FORMATION OF AN ACID-LABILE MALTOSYL-LIPID BY ENZYME PREPARATIONS FROM *ACETOBACTER XYLINUM*

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1. Introduction

Acetobacter xylinum is a gram-negative bacterium depositing large amounts of crystalline cellulose in its culture medium [1]. A glycogen-like polymer and a lipopolysaccharide of the mannan-type have also recently been isolated from *A. xylinum* [2]. Membrane preparations of the organism catalyze the transfer of D-glucose from UDP-glucose to small amounts of cellulose-like material [3–5], as well as to a major non-cellulosic polymer fraction [2]. It has, however, remained open whether UDP-glucose, or a compound derived from it, act as the immediate glucose donor in these transfer reactions.

More recent results indicate that poly-isoprenyl-diphospho-derivatives of D-glucose and cellobiose may play a role in polysaccharide biosynthesis by *A. xylinum* [6–8].

The glucolipid fraction has now been prepared using EDTA-treated cells [8], as well as a membrane preparation [9] from *A. xylinum*. The major sugar constituents have been identified as D-glucose and maltose.

2. Experimental

2.1. *Materials*

Cells of a cellulose-producing strain of *A. xylinum* (obtained from the collection of Dr J. R. Colvin, Ottawa, through Dr W. Herth, Heidelberg) were isolated by the method of Hestrin and Schramm [10]. EDTA-treated cells [8] and a total membrane fraction [9] were prepared and stored at −20°C and −196°C, respectively.

2.2. *Separation methods*

Descending paper chromatography was carried out using the following solvent systems (parts per volume): (A) Isobutyric acid / 1 M ammonia, 5:3. (B) Ethyl acetate / pyridine / acetic acid / water, 5 : 5 : 1 : 3. (C) Butanol-1 / pyridine / water, 6 : 5 : 5. (D) Ethyl acetate / acetic acid / water, 8 : 2 : 1. (E) Solvent system (D), with addition of 0.5% (w/v) phenylboronic acid. (F) Phenol, saturated at 25°C with water. High-voltage paper electrophoresis was carried out using buffer system (G). Sodium molybdate, 0.1 M, adjusted to pH 5.0 with concentrated sulfuric acid [11] (run at 100 mA, 900 V). Sugars were detected using either anilinium phthalate or silver nitrate spray reagents.

2.3. *Preparation of a glucolipid fraction (cf. ref. [8])*

The incubation mixture contained 100 mM Tris–HCl, 12 mM $MgCl_2$, 4 mM EDTA, 120 μM UDP-[^{14}C]glucose (about 1 μCi), at final pH 7.8. The final volume for incubation of EDTA-treated cells was 530 μl and 40 parallel incubations were performed, each containing about 25 mg total protein. The final volume for incubation of the membrane preparation was 230 μl and 11 parallel incubations were performed, each containing 7 mg total protein. After incubation for 30 min at 30°C, the reactions were terminated by extraction with 1.5 vol. ice-cold water-saturated butan-1-ol. This extraction was repeated two more times. The butan-1-ol phases from each set of parallel incubations were combined and washed twice with equal volumes of ice-cold water, followed by back-extraction with butan-1-ol. Aliquots of the final butan-1-ol phases were immediately examined in solvent system (A). Single relatively broad peaks

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of radioactivity were observed at R_F-values of 0.8 (membrane preparation) and 0.6 (EDTA-cells), respectively.

2.4. *Mild acid hydrolysis*

Non-radioactive carrier sugars (200 μl each of 10 mg/ml cellobiose, maltose and glucose) were added to the butanolic glucolipid extracts (about 20 ml), followed by addition of 50 μl trifluoroacetic acid (40 mM final concentration). The mixtures were heated for 15 min at 90°C. Most of the organic solvent was then removed at a rotary evaporator (below 40°C), followed by fractionation in solvent system (B).

