Studies on the Structure of Yeast Mannan

I. PURIFICATION AND SOME PROPERTIES OF AN α-MANNOSIDASE FROM AN ARTHROBACTER SPECIES*

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SUMMARY

A soil microorganism, designated Arthrobacter GJM-1, has been isolated which is capable of growing on Saccharomyces cerevisiae mannan as the carbon source. When grown on mannan, the microorganism secretes an α-mannosidase into the cultural medium. The enzyme is not found when mannose, glucose, or glycerol is substituted for mannan, but is present when oligosaccharides obtained by acetylation of mannan are used as carbon sources. The α-mannosidase has been isolated and partially purified. It was free of contaminating glycosidase, phosphatase, and protease activities.

The enzyme is active on mannans from several different yeasts and on the oligosaccharides produced by acetylation of yeast mannans, but has a low activity on α-mannosides with aromatic or aliphatic aglycons. This is in contrast to results obtained with other α-mannosidases and may reflect the different metabolic roles of the respective enzymes.

The $K_m$ values for the di-, tri-, tetra-, penta-, and hexasaccharides from S. cerevisiae mannan varied between 0.5 and 2 mM. Because of the structural heterogeneity of these oligosaccharides, it is not certain what features contribute to the differences observed. The $K_m$ value for intact mannan was $1 \times 10^{-3}$ M, but this value probably reflects the higher concentration of side chains in the mannan molecule.

Mannan is an important structural component of the yeast cell wall (1, 2). The structure of the mannan from Saccharomyces cerevisiae, or baker's yeast, has been studied by methylation (3) and by the degradative technique of acetylation (4, 5). With acetylation, a series of oligosaccharides containing α-(1 → 2)-, α-(1 → 3)-, and α-(1 → 6)-linkages has been isolated from S. cerevisiae mannan. The acetylation and methylation data indicate that the mannan is a highly branched polysaccharide with α-(1 → 2)- and α-(1 → 3)-linked side chains attached to an α-(1 → 6)-linked backbone (5). No evidence for a simple repeating unit in mannan has been advanced.

Mannans have been isolated from the cell walls of many yeasts as well as from cultural filtrates and from the cell sap of some species (2, 6, 7). These mannans usually contain phosphorus and nitrogen (2). The phosphorus in mannans is diesterified and phosphorus-containing oligosaccharides have been isolated from acetolyses of Kloeckera brevis mannan (8). The nitrogen is present as hexosamine and protein (1, 2). Although there are reports of the isolation of mannan protein complexes from yeast cell walls (9, 10), not much is known about the way in which the carbohydrate and protein are associated. The enzyme, yeast invertase, is perhaps the best known example of a mannan-protein (7).

Most studies on the structure of mannans have been performed with chemical techniques. It was felt that a significant contribution to the knowledge of mannan structure could be made by combining chemical studies with enzymatic techniques. Several known α-mannosidases are relatively inactive on yeast mannan (11, 12). Consequently, a search was instituted for an enzyme capable of digesting this polysaccharide. Such an enzyme has been obtained from a soil microorganism grown on a mannan-salts medium. This report describes the isolation, partial purification, and some properties of this enzyme. A preliminary account of this work has appeared (13).

EXPERIMENTAL PROCEDURE

Substrates—Mannan was prepared by citrate buffer extraction (14) of pressed baker's yeast (Red Star Company, Oakland, California) or by extraction of fresh cells of K. brevis. The mannans of Candida albicans, Candida stellatoidea, and Candida tropicalis were donated by Dr. H. F. Hasenclever, National Institutes of Health, Bethesda, Maryland. p-Nitrophenyl α-D-mannoside was a gift of Dr. Y. C. Lee, McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland. Oligosaccharides were prepared from different mannans by acetylation and purification of the products by gel filtration (4, 5). See Fig. 1 for the structures of these oligosaccharides. The

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Fig. 1. Structures of the oligosaccharides isolated from acetylates of S. cerevisiae mannan. M denotes an α-α-mannopyranosyl residue. After Stewart, Mendezhausen, and Ballou (5).

