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Isolation and Properties of β-Glucosidase from *Ruminococcus albus*

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An enzyme active against *p*-nitrophenyl-β-D-glucoside was purified from logarithmic-phase cells of *Ruminococcus albus* cultivated in a medium containing ball-milled cellulose. The purification yielded homogeneous enzyme after an approximately 520-fold increase in specific activity and a 9% yield. The enzyme was identified as a β-glucosidase because it can hydrolyze cellobiose and cellooligosaccharides to glucose from the nonreducing ends.

Recently, anaerobic bacteria with cellulolytic activity have become of great interest because of possible commercial applications such as production of ethanol or single cell proteins or both (2, 7, 12). A rumen anaerobe, *Ruminococcus albus*, has been studied to determine the cultivation conditions for growth on ball-milled cellulose (BMC) in test tubes (17) and in jar fermentors (18). Additional studies showed that the anaerobe could solubilize BMC (80 g liter^{-1}) within 3 days, producing bacterial cells, cellulases, and other compounds of economic value (13). The preparation of large amounts of cellulase-containing culture broth provided a ready source for studies on the putative enzymes.

In our previous report (14) we described a cellobiosidase purified from the culture supernatant of *R. albus*. In the present report we describe an enzyme having activity against *p*-nitrophenyl-β-D-glucoside (PNPG) which was purified to homogeneity and further characterized.

R. albus from bovine rumen was cultivated in a medium containing 1.5% BMC at pH 6.5 and 30°C for a given period. Details of the medium and the cultivation conditions are described in a previous paper (14). For the enzyme assay, PNPG (Nakarai Chemicals Co.) was mainly used as the substrate, because it was found to provide a very sensitive index of the enzyme activity in this experiment. PNPG solution (2 mM; 250 μl) and enzyme sample (700 μl) were added to the potassium phosphate buffer (1 M, pH 6.8; 50 μl), and incubated at 30°C for 1 h. *p*-Nitrophenyl-β-D-cellobioside (PNPC) was also used under the same conditions. In both cases, after the reaction was stopped by the addition of Na_2CO_3 (2 M; 250 μl), the absorbance of the released *p*-nitrophenol was measured at 405 nm. One unit of enzymatic activity was defined as the amount of enzyme catalyzing the release of 1 μmol of *p*-nitrophenol per min. The enzyme with activity against PNPG is denoted as PNPGase. For experiments where cellooligosaccharides were used as substrates, the amounts of glucose released were determined with glucose oxidase colorimetric reagent purchased from Fujisawa Medical Supply Co.

Cells of *R. albus* harvested after 48 h of cultivation in the cellulose medium were used to establish optimal conditions for enzyme extraction. The highest overall activity was obtained from frozen cells at −20°C by mechanical cell disruption with Al_2O_3, but the specific activity was less than those obtained by sequential rinsing of frozen cells with distilled water, 0.9% NaCl solution, potassium phosphate buffer, and mineral medium solution. Extraction of the enzyme from intact cells was negligible by these rinse methods. The solvent most effective for enzyme extraction from frozen cells and for the enzymatic reaction was 10 mM potassium phosphate buffer (pH 6.8). Therefore, the rinse method with the buffer (500 ml) was employed for preparing crude enzyme solution from frozen cells (17 g).

The time courses for growth, remaining BMC, and PNPGase activities both in the supernatant and in the cells were determined during cultivation of *R. albus* on BMC (Fig. 1). The residual amount of BMC was quantified with the anthrone-sulfuric acid reagent after hydrolysis with 60% sulfuric acid. Dry cell weights were calculated from the nitrogen content of the cells, which was measured with Nessler's reagent after the decomposition of the cells in a Kjeldahl flask with 98% sulfuric acid (19). Almost all of the BMC was solubilized within 30 h, and bacterial cells increased to maximum at about 30 h of cultivation. PNPG-hydrolyzing activity from the cells was the highest when cells were harvested in the logarithmic growth phase at about 20 h of cultivation, at which time about 50% of the initial cellulose content was consumed. PNPGase activity in the supernatant was slightly detected when the cell concentration began to decrease owing to autolysis.

