APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1986, p. 902–904
0099-2240/86/100902-03$02.00/0

Vol. 52, No. 4

Synthesis of [^{14}C]Cellobiose with *Clostridium thermocellum* Cellobiose Phosphorylase

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Received 4 November 1985/Accepted 23 June 1986

Cellobiose labeled either at the reducing end (4-*O*-β-D-glucopyranosyl-D-[U-^{14}C]glucopyranose) or at the nonreducing end (4-*O*-β-D-[U-^{14}C]glucopyranosyl-D-glucopyranose) was synthesized with *Clostridium thermocellum* cellobiose phosphorylase at greater than 98% purity. The radioactive cellobioses were identical to authentic cellobiose in melting point, optical rotation, isotopic dilution, and chromatographic properties.

Cellobiose is an important hydrolytic end product of cellulose degradation and is formed in large amounts by cellobiohydrolyase and in smaller amounts from endoglucanase activity (5). The metabolism of cellobiose by cellulolytic and noncellulolytic microorganisms often involves cellobiase, which cleaves the substrate into two molecules of glucose. However, several microorganisms use cellobiose phosphorylase, which catalyzes the phosphorolytic cleavage of cellobiose to glucose-1-phosphate plus glucose (3, 7, 16). Previous studies with *Cellvibrio gilvus* ATCC 13127 indicated that the two glucose moieties formed by cellobiose phosphorylase are subsequently metabolized via different routes. The nonreducing glucose moiety is converted mainly to carbon dioxide, whereas the reducing glucose moiety is incorporated into cell material (6, 7, 19). Similar observations were reported for *Ruminococcus flavefaciens* (3).

Clostridium thermocellum contains cellobiose phosphorylase (16). During growth, some strains preferentially consume cellobiose over glucose (9, 10, 13), even though glucose is also part of the immediate products of cellobiose metabolism (12). To study the mechanism of cellobiose and glucose in *C. thermocellum* strains, it was necessary to prepare radioactive cellobiose ^{14}C labeled in the different glucose moieties of the molecule. [^{14}C]cellobiose labeled only at the C-1 position of the reducing end of the molecule was prepared by a tedious chemical reaction (4). Previous studies on enzymatic synthesis of cellobiose by using crude cellobiose phosphorylase were not detailed in regard to product yield or specific properties. The present report describes a procedure for the enzymatic synthesis of 4-*O*-β-D-[U-^{14}C]glucopyranosyl-D-glucopyranose and 4-*O*-β-D-glucopyranose-D-[U-^{14}C]glucopyranose at high yields by using purified *C. thermocellum* cellobiose phosphorylase.

MATERIALS AND METHODS

Chemicals. All chemicals used were reagent grade unless stated otherwise. Others were obtained as follows: Charcoal Darco G60 from MCB Manufacturing Chemists; Celite 545 from Fisher Scientific Co.; cellobiose from Merck & Co., Inc.; and α-D-[U-^{14}]glucose (specific activity, 323 mCi/mmol) and α-D-[U-^{14}C]glucose-1-phosphate, dipotassium salt (specific activity, 294 mCi/mmol), from New England Nuclear Corp.

Enzyme preparation. Crude cell extracts of *C. thermocellum* LQRI grown on GS medium with cellobiose as the energy source were prepared as described previously (13). Cellobiose phosphorylase was then purified from the cell extract by the procedure of Alexander (1). Purified cellobiose phosphorylase was stored in 100 mM Tris hydrochloride buffer (pH 7.0) with 3 M ammonium sulfate at 4°C without loss of activity for over 3 months.

