Studies on the Structure of Yeast Mannan

II. MODE OF ACTION OF THE ARTHROBACTER α-MANNOSIDASE ON YEAST MANNAN*

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SUMMARY

The mode of action of the extracellular α-mannosidase from Arthrobacter GJM-1 has been studied with several yeast mannans as substrates. In vitro, the enzyme cleaves most of the α-(1 → 2)- and α-(1 → 3)-linked side chains from Saccharomyces cerevisiae mannan, producing free mannose and leaving a resistant polymer containing mainly α-(1 → 6)-linkages. This material cannot be further degraded by the enzyme. The extent to which mannans from several Candida species are digested by the enzyme is inversely proportional to their phosphorus content. Kloeckera brevis mannan, although highly phosphorylated, is extensively digested by the enzyme. The reason for this discrepancy is unclear, but it may be related to the positions of the phosphate residues in the respective mannans.

Growth of Arthrobacter GJM-1 on S. cerevisiae mannan results in the accumulation of an undigested residue in the cultural filtrate. This residue remains in the supernatant after ammonium sulfate precipitation of the α-mannosidase. It has been purified by gel filtration and precipitation with Fehling's solution. The structure of this residue has been studied by acidolysis, proton magnetic resonance spectrometry, and methylation. These studies indicate that the product is exclusively α-(1 → 6)-linked and represents the mannan backbone. The data suggest that the α-mannosidase from Arthrobacter GJM-1 is an exoglycosidase which acts by splitting off single mannose residues from the nonreducing ends of the side chains of the mannan molecule.

The structure of cell walls has been of interest to biologists for some time. The general peptidoglycan structure of the bacterial cell envelope of Staphylococcus aureus has been worked out by Strominger et al. (1). In many ways the yeast cell wall provides a simpler system for study. Ninety per cent of the dry weight of yeast cell walls is accounted for by only two constituents, glucan and mannan (2). Enzymes which are active on the glucan component (glycosidases and glucanases) are well known (3, 4), but most mannosidases are inactive on yeast mannan (5, 6).

The preceding paper described the partial purification and some properties of a mannosidase which was capable of extensive digestion of Saccharomyces cerevisiae mannan (7). The present report deals with the mode of action of the enzyme on S. cerevisiae mannan and on the mannans of other yeast species. It is concluded that the mannosidase acts by stripping off the side chains from the mannan molecule to leave the α-(1 → 6)-linked backbone intact. The mannans of the other yeasts studied have structures which are similar to S. cerevisiae mannan, but significant differences do exist within and between species.

EXPERIMENTAL PROCEDURE

Analytical procedures for total carbohydrate (8), reducing sugar (9), and protein (10) have been described (7). Nitrogen was determined by the method of Long and Staples (11) and phosphorus according to Bartlett (12). Yeast mannans were obtained as described (7).

Descending paper chromatography was performed on Whatman No. 1 filter paper in the following solvents: A, ethyl acetate-pyridine-water (5:3:2); B, 1-butyl alcohol-pyridine-water (10:3:3); C, ethyl acetate-pyridine-water (8:2:1). Sugar spots were detected with the silver nitrate-sodium hydroxide dip.

Thin layer chromatography was carried out on Silica Gel H (Merck, Darmstadt, Germany). Acetylated oligosaccharides were chromatographed in Solvent D, benzene containing 7% methanol, and they were detected with the hydroxylamine-ferric chloride spray described by Tate and Bishop (13).

Oligosaccharides were methylated by the procedure of Hakomori (14) as described by Sandford and Conrad (15). Methanalysis was carried out in a sealed ampoule for at least 8 hours at 100°. The solvent was removed by evaporation in a desiccator containing potassium hydroxide pellets and anhydrous calcium chloride. Acetylation of mannans and acetylation of the...
acetylated products were performed as described (7). Deacetylated acetylation products were separated on Sephadex G-25.

