Carbohydrate Research, 37 (1974) 23–35

CHARACTERIZATION OF A YEAST D-MANNAN WITH AN α-D-GLUCOSYL PHOSPHATE RESIDUE AS AN IMPORTANT IMMUNOCHEMICAL DETERMINANT*

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(Received January 30th, 1974; accepted April 10th, 1974)

ABSTRACT

Rabbit antiserum against whole heat-killed cells of the yeast *Hansenula polymorpha* gives with the isolated cell-wall D-mannan a precipitin reaction that shows a 50-percent inhibition by α-D-glucosyl phosphate. The antiserum does not cross-react with *Streptococcus faecalis* glycan phosphate, which contains a glucosyl phosphate antigenic determinant, indicating an α-D anomeric configuration for the determinant in the *H. polymorpha* mannan and β-D for that of the *S. faecalis* determinant. The homologous *H. polymorpha* immune reaction is also partly inhibited by large fragments, obtained from the mannan by partial acetolysis, a reaction that cleaves preferentially (1→6) linkages to yield oligosaccharides of mannose up to the pentasaccharide that contain mostly α-D-(1→2) linkages. Digestion of the intact mannan with a bacterial exo-α-mannanase releases 40 percent of the D-mannose, but the acetolysis pattern of the resulting enzyme-resistant fragment is not significantly altered from that of the undigested mannan, thus showing that the structure is different from that of the mannan of *Saccharomyces cerevisiae*, which has an α-D-(1→6)-linked backbone with α-(1→2)- and α-(1→3)-linked side chains. The *H. polymorpha* mannan appears to have a modified block-type structure in which the acetolysis-stable oligosaccharides are connected by (1→6) linkages, some of the (1→2)-linked units forming a part of the backbone. Methylation analysis showed that most of the (1→6)-linked D-mannose residues are otherwise unsubstituted, but the presence of a small amount of 3,4-di-*O*-methyl-D-mannose showed that the branch-points in the mannan involve positions 2 and 6 with an average chain-length of 8 mannose units. P.m.r. of the D-mannan with intact phosphate diester linkages showed the expected signal for the anomeric proton of an α-D-glucosyl phosphate group; the signal disappeared after cleavage of the D-glucosyl phosphate diester bond in acid.

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*Dedicated to the memory of Professor W. Z. Hassid.
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INTRODUCTION

Mannan–proteins are a major constituent of yeast cell-walls. They serve as structural elements, for immobilization of cell-wall enzymes, and as sites for surface recognition. The polysaccharide portion of mannan–proteins can be classified into several types[1]. Many common yeasts have a D-mannan like that of *Saccharomyces cerevisiae*, consisting of a linear backbone chain of α-(1→6)-linked D-mannose residues[2] to which are attached short side chains of (1→2)- and (1→3)-linked residues[3]. Some other yeasts have mannans that lack side-chains and contain (1→3)- and (1→6)-linked residues in a linear array called a "block" structure by Gorin and Spencer[4]. Finally, there are the extracellular mannan phosphates of *Hansenula holstii* and *Hansenula capsulata*, polymers composed of short chains of mannose residues linked through phosphate diester bonds[5] in a manner analogous to the teichoic acids[6].

Gorin and Spencer[7] have classified yeasts by using the proton magnetic resonance spectra of the mannans. In a different approach, we have used chemical[8] and immunochemical[9] analyses for their comparison. These latter studies have shown that the mannans of *Kloeckera brevis*[10,11], *Kluyveromyces lactis*[12], and *Candida parapsilosis*[13] have the *S. cerevisiae*-type structure; whereas the mannans from *Pichia bispora*[13] and *Hansenula wingei*[14] have structures similar to each other and to that of *Hansenula polymorpha* (formerly *Hansenula angusta*)[13]. We now report that the mannan of this last species contains a modified block structure, having α-(1→2)- and α-(1→6)-linked D-mannose residues interspersed in the backbone, with some of the units extending from the backbone to form side chains. Consequently, the acetolysis-stable fragments are located both in side chain and in backbone structures. Immunochemical studies have revealed the presence of α-D-glucosyl phosphate determinants in the D-mannan, a feature also found in that of *H. wingei*[14].

