ENZYMATIC SYNTHESIS OF A GROWTH FACTOR FOR LACTOBACILLUS BIFIDUS VAR. PENN*

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It has been shown (1, 2) that in human milk there are N-acetyl-D-glucosamine-containing oligosaccharides which promote the growth of Lactobacillus bifidus var. Penn. In the search for substances with lower molecular weight which would still show a growth-promoting effect in the microbiological test, activity was found in mildly hydrolyzed chitin of crab and lobster shells,1 in β-methyl N-acetyl-D-glucosaminide, but not in the pure corresponding α-methyl glycoside (3) and in a crystallized disaccharide composed of N-acetyl-D-glucosamine and galactose, isolated from acid-hydrolyzed gastric mucin (4). A crude, cell-free enzyme preparation, prepared from cells of L. bifidus var. Penn by grinding with alumina, extracting with 0.01 M phosphate buffer at pH 6.3, and lyophilizing the supernatant fluid, hydrolyzes these growth factors with liberation of N-acetyl-D-glucosamine which is only slightly active microbiologically (5). This same enzyme preparation, permitted to act upon a mixture of N-acetyl-D-glucosamine and lactose, may increase the apparent activity of N-acetyl-D-glucosamine up to 300 per cent. A very active N-acetyl-D-glucosamine-containing disaccharide has been found in such digests. The isolation and characterization of this compound and of an isomeric disaccharide are described in the present communication.

EXPERIMENTAL

Conditions for Optimal Synthesis

Various amounts of lactose, N-acetyl-D-glucosamine, and enzyme were incubated under toluene in phosphate and acetate buffers ranging from pH 4 to 8 and at temperatures from 20-45°. Samples were taken from the digests at intervals up to 96 hours. The enzyme action was stopped by heating for 30 minutes at 100°. The resulting precipitate was removed by centrifugation and the supernatant fluid was checked for growth-promoting

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1 Unpublished results.
activity for *L. bifidus* var. *Penn* in the microbiological assay developed by György and collaborators (6).

**Fig. 1** shows that the activity of such enzymatic digests in the microbiological test is dependent upon pH and incubation time. As can be seen, the stimulating effect was generally lost after 24 hours. The highest activation of N-acetyl-D-glucosamine was obtained in a mixture containing 48 mg. of lactose, 29 mg. of N-acetyl-D-glucosamine, and 4 mg. of crude enzyme per ml. of 0.1 M phosphate buffer at pH 5.4 after 4 hours of incubation at 37°. These conditions were followed in preparing digests for further studies.

**Paper Chromatographic Studies**

The paper chromatography was done on Whatman paper No. 1 at 25°. The upper layer from ethyl acetate, pyridine, and water (2:1:2) was used as solvent in the descending technique. The running time was between 24 and 40 hours. The paper chromatograms were then dried in air at room temperature for at least 12 hours. A typical chromatogram of an enzymatic digest is diagramed in **Fig. 2**.

When the chromatogram was sprayed with aniline oxalate, several spots appeared which might be attributed to hydrolytic or transglycosidic action on lactose: glucose and galactose, as well as more slowly moving spots which
correspond in position with the galactobiose and lactotriose observed by Wallenfels (7). In addition, two other reducing spots were found, occasionally weak, but always clearly visible under ultraviolet light. These spots, which will be referred to as $D_1$ and $D_{II}$, are located between lactose and galactose. The relative mobility was found to be lactose to $D_1$ to $D_{II} = 1.00:1.13:1.30$. The Morgan-Elson reagent (8) was used as a test for N-acetylamino sugars. When the reagent was applied after treatment of the paper with 50 per cent ethanolic Na$_2$CO$_3$ for 5 minutes at 100°, $D_1$ appeared as a violet spot while $D_{II}$ did not become visible. Without pretreatment with alkali, $D_1$ did not develop color when it was sprayed with the Morgan-Elson reagent, indicating that the compound itself did not possess the chromogenic structure. In the test for $-$NH$-$CO$-$ compounds, according to the method of Rydon and Smith (9) as modified by Gauhe,$^2$ both $D_1$ and $D_{II}$ appeared as blue spots. When the lactose was

$^2$ Private communication.
replaced by galactose in the enzymatic digest, neither $D_1$ nor $D_{II}$ was detectable.

To determine where the microbiological activity of the digests was located, 0.06 ml. of the digest was placed in a band across a strip of filter paper 10 cm. in width and run for 40 hours. Sections of the chromatograms were cut out and tested for microbiological activity; the sections corresponding to the position of $D_{II}$ were found to be the most active.