Gas-liquid chromatography was carried out with a Varian Aerograph Series 1200 (Wilken Instrument Company) equipped with a hydrogen flame detector. Peak area was determined with a digital integrator Infotronics model CRS 11 HSV connected directly to the electrometer of the gas chromatograph. The column was stainless steel, 5 feet \( \times \) 2 inch, packed with 10% Carbowax on Aeropak (Wilken Instrument Company). Flow rates of 20 to 25 ml of \( \text{N}_2 \) per min were used. Peaks were identified with standard methylated sugars.

Ultracentrifugations were done in a Spinco model A-60 spectrometer. Infrared spectra were taken with a Perkin-Elmer model 257 grating infrared spectrophotometer and optical rotations were determined with a Rudolph photoelectric polarimeter. Absorbances were read in a Zeiss PMQ II spectrophotometer and a Packard Tri-Carb liquid scintillation spectrometer was used to determine the radioactivity of labeled samples.

RESULTS

Mode of Action of Enzyme on S. cerevisiae Mannan

A large scale reaction mixture containing 200 mg of mannan, 10 \( \mu \)moles of \( \text{CaCl}_2 \), and 2 mg of enzyme protein (enzyme source: concentrated DEAE-cellulose fraction equivalent to 740 units of enzyme) in 100 ml of 0.05 M potassium phosphate buffer, pH 6.8, was incubated at 37°. Aliquots of the reaction mixture were removed at 2-hour intervals and assayed for the release of reducing sugar. Further additions of enzyme (1 mg of protein, 370 units) were made every 2 hours. Sixty-five per cent of the mannose in the polymer was released as reducing sugar after 8 hours with three additions of enzyme. Longer incubation or further additions of enzyme did not increase the extent of hydrolysis. The fraction of the polysaccharide remaining after exhaustive digestion (35%) was identical with the fraction of mannose bound by \( \alpha-(1 \rightarrow 6) \)-linkages in the original mannan molecule.

After 12 hours, the reaction was stopped by boiling the mixture, the solution was concentrated to about 2 ml on a rotary evaporator, and the precipitated protein was removed by centrifugation. The supernatant was applied to a column, 2 \( \times \) 150 cm, of Sephadex G-25 (irregular form) and eluted with 0.2 M \( \text{NH}_4\text{HCO}_3 \). The results are shown in Fig. 1. The only carbohydrates recovered from the mixture were an excluded residue in the void volume and a low molecular weight compound identified as mannose by paper chromatography (Solvents A and B). Oligosaccharides of intermediate size were not detected.

To test the possibility that oligosaccharides might be produced initially and subsequently be digested to monosaccharide, a large scale incubation similar to the one described above was carried out in dialysis tubing. Throughout the course of the reaction the contents of the tubing were dialyzed against 0.1 M potassium phosphate buffer, pH 6.8. Aliquots of the dialysis medium were removed at various times after the addition of enzyme; the material was concentrated and examined by paper chromatography and by gel filtration on Sephadex G-25. The only product detected at any time during the reaction was mannose.

The structure of the residue from enzyme digestion was examined by the technique of acetolysis. Acetolysis of intact mannan for 13 hours at 40° produces mannose, \( M_5 \), \( M_3 \), and \( M_4 \) in the ratios 1:2:2:1 (17). The oligosaccharide products are \( \alpha-(1 \rightarrow 2)- \) and \( \alpha-(1 \rightarrow 3)- \)-linked and arise by selective cleavage of \( \alpha-(1 \rightarrow 6)- \) linkages. Acetolysis of the residue from enzyme digestion would be expected to yield stable oligosaccharides if \( (1 \rightarrow 2)- \) and \( (1 \rightarrow 3)\)-linkages remain after digestion, but only mannose if these linkages were all hydrolyzed by the enzyme. The results of acetolysis of the residue from enzyme digestion are shown in Fig. 2A. The pattern obtained from intact mannan is shown for comparison. The predominant product was mannose and only small amounts of oligosaccharides were observed. These are thought to result from incomplete digestion of the side chains near phosphate groups in the molecule which could sterically hinder the enzyme (see below). The results do indicate that the enzyme hydrolyzes most of the \( \alpha-(1 \rightarrow 2)- \) and \( \alpha-(1 \rightarrow 3)- \)-linkages in intact mannan.

