Structure and Immunochemistry of *Hansenula wingei* Y-2340 Mannan†

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ABSTRACT: As a preliminary to characterization of the sexual agglutination factors of the yeast *Hansenula wingei* NRRL Y-2340, the total cell wall mannan from the haploid mating type 5 cell was analyzed by chemical and immunochemical methods. Methylation of the mannan showed that it was highly branched. Acetolysis of the polysaccharide gave five neutral fragments, mannose, mannobiose, mannotriose, mannotetraose, and mannopentaose, and methylation of these oligosaccharides revealed that they were isomeric mixtures which differed in the position and the amount of 1→2 and 1→3 linkages. The optical rotations and the nuclear magnetic resonance spectra established that the mannose units in the oligosaccharides were α linked. The oligosaccharides formed by acetolysis were partially protected from *exo*-α-mannanase digestion while in the intact mannan, either by substitution of the mannose units with some acid-labile group or by the linkage of these oligosaccharides in the molecule. Thus, in contrast to the 1→6-linked backbone in *Saccharomyces cerevisiae* mannan, the Y-5 mannan appears to have a main chain in which 1→2, 1→3, and 1→6 linkages all are present. Less than 10% of the carbohydrate was released as oligosaccharides by alkaline treatment (β elimination), indicating that most of the mannose was present in the form of large polysaccharide units probably attached to asparagine in the protein. Immunochemical studies of Y-5 mannan revealed that about 30% of the homologous precipitin reaction involved the terminal αMan(1→3)αMan(1→3)Man trisaccharide unit of the side chains, whereas the remaining 70% involved some acid-labile structure in the mannan. Part of the acid-labile antigenic reactivity was accounted for by the α-D-glucosyl phosphodiester determinant but the chemical structure of the rest is not known.

The yeast *Hansenula wingei* NRRL Y-2340 is known for the strong sexual agglutination reaction that occurs when the two cells of opposite mating type, called 5 and 21 cells, are intermixed (Wickerham, 1956). This agglutination is due to complementary molecules on the surfaces of the two cell types (Crandall and Brock, 1968), and the system provides a general model for the study of specific cell–cell interaction. An agglutinin is released from the surface of 5 cells by proteolytic enzymes, and the soluble factor is a mannan–protein complex containing 4% protein (Taylor and Orton, 1967). The cell wall of many yeasts contains a mannan–protein coat (Matile *et al.*, 1969), and the characterization of the agglutination process requires distinction between the specific agglutinin and the total cell wall mannan.

The mannan component is the principal antigen of the yeast cell (Hasenclever and Mitchell, 1964), and its structure is strain specific. Mannan from *Saccharomyces cerevisiae* consists of a linear α(1→6)-linked mannose backbone to which side chains are attached by α(1→2) and α(1→3) linkages (Lee and Ballou, 1965; Jones and Ballou, 1968). A similar structure occurs in other yeast mannans (Gorin and Spencer, 1970a). Alternative structures, such as a branched mannan with a backbone of mixed α(1→3) and α(1→6) linkages (Gorin and Spencer, 1970b), unbranched mannan with a chain of alternating 1→3 and 1→4 linkages (Gorin *et al.*, 1965), and β mannans (Sikl *et al.*, 1970), have also been found. Many yeast mannans also contain phosphate in diester linkage between short oligosaccharides, forming long polymers (Jeanes *et al.*, 1961). In other mannans, the phosphate links mono- and disaccharide units of mannose as side chains attached to the main chain (Thieme and Ballou, 1971). In mannan–proteins, the carbohydrate is attached as short oligosaccharide chains to asparagine (Phaff, 1971).

This report describes the partial chemical and immunological characterization of the cell wall mannan from *H. wingei* 5 cells. This mannan is a branched polysaccharide in which the backbone contains α(1→2), α(1→3) and α(1→6) linkages. The mannose in the side chains is substituted by an acid-labile group of unknown nature which forms the principal antigenic determinant in the molecule. In the following paper are reported the chemical characterization of the 5-agglutinin and its comparison with the total cell wall mannan.

Experimental Section

Materials. Cultures of *Hansenula wingei* NRRL Y-2340 diploid, and the haploid mating types 5 and 21, were obtained from C. P. Kurtzman of Northern Utilization Research and Development Division of U. S. Department of Agriculture. Mannans were isolated from these cells by precipitation with ethanol (Stewart and Ballou, 1968) or Fehling's solution (Kocourek and Ballou, 1969). Samples of α- and β-D-glucose 1-phosphate were obtained from W. Z. Hassid, and α-D-mannose 1-phosphate came from W. C. Raschke, Department of Biochemistry, University of California, Berkeley. 2,4,6-Tripyridyl-*s*-triazine was from G. F. Smith Chemical Co. and sodium borotritide (200 Ci/mol) from New England Nuclear. Bio-Gel P-4 (200–400 mesh) and A 5m (200–400 mesh) were obtained from Bio-Rad Corp., and DEAE-Sephadex A-25 and Sephadex G-100 from Pharmacia.

General Procedures. Total carbohydrate was measured by the phenol–sulfuric acid method (Dubois *et al.*, 1956), total phosphate by the procedure of Bartlett (1959), and reducing sugar according to Paleg (1959). Periodate was measured with 2,4,6-tripyridyl-*s*-triazine (Avigad, 1969), and protein was

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determined by the Lowry method (Lowry *et al.*, 1951), using bovine serum albumin as the standard.

