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Solubilization of a Mannose-Polymerizing Enzyme from *Phaseolus aureus*

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A soluble enzyme preparation, which catalyses the polymerization of mannose, was obtained by Triton X-100 extraction of a particulate fraction derived from *Phaseolus aureus* hypocotyls. The product that resulted when GDP-α-D-mannose was used as a substrate was a β-(1→4)-linked mannan, about three-quarters of which was alkali-insoluble. The mannose-polymerizing enzyme activity was at least as great in the soluble preparation as in the particulate preparation, and the specific activity of the solubilized enzyme was greater by a factor of at least 3.5. Kinetic studies of the soluble enzyme indicate that the apparent K_m is 55–62 μM, and a disproportionate increase in rate is observed at high concentrations. GDP-α-D-glucose is a strong competitive inhibitor of the mannose-polymerizing reaction, with an apparent K_i of 6.2 μM. The soluble enzyme is relatively unstable, losing about two-thirds of its original activity in 5h at 0°C or in 24h at −20°C. A solvent (acetone, butanol, diethyl ether)-extracted particulate preparation, which also exhibits the same enzyme activity, is more stable, retaining full activity for at least 5 days at −20°C. There was no polymerizing-enzyme activity in the soluble enzyme preparation when UDP-D-glucose, UDP-D-galactose, UDP-D-xylose, UDP-L-arabinose or UDP-D-glucuronic acid were used as substrates. However, the soluble enzyme preparation would catalyse the polymerization of glucose, with GDP-D-glucose as substrate.

Enzyme systems have been isolated from *Phaseolus aureus* that will catalyse the incorporation of radioactive sugars into polysaccharides (Feingold *et al.*, 1958; Barber *et al.*, 1964; Villemez *et al.*, 1966, 1967, 1968; Kauss, 1967; McNab *et al.*, 1968). These enzymes reside in crude particulate fractions, and all use sugar nucleotides as substrates. Only one attempt to purify the particulate enzymes from *Phaseolus aureus* has been reported (Villemez *et al.*, 1968), and the results from this attempt indicate that the resolution of these particulate enzyme systems will be difficult. However, more promising results have been reported in efforts to solubilize individual glycosyltransferases from these enzyme systems. Feingold *et al.* (1958) reported the solubilization of a glucosyltransferase, which catalyses the formation of a β-(1→3)-linked glucan with UDP-α-D-glucose as substrate. Liu & Hassid (1970) also reported the solubilization of a glucosyltransferase from the *Phaseolus aureus* particulate preparation, which used GDP-α-D-glucose as substrate in the formation of a β-(1→4)-linked glucan. The present paper contributes a method for the solubilization of another polymerizing enzyme, which catalyses the formation of a β-(1→4)-linked mannan with GDP-α-D-mannose as a substrate.

The plant-enzyme-catalysed incorporation of [^{14}C]mannose from GDP-α-D-[^{14}C]mannose into polysaccharides was reported by Barber *et al.* (1964). These researchers utilized a particulate enzyme preparation derived from *Phaseolus aureus* seedlings, and suggested that this enzyme preparation catalysed the synthesis of two mannose-containing polysaccharides, a mannan from GDP-α-D-mannose, and a glucomannan from the guanosine derivatives of glucose and mannose. A later study (Elbein, 1969) demonstrated the *Phaseolus aureus* particulate-enzyme-catalysed incorporation of [^{14}C]mannose into a glucomannan from GDP-α-D-[^{14}C]mannose, the glucose residues in the polysaccharide being derived from the particulate enzyme preparation. On the basis of kinetic results, Villemez (1971) concluded that at least two polysaccharides were being formed simultaneously from GDP-α-D-mannose in reactions catalysed by a *Phaseolus aureus* particulate enzyme preparation. However, the polysaccharide mixture could not be resolved by selective extraction with alkali. The production, by partial hydrolysis of radioactive polysaccharide material (Elbein, 1969), of radioactive oligosaccharides containing glucose and mannose demonstrated directly that a glucomannan had been formed from GDP-α-D-[^{14}C]mannose. But the isolation of a small amount of radioactive mannose-containing oligosaccharide from the same hydrolysis was inconclusive with regard to the formation of a mannan, as these could have been derived from portions of a glucomannan. To demonstrate homopolymerization of mannose, the mixture of radioactive polysaccharides must be resolved and a pure radioactive mannan isolated, or

an enzyme preparation that forms only radioactive mannan from GDP-α-D-[^{14}C]mannose must be isolated. In the present paper we describe two enzyme preparations from *Phaseolus aureus* hypocotyls that catalyse the formation of a β-(1→4)-linked [^{14}C]-mannan as the only radioactive polysaccharide product when GDP-α-D-[^{14}C]mannose is the only substrate provided. One of these enzyme preparations is particulate, and is prepared by extracting a *Phaseolus aureus* particulate enzyme preparation sequentially with acetone, butanol and diethyl ether at low temperatures. The other enzyme preparation is soluble, and was obtained from a *Phaseolus aureus* particulate enzyme preparation by treatment with an aqueous solution of Triton X-100.