[^{14}C]cellobiose preparation. 4-*O*-β-D-[U-^{14}C]glucopyranosyl-D-glucopyranose was prepared by adding 100 μCi of α-D-[U-^{14}C]glucose-1-phosphate, 10.0 μmol of glucose-1-phosphate, dipotassium salt, 200 μmol of α-D-glucose, and 2.5 U of cellobiose phosphorylase into 1 ml of reaction mixture (100 mM Tris hydrochloride [pH 7.0], 10 mM MgCl, 2 mM dithiothreitol). The reaction mixture was incubated at 60°C for 30 min, and the reaction was terminated by immersion in boiling water for 3 min. 4-*O*-β-D-Glucopyranosyl-D-[U-^{14}C]glucose was prepared similarly, except that 200 μCi of α-D-[U-^{14}C]glucose, 10.0 μmol of glucose, and 200 μmol of α-D-glucose-1-phosphate, dipotassium salt, were used in the reaction. Nonlabeled cellobiose was also prepared by using a 20-ml reaction mixture that contained 400 μmol each of glucose-1-phosphate and α-D-glucose and 5 U of enzyme; the preparation was incubated for 4 h. Upon termination of synthesis, the reaction mixtures were centrifuged at 15,000 × *g* for 10 min and the supernatant was collected.

Cellobiose was separated from the supernatant by a modification of the Celite-charcoal chromatography procedure described by Miller et al. (10). Samples (1 ml) were applied to a Celite-charcoal column (bed volume, 2.6 by 4 cm) in distilled water. Glucose and contaminating materials were removed by elution with 80 ml of distilled water. Cellobiose was then eluted with 8% ethanol (vol/vol). Fractions with high radioactivity were pooled and concentrated by evaporation under vacuum at 40°C. The concentrated fractions were rechromatographed on a new Celite-charcoal column as described above. The fractions were again concentrated and kept at −20°C until used.

Analytical procedures. Thin-layer chromatography of cellodextrins was performed on Silica Gel 60 plates (20 by 20 cm; EM Science) with a 0.25-mm layer gel thickness. Chromatograms were developed at 25°C in isopropanol-water-ethyl acetate (0.65:0.35:1.00 [vol/vol/vol]). Developed

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TABLE 1. Physical properties of [^{14}C]cellobiose synthesized with *C. thermocellum* cellobiose phosphorylase

Property	4-*O*-β-D-[U-^{14}C] glucopyranosyl-D-glucopyranose	4-*O*-β-D-Glucopyranose-D-[U-^{14}C] glucopyranose	Authentic cellobiose
Melting point (°C)[a]	222–224	222–224	224–225
Optical rotation $[\alpha]_D^{25}$[a]	+36.5	+37.0	+36.0
R_f (cm)	0.21	0.22	0.21
Sp act (dpm/μmol)[b]	6,846	6,846	

[a] Analysis was performed on unlabeled synthesized cellobiose.
[b] The value did not change after five successive crystallizations.

plates were air dried ovenight. Autoradiography was performed by placing the plates in a Du Pont Cornex X-ray holder and exposing them to Kodak SB-5 X-ray film. The film was processed by Kodak developer and fixer. Sugars were visualized by spraying the plates with aniline phthalate solution (14). Chromatograms and autoradiograms were scanned with a Zenith Laser Scanning Densitometer (LKB Instruments). The melting point of cellobiose was determined on a Fisher-Johns melting point apparatus. A 1% cellobiose solution in water was placed in a 20-cm microtube, and optical rotation was measured with a PolyScience Polarimeter model SR-6.

The purity of the cellobiose was analyzed by isotopic dilution. [^{14}C]cellobiose (2×10^6 dpm) was added to 1 ml of 0.292 M authentic cellobiose solution in a microsolution in a microtube. Cellobiose was then precipitated by the addition of 99% ethanol (1 ml) at −4°C. The precipitate was collected by centrifugation, washed twice with 1 ml of absolute ethanol, and redissolved in 0.9 ml of water, and the specific activity of the cellobiose was determined. The amount of cellobiose was determined by first hydrolyzing it to glucose with almond β-glucosidase (Sigma Chemical Co.), and then measuring the glucose with a Glucostat (Worthington Diagnostics). Radioactivity was quantified by placing samples in vials that contained Instagel scintillation fluid (Packard Instrument Co.) and counting with either a Packard PLD Tri-Carb or a Beckman LS9000 liquid scintillation spectrometer.