Descending paper chromatography was carried out on Whatman No. 1 filter paper using the following solvent systems (in volume ratios): (A) 1-butanol–pyridine–water (10:3:3); and (B) ethyl acetate–pyridine–water (5:3:2). Whatman No. 3 MM paper was used for preparative chromatograms. Sugars and sugar alcohols were detected on paper chromatograms with the silver nitrate–sodium hydroxide reagent (Trevelyan *et al.*, 1950), phosphate with the Hanes–Isherwood reagent (Bandurski and Axelrod, 1951), and amino acids and amino sugars with 1% ninhydrin in acetone.

Proton magnetic resonance spectra were measured at room temperature with a Varian A-60 spectrometer using tetramethylsilane as an external standard. Gas chromatography of partially methylated mannose derivatives was carried out at 195° on a 5-ft Carbowax 20M column on a Varian Aerograph 1200 instrument, while partially methylated alditol acetates were analyzed on a 2.5-ft Carbowax 20M column on a Varian Aerograph 1400 instrument equipped with a Du Pont 21-491 mass spectrometer operating at an ionizing voltage of 70 eV. Amino acid analysis was carried out on a Beckman Model 120C amino acid analyzer. Optical rotation was determined with a Series 1100 Bendix automatic polarimeter, absorbances were read on a Zeiss PMQ or B & L Spectronic 20 spectrophotometer and radioactivity was measured on a Packard TriCarb liquid scintillation spectrometer.

Growth of Cells. Hansenula wingei Y-2340 diploid and mating types 5 and 21 cells were grown with aeration at 30° in a medium containing 5 g of KH_2PO_4, 7 g of Difco yeast extract, and 30 g of glucose per liter of water. For slants, 2% Bacto-agar was added.

Acetolysis. Acetylation and acetolysis of mannan were carried out according to Kocourek and Ballou (1969) except that 10 ml of dry pyridine and 15 ml of acetic anhydride were used to acetylate 100 mg of mannan. The higher concentration of acetic anhydride was needed to promote acetylation of the phosphomannan.

Methylation of oligosaccharides was done with methyl iodide and silver oxide in dimethylformamide (Kuhn *et al.*, 1955), and the products were methanolyzed in 2% methanolic hydrogen chloride at 100° for 24 hr. Mannan was methylated first according to Hakomori (1964), followed by the Kuhn procedure (Kuhn *et al.*, 1955). The methylsulfinyl anion was prepared according to Sandford and Conrad (1966), and the methylation conditions followed those of Hellerqvist *et al.* (1968). Methylated mannans were purified by dialysis against water and were hydrolyzed according to Garegg and Lindberg (1960). The hydrolysates were neutralized with $BaCO_3$ and the solids were removed by centrifugation. For gas chromatography, the products were converted to alditol acetates by reduction with sodium borohydride followed by acetylation with acetic anhydride and pyridine.

Acid hydrolysis of mannan was carried out in 2 N HCl at 100° for 4 hr. The hydrochloric acid was removed by rotary evaporation under vacuum and the residue was taken up in water and chromatographed on paper with solvent system A. In the amino acid analyses, hydrolysis was done at high dilution (1 mg of mannan in 5 ml of 6 N HCl) to minimize reaction between amino acids and sugars. The hydrolysis was performed at 110° for 24 hr, and the rate of destruction of individual amino acids was determined by hydrolysis of mannan for times from 12 to 72 hr. All hydrolyses were done in sealed tubes under nitrogen.

β Elimination of mannans was done by dissolving 30 mg of mannan in 100 ml of 0.1 N NaOH containing 0.3 M $NaBH_4$ (or a few crystals of $NaBT_4$). The reaction was left at room temperature for 24 hr and was stopped by neutralization with 1 N acetic acid. For amino acid analysis of the products, the reaction mixture was dialyzed extensively against water before being hydrolyzed with 6 N HCl. For studies on the polysaccharide chains that were released, the reaction mixture was concentrated on a rotary evaporator. Boric acid was removed by repeated addition of methanol and evaporation to dryness. The residue was taken up in 0.1 M NH_4HCO_3 and applied to a Bio-Gel A 1.5m column (2 × 100 cm) or P-4 column (2 × 200 cm) which was eluted with 0.1 M NH_4HCO_3.

Enzymes. Exo-α-mannanase was purified from Arthrobacter GJM-1 through the 60% ammonium sulfate step (Jones and Ballou, 1968). Protease-free exo-α-mannanase was prepared by further purification on a Sephadex G-100 column. Exo-α-mannanase assay and the digestion of mannan with the enzyme were carried out according to Jones and Ballou (1968). Proteolytic activity was assayed with denatured hemoglobin as the substrate (Davis and Smith, 1955).

Immunological Methods. Antisera against 5 and 21 cells were prepared by injecting heat-killed cells (1 mg of lyophilized cells in 1 ml of saline) into 2–3-kg rabbits through the marginal ear vein three times a week for 1 month. After resting the animals for 1 month, the injection procedure was continued for another month. The rabbits were bled 1 week after the last injection, and the blood was allowed to clot at room temperature overnight. The clot was removed and the remaining cells were separated by centrifugation. Merthiolate was added as a preservative.

Precipitin reactions between antisera and mannan were done according to Ballou (1970), with 15 μl of serum in a final volume of 0.5 ml. For inhibition studies of the precipitin reaction, 20 μg of mannan was used.

Results

Chemical Composition. Mannan from *H. wingei* cells (Y-5 mannan) contained more than 90% carbohydrate, 2–5% protein, and variable amount of phosphate. Mannose was the major carbohydrate component, although traces of glucose and glucosamine were present. The mannose to phosphate molar ratio varied between 15 and 30. One-third of the amino acid residues in Y-5 mannan were serine and threonine (Table I). Alanine was the next most abundant amino acid, while very few basic amino acids were found. The presence of glucosamine suggested that some of the carbohydrate chains in the mannan molecules might be linked to the protein through the *N*-aspartylglucosylamine linkage (Neuberger *et al.*, 1966).

