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INCORPORATION OF MANNOSE INTO HEMICELLULOSE A IN MUNG BEAN SEEDLINGS

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Abstract—A particulate enzyme system from mung bean seedlings (*Phaseolus aureus*) catalyzed the incorporation of radioactivity from GDP-D-[^{14}C]mannose, GDP-D-[^{14}C]glucose and UDP-D-[^{14}C]glucose into a presumed hemicellulose A component. Both GDP-D-[^{14}C]mannose and GDP-D-[^{14}C]glucose utilization reached a maximum in 2- to 3-day-old seedlings and then decreased, while UDP-[^{14}C]glucose utilization remained constant. Mg^{2+} was required with the optimum concentration being 4×10^{-3}M. The K_m for GDP-D-[^{14}C]mannose was about 5×10^{-4}M.

Complete hydrolysis of the hemicellulose A fraction, synthesized from GDP-[^{14}C]mannose, released all the radioactivity as mannose. Enzymatic hydrolysis with a hemicellulase released three neutral [^{14}C]oligosaccharides. One of these was tentatively identified as β-D-mannosyl-D-glucose. On Smith degradation the larger-[^{14}C]oligosaccharides yielded mannose, glycerol and only a small amount of [^{14}C]erythritol, suggesting either β(1–3) linkage, or a branched structure.

INTRODUCTION

THE HEMICELLULOSES comprise mixtures of alkali-soluble polymers which make up a major portion of the plant cell wall and which yield mixtures of monosaccharides on hydrolysis. Among the polymers containing mannose are glucomannan, galactomannan, glucogalactomannan and mannan. Many of these polysaccharides have been isolated and characterized from a variety of plants and seeds.[1–6] In most cases they contain mannose linked by β(1–4) linkage. However, other linkages have occasionally been reported, e.g. galactose has been found linked to mannose in a(1–6) linkage.[7–9]

GDP-D-mannose is a very effective mannosyl donor involved in the biosynthesis of mannose-containing polysaccharides in higher plants.[10–12] Recently Elbein[10] showed the biosynthesis of a glucomannan from GDP-D-[^{14}C]mannose in mung bean seedlings and the

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polymer synthesized from GDP-D-mannose was characterized as β(1–4)-linked alkali-insoluble glucomannan. It was also pointed out that a significant amount of radioactivity was incorporated into alkali-soluble polysaccharides from GDP-D-[^{14}C]mannose. However, the nature of the alkali-soluble polysaccharides was not characterized. The present paper describes some properties of the enzyme system which incorporates GDP-D-[^{14}C]mannose into hemicellulose A and some evidence is also presented on the nature of the hemicellulose A synthesized using GDP-D-[^{14}C]mannose as substrate.

RESULTS

The incorporation of [^{14}C]mannose from GDP-D-[^{14}C]mannose into hemicellulose A was proportional to GDP-D-[14C]mannose concentrations and the K_m for the GDP-D-[^{14}C]mannose was estimated to be about 5×10^{-4}M. Magnesium stimulated the reaction markedly and the optimum concentration of Mg^{2+} was found to be 4×10^{-3}M, no further increase in activity being found above this concentration.

Figures 1 and 2 show the incorporation of GDP-D-[^{14}C]mannose, GDP-D-[^{14}C]glucose and UDP-D-[^{14}C]glucose into an alkali-insoluble fraction and an alkali-soluble hemicellulose A as a function of age of the seedlings. GDP-D-[^{14}C]glucose and GDP-D-[^{14}C]mannose utilization decreased markedly with the age of the seedlings and became almost negligible after 7 days. The optimum age of seedlings for the incorporation of GDP-D-[^{14}C]mannose and GDP-D-[^{14}C]glucose was found to be 2–3 days. UDP-D-[^{14}C]glucose incorporation did not show any significant differences with the age of the seedlings.

Analysis of mung bean seeds and seedlings showed the presence of mannose in trace amounts indicating that mannose was a very minor natural substrate during the early stages of the biosynthesis of cell wall. It should also be noted that 2- to 3-days-old seedlings are not

