CHARACTERIZATION OF A MANNAN-LIKE OLIGOSACCHARIDE FROM *MYCOBACTERIUM SMEGMATIS*

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SUMMARY: A nitrogen-free neutral mannooligosaccharide, similar in structure to the polysaccharide component of yeast mannoproteins, has been isolated from *Mycobacterium smegmatis* ATCC-356. It has a molecular weight of 3200 and is terminated at the reducing end by mannose. The evidence from nuclear magentic resonance spectroscopy, methylation analysis, selective enzymic degradation and acetolysis indicates that the molecule consists of an α1→6-linked backbone to which single mannose units are attached in α1→2 linkage as sidechains.

Yeast cell wall mannans are glycoproteins containing 50-90% by weight of D-mannose (1), whereas succinylated mannans occur in the cell wall of bacteria (2). The presence of mannan-like molecules in *Mycobacterium smegmatis* and some biosynthetic work on a mannoprotein molecule (3) have been reported, but with little characterization. Here we describe an oligosaccharide from *M. smegmatis* that is composed of nine α1→2-linked mannose units attached to a chain of eleven α1→6-linked mannoses.

EXPERIMENTAL

Materials--Sodium borotritide (1 mCi/μmole) was from I.C.N., and Bio-Gel P-4 (minus 400 mesh) and P-2 (200-400 mesh) were from Bio-Rad. All other chemicals were reagent grade obtained from commercial sources. Exo-α-mannanase was purified from the cultural filtrate of Arthrobacter GJM-1 (4). endo-α1→6-mannanase from *Bacillus circulans* ATCC-29101 (5), and the methyl-mannose polysaccharide from *Mycobacterium smegmatis* ATCC-356 (6).

Analysis of Sugars--Descending chromatography was done on Whatman No. 1 paper in the following solvents (compositions in volume ratios): Solvent A, nitromethane-ethanol-water, 45:36:19; Solvent B, butanol-pyridine-water, 10:3:3; Solvent C, methyl ethyl ketone-boric acid saturated water-acetic acid, 9:1:1; Solvent D, 1-butanol-pyridine-water, 6:4:3. Sugars were detected with an alkaline silver nitrate reagent (7) and the radioactive sugars on the chromatogram were counted in 10 ml of Bray's solution (8) by cutting the paper in 1 cm strips. Reducing power was determined according to Park and Johnson (9). Acetolysis of mannan was done according to Kocourek and Ballou (10).

Gas Chromatography and Mass Spectrometry--Sugars were characterized as their alditol derivatives on an ECNSS column (6 ft x 1/8 in) with a Varian

1400 gas chromatograph. About 2 μmoles of mannan was methylated according to Hakomori (11). The product was dialyzed against water, remethylated and purified on a Sephadex LH-20 column (1 x 20 cm) (12). The methylated polysaccharide was hydrolyzed and the monosaccharide units were reduced with sodium borohydride and acetylated. Methylated sugar alditol derivatives were separated on an OV-210 column (4 ft x 1/8 in). Mass spectra were obtained on a DuPont model 21-491 instrument coupled to a Varian 1400 gas chromatograph.

Physical Measurements--Synthetic boundary and sedimentation equilibrium measurements were done on mannan at 5 mg per ml with a Beckman Model E analytical ultracentrifuge. The concentration at the meniscus was determined and the molecular weight was calculated from a plot of ln C against r^2 (13,14).

The NMR spectrum of mannan was obtained in D_2O, with the internal standard trimethylsilylpentane sulfonic acid, using a 270 MHz Brucker magnet attached to Nicholet 1080 system for Fourier transformation. A 90° pulse of 30 μsec with a delay time of 10 sec was used.

