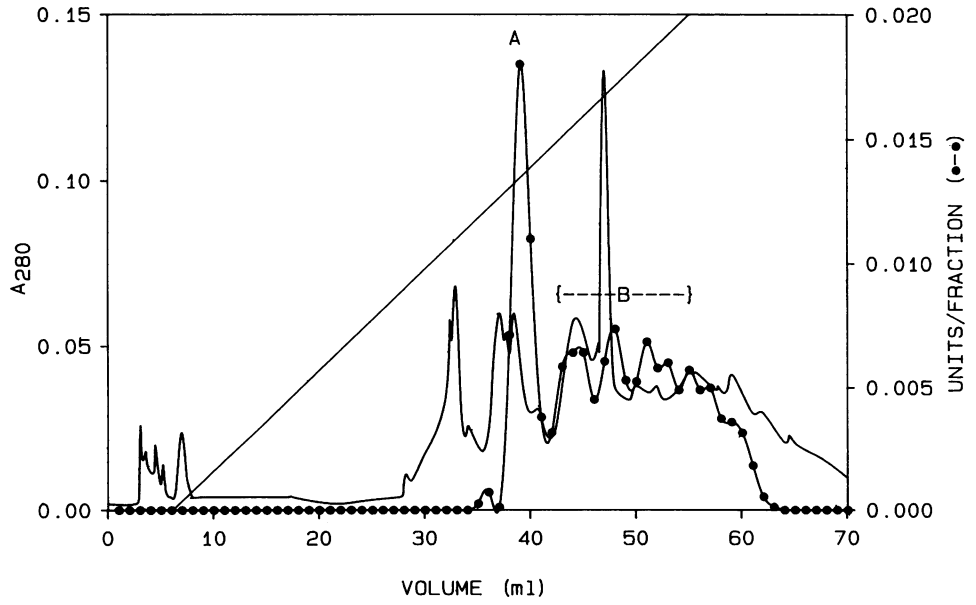

FIG. 1. Spherogel TSK DEAE-5PW HPLC chromatography of the ≥300,000-M_r retentate. The column was equilibrated with 0.05 M sodium phosphate (pH 8.0) at a flow rate of 1.0 ml/min. The protein was eluted with a linear gradient of 0 to 0.5 M NaCl in 55 min. NPC activity (●) was assayed as described in Materials and Methods. A, Peak A; B, region B.

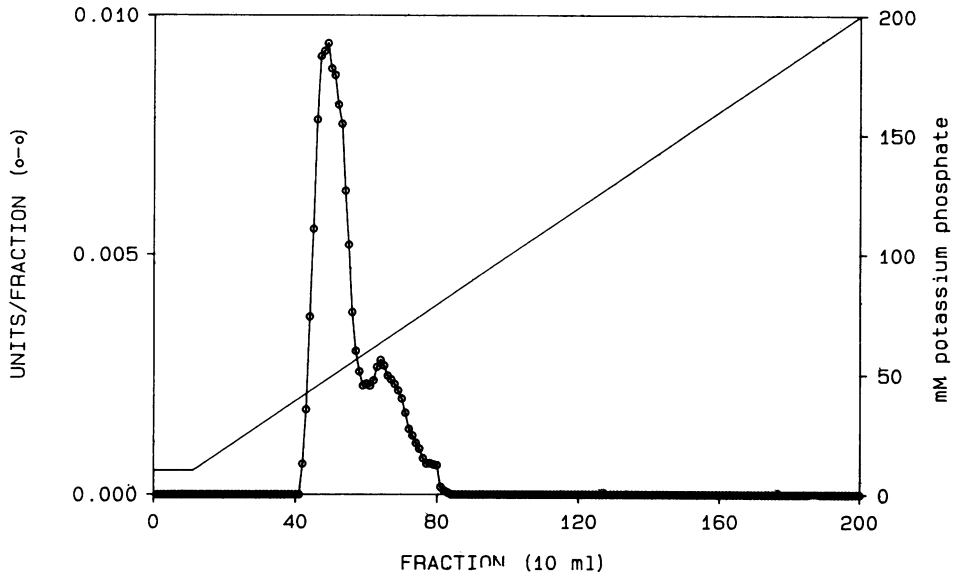

FIG. 2. HPT column chromatography of peak A. A 2-liter linear gradient of 20 to 200 mM potassium phosphate (pH 7.0) was used to elute peak A. NPC activity (○) was assayed as described in Materials and Methods.

more, as there were multiple NPC-utilizing enzymes (at least seven) present in the culture filtrate, the final fold purification and yield of Exo A were also low estimates. The purified enzyme was stored at −20°C for at least 1 month without loss of activity. The enzyme was stored at 4°C for at least 3 weeks without any detectable change in activity or molecular weight.