Materials and Methods

Materials

GDP-α-D-[U-^{14}C]glucose (sp. radioactivity 124 or 191 μCi/μmol), UDP-α-D-[U-^{14}C]glucuronic acid (sp. radioactivity 237 μCi/μmol), UDP-L-[U-^{14}C]arabinose and UDP-α-D-[U-^{14}C]xylose (sp. radioactivity 184 μCi/μmol) were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. GDP-α-D-[U-^{14}C]mannose (sp. radioactivity 65 or 154 μCi/μmol) was obtained from International Chemical and Nuclear Corp., Irvine, Calif., U.S.A. UDP-α-D-[U-^{14}C]glucose (sp. radioactivity 35 μCi/μmol) was obtained from Calatomic, Los Angeles, Calif., U.S.A. Triton X-100 was obtained from Calbiochem, Los Angeles, Calif., U.S.A.

Methods

Preparation of particulate enzyme. The particulate enzyme from *Phaseolus aureus* seedlings was prepared in a manner similar to that described by Villemez & Clark (1969). Mung-bean hypocotyls (3–4cm long) were homogenized in a cold mortar with sand, in the presence of an equal weight of potassium phosphate buffer (0.05M, pH7.3, 0°C) containing 1% (w/v) bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.), 5mM-dithiothreitol (Calbiochem) and 1mM-$MgCl_2$. The homogenate was strained through two layers of Miracloth (Calbiochem) and the filtrate was centrifuged at 1000*g* for 10min. The material sedimented from the 1000*g* supernatant solution at 49000*g* in 15min was resuspended in 0.5ml (per 10g of hypocotyls) of 0.05M-potassium phosphate buffer, pH7.3, containing 0.01M-$MgCl_2$, 0.4M-sucrose, 5mM-dithiothreitol and 1% bovine serum albumin. The particulate enzyme was stored at −20°C for later use.

Preparation of Triton-solubilized enzyme. Frozen particulate enzyme (1ml) was thawed at 0°C, and 40μl of an aqueous solution of Triton X-100 (12.5%, w/v) was added. The mixture was stirred for 10min at 0°C and then was centrifuged at 300000*g* for 40min. The pellet was discarded and the supernatant solution was used as an enzyme source.

Solvent-extracted particulate enzyme. The extractions were performed, with minor modifications as described by Troy *et al.* (1971). Particulate enzyme (3ml) was added dropwise to 120ml of acetone at −22°C and stirred for 5min. The insoluble material was isolated by centrifugation at −15°C and extracted at −20°C with 60ml of butanol for 2min with stirring. The insoluble material was isolated by centrifugation at −15°C and extracted successively with 120ml of acetone at −20°C and twice with 120ml portions of diethyl ether at −10°C. Residual solvent was removed under reduced pressure at room temperature and the dry powder was stored in a desiccator at −20°C. Powder (25mg) was resuspended in 0.5ml of the resuspending buffer described above for use as an enzyme source.

Enzyme reaction. Enzyme (50μl) was incubated at 22–24°C for 30s. Radioactive sugar nucleotides and any other additives (in 50μl of water) were added to produce a final volume of 100μl. After the desired time-interval, the reaction was terminated by the addition of 50μl of aq. 15% (w/v) trichloroacetic acid. Approx. 10mg of powder cellulose (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was added to each sample to provide a supporting medium. The insoluble material was extracted three times with 1ml of water to remove unchanged substrate and other water-soluble material. The water-insoluble residue was extracted with two 1ml portions of aq. 50% (v/v) butanol and then with two 1ml portions of aq. 45% (w/v) phenol. The insoluble residue was washed with 1ml of water, 1ml of acetone and then dispersed in 0.5ml of methanolic 1M-Hyamine. This suspension, and the analysis tube containing it, were placed in a counting vial for radioactivity determinations. Radioactivity was measured by scintillation counting and all samples were corrected for quenching by external standardization (Villemez, 1971).

Preparation of radioactive polysaccharide. Enzyme (100μl) and 100μl of GDP-D-[^{14}C]mannose (450000c.p.m., 5.6nmol) were incubated at 24°C for 1.5h. Radioactive polysaccharide was isolated as described above.

Analytical methods. Periodate oxidations were performed by the modification of the procedure of Hay *et al.* (1965) described by Elbein (1969). Protein was determined by the biuret method (Gornall *et al.*, 1949).

Radioactive oligosaccharides in 100μl of water were reduced by treatment with 1ml of 0.1M-$NaBH_4$ and stirred overnight at room temperature. The reaction mixture was adjusted to pH3.6 with Dowex

50 (H^+ form) and evaporated to dryness after removal of the Dowex 50. Boric acid was removed by repeated evaporation to dryness in the presence of acidic methanol. The reduced oligosaccharides were hydrolysed in 2M-HCl at 100°C for 2h. The hydrolysate was concentrated to dryness at reduced pressure over NaOH.

Linkage configuration in the elucidation of the polysaccharide structure was determined with a β-mannanase, QM477 (Reese & Shibata, 1965), kindly provided by Dr. Elwyn Reese.

Chromatographic methods. Descending paper chromatography was performed with Whatman no. 1 and no. 4 paper. The following solvent systems were used: (I) propan-1-ol–ethyl acetate–water (7:1:2, by vol.); (II) butan-1-ol–pyridine–0.1M-HCl (5:3:2, by vol.). The chromatographs were developed for 19–24h. Paper electrophoresis was performed on Whatman no. 4 paper saturated with 0.05M-sodium tetraborate, pH9.2. The sugar standards were made visible by the periodate–benzidine procedure described by Gordon (1959), or with an alkaline silver nitrate reagent.