Purification of Mannan--Mycobacterium smegmatis ATCC-356 was grown in a glycerol medium (15) in 10 liter batches and harvested after 48 hours. About 100 gm of cells was refluxed in 70% ethanol for 3 hours. The ethanol extract was concentrated and reextracted with chloroform-methanol-water, 8:4:3. The concentrated aqueous layer was passed through a Sephadex G-50 column (2 x 190 cm) equilibrated with 0.1 M acetic acid. The fractions containing methylmannose polysaccharide and methylglucose lipopolysaccharide were pooled and lyophilized. This material was then extracted with chloroform-methanol 4:1, and the insoluble residue which contained the mannan was dissolved in water and passed through a Sephadex A-25 column (1 x 20 cm). The neutral fraction was pooled and concentrated. Paper chromatography of the hydrolyzed neutral fraction showed 3-O-methylmannose, mannose and glucose. The neutral fraction was streaked on Whatman No. 3 MM paper and chromatographed in solvent A for 12 hours. The chromatogram gave spots corresponding to glucose, maltose, and oligosaccharides of glucose, ranging from Glc_3 to Glc_{12}, and a spot at the origin. The material at the origin was eluted, and on hydrolysis it gave mannose as the major product with a trace of glucose. This material was passed through a Sephadex G-50 column (2 x 190 cm) and 160 drops were collected per fraction. The peak fractions (40-50) were pooled, concentrated and passed through a Bio-Gel P-2 column (2 x 180 cm).

RESULTS AND DISCUSSION

The mannan-like material was isolated from *Mycobacterium smegmatis* in a yield of about 5 mg per 100 g of wet cell paste. Acid hydrolysis of the polysaccharide yielded only mannose. The substance gave a positive reducing test, and from the reducing power it was estimated to contain about 22 mannose units. Since reduction with $NaBT_4$ followed by acid hydrolysis yielded radioactive mannitol (Fig. 1), the molecule must have mannose at the reducing end. The presence of N-acetylglucosamine was not detected in the hydrolysate by chromatography in Solvent D.

The $NaBT_4$-reduced mannan gave a broad symmetrical peak on the Bio-Gel

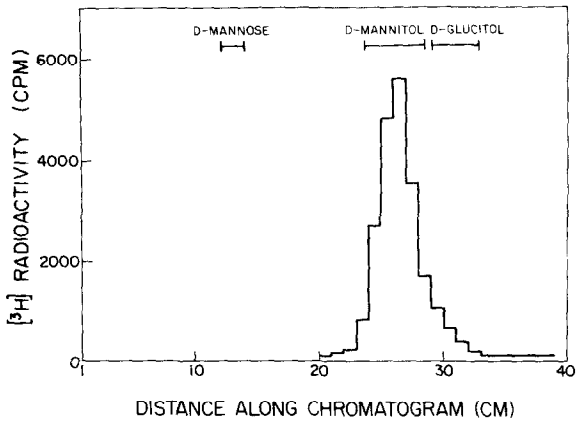

Fig. 1. Identification of the sugar at the reducing end of the mannan. The sample was reduced with $NaBT_4$, hydrolyzed in acid and the product was chromatographed in solvent C for 24 hours.

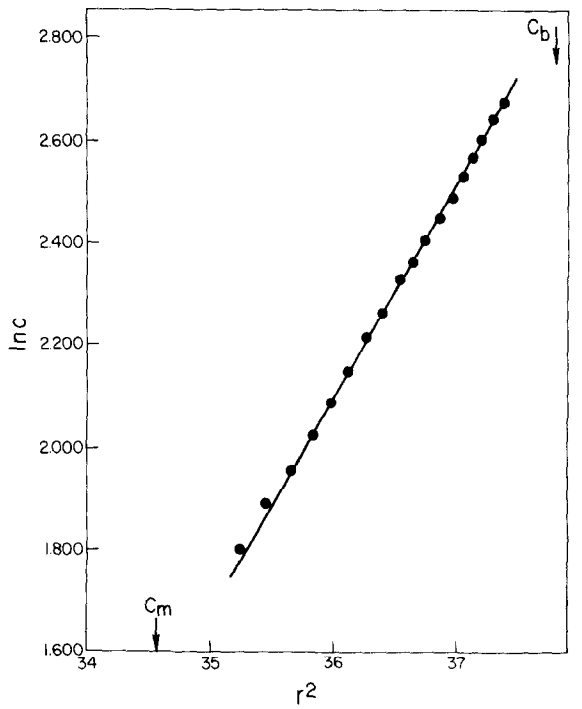

Fig. 2. Molecular weight estimation of the mannan. The sedimentation equilibrium run was carried out on the sample with an AN-D rotor at 42,000 rpm. The plot of ln C vs r^2 (cm) was obtained at 42.5 hours from zero time.