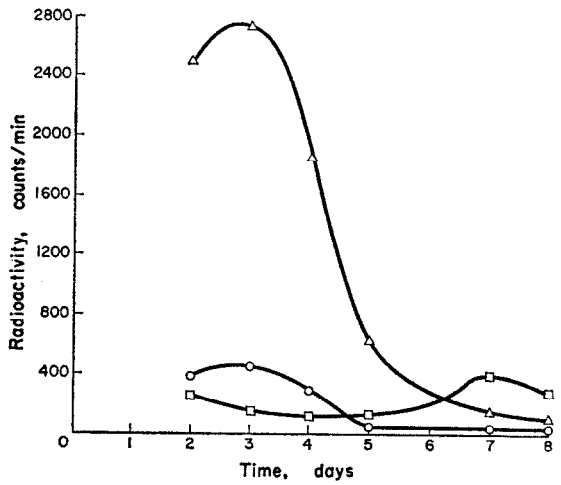

FIG. 1. EFFECT OF THE AGE OF SEEDLINGS ON THE INCORPORATION OF RADIOACTIVITY FROM GDP D-[^{14}C]MANNOSE, GDP-D-[^{14}C]GLUCOSE AND UDP-D-[^{14}C]GLUCOSE INTO ALKALI-INSOLUBLE POLYMER. INCUBATION MIXTURES WERE AS DESCRIBED UNDER EXPERIMENTAL. ALKALI-INSOLUBLE POLYMER WAS ISOLATED AND RADIOACTIVITY WAS COUNTED. RESULTS WERE EXPRESSED ON THE BASIS OF 10 mg DRIED PARTICULATE ENZYME PREPARATION.

△—GDP-D[^{14}C[MANNOSE; □—UDP-D[^{14}C]GLUCOSE; ○—GDP-D[^{14}C]GLUCOSE.

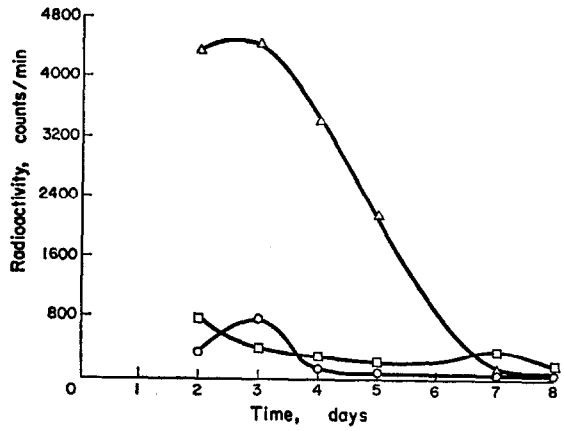

FIG. 2. THE EFFECT OF AGE OF THE SEEDLINGS ON THE INCORPORATION OF RADIOACTIVITY FROM GDP-D-[^{14}C]MANNOSE, GDP-D-[^{14}C]GLUCOSE AND UDP-D-[^{14}C]GLUCOSE INTO HEMICELLULOSE A. CONDITIONS OF THE EXPERIMENT AND METHOD OF ISOLATION OF HEMICELLULOSE A WERE AS DESCRIBED UNDER EXPERIMENTAL. RESULTS WERE EXPRESSED ON THE BASIS OF 10 mg DRIED PARTICULATE ENZYME PREPARATION.

able to synthesise their own carbohydrates through photosynthesis when grown in the dark. It was observed that the rate of incorporation of GDP-D-[^{14}C]mannose added as substrate was much higher as compared with the rate of incorporation of GDP-D-[^{14}C]glucose or UDP-D-[^{14}C]glucose in 2- to 3-day-old seedlings (Figs. 1 and 2). The physiological significance of the high activity of the enzyme which incorporated GDP-D-[^{14}C]mannose added as substrate into polysaccharides is not clear during the early stages of cell wall biosynthesis. Since mannose was found to be present as a minor constituent in the seeds, it may be possible that small amounts of glucomannan may be important during the early stages of cell wall formation.

In order to characterize the hemicellulose A synthesized from GDP-D-[^{14}C]mannose large scale incubation mixtures were made. Complete hydrolysis of hemicellulose A released a number of neutral sugars which were identified as galactose, glucose, mannose, arabinose and xylose by paper chromatography in four different solvents. The amounts of hexoses, pentoses, uronic acids and proteins in hemicellulose A and particulate enzyme preparation

TABLE 1. COMPOSITION OF HEMICELLULOSE A AND PARTICULATE ENZYME PREPARATION

Compounds	Particles (expressed as mg/100 mg)	Hemicellulose A (expressed as mg/100 mg)
Hexoses	2·81	1·44
Pentoses	0·83	0·56
Uronic acid	0·54	0·38
Proteins	86	81·0

Hemicellulose A was isolated from the particulate enzyme preparation. Proteins were determined by difference using pronase digestion in 0·05 M Tris HCl buffer, pH 7·5 at 37° for 48 hr in a dialysis bag. The non-protein residue left after this treatment was isolated by centrifugation and weighed.

are shown in Table 1. All of the radioactivity in the hydrolysates of hemicellulose A was found in mannose when GDP-D-[^{14}C]mannose was used as substrate in the incubation.

