AN APPROACH TO THE SEQUENCING OF YEAST MANNAN

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SUMMARY: A "nearest neighbor" analysis of the products of partial acetolysis of yeast mannan was carried out in an attempt to learn whether the side chains in the polysaccharide are ordered in a sequence or occur randomly. The results suggest that the arrangement is nonrandom, but they do not give unequivocal support to a single repeating sequence.

Acetolysis of *Saccharomyces cerevisiae* 4484-24D mannan, under conditions that break only 1→6 linkages (1,2), yields mannose, mannobiose and mannotriose in a molar ratio approximating 1:2:1. Assuming that the mannan is made of the smallest ordered repeating unit of these four fragments connected by 1→6 linkages, one can construct just three sequences: (A) --M→M_2→M_2→M_3--, (B) --M→M_2→M_3→M_2--, and (C) --M→M_3→M_2→M_2-- (Fig. 1).

To test this model, the mannan was subjected to partial acetolysis under conditions in which some fragments with intact 1→6 linkages remain (3). These fragments were separated, and each was reduced with sodium borotritide and then subjected to further acetolysis to cleave the 1→6 linkage. Characterization of the products gave the order of the side chain units in each fragment. Very little tetrasaccharide fragment with intact 1→6 linkage was obtained, a result expected for sequence B. The pentasaccharide fragment was mostly M_2→M_3 which

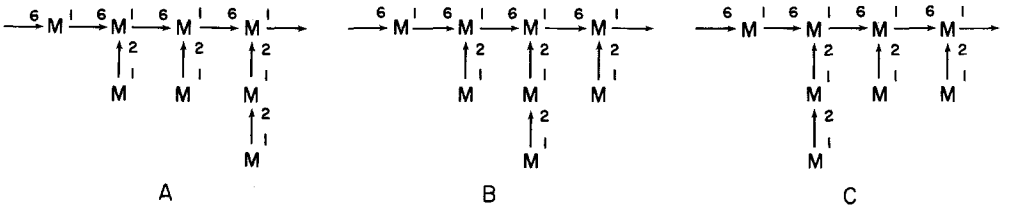


Fig. 1. Three possible repeating sequences for *S. cerevisiae* 4484-24D mannan. Sequence A is --M→M_2→M_2→M_3--, B is --M→M_2→M_3→M_2--, and C is --M→M_3→M_2→M_2--. The 1→6 linkages can be broken selectively by acetolysis. Limited acetolysis leaves some intact 1→6 linkages, and the sequence in the resulting fragments can be determined by reduction and further acetolysis.

is consistent with sequence A, or B if there is some steric selectivity in the acetolysis reaction.

Although all of the assumptions on which this analysis was based have not been rigorously tested, the method does give results that support a nonrandom order of side chains in the mannan polymer. This implies that some directing influence, perhaps derived from the specificity of the various mannosyltransferases, regulates both the amount and the order of mannan side chains.

EXPERIMENTAL

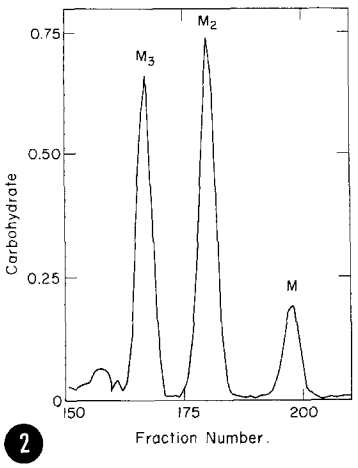
Saccharomyces cerevisiae 4484-24D (4,5) was obtained from Dr. S. Fogel. The yeast was grown to stationary phase on the medium used by Stewart and Ballou (6). Mannan was extracted (7) and purified by published procedures (8). The mannan used in the following experiments was eluted from a DEAE-Sephadex A-25 column with 0.2 M KCl and had a mannose to phosphate ratio of 36.

Complete acetolysis of the acetylated mannan was carried out at 40° for 10 hours, whereas partial acetolysis was done for 1 hour (9,10). The deacetylated products from acetolysis were separated by gel filtration on a 2 x 200 cm Bio-Gel P-2 column, and the peaks were detected by the phenol-sulfuric acid method (11). Acetolysis rates of reduced disaccharides were measured as described elsewhere (10).

Reduction of the partial acetolysis fragments with $NaBT_4$ (New England Nuclear, 200 Ci/mole) for 2 hours in 0.05 M NH_4HCO_3 was followed by reduction with excess $NaBH_4$ (Alfa Inorganics) overnight (12). The reduction was stopped and cations were removed with Dowex AG 50 (H^+), after which the borate was removed from the combined filtrate and washings by repetitive evaporation with methanol.