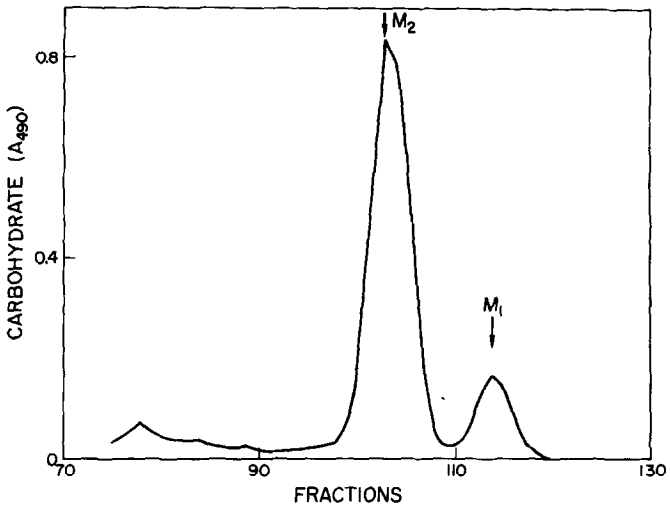

Fig. 3. Acetolysis pattern of the mannan. The deacylated acetolyzed product (5 mg) was applied to a Bio-Gel P-2 column (1 x 100 cm) and eluted with 0.1 M ammonium bicarbonate. The arrows indicate the elution position of mannose (M_1), mannobiose (M_2).

P-4 column, a suggestion that it could be heterogeneous. However, sedimentation equilibrium studies gave a linear plot of ln C vs r^2 (Fig. 2), from which a molecular weight of 3180 ± 100 was calculated, assuming a partial specific volume of 0.69 (16). This indicates a size of 20 mannose units and agrees with 22 units found by the reducing determination.

The NMR spectrum revealed several anomeric proton signals, whereas the ring protons appeared at 5.93 to 6.22 τ. The major peaks at 4.89 τ and 4.96 τ are attributed to α1→2 and α1→6 linkages, respectively. A small signal upfield from the proton signal at 4.89 τ probably comes from the mannose at the reducing end of the molecule. Based on these assignments, the ratios of the anomeric signals for α1→2:α1→6:α1→6-reducing are 9:11:1. The optical rotation $[\alpha]_{26} = +120°$ confirms the α configuration of the glycosidic linkages

The presence of α1→6 linkages was confirmed by acetolysis, and the molar ratio of mannobiose to mannose produced in this reaction was approximately 9

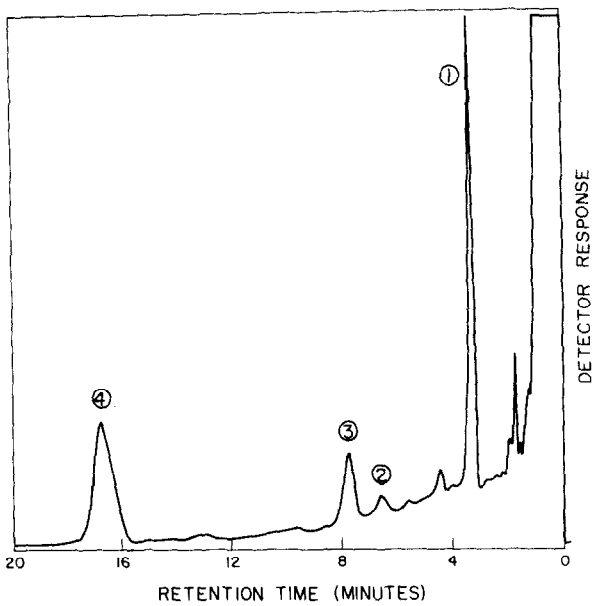

Fig. 4. Methylation analysis of the mannan. Gas chromatographic tracing of the methylated alditol acetates of the permethylated mannan; (1) 2,3,4,6-tetra-O-methylmannose, (2) 3,4,6-tri-O-methylmannose, (3) 2,3,4,tri-O-methylmannose, and (4) 3,4-di-O-methylmannose.

to 2 (Fig. 3). Taken together, these results suggest that the mannooligosaccharide has a structure like the following.