3. Results and discussion

3.1. *Isolation of sugar constituents*

A cellulose-producing strain of *Acetobacter xylinum* was employed to prepare EDTA-treated cells [8] and a membrane fraction [9]. These preparations were incubated with UDP-[^{14}C]glucose, and glucolipid fractions were isolated by extraction with butan-1-ol. In order to minimize the decomposition to cyclic sugar phosphate esters, the glucolipid extracts were immediately subjected to mild acid hydrolysis, followed by paper chromatographic fractionation in solvent system (B). The amounts of released [^{14}C] sugar constituents were calculated from the distributions of radioactivity, and are shown in table 1.

3.2. *Characterization of sugar constituents*

3.2.1. D-Glucose

Upon elution of the radioactive material with $R_{glucose}$, 1.0, from the initial paper chromatogram, this fraction was found to co-chromatograph with authentic glucose in solvent systems (D,E) and (F). Treatment with glucose oxidase led to complete conversion to a product which co-chromatographed with authentic gluconic acid (solvent system (B); $R_{glucose}$, 0.45).

Table 1
Distribution of radioactivity between the products of mild acid hydrolysis of the [^{14}C]glucolipid fractions formed by EDTA-treated cells and by a membrane preparation of *Acetobacter xylinum*

$R_{glucose}$-value of product	Presumed nature of product	Amount of product (nmol; in brackets, % total)	
		EDTA-treated cells	Membrane preparation
Below 0.5	Phosphate esters[a]	0.2 (5)	0.02 (1)
0.5	Trisaccharide[b]	0.4 (10)	[c]
0.7	Disaccharide	2.0 (50)	0.2 (14)
0.9	Galactose	[d]	[d]
1.4 (Cells) 1.9 (Membrane)	Butyl-glucopyranoside and -disaccharide (mixture)	0.9 (23)	0.3 (21)

[a] Predominantly cyclic sugar phosphate esters

[b] The trisaccharide has not been identified, but was clearly distinguished from maltotriose

[c] If present, the amount was below 5 pmol

[d] To resolve galactose from excess glucose, rechromatography was performed in solvent system (F) (galactose, $R_{glucose}$, 1.1). Galactose could not be positively identified. If present, its amount was below 10 (EDTA-treated cells) and 50 pmol (membrane preparation), respectively

Radioactivity was determined by scintillation counting of segments of the initial paper chromatograms of the hydrolyzates (solvent system (B)). One nmol of [^{14}C]glucose incorporated corresponded to about 60 000 cpm. Analytical results obtained in subsequent experiments have been taken into account to calculate the amounts of product present in areas of the chromatograms where the main peaks of radioactivity were overlapping

3.2.2. Disaccharide fraction

Upon acid hydrolysis (2 N HCl, 2 h, 110°C) a single product co-chromatographing with glucose (solvent system (B)) was released. This product was completely converted to gluconic acid upon treatment with glucose oxidase. The disaccharide fraction was also completely converted to glucose when incubated with α-glucosidase or amyloglucosidase (fig.1). The product of enzymatic cleavage was converted to gluconic acid by treatment with glucose oxidase (fig.1). Identical results were obtained for the disaccharide isolated from the glucolipid formed by EDTA-treated cells (fig.1) and for the corresponding product of the membrane preparation. Cleavage of the disaccharide also occurred with a crude almond β-glucosidase preparation (NBC, No. 1280; cf. ref. [8]). However, the disaccharides were resistant against purified almond β-glucosidase.

The disaccharide fraction was further characterized by borohydride reduction, followed by electrophoresis in molybdate buffer [8,11]. The disaccharide alcohol migrated like authentic maltitol, and was clearly separated from cellobiitol, as well as from the α-(1.2), α-(1.3) and α-(1.6) linked sugar alcohols (fig.2).