EXPERIMENTAL

Materials. — *Hansenula polymorpha* 52-251 was obtained from Dr. H. J. Phaff, University of California, Davis, and was grown in a complete medium as reported[15]. D-Mannan was purified by extraction of the cells with hot citrate buffer, pH 7.0, followed by precipitation either with an alkaline copper reagent (Fehling's solution)[16] or hexadecyltrimethylammonium bromide (Cetavlon) in the presence of borate ions[17]. The latter procedure avoids strong alkaline and acid conditions that may degrade the D-mannan.

α-D-Mannopyranosyl phosphate was obtained commercially, whereas α- and β-D-glucopyranosyl phosphates were gifts from Dr. W. Z. Hassid in this department. Glucobioses, β-(1→4)-linked D-mannotetraose from ivory nut mannan, and α-(1→6)-linked D-mannotriose from *S. cerevisiae* mannan were obtained from Dr. Louis Rosenfeld in this laboratory. α-D-Man-(1→3)-α-D-Man-(1→2)-α-D-Man-(1→2)-D-Man was prepared by acetolysis of *S. cerevisiae* D-mannan[3]. *Streptococcus faecalis* glycan and antiserum[18] were gifts from Dr. J. H. Pazur, Pennsylvania State University, University Park. All other chemicals were commercially available reagent grade.

Bacterial and chicken alkaline phosphatases (EC 3.1.3.1) and chicken acid phosphatase (EC 3.1.3.2) were obtained from Worthington Biochemical Corp. (Freehold, N.J. 07728). Exo-α-mannanase was purified through the ammonium sulfate step from *Arthrobacter* GJM-1 by the method of Jones and Ballou[19]. The enzyme preparation was free from protease activity but it did hydrolyze maltose. D-Mannan (200 mg) was digested with 200 units of enzyme[20], and after 24 h an additional 100 units of exo-α-mannanase was added. When no additional release of reducing material was observed after 12 h, the reaction mixture was boiled to inactivate the remaining enzyme and then was dialyzed against distilled water.

Methods. — Descending paper chromatography was done on Whatman No. 1 paper in the following solvent systems: (A) 5:3:2 (v/v) ethyl acetate–pyridine–water; (B) 8:2:1 (v/v) ethyl acetate–pyridine–water; (C) 10:3:3 (v/v) 1-butanol–pyridine–water; (D) 18:3:1:4 (v/v) ethyl acetate–acetic acid–formic acid–water; (E) 3:3:1 (v/v) ethyl acetate–acetic acid–water; (F) 20:5:1 (v/v/w) 2-methyl-2-propanol–water–picric acid. Sugars were detected by the alkaline silver nitrate dip-reagent[21] and phosphate ions by spraying with the Hanes–Isherwood reagent[22].

Gel filtration was carried out in water on Bio-Gel P-2 (200–400 mesh) (Bio-Rad Laboratories, Richmond, Calif. 94804) and in 0.1M ammonium acetate on Bio-Gel A5m (Bio-Rad Laboratories). All columns were 2.0 × 180 cm.

Acetolysis was performed according to Kocourek and Ballou[8]. In this procedure, the material was acetylated and treated with the reagent, 10:10:1 (v/v) acetic anhydride–acetic acid–conc. sulfuric acid, at 40° for 12 h. The reaction mixture was neutralized with pyridine and then partitioned between benzene and water. The benzene layer was separated and evaporated to dryness. The residue, which contained acetylated neutral oligosaccharides, was deacetylated in dry methanol with 25mM sodium methoxide. Acidic oligosaccharides were removed during the water wash of the benzene extract, and thus were not present in the final mixture of products.

Both intact D-mannan and acetolysis fragments were characterized by methylation analysis[23]. Whole mannan was methylated twice by the method of Hakomori[24], and then twice according to Kuhn *et al.*[25]. The sample was hydrolyzed, reduced with sodium borohydride and acetylated. Oligosaccharides produced by acetolysis were reduced, methylated twice[25], hydrolyzed, reduced, and acetylated by procedures of Raschke and Ballou[12]. Methylation was judged to be complete because of the absence of mono-*O*-methyl penta-*O*-acetyl derivatives from the methylated intact mannan, and the absence of di-*O*-methyl tetra-*O*-acetyl derivatives from the methylated oligosaccharides. The partially methylated alditol acetates were separated on a column (0.3 × 90 cm) of OV-210 or Carbowax 20M in a Varian 1400 gas chromatograph, coupled to a DuPont 21-491 mass spectrometer.