Acetolysis of Y-5 mannan, under conditions in which the 1→6-linked glycosidic bonds were broken preferentially (Lee and Ballou, 1965) gave five neutral fragments which were separated on a Bio-Gel P-4 column (Figure 1). These corresponded to mannose, mannobiose, mannotriose, mannotetraose, and mannopentaose. Mannan from the diploid and 21 cells gave similar acetolysis patterns, although the relative amounts of the oligosaccharides varied slightly with the strain and with the growth phase.

The acetolysis oligosaccharides gave only mannose on acid hydrolysis. Three peaks were observed in the gas chromatographic patterns of their methylated products, which corresponded to methyl 2,3,4,6-tetra-*O*-methylmannoside, methyl 3,4,6-tri-*O*-methylmannoside, and methyl 2,4,6-tri-*O*-methylmannoside. The molar ratios of these methyl derivatives dif-

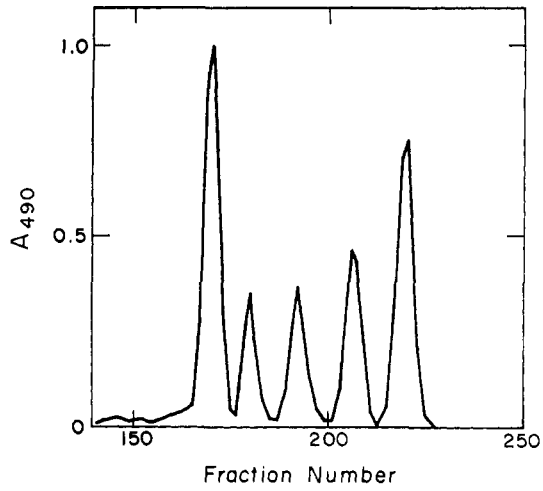

FIGURE 1: Neutral acetolysis fragments of *H. wingei* mannan from early stationary phase Y-5 cells. The peaks, from right to left, correspond to mannose, mannobiose, mannotriose, mannotetraose, and mannopentaose. A 2 × 200 cm column of Bio-Gel P-4 (200–400 mesh) was used and was eluted with water. The tracing represents the weight ratios based on the carbohydrate assay.

fered in the oligosaccharides (Table II). The presence of both trimethyl derivatives in the methylation products of the disaccharide indicated that both Man(1→2)Man and Man(1→3)Man were present. There are four possible combinations of 1→2 and 1→3 linkages in mannotriose: Man(1→3)Man(1→3)Man, Man(1→2)Man(1→2)Man, Man(1→3)Man(1→2)Man, and Man(1→2)Man(1→3)Man. The presence of Man(1→3)Man(1→3)Man is without question, since there was more 1→3 linkage than 1→2 linkage in the mannotriose sample. Methylation analysis of the reduced mannotriose gave both 3,4,6-tri-*O*-methyl and 2,4,6-tri-*O*-methyl derivatives in a ratio of 1:3, indicating that the mannose at the nonreducing end was linked to the internal

TABLE I: Amino Acid Composition of Y-5 Mannan Before and After β Elimination.

Amino Acid	Percentage[a]	
	Before	After
Aspartic acid	6.32	8.72
Threonine	11.20	5.15
Serine	27.57	8.70
Glutamic acid	7.34	9.13
Proline	2.08	2.28
Glycine	5.55	7.75
Alanine	14.50	26.36
α-Aminobutyric acid	0.0	1.78
Valine	7.71	8.53
Methionine	0.17	0.00
Isoleucine	3.05	3.60
Leucine	3.31	4.11
Tyrosine	2.67	3.16
Phenylalanine	1.46	1.82
Histidine	0.61	0.93
Lysine	1.69	1.65
Arginine	0.95	1.23
Glucosamine	3.78	5.03

[a] Residues per 100 residues.

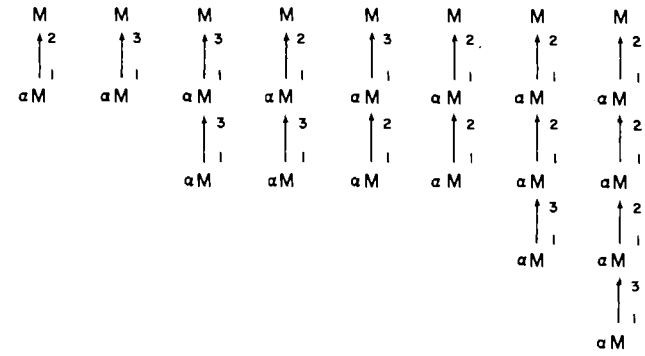

FIGURE 2: Structures of the acetolysis oligosaccharides obtained from Y-5 mannan.

mannose through both 1→2 and 1→3 linkages. In mannotetraose and mannopentaose, the 1→2 linkage predominated. The immunochemical studies reported below suggest that a 1→3 linkage is located at the nonreducing end in the latter two fragments. The structures of all of the fragments are illustrated in Figure 2.

Table III gives the specific rotations of the *H. wingei* acetolysis fragments. For comparison, optical rotations of the *S. cerevisiae* oligosaccharides and the structures proposed by Lee and Ballou (1965) are also shown. The high positive values indicate that the mannose units in the oligosaccharides were α linked. Proton magnetic resonance spectra of *H. wingei* oligosaccharides revealed the expected number of anomeric hydrogen signals in the region τ 4.5–5 and confirmed the finding that these sugars contained only α linkages (Van der Veen, 1963).