Enzyme Assay—The assay mixture contained 250 μg of mannan, 0.50 ml of 0.1 M potassium phosphate buffer (pH 6.8), 0.1 μmole of CaCl₂, 50 μg of bovine serum albumin, 1 to 3 μg of enzyme protein, and water to 1.0 ml. The mixture was incubated for 1 hour at 37°, when the reaction was stopped by the addition of 1.0 ml of the alkaline copper reagent described by Paleg (19). The tubes were heated in a boiling water bath for 25 min. After the tubes cooled, 1.0 ml of the ammonium sulfate reagent was added, the tubes were stirred vigorously, and the contents were diluted to 10 ml. After 15 min, the absorbance was read at 560 nm in a Zeiss PMQ II spectrophotometer. Enzyme or zero time controls were subtracted from the sample values. The amount of reducing sugar released was determined from curves with mannose as the standard. One unit of activity was defined as the release of 1 μmole of mannose eq per hour under the assay conditions.

Enzyme samples were assayed in triplicate with zero time or boiled enzyme blanks for each determination. The assay was linear up to 100 μg of reducing sugar. Enzyme samples were diluted so that 23 to 65 μg of reducing sugar were released under the assay conditions. Phosphate buffer containing 1.0 mg per ml of bovine serum albumin was used as diluant.

RESULTS

Isolation and Identification of Mannosidase-producing Microorganism

A soil microorganism which was capable of growing on yeast mannan was isolated by enrichment culture techniques (13). The organism was grown on a medium of the following composition: 1 g of yeast mannan, 500 mg of (NH₄)₂SO₄, 200 mg of MgSO₄, 10 mg of FeSO₄·7H₂O, 50 mg of CaCl₂·2H₂O, 500 mg of potassium phosphate buffer, pH 6.8, were mixed and layered on the lower gel. The Tris-glycine buffer was carefully layered over this solution and electrophoresis carried out. Gels were stained with 1% Amido black in 7% acetic acid.

Oligosaccharides were acetylated in a 1:1 mixture of anhydrous pyridine and acetic anhydride, 10 to 50 mg of saccharide in 5 ml of the mixture, by heating on a steam bath for 12 hours. The solvent was evaporated and the residue extracted with chloroform, which was washed three times with water and then evaporated to dryness. The acetylated oligosaccharide was dissolved in 2 ml of acetylum medium, a mixture of acetic acid, acetic anhydride, and concentrated sulfuric acid in the ratios 10:10:1, v/v. The solution was warmed in an oil bath at 49°. To obtain complete cleavage of α-(1 → 6)-linkages, acetylation was carried out for 13 hours (5). The reaction was stopped by the addition of 2 volumes of anhydrous pyridine to the solution. The solvent was evaporated and the oily residue was taken up in chloroform. The chloroform extract was washed three times with water and the water wash was extracted three times with chloroform. The combined chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in a few milliliters of anhydrous methanol and barium methoxide in methanol was added dropwise until the solution became alkaline. After 15 min, solid CO₂ was added to neutralize the base and the methanol was evaporated. The deacetylated oligosaccharides were dissolved in 2 ml of water and the barium carbonate which formed was removed by centrifugation. Deacetylated acetylation products were separated by gel filtration.

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Dialyzed for 24 hours against 0.01 M potassium phosphate buffer, pH 6.8, containing 10^{-4} M Ca^{++}, and water to 1 liter. The cells were grown at 30° with shaking, and the growth curve is shown in Fig. 2. It was necessary to incubate the cells for 36 hours to obtain maximal production of enzyme and digestion of mannan. Analysis of cultural filtrates and of harvested cells, which had been disrupted by sonic radiation, showed that 95% of the total α-mannosidase activity was in the cultural filtrate.

The mannosidase-producing microorganism was an obligate aerobe which did not form spores. The cells were long, slender rods in young cultures and were nonmotile. The long rods were replaced by shorter rods and coccolid shapes in older cultures. Stationary phase cell sizes were 2 to 3 μ in length and were about 1 μ in width. Colonies of the microorganism were round and yellow and they showed pseudomycelial growth.