Proton magnetic resonance (p.m.r.) spectra were determined on a Varian A-60 spectrometer equipped with a Model C-1024 time averaging computer. Samples were prepared by repeated evaporation from deuterium oxide, and measurements were made in deuterium oxide solution. Optical rotations were measured with a Bendix 1100 polarimeter.

D-Mannans were titrated with carbonate-free 10mM sodium hydroxide with a Radiometer Model 4D pH meter. A sample of mannan was dissolved in 10mM hydrochloric acid and divided into two portions. One was heated at 100° for 30 min to hydrolyze the glycosyl phosphate bonds before titration. All values were corrected for those determined on untreated mannan.

Protein content was determined by the method of Lowry *et al.*[26], carbohydrate content by the phenol–sulfuric acid method[27], D-glucose content with the Glucostat Reagent Special (Worthington Biochemicals), total and inorganic phosphate by the method of Bartlett[28], and reducing sugars by the Nelson–Somogyi procedure[29]. Hydrolysis of saccharides was performed in M trifluoroacetic acid for 90 min at 120°; the hydrolyzates were cooled and evaporated repeatedly from water under reduced pressure to remove the acid.

Precipitin reactions were carried out by the method of Ballou[9] with 25 μl of antiserum. Methods for preparation of antisera are given in the same reference.

RESULTS

Structural studies. — All D-mannan preparations from *H. polymorpha* contained mannose and glucose, as revealed by acid hydrolysis and chromatography in solvent systems A, B, and C. Methylation analysis of the intact mannan (Fig. 1) gave the results summarized in Table I. The mannan contained predominantly (1→2) linkages, with about a third of the residues in (1→6) linkage. Most of the latter residues were otherwise unsubstituted and yielded 2,3,4-tri-*O*-methylmannitol. The mean number of residues per branch point (calculated from the ratio of trimethyl to tetramethyl derivatives) was about 8, twice that found for *Saccharomyces cerevisiae* D-mannan[3].

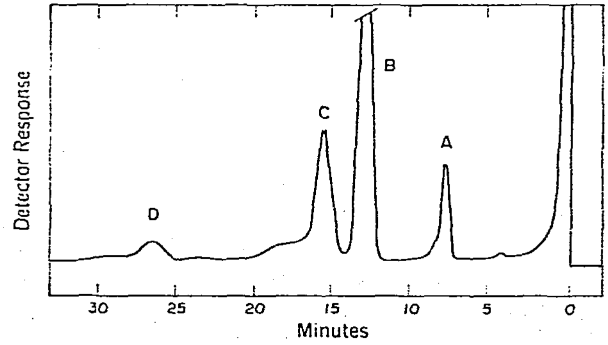


Fig. 1. Gas chromatography of the partially methylated alditol acetates from *Hansenula polymorpha* mannan, preparation A. The peaks identified by mass spectrometry are: (A) 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol; (B) 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol; (C) 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol; and (D) 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methylmannitol.

TABLE I

METHYLATION ANALYSIS OF INTACT *H. polymorpha* MANNAN PREPARATIONS

O-Methylmannitol derivatives[a]	*Linkage*	*Molar ratios*[b]	
		Mannan A	*Mannan C*
2,3,4,6-	Man-(1→	0.9	1.3
3,4,6-	→2)-Man-(1→	4.8	3.5
2,3,4-	→6)-Man-(1→	1.6	2.6
3,4-	→6) →2]-Man-(1→	1.0	1.0

[a] Isolated and determined as per-*O*-acetylated derivatives. The D-glucosyl phosphate group was split during methylation and the *O*-methyl-D-glucose lost during the dialysis. [b] Ratios based on the 3,4-di-*O*-methyl derivative.