The M_r of Exo A was estimated to be 230,000 by gel filtration chromatography with a TSK 4000-SW HPLC column equilibrated with 0.05 M HEPES (pH 7.0) containing 0.1 M NaCl. Exo A was tested for purity by nondenaturing PAGE and SDS-PAGE (Fig. 4). Both SDS-PAGE (Fig. 4A) and nondenaturing PAGE (Fig. 4B) showed single bands of protein when visualized by silver staining. The single polypeptide detected by SDS-PAGE was estimated to have an M_r of 118,000, indicating that the enzyme existed as a dimer. Exo A was not a glycoprotein, as the purified protein did not stain positively with the commonly used reagents (9, 35). The N-terminal amino acid sequence of Exo A was NH_3-Ala-Gln-Val-Leu-Gly-Asn-Gly-Asp-Phe-Asp-Asp-Gly-Thr-Ala-Leu-Pro-Trp-Gln-Thr-X-Glu-Asp-Gln-Pro-Ala-Gly. Amino acid 20 could not be identified by this procedure due to high background on the chromatogram.

Catalytic properties of Exo A. The effect of divalent cations on Exo A was examined by first treating the enzyme with chelating agents (EDTA and EGTA) to remove any endog-

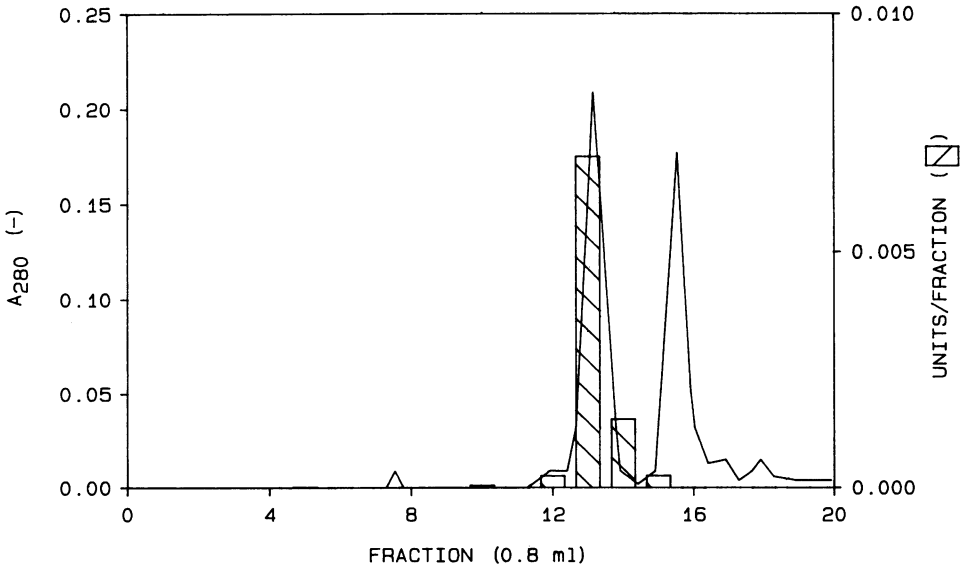

FIG. 3. Spherogel TSK 4000-SW HPLC chromatography of peak A. The column was equilibrated with 0.05 M HEPES (pH 7.0) containing 0.1 M NaCl at a flow rate of 0.8 ml/min. The 0.09 mg applied to the column and the activity eluted isocratically. NPC activity (hatched bars) was assayed as described in Materials and Methods.

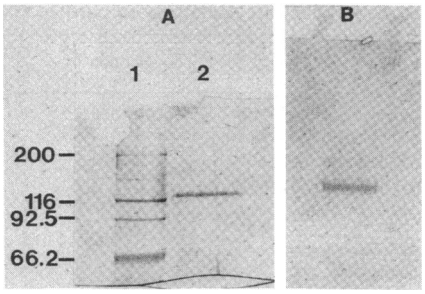

FIG. 4. Silver stains of SDS-PAGE (A) and nondenaturing PAGE (B) gels of purified Exo A. Lanes: (A) 1, molecular mass markers as indicated (in kilodaltons); 2, 0.5 μg of purified Exo A; (B) 0.5 μg of purified Exo A. PAGE was performed as described in Materials and Methods.