The microorganism was strongly gram-positive but was not acid-fast at any stage of growth between 0 and 96 hours of incubation. The cells grew well at 30° and 37° on media containing 0.5% glucose or mannose, supplemented with 0.05% yeast extract. The microorganism grew well on 0.5% yeast extract plus salts, and on the glycerol medium used for Mycobacteria (22), but did not grow as well on the latter medium as on glucose, mannose, or mannan. The organism secreted a yellow pigment into the cultural medium which showed a bluish green fluorescence when irradiated with ultraviolet light.

Two liters of cells were grown on the glycerol medium used for Mycobacteria (22) and were extracted with 70% ethanol in a search for the 6-O-methylglucose-containing lipopolysaccharide found to be characteristic of several species of Mycobacteria (23). The extract was worked up as described by Saier and Ballou (24). Carbohydrate-containing fractions from the Sephadex G-50 column were pooled, concentrated, and hydrolyzed in 2 N H_2SO_4 at 100°. The neutralized hydrolysates were chromatographed on paper in Solvents B, C, and D. No spot corresponding to 6-O-methylglucose was observed. The hydrolysates contained substantial amounts of inositol and glycerol, but only a small amount of mannose, indicating that the mannophosphosides which are found in Mycobacteria, Corynebacteria, and Nocardia (23, 25, 26) were present in very small amounts, if at all. On the basis of these findings, the organism was identified as a member of the genus Arthrobacter, and was designated Arthrobacter GJM-1.

**Isolation and Purification of α-Mannosidase**

**Cultural Filtrate**—Five cultures of 1 liter each were grown on the mannus-salts medium described above. All subsequent steps were carried out at 4°. The cells were harvested after 36 hours by centrifugation in a Sorvall automatic refrigerated centrifuge at 10,000 rpm for 30 min. The cultural filtrate was dialyzed for 24 hours against 0.01 M potassium phosphate, pH 6.8, containing 10^{-4} M Ca^{++} (Buffer A). Aliquots, 500 ml, of the cultural filtrate were placed in suitable lengths of cellulose dialysis tubing (1 inch diameter, Union Carbide). Five such sacs were placed in a 15-liter bell jar containing 10 liters of Buffer A. The dialysate was replaced with fresh buffer after about 12 hours. Finally, the solution in the dialysis sacs was lyophilized in 2.5-ml batches to a concentration of about 20 mg per ml. About 200 ml of buffer were used. The enzyme was precipitated from this solution by the addition of solid ammonium sulfate (610 mg per ml). The protein suspension was allowed to stand at 4° overnight and then was centrifuged at 12,000 rpm for 30 min. The resulting precipitate was extracted with 50 ml of a 0.01 M ammonium sulfate solution. After standing for several hours at 4°, the precipitate was collected by centrifugation at 15,000 rpm for 30 min. Two centrifugations were necessary for complete precipitation of the enzyme.

**Sephadex G-100 Gel Filtration**—The ammonium sulfate precipitate was dissolved in 10 ml of Buffer B to give a final protein concentration of about 20 mg per ml. This solution was applied in 2.5 ml batchwise to a column, 2 × 100 cm, of Sephadex G-100, previously equilibrated with Buffer B. The elution pattern from this column is shown in Fig. 3. The mannoside-containing fractions were pooled and concentrated to 25 ml in an Amicon model 400 ultrafilter at an argon pressure of 70 psi with the UM-1 membrane.