Partial acetolysis, which cleaves the (1→6) linkages preferentially[3,30], gave "fingerprints" for this mannan that varied substantially with different preparations made over the five years that we have studied it, in contrast with those of other species which are almost invariant (Fig. 2). We will refer to the various D-mannan preparations by the letters assigned in this Figure. The principal difference was in the

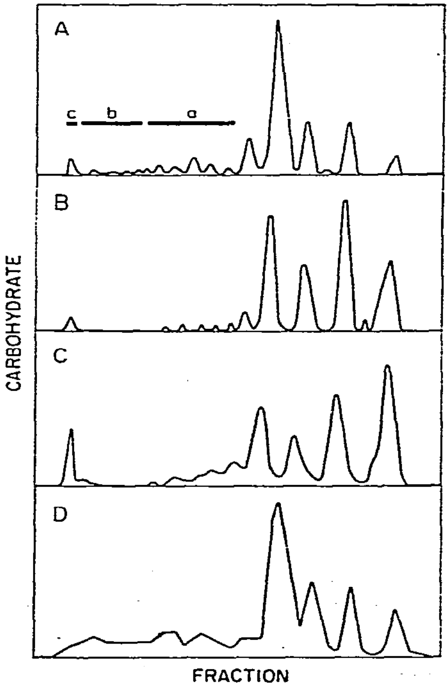
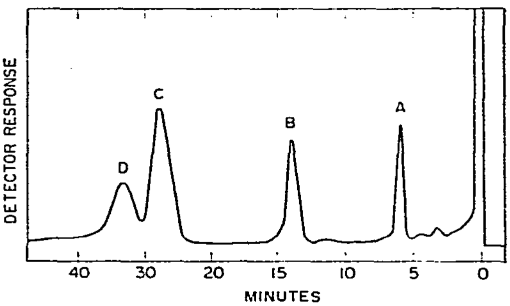


Fig. 2. Acetolysis patterns of several *H. polymorpha* mannan preparations that illustrate variability in structure. The major peaks from right to left are monosaccharide, mannobiose, mannotriose, mannotetraose, and mannopentaose. All the patterns show small amounts of larger fragments.

relative amount of the mannotetraose fragment. The presence of acetolysis oligosaccharides larger than mannopentaose is another unusual feature. The di-, tri-, tetra-, and penta-saccharides revealed a single component on chromatography in solvent systems A and D. The monosaccharide peak contained both mannose and glucose. Mannose was the only product obtained by acid hydrolysis of the oligosaccharides. Methylation analysis of the di-, tri-, and tetra-saccharide peaks showed only (1→2) linkages. However, the pentasaccharide fragment from mannan A contained a (1→3) linkage (Fig. 3).



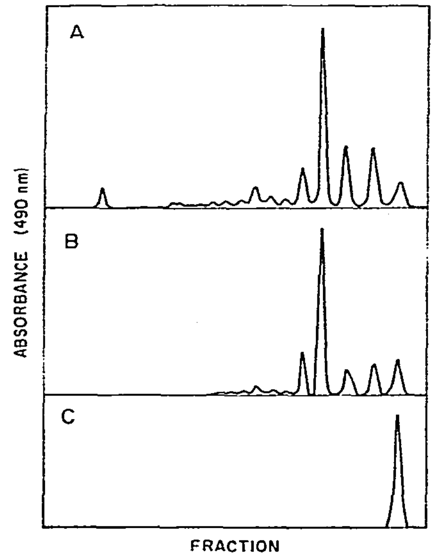


Fig. 3. Gas chromatography of the partially methylated alditol acetates from the pentasaccharide isolated by acetolysis of mannan preparation A. The peaks identified by mass spectrometry are: (A) 2-*O*-acetyl-1,3,4,5,6-penta-*O*-methylmannitol; (B) 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol; (C) 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol; and (D) 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol.

Fig. 4. Comparison by gel filtration on Bio-Gel P-2 of the acetolysis patterns of intact *H. polymorpha* mannan (A) and exo-α-mannanase-digested mannan (B). The fragments from acetolysis of intact mannan were converted completely to D-mannose by the action of the enzyme (C).

The mannans of *H. polymorpha* have mostly α-D linkages as shown by a high-positive specific rotation. The $[\alpha]_{546}$ of mannan A was +92.8°, of mannan B +81.2°, and of mannan C +105°. Furthermore, the p.m.r. spectra[3,7] revealed no β-D anomeric signals in either the intact mannans or in the acetolysis oligosaccharides.