Preparation of radioactive oligosaccharides. Oligosaccharides were obtained by acetolysis of the polysaccharide under the conditions described by Scher & Lennarz (1969). Sugars were deacetylated by dissolving them in dry methanol, adding a catalytic amount of barium methoxide, and leaving them to react for 30min at room temperature (Steward *et al.*, 1968). Barium was removed by bubbling CO_2 through the solution and filtering off the $BaCO_3$ precipitate.

Results

The addition of Triton X-100, a neutral detergent, to particulate enzyme preparations from *Phaseolus aureus* has an effect on incorporation of GDP-α-D-[^{14}C]mannose into polysaccharide material. Final concentrations of Triton X-100 in the reaction mixtures of up to 0.5% (w/v) increase incorporation slightly. For example, in a typical experiment, a final concentration of 0.5% Triton X-100 produced an incorporation that was 10% greater than in control reactions. However, Triton X-100 concentrations above 0.5% cause an inhibition of incorporation that is increasingly severe as the detergent concentration increases. For example, in comparison with control values in a typical experiment, 1% Triton X-100 produced a 5% inhibition, and 5% Triton X-100 caused a 90% inhibition of incorporation. Examination of the supernatant solution that resulted from Triton X-100 treatment of particulate enzyme followed by removal of the insoluble material by centrifugation indicated that some of the mannose-polymerizing-enzyme activity was solubilized. Also, a portion of the mannose-polymerizing-enzyme activity remained in the insoluble residue after treatment with Triton X-100 at final concentrations up to 1%. For example, after treatment with 1% Triton X-100 one enzyme-preparation retained 30% of the original mannose-polymerizing-enzyme activity in the detergent-insoluble residue.

An experiment designed to obtain the conditions required for solubilization of maximum mannose-polymerizing-enzyme activity is presented in Fig. 1. Treatment of the particulate enzyme preparation with 0.5% detergent produces slightly more solubilized enzyme activity than treatment with 0.25% detergent, but treatment with 0.75% Triton X-100 resulted in considerably less solubilized enzyme activity. Consequently, treatment with 0.5% Triton X-100 was used as a routine to prepare soluble mannose-polymerizing-enzyme activity. Under these conditions, the soluble enzyme activity usually represented two-thirds to three-quarters of the original activity present in the particulate enzyme preparation (Fig. 2). Solubilization by treatment with Triton X-100 resulted in more soluble mannose-polymerizing-enzyme activity, by a factor of 15–20, than treatment of the particulate enzyme preparation with digitonin (Fig. 3) under conditions described by Liu & Hassid (1970), even though there is slightly more plant protein in the digitonin-solubilized preparation. After

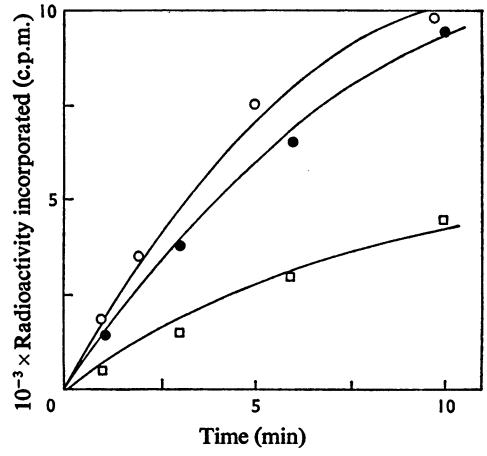

Fig. 1. *Effect of concentration of Triton X-100 in the solubilization of mannose-polymerizing-enzyme activity*

GDP-D-[^{14}C]mannose (40000c.p.m., 0.39nmol) was added to each sample, which was allowed to react at 24°C. The soluble enzyme was prepared as described in the text, except that different concentrations of Triton X-100 were used: ●, 0.25%; ○, 0.50%; □, 0.75%. Reaction conditions and analysis were performed as described in the text.

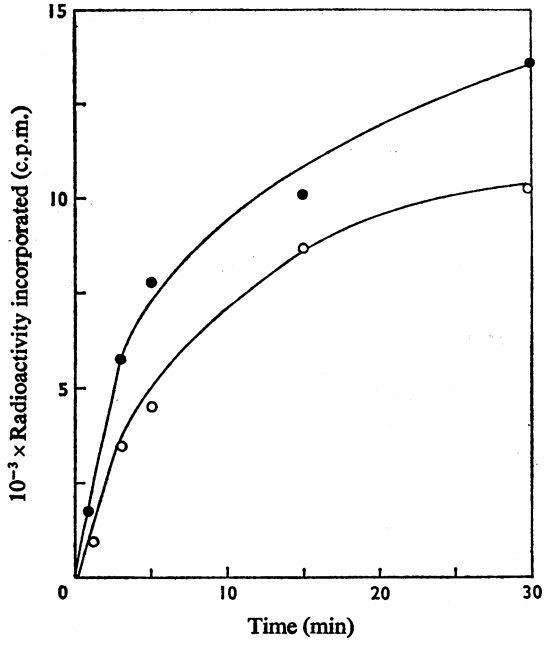

Fig. 2. *Catalysis of mannose polymerization by particulate and soluble enzymes*

Enzyme preparation, reaction conditions and analysis were as described in the text, except that the alkali-soluble material was also removed from the product. Comparable quantities of enzyme preparation were used in each case, based on a constant amount of initial plant material. GDP-D-[^{14}C]mannose (44000c.p.m., 0.43nmol), was added to each sample, which was allowed to react at 24°C. ●, Particulate enzyme; o, Triton-solubilized enzyme.

