JOURNAL OF BACTERIOLOGY, Apr. 1982, p. 407–409
0021-9193/82/040407-03$02.00/0

Vol. 150, No. 1

Purification and Properties of Cellobiosidase from *Ruminococcus albus*

KUNIO OHMIYA,* MASATOSHI SHIMIZU, MASAHITO TAYA, AND SHOICHI SHIMIZU

Department of Food Science and Technology, Faculty of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan

Received 17 August 1981/Accepted 4 December 1981

An enzyme active against *p*-nitrophenyl-β-D-cellobioside was purified from *Ruminococcus albus* to homogeneity. The enzyme was identified as a cellobiosidase.

Extensive literature has accumulated during the past decade regarding cellulolytic aerobes and their cellulases (3, 6, 9). In contrast, cellulolytic enzymes of rumen anaerobes have not been as widely examined (1, 4, 8) despite the fact that anaerobes are well known for their role in cellulose utilization by ruminants for energy generation. Our earlier reports indicated that *Ruminococcus albus* could grow in a synthetic medium without rumen fluid in a jar fermentor at pH 6.5 (11, 12, 13) and that this cultivation is a convenient method for obtaining cellulolytic enzyme proteins in bulk. The present paper describes a procedure for isolating an electrophoretically homogeneous cellulolytic enzyme from the culture supernatant of *R. albus* grown in a medium containing ball-milled cellulose (BMC) as the main carbon source. Characterization of the enzyme is also provided.

R. albus isolated from bovine rumen was cultivated in a medium containing 1.5% BMC at pH 6.5 and 37°C for 50 h. Details of the medium and the cultivation conditions are described in a previous paper (13). For determination of enzyme activity, *p*-nitrophenyl-β-D-cellobioside (PNPC) synthesized by the method of Nishizawa and Wakabayashi (7) was usually used as the substrate, as it was found to provide a very sensitive index of enzyme activity in preliminary experiments. PNPC solution (2 mM; 250 μl) and an enzyme sample (700 μl) were added to potassium phosphate buffer (1 M; pH 6.8; 50 μl) and incubated at 37°C for 1 h. *p*-Nitrophenyl-β-D-glucoside (Senn Chemicals Co.) was also used, under the same conditions, for measurement of enzyme activity. After the reaction was stopped by the addition of Na_2CO_3 (2 M; 250 μl), the absorbance of released *p*-nitrophenol was measured at 405 nm. For determining β-1,4-glucanase activity, a reaction mixture of potassium phosphate buffer (50 mM; pH 6.8; 1 ml) containing 1% BMC and enzyme solution (0.5 ml) from column chromatographic fractions was allowed to stand at 37°C for 2 h. The reaction mixture was placed for 10 min in boiling water, and then insoluble material was removed by filtration. The amount of reducing sugar in the filtrate was determined by the Somogyi-Nelson method (5, 10). Protein concentrations were determined either by measuring the absorbance at 280 nm or by the method of Lowry et al. (2). Polyacrylamide disc gel electrophoresis was carried out by the procedure of Weber and Osborn (14). Purified protein samples (17 and 6 μg, respectively) were applied on 5% gels in the presence and absence of 0.2% sodium dodecyl sulfate (SDS). Electrophoresis was conducted at a constant current of 8 mA per gel for 4 h at room temperature in the presence of SDS and at 3 mA per gel for 2 h at 4°C in the absence of SDS. Protein was stained with Coomassie brilliant blue (R-250). For detection of enzyme activity in the SDS-free gel, the gel was sliced at 4°C into 30 pieces, each 3 mm in length, immediately after electrophoresis. Substrate solution (2 mM PNPC; 0.25 ml) was added to the solution made when the enzyme was extracted from each piece by immersion in phosphate buffer (0.75 ml) overnight; the resulting solution was then incubated at 37°C for 3 h.