The high percentage of unsubstituted mannose residues with (1→6) linkage found by methylation suggests that the *H. polymorpha* D-mannan is not of the (1→6)-linked-backbone and (1→2)-linked-side-chain type. To test this hypothesis, the D-mannan was digested with the exo-α-mannanase[2] from Arthrobacter GJM-1. This enzyme hydrolyzes α-(1→2)- and α-(1→3)-linked side-chains of *S. cerevisiae* D-mannan, but leaves the α-(1→6)-linked backbone essentially intact[20]. Phosphorylated D-mannosyl residues are not attacked. The nondialyzable, enzyme-resistant fraction was subjected to acetolysis, and the gel-filtration profile of the resulting neutral saccharides is shown in Fig. 4B. No significant change from the pattern for intact mannan was observed (compare Fig. 4A and 4B). Digestion with exo-α-mannanase of the pooled, deacetylated products of acetolysis resulted in their complete hydrolysis to D-mannose (Fig. 4C), which shows that the original resistance to enzymic digestion did not arise from the presence of β-D linkages or other refractory components in the oligosaccharides.

We were unable do demonstrate that phosphate groups were responsible for the protection of the mannanase-resistant core, because neither acid nor alkaline phosphatase would remove the phosphate groups from mild acid-treated mannan. Other mannan phosphates are also resistant to these enzymes[10].

Nature of the phosphate moiety. — Titration of intact mannan preparations A and B indicated that the phosphate groups are diesterified (Fig. 5). The titration curve for the mild acid-hydrolyzed mannan showed that a substantial portion of the

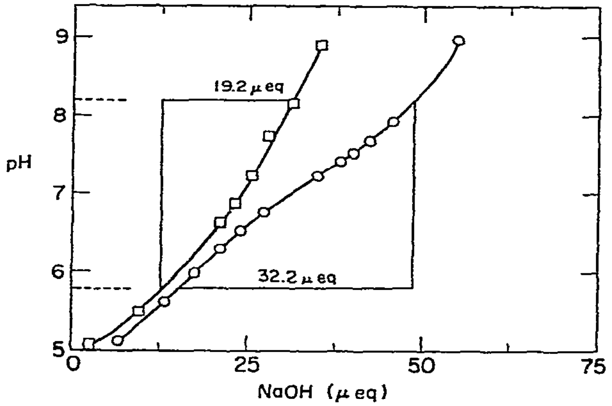


Fig. 5. Titration curves of intact (□) and mild acid-hydrolyzed (○) *H. polymorpha* mannan which demonstrate the conversion from di- to mono-esterified phosphate. Each sample contained 24 μequiv. of phosphate group.

TABLE II

ACID LABILITY OF PHOSPHATE ESTER LINKAGE IN *H. polymorpha* MANNANS

Mannan preparation	*Method of purification*	*Molar ratios*			
		Man/P	*Man/Glc*	*Acid-labile P/ total P*	*Glc released/ acid-labile P*
A	Fehling	30	24	0.78	0.68
B	Cetavlon	13	14	0.55	0.96
C	Fehling	13			

phosphate groups was converted from a di- into a mono-ester form (Table II). The elution profile of the mannan on a Bio-Gel A5m column was not altered by mild acid hydrolysis, indicating that other glycosidic bonds were not hydrolyzed. Gel filtration of the products of acid hydrolysis on Bio-Gel P-2 revealed only a monosaccharide peak and carbohydrate that was eluted in the void volume. Chromatographic analysis in solvent systems A and C of the same material showed that glucose, and no other sugar molecule smaller than a hexasaccharide, was present. The amount of D-glucose released was measured enzymically (Table II). The known lability of the phosphate diester linkage and the concomitant release of D-glucose suggest that the sugar was attached by a D-glucosyl phosphate bond.

The proton magnetic resonance spectrum in the region between τ 4.6 and 5.0 showed two strong signals for the α-(1→2)- and α-(1→6)-linked D-mannose residues. At τ 4.5 appeared two poorly resolved doublets, separated by about 8 Hz, corresponding to the anomeric proton of the α-D-glucosyl phosphate groups which should be split about 2 Hz by H-2 and by 8–10 Hz by the phosphorus atom[10]. This signal, the area of which was about 6 percent of that of the other anomeric protons, was absent in the spectrum of the mannan subjected to mild acid hydrolysis.

