SYNTHESIS OF 4-O-β-D-GALACTOPYRANOSYL-N-ACETYL-D-GLUCOSAMINE BY INTACT CELLS OF LACTOBACILLUS BIFIDUS VAR. PENNSYLVANICUS*

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Recently we have described the enzymatic synthesis of two isomeric β-D-galactosides of N-acetyl-D-glucosamine from lactose and N-acetyl-D-glucosamine by means of a crude enzyme from Lactobacillus bifidus var. pennsylvanicus (1). One of the crystallized disaccharides (DII) markedly stimulated growth of this microorganism, whereas the other (DI) was nearly inactive. We have established the structure of DII as 4-O-β-D-galactopyranosyl-N-acetyl-D-glucosamine (2). DI represents the 6-O-β-D isomer.1 When the synthesis was performed with the crude enzyme, the ratio DI: DII was estimated as approximately 1:1. The yield of microbiologically active DII was less than 0.5 per cent of the theoretical. Optimal conditions for the formation of DII included 5 hours of incubation at 37° in a phosphate buffer of pH 5.4. When incubation was extended beyond 5 hours, DII began to disappear from the system.

It seemed desirable to find conditions for a more selective synthesis of the microbiologically active disaccharide. A remarkable increase in yield, a nearly selective synthesis, and an accumulation of DII were achieved when the synthesis was performed with intact cells of L. bifidus var. pennsylvanicus.

EXPERIMENTAL

Preparation of Cells—L. bifidus var. pennsylvanicus was grown as previously described (3). Cells from 40 hour cultures were collected by centrifugation and were washed twice with 0.85 per cent saline and once with m/15 phosphate buffer of pH 5.4.

Conditions for Synthesis—The amounts of lactose and N-acetyl-D-glucosamine, as well as the pH determined as optimal for our previous work with the crude enzyme from L. bifidus var. pennsylvanicus, were adopted for this synthesis with intact cells.

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1 Unpublished data.
Cells prepared as described above were suspended in m/15 phosphate buffer, so that 3 ml. of the cell suspension represented cells from 100 ml. of the culture. 3 ml. aliquots of this cell suspension were added to 10 ml. portions of m/15 phosphate buffer containing 382 mg. of N-acetyl-D-glucosamine and 625 mg. of lactose (monohydrate). These cell-substrate suspensions were incubated at 37° in cotton-plugged 50 ml. Erlenmeyer flasks under anaerobic and aerobic conditions. Anaerobic incubation was accomplished in Brewer type jars which were first evacuated and then thrice filled with N₂ and evacuated before atmospheric pressure was finally restored by the addition of 10 per cent CO₂ and 90 per cent N₂. For aerobic incubation, the flasks were allowed to stand in the atmosphere without agitation.

Samples were taken at intervals from 0 to 48 hours of incubation. Each sample was heated for 10 minutes in a boiling water bath, cooled, and centrifuged. The supernatant fluid was analyzed for reducing sugars and for N-acetylamino sugars by paper chromatographic methods, as described previously (1).

The greatest yield of D₁ was obtained under anaerobic conditions, while D₂ was formed in trace amount barely detectable by the methods employed. Under aerobic conditions, the yield of D₁ was less than that obtained anaerobically, and D₁ appeared in larger amount but still in very small yield when compared to the amount of D₁₁ formed. The conversion of lactose into glucose and galactose was much more pronounced under aerobic conditions.

Assay with L. bifidus var. pennsylvanicus (3) confirmed the chromatographic analysis for the microbiologically active D₁₁. Formation of this active disaccharide is indicated by the increase in growth-promoting activity for the lactobacillus as expressed in growth units per ml. of the supernatant fluid assayed. The initial activity was 6 units per ml. and is attributed to the N-acetyl-D-glucosamine present. Within 24 hours of anaerobic incubation, the activity increased to 30 units per ml. and remained at this value throughout 48 hours of incubation. Under aerobic conditions the activity was only 20 units per ml. in 24 hours and 16 units per ml. after 48 hours of continuous incubation.

These data indicate anaerobic conditions as optimal for the synthesis of microbiologically active D₁₁ by intact cells of L. bifidus var. pennsylvanicus. The same amount of growth-promoting activity was produced by 30 per cent of the cell concentration described above, and synthesis occurred even with 1 per cent of the cell concentration originally used.