During the cultivation of *R. albus* for 20 to 40 h, almost all of the BMC was solubilized (Fig. 1), and the bacterial dry cell weight increased to a maximum. The enzyme activity against PNPC increased in the supernatant from the broth to a maximum at around 50 h of cultivation. This suggested that the enzyme may be synthesized and released from the cells once their growth has stopped. Details about the release of the enzyme from the cells have to be studied in future. After 50 h of cultivation, the supernatant from the broth was dialyzed within a cellophane membrane against 1,000 volumes of Tris-hydrochloride buffer (0.01 M; pH 7.2). The activity in the

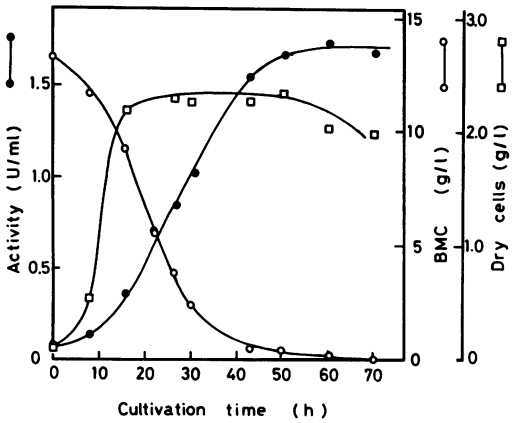

FIG. 1. Time courses of the enzyme activity against PNPC (●), BMC consumption (○), and bacterial cell numbers (□) in *R. albus* cultivated in a jar-fermentor with synthetic medium. The amount of BMC and the number of bacterial cells in the culture were determined with anthrone reagent (7) and Nessler reagent (4), respectively. One unit of enzyme activity equals 1 nmol of *p*-nitrophenol released from PNPC per minute.

dialysate was 80% of the original activity. The dialysate (1,000 ml) was loaded on a DEAE-Sephadex A-25 column (2.5 by 22 cm) equilibrated with the same buffer. A linear gradient elution was done with 1 liter of the buffer containing KCl (0 to 1.0 M; 10-ml fractions). The chromatography provided one major and two minor peaks with activity against PNPC eluting at KCl concentrations of 0.1, 0.3, and 0.5 M, respectively. The fraction that eluted with KCl at 0.5 M and that had major activity against BMC was denoted as β-1,4-glucanase-1 and was used for the determination of viscosity changes of carboxymethyl cellulose (CMC; Dai-ichi Pharmaceutical Co.) solutions.

The major fraction with activity against PNPC eluting at a KCl concentration of 0.1 M was chromatographed further on an ω-aminohexyl Sepharose 6B column (1.6 by 2.5 cm). A broadly spread protein peak with the activity was eluted around 0.3 M KCl. The fraction (15 ml) with the highest activity was dialyzed against 600 volumes of the Tris buffer containing 2 mM mercaptoethanol.

Isoelectric focusing was done with Pharmalyte (company), covering the pH range of 4 to 6.5, in a sucrose gradient at 4°C with constant voltage (1,300 V) for 85 h. Sucrose and Pharmalyte in the active fractions were replaced with 0.5 M KCl in the Tris buffer by gel filtration on a column (1.7 by 50 cm) containing equal volumes of Sephadex G-15 and G-50. Isoelectric focusing of fractions with enzyme activity provided a sharp protein peak coinciding with enzyme activity at a pI of 5.3. The protein in the peak fraction was homogeneous, as determined by polyacrylamide disc gel electrophoresis both in the presence (Fig. 2A) and absence (Fig. 2B) of SDS. The enzyme activity corresponded to the protein band found by standard gel electrophoresis in the presence of 10 mM mercaptoethanol. This three-step purification procedure yielded a 39-fold enrichment of the enzyme (Table 1). The amount of purified protein obtained was about 2 mg. All subsequent studies were carried out with purified enzyme.

For the identification of the compounds released from PNPC, a reaction mixture of enzyme (50 μg/ml; 0.5 ml) and substrate (0.45%; 1 ml) was incubated at 37°C for 2 h after the solution was boiled for 10 min and the supernatants were spotted on silica gel-precoated thin-layer plates (5 by 20 cm; Yamato replate-50 plates; Yamato Scientific Co. Ltd.). After the plates were developed for 5 h at room temperature with a 1-butanol–water–pyridine (6:3:4 [vol/vol]) solution, *p*-nitrophenol was detected by its yellow color, and the other compounds were detected as dark spots when the plates, which had been sprayed with 15% H_2SO_4 solution, were heated. Reaction products released from PNPC were identified as cellobiose and *p*-nitrophenol. In the case of BMC, the enzymatic reaction was continued overnight with twice the usual concentration of purified enzyme, and under this condition small amounts of cellobiose were detected, but glucose was not. *p*-Nitrophenyl-β-D-glucoside was not hydrolyzed even after overnight reaction. Viscosity changes in CMC solutions resulting from the action of the enzyme or from β-1,4-glucanase-1 (a fraction from DEAE-Sephadex A-25 chromatography) were determined with an Ostwald viscometer (Iwaki Glass Co. Ltd.) at 37°C. The changes in fluidity ($1/\eta_{sp}$ [the reciprocal of the specific