Eighty to ninety percent of the radioactivity of hemicellulose A synthesized from GDP-D-mannose became diffusable with hemicellulase treatment in 24 hr. Enzymatic hydrolysis released 3 major neutral radioactive oligosaccharides in addition to mannose. Figure 3 shows a scan of the radioactive oligosaccharides obtained on a paper chromatogram from

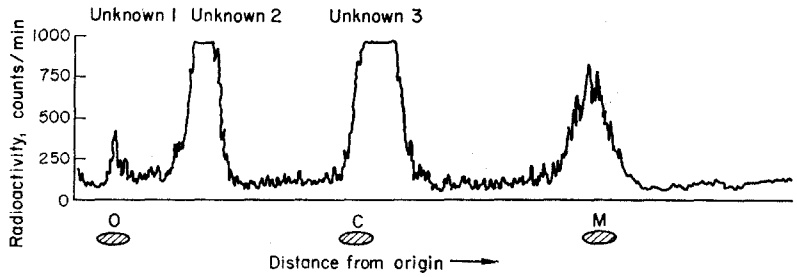

FIG. 3. RADIOACTIVE TRACING, SHOWING SOLUBLE OLIGOSACCHARIDES RELEASED BY ENZYMATIC HYDROLYSIS WITH A HEMICELLULASE TREATMENT AS DESCRIBED UNDER EXPERIMENTAL. THE SOLUBLE OLIGOSACCHARIDES WERE SEPARATED BY PAPER CHROMATOGRAPHY IN SOLVENT No. III. STANDARD COMPOUNDS SHOWN ARE (C)—CELLOBIOSE; (M)—MANNOSE AND (O)—INDICATE ORIGIN.

the hydrolysis of hemicellulose A using GDP-D-[^{14}C]mannose as substrate. These oligosaccharides were purified in 3 different solvents (I, II, III) and appeared homogeneous when rechromatographed in solvent No. III for the fourth time. The mobilities, relative to cellobiose, of unknowns 1, 2 and 3 in solvent II were 0·0, 0·35 and 1·0, and in solvent III 0·0, 0·37 and 1·07 respectively. Each of the radioactive oligosaccharides was hydrolyzed with 3 N H_2SO_4 or HCl for 1 hr at 100° and then chromatographed in solvents No. II and III. Glucose and mannose were identified in the oligosaccharide hydrolysates.

The disaccharide which had a mobility very close to cellobiose was reduced with $NaBH_4$. This reduced disaccharide liberated [^{14}C]mannose after acid hydrolysis, indicating that mannose was not at the reducing ends. The $NaBH_4$ reduced disaccharide, after acid hydrolysis, failed to react with glucose oxidase while the unreduced disaccharide after acid hydrolysis reacted with glucose oxidase. These observations indicated that glucose was at the reducing end of disaccharide. This was also confirmed by treatment with enzymes such as β-glucosidase and β-mannosidase. β-Glucosidase did not release radioactive mannose and all the radioactivity remained in the disaccharide while β-mannosidase liberated all the radioactive mannose. β-Mannosidase also released glucose which was confirmed by glucose oxidase. It appears that mannose is attached to the glucose and is linked by β-configuration to glucose.

The higher radioactive oligosaccharides which remained at the origin were subjected to periodate oxidation, followed by reduction with $NaBH_4$ and acid hydrolysis. The alcohols produced were separated by paper chromatography in the solvents No. II and III using authentic standards. [^{14}C]mannose, [^{14}C]glycerol and [^{14}C]erythritol were formed by this treatment. [^{14}C]mannose (235 counts/min) and [^{14}C]glycerol] 231 counts/min) were found in greater amounts as compared with [^{14}C]erythritol (95 counts/min). The large proportion of radioactivity in mannose suggests periodate stable linkages (1–3) or incomplete oxidation. Radioactive glycerol showed that mannose was either attached at the terminal ends or was linked by 1–6 or 1–2 linkages. Small amounts of [^{14}C]erythritol indicated that some mannose

was also linked as 1–4 linkage, but the major linkage appears to be 1–3 as evidenced by the fact that [^{14}C]mannose was present in greater amounts as compared with [^{14}C]erythritol.