Smith degradation[31] of the intact D-mannan was employed to characterize the other position to which the phosphate group was linked. A nonreducing terminal D-mannose residue with a phosphate ester at C-6 would yield glycerol 1-phosphate, whereas derivatives having a phosphate group at C-4 would result in erythritol 2-phosphate, at C-3 would give D-mannose 3-phosphate, and at C-2 would give glyceraldehyde 2-phosphate. The D-mannan (30 mg) was oxidized in 40mM sodium periodate for 40 h at 20° during which 1.6 moles of periodate were consumed per mole of hexose. The reaction mixture was processed according to Thieme and Ballou[10], and the degradation products were chromatographed in systems A, E, and F. Glycerol 1-phosphate was the only phosphorylated material detected. Thus, the linkage appears to have been to C-6 of another hexose residue in the D-mannan.

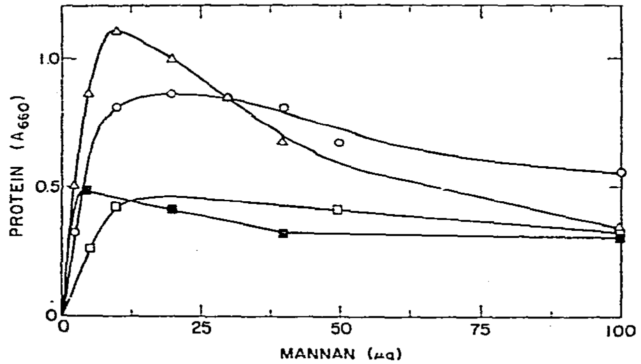


Fig. 6. Precipitin curves of various yeast mannans with anti-*H. polymorpha* sera. The mannans are (○) *H. polymorpha* preparation A, (△) *H. polymorpha* preparation B, (□) *H. wingei*, and (■) *Pichia bispora*.

Immunochemistry. — Antisera[9] prepared against the yeasts from which D-mannans A and C were isolated had identical specificities. *Hansenula wingei* and *Pichia bispora* mannans showed 50 percent cross-reaction (Fig. 6). The anti-*H. polymorpha* sera did not cross-react with *S. cerevisiae* X2180 mannan or with *Streptococcus faecalis* glycan phosphate, a polymer known to contain a D-glucosyl phosphate determinant[18]. Thus the D-glucosyl phosphate anomeric linkage in this glycan phosphate is probably of configuration opposite to that in the mannan. *H. polymorpha* mannan A showed a 10-percent cross-reaction with anti-*H. wingei*, a yeast which also contains an α-D-glucosyl phosphate determinant[14], but it did not cross-react with antiserum against the *Streptococcus faecalis* glycan.

The specificity of the homologous precipitin reaction was investigated by inhibition studies (Fig. 7). α-D-Glucopyranosyl phosphate inhibited 50 percent of the reaction, as did the high-molecular-weight acetolysis fragments A, B, and C shown in Fig. 2. Acetolysis oligosaccharides, penta and smaller saccharides, had no inhibitory activity. A combination of α-D-glucopyranosyl phosphate and the high-molecular-weight acetolysis products gave complete inhibition (Fig. 7). Compounds that showed no inhibitory activity included D-mannose, D-glucose, β-D-glucopyranosyl phosphate, D-mannose 6-phosphate, D-glucose 6-phosphate, *S. cerevisiae* D-mannotetraose, α-(1→6)-D-mannotriose, β-(1→4)-D-mannotetraose, maltose, nigerose, kojibiose, isomaltose, sophorose, laminaribiose, cellobiose, and gentiobiose.