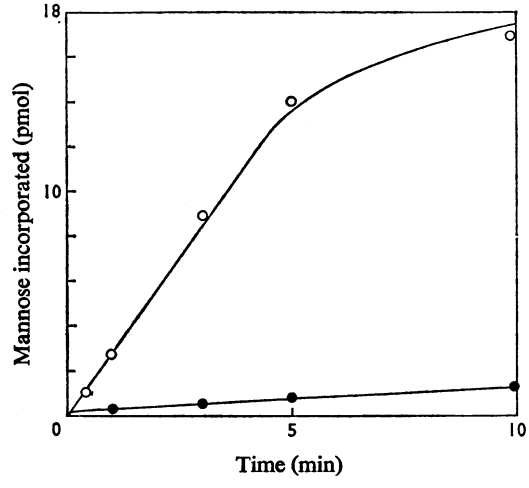

Fig. 3. *Catalysis of mannose polymerization by Triton X-100-solubilized and digitonin-solubilized enzymes*

Enzymes were prepared as described in the text, from equivalent quantities of plant material. Reaction conditions and analysis were as described in the text. GDP-D-[^{14}C]mannose (49800c.p.m., 0.49nmol) was added to each sample, which was then allowed to react for the time indicated at 24°C. ●, Digitonin-solubilized enzyme; o, Triton-solubilized enzyme.

being adjusted to comparable volumes with respect to the quantity of original particulate enzyme preparation extracted, the volume of enzyme preparation assayed (50μl) contained 90μg of plant protein when solubilized by Triton X-100 and 99μg when solubilized by digitonin treatment. The comparable value for the original particulate enzyme preparation was typically 460μg of plant protein/50μl of enzyme preparation. Results comparing the relative specific activity of original particulate enzyme preparation and Triton X-100-solubilized enzyme were not obtained, as (1) the K_m of the soluble enzyme is 50–100-fold higher than the particulate enzyme, and (2) the particulate enzyme preparation apparently exhibits more than one type of mannose-polymerizing-enzyme activity, whereas the soluble enzyme preparation appears to contain only one. However, from the protein concentrations given above and an examination of Fig. 2, it is apparent that the specific activity of the soluble mannose-polymerizing-enzyme is at least 4-fold greater, and probably considerably more, than that of the comparable enzyme in the particulate enzyme preparation.

Stability and substrate utilization

The mannose-polymerizing activity of the Triton-solubilized enzyme preparation was relatively unstable. Storage at 0°C resulted in a 62% decrease in activity in 5h, and a 95% decrease in activity in 24h. Lower temperatures slowed, but did not prevent, loss of activity. Storage at −20°C resulted in a 60% decrease in activity in 24h and an 80% decrease in activity in 48h. However, the Triton X-100 extraction of soluble mannose-polymerizing-enzyme activity was as effective on stored as on fresh particulate enzyme preparations. The particulate enzyme can be stored for several weeks with little loss of enzyme activity.

No radioactivity in insoluble polysaccharide was observed when UDP-D-[^{14}C]glucose, UDP-D-[^{14}C]-galactose, UDP-D-[^{14}C]xylose, UDP-D-[^{14}C]glucuronic acid or UDP-L-[^{14}C]arabinose were used as substrates with the solubilized enzyme preparation. However, an insoluble radioactive polysaccharide

was produced when GDP-D-[^{14}C]glucose was used as a substrate. The polysaccharide obtained with GDP-D-[^{14}C]glucose as a substrate produced what appeared to be a cellodextrin series on chromatography of an oligosaccharide mixture subjected to partial acetolysis. From these results, and from solubility characteristics, the radioactive polysaccharide that results when GDP-D-[^{14}C]glucose is used as a substrate is probably a β-(1→4)-linked glucan. The quantity of [^{14}C]glucan that resulted when the Triton-solubilized enzyme preparation was used with GDP-D-[^{14}C]-glucose as substrate is considerably less than the quantity of [^{14}C]mannose polysaccharide that resulted when GDP-D-[^{14}C]mannose was used as a substrate (Fig. 4).

Kinetics of the reaction

A reciprocal plot (Lineweaver & Burk, 1934) of initial rate as a function of GDP-D-[^{14}C]mannose concentration indicated an apparent K_m of 55–62μM (Fig. 5). The K_m of individual enzyme preparations differed somewhat, and the range listed above encompasses the values obtained from two different preparations. At high concentrations of GDP-D-[^{14}C]mannose, an unexpected increase in rate was observed. The disparate slope of the reciprocal plot at high concentrations of substrate, if extrapolated to its intercept on the negative side of the substrate axis, resulted in an intercept on the substrate axis equivalent to a negative substrate concentration of 0.5–1.25mM (Fig. 5).

The addition of GDP-D-glucose to a reaction mixture containing Triton-solubilized enzyme preparation and GDP-D-[^{14}C]mannose results in an inhibition of mannose polymerization (Fig. 6). In separate experiments, by either a reciprocal-plot method (Lineweaver & Burk, 1934) with constant inhibitor concentration and various substrate concentrations, or the plotting technique suggested by Dixon (1953) with a constant substrate concentration and various inhibitor concentrations, GDP-D-glucose produces the kinetic effects of a classical competitive inhibitor on the enzyme catalysing the polymerization of D-mannose. The K_i of GDP-D-glucose, as determined by either experimental technique, is approx. 6.2μM. Therefore GDP-D-glucose is quite a potent competitive inhibitor of the catalysed reaction resulting in the polymerization of D-mannose.