Isolation of Mannan Residue from Cultural Filtrate

The supernatant from the first ammonium sulfate precipitation of the enzyme was used as the source of the mannan residue left after growth of the mannosidase-producing microorganism (7). This solution was dialyzed against distilled water and concentrated on a rotary evaporator, and the mannan residue was isolated by gel filtration on a column, 3 \( \times \) 150 cm, of Sephadex G-25. The carbohydrate appeared in the void volume of the column. The carbohydrate-containing fractions were pooled and evaporated to give a carbohydrate concentration of about 50 mg per ml. The solution was made alkaline with sodium hydroxide and the mannan residue was precipitated by dropwise addition of Fehling's solution. The resulting precipitate was dissolved in 1 N \( \text{HCl} \) and the solution was dialyzed against distilled water for 18 hours. Residual copper was removed from

![Fig. 1. Sephadex G-25 column chromatography of the products of the action of \( \alpha \)-mannosidase on baker's yeast mannan. Conditions are given in the text.](http://www.jbc.org/DownloadedFrom)
Cu-(1+3)-linked side chains were cleaved from the mannan. It oligosaccharide was detected. Thus, all of the (1→2)- and products showed mannose as the only product (Fig. 2B). No H+ and RCF was collected by lyophilizing the eluate. Molecular weight! Acetolysisc products Nitrogen*

The preparation by passage through a small column of Dowex 50 (H+) and RCF was collected by lyophilizing the eluate. The molecular weight of RCF, determined by the high speed equilibrium technique of Yphantis (16), was 8,100; the values for the residue from enzyme digestion and mannan were 8,800 and 22,000 respectively. The δ of sucrose (0.618) was used in the calculations and plots of log c against x² were linear. The ratio of mannose to phosphorus in undigested mannan was 77:1, that for RCF was 69:1, and that for the residue from enzyme digestion was 37:1. RCF had a specific rotation of +67.7° in water while mannan gave +72.7°. Thus most glycosidic linkages had the α configuration.

The proton magnetic resonance spectrum of RCF is shown in Fig. 3. The peak at 4.92 ppm represents the resonance due to the anomeric proton, and its position is characteristic of α-(1→6)-glycosidic linkages (18). RCF was methylated twice by the procedure of Hakomori (14). After two methylations no hydroxyl absorption was observed in the infrared spectrum. The product was methanolyzed and the methanolysis products were purified by passing the mixture in methanol through a column of Sephadex LH-20. The methanol was evaporated carefully and the products were examined by gas-liquid chromatography. The pattern obtained is shown in Fig. 4. The products were identified as methyl 2,3,4,6-tetra-O-methyl-D-mannoside and a methyl tri-O-methyl-D-mannoside peak which could have been the 2,3,4 or 3,4,6 isomer, both of which have the same retention time on the column used. The tetramethyl ether represents the end group of the polymer chain. Any (1→2)- or (1→3)-linked side chains remaining after enzyme digestion would give rise to tetramethyl mannose on methylation. The ratio of tri- to tetramethylmannoside expected for a linear polymer of molecular weight 8000 is about 50:1. The ratio obtained experimentally was 34:1. Thus, RCF is at least 97% α-(1→6)-linked. The acetolysis results and the proton magnetic resonance spectrum confirm this conclusion. In addition, since no dimethyl mannoside was detected in the gas chromatogram, the RCF molecule must be unbranched.