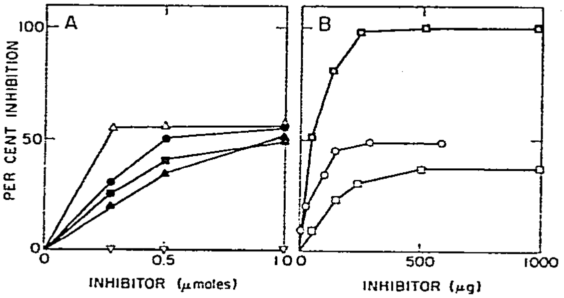


Fig. 7. Inhibition by mannan fragments of the homologous precipitin reaction of *H. polymorpha*. In the acetolysis procedure, the acetylated fragments were separated into a water-soluble fraction that presumably contained charged molecules, and a benzene-soluble fraction that contained neutral, acetylated oligosaccharides. Following deacetylation, the latter were fractionated by gel filtration whereas the former was not further resolved. The inhibitors are in part A: (△) water-soluble fraction of acetolyzate; (●) benzene-soluble fraction of acetolyzate (fraction C of Figure 2A), (■) fraction B of Figure 2A, (▲) fraction A of Figure 2A, and (▽) all other inhibitors, including acetolysis oligosaccharides; in part B: (□) total acetolyzate, (○) α-D-glucopyranosyl phosphate, ⧇ total acetolyzate plus α-D-glucopyranosyl phosphate.

Mild acid-hydrolyzed[10] or mannanase-treated[2] mannan reacted half as well as the native antigen in the homologous precipitin system, and the precipitation of both could be inhibited completely by the larger acetolysis fragments[13]. Apparently both treatments resulted in hydrolysis of the D-glucosyl phosphate bond, the latter because the mannanase preparation contained a glucosidase contaminant. *H. polymorpha* mannan treated with 0.1M sodium hydroxide at 25° for 30 min was precipitated to the same extent as the untreated mannan. Because complete inhibition of the precipitin reaction with intact mannan was not obtained either with α-D-glucopyranosyl phosphate or with the acetolysis fragments, we conclude that the reaction involves at least two determinants.

DISCUSSION

Four D-mannan preparations of different composition have been isolated from the same *H. polymorpha* strain over a period of several years. They varied in the ratios of mannose residues to phosphate groups and in their acetolysis fingerprints[8]. However, the macromolecular structures have remained much the same. The methylation products of the intact mannans have been constant in composition (Table I), and all of the D-mannans contained a majority of α-(1→2) and α-(1→6) linkages. The phosphate group was uniformly diesterified. The immunochemical properties were the same; antisera against mannans A and C gave identical precipitin and inhibition reactions with mannans A, B, and C.

The change in the acetolysis patterns is related to the time of isolation of the mannan. Preparation D was made in 1968 and was purified by precipitation with alkaline copper reagent. The predominant component is the tetrasaccharide. The same is true of preparation A, which was made in 1969. In 1972, when we isolated preparation B, the relative amounts of the acetolysis fragments had altered. There was less tetrasaccharide and an increased proportion of the di- and mono-saccharides. There was also a decrease in the mannose to phosphate ratio (Table II). The same results were obtained with mannans purified by both methods. The unexpected findings led us to obtain a new stock culture from Dr. Phaff. Preparation C was made from this organism in 1973. The acetolysis fingerprint of this mannan is similar to that of preparation B, except that there is a larger proportion of monosaccharide. The mannose to phosphate ratio was similarly lowered.

The differences in the *H. polymorpha* mannans have not been traceable to differences in the growth of the culture from which each was prepared. It appears that some component of the mannan with a large amount of neutral acetolysis products is absent from our more recent preparations, and as a result the acetolysis pattern has changed and the mannose to phosphate ratio decreased. It is known that this yeast changes readily from diploid to haploid forms during growth[32], and it is possible that we have obtained mannan from these two different forms on different occasions.

The analyses show that *H. polymorpha* mannan does not have a uniform, (1→6)-linked backbone with (1→2)-linked side chains. The methylation data give an average chain length of eight units, whereas the acetolysis products show that the average number of sequential (1→2) linkages is three to four. Thus, there must be (1→6) linkages interspersed with the (1→2) linkages. This condition is met by the models of Fig. 8. The model B (Fig. 8) is unlikely, for it is inconsistent with the mannanase digestion experiments. The enzyme should remove all of the side chains from such a molecule and leave the α-(1→6)-linked backbone intact. On the other hand, structure A (Fig. 8) is reasonable, because the exo-α-mannanase should digest the mannan up to an inhibiting structure nearer the core. Since all of the structural units are present in the core of this mannan model, no one class of oligosaccharide would be digested preferentially, and the acetolysis pattern would be essentially unaltered. Exo-α-mannanase digestion does not substantially alter the acetolysis pattern of the mannan; therefore, the resistant core must have the same overall structure as the native material.