When the organisms were cultivated on cellobiose, its consumption was almost completed within 20 h: the maximum enzyme activity in the cells appeared at 8 to 9 h of cultivation. During growth on glucose, the activity from the cells was maximum after around 15 h of cultivation. These data suggest that the presence of cellulose is not essential for the enzyme formation.

On the basis of these results, *R. albus* cells were harvested at the logarithmic growth phase for preparing the crude enzyme solution when BMC in the medium was reduced to half of the initial concentration. The mixture of cells and BMC was frozen at −20°C until used.

The crude PNPGase extracted from the defrosted cell mixture (100 g) in potassium phosphate buffer (10 mM, pH 6.8; 1 liter) containing 10 mM mercaptoethanol was completely adsorbed on an aminohexyl Sepharose 6B column (2.4 by 25 cm) previously equilibrated with the same buffer, but large amounts of other proteins were passed through the column. The enzyme protein was eluted with a KCl linear gradient (0 to 0.6 M) in the same buffer (1.2 liters). A single peak of the enzyme activity was eluted at about 0.25 M KCl between two large protein peaks. The purity was increased sixfold by the first step. Fractions (13 ml each) with higher activity were combined and equilibrated with 20% saturated $(NH_4)_2SO_4$ and chromatographed through an aminooctyl

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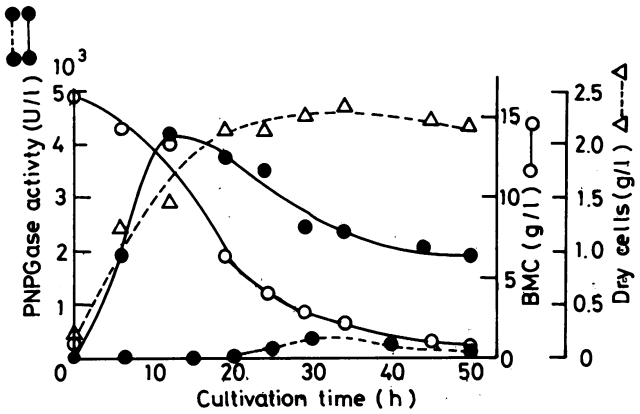

FIG. 1. Time courses of activities against PNPG of the enzyme from cells (—●—) and from culture supernatant (- - -●- - -) and the consumption of BMC (—○—) and dry cells (—△—) in a jar fermentor cultivation of *R. albus* on BMC as a carbon source.

Sepharose 6B column (1.6 by 14 cm) as the second step. Although almost all of the proteins in the sample loaded on the column were passed through the column, enzyme protein having PNPG-hydrolyzing activity was adsorbed on the hydrophobic column and eluted by a dual linear gradient of $(NH_4)_2SO_4$ (20% saturated to 0%) and ethylene glycol (0 to 40%) (15). Thereafter, the eluate was loaded on a DEAE–Bio-Gel (Bio-Rad Laboratories) column (1.6 by 20 cm) and eluted with phosphate buffer at 0.15 M KCl. Isoelectric focusing analysis, with pharmalyte covering the pH range 2.5 to 5.0 in a sucrose gradient at 4°C with constant voltage (1,000 V) for 90 h, revealed a sharp symmetrical peak with activity corresponding to that a small protein peak at around pI 4.4, indicating the absence of isoenzyme. The final protein yield was 0.12 mg, with 520-fold purification and recovery of about 9% (Table 1). Disk gel electrophoresis showed that the enzyme with activity against PNPG was a homogeneous protein having a relative mobility of 0.6.

The molecular weight of the purified enzyme was estimated to be 82,000 by gradient slab polyacrylamide gel electrophoresis and by gel filtration with Bio-Gel P-300. From the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, this purified enzyme seems to consist of one polypeptide chain because of the appearance of a single band on the gel. The estimate of the polypeptide molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 116,000, which might be overestimated due to the high content of carbohydrate in the enzyme as reported by Ng and Zeikus (11). Maximum activity of the enzyme was observed at pH 6.5 and at 30 to 35°C. The values were similar to the optimal conditions for cultivating *R. albus*. The enzyme incubated at pH 6.8 for 10 min maintained almost all of the initial activity at temperatures below 30°C, and about 20% of the initial activity was lost at 37°C. The enzyme was less stable at 37°C than β-glucosidases from other microorganisms such as *Clostridium thermocellum*, *Trichoderma reesei*, and *Thermoasscus auranticus* (21).