Mild Acid Treatment. Gel filtration of Y-5 mannan on a Bio-Gel A 5m column gave a void volume peak and a broad peak included in the column (Figure 3A). Treatment of the material in fractions 37–45 with 0.1 N HCl at 100° for 30 min released 5.5% of the carbohydrate in dialyzable form, and the molecular weight of the nondialyzable part decreased significantly (Figure 3B). Gel filtration on a P-4 column, and paper chromatography with solvent B, revealed that the dialyzable material contained mannose, glucose, mannobiose, some phosphorylated oligosaccharides, and glucosamine-containing oligosaccharides. If 0.01 N HCl was employed instead of 0.1 N HCl, the molecular weight did not change much (Figure 3C), and paper chromatography of the dialysate revealed glucose and an unidentified spot.

Mannanase Digestion of Y-5 Mannan. Exo-α-mannanase released 22% of the carbohydrate in dialyzable form from Y-5 mannan after 30-hr digestion. Enzymic digestion of mannan, previously treated with acid (0.1 N HCl, 100°, 30 min), released 44% of the carbohydrate. Table IV compares the molar ratios of the acetolysis products of the original mannan, the mild acid-treated mannan, and the mannanase-resistant mannan. Neither mild acid treatment nor mannanase

TABLE II: Methylation Analysis of Acetolysis Products of Y-5 Mannan.

Methyl Mannoside Derivative	Molar Ratios			
	Man_2	Man_3	Man_4	Man_5
2,3,4,6-Tetra-*O*-methyl	1.00	1.00	1.00	1.00
3,4,6-Tri-*O*-methyl	0.40	0.82	2.27	2.70
2,4,6-Tri-*O*-methyl	0.67	1.45	0.58	1.07

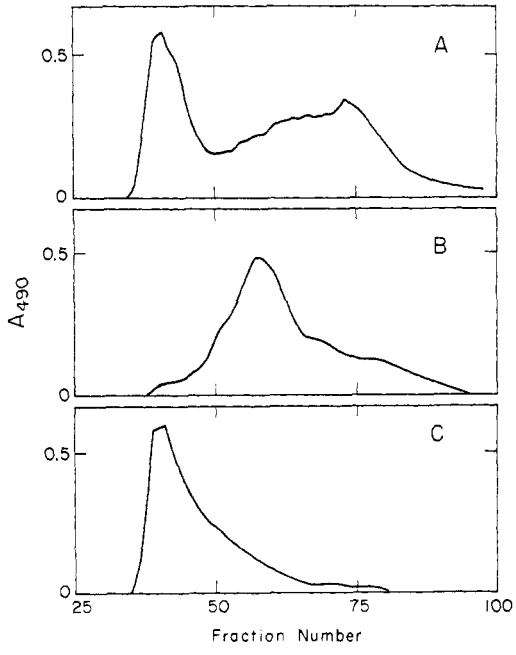

FIGURE 3: (A) Gel filtration of Y-5 mannan on a Bio-Gel A 5m column (2 × 200 cm) eluted with 0.1 N NH_4HCO_3. The void peak material was then used for treatment with (B) 0.1 N HCl, or (C) 0.01 N HCl, both at 100° for 30 min, and rerun on the same column.

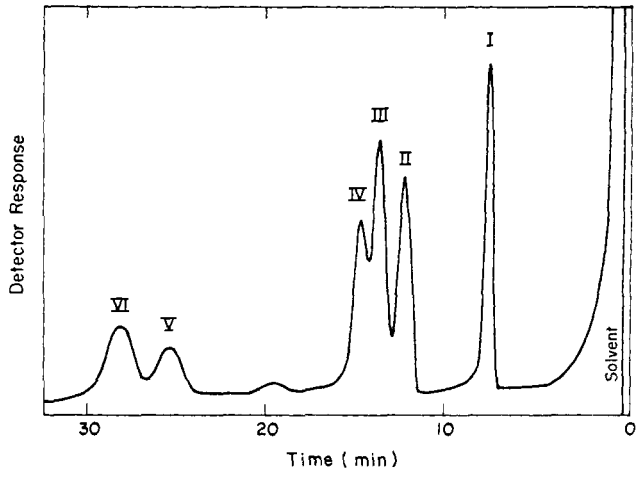

FIGURE 4: Gas chromatography of partially methylated alditol acetates from permethylated *H. wingei* Y-5 mannan. The peaks identified by mass spectrometry are (I) 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol, (II) 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol, (III) 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol, (IV) 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol, (V) 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methylmannitol, and (VI) 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylmannitol.

digestion alone affected the acetolysis patterns, while mild acid treatment followed by mannanase digestion increased the mannose peak significantly. This suggests that acid treatment exposed more of the side chains to enzymic attack. The isolated oligosaccharides were not themselves mannanase resistant because only mannose was found after the acetolysis products were subjected to mannanase digestion.

Methylation of Y-5 Mannan. Y-5 mannan was methylated by the Hakomori method (1964) followed by Purdie's reagent (Kuhn *et al.*, 1955). After hydrolysis, the products were converted to alditol acetates. Figure 4 shows one of the gas chromatographic patterns. The peaks, from right to left, were identified by mass spectrometry as the acetates of 2,3,4,6-tetra-*O*-methylmannitol, 3,4,6-tri-*O*-methylmannitol, 2,4,6-tri-*O*-methylmannitol, 2,3,4-tri-*O*-methylmannitol, 3,4-di-*O*-methylmannitol, and 2,4-di-*O*-methylmannitol. Table V gives the molar ratios of these derivatives for different mannan preparations. The finding of both 3,4- and 2,4-di-*O*-methyl derivatives indicates that both 1→2 and 1→3 linkages were present at branch points. The ratio of tri-*O*-methyl derivatives to the tetra-*O*-methyl derivative was about 3, giving an average chain length of 5. A value of 4.3 was calculated from the ratios of the various acetolysis products, assuming that the backbone side chain structure is similar to that in *S. cerevisiae*. Mild acid or mannanase treatment did not change the average chain length.