**DEAE-cellulose Column Chromatography**—The concentrated Sephadex G-100 fraction was dialyzed overnight against 0.05 M Tris-chloride buffer, pH 9.0, containing 10^{-4} M Ca^{++} (Buffer C) and the dialyzed protein was applied to a column, 1.8 × 12 cm, of DEAE-cellulose equilibrated with the same buffer. The **Fig. 2.** Growth curve of the α-mannosidase-producing microorganism. One liter of the cultural medium was inoculated with 10 ml of an 18-hour liquid culture and grown in a conical flask at 30° on a New Brunswick gyratory shaker at 150 rpm. Aliquots were removed aseptically at intervals and analyzed for cell dry weight and for mannan and enzyme concentrations. Cell dry weight was determined on samples which had been dried at 120° for 15 hours, and is represented in the figure by the solid line. The dashed line represents the mannose concentration (milligrams per ml) at various stages of growth and the dotted line represents the enzymatic activity (micrograms of mannose equivalent released per hour by 1 ml of cultural medium).
The enzyme was purified approximately 10-fold with about 40% recovery. Although the DEAE-cellulose step resulted in a 50% loss of activity, it removed an acidic, yellow protein contaminant from the enzyme preparation. This was the major protein contaminant at all stages of the purification prior to the DEAE-cellulose column and it could not be shown to possess any enzymatic activity. The degree of purification of the enzyme was not increased by substitution of Sephadex G-150 or G-200 for G-100 or by chromatography of the enzyme on hydroxylapatite, although both procedures could be carried out with good recovery of enzymatic activity.

**Properties of Enzyme**

The purity of the enzyme was assessed at each stage of the purification by disc electrophoresis on 8% anionic polyacrylamide gels. The concentrated enzyme from the DEAE-cellulose column showed two protein bands which stained with about equal intensities. The two bands had very similar mobilities and were only 1 mm apart after 1.5 hours of electrophoresis. Attempts to elute the enzyme from the gel were only slightly successful. In these experiments a gel, to which 100 μg of protein had been applied, was cut in half after electrophoresis. One half was stained for protein with Amido black. The other half was cut into 0.5-cm strips and each strip was placed in a small test tube containing 1 ml of a 0.1% solution of bovine serum albumin in Buffer B. The gel strips were macerated with a spatula and the suspensions were allowed to stand at room temperature for 1 hour. The gel fragments were removed by centrifugation and the supernatant liquid was dialyzed for 12 to 24 hours at 4° against Buffer B and then assayed. Only a small fraction (≤5%) of activity was recovered by this technique, but other methods (e.g. freezing and thawing) resulted in even lower recoveries. The activity recovered was located on the same strip as the two protein bands. No other strip contained activity or protein. Since it was not possible to determine which of the two bands represented the enzyme, it may be only 50% pure.

The purified enzyme was free of other glycosidase activities (see below) and was not contaminated with phosphatase or protease activity. It digested mannan to the same extent as

A summary of the purification procedure is given in Table I. The enzyme was purified approximately 10-fold with about 40% recovery. Although the DEAE-cellulose step resulted in a 50% loss of activity, it removed an acidic, yellow protein contaminant from the enzyme preparation. This was the major protein contaminant at all stages of the purification prior to the DEAE-cellulose column and it could not be shown to possess any enzymatic activity. The degree of purification of the enzyme was not increased by substitution of Sephadex G-150 or G-200 for G-100 or by chromatography of the enzyme on hydroxylapatite, although both procedures could be carried out with good recovery of enzymatic activity.

**Table I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultural filtrate</td>
<td>4890</td>
<td>875</td>
<td>2.88</td>
<td>33</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Dialysis and lyophiliza-</td>
<td>245</td>
<td>610</td>
<td>2.78</td>
<td>46</td>
<td>1.4</td>
<td>97</td>
</tr>
<tr>
<td>60% Ammonium sulfate pre-</td>
<td>9.5</td>
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<td>2.56</td>
<td>128</td>
<td>3.9</td>
<td>89</td>
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<td>Sephadex G-100</td>
<td>130</td>
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<tr>
<td>Concentration</td>
<td>15</td>
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- One unit is defined as the release of 1 μmole of mannose per hour under the assay conditions described in the text.
- This column gives the increase in specific activity over that of the cultural filtrate at each stage of the purification.