Isolation of Active Compound

For the isolation of $D_1$ and $D_{II}$, 800 mg. of crude enzyme preparation were added to 10 gm. of lactose and 5 gm. of $N$-acetyl-$d$-glucosamine dissolved in 180 ml. of 0.1 m phosphate buffer at pH 5.4. The mixture was incubated under toluene at 37° for 4 hours. The enzyme action was then stopped by heating the digest for 1 hour at 100°. The digest was centrifuged, and the supernatant fluid was deproteinized by shaking it mechanically for 30 minutes with 40 ml. of chloroform and 10 ml. of isoamyl alcohol, centrifuging for 20 minutes at 1500 r.p.m., and decanting the supernatant fluid through wet filter paper (10, 11). The filtrate was concentrated in vacuo to 30 to 50 ml. volume and this concentrate was adsorbed on a charcoal-Celite (1:1) column 60 mm. in diameter and 600 mm. in length. The column was eluted first with 14 liters of distilled water and then with ethanol: 21 liters of 2.5 per cent, 9 liters of 5 per cent, 18 liters of 7.5 per cent, 8 liters of 10 per cent, 4 liters of 15 per cent, and finally 4 liters of 30 per cent. The column was kept at 25° and the solvent was run through continuously at a rate of 1.5 to 2 ml. per minute. All fractions were tested for reducing power with anthrone (12) (0.1 per cent in concentrated H$_2$SO$_4$). Each eluate was concentrated to 1/100 of its original volume and then analyzed by means of paper chromatography. Paper chromatographic analysis of the eluates indicated that $D_1$ and $D_{II}$, nearly free of other sugars, were present in the last 6 to 8 liters of the 7.5 per cent ethanolic fractions.

Characterization of $D_1$ and $D_{II}$

The combined 7.5 per cent alcoholic eluates which contain only $D_1$ and $D_{II}$ were evaporated in vacuo to dryness. The amorphous residue weighed 750 mg. and had an optical rotation of [α]$^\circ_{D}$ +27.5° (H$_2$O; c = 1; α = 0.55; l = 2 dm.) and a nitrogen content of 3.66 per cent. 700 mg. of the residue were peracetylated with 8 ml. of acetic anhydride and 2 ml. of dry pyridine. After the mixture had stood for 24 hours at room temperature, the excess of pyridine and acetic acid was removed. The residue was dissolved in 50 ml. of dry chloroform, and petroleum ether was added until there was a lasting turbidity. After standing for a few days in the re-
frigerator, the amorphous precipitate was removed by centrifuging and 35 ml. of petroleum ether were added to the supernatant fluid. After a few days, 225 mg. of long needles with the sharp melting point at 222-223° (Berl; uncorrected) were obtained. The optical rotation was \([\alpha]_{D}^{30} + 61.5°\) (CHCl₃; \(c = 1; \alpha = 1.23; l = 2\) dm.). After a second recrystallization from dry ethanol, the melting point and the optical rotation remained unchanged.

The analysis agrees with that calculated for an octaacetyl derivative of a disaccharide composed of N-acetylglucosamine and an aldohexose such as galactose or glucose.

\[
C_{26}H_{39}O_{13}N. \quad \text{Calculated.} \quad C \ 49.61, \ H \ 5.81, \ N \ 2.07, \ \text{acetyl} \ 50.09
\]
\[
\text{Found.} \quad " \ 49.65 " \ 6.09 " \ 2.08 " \ 51.02
\]

The product obtained by saponification of the octaacetyl derivative in ammonia-saturated dry methanol was crystallized from ethanol in thin needles. After two recrystallizations from n-butanol, the melting point was 172°. The equilibrium rotation, reached after 50 minutes, was \([\alpha]_{D}^{30} + 40.5°\) (H₂O; \(c = 0.5; l = 2\) dm.). The analysis gave C 43.84, H 6.58, N 3.65 per cent. The calculated values for a disaccharide composed of N-acetylglucosamine and galactose or glucose would be for C 43.85, H 6.54, N 3.66 per cent.