RESULTS

Total acetolysis of 1→6 linkages in the mannan yielded the pattern in Fig. 2. A small mannotetraose peak and the mannotriose phosphate peak (not shown) were ignored in this analysis. The partial acetolysis pattern (Fig. 3) showed a void volume peak of undegraded material and only small amounts of oligosaccharides



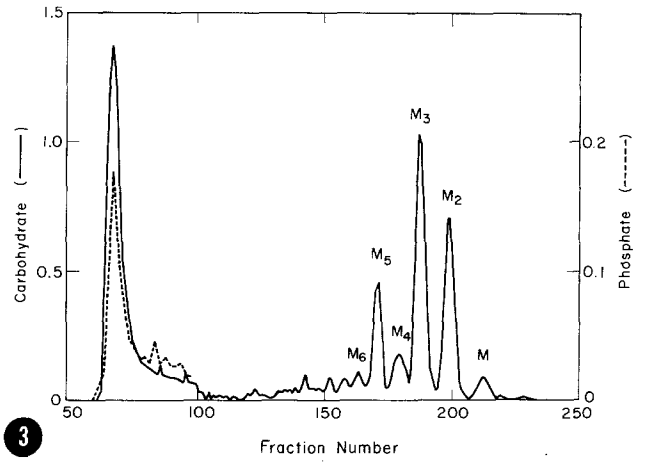


Fig. 2. Total acetolysis of *S. cerevisiae* 4484-24D phosphomannan. The ratio of mannose (M) to mannobiose (M_2) to mannotriose (M_3) is 1.0 to 1.83 to 1.05 On this scale, the small fourth peak at about fraction number 157 is 0.09 and the mannotriose phosphate (not shown) was about 0.1. These two components were ignored for this analysis.

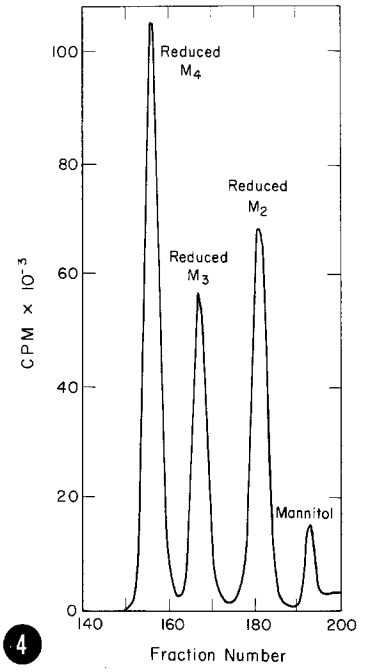
Fig. 3. Partial acetolysis (1 hour) of *S. cerevisiae* 4484-24D phosphomannan. The areas under the peaks up to mannohexaose (M_6) were used to calculate the molar fractions M: M_2: M_3: M_4: M_5: M_6 to be 0.13: 0.43: 0.35: 0.02: 0.07: 0.003.

TABLE I

Molar ratios of tetra- and pentasaccharide fragments with one 1→6 linkage

Sequence	$M_2 \rightarrow M_2$	$M \rightarrow M_3$	$M_3 \rightarrow M$	Total M_4	$M_2 \rightarrow M_3$	$M_3 \rightarrow M_2$	Total M_5
Predicted							
Sequence A	1	0	1	2	1	0	1
Sequence B	0	0	0	0	1	1	2
Sequence C	1	1	0	2	0	1	1
Random	4	1	1	6	2	2	4
Observed[a]	0.17	0.11	0.03	0.31	1	0.22	1.22

[a]Corrected for the presence of the M_4 fragment that resisted reacetolysis, and for nonspecific acetolysis of 1→2 linkages during the reaction.



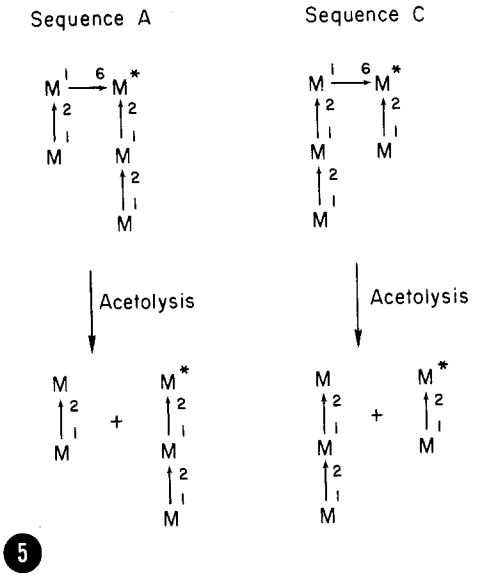


Fig. 4. Reacetolysis (7 hours) of $NaBT_4$-reduced M_4 From Fig. 3. The ratio of radioactivity for mannitol: reduced M_2: reduced M_3: reduced M_4 is 1.05: 5.8: 4.4: 8.1, respectively. After correction for nonspecific acetolysis, the ratios were 1.06: 6.4: 4.3: 12.7. The acetolysis-resistant M_4 is 52 percent of the total and may be derived from the inner core of the mannan (13). These ratios, excluding the acetolysis-resistant M_4, were used to calculate the values presented in Table I.