**Fig. 3.** Chromatography of crude α-mannosidase (60% ammonium sulfate fraction) on Sephadex G-100. The column was loaded with 2.5 ml of enzyme solution (equivalent to 50 mg of protein) and eluted with 0.1 M potassium phosphate buffer, pH 6.8, containing 10^{-4} M Ca^{2+}. The solid line represents the absorbance at 280 μm (protein) and the dashed line the absorbance at 560 μm (enzyme activity). The most active fractions were pooled and purified further.

**Fig. 4.** DEAE-cellulose chromatography of α-mannosidase. The concentrated Sephadex G-100 fraction was dialyzed against 0.05 M Tris buffer, pH 8.0, containing 10^{-4} M Ca^{2+}. The dialyzed solution was applied to a column, 1.8 × 12 cm, of DEAE-cellulose equilibrated with the same buffer. Elution was accomplished with a linear gradient of 0 to 0.6 M KCl. The solid line represents the absorbance at 280 μm (protein), and the dashed line the absorbance at 560 μm (enzyme activity). The shape of the KCl gradient is also depicted in the figure. Active fractions were pooled and concentrated.

**Enzyme activity**

A summary of the purification procedure is given in Table I. The enzyme was purified approximately 10-fold with about 40% recovery. Although the DEAE-cellulose step resulted in a 50% loss of activity, it removed an acidic, yellow protein contaminant from the enzyme preparation. This was the major protein contaminant at all stages of the purification prior to the DEAE-cellulose column and it could not be shown to possess any enzymatic activity. The degree of purification of the enzyme was not increased by substitution of Sephadex G-150 or G-200 for G-100 or by chromatography of the enzyme on hydroxylapatite, although both procedures could be carried out with good recovery of enzymatic activity.

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did the crude preparation and the digestion products were the same. Thus, no enzyme of a particular specificity was lost during the purification procedure.

**pH Optimum and Protein, Substrate, and Time Dependence**
The enzyme was active between pH 5 and 9 with an optimum between 6.5 and 7.0. Maximal activity was obtained in potassium phosphate buffers. The mannosidase activity was dependent on added enzyme, substrate, and time of incubation (Figs. 5 to 7). The added mannan was about 40% hydrolyzed after 3 hours of incubation.

**Stability**—The enzyme was stable to repeated freezing and thawing and could be stored at −10°C indefinitely with only a slight decrease in activity. It was stable at 4°C for at least 2 days and could be kept at room temperature under 1 drop of toluene for 1 day with only a 15% decline in activity. No loss of activity occurred when the enzyme was heated at 50°C for 5 min, whereas the enzymatic activity was completely destroyed by heating at 70°C for the same length of time.

**Effects of Added Divalent Cations**—The effects of Ca++, Mg++, Cu++, and Zn++ on the mannosidase are shown in Table II. All cations were added as the chloride. Added KCl was without effect. The activity of the partially purified enzyme was stimulated by added Ca++ ions. In contrast to crude preparations, which had maximal activity in the presence of 10⁻⁴ M Ca++, the purified preparation exhibited maximum activity at 10⁻⁵ M. Therefore, this concentration of Ca++ was used in assays of the partially purified enzyme. The enzyme was inhibited by Mg++, Cu++, and Zn++. Zinc ion has been shown to stimulate the mannosidases of rat epididymis and jack bean meal (27).

To determine whether the purified enzyme required added divalent cation, an aliquot was dialyzed against 10⁻⁴ M EDTA in 0.05 M Tris-chloride buffer, pH 7.2. After 24 hours, the

![Fig. 5. Dependence of the activity of partially purified α-mannosidase on added protein. Assay mixtures contained the following ingredients: mannan; 250 μg; CaCl₂, 10 μmol; 0.1 M potassium phosphate buffer (pH 6.8), 0.50 ml; bovine serum albumin, 50 μg; and water to 1.0 ml. Incubation was carried out at 37°C for 1 hour. The reaction mixtures were analyzed as described in the text under "Enzyme Assay."](image1)

![Fig. 6. Dependence of the activity of α-mannosidase on added substrate. Assay mixtures were prepared as described in Fig. 5 except that the amount of substrate added was varied.](image2)