Periodate Oxidation. In mannan, each mole of mannose at a nonreducing end, or one with only position 6 substituted, should consume 2 mol of periodate. A mannose unit with position 2 or 4 substituted will consume 1 mol of periodate, while one with position 3 substituted would not be attacked. From these relationships, periodate consumption per mole of mannose unit of Y-5 mannan was calculated to be 0.97 from the methylation data in Table V. The experimental finding was 0.80. The low result suggests that some of the mannose units were substituted in a manner that prevented periodate oxidation but did not interfere with methylation. One possibility would be by phosphate groups that became labilized and were lost during methylation.

β Elimination. In glycoproteins several types of linkage

TABLE III: Optical Rotation of Acetolysis Fragments.[a]

H. wingei Y-5 Mannan	*S. cerevisiae* Mannan	
$[\alpha]_{546}^{25}$ (deg)	$[\alpha]_{546}^{25}$ (deg)	Structure
Man_2 +86	+63	αMan(1→2)Man
Man_3 +120	+73	αMan(1→2)αMan(1→2)Man
Man_4 +94	+92	αMan(1→3)αMan(1→2)αMan(1→2)Man
Man_5 +111		

[a] The Man_4 fragments from *H. wingei* and *S. cerevisiae* have identical structures, whereas the higher positive rotations of the Man_2 and Man_3 from *H. wingei* reflect the presence of α(1→3) linkages in these fragments.

TABLE IV: Molar Ratios of Acetolysis Products of Various Y-5 Mannans.

	Acetolysis Fragment					
Mannan	Man_5	Man_4	Man_3	Man_2	Mannose	Av Chain Length[a]
Y-5 Mannan	1.5	0.7	1.0	2.1	5.2	4.3
Mannanase digested	1.3	0.8	1.0	1.8	7.2	
Acid treated	1.3	0.7	1.0	2.2	6.5	
Acid treated, mannase digested	1.0	1.3	1.0	3.5	18.7	5.7

[a] Calculated assuming a structure for the mannan similar to that in Figure 9A.

TABLE V: Methylation Analysis of Various Y-5 Mannan Preparations.

Mannan	Molar Ratios of the Partially Methylated Alditol Acetates[a]							
	Tetra-*O*-methyl	Tri-*O*-methyl				Di-*O*-methyl		
	2,3,4,6	3,4,6	2,4,6	2,3,4	Total	3,4	2,4	Total
Ethanol precipitated	1.00	1.32	1.32	0.74	3.38	0.34	0.56	0.90
Acid treated	1.00	1.40	1.08	0.80	3.29	0.45	0.55	1.00
Fehling's precipitated	1.00	1.39	1.41	0.86	3.66	0.42	0.65	1.07
Fehling's precipitated and mannanase digested	1.00	0.91	1.00	1.28	3.19	0.43	0.57	1.00

[a] All values are calculated in reference to the tetramethyl derivative.

have been observed between the carbohydrate and the peptide chain (Neuberger *et al.*, 1966). Two of these are found in mannans. One is the O-glycosidic linkage involving the hydroxyl groups of serine and threonine, which are broken in mild alkali by β elimination. The other linkage is the *N*-acylglycosylamine linkage involving the amide-N of asparagine, which is relatively stable to both acid and alkali. When Y-5 mannan from the void peak of a Bio-Gel A 5m column was treated with 0.1 N NaOH in the presence of 0.3 M $NaBH_4$ at 22°, 9% of the carbohydrate was released as short oligosaccharide chains which ranged in size from mono- to octasaccharide on the basis of gel filtration properties (Figure 5). Most of the carbohydrate in the material from the Bio-Gel P-4 void volume peak was eluted at the void volume of a Bio-Gel A 5m column. Thus, it was still of large size. The amino acid compositions before and after β elimination (Table I) showed that 68% of the serine was destroyed during the reaction, of which 63% was converted to alanine by reduction. The threonine content decreased 54%, of which 29% was recovered as α-aminobutyric acid. Thus, in Y-5 mannan 39% of the amino acid residues were serine or threonine, and 64% of these were substituted by small oligosaccharide chains. However, these short chains accounted for less than 10% of the total carbohydrate, and the majority was apparently linked to protein through the *N*-acylglycosylamine linkage. From the content of glucosamine in the mannan, the average size of the polysaccharide chains linked to asparagine was calculated to be 100–200 mannose units.

Immunochemistry of Y-5 Mannan. Both Y-5 and Y-21 antisera gave about the same precipitin reaction with mannans from 5, 21, or diploid cells, or mannans from cells at different stages of growth. After adsorption with diploid cells, Y-21 antiserum no longer agglutinated 5 or 21 cells. However, Y-5 antiserum adsorbed with diploid cells still agglutinated specifically with 5 cells, although no precipitation was detected when this adsorbed antiserum was incubated with 5-agglutinin or Y-5 mannan. These results suggest that in Y-5 antiserum most of the antibodies are against the bulk cell wall mannan, the structure of which is the same for 5, 21, and diploid cells. However, there also is a small population of antibodies that are specific for 5 cells, and which may be directed against some cell wall structure which is related to the 5 mating type.