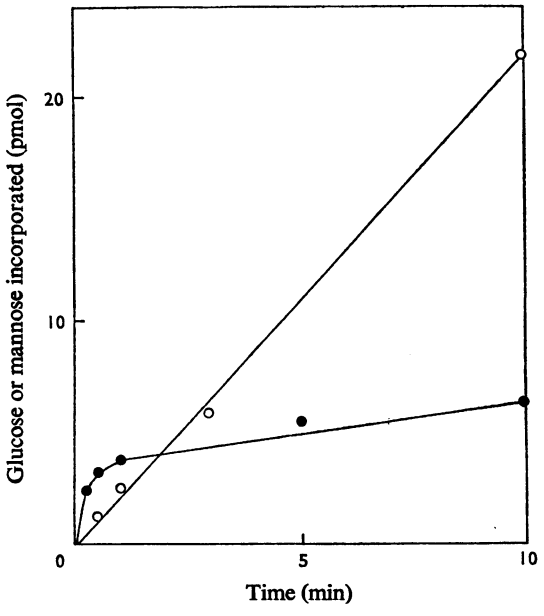

Fig. 4. *Polymerization of* D-*mannose or* D-*glucose by Triton X*-100-*solubilized enzyme*

Reaction conditions and analysis were as described in the text. GDP-D-[^{14}C]mannose (26000c.p.m., 0.32nmol) or GDP-D-[^{14}C]glucose (86000c.p.m., 0.33nmol) was added to each sample, which was allowed to react at 24°C for the indicated time periods. ●, Glucan; ○, mannan.

Structure of the mannose-containing polysaccharide

Catalysis by soluble enzyme. The water-insoluble radioactive polysaccharide produced by using GDP-D-[^{14}C]mannose as a substrate for the Triton-solubilized enzyme is resistant to extraction with

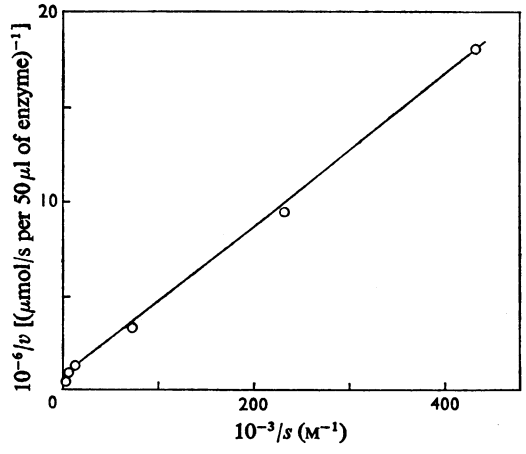

Fig. 5. *Kinetics of* ^{14}*C-labelled polysaccharide formation from GDP-*α-D-[^{14}*C]mannose*

Reaction times for the determination of the initial rate were 30s. Velocity (v) is given as μmol/s per 50μl of enzyme source. Substrate concentration (s) is given as mol/l.

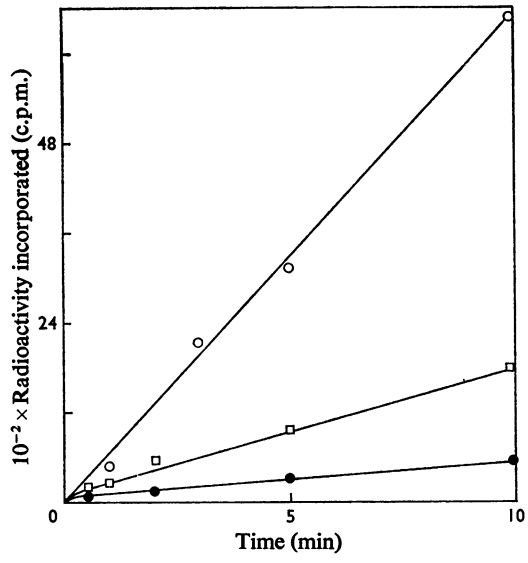

Fig. 6. *Inhibition of mannan formation by GDP-D-glucose*

GDP-D-[^{14}C]mannose (39300c.p.m., 0.39nmol) was added to each sample, alone (○), or in the presence of 0.14nmol of GDP-D-glucose (□) or in the presence of 0.35nmol of GDP-D-glucose (●). Reaction conditions and analysis are described in the text.

alkaline solutions. Only about 25% of the radioactivity is extracted by two treatments with aq. 2% NaOH at 100°C. A chromatographic separation of the products obtained by partial acetolysis of the water-insoluble radioactive polysaccharide is depicted in Fig. 7 (chromatogram I). Three major radioactive peaks were present (chromatogram I, a, c and d). Peak d of chromatogram I had the chromatographic mobility expected of a monosaccharide, and on elution and re-chromatography all the radioactivity had the same mobility as that of D-mannose. Also, all the radioactivity in peak d of chromatogram I, after reduction with $NaBH_4$ and paper electrophoresis in sodium tetraborate buffer (pH9.2), exhibits an electrophoretic mobility identical with that of D-mannitol. Since this electrophoretic method separates D-glucitol, D-galactitol and D-mannitol, peak d of chromatogram I consists exclusively of D-[^{14}C]mannose. Peak c of chromatogram I has a mobility somewhat greater than cellobiose, i.e. that expected of a disaccharide. On elution and reduction with $NaBH_4$, followed by acid hydrolysis and paper electrophoresis of the products from peak c of chromatogram I, D-mannose and D-mannitol in equivalent amounts were found to be the only radioactive components (Table 1). A similar treatment of the material in peak a of chromatogram I (Fig. 8) resulted in an approx. 2:1 ratio of D-[^{14}C]mannose to D-[^{14}C]mannitol (Table 1), with no other radioactive components detectable. Therefore the materials in peaks a and c of chromatogram I are a D-[U-^{14}C]mannotriose and a D-[U-^{14}C]mannobiose respectively.