Fig. 5. Expected products from the reacetolysis of $NaBT_4$-reduced M_5 from mannan with sequence A and sequence C. Sequence B is expected to yield equal amounts of both M_5 fragments, assuming that the acetolysis is random.

above M_5. This suggests that only those products with at most one 1→6 linkage survived partial acetolysis. Very little M_4 with a single 1→6 linkage remained after the 1-hour acetolysis (Table I, see below), which suggests that the order of side chains approximated Sequence B. The molar fractions of the first six peaks from a 1-hour acetolysis are listed in the legend to Fig. 3. Total acetolysis (7 hours) of the $NaBT_4$-reduced M_4 fragment (Fig. 4) revealed that 50 percent of it was resistant to further acetolysis, so the amount of the M_4 fragment with an intact 1→6 linkage was reduced by this amount.

Partial acetolysis of mannans with the repeating sequences shown in Fig. 1 should yield oligosaccharides with one intact 1→6 linkage that have different

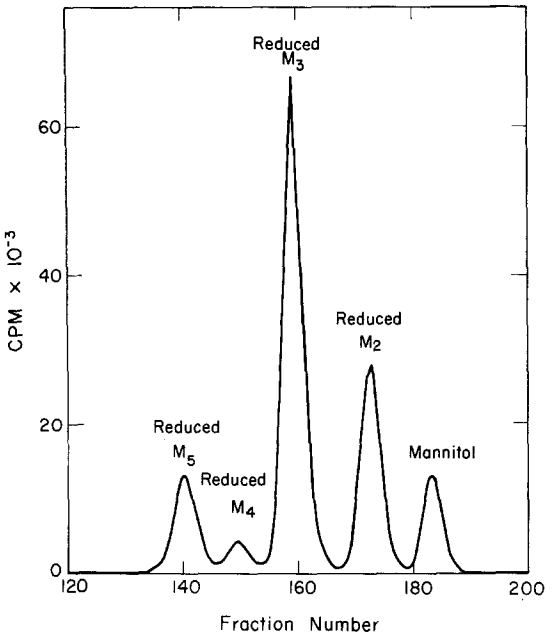


Fig. 6. Reacetolysis (10 hours) of $NaBT_4$-reduced M_5 from Fig. 3. The ratio of tritium in mannitol: reduced M_2: reduced M_3: reduced M_4: reduced M_5 is 1.02: 2.18: 4.07: 0.36: 1.17. After correction for nonspecific acetolysis, the ratio of M: M_2: M_3 became 1.0: 1.96: 8.95. These ratios were used to calculate the values in Table I. The remaining M_4 and M_5 were not characterized.

orders of side chain units. For example, M_5 fragment from sequence A (Fig. 5) would give reduced radioactive M_3 when treated with $NaBT_4$ and then reacetolyzed. Assuming acetolysis to cleave randomly, the M_5 from sequence B should give equal amounts of reduced radioactive M_2 and M_3, whereas similar treatment of the M_5 from sequence C would yield M_2 as the only radioactive fragment. In this preliminary report, only the M_4 and M_5 peaks have been analyzed in this manner. Gel filtration of each reaction product gave the ratios of radioactive fragments listed in the legends of Fig. 4 and 6. The M_4 ratios were normalized to the M_5 ratios from the observation (Fig. 3) that the partial acetolysis pattern revealed four times as much M_5 as M_4. The ratios in Fig. 4 and 6 were corrected for non-specific acetolysis by amounts determined in control experiments with an M_3 that contained only $\alpha 1 \rightarrow 2$ linkages (Fig. 7). The experimental values, along with the ratios predicted from each of the proposed sequences and from a random order of side chains are listed in Table I.

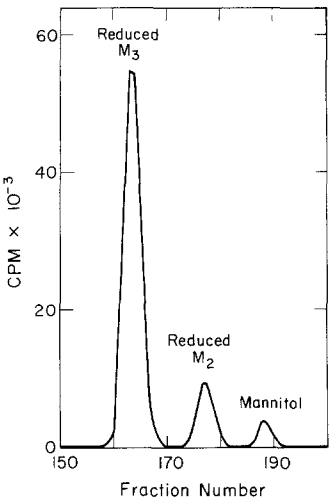


Fig. 7. Reacetolysis (10 hours) of the $NaBT_4$-reduced M_3 peak from Fig. 2. The molar fractions obtained from this experiment were used to correct the other results for nonspecific acetolysis.