$$\begin{array}{l} \alpha M\rightarrow 6\alpha M\rightarrow 6\alpha M\rightarrow 6\alpha M\rightarrow 6\alpha M\rightarrow 6\alpha M\rightarrow 6\alpha M\rightarrow 6\alpha M\rightarrow 6\alpha M\rightarrow 6\alpha M \\ \quad\uparrow 2\quad\uparrow 2\quad\uparrow 2\quad\uparrow 2\quad\uparrow 2\quad\uparrow 2\quad\uparrow 2\quad\uparrow 2\quad\uparrow 2 \\ \alpha M\quad\alpha M\quad\alpha M\quad\alpha M\quad\alpha M\quad\alpha M\quad\alpha M\quad\alpha M\quad\alpha M \end{array}$$

This structure would give an anomeric proton ratio for α1→2:α1→6:α1→6-reducing of 9:10:1, which agrees with the observed values.

Methylation analysis gave 2,3,4,6-tetra, 2,3,4-tri, 3,4,6-tri and 3,4-di-O-methylmannose in the molar ratios of 7:2:1:9 (Fig. 4), whereas the above structure would predict ratios of 9:2:1:8. The low value for the tetramethyl derivative is commonly observed owing to its volatility that leads to losses during processing of the reaction products.

The nature of branching was investigated with the bacterial exo-α-mannanase (4) and endo-α1→6-mannanase (5). The former enzyme cleaves the α1→2 linkages, leaving the α1→6-backbone intact; and the latter enzyme attacks only the unsubstituted backbone and produces mainly mannobiose units (4).

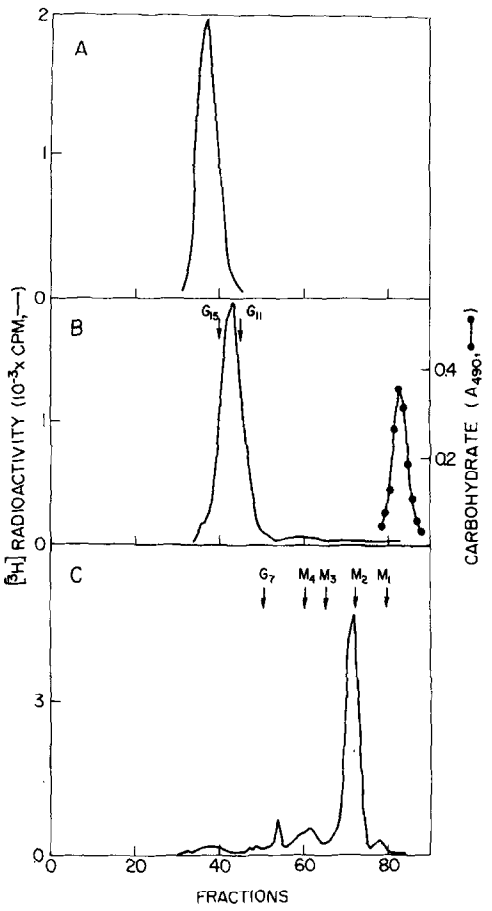

Fig. 5. Enzymic degradation of the mannooligosaccharide. (A) Sodium borotritide-reduced oligosaccharide (2.2 mg) was eluted with water through a Bio-Gel P-2 column (2 x 95 cm). (B) The peak fractions in A were pooled and digested with the exo-α-mannanase for 4 hours at 37° and then fractionated on the same column. The peak at fraction 80 was identified as mannose by paper chromatography. (C) Pooled fractions 38 to 50 were digested with endo-α1→6-mannanase for 2 hours at 50° and the products fractionated on the same column. The major peak has the elution position of a reduced disaccharide and accounts for 77% of the total sugar. The arrows indicate the elution positions of mannooligosaccharides M_1 to M_4 and glucooligosaccharides G_7, G_{11} and G_{15}.