TABLE 1. Summary of purification of PNPGase from *R. albus*

Purificaton step	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Recovery of activity (%)	Purification (fold)
Crude extract	17	690	0.025	100	1
Aminohexyl Sepharose 6B	9.9	69	0.14	58	6
Aminooctyl Sepharose 6B	7.8	7.4	1.1	46	44
DEAE Bio-Gel A	5.9	2.0	3.0	35	120
Isoelectric forcusing	1.6	0.12	13	9	520

The effects of chemical reagents on the activity were tested. All of the reagents (1 mM) were added before the reaction was initiated by the action of enzyme at 37°C for 1 h. Ca^{2+}, Co^{2+}, and Mg^{2+} had little effect on enzymatic activity, whereas Zn^{2+}, Hg^{2+}, and Cu^{2+} depressed the activity remarkably. Reducing reagents such as mercaptoethanol, dithiothreitol, glutathione, and cysteine-hydrochloride activated the enzymatic activity slightly at a concentration of 1 mM. Sulfhydryl-reacting reagents (*p*-chloromercuribenzoate and iodoacetoamide) strongly inhibited the activity. The cells from *C. thermocellum* (10) and the cellobiosidase from *R. albus* (14) showed similar properties against Hg^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, and Ca^{2+}. These properties were completely different from the cellulase from *T. ressei*, since it was not inhibited by Hg^{2+}, Cu^{2+}, and Zn^{2+}, but was inhibited by Mg^{2+} and Ca^{2+}.

The K_m value of the enzyme against PNPG was estimated to be 2.2 mM from Lineweaver-Burk plots. When cellobiose was used as a substrate, the value was 26 mM. Both values were about 10 times greater than those of β-glucosidase from fungi (3, 21) such as *Trichoderma viride* cultivated on each substrate, but were comparable to the values of the enzyme from *C. thermocellum* (1).

The rates of enzymatic reactions against PNPG and PNPC were 1.3 and 1.4 mol min^{-1} ml^{-1}, respectively. The rates of the enzyme reactions against salicine and against other cellooligomers (G_2, G_3, G_4, and G_5) were 0.2 and 0.04 mol min^{-1} ml^{-1}, respectively. Higher-molecular-weight cellulose or sugars having no β-1,4-glucoside linkage such as maltose, sucrose, and lactose were not degraded. On thin-layer chromatography, any hydrolytic products of CMC and BMC were not detected. The carbohydrate content in the present enzyme, determined by the phenol sulfuric acid method (4), was 12%.

The hydrolyzed products of cellooligosaccharides and their derivatives were identified by thin-layer chromatography (Fig. 2). A mixture of enzyme (50 μg/ml; 0.5 ml) and each cellooligosaccharide (1%; 0.5 ml) were incubated at 30°C for a given period. After the reaction mixture was boiled for 10 min, it was spotted on silica gel-precoated thin-layer plates (5 by 20 cm; Yamato replate-50). Samples were developed for 3.5 h at room temperature with an *n*-buthanol–water–pyridine (6:3:4, vol/vol) solution. The position of *p*-nitrophenol with yellow color at alkaline pH was indicated by drawing a circle. The other compounds were detected as dark spots by heating the plates after they were sprayed with 15% sulfuric acid solution. Glucose was the product released from all susceptible substrates. Thin-layer chromatograms of cellooligomers revealed that every glucose unit was released one by one from the oligomers to smaller oligomers (Fig. 2, lanes A, B, C, and D). This was supported by the chromatograms of hydrolyzates of PNPC (Fig. 2, lanes F, G, and H). Thus, PNPC was hydrolyzed to glucose and PNPG (Fig. 2, lane G; a spot on a circle), and the resulting PNPG was then hydrolyzed to glucose and *p*-nitrophenol (a circle) by further enzymatic reaction (Fig. 2, lane H), but cellobiose was not detected. These results lead to the conclusion that the enzyme cleaves β-glucoside linkages one by one. Therefore, the purified enzyme is