The linkage type joining the sugar moieties of the disaccharide c of chromatogram I was established as being (1→4), by periodate oxidation followed by reduction, hydrolysis and identification of the reaction products by chromatography (Fig. 9). The only reaction products were those predicted (Hay *et al.*, 1965) for hexopyranoses joined by (1→4) linkages, i.e. erythritol and glycerol. Treatment of the mannobiose with a β-mannosidase resulted in complete hydrolysis, indicating a β-D-linkage.

Catalysis by particulate enzyme. The structure of the radioactive polysaccharide that was formed by catalysis of the hypocotyl particulate preparation, with GDP-α-D-[^{14}C]mannose as substrate, was investigated in a manner similar to that used for the [^{14}C]polysaccharide resulting from soluble enzyme catalysis. The products resulting from a partial acetolysis procedure were, with one exception, the same as those produced from the [^{14}C]polysaccharide produced by the soluble enzyme (Figs. 7, 8 and 9; Table 1). The exception was an additional component (peak b, chromatogram II), with a chromatographic mobility slightly less than that of cellobiose (Fig. 7). This component yielded [^{14}C]-mannose as the only radioactive component after reduction and hydrolysis (Table 1), indicating that no [^{14}C]mannose residues exist on the reducing end of this oligosaccharide. Further, since the chromatographic mobility demonstrates that peak b of chromatogram II is not mannobiose or mannotriose, the reducing sugar must be other than mannose. Therefore, this extra component found in the polysaccharide must be a mannosyl (or mannobiosyl) derivative of another sugar, probably D-glucose. The reducing sugar of this oligosaccharide was not radioactive, indicating that it was derived from the particulate enzyme preparation.

Catalysis by solvent-extracted particulate enzyme. Low-temperature extraction of the particulate enzyme with acetone, butanol and diethyl ether resulted in an enzyme preparation that was active in the utilization of GDP-α-D-[^{14}C]mannose. Characterization of this [^{14}C]polysaccharide was accomplished as indicated above. The products of partial acetolysis (Fig. 10) were the same as those from the [^{14}C] mannan produced by the soluble enzyme preparation, i.e. [^{14}C]mannose, [U-^{14}C]mannobiose, and [U-^{14}C]-mannotriose (Table 1). There was no indication of any other oligosaccharide, particularly none with mobility similar to that of peak b, chromatogram II (Fig. 7). Elution and re-chromatography of the area

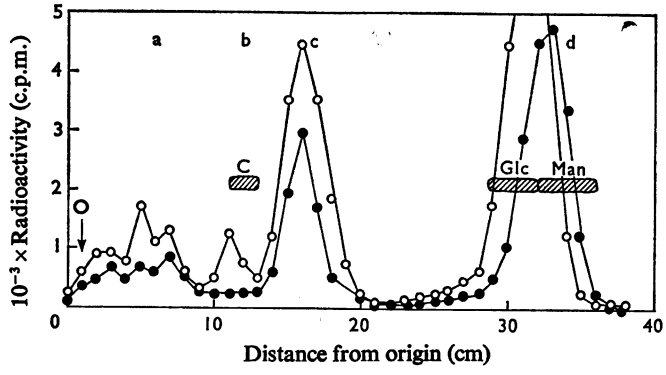

Fig. 7. *Acetolysis of [^{14}C]mannose-containing polysaccharides formed by particulate-enzyme or Triton X-100-solubilized-enzyme catalysis*

The radioactive polysaccharides were prepared and subjected to acetolysis as described in the text. The resulting oligosaccharide mixtures were chromatographed on Whatman no. 4 paper in solvent I. Standard compounds shown are: C, cellobiose; Glc, glucose; Man, mannose. Chromatogram I (●) is that resulting from Triton X-100-solubilized enzyme-catalysed polysaccharide, whereas II (○) is that resulting from particulate-enzyme-catalysed polysaccharide. The small letters indicate peaks that were isolated for further analysis. The arrow indicates the origin (O).

Table 1. *Separation of acetolysis products from radioactive polysaccharide synthesized from GDP-α-D-[^{14}C]mannose*

Radioactive polysaccharide was prepared by using GDP-α-D-[^{14}C]mannose and three different enzyme preparations. The three radioactive polysaccharides were subjected to acetolysis and the products were separated individually by paper chromatography in solvent I. The chromatographic peaks were eluted from the paper, reduced with $NaBH_4$, and hydrolysed with acid (except peak d, which was not hydrolysed). The radioactive products were identified as mannose and mannitol by electrophoresis in sodium tetraborate buffer, and a ratio of the radioactivity present in the two components was calculated. See Fig. 7 for the identity of the peaks.