When the mannan was digested with exo-α-mannanase, mannose was released and the size of the oligosaccharide was reduced to about 12 sugar units (Fig. 5 A,B). The $NaBT_4$-reduced fragment (fractions 39 to 50), supposedly α1→6-backbone, was then digested with endo-α1→6-mannanase which yielded a series of products, but mainly mannobiose (Fig. 5C) as would be expected from the

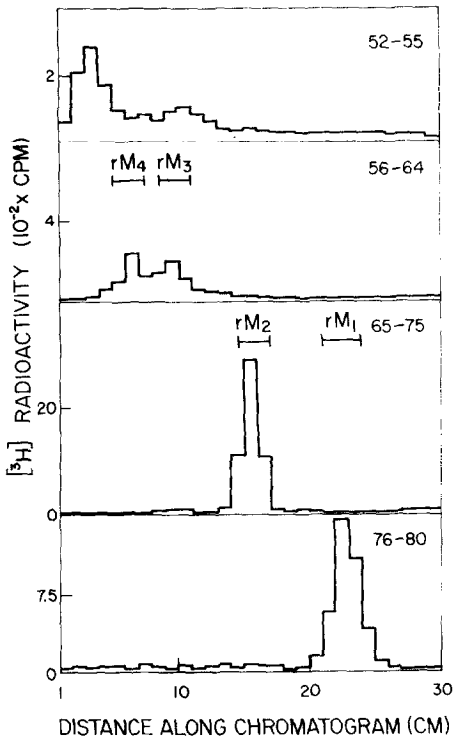

Fig. 6. Identification of the fractions in Fig. 5C. Paper chromatography was done in Solvent A, and the positions of reduced reference oligosaccharides M, M_2, M_3, and M_4 are indicated. The major peak in Figure 5C (fractions 65-75) corresponds to a reduced disaccharide, indicating that the oligosaccharide backbone was almost quantitatively degraded to yield this product.

postulated oligosaccharide structure and the specificity of this enzyme (4). Paper chromatography confirmed the identity of the various fractions (Fig. 6).

This mannan fragment shows some structural similarity to the outer chain of yeast mannan (17), and differs in that it is smaller and that the side-chains are limited to a single mannose unit. We feel it unlikely that the oligosaccharide is produced by degradation of a larger polysaccharide or that it is released by β-elimination from attachment to a hydroxyamino acid of a protein. It could result from degradation of a glycosyl-pyrophosphoryl-lipid derivative, but there is no reason to suspect the existence of such a compound in *Mycobacteria*. Regardless of the origin, it is routinely observed as a

contaminant in methylmannose polysaccharide preparations purified only by gel filtration, and is most effectively removed by high pressure liquid chromatography (unpublished observation). Its presence in the methylmannose polysaccharide preparations accounts for the earlier conclusion that this polymer contained an α1→2-linked mannobiose sidechain (16).

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REFERENCES

1. Ballou, C. E. and Raschke, W. (1974) *Science 184*, 127-134.
2. Powell, D. A., Duckworth, M. and Baddiley, J. (1975) *Biochem. J. 151*, 387-397.
3. Schultz, J. C. and Takayama, K. (1975) *Biochem. Biophys. Acta 381*, 175-184.
4. Nakajima, T., Maitra, S. K. and Ballou, C. E. (1976) *J. Biol. Chem. 251*, 174-181.
5. Jones, G. H. and Ballou, C. E. (1969) *J. Biol. Chem. 244*, 1043-1051.
6. Maitra, S. K. and Ballou, C. E. (1974) *Fed. Proc. 33*, 1452.
7. Anet, E. F. L. J. and Reynolds, T. M. (1954) *Nature 174*, 930.
8. Bray, G. A. (1960) *Anal. Biochem. 1*, 279-285.
9. Park, J. T. and Johnson, M. J. (1949) *J. Biol. Chem. 181*, 149-151.
10. Kocourek, J. and Ballou, C. E. (1969) *J. Bacteriol. 100*, 1175-1181.
11. Hakomori, S. I. (1964) *J. Biochem. (Tokyo) 55*, 205-208.
12. Stellner, K., Siato, H. and Hakomori, S. I. (1973) *Arch. Biochem. Biophys. 155*, 464-472.
13. Richards, E. G. and Schachman, H. K. (1959) *J. Phys. Chem. 63*, 1578-1591.
14. Edelstein, S. J. and Schachman, H. K. (1967) *J. Biol. Chem. 242*, 306-311.
15. Gray, G. R. and Ballou, C. E. (1972) *Methods in Enzymol. 35B*, 90-95.
16. Gray, G. R. and Ballou, C. E. (1971) *J. Biol. Chem. 246*, 6835-6842.
17. Nakajima, T. and Ballou, C. E. (1974) *J. Biol. Chem. 249*, 7679-7684.