TABLE 2. Effect of divalent cations on purified Exo A[a]

Divalent cation (4 mM)	Enzyme activity (% of control)
None (control)	100.0
Ca^{2+}	190.0
Zn^{2+}	0.3
Mg^{2+}	94.8
Mn^{2+}	59.1
Co^{2+}	29.7
Fe^{2+}	7.7
Ni^{2+}	69.1

[a] Assays were done at 39°C in 0.1 M sodium phosphate buffer (pH 5.0) with 4.2 μg of purified enzyme. Enzyme preparation was treated with 10 mM EDTA and 10 mM EGTA for 1 h prior to the assay. The chelating agents were removed by ultrafiltration (see Materials and Methods) before testing the effects of metal ions. Control (100%) activity equals 0.14 μmol of NPC hydrolyzed per min per mg of protein.

enous divalent cations, followed by adding various divalent cations to the reaction mixture. Ca^{2+} (4 mM) stimulated Exo A activity approximately 1.9-fold, but none of the other divalent cations tested were stimulatory (Table 2). Zn^{2+} (4 mM) and Fe^{2+} (4 mM) were strongly inhibitory, while the other divalent cations tested showed either no effect or moderate levels of inhibition. These results show that Exo A required Ca^{2+} for maximal activity.

The pH dependence for Exo A activity towards NPC in the presence of Ca^{2+} was determined by measuring the activity in 0.1 M sodium citrate–sodium phosphate buffer and 0.1 M sodium phosphate buffer at various pH (Fig. 5). Maximal enzyme activity was found at pH 5.0, with less than 20% maximal activity detected at pH above 6.5, under the conditions used. The effect of temperature on Exo A activity was measured in 0.1 M sodium phosphate buffer (pH 5.0) containing 4 mM Ca^{2+}. Maximal enzyme activity was detected at temperatures between 39 and 45°C. At 23°C, only 22% of the maximal activity was detected, whereas at 50°C, 50% of the maximal activity was detected. The enzyme was inactive at temperatures above 55°C.

The substrate saturation kinetics for the hydrolysis of NPC by Exo A in the presence of 4 mM Ca^{2+} at pH 5.0 were determined. The saturation curve was hyperbolic, and the double-reciprocal plot of Lineweaver and Burke (18) yielded an apparent K_m of 3.08 mM for NPC, with a V_{max} of 0.298 μmol/min per mg of protein.

The influence of several commonly used inhibitors on Exo A was then tested (Table 3). Sulfhydryl inhibitors such as *N*-ethylmaleimide, iodoacetate, and p-chloromercuribenzoate were all inhibitory, with p-chloromercuribenzoate providing the greatest inhibition. The chelating agent EDTA was slightly inhibitory, whereas EGTA, a more efficient chelator of Ca^{2+}, inhibited the enzyme by approximately 32%. Exo A was inhibited by almost 85% by *O*-phenanthroline, a result consistent with the apparent requirement of the enzyme for Ca^{2+}. Glucose had little if any effect on the enzyme, whereas cellobiose, the end product of the reaction, inhibited the enzyme by 50% at a concentration of 1 mM. Cellobiose at 10 mM inhibited the enzyme by 85%.

Exo A was tested for the ability to hydrolyze various carbohydrates and aryl-glycosides in the presence of 4 mM Ca^{2+} at pH 5.0 (Table 4). Of the NPC derivative substrates tested, NPC, p-nitrophenyl-β-D-lactoside, and p-nitro-