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The properties of RCF are summarized in Table I. Those of the residue from enzyme digestion and intact mannan are included for comparison. The residue from enzyme digestion and RCF were homogeneous in the ultracentrifuge and on Sephadex G-25 and G-50. The molecular weight of RCF, determined by the high speed equilibrium technique of Yphantis (16), was 8,100; the values for the residue from enzyme digestion and mannan were 8,800 and 22,000 respectively. The δ of sucrose (0.618) was used in the calculations and plots of log c against x² were linear. The ratio of mannose to phosphorus in undigested mannan was 77:1, that for RCF was 69:1, and that for the residue from enzyme digestion was 37:1. RCF had a specific rotation of +67.7° in water while mannan gave +72.7°. Thus most glycosidic linkages had the α configuration.

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**Table I**

Properties of intact mannan and of backbone residues from cultural filtrate and from action in vitro of mannosidase on mannan

<table>
<thead>
<tr>
<th></th>
<th>Mannan</th>
<th>Residue from cultural filtrate</th>
<th>Residue from enzyme digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moles of mannose per mole of phosphorus</td>
<td>77</td>
<td>69</td>
<td>37</td>
</tr>
<tr>
<td>Nitrogen*</td>
<td>0.86</td>
<td>1.20</td>
<td>1.15</td>
</tr>
<tr>
<td>Specific rotation</td>
<td>72.7°</td>
<td>67.7°</td>
<td>68.0°</td>
</tr>
<tr>
<td>Molecular weight*</td>
<td>22,000</td>
<td>8,100</td>
<td>8,800</td>
</tr>
<tr>
<td>Acetolysis products</td>
<td>M₁, M₂, M₃</td>
<td>M₂, M₃, M₄</td>
<td>M₄</td>
</tr>
<tr>
<td></td>
<td>Mannose</td>
<td>Mannose</td>
<td>Mannose</td>
</tr>
</tbody>
</table>

* Percentage by weight.

* Determined by sedimentation equilibrium with δ = 0.618.

* For 13 hours at 40°.

* Only traces of M₂ and M₃ were obtained.

It is believed that phosphatases associated with the cells removed phosphate groups from the mannan, allowing the mannosidase to attack those side chains which are inaccessible in the residue during digestion *in vitro*.

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**Fig. 2.** Separation on Sephadex G-25 of the products from acetolysis of mannan and of the mannosidase-resistant mannan core polymer. Acetolysis cleaves only (1→6)-linkages, so a polymer composed of only (1→6)-linkages will yield free mannose while one with (1→2)- and (1→3)-linked side chains will yield resistant fragments of a size corresponding to the length of these chains. The dashed line in A shows the pattern of acetolysis-resistant fragments from intact mannan and the solid line in A shows the products from acetolysis of the high molecular weight residue from the digestion of mannan with partially purified α-mannosidase. B shows the product from the acetolysis of the high molecular weight mannan residue isolated from the cultural medium after growth of *Arthrobacter* strain GJM-1 on mannan as the sole carbon source.

**Fig. 3.** Proton magnetic resonance spectrum (in D₂O) of the mannan residue isolated from cultural filtrates of *Arthrobacter* strain GJM-1.
The results summarized above indicate that the *Arthrobacter* α-mannosidase acts by hydrolyzing the α-(1 → 2) and α-(1 → 3)-linkages in the side chains of the mannan molecule. These data are consistent with the proposed structure of *S. cerevisiae* mannan (17, 19) in which (1 → 2)- and (1 → 3)-linked side chains are attached to a (1 → 6)-linked backbone. The fact that no oligosaccharide is produced indicates that the enzyme is an exoglycosidase which attacks from the nonreducing ends of the side chains. To determine whether the (1 → 6)-linkages in RCF could be broken by the enzyme, the isolated material was subjected to further treatment with excess enzyme. No reducing sugar was released after a 24-hour incubation with several additions of enzyme.