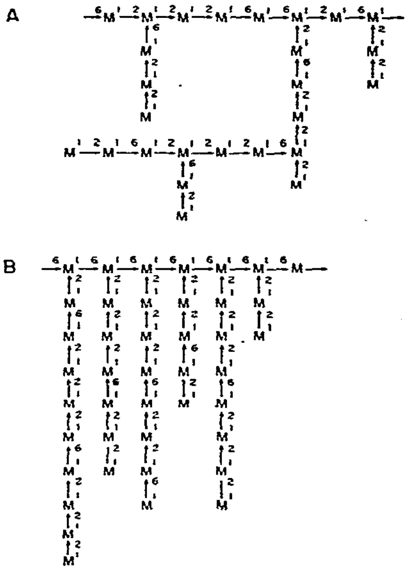


Fig. 8. Possible models of the structure of the neutral polysaccharide of *H. polymorpha* D-mannan. The α-D-glucosyl phosphate groups are not shown, because their location in the mannan is not established.

The fact that very little mannose is released by acetolysis following digestion with the exo-α-mannanase demonstrates that a substantial proportion of the (1→6)-linked residues are not in sequence and confirms the absence of a (1→6)-linked backbone. If present, it must constitute only 16 percent of the total residues, which corresponds to the fraction of branch-point residues detected by methylation. The other (1→6)-linked residues would be placed between the (1→2)-linked oligosaccharides.

The majority of the phosphate groups in the mannan appears to be diesterified. From the titration data, about 60 percent of the phosphate groups can be converted into the monoester form by mild acid hydrolysis, with the simultaneous release of D-glucose in an amount equimolar to the secondary phosphoric protons that become titratable (Table II). The results of Smith degradation indicated that the other phosphate linkage is to C-6 of the hexose units in the mannan polymer. This assignment of a D-glucosyl phosphate structure was confirmed by the immunochemical study, which showed that α-D-glucopyranosyl phosphate was a good inhibitor of the precipitin reaction between intact mannan and the specific antiserum. The α-D-glucosyl linkage was supported by the proton magnetic resonance spectrum that showed a signal in the characteristic position of the α-D-glycosyl phosphate anomeric proton, downfield from the other anomeric proton signals. It appeared to consist of a quartet with two poorly resolved doublets split by about 8 Hz owing to coupling to the phosphorus atom[10].

The immunochemistry of this D-mannan is unusual in two respects. First, the α-D-glucosyl phosphate group is an important determinant. Second, the rest of the determinants are resistant to acetolysis, mild acid and base hydrolysis, and exo-α-mannanase digestion. α-D-Glucosyl phosphate has been reported previously as an immunogenic determinant in *Hansenula wingei*[14]. This determinant in *H. polymorpha* mannan is destroyed by mild acid hydrolysis, which results in a 50 percent decrease in the precipitin reaction and a concomitant rise in the degree of inhibition by the large acetolysis fragments. The identical effect of mannanase digestion is due apparently to hydrolysis of the D-glucosyl phosphate bonds by a contaminating glucosidase.

Pazur *et al.*[18] have isolated a glycan phosphate from *Streptococcus faecalis* strain N that contains a D-glucosyl phosphate antigenic determinant, but the anomeric configuration of the D-glycosyl phosphate bond was not reported. This glycan phosphate does not cross-react with antiserum prepared against *H. polymorpha*, nor does the antiserum against the glycan phosphate react with *H. polymorpha* or *H. wingei* mannans. Thus, the *S. faecalis* determinant probably has the β-anomeric configuration. *H. wingei* mannan does cross-react with anti-*H. polymorpha* serum, and this must involve the α-D-glucosyl phosphate determinant that they have in common, because the cross-reaction was completely inhibited by α-D-glucopyranosyl phosphate. However, antiserum prepared against *H. wingei* does not contain a large antibody population directed against this determinant, because the homologous precipitin reaction is inhibited at less than 10 percent by α-D-glucopyranosyl phosphate, and *H. polymorpha* mannan cross-reacts only weakly. Clearly, the α-D-glucosyl phosphate determinants plays a quantitatively different role in these two yeasts.

ACKNOWLEDGMENTS

We thank Dr. S. Maitra for assistance with the p.m.r. spectra. This work was supported by grants from the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, U.S.P.H.S. (AM-884) and from the National Science Foundation (GB-35229X).

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