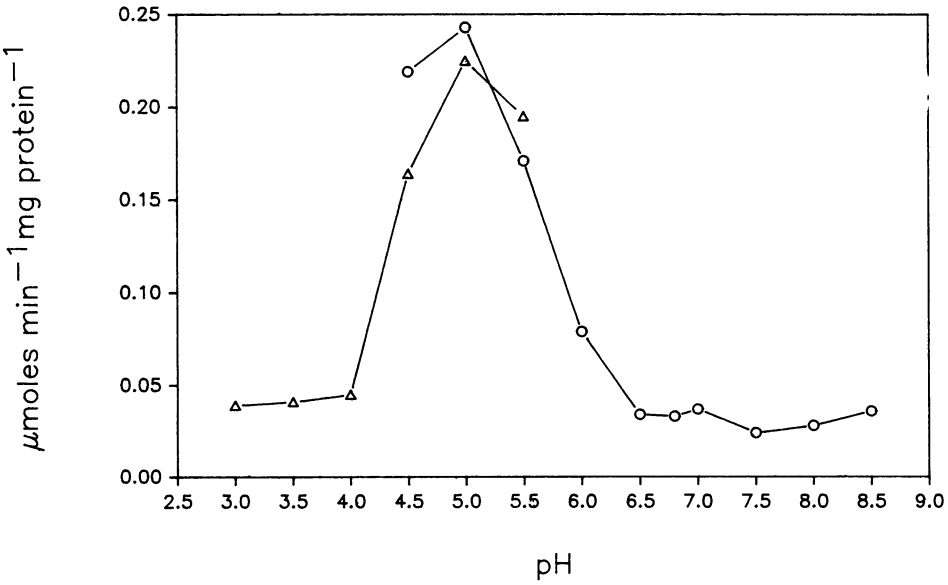

FIG. 5. Effect of pH on purified Exo A activity. Sodium citrate-phosphate buffer (0.1 M) (△) and 0.1 M sodium phosphate buffer (○) were used. NPC activity was assayed as described in Materials and Methods.

TABLE 3. Effect of inhibitors on purified Exo A[a]

Addition	Concn (mM)	Enzyme activity (% of control)
None (control)		100.0
EDTA	10	80.1
	1	91.3
EGTA	10	47.7
	1	68.5
N-Ethylmaleimide	10	62.0
	1	85.7
Iodoacetate	10	3.9
	1	38.0
O-Phenanthroline	10	15.8
	1	38.6
p-Chloromercuribenzoate	10	1.6
	1	13.2
Glucose	10	92.5
	1	85.9
Cellobiose	10	15.0
	1	50.1

[a] Assays were done at 39°C in 0.1 M sodium phosphate buffer (pH 5.0) with 4 mM Ca^{2+} and 4.2 μg of purified enzyme. Control (100%) activity equals 0.266 μmol of NPC hydrolyzed per min per mg of protein.

TABLE 4. Substrate specificity of purified Exo A[a]

Substrate	Concn	Activity (μmol/min per mg of protein)
p-Nitrophenyl-β-D-cellobioside	8 mM	0.298
p-Nitrophenyl-β-D-glucopyranoside	8 mM	0
p-Nitrophenyl-β-D-lactoside	8 mM	0.09
p-Nitrophenyl-β-D-xylopyranoside	8 mM	0.01
CMC	1%	0
Sigmacell	1%	0.049
Acid-swollen filter paper	1%	0.025
Filter paper	1%	0.019

[a] Assays were done at 39°C in 0.1 M sodium phosphate buffer (pH 5.0) with 4 mM Ca^{2+} and 4.2 μg of purified enzyme.

phenyl-β-D-xylopyranoside were hydrolyzed with various efficiencies. Activity against ρ-nitrophenyl-β-D-lactoside was only 30% of that obtained with NPC, whereas activity against ρ-nitrophenyl-β-D-xylopyranoside was only 3% of the activity against NPC. Reducing sugars were released from Sigma-cell, acid-swollen filter paper, and filter paper, but not from CMC. The substrate specificity results are consistent with the classification of peak A as an exoglucanase.

To confirm that the enzyme was indeed an exoglucanase, the hydrolytic end products from NPC and filter paper were analyzed by TLC. Analysis of the NPC hydrolysate indicated that cellobiose was the only product, demonstrating that the agluconic bond and not the holosidic bond was cleaved by the enzyme. Furthermore, cellobiose was the only detectable product released from filter paper by Exo A. This provides additional evidence that peak A is an exoglucanase.

DISCUSSION

The cellulolytic enzymes from *R. flavefaciens* have been reported to exist in three forms: a high-molecular-weight (≥300,000) enzyme complex, as well as smaller fractions with molecular weights of ≥800,000 and approximately 89,000 (29). The question arises as to the origin of the different-molecular-weight complexes and the type of cellulase enzymes present. Is the high-molecular-weight enzyme complex an aggregate of enzymes and the low-molecular-weight activity a breakdown product of this larger complex, or are different cellulolytic enzymes produced and incorporated into different complexes? One approach to resolving this question is to compare purified enzymes from different complexes at the subunit level. In this report, we present data concerning the identification, purification, and characterization of an exoglucanase from *R. flavefaciens*. This is the first report in the literature of the purification of an exoglucanase from this bacterial species.