**Nature of Reducing End of RCF**

Ten milligrams of sodium borohydride and a small crystal of sodium borotritide (New England, 200 mC per mmole) were dissolved in 1 ml of 0.05 M NH₄HCO₃ (pH about 8). The samples of mannan and M₄ to be reduced were each dissolved in 1 ml of 0.05 M NH₄HCO₃. To each sample solution was added 0.5 ml of the borohydride-borotritide mixture. The mixtures, in capped tubes, were heated at 60° for 1.5 hours, when Dowex 50 (H⁺) resin was added. The resin slurry was shaken at room temperature for 5 min and the resin was removed by filtration and washed with several portions of 50% methanol. The combined filtrate was evaporated to dryness, the residue was dissolved in 2 ml of distilled water, and the reduced products were purified by gel filtration on a Sephadex G-25 column. The carbohydrate-containing fractions from the column were pooled, concentrated to dryness, and hydrolyzed in 2 M H₂SO₄ for 4 hours at 100°. The hydrolysates were assayed for carbohydrate and radioactivity.

The results of the analyses are shown in Table II. From the specific activities of M₄ and reduced RCF, a molecular weight of about 7,100 was calculated for the RCF polymer. Since RCF represents 35% of the intact mannan, a molecular weight of 20,000 is indicated for this compound. Values of 8,100 and 22,000, respectively, were determined for RCF and mannan by sedimentation equilibrium. The gas chromatographic data on methylated RCF indicate that the molecule contains about 35 sugar residues, which corresponds to a molecular weight of about 6,500. Thus, all results suggest about the same molecular weight for RCF.

The hydrolysate of RCF was neutralized by shaking it with mixed bed resin (carbonate form), and the solution was concentrated and chromatographed on Whatman No. 1 papers in Solvents A and C. The carbohydrate products were detected with the silver nitrate-sodium hydroxide reagents and the radioactivity was located on the paper by cutting it into 0.5-inch strips and counting each strip in a vial containing 10 ml of a scintillation fluid composed of 12 g of 2,5-diphenyloxazole and 0.6 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 3.5 liters of toluene. Mannose and mannitol were the only carbohydrates observed on the chromatograms. The majority of the radioactivity in each case was localized in the position in which mannitol migrated (Fig. 5). The results confirm the existence of mannose at the reducing end of RCF and, presumably, also at the reducing end of the intact mannan molecule.

**Preparation of α-(1 → 6)-linked Oligosaccharides**

Since the mannose residues of RCF are joined exclusively by α-(1 → 6)-linkages, this material is a potential source of α-(1 → 6)-linked oligosaccharides. Accordingly, 5 mg of acetylated RCF were subjected to acetolysis in 5 ml of medium. Aliquots of 0.1 ml were removed from the mixture at different times after initiating the reaction. Each aliquot was pipetted into 0.2 ml of anhydrous pyridine, the solvent was evaporated, and the residue was dissolved in 1 ml of chloroform. The chloroform extract was washed with water and the acetolysis products were examined by thin layer chromatography in Solvent D, which indicated that the maximum production of oligosaccharides occurred after about 30 min of acetolysis. The deacetylated acetolysis products from 50 mg of starting material were separated by gel filtration. The elution patterns for a 20-min and a 35-min acetolysis are shown in Fig. 6. Di- to heptasaccharide were obtained by this procedure. As expected, the yield of oligosaccharide decreased with increasing chain length. The sharpness of the peaks obtained from RCF (compare the pattern of intact mannan in Fig. 2A) indicates that these fractions were homologous in structure. The shorter acetolysis resulted in a lower over-all yield.

The crude column fractions were pooled and purified further by preparative paper chromatography in Solvent A. Rechromatography on Sephadex G-25 yielded pure di- to heptasaccharide. The properties of these compounds are summarized in Table III. The oligosaccharides had slightly higher reduced Rₕ values than the correponding acetolysis products from methylated mannan. The sharpness of the peaks obtained from RCF (compare the pattern of intact mannan in Fig. 2A) indicates that these fractions were homologous in structure. The shorter acetolysis resulted in a lower over-all yield.