Different preparations of Y-5 mannan reacted differently with Y-5 antiserum (Figure 6). Mannan with intact phosphodiester linkages, isolated by ethanol precipitation, gave a higher precipitin reaction than that isolated by Fehling's precipitation. This indicates that some of the antigenic determinants

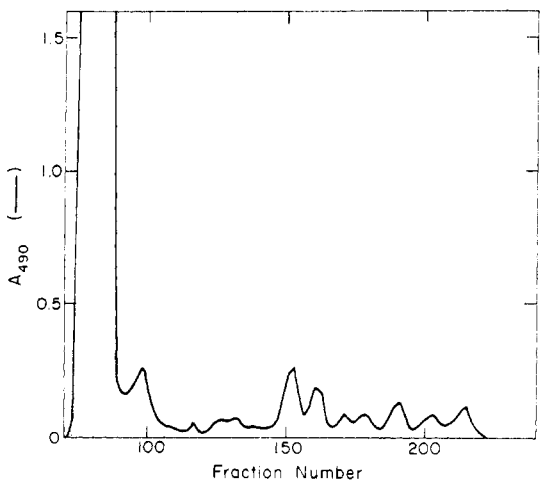

FIGURE 5: Gel filtration of the β-elimination products of Y-5 mannan obtained from the void volume of a Bio-Gel A 5m column. A 2 × 200 cm column of Bio-Gel P-4 (200–400 mesh) was eluted with 0.1 N NH_4HCO_3. The small fragments correspond to di- to octasaccharides.

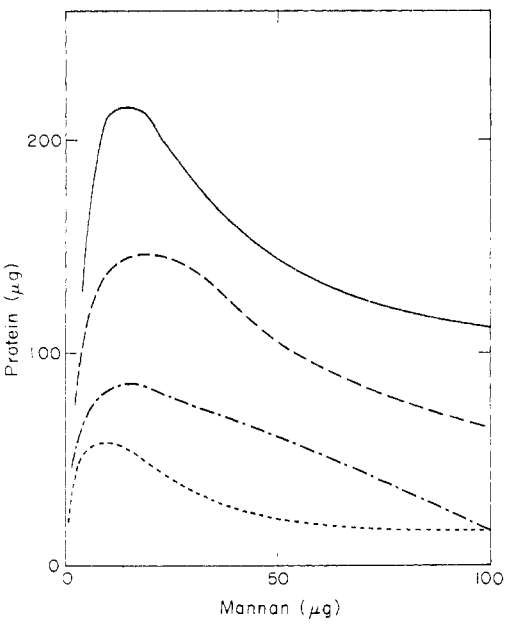

FIGURE 6: Precipitin curves of *H. wingei* Y-5 antiserum with Y-5 mannan isolated by ethanol precipitation (—), by Fehling's precipitation (- -), Y-5 mannan treated with 0.1 N HCl (· · ·), and with 0.01 N HCl (–·–), both at 100° for 30 min. For each assay, 15 μl of antiserum was used.

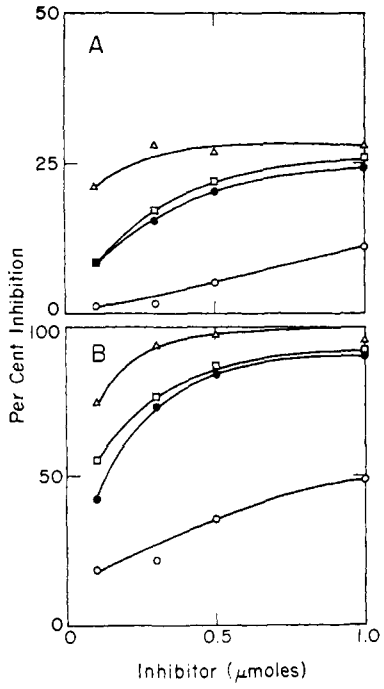

FIGURE 7: Inhibition of the precipitin reactions between (A) Y-5 antiserum and Y-5 mannan, and (B) Y-5 antiserum and mild acid-treated Y-5 mannan. The inhibitors are mannobiose (○), mannotriose (△), mannotetraose (□), and mannopentaose (●) from the acetolysis of *H. wingei* Y-5 mannan.

were destroyed in strong alkali. Although mannanase released 22% of the carbohydrate from Y-5 mannan, the reaction of the resulting mannan with Y-5 antiserum was only 12% lower than that of the original mannan. Thus, this treatment did not remove important determinants.

Mild acid treatment (0.01 N HCl, 100°, 30 min) destroyed about 70% of the antigenic reactivity in Y-5 mannan (Figure 6). In contrast, mild base treatment (0.1 N NaOH, 100°, 30 min) affected the antigenic reactivity of Y-5 mannan only slightly.

None of the oligosaccharides from the acetolysis of Y-5 mannan inhibited the precipitin reaction between Y-5 antiserum and Y-5 mannan more than 30% (Figure 7A). Of these oligosaccharides, mannotriose was a better inhibitor than either mannotetraose or mannopentaose. *S. italicus* mannopentaose, which has the structure αMan(1→3)αMan(1→3)-αMan(1→2)αMan(1→2)Man (W. Lipke, W. C. Raschke, and C. E. Ballou, unpublished data), inhibited even better than the mannotriose. Thus, two contiguous 1→3 linkages appear to be important. *K. lactis* mannotetraose, with only one 1→3-linked terminal mannose unit (Raschke and Ballou, 1972), inhibited about the same as *H. wingei* mannotetraose and mannopentaose. While only 30% of the precipitin reaction between Y-5 antiserum and Y-5 mannan was inhibited by the acetolysis oligosaccharides, the reaction between Y-5 antiserum and acid-treated mannan was inhibited completely (Figure 7B). Thus, 70% of the Y-5 antiserum activity is related to acid-labile determinants, whereas the rest is directed against the terminal αMan(1→3)αMan(1→3)Man structure of the polysaccharide side chains.