The crystals reduced Fehling’s solution upon heating. The reducing value according to Macleod and Robison (13) was found to be 49.1 per cent that of glucose. The Morgan-Elson reaction with and without alkali pretreatment was negative. Paper chromatographic analysis of a 20 per cent solution indicated that the preparation was homogeneous. The relative mobility was found to be \(R_{\text{lactose}} = 1.30\). After hydrolysis with \(L. \ bifidus \ \text{var. Penn enzyme, N-acetylglucosamine and galactose were found by means of paper chromatography. After hydrolysis with 2 N H}_2\text{SO}_4 \text{ for 4 hours at 100°, glucosamine and galactose were found. By oxidation with NaOCl, followed by acid hydrolysis, galactose and glucosaminic acid were obtained. In the microbiological test the preparation contained the unit of activity in 80 \(\gamma\). The crystals were found to have optical rotation, \(R_{\theta}\) value, and melting point identical with those of the crystallized disaccharide isolated from hog mucin by Tomarelli et al. (4). The octaacetyl derivative of \(D_{\Pi}\) and the compound obtained from Tomarelli’s disaccharide by peracetylation possessed identical properties. No depression was observed in mixed melting points of the two peracetylated compounds.

For the isolation of \(D_{\Pi}\), the combined mother liquors of the first peracylation experiment were saponified with ammonia-saturated dry methanol and evaporated to dryness. After one reprecipitation from dry ethanol, an amorphous product was obtained which was composed of \(D_{\Pi}\) and small
amounts of \( D_{II} \). After a second precipitation from dry ethanol, irregular crystals were obtained with a melting point of 157–159°. The equilibrium optical rotation was found to be \( [\alpha]_{D}^{\text{aq}} +15.0^\circ \) (H\(_2\)O; \( c = 0.5; l = 2 \text{ dm.} \)). The analysis was for C 43.62, H 6.72, N 3.85 per cent. The substance reduced Fehling’s solution upon heating. The reduction value according to Macleod and Robison was found to be 51.1 per cent that of glucose. After pretreatment with alkali, a positive Morgan-Elson reaction was obtained. The relative mobility was \( R_{\text{lactose}} = 1.13 \). After hydrolysis with \( L. \text{bifidus} \) var. Penn enzyme, only N-acetylglucosamine and galactose were found by paper chromatography. The unit of microbiological activity was found in 1 to 1.2 mg.

**DISCUSSION**

In the presence of an enzyme preparation from \( L. \text{bifidus} \) var. Penn, it has been possible to synthesize from lactose and N-acetyl-D-glucosamine a disaccharide (\( D_{II} \)), highly active as \( L. \text{bifidus} \) factor. On the basis of the elementary analysis, hydrolysis products, reducing value, and reaction to the Morgan-Elson reagent, this compound appears to be identical with the active disaccharide which Tomarelli et al. (4) isolated from hog mucin. The formation of galactose and glucosaminic acid on treatment with sodium hypoiodite indicates that the compound is galactosido-N-acetyl-D-glucosamine, but the negative reaction to the Morgan-Elson reagent is not readily explained. The other disaccharide isolated from the digestion mixture, which was much less active as \( L. \text{bifidus} \) factor, gives the positive reaction to the Morgan-Elson reagent expected of galactosido-N-acetyl-D-glucosamine.

The enzyme preparation from \( L. \text{bifidus} \) var. Penn had strong lactase activity. In addition to glucose and galactose, the presence of oligosaccharides was observed. The occurrence of transglycosidation in conjunction with the hydrolysis of disaccharides has been studied extensively during the past few years in enzyme preparations with invertase (12, 14), maltase (15, 16), and lactase (17, 18) activity. It is probable that the disaccharides of N-acetyl-D-glucosamine and galactose were formed by a transgalactosidation. That this type of mechanism is involved is supported by the fact that no N-containing disaccharides were formed when the lactose in the digestion mixture was replaced by galactose. Since this work was undertaken, a brief report has appeared (19) that Wallenfels claims to have obtained galactosido-N-acetyl-D-glucosamine from lactose and N-acetyl-D-glucosamine by means of a lactase from \( E\). coli. It is of interest that in the present study two isomeric disaccharides were formed of which one only has high microbiological activity.
SUMMARY

Two isomeric disaccharides of galactose and N-acetyl-D-glucosamine have been synthesized from lactose and N-acetyl-D-glucosamine by means of an enzyme preparation from Lactobacillus bifidus var. Penn. Both appear to be galactosido-N-acetyl-D-glucosamine, but they differ in their response to the Morgan-Elson reagent. The Morgan-Elson-negative component possessed a high growth-promoting activity for L. bifidus var. Penn. It was purified by means of the octaacetyl derivative and was found to be identical with a disaccharide obtained by Tomarelli et al. (4), while the Morgan-Elson-positive compound was relatively inactive in the microbiological test.

Physical constants of the compounds were determined.

BIBLIOGRAPHY

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