![Fig. 7. Dependence of the activity of α-mannosidase on the time of incubation. Assay mixtures were prepared as described in Fig. 5. Tubes were removed at various times after incubation began and were assayed for the release of mannose.](image3)
dialysis medium was changed to 0.05 M potassium phosphate buffer, pH 6.8. Dialysis was continued for an additional 24 hours. Assay of the dialyzed enzyme showed that about 40% of the activity had been lost. The activity was completely restored when the enzyme was assayed in the presence of $10^{-2}$ M Ca++. 

**FIG. 8.** Photograph of a descending paper chromatogram of the products of the mannosidase digestion of oligosaccharides (ethyl acetate-pyridine-water, 5:3:2). The standards are $\alpha$-(1 → 6)-linked mannobiore and the di-, tri-, tetra-, and pentasaccharides isolated from yeast mannan by acetolysis (5). See Fig. 1 for exact structures. The oligosaccharides obtained by mannosidase digestion result from the cleavage of single mannose residues from the substrates. In the case of $M_4$ and $M_5$, these products have structures identical with the corresponding oligosaccharide from yeast mannan acetolyses. Thus, $M_4$ yields $M_5$ and $M_3$ yields $M_2$ and mannose. $M_1$ digestion yields a mixture of di-, tri-, and tetrasaccharides containing $\alpha$-(1 → 6)- and $\alpha$-(1 → 2)-linkages. $M'_4$ is $\beta$-$\alpha$-mannopyranosyl-(1 → 6)-$\beta$-mannopyranose.

**FIG. 9.** Schematic representation of a method for characterizing the products of $\alpha$-mannosidase digestion of $M_4$. Tri- and tetrasaccharide fractions obtained by mannosidase digestion were examined by acetolysis. If both nonreducing ends of $M_4$ were attacked by the mannosidase, the tetrasaccharide fraction would yield $M_5$, $M_3$, and mannose on acetolysis. If only one nonreducing end is attacked by the enzyme, the acetolysis products would contain $M_5$, or $M_3$ plus mannose, but not all three. Similarly, if all of the nonreducing ends of the tetrasaccharide were attacked, acetolysis of the trisaccharide fraction should yield $M_5$, mannose, and some $M_4$ if the terminal $\alpha$-(1 → 6)-linkage of one isomer is broken.

**Specificity of $\alpha$-Mannosidase**—The enzyme was active on the mannans of different strains of *S. cerevisiae* and on the mannans of *K. brevis*, *C. albicans*, *C. stellatoidea*, and *C. tropicalis*. It was also active on the oligosaccharides obtained by acetolysis of yeast mannans and on a series of $\alpha$-(1 → 6)-linked oligomannosides (di- to hexa saccharide). The preparation of the latter oligosaccharides and the mode of action of the enzyme on intact mannans are described in an accompanying paper (28).

The $\alpha$-mannosidase hydrolyzed $p$-nitrophenyl $\alpha$-d-mannoside at a very slow rate, and methyl $\alpha$-d-mannoside, glycogen, amylose, maltose, and cellobiose not at all. It was also inactive on a sample of crude *Mycobacterium phlei* mannolipid (22) and on mycoinositol 2-O-$\alpha$-d-mannoside and glycerylphosphorylmycoinositol dimannoside from this mannolipid fraction.
Action of Enzyme on Oligosaccharide Substrates

