Amylooligosaccharides from *Mycobacterium smegmatis*

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In early stationary phase of growth, *Mycobacterium smegmatis* cultures accumulate amylooligosaccharides (α\(1 \rightarrow 4\)-glucooligosaccharides) up to the undeca-saccharide. Although *M. smegmatis* also makes an acylated polymethylpolysaccharide that is predominantly an α\(1 \rightarrow 4\)-glucan, we conclude that these oligosaccharides are precursors of glycogen rather than lipopolysaccharide.

*Mycobacterium smegmatis* synthesizes a methylated and acylated lipopolysaccharide that is composed predominantly of α\(1 \rightarrow 4\)-linked \(\beta\)-glucose units (2). The observation that partially acylated amylooligosaccharides can serve as methyl acceptors from \(S\)-adenosylmethionine with a particulate enzyme preparation from the bacterium (3, 5) suggested that small precursors of the lipopolysaccharide might be detected in the cell. Fractionation of the 70% ethanol extract of *M. smegmatis* has failed to reveal such intermediates, although a homologous series of amylooligosaccharides does appear in the extracts of cells harvested in stationary phase. Since mycobacteria are known to accumulate glycogen when growth is restricted (1), we postulate that these are intermediates in glycogen synthesis.

Reference amylooligosaccharides were obtained by partial acidolysis of amylpectin (5), and 3-\(O\)-methylmannose oligosaccharides were isolated from *M. smegmatis* ATCC 356 ATCC. Descending paper chromatography was done on Whatman no. 1 paper in nitromethane–ethanol–water (45:36:19) or \(n\)-butanol–pyridine–water (10:3:3), and the sugars were detected with alkaline silver nitrate. Oligosaccharides were hydrolyzed in 1 M trifluoroacetic acid at 120°C for 2 h. For gas chromatography, sugars were converted to their alditol acetates and separated on a 3% OV-210 column (4 feet by \(\frac{1}{8}\) inch; ca. 1.2 m by 3.2 mm) with a temperature gradient of 2°C/min from 175 to 225°C.

*M. smegmatis* was grown in a glycerol medium (4) in a 150-liter fermentor, and the cells were harvested after 30 h (log phase) or 45 h (stationary phase). The cells were refluxed with 70% ethanol for 3 h, and the ethanol extract was separated from the cells and evaporated to yield a residue that was extracted with a mixture of chloroform–methanol–water (8:4:3, vol/vol/vol) (4). The insoluble fraction was dissolved in a small amount of water to give a thick solution that was fractionated on a Sephadex G-50 column (2 by 190 cm) in 0.1 M acetic acid, and the sugar composition of the different fractions was determined by chromatography of the acid hydrolysat. The fractions containing methylmannose polysaccharide (MMP) and methylglucose lipopolysaccharide (MGLP) were pooled, lyophilized, and further fractionated (4). The Sephadex G-50 fractions that contained smaller oligosaccharides, precursors of MMP (7) and potential precursors of MGLP, were passed through a DEAE-Sephadex A-25 column (1 by 20 cm) to obtain the neutral material. These oligosaccharides were then fractionated on a Bio-Gel P-4 column (2 by 190 cm). Individual peaks were further fractionated by high-pressure liquid chromatography (HPLC) on a Waters Associates µ-Bondapak/Carbohydrate column (0.4 by 30 cm) with an acetonitrile–water mixture, 65:35 to 75:25 (vol/vol), depending on the efficiency of the column.

The 70% ethanol extract of *M. smegmatis* cells cultured until mid log phase gave the pattern in Fig. 1. Acid hydrolysis of fraction I revealed glucose, 3-\(O\)-methylglucose, 6-\(O\)-methylglucose, mannose, and 3-\(O\)-methylmannose, characteristic of MMP and MGLP; fraction II gave glucose, mannose, and 3-\(O\)-methylmannose, as expected for the MMP precursors; and fraction III gave glucose (mainly from trehalose), methionine, and other unidentified substances. In contrast, the 70% ethanol extract from cells grown to late stationary phase gave the pattern in Fig. 2. Component sugars of fractions I and III were similar to those from mid log-phase cells, but the sugar content of fraction II was increased and a peak was observed that gave mainly glucose after hydrolysis. This glucose-rich peak was passed through a DEAE-Sephadex A-25 column to isolate the neutral material, which was then fractionated on a Bio-Gel P-4 column (Fig. 3). Fractions were combined as indicated on the figure and analyzed by HPLC.
Fig. 1. Sephadex G-50 column chromatography of the 70% ethanol extract of M. smegmatis cells grown to mid log phase. Peak I contained MMPs and MGLPs; peak II contained MMP precursors; and peak III contained trehalose and methionine. Absorbance at 490 nm is by the phenol-sulfuric procedure for carbohydrate.

The HPLC pattern from fraction I of Fig. 3 (which yielded only glucose after hydrolysis) is shown in Fig. 4. Eight peaks were observed which correspond in elution position exactly with the reference series of amylooligosaccharides from maltose to the octasaccharide. None of the peaks corresponded to any MMP homolog. Each of the other Bio-Gel P-4 fractions contained mainly a single oligosaccharide: fraction II agreed in position with the octasaccharide, fraction III agreed with the nonasaccharide, and fraction IV agreed with the decasaccharide and the undecasaccharide.

All of the oligosaccharides larger than the disaccharide were degraded by α-amylase, suggesting that they were α1→4-glucooligosaccharides. The products from exhaustive digestion of the nonasaccharide were reduced with sodium borotritide and subjected to paper chromatography. The main product had the Rf of maltitol, although a small amount of glucitol and a trace of a reduced trisaccharide were also observed (Fig. 5). The ratio of label in maltitol to that in glucitol was 3.4; for quantitative amylolysis of a nonasaccharide, a ratio of 4.0 is expected. Amylase digestion of the octasaccharide gave almost exclusively maltitol (data not shown).

Fraction V contained glucose as the only component sugar, but the elution pattern on HPLC showed several peaks, none of which corresponded to any amylooligosaccharide reference. Fraction VI contained MMP precursors, as evidenced by the fact that mannose and 3-O-methylmannose were obtained on acid hydrolysis (data not shown).

In the standard preparation of polymethylpolysaccharides from M. smegmatis (4), the 70% ethanol extract is treated with amylase to de-
Fig. 5. Paper chromatography of the sodium barbital-reduced products from exhaustive digestion of the purified nonasaccharide. A is the position of a glucitol standard, B is a maltitol standard, and C is a maltotriitol standard.

stroy the glucans and to facilitate the isolation of oligosaccharides free of such contamination. If this step is eliminated, we see that a series of α1→4-linked glucooligosaccharides can be isolated. Although these compounds are structurally related to MGLP, we found no evidence that they were methylated or acylated. The lack of methylation is consistent with a previous observation (5) that cell extracts of M. smegmatis methylate amyloooligosaccharides only if they have been partially acylated. These results suggest that the biosynthesis of MGLP must involve some alternative pathway, perhaps by way of a lipid-linked oligosaccharide.

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LITERATURE CITED