The distribution of radioactivity between the reducing and non-reducing glucose moieties was deter-

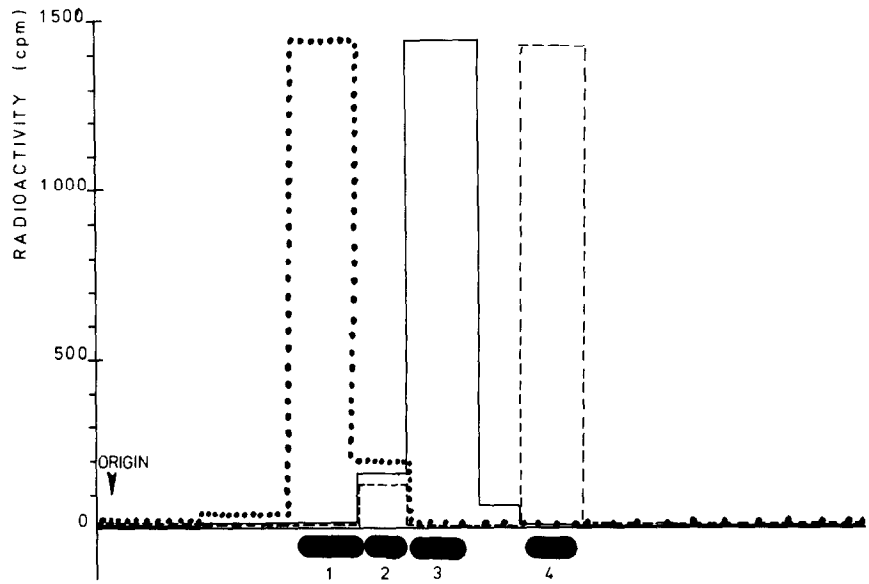

Fig.1. Enzymatic treatment of the disaccharide isolated from the glucolipid fraction formed by EDTA-treated cells of *A. xylinum*. The distribution of radioactivity (cpm) is plotted against the distance moved on paper chromatograms (solvent system (B); drawn to scale). The following reference sugars were employed: (1) Gluconic acid; (2) Maltotriose; (3) Maltose; (4) Glucose. (———) No treatment. The same distribution of radioactivity was obtained after treatment with purified β-glucosidase or β-amylase. (– – – – – –) Treatment with α-glucosidase. The same distribution of radioactivity was obtained after treatment with amyloglucosidase or crude emulsin. (· · · · · · · · ·) Treatment with amyloglucosidase or α-glucosidase, followed by treatment with glucose oxidase. The following procedures were used. β-Glucosidase: The sugar sample (containing 10 μg each of carrier cellobiose and maltose) was lyophilized and 25 μl reagent solution was added (100 mM sodium acetate, pH 5.0, containing 10 μg purified almond β-glucosidase, Boehringer No. 15399). After incubation for 60 min at 30°C, the reaction mixture was fractionated in solvent system (B). Under these conditions, authentic cellobiose (250 μg) was completely cleaved, whereas maltose or maltotriose (250 μg each) remained unchanged. α-Glucosidase, amyloglucosidase, β-amylase: The above procedure was followed using 10 μg *Aspergillus niger* amyloglucosidase (Boehringer No. 15043), potato β-amylase (Boehringer No. 15471) or yeast α-glucosidase (Boehringer No. 15018). With the latter enzyme, the buffer was 100 mM sodium phosphate, pH 7.0. Authentic maltose (250 μg) or maltotriose (250 μg) were completely cleaved by both α-glucosidase and amyloglucosidase, whereas cellobiose (250 μg) remained unchanged. β-Amylase failed to cleave any of the oligosaccharides. Glucose oxidase: Carrier D-glucose (200 μg) was added to the lyophilized sample to be tested, followed by 25 μl reagent solution (100 mM sodium phosphate, pH 7.0, containing 50 μg glucose oxidase, Boehringer No. 15422 and 50 μg catalase, Boehringer No. 15674). After incubation for 60 min at 25°C, the reaction mixture was fractionated in solvent system (B).