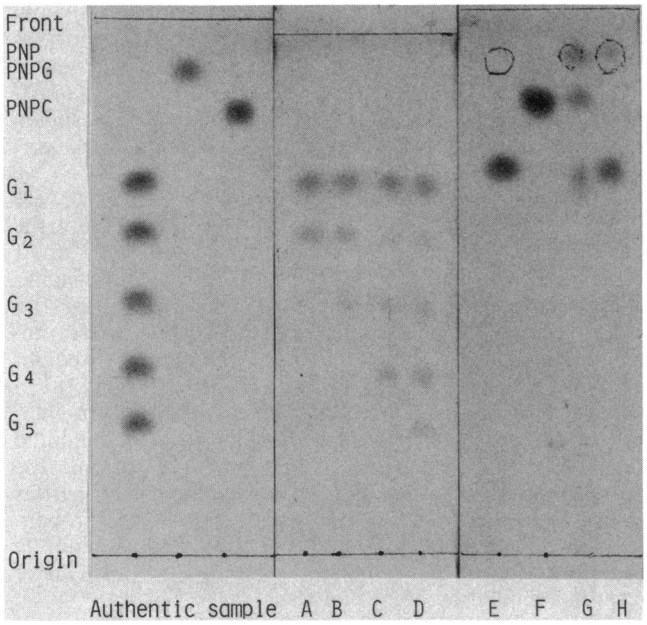

FIG. 2. Thin-layer chromatograms of the water-soluble products released from cellooligosaccharides PNPG and PNPC. Abbreviations: G_1, glucose; G_2, cellobiose; G_3, cellotriose; G_4, cellotetraose; G_5, cellopentaose; PNP, *p*-nitrophenol. Enzyme hydrolysates of cellooligosaccharides (reaction hour): A, G_2 (15 h); B, G_3 (15 h); C, G_4 (15 h); D, G_5 (15 h); E, PNPG (2 h); F, PNPC (0.5 h); G, PNPC (1 h); H, PNPC (2 h). The circles indicate the positions of *p*-nitrophenol recognized before spraying the H_2SO_4 solution.

referred as a β-glucosidase. The enzyme can not hydrolyze lactose to galactose and glucose, suggesting that the enzyme does not recognize glucose at the reducing end of substrate. Therefore, the enzyme can be characterized by hydrolysis of cellooligomers at the non-reducing end only.

The β-glucosidase of *R. albus* seems to be cell associated. Therefore, the enzyme was readily released from cells by a simple freeze-thawing procedure. β-Glucosidases from rumen microorganisms such as *Bacteroides succinogenes* (5) and *Ruminococcus flavefaciens* (16), and *C. thermocellum* (1) were also found to be cell associated. The enzyme from *Acetivibrio cellulolyticus* is similar to that from *R. albus* in that the enzyme is readily released by simple freeze-thawing (8). β-Glucosidases from *Talaromyces emersonii* (9), *T. viride* (3), and *Trichoderma koningii* (20) were released from the cells into culture supernatant. Thus, bacterial enzyme seems to be retained in or on the cells, whereas the fungal enzyme is released from the cells. Localization of β-glucosidase in the cells seems to be advantageous for releasing from glucose inhibition, since glucose does not accumulate outside of the cells and can be converted effectively to the other compounds by Embden-Meyerhof-Parnas pathway (6) in the cells. This β-glucosidase may be a key enzyme in generating glucose as an energy source for *R. albus* growth.