Enzyme preparation	Chromatographic peak	$R_{cellobiose}$	Mannose/mannitol ratio
Particulate	a	0.42	1.82:1
	b	0.92	(100% mannose)
	c	1.32	1.06:1
	d	2.56	(100% mannitol)
Solvent-extracted particulate	a	0.46	1.91:1
	b	(no peak)	—
	c	1.35	0.92:1
	d	2.42	(100% mannitol)
Soluble	a	0.42	1.82:1
	b	(no peak)	—
	c	1.32	1.06:1
	d	2.66	(100% mannitol)

between peaks a and c (Fig. 10) resulted in a small amount of radioactivity with a mobility coincident with that of the material in peak c of chromatogram I. Reduction, followed by acid hydrolysis and electrophoresis in sodium tetraborate buffer, established that this small amount of radioactivity in the inter-peak area of the chromatogram was the tailing of peak c, i.e. was D-[U-^{14}C]mannobiose.

Discussion

The evidence presented in this paper indicates that a radioactive β-(1→4)-linked mannan results from the catalytic action of a Triton-solubilized enzyme on GDP-α-D-[^{14}C]mannose. The results indicating structure are: (1) the product is largely insoluble in hot alkaline solutions, suggesting an unbranched

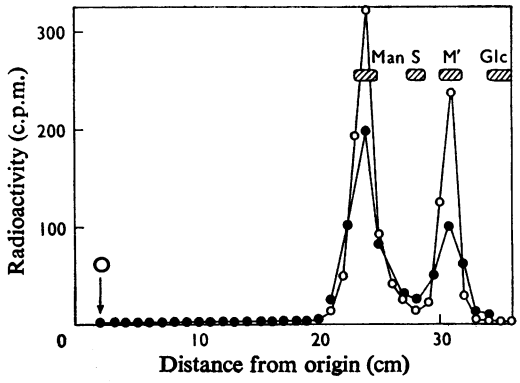

Fig. 8. *Reduction and hydrolysis of trisaccharides obtained from partial acetolysis*

Samples of the two trisaccharides (peak a of chromatograms I and II, see Fig. 7), isolated by the procedure described in Fig. 7, were reduced and hydrolysed as described in the text. The products were subjected to paper electrophoresis in 0.05M-sodium borate buffer, pH9.2. The standard compounds shown are: Man, mannose; S, sorbitol; M', mannitol; Glc, glucose. (●) Ia and (○) IIa (see Fig. 7). The arrow indicates the origin (O).

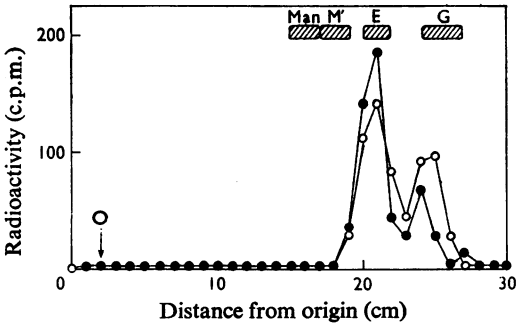

Fig. 9. *Paper chromatography of periodate-oxidation products*

The disaccharides (peak c of chromatograms I and II, see Fig. 7), isolated by the procedure described in Fig. 7, were oxidized, reduced and hydrolysed as described in the text. The resulting alcohols were chromatographed on Whatman no. 4 paper in solvent II. The standard compounds shown are: Man, mannose; M', mannitol; E, erythritol; G, glycerol. (●) Ic and (○) IIc (see Fig. 7). The arrow indicates the origin (O).

molecule with the subunits joined by β-D-glycosidic linkages; (2) complete hydrolysis demonstrates that [^{14}C]mannose is the only radioactive product; (3)

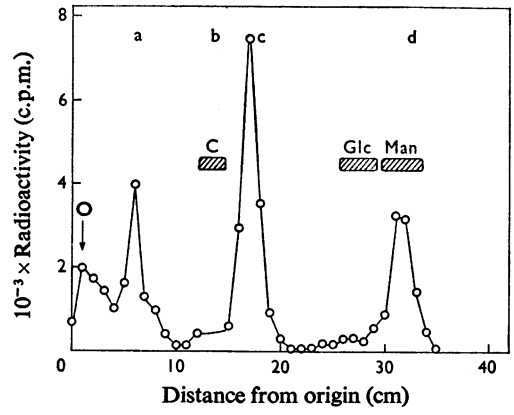

Fig. 10. *Separation of partial acetolysis products derived from [^{14}C]mannan formed by catalysis with solvent-extracted particulate enzyme*

The conditions and symbols are those described in Fig. 7, except that the solvent-extracted particulate enzyme, described in the text, was used as enzyme. The standard compounds shown are: C, cellobiose; Man, mannose; Glc, glucose. The small letters indicate peaks that were isolated for further analysis. The arrow indicates the origin (O).

partial acetolysis produces a typical oligosaccharide series with the chromatographic mobilities expected of mannose-containing oligosaccharides (Fig. 7); (4) no other oligosaccharides result from partial acetolysis; (5) reduction of the di- and tri-saccharide, followed by hydrolysis, produces the ratios of [^{14}C]-mannose and [^{14}C]mannitol expected of mannobiose and mannotriose respectively (Fig. 8; Table 1); no other radioactive products are detectable; (6) periodate oxidation of the disaccharide, followed by reduction and hydrolysis, produce erythritol and glycerol (Fig. 9), demonstrating that the glycosidic linkage is 1→4; these are the only detectable radioactive products of the oxidation reaction; (7) hydrolysis of the disaccharide with a β-mannosidase supports the indications of the solubility and chromatographic data that the sugars are joined by a β-D-glycosidic linkage.