Isolation of 4-O-β-D-Galactopyranosyl-N-acetyl-D-glucosamine—L. bifidus var. pennsylvanicus cells obtained from 8 liters of medium and prepared as described above were incubated for 24 hours with 16.5 gm. of lactose (monohydrate) and 10.1 gm. of N-acetyl-D-glucosamine in 345 ml. of m/15 phos-
Phosphate buffer at pH 5.4. The digest was heated for 1 hour in an oven at 100° and centrifuged. The supernatant solution was concentrated in vacuo to about 50 ml. of volume. The concentrate was adsorbed on a column composed of 320 gm. of Norit A (Pfanstiehl Chemical Company) and 160 gm. of Celite No. 535 (Johns-Manville). The column was eluted with water and aqueous ethanol, as described previously (1). The 7.5 per cent ethanolic eluates were evaporated to dryness in vacuo. The amorphous residue was dried over P₂O₅ in a desiccator and then dissolved in 80 ml. of hot dry methanol. The hot methanolic solution was filtered and kept at room temperature. Crystallization of nearly square platelets started immediately and was completed after a few hours standing at +5°. After recrystallization from a minimal volume of dry methanol, 1.02 gm. of disaccharide, or a 5.4 per cent yield, were obtained with a melting point of 172° (Berl; uncorrected) and a specific rotation of [α]₂⁰ +27.8° (H₂O; c = 1, equilibrium rotation reached after 180 minutes); extrapolated to zero time [α]₂⁰ +51.2°. The disaccharide crystallizes with 1 mole of methanol and analyzes for

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C₁₄H₂₂O₁₄N + CH₃OH. \text{ Calculated. C 43.37, H 7.03, N 3.37, OCH₃ 7.46} \\
(415.4) \text{ Found. C 43.25, H 6.88, N 3.30, OCH₃ 7.62}
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No depression was found in a mixed melting point of the disaccharide with pure 4-O-β-D-galactopyranosyl-N-acetyl-D-glucosamine obtained from hog stomach mucin (2, 4). The physical constants determined as well as the microbiological activity were identical for both compounds.

**DISCUSSION**

The 4-O-β-D-galactopyranosyl-N-acetyl-D-glucosamine is only one of three crystalline isomeric β-galactosides of N-acetyl-D-glucosamine synthesized in our laboratories from lactose and N-acetyl-D-glucosamine by means of crude enzyme preparations with high lactase activity. In addition to *L. bifidus* var. *pennsylvanicus*, yeast² and bull testes³ have been used as sources of crude lactase. It was surprising to find in the extracts of bull testes a highly active β-galactosidase in addition to hyaluronidase. In preparations of approximately identical lactase activity, the crude enzyme from yeast produced chiefly 6-O-β-D-galactopyranosyl-N-acetyl-D-glucosamine,¹ while the extract from bull testes led to 3-O-β-D-galactopyranosyl-N-acetyl-D-glucosamine with traces of the 4-O-β-D-galactoside.¹ With intact cells of *L. bifidus* var. *pennsylvanicus*, the 4-O-β-D-galactoside was formed almost exclusively. In experiments carried out previously, utilizing a cell-free extract of *L. bifidus* var. *pennsylvanicus*, the 4-O-β-D-galactoside and the 6-O-β-D isomer were formed in approximately equal amount, and

² Rohm and Haas.

³ Crude hyaluronidase preparations, Wyeth Laboratories.
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these galactosides accumulated in the digest only for a few hours and then gradually disappeared. In contrast, with the intact cells the formation of the 4-O-β-D-galactoside was continuous through 24 hours and there was no evidence of loss throughout 48 hours of incubation. Of these three β-D-galactosides of N-acetyl-D-glucosamine, only the 4-O-β-D isomer exhibited high growth-promoting activity for L. bifidus var. pennsylvanianus.

The hydrolysis of lactose by means of various lactase preparations has been studied intensively by Wallenfels during the past few years (5). He has shown that the emergence of glucose and galactose as enzymatic breakdown products of lactose is accompanied by the synthesis of new oligosaccharides such as lactobiose, galactobiose, and lactotriose. These were formed through the transfer of galactose to lactose or to its split-products and were, in general, related to the over all β-galactosidase effect residing in the enzyme preparations. Inasmuch as Wallenfels was unable, by fractionation of the crude lactase preparations, to separate hydrolytic and synthesizing enzymes, he questioned the existence of a specific β-transgalactosidase.

In our own studies crude lactase preparations from different sources have shown remarkable and reproducible differences in their ability to transfer the galactose moiety of lactose to N-acetyl-D-glucosamine. This fact, with the further observation that these preparations favor the synthesis of different isomers of β-D-galactopyranosyl-N-acetyl-D-glucosamine, suggests the presence of specific β-transgalactosidases. A definite answer to this question may be obtained only with highly purified enzymes.

SUMMARY

A nearly selective synthesis of microbiologically active 4-O-β-D-galactopyranosyl-N-acetyl-D-glucosamine (D11) has been achieved by incubation of intact cells of Lactobacillus bifidus var. pennsylvaniaicus with lactose and N-acetyl-D-glucosamine. The disaccharide has been isolated in crystalline form in a yield 5.4 per cent of the theoretical. In contrast to results obtained with a crude enzyme preparation from the same organism, only negligible amounts of the isomeric 6-O-β-D-galactoside were formed, and there was no loss of active disaccharide on prolonged incubation.

BIBLIOGRAPHY