Cellobiose was reduced to cellobiotol by sodium borohydride by the procedure described by Storvick et al. (18) and then hydrolyzed to glucose and sorbitol with 3 N HCl under reflux at 100°C for 2 h. Glucose or sorbitol was separated and quantified by high-performance liquid chromatography. The separation system consisted of a Perkin-Elmer series B liquid chromatograph, a Whatman Partisil 10 PAC PXS 10/25 column, a Laboratory Data Control differential refractometer, and a Perkin-Elmer Sigma 10 BASIC data acquisition system. Acetonitrile–0.25 M sodium acetate (65:35; pH adjusted to 5.0 with acetic acid) was used as the mobile phase at a flow rate of 0.5 ml/min and at a pressure of 0.4 to 0.5 MPa.

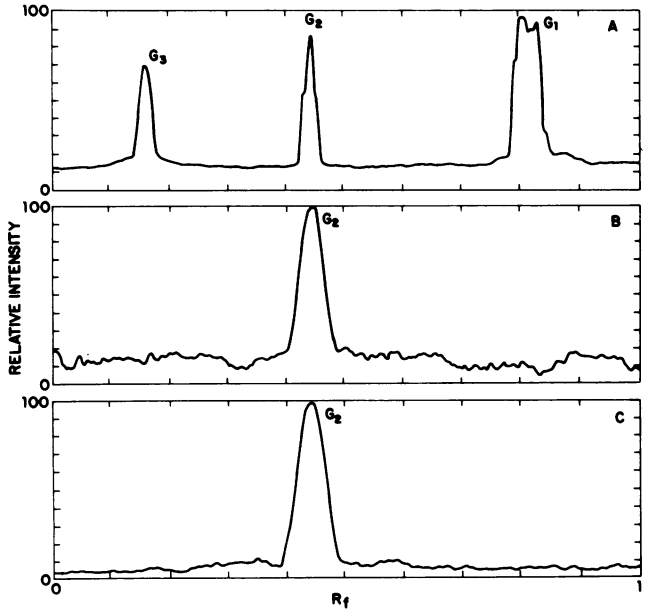

FIG. 1. Autoradiogram showing the position of [^{14}C]cellobiose synthesized with *C. thermocellum* cellobiose phosphorylase. (A) Standards: glucose (G_1); cellobiose (G_2); cellotriose (G_3). (B) 4-*O*-β-D-glucopyranosyl-D-[U-^{14}C]glucopyranose. (C) 4-*O*-β-D-[U-^{14}C]glucopyranosyl-D-glucopyranose. [^{14}C]cellobiose was purified from reaction mixtures and subjected to thin-layer chromatography on silica gel plates.

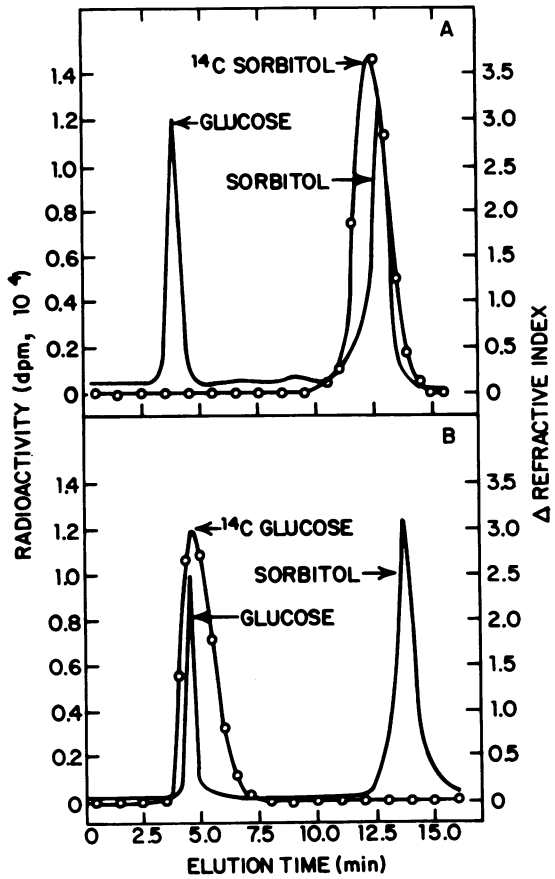

FIG. 2. Separation of [^{14}C]cellobiotol acid hydrolysis products by high-performance liquid chromatography. —○—○—○—, ^{14}C radioactivity. (A) Degradation products of 4-*O*-β-D-glucopyranosyl-D-[U-^{14}C]glucopyranose. (B) Degradation products of 4-*O*-β-D-[U-^{14}C]glucopyranosyl-D-glucopyranose.