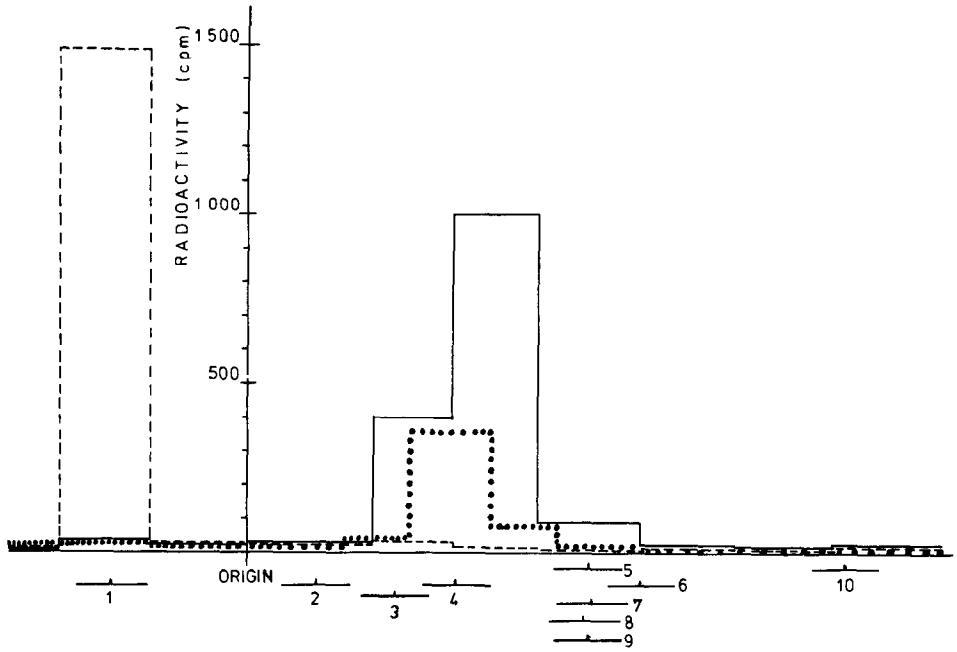

Fig.2. High-voltage paper electrophoresis (buffer system I) of the borohydride-reduced disaccharide isolated from the glucolipid fractions formed by EDTA-treated cells (————) or by a membrane preparation (· · · · · · · ·) of *A. xylinum*. In a control experiment (– – – – – –), the disaccharide formed by EDTA-treated cells was run without prior borohydride reduction. The distributions of radioactivity (cpm) are plotted against the positions (drawn to scale) of the following reference compounds. (1) Nigeritol. The neutral sugars, glucose, rhamnose, α,α-trehalose and gentiobiose were found at the same position. (2). Cellobiitol. (3) Maltotriitol. (4) Maltitol. (5) Picrate (visible standard). (6) Isomaltitol. (7) Gentiobiitol. (8) Kojibiitol. (9) Sophorositol. (10) Sorbitol. Reduction procedure: Sodium borohydride (20 μl, 100 mg/ml) was added to 20 μl aqueous sugar solution, containing less than 1 mg sugar material. After incubation for 30 min at 25°C, the solution was brought to pH 4 by addition of about 5–15 μl glacial acetic acid and applied to electrophoresis paper.

mined by borohydride reduction and strong acid hydrolysis (2 N HCl, 2 h, 110°C), followed by the electrophoretic separation of glucose and sorbitol in buffer system (G). The reducing glucose moiety of maltose (determined as sorbitol) in both cases contained about 15% of the total radioactivity, the remaining radioactivity being associated with glucose. This marked asymmetry of labeling may indicate that maltose was formed predominantly by transfer of the non-reducing D-glucose moiety from UDP-glucose to glucosyl-lipid already present in the membranes used for incubation.

4. Conclusion

In contrast to previous reports [6–8] the lipid-linked disaccharide formed by enzyme preparations from *A. xylinum* has been identified as maltose (4-*O*-α-D-glucopyranosyl-D-glucose) rather than cellobiose (4-*O*-β-D-glucopyranosyl-D-glucose). An acid-labile maltosyl-lipid having properties of isoprenoid carrier lipids has apparently so far not been isolated from any source. This lipid could conceivably provide a primer for α-glucan biosynthesis, perhaps by transfer to a protein. Alternatively, it could be involved in the biosynthesis of side-chains of a cellulose precursor molecule. No decision between these possibilities can be made at present.

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