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**Table II**

<table>
<thead>
<tr>
<th>Compound reduced</th>
<th>Tritium incorporated (cpm)</th>
<th>Specific activity (cpm/μg)</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannotetraose</td>
<td>73,000</td>
<td>183</td>
<td>666</td>
</tr>
<tr>
<td>Residue from cultural filtrate</td>
<td>18,400</td>
<td>17.2</td>
<td>7100</td>
</tr>
</tbody>
</table>

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**Fig. 4.** Gas chromatographic tracing of the separation of the methanolysis products of methylated mannan residue from the cultural filtrate. The peaks were methyl 2,3,4,6-tetra-O-methylmannoside (I) and 2,3,4-tri-O-methylmannoside (II). The column temperature was 49°.
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Fig. 5. Identification of sugar unit at the reducing end of the mannan backbone. After reduction with sodium borotritide and hydrolysis, aliquots of the neutralized hydrolysate were chromatographed on Whatman No. 1 paper with ethyl acetate-pyridine-water (5:3:2) (A) and ethyl acetate-pyridine-water (8:2:1) (B). The cross-hatched blocks represent the positions in which the standards migrate. In both solvents the major radioactive peak had the chromatographic mobility of mannitol.

values than the α-(1 → 2)- and α-(1 → 3)-linked oligosaccharides from whole mannan. All of the oligosaccharides were reducing and were converted to mannose after acetylation for 13 hours, properties which would be expected of (1 → 6)-linked compounds. The yield of purified oligosaccharide from 50 mg of starting material was 0.3 to 4 mg. Fig. 7 shows semilogarithmic plots of the elution volumes (from Sephadex G-25) and the $R_{max}$ values of the oligosaccharides (ethyl acetate-pyridine-water, 5:3:2) against their degree of polymerization. In each case a straight line was obtained indicating that the oligosaccharides were members of an homologous series. These oligosaccharides were substrates for the Arthrobacter α-mannosidase, but were not as good as their α-(1 → 2)- and α-(1 → 3)-linked counterparts from intact yeast mannan.

Studies on Structures of Other Mannans

Acetolysis Studies—The mannans available as test substrates for the α-mannosidase included those from Kloechera brevis, Candida albicans (B-792), C. albicans (AB-311), Candida stellatoidea, and Candida tropicalis. The structures of K. brevis, C. albicans (B-792), and C. stellatoidea mannans have previously been studied by the technique of acetolysis (19). All of these were investigated in the present work. The gel filtration elution profile of the deacetylated products from 13-hour acetolyses of each of these mannans is shown in Fig. 8. The pattern for K. brevis shows peaks corresponding to mono-, di-, and trisaccharide only, indicating that tetrascarbohydrate is not a part of the structure of this mannan. Thus, all α-(1 → 3)-linkages in its structure are those found in the isomeric $M_3$. The most striking feature of the patterns of the Candida mannans is the large amount of material of molecular size greater than $M_4$. Such material is not found in S. cerevisiae or K. brevis mannans. Since acetolysis for 13 hours cleaves all α-(1 → 6)-linkages, this larger material must contain α-(1 → 2)- and α-(1 → 3)-linkages. Stewart and Ballou (19) have studied the tetra-, penta-, and hexasaccharides from C. stellatoidea mannan. The tetrascarbohydrate was found to be exclusively α-(1 → 2)-linked; the penta- and hexasaccharides were branched and contained α-(1 → 2)- and α-(1 → 3)-linkages in the ratios of 3:1 and 3:2, respectively.

The large acetolysis-stable material in these mannans indicates that they are more highly branched than S. cerevisiae mannan.
The relatively high proportion of mannose in these acetolysis patterns indicated that, in addition to the extensive branching, there are regions in these mannans which are composed of unsubstituted mannose residues. The acetolysis patterns show that significant differences in the structures exist within the genus Candida, and even between the two strains of C. albicans.