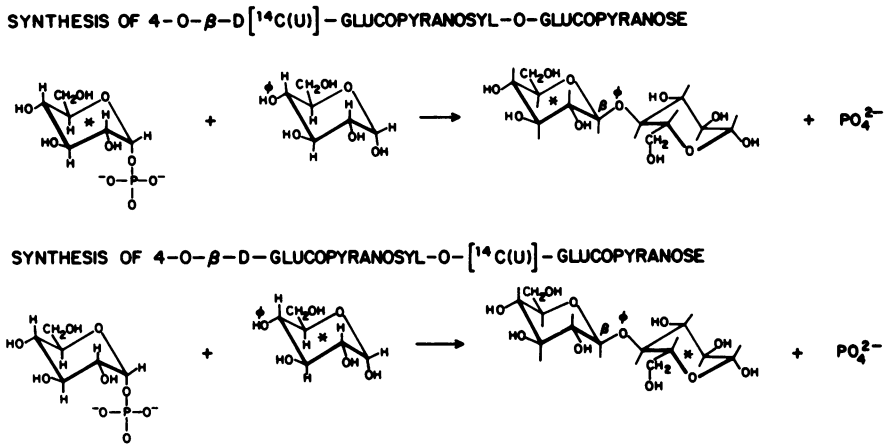

FIG. 3. General scheme for synthesis of differentially labeled [^{14}C]cellobiose with *C. thermocellum* cellobiose phosphorylase. *, ^{14}C isotope; ø, oxygen origin.

RESULTS AND DISCUSSION

Enzymatic synthesis of radioactive cellobiose yielded approximately 8 μmol (80% yield) of 4-*O*-β-D-glucopyranosyl-D-[U-^{14}C]glucopyranose with a specific activity of 18.9 mCi/mmol and approximately 7.3 μmol (73% yield) of 4-*O*-β-D-[U-^{14}C]glucopyranosyl-D-glucopyranose with a specific activity of 9.4 mCi/mmol. The prepared cellobiose was physically and chemically indistinguishable from authentic cellobiose (Table 1). The melting point (222 to 224°C) and optical rotation (+36.5; +37.0) were similar to the values 224 to 225°C and +36.0 reported previously (8). The radioactive materials coprecipitated with authentic cellobiose in five consecutive crystallizations, as indicated by a constant specific activity. Synthetic cellobiose samples comigrated with authentic cellobiose on Silica Gel 60 plates with isopropanol-water-ethyl acetate as the solvent. The preparations were >98% pure and did not contain glucose or cellodextrins, as indicated by autoradiography and measurement of the label recovered as cellobiose (Fig. 1).

The distribution of radioactivity between the two glucose moieties of cellobiose was analyzed after reduction to cellobiotol (reducing-end glucose reduced to sorbitol) followed by acid cleavage (Fig. 2). These results indicated that [^{14}C]sorbitol was detected in cellobiose synthesized from [^{14}C]glucose, whereas only [^{14}C]glucose was detected when [^{14}C]glucose-1-phosphate was formed.

In summary, high-purity radioactive cellobiose, differentially labeled in either the reducing or the nonreducing glucose moiety, was efficiently synthesized by the procedures described above and illustrated in Fig. 3. The use of purified cellobiose phosphorylase eliminated the possibility of cellotriose contaminating the preparation by the presence of cellodextrin phosphorylase (15), which can be associated with other described procedures (3, 17, 19). Solving this problem simplified the purification procedure from gradient (10) to stepwise elution. Besides being useful for studies of cellobiose metabolism, radioactive cellobiose could also be used as the starting compound for the enzymatic synthesis of cellodextrins, which are valuable in the investigation of cellulase action (5) by purified cellodextrin phosphorylase (2). The ease of synthesis, high purity, and yield of synthesized [^{14}C]cellobiose suggests that this method may have commercial viability.