The portion of the M_4 peak that underwent further acetolysis gave reduced M_2 (55%), reduced M_3 (36%), and mannitol (9%). The result suggests that the M_4 fragment with one intact 1→6 linkage was from a region of the mannan predominantly, although not exclusively, with sequence C. Similarly, reduced M_3 (75%) was shown to be the major radioactive oligosaccharide from the reacetolysis of reduced M_5 (Fig. 6). Reduced M_2 (17%) and mannitol (8%) were obtained in smaller amount. Thus, most of the M_5, which constituted 7 percent of the oligosaccharide fragments, came from a region of the mannan that resembled sequence A. If, owing to steric control, the acetolysis reaction selectively degraded the M_3→M_2 fragment, sequence B would be indicated.

Acetolysis rates for linkages adjacent to reduced sugars should differ from the rates of nonreduced sugars, owing to a change in the number and type of electronegative groups (10). Since the oligosaccharides were reduced with $NaBT_4$ in the nearest neighbor analysis, it was important to show that the acetolysis reaction still cleaved α1→6 linkages more rapidly than α1→2 linkages. Therefore, the rates of acetolysis of the disaccharides α1→2-mannobiose and α1→6-mannobiose were compared in the 10:10:4 system and 10:10:1 system before

and after reduction (10). The reduced α1→6-mannobiose was cleaved 4.3 times more slowly than the native disaccharide, and the reduced α1→2-mannobiose was acetolyzed 3.9 times faster than the original oligosaccharide. We have already shown that the unreduced α1→6-mannobiose cleaves 300 times faster than the α1→2-mannobiose (10). Consequently, the reduced 1→6 linkage was cleaved 17 times faster than the reduced 1→2 linkage. Although specificity of the acetolysis reaction for the 1→6 linkage is clearly preserved upon reduction, the difference in rates is decreased from that of the unreduced pair.

DISCUSSION

A critical assumption in this study is that the acetolysis reaction cleaves all 1→6 linkages in a relatively unselective way. Although the low yield of tetrasaccharide fragment with an intact 1→6 linkage could reflect the infrequent occurrence of such units in the polysaccharide, it may result from their preferential degradation during acetolysis. This might occur if the shorter side chains in the region of such 1→6 linkages reduced the steric hindrance to attack by the acetolysis reagent. Within any pair of isomeric fragments, such as $M_2 \rightarrow M_3$ and $M_3 \rightarrow M_2$ or $M \rightarrow M_3$ and $M_3 \rightarrow M$, we note that the ratio favors that fragment with the longest side chain at the reducing end of the unit. Whether this would lead to steric hindrance we do not know, but intuitively it seems more reasonable that a substituent on position 2 of the mannose unit undergoing attack would be more critical. By this argument, steric hindrance should favor accumulation of $M_3 \rightarrow M_3$ fragments if they occur, but none was found.

The low yield of tetrasaccharide fragment supports sequence B, Fig. 1. However, this sequence should give equal amounts of the two pentasaccharide fragments, which was not found. Some uncertainty is inherent in the determination of the ratios of the two kinds of pentasaccharide units because of the contribution of nonspecific acetolysis of the reduced oligosaccharides. Whereas the rates of acetolysis of α1→2-mannobiose and α1→6-mannobiose differ by a factor of about 300, the difference for the two reduced disaccharides is only about 17. Thus, specificity for acetolysis of the 1→6 linkage is decreased by reduction,

owing apparently to a change in the number of electronegative groups in the vicinity of the glycosidic bond. We have tried to make corrections for this effect, but their reliability is not certain.

Although this study leaves many questions unresolved, it clearly suggests that the order of side chains in *S. cerevisiae* mannan is not random. Other polysaccharides, such as the O-antigen chain of *Salmonella* lipopolysaccharides and the capsular polysaccharide of *Aerobacter aerogenes*, have repeating sequences of three or four sugars, and it is easy to understand the formation of such repeating sequences in terms of the mechanism of biosynthesis from a lipid-linked oligosaccharide intermediate (14). The synthesis of a repeating sequence for mannan poses a slightly different problem because the units extend to form side chains rather than the backbone of the polysaccharide. There are several ways in which the specificity of glycosyltransferases might be channeled to yield such repeating sequences, and the answer should be attainable from a study of the relevant enzymic reactions in cell-free systems.

An interesting feature of sequence B is that the side chains project to form a symmetrical wave along the backbone. This leads to a regularity of shape that could be important for the hydrogen bonded association of two such polymer chains. Such interaction could serve to immobilize the mannan-proteins in the cell wall (15).

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