In *K. brevis* mannan, the α-D-mannosyl phosphodiester structure is an acid-labile determinant (Raschke and Ballou, 1971). The antigenic determinant of *H. polymorpha* mannan is also acid labile (Raschke, 1972), and has been shown to be due to the α-D-glucosyl phosphodiester group (W. Lipke, unpublished data). These acid-labile determinants were compared in studies of the cross-reactions of Y-5 antiserum and *K. brevis* antiserum with mannans from *H. wingei*, *K. brevis*, and *H. polymorpha*. The precipitin reaction of Y-5 antiserum with *H. polymorpha* mannan was only 6% of the homologous reaction, whereas *K. brevis* mannan did not show any cross-reaction. The reaction of Y-5 mannan with *K. brevis* antiserum was 20% of the homologous reaction, and that with *H. polymorpha* antiserum was 44% of the homologous reaction (W. H. Lipke, unpublished data). α-D-Mannose 1-phosphate inhibited 70% of the precipitin reaction between *K. brevis* antiserum and *K. brevis* mannan, but it had no effect on the other two homologous reactions. Thus, the acid-labile antigenic determinants are different in these mannans.

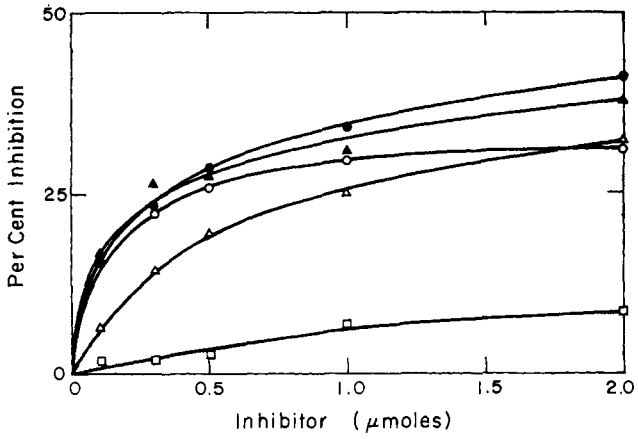

FIGURE 8: Inhibition of the precipitin reaction between Y-5 antiserum and Y-5 mannan. The inhibitors are Y-5 mannan neutral acetolysis fraction (○), dialysate of Y-5 mannan after treatment with 0.1 N HCl at 100° for 30 min (△), α-D-glucose 1-phosphate (□), neutral acetolysis fraction + dialysate of acid treatment (▲), and neutral acetolysis fraction + dialysate of acid treatment + glucose 1-phosphate (●).

The dialysate from acid-treated Y-5 mannan (0.1 N HCl, 100°, 30 min) inhibited the precipitin reaction to the same extent as the neutral acetolysis fraction (Figure 8), and a combination of these two fractions only increased the inhibition slightly. α-D-Glucose 1-phosphate inhibited 10% of the reaction, whereas β-D-glucose 1-phosphate or α-D-mannose 1-phosphate gave no inhibition. The maximum inhibition was 40% when the neutral acetolysis fraction, the dialysate of acid treatment, and α-D-glucose 1-phosphate were all present. Glucose, mannose, galactose, glucosamine, inositol, fructose, and arabinose were tested at high concentrations, but none showed a specific inhibition.

Discussion

This investigation has dealt with the structure of the polysaccharide part of *Hansenula wingei* Y-5 mannan, mainly with a characterization of the acetolysis fragments and identification of the antigenic determinants. The oligosaccharides obtained by acetolysis of the mannan are detailed in Figure 2, and the structure one can infer for the mannan from which they were derived depends on the manner in which these fragments are linked together. There are three ways of doing this: one oligosaccharide could be linked glycosidically to position 6 of the reducing end mannose of the next oligosaccharide, as in *S. cerevisiae* mannan (Figure 9A); to position 6 of an internal mannose unit (Figure 9B); or to position 6 of the mannose at the nonreducing end (Figure 9C) in what has been called a "block type" structure (Gorin and Spencer, 1970b). These three types are potentially distinguishable on

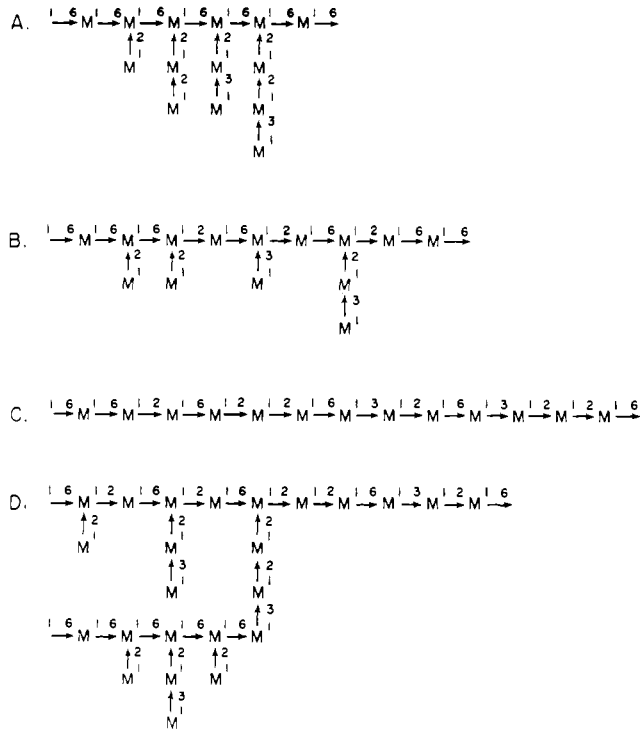

FIGURE 9: The four possible ways of linking acetolysis oligosaccharides to form the mannan molecule. M denotes mannose units and they are all α linked. The average chain length and exo-α-mannanase susceptibility change inversely in going from model A to B to C. D is a combined structure encompassing all three forms.

the basis of the products of mannanase digestion. The acetolysis oligosaccharides in structure A are completely susceptible to mannanase digestion, those of structure B are partly protected from the action of the enzyme, while those in structure C are immune to attack except from the single nonreducing end of the chain. Consequently, a comparison of the acetolysis fragments obtained before and after mannanase digestion should be helpful in deciding between these possibilities.