ACKNOWLEDGMENTS

This research was supported by grant no. PRF 80-10084 from the National Science Foundation awarded to J. G. Zeikus.

We thank L. Savage for aid in preparation and typing of the manuscript.

LITERATURE CITED

1. **Alexander, J. K.** 1972. Cellobiose phosphorylase from *Clostridium thermocellum*. Methods Enzymol. **28**:944–948.
2. **Alexander, J. K.** 1972. Cellodextrin phosphorylase from *Clostridium thermocellum*. Methods Enzymol. **28**:948–953.
3. **Ayers, W. A.** 1959. Phosphorolysis and synthesis of cellobiose by cell extracts from *Ruminococcus flavefaciens*. J. Biol. Chem. **234**:2819–2822.
4. **Feather, M. S., and J. F. Harris.** 1967. Chemical synthesis of [^{14}C]cellobiose, p. 1. U.S. Forest Service research note FPL-0161. U.S. Forest Service, Washington, D.C.
5. **Haliwell, G.** 1979. Microbial β-glucanases. Prog. Ind. Microbiol. **15**:1–60.
6. **Hulcher, F. H., and K. W. King.** 1958. Disaccharide preference of an aerobic cellulolytic bacterium, *Cellvibrio gilvus* n. sp. J. Bacteriol. **76**:565–570.
7. **Hulcher, F. H., and K. W. King.** 1958. Metabolic basis for disaccharide preference in a *Cellvibrio*. J. Bacteriol. **76**:571–577.
8. **Inhat, M., and D. A. I. Goring.** 1967. Shape of the cellodextrins in aqueous solution at 25°C. Can. J. Chem. **45**:2353–2358.
9. **McBee, R. H.** 1948. The culture and physiology of a thermophilic cellulose-fermenting bacterium. J. Bacteriol. **56**:653–663.
10. **Miller, G. L., J. Dean, and R. Blum.** 1960. A study of methods for preparing oligosaccharides from cellulose. Arch. Biochem. Biophys. **91**:21–26.
11. **Ng, T. K., P. J. Weimer, and J. G. Zeikus.** 1977. Cellulolytic and physiological properties of *Clostridium thermocellum*. Arch. Microbiol. **114**:1–7.
12. **Ng, T. K., and J. G. Zeikus.** 1981. Comparison of extracellular cellulase activities of *Clostridium thermocellum* LQRI and *Trichoderma reesei* QM9414. Appl. Environ. Microbiol. **42**:231–240.
13. **Ng, T. K., and J. G. Zeikus.** 1982. Differential metabolism of cellobiose and glucose by *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. J. Bacteriol. **150**:1391–1399.
14. **Scott, R. W.** 1970. Quantitative recovery of sugars from silica gel thin layers. J. Chromatogr. **49**:473–481.
15. **Sheth, K., and J. K. Alexander.** 1967. Cellodextrin phosphorylase from *Clostridium thermocellum*. Biochim. Biophys. Acta **148**:808–810.
16. **Sih, C. J., and R. H. McBee.** 1966. A cellobiose phosphorylase in *Clostridium thermocellum*. Proc. Mont. Acad. Sci. **15**:21–22.
17. **Sih, C. J., N. M. Nelson, and R. H. McBee.** 1957. Biological synthesis of cellobiose. Science **126**:1116–1117.
18. **Storvick, W. O., F. E. Cole, and K. W. King.** 1963. Mode of action of a cellulase component from *Cellvibrio gilvus*. Biochemistry **2**:1106–1109.
19. **Swisher, E. J., W. O. Storvick, and K. W. King.** 1964. Metabolic nonequivalence of the two glucose moieties of cellobiose in *Cellvibro gilvus*. J. Bacteriol. **88**:817–820.