Effects of α-Mannosidase Digestion on Mannans from Kloeckera and Candida—These mannans were subjected to digestion by the purified α-mannosidase to determine whether they contained an α-(1 → 6)-linked, mannosidase-resistant core similar to that isolated from S. cerevisiae mannan and to determine what effect the phosphate groups in the mannans would have on the activity of the enzyme. The results of these experiments, and some properties of the mannans, are summarized in Table IV. The data show that these mannans differ in molecular size, in their contents of nitrogen and phosphorus, and in their susceptibility to mannosidase digestion. The extent to which Candida mannans were digested was inversely proportional to their phosphorus content. This result suggests that the phosphate groups in these mannans inhibit the action of the mannosidase. Further evidence for this contention is presented below. Surprisingly, K. brevis mannan, which was the most heavily phosphorylated mannan studied, was 50% digested by the enzyme. The reason for the discrepancy between the results obtained for the Kloeckera and Candida mannans is unclear, but may be related to a difference in the location of the phosphate groups in the mannans of the two genera.

The mannosidase-resistant residues were isolated by gel filtration of the digest of K. brevis and C. stellatoidea mannans. The structures of these residues were analyzed by acetolysis. Only mannose was obtained from the K. brevis residue, whereas in addition to mannose significant amounts of oligosaccharides were found among the C. stellatoidea products (Fig. 9). The results do indicate that both K. brevis and C. stellatoidea mannans are degraded to resistant residues which contain mainly α-(1 → 6)-linked mannose units. In this respect they are similar to S. cerevisiae mannan. The mannosidase to phosphorus ratios for the residues from K. brevis and C. stellatoidea were 6:1 and 30:1, respectively. These may be compared with values of 10:1 and 70:1 for the intact polysaccharides, results which indicate that much of the phosphate in these mannans remains associated with the mannosidase-resistant residues.

Effect of Phosphate Groups on Action of α-Mannosidase—To

![Figure 7](image_url)  
**Fig. 7.** Semilogarithmic plots of the elution volumes ($V_e$ in A) and $R_{max}$ values in Solvent A (B) of the α-(1 → 6)-linked oligosaccharides from the mannan backbone against their degree of polymerization. The fact that straight lines were obtained in both cases indicates that the oligosaccharides are members of a homologous series.

![Figure 8](image_url)  
**Fig. 8.** Separation on Sephadex G-25 of the acetolysis products of various mannans. The patterns represent the mannans of: A, K. brevis; B, C. albicans (B-792); C, C. albicans (A-B311); D, C. stellatoidea; and E, C. tropicalis. Peaks I to VI represent monosaccharide fractions, respectively. The exact structures of some of the oligosaccharides remain to be determined.
TABLE IV  
Properties of mannans from various yeasts

<table>
<thead>
<tr>
<th>Mannan source</th>
<th>Molecular weight&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mannose</th>
<th>N</th>
<th>Extent of digestion with α-mannosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>moles/mole phosphorus</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>22,000</td>
<td>77</td>
<td>0.86</td>
<td>65</td>
</tr>
<tr>
<td>K. brevis</td>
<td>36,000</td>
<td>9</td>
<td>0.11</td>
<td>54</td>
</tr>
<tr>
<td>C. albicans B-792</td>
<td>47,000</td>
<td>22</td>
<td>1.49</td>
<td>20</td>
</tr>
<tr>
<td>C. albicans AB-311</td>
<td>47,000</td>
<td>35</td>
<td>0.24</td>
<td>22</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>54,000</td>
<td>20</td>
<td>0.46</td>
<td>6</td>
</tr>
<tr>
<td>C. stellatoidea</td>
<td>52,000</td>
<td>70</td>
<td>0.53</td>
<td>46</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by high speed sedimentation equilibrium with $\bar{v} = 0.618$.

Fig. 9. Separation on Sephadex G-25 of the products of acetolysis of the C. stellatoidea mannan backbone polymer. In addition to mannose, small oligosaccharides and a high molecular weight product were obtained (HMWP).