The results indicate that Y-5 mannan is a mixture of polysaccharides of all three types, or else it encompasses features of all three in a single molecular species (structure D). Only half as much mannose was released by the mannanase as would be predicted for structure A, and the undigested residue gave an acetolysis pattern unchanged from that of the original mannan. Thus, a large fraction of the acetolysis oligosaccharides are protected from enzymic attack. Methylation analysis of the ethanol-precipitated Y-5 mannan indicated that the average chain length of the molecule was about 5 mannose units (Table V), while the average chain length of the isolated acetolysis fragments was only about 4 sugar units (Table IV). This means that some of the nonreducing ends of the acetolysis oligosaccharides were substituted in the intact mannan so that they did not yield tetramethylmannose. This result is characteristic of a polysaccharide with the structure shown in Figure 9D.

In addition to the protection afforded by the interglycosidic linkages discussed above, it also is clear that some acid-labile structure was involved. Thus, mild acid hydrolysis of the mannan, under conditions that should not cleave glycopyranosidic bonds but would split glycosyl phosphate linkages, more than doubled the release of mannose by the mannanase. The acetolysis pattern of the residue showed an increased amount of mannose, meaning that side chains were removed by the enzyme to increase the amount of unsubstituted 1→6-linked mannose in the polysaccharide. In addition to increasing the mannanase susceptibility of the Y-5 mannan, the mild acid hydrolysis eliminated a major antigenic determinant, a result that is discussed in more detail below. The acid treatment led to release of some glucose that probably was attached by a glucosyl phosphate linkage to side chains in the mannan. If the phosphate groups were esterified to mannose units in the side chains, as they are in *K. brevis* mannan (Thieme and Ballou, 1971), these side chains would be protected from mannanase digestion. However, the acid treatment would not remove the phosphate from the mannan, since it is attached by a relatively stable ester linkage. Therefore, the acid treatment must increase the mannanase susceptibility of the mannan by a process other than the hydrolysis of phosphodiester bonds. The results suggest that the acetolysis oligosaccharides in *H. wingei* mannan are protected from mannanase in two ways. One involves substituents other than the phosphate group, probably at positions other than 6 of the mannose units because of the results of periodate oxidation. Mild acid releases this unidentified substituent and exposes new sites for mannanase digestion. However, since the residue from mannanase digestion of the mild acid-treated mannan still gave the same five peaks upon acetolysis, the acetolysis oligosaccharides are also protected by their interglycosidic linkage in the polymer.

The small yield of oligosaccharides in the β-elimination reaction (Figure 5) suggests that most of the carbohydrate in Y-5 mannan is present as large polysaccharide units perhaps attached to asparagine units in the protein. It is possible that the oligosaccharides released by alkali came from a small amount of a special mannan fraction that was isolated along with the bulk of the cell wall mannan. There is now sufficient reason to believe that the whole yeast mannan, isolated in the manner we have used, is a mixture of many different molecular species. Although the carbohydrate structures of these different species, as represented by the acetolysis fragments, are probably very similar or identical, the overall structures of the macromolecules may be very different.

H. wingei mannan has a mannose to phosphate molar ratio of about 15. We do not know where in the molecule the phosphate group is located. The phosphodiester linkages in *H. capsulata* (Slodki, 1963) and in *H. holstii* (Jeanes *et al.*, 1961) are apparently involved in linking short oligosaccharides together to form long teichoic acid like polymers. This does not seem to be the case in *H. wingei* phosphomannan because acid treatment under conditions which break the phosphodiester linkage did not cause a significant decrease in size or result in a release of oligosaccharides.

Mannans from the diploid and the 5 and 21 haploid cells of *H. wingei* gave essentially the same acetolysis patterns, and they reacted similarly with both Y-5 and Y-21 antiserum. Thus, they must be fundamentally very similar. However, Y-5 antiserum did contain an antibody population that appeared to be specific for some determinant on the surface of 5 cells. This is in contrast to the results of Brookbank and Heisler (1963) who failed to obtain antisera that were specific for the *H. wingei* mating types.

The cross-reaction we observed between Y-5 mannan and *K. brevis* antiserum is probably due to the αMan(1→2)-αMan(1→2)Man structure they have in common (Raschke and Ballou, 1971). Although this side chain is also present in Y-5 mannan, it is such a weakly immunogenic group compared to the trisaccharide αMan(1→3)αMan(1→3)Man and the acid-labile determinant that antibodies were not formed against it. This probably explains why *K. brevis* mannan did

not precipitate with *H. wingei* antiserum even though the reverse reaction was detected.

The chemical nature of the acid-labile immunodominant determinant of Y-5 mannan is poorly understood. α-D-Glucose 1-phosphate inhibited only 10% of the antigenic reactivity in Y-5 mannan and attempts to identify the other acid-labile determinant were not successful. Some mannose was released by mild acid hydrolysis and could have come from terminal mannofuranose units, but there is no other evidence supporting this idea. The occurrence of pyruvic acid as a covalently linked ketal unit in polysaccharides has been reported (Dutton and Yang, 1972) and in certain cases this acid may be immunochemically active (Heidelberger *et al.*, 1970). Since ketal groups are stable to base and are readily hydrolyzed by acid, it was a possible candidate for the acid-labile determinant. However, we found no evidence for such a structure in Y-5 mannan.

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