Oligosaccharides from S. cerevisiae Mannan—Assay mixtures were scaled up to 3 ml, with 200 μg per ml of a given oligosaccharide as substrate instead of mannan. Enzyme (6 μg) was added and the mixture was incubated for 1 hour, when the reaction was stopped by boiling. Two milliliters of the mixture were used to assay for the release of reducing sugar. The remaining 1 ml was desalted by shaking it with mixed bed resin (carbonate form), after which it was concentrated and applied to Whatman No. 1 paper for chromatography with Solvent A. The results of this experiment are shown in Fig. 8. Reducing sugar was released from all of the oligosaccharides, although at different rates. Fig. 8 shows that the products of digestion were mannose, starting material, and the oligosaccharides which would be produced by cleavage of mannos residues from the starting material. For example, M3 and M5 yielded mannose only, M4 yielded M2 and mannose, and M4 yielded mainly M2 and mannose. M4 is a branched oligosaccharide composed of two structural isomers (see Fig. 1). Digestion of M4 yielded a mixture of products with the chromatographic mobilities of di-, tri-, and tetrasaccharides. These oligosaccharides contain α-(1→6)- as well as α-(1→2)-linkages and are produced by sequential cleavage of single mannose units from both nonreducing termini of the starting material. The exact structures of these products are described below. At times much shorter than 1 hour, M5 digestion yielded only the tetrasaccharide fraction and mannose, confirming that the lower oligosaccharides were produced by stepwise cleavage of mannose residues from the starting material. When the reactions were allowed to proceed for 6 hours with additions of enzyme (6 μg each time) at 2-hour intervals, each oligosaccharide was completely degraded to mannose. Thus, the enzyme can hydrolyze (1→2)-, (1→3)-, and (1→6)-linkages in small substrates. Boiled enzyme controls showed only starting material on paper chromatography.

Characterization of Products of M5 Digestion—As mentioned above, mannosidase digestion of M5 produced a mixture of lower oligosaccharides. These compounds were examined to determine whether the enzyme was capable of attacking both nonreducing ends of the M5 molecule. This was done by acetylation of the tri- and tetrasaccharide fractions produced by digesting M5 with α-mannosidase. The products expected from the digestion are shown in Fig. 9. If both nonreducing ends of M5 were attacked, three different tetrasaccharides would be produced, two containing mannose linked α-(1→6)- to M5 and one containing two disaccharides joined by an α-(1→6)-linkage. Acetylation of the tetrasaccharide fraction would yield M5, M4, and mannose. If only one of the nonreducing ends of M5 were attacked, these three disaccharides would be produced, two containing mannose linked α-(1→6)- to M5 and one containing two disaccharides joined by an α-(1→6)-linkage. Acetylation of the tetrasaccharide fraction would yield M5, M4, and mannose. If only two of the nonreducing ends of the tetrasaccharide intermediates were attacked, acetylation of the resulting disaccharide fraction should yield M5, M4, and mannose, and some M4 if the terminal α-(1→6)-linkage of one isomer is broken (Fig. 9).

To test the above hypothesis, 10 mg of M5 were digested for 1 hour with a limiting amount of enzyme. The reaction was stopped by boiling the mixture and the digestion products were isolated by gel filtration. The products obtained were starting...
Table III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (μmol/mg enzyme protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ma</td>
<td>2.3</td>
<td>15</td>
</tr>
<tr>
<td>M2</td>
<td>1.9</td>
<td>14</td>
</tr>
<tr>
<td>M3</td>
<td>0.4</td>
<td>5.1</td>
</tr>
<tr>
<td>M5</td>
<td>0.5</td>
<td>13.6</td>
</tr>
<tr>
<td>M4</td>
<td>0.4</td>
<td>10.6</td>
</tr>
<tr>
<td>M2*</td>
<td>6.9</td>
<td>29.0</td>
</tr>
<tr>
<td>Mannan</td>
<td>$1.0 \times 10^{-2}$</td>
<td>5.4</td>
</tr>
</tbody>
</table>

* Isolated from C. stellatoidea mannan.

The enzyme described in this report is very active on yeast mannan. Many α-mannosidases are known, and of these, the enzymes of rat epididymis, jack bean meal, and marine gastropods have been studied extensively (11, 12, 29). Significant differences exist between these enzymes and the bacterial enzyme described in this paper. One notable difference is that the bacterial enzyme was not very active on α-L-mannosides with the elution pattern shown in Fig. 10. M3, M5, and mannose were obtained from the tetrasaccharide fraction, indicating that the enzyme split both terminal (1 → 2)-linkages in the tetrasaccharide, the terminal (1 → 6)-linkage was hydrolyzed at a much slower rate.