Formation of the same radioactive polysaccharide appears to be catalysed by a solvent-extracted particulate preparation. An identical oligosaccharide series is produced on partial acetolysis of this radioactive polysaccharide (Fig. 10), and the di- and trisaccharides have been identified as mannobiose and mannotriose (Table 1). These are the only radioactive oligosaccharides produced on partial acetolysis of this polysaccharide. In contrast, the untreated particulate enzyme preparation catalyses the produc-

tion of a polysaccharide from GDP-D-mannose that contains sugar residues other than mannose. Partial acetolysis of this polysaccharide produces, in addition to mannobiose and mannotriose, an oligosaccharide (peak b of chromatogram II, Fig. 7) with a chromatographic mobility slightly less than that of cellobiose. On reduction and hydrolysis, this oligosaccharide produces only radioactive mannose (Table 1), indicating that the reducing position is occupied by a non-radioactive sugar. This oligosaccharide has a chromatographic mobility similar to one identified by Elbein (1969) as β-D-mannobiosyl-D-glucose. As Elbein (1969) studied a radioactive polysaccharide produced from GDP-α-D-[^{14}C]mannose and catalysed by a particulate enzyme preparation derived from the same plant source (*Phaseolus aureus*), it seems likely that the unidentified sugar mentioned above is glucose.

Elbein (1969) characterized the alkali-insoluble radioactive polysaccharide that resulted from utilization of GDP-D-[^{14}C]mannose catalysed by the *Phaseolus aureus* particulate enzyme. The oligosaccharides that resulted from partial acid and enzymic hydrolysis, as well as partial acetolysis, of the radioactive polysaccharide were predominantly glucose and mannose-containing oligosaccharides. A small quantity of a material that appeared to be mannobiose was present in the enzymic hydrolysate, but this disaccharide was present in too small a quantity to be characterized completely. Therefore, at least one component of the [^{14}C]polysaccharide was demonstrated to be a glucomannan (Elbein, 1969). Further, the overall ratio of mannose to glucose (3 or 4:1) in the polysaccharide would make mannobiose an expected product of the partial hydrolysis of such a glucomannan. Villemez (1971), however, reported kinetic results on the incorporation of [^{14}C]mannose from GDP-D-[^{14}C]mannose by *Phaseolus aureus* particulate preparations, which indicated that GDP-D-mannose was a precursor for at least two polysaccharides. Partial acetolysis of the [^{14}C]polysaccharide resulting from the particulate-enzyme-catalysed reaction reported here produced an oligosaccharide (peak b, chromatogram II, Fig. 7) that is consistent with Elbein's (1969) findings. This oligosaccharide has a chromatographic mobility similar to one identified by Elbein (1969) as mannobiosylglucose. Also, the reducing sugar of the oligosaccharide is not mannose (Table 1), nor is it radioactive. Therefore the reducing-sugar moiety of this oligosaccharide was obtained from the particulate enzyme preparation, and is very probably glucose. However, we also obtain β-D-(1→4)-linked mannobiose and mannotriose (Fig. 7). Mannobiose is the major oligosaccharide that we obtain from the particulate-enzyme-catalysed [^{14}C]polysaccharide. The difference in the relative proportion of oligosaccharides produced by partial acetolysis of the particulate-enzyme-derived [^{14}C]polysaccharide as reported here, in comparison with that reported by Elbein (1969), could be due to subtle differences in the acetolysis procedure. It could also be due to different proportionation in the components of a [^{14}C]polysaccharide mixture, resulting from subtle differences in the preparation of the particulate enzyme. We favour the latter explanation as being more probable. As indicated by the results in this report, the *Phaseolus aureus* particulate enzyme system loses the ability to form glucomannan from GDP-D-mannose alone when (1) treated with a neutral surface-active agent, or (2) extracted with organic solvents. These treated enzyme preparations will, however, catalyse the formation of β-[^{14}C]-mannan from GDP-D-[^{14}C]mannose. Therefore it seems probable that subtle differences in enzyme preparation could produce the same type of changes, but to a lesser degree, as that produced by solvent extraction or treatment with a surface active agent.

The biological significance of this soluble mannose-polymerizing enzyme is as difficult to assign as is that of all the other cell-free plant polysaccharide-synthesizing systems reported (Feingold *et al.*, 1958; Barber *et al.*, 1964; Villemez *et al.*, 1966, 1967, 1968; Kauss, 1967; McNab *et al.*, 1968). Certainly, mannans have been shown to be cell-wall components of a variety of plants (Percival, 1966), and their presence has been indicated in *Phaseolus aureus* seedlings (Bailey *et al.*, 1967). This mannose-polymerizing enzyme, as with the other glycosyltransferases isolated from this plant system, almost certainly is involved in cell-wall formation. Also, there is no doubt that this enzyme will catalyse the polymerization of mannose from GDP-D-mannose *in vitro*. The question that is impossible to answer with the available information is whether this enzyme is actually responsible for the synthesis of a pure mannan *in vivo*, or is only one component of a system that forms a heteropolysaccharide. A similar question has been raised with regard to the synthesis of cellulose from GDP-D-glucose *in vitro* (Villemez & Heller, 1970; Villemez, 1971). To obtain information that could help resolve this type of question, studies could be performed on the glycosyl acceptor specificity of these *Phaseolus aureus* glycosyltransferases. Unfortunately, the identity of the glycosyl acceptors is not known, as they are already contained in the enzyme preparations, most of which have been particulate enzymes. However, the existence of soluble enzyme systems such as the one reported here should make the task of isolating the glycosyl acceptors easier.

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