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LITERATURE CITED

1. **Ait, N., N. Creuzet, and J. Cattaneo.** 1982. Properties of β-glucosidase purified from *Clostridium thermocellum*. J. Gen. Microbiol. **128**:569–577.
2. **Akin, D. E., and H. E. Amos.** 1975. Rumen bacterial degradation of forage cell walls investigated by electron microscopy. Appl. Microbiol. **29**:692–701.
3. **Berghem, L. E. R., and L. G. Petterson.** 1974. The mechanism of enzymatic cellulose degradation. Isolation and some properties of a β-glucosidase from *Trichoderma viride*. Eur. J. Biochem. **46**:295–305.
4. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for the determination of sugar and related substances. Anal. Chem. **28**:350–356.
5. **Groleau, D., and C. W. Forsberg.** 1981. Cellulolytic activity of rumen bacterium *Bacteroides succinogenes*. Can. J. Microbiol. **27**:517–530.
6. **Joyer, A. E., Jr., and R. L. Baldwin.** 1966. Enzymatic studies of pure cultures of rumen microorganisms. J. Bacteriol. **92**:1321–1330.
7. **Leatherwood, J. M.** 1973. Cellulose degradation by *Ruminococcus*. Fed. Proc. **32**:1814–1818.
8. **MacKenzie, C. R., and D. Bilous.** 1982. Location and kinetic properties of the cellulase system of *Acetivibrio cellulolyticus*. Can. J. Microbiol. **28**:1158–1164.
9. **McHale, A., and M. Coughlan.** 1982. Properties of the β-glucosidase of *Talaromyces emersonii*. J. Gen. Microbiol. **128**:2327–2331.
10. **Ng, T. K., and J. G. Zeikus.** 1981. Comparison of extracellular cellulase activities of *Clostridium thermocellum* LQRI and *Trichoderma reesei* QM 9414. Appl. Environ. Microbiol. **42**:231–240.
11. **Ng, T. K., and J. G. Zeikus.** 1981. Purification and characterization of an endoglucanase (1,4-β-D-glucan glucanohydrolase) from *Clostridium thermocellum*. Biochem. J. **199**:341–350.
12. **Ohmiya, K.** 1977. Conversion of cellulose to single cell protein from rumen bacteria. New Food Indust. **19**:41–51.
13. **Ohmiya K., K. Nokura, and S. Shimizu.** 1983. Enhancement of cellulose degradation by *Ruminococcus albus* at high cellulose concentration. J. Ferment. Technol. **61**:25–30.
14. **Ohmiya, K., M. Shimizu, M. Taya, and S. Shimizu.** 1982. Purification and properties of cellobiosidase from *Ruminococcus albus*. J. Bacteriol. **150**:407–409.
15. **Pahlman, S., J. Rosengren, and S. Hjerten.** 1977. Hydrophobic interaction chromatography on uncharged Sepharose derivatives. Effects of neutral salts on the adsorption of proteins. J. Chromatogr. **131**:99–108.
16. **Pettipher, G. L., and M. J. Latham.** 1979. Production of enzymes degrading plant cell walls and fermentation of cellobiose by *Ruminococcus flavefaciens* in batch and continuous culture. J. Gen. Microbiol. **110**:29–38.
17. **Taya, M., Y. Ito, K. Ohmiya, T. Kobayashi, and S. Shimizu.** 1978. Cellulose digestion with anaerobic microorganisms. Nippon Nogei Kagaku Kaishi **52**:567–574.
18. **Taya, M., Y. Ito, K. Ohmiya, T. Kobayashi, and S. Shimizu.** 1979. Anaerobic digestion of cellulose and utilization of digestion products in a consecutive cultivation system. J. Ferment. Technol. **57**:178–185.
19. **Umbreit, W. W., R. H. Burris, and J. F. Stauffer.** 1957. Method for nitrogen, p. 238. *In* Manometric techniques—a manual describing methods applicable to the study of tissue metabolism. Burgress Publishing Co., Minneapolis.
20. **Wood, T. M., and S. I. McCrae.** 1982. Purification and some properties of the extracellular β-D-glucosidase of the cellulolytic fungus *Trichoderma koningii*. J. Gen. Microbiol. **128**:2973–2982.
21. **Woodward, J., and A. Wiseman.** 1982. Fungal and other β-D-glucosidases—their properties and applications. Enzyme Microb. Technol. **4**:73–79.