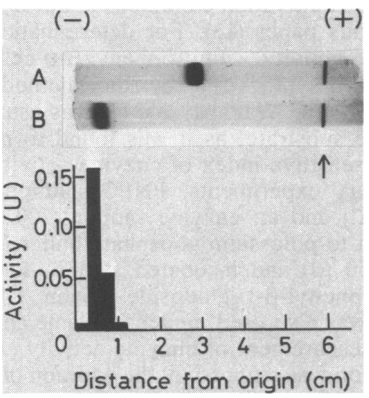

FIG. 2. Polyacrylamide disc gel electrophoresis of the enzyme. Gels of the enzyme were stained in the presence (A) and absence (B) of SDS. Protein migrated from the cathode (−) to the anode (+). The arrow indicates the position of bromophenol blue.

TABLE 1. Summary of purification of the enzyme from *R. albus*[a]

Purification step	Total volume (ml)	Total protein (mg)	Total enzyme activity (OD_{405})	Sp act (OD_{405}/mg)	% Recovery of enzyme activity	Fold purification
Culture supernatant (dialysate)	1,000	145.0	350	2.4	100	1
DEAE-Sephadex A-25	70	7.6	98	12.9	28	19
ω-Aminohexyl Sepharose 6B	600	4.7	95	20.2	27	31
Isoelectric focusing	5	3.7	77	20.8	22	39

[a] All fractionation procedures were performed at 4°C. OD_{405}, Optical density at 405 nm.

viscosity]) generated by the enzyme were compared with those generated by β-1,4-glucanase-1 after a reaction period during which the amounts of reducing sugar released from CMC by either of these enzymes were the same (Fig. 3). Fluidity ($1/\eta_{sp}$) of CMC after enzyme action increased linearly with the amount of reducing sugar released. The slope of this line was smaller than that obtained after the action of β-1,4-glucanase-1 on CMC. These results suggested that the purified enzyme cleaves the penultimate glucosidic linkage and can be described as an exoenzyme, whereas β-1,4-glucanase-1 is classified as an endoenzyme. Therefore, the former enzyme is termed a cellobiosidase.

The molecular weight of the enzyme in the presence of SDS was estimated to be 100,000 by disc gel electrophoresis. The estimated molecular weight by gel filtration analysis with a Bio-Gel P-300 column (1.7 by 60 cm; Bio-Rad Laboratories) in the absence of SDS was 200,000. Therefore, the native enzyme may be a dimer of two polypeptide chains of similar molecular weight. Maximum activities were noted at pH 6.8 and 37°C. Maximum stability was noted at pH values between pH 5.5 and 8.0 when the enzyme was stored at each pH for 15 h. Activity decreased greatly at temperatures above 37°C. The temperature required for 50% maximum activity was 40°C after the enzyme was held for 15 h in the Tris buffer (10 mM; pH 7.2).

This enzyme is quite active against PNPC but not very active against cellulose. Therefore, its function as a cellulolytic enzyme is not clear at this time.

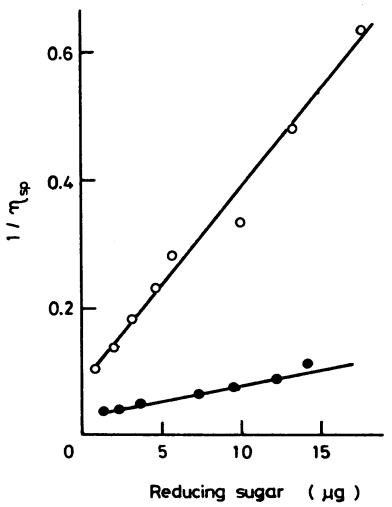

FIG. 3. Fluidity changes of CMC versus the amounts of reducing sugar released by the action of the purified enzyme (●) and by the action of β-1,4-glucanase-1 (○) at pH 6.8 and 37°C.

We are indebted to Keiji Ogimoto, Tohoku University, for providing a strain of *R. albus*, and to Hajime Minato, National Institute of Animal Health, for guidance in the cultivation of rumen anaerobes. We appreciate the advice on the synthesis of PNPC provided by Takahisa Kanda and Kazumasa Wakabayashi, Shinshu University.

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