Fig. 10. Separation of the neutral and acidic components of C. albicans (B-792) mannan on DEAE-cellulose. Twenty milligrams of the mannan in distilled water were applied to a column, 1.8 x 8 cm, of DEAE-cellulose (bicarbonate form). The column was washed with water and then eluted with a linear gradient of 0 to 0.5 M NH₄HCO₃. The elution pattern (Fig. 10) shows a small peak (2.5% of the total carbohydrate) of neutral carbohydrate and a broad peak of material which eluted after the gradient was applied. The neutral material was subjected to digestion by the α-mannosidase. After 8 hours, with three additions of enzyme, 57% of this material had been released as mannose, whereas only 20% of the mannose of the acidic fraction was released. To confirm this result, a sample of the acidic mannan fraction was treated with alkaline phosphatase for 12 hours. This treatment released inorganic phosphate into the medium and resulted in a product which was digested to the extent of 29% by the mannosidase. These findings suggest that the phosphate groups are inhibitors of the enzymatic digestion of the Candida mannan.

DISCUSSION

The α-mannosidase isolated from Arthrobacter GJM-1 has been shown to be an exoglycosidase which removes the α-(1 → 2)- and α-(1 → 3)-linked side chains of S. cerevisiae mannan, leaving a backbone which is exclusively α-(1 → 6)-linked. Mannose has been identified as the sugar at the reducing end of this backbone. The backbone itself is not attacked by the enzyme at a measurable rate, although the enzyme does hydrolyze the α-(1 → 6)-linked oligosaccharides obtained by short term acetolysis of the backbone. Formerly, α-(1 → 6)-linked oligosaccharides have been isolated by acid hydrolysis of yeast mannan or by acid reversal of free mannose (20). The purification of oligosaccharides from these reaction mixtures was difficult and yields were usually not very high. Acetolysis of the yeast mannan backbone provides an excellent source for this series of oligosaccharides.

Both S. cerevisiae mannan and the mannan backbone appeared to be relatively homogeneous in the ultracentrifuge. Molecular weights of 22,000 and 8,100 were calculated for the mannan and its backbone, respectively. The value for mannan is lower than that reported by other workers. McLellan and Lampen (21) have reported a molecular weight of >200,000 for the mannan released from yeast cell walls by enzyme treatment. The lower molecular weight of the mannan used in the studies reported above may have resulted from fragmentation of the native molecule during the isolation and purification procedure. The molecular weights of the backbone, obtained by three independent methods (sedimentation equilibrium, sodium borotritide reduction, and methylation), were all consistent with a value of about 20,000 for the mannan used in the studies described above. The studies on C. stellatoidea and K. brevis mannans indicate that the mannosidase digestion produces resistant residues which are predominantly α-(1 → 6)-linked, and are similar to the backbone from S. cerevisiae mannan.

At this stage of the study not much can be said about the positions of the phosphate groups in the Candida mannans. The studies on C. stellatoidea and K. brevis mannans indicate that the mannosidase digestion produces resistant residues which are predominantly α-(1 → 6)-linked, and are similar to the backbone from S. cerevisiae mannan. They showed that the phosphate groups were attached to the mannosyl unit at the reducing end of the oligosaccharides. This unit is a part of the backbone in the intact polysaccharide which is composed of a string of α-(1 → 6)-linked mannose residues. Therefore, the phosphate groups must be attached to carbon 3 or 4 of these mannosyl residues. The data presented in the present study are consistent with this conclusion. In the three
mannans studied most carefully, most of the phosphorus is associated with the material which was resistant to mannosidase digestion. It was not possible to remove completely the side chains of the mannans of the Candida species probably because of the inhibitory effects of the phosphate groups in these mannans. The extents to which these mannans were digested varied inversely with their phosphorus contents, and the susceptibility of the phosphorylated fraction to mannosidase digestion was increased by treating it with alkaline phosphatase.

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