NPC-hydrolyzing activity (cell associated or free in the culture supernatant) was not maximal until a majority of the insoluble carbohydrate was degraded. The N-terminal amino acid of Exo A and its supernatant location in the later stages of cell growth support the hypothesis that the enzyme is secreted. If the enzyme is cell associated in the early stages of growth, it must be wall associated, as has been reported by Pettipher and Latham (29). Alternatively, the enzyme could be tightly bound to the cellulose and the soluble chromophoric substrate may not be accessible to the enzyme, as has been suggested for cellulases from *Clostridium thermocellum* (19). Although the filter paper disks were covered with attached *R. flavefaciens* cells (as evidenced by the yellow color associated with the disks after 24 h of growth), there were many bacteria free in the supernatant (OD_{660}, 1.2). It is not known whether, in actively growing cells, the cellulolytic enzymes are cell bound or free in the culture supernatant. A cellobiosidase from *R. albus* has also been reported to be free in the supernatant once the cells have reached stationary phase (26). Pettipher and Latham (29) reported that, with *R. flavefaciens* 67, CMCase activity was associated with the cell fraction even into late stationary phase. In this study, over 85% of the total NPC-hydrolyzing activity was found free in the culture supernatant once the cells cleared a majority of the cellulose and were in the stationary phase. It should be noted that in this report a different strain of *R. flavefaciens* was used. Furthermore, the substrate used was more specific for exoglucanases, even though many endocellulases have been reported that use this substrate (7). The endoglucanases produced by *R. flavefaciens* FD-1 are currently under investigation, and it appears that they are also found in the culture supernatant once the cells have reached stationary phase (data not presented).

The catalytic properties of Exo A from *R. flavefaciens* FD-1 are similar to those reported previously for the cellulase complex of *R. flavefaciens* 67 (29). However, Exo A was only stimulated by Ca^{2+} and not by Mg^{2+}, suggesting that another cellulase component is activated by Mg^{2+}.

The pH optimum for Exo A was 5.0, whereas the pH optimum for the cellobiosidase from *R. albus* is 6.8. The pH optimum for Exo A was more similar to pH optima reported for fungal cellulases. The temperature optimum for Exo A was 39 to 45°C, compared with 37°C for the *R. albus* cellobiosidase. Exo A had a relatively high K_m (3.08 mM) for NPC. The K_m for NPC of the *R. albus* cellobiosidase was not reported, but Desphande et al. (7) reported that an exoglucanase from *Sporotichum pulverulentum* has a K_m for NPC of 1.67 mM and that two exoglucanases from *Trichoderma reesei* had apparent K_ms of 0.252 and 2.51 mM.

Exo A is a 230,000-M_r dimer made up of 118,000-M_r subunits. The *R. albus* cellobiosidase is a 200,000-M_r dimer made up of 100,000-M_r subunits (26). The exoglucanase of *A. cellulolyticus* was reported to be 33,000 M_r as determined by SDS-PAGE and 87,500 M_r in its native state (31). Fungal

exoglucanases range in size from 4,800 to 65,000 M_r (15, 32, 34). Therefore, Exo A is the largest exoglucanase that has been reported for either bacterial or fungal cellulolytic systems.

The substrate specificity of Exo A and the hydrolysis products from filter paper and NPC are indicative of exoglucanase activity. As has been reported for exoglucanases from both fungal and bacterial systems, Exo A showed little activity towards CMC (6, 21, 24, 36). Carboxymethyl substitution of cellulose yielding three or more adjacent substituted glucosyl residues is very resistant to enzymatic attack (8, 16). Exoglucanases remove unsubstituted residues from the ends of CMC and cannot remover further residues once a substituted moiety is encountered. Endoglucanases are much more active on CMC, as there are many more unsubstituted glucosyl residues available for this randomly acting enzyme. These results indicate that the enzyme described here is an exo-β-1,4-glucanase.

The identification, purification, and characterization of an exoglucanase are the initial steps in elucidating the mechanism of cellulose degradation and the cellulase complex composition of *R. flavefaciens*. We are currently in the process of purifying the minor NPC-positive peaks from the DEAE purification step (region B) and the endoglucanase components of *R. flavefaciens* FD-1 so that the cellulase complex can be completely described and reconstituted in vitro. Furthermore, monoclonal antibodies against the homogeneous enzyme are currently being used in experiments aimed at determining the location and distribution of Exo A during the growth phase.

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