Kinetic Studies of Action of α-Mannosidase on Oligosaccharides of S. cerevisiae Manann—The $K_m$ and $V_{max}$ values for the baker’s yeast mannan oligosaccharides were determined with the following assay mixtures: 0 to 500 μg of substrate, 0.5 ml of 0.1 M potassium phosphate buffer (pH 6.8), 10 μmole of CaCl2, 1.72 μg of enzyme protein, and water to 1.0 ml. After incubation for 15 min at 37°C, reducing sugar was determined. Lineweaver-Burk plots of the data are given in Fig. 11 and the $K_m$ values are listed in Table III.

Discussion

The enzyme described in this report is very active on yeast mannan. Many α-mannosidases are known, and of these, the enzymes of rat epididymis, jack bean meal, and marine gastropods have been studied extensively (11, 12, 29). Significant differences exist between these enzymes and the bacterial enzyme described in this paper. One notable difference is that the bacterial enzyme was not very active on α-mannosides with aromatic aglycons, whereas these compounds are good substrates for the other three enzymes. This finding could be related to the different metabolic roles of the various enzymes. For example, the rat, jack bean, and gastropod enzymes are involved exclusively to other polar mannose residues. Thus, the mannosidase might be expected to be less active on mannosides containing nonpolar aglycons.

Another significant difference between the bacterial enzyme and the other three has to do with the effects of cations on their activity. Most reports of studies on the rat, gastropod, and jack bean enzymes do not indicate any dependence on, or stimulation by, added divalent cations. It has been reported recently, however, that zinc ion stimulates the rat and jack bean enzymes, prevents their inactivation at 37°C, reactivates activity which has been lost by incubation, and counteracts the inhibitory effects of Cu++ and Cd++ (27). As can be seen from Table II, zinc ion was a potent inhibitor of the Arthrobacter enzyme at concentrations as low as $10^{-3}$ mM, and did not stimulate at any concentration tested. Calcium ion stimulated both the crude and purified enzyme and was able to restore activity which had been lost by dialysis against EDTA. This finding implicates the divalent cation in the mechanism of action of the enzyme. Calcium ion inhibits the mannosidase of the marine gastropod, Charonia lampas (11).

Studies with the oligosaccharides obtained by acetylation of S. cerevisiae mannan have shown that the enzyme cleaves α-(1 → 3), α-(1 → 4), and α-(1 → 6)-linkages in these substrates, releasing mannose residues from their nonreducing ends. It was surprising to find that the enzyme was not able to hydrolyze mycosinol 2-α-O-mannoside since this compound is very similar in structure and presumably possesses the same conformation as M2 (30). This finding further suggests that the enzyme is very sensitive to the structure of the aglycon.

Because of the structural heterogeneity of M3, M2, and M4 it was not possible to define exactly the factors which contribute to the differences in $K_m$ and $V_{max}$ obtained for the oligosaccharides. It does seem likely that the terminal α-(1 → 3)-linkage is responsible for the low $V_{max}$ observed for M4. It is also probable that the smaller $K_m$ values of M5 and M6 result from a combination of increased molecular size and the introduction of the branch points.

The $K_m$ value for intact S. cerevisiae mannan is $1 \times 10^{-4}$ mM, based on a molecular weight of 22,000 (13). However, the molar concentration of side chains in the mannan molecule is about 50 times higher than the concentration of the mannan itself so that many more end groups are available for enzymatic attack. When the $K_m$ for mannan is multiplied by this factor, a value of $5 \times 10^{-4}$ mM is obtained, which is close to those determined for the oligosaccharide substrates.

In the accompanying paper (28), the mode of action of the enzyme on intact mannans from S. cerevisiae and other yeasts is described.

References

Studies on the Structure of Yeast Mannan: I. PURIFICATION AND SOME PROPERTIES OF AN α-MANNOSIDASE FROM AN ARTHROBACTER SPECIES

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