DISCUSSION

A particulate enzyme preparation from mung bean seedlings was shown to catalyse the transfer of mannose from GDP-D-[^{14}C]mannose into hemicellulose A which appears to be a component of plant cell wall. The polysaccharide synthesized from GDP-D-[^{14}C]mannose and incorporated into hemicellulose A seems to be a β(1–3) and β(1–4) linked glucomannan. Complete oxidation of the higher oligosaccharide by periodate indicated that glucomannan incorporated into hemicellulose A contain β(1–3) major linkages and to a lesser extent β(1–4) linkages. At present we are not able to establish whether the product is a mixture of β(1–3) glucomannan and β(1–4) glucomannan or a single polysaccharide containing β(1–3) and β(1–4) linked glucomannan. Several workers have reported the biosynthesis of β(1–3) glucan from UDP-D-glucose in higher plants, but there is no evidence on β(1–3) linked glucomannan biosynthesis in higher plants.[13–15] Yeast mannans which are highly branched are known to contain (1–6), (1–2) and (1–3) linkages[16,17] but the mannan and glucomannan containing other than β(1–4) linkages are not reported in higher plants. β(1–4) linked glucomannan have been isolated and characterized in a variety of plants and seeds.[2,4,10,17] Reduction of the disaccharide isolated from hemicellulose A and enzymatic hydrolysis by β-glucosidase and β-mannosidase indicated that mannose was attached to glucose by β-anomeric configuration. The structure of the disaccharide appears to be β-D-mannosyl-D-glucose.

A study was also made to determine how the age of the seedlings affects the utilization of different nucleoside diphosphate sugars. GDP-D-[^{14}C]mannose and GDP-D-[^{14}C]glucose incorporation into alkali-insoluble polymer and hemicellulose A was found to be maximum in 2- to 3-day-old seedlings and then decreased, while UDP-D-[^{14}C]glucose utilization remained constant. It appears that various polysaccharide synthetase activities are at a maximum at different stages of growth. The changes of enzymes with the age of seedlings may be important for understanding the mechanism of biosynthesis of cell wall components and for determining the ways in which the processes of biosynthesis of different polysaccharides are controlled.

EXPERIMENTAL

Materials. GDP-D-[^{14}C]mannose and GDP-D-[^{14}C]glucose were prepared by a modification[18] of the method of Roseman *et al.*[19] GDP-D-[^{14}C]mannose was purchased from New England Nuclear Co. All other sugar nucleotides were prepared as described.[18] Partially purified β-mannosidase containing slight β-glucosidase activity was kindly donated by Dr. Elwyne Reese, U.S. Quartermaster Corps, Natrick, Mass.

Analytical method. Hexoses were determined by the anthrone method,[20] pentoses by the orcinol method,[21] D-glucose by oxidation with glucose oxidase (Worthington), and uronic acid by using carbazole method.[22] Radioactivity on paper chromatograms was located with a Packard Radiochromatogram Scanner and was quantitatively determined by liquid scintillation.

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Chromatographic method. Descending paper chromatography was performed on Whatman No. 1 or Whatman No. 3 MM paper. The following solvents were used in the ratio (v/v) as indicated: (I) Butan-1-ol–pyridine–H_2O (6:4:3); (II) Butan-1-ol–pyridine–0·1 *N* HCl (5:3:2); (III) Propan-1-ol–ethylacetate–H_2O (7:1:2); (IV) Ethyl acetate–pyridine–H_2O (8:2:1). Chromatograms were usually developed for 48–72 hr for the separation of oligosaccharides. Sugars and alcohols were detected with alkaline silver nitrate.[23] Pentoses were located with acid aniline phthalate.[24]

Enzyme preparation and incubation mixtures. Mung beans (*Phaseolus aureus*) were grown in the dark. The particulate enzyme was prepared from 3- to 4-day-old seedlings as previously described.[11] For time studies, 2- to 7-day-old seedlings were used.

Incubation mixtures for the incorporation of mannose contained GDP-D[^{14}C]mannose 0·3 μmole (46,000 counts/min); $MgCl_2$ 5 μmoles; tris HCl buffer pH 7·5, 5 μmoles and 0·2 ml of particulate enzyme (about 200–300 μg protein) in a final volume of 0·3 ml. In some experiments UDP-D-[^{14}C]glucose 0·3 μmole (45,000 counts/min) and GDP-D-[^{14}C]glucose 0·3 μmole (42,000 counts/min) were also used as substrates. Tubes were incubated at 37° for 15 min unless otherwise specified and the reaction was stopped by heating at 100° for 5 min. Water (1 ml) was added to the reaction mixture, and the precipitate was isolated by centrifugation. The pellets were resuspended in 2 ml H_2O and washed several times by recentrifugation until the supernatant was free of radioactivity (3–4 washings usually being sufficient). The insoluble material was digested with 2% KOH at 100° for 3 min to solubilize the hemicelluloses. The supernatant after this treatment contained hemicellulose A and B. Hemicellulose A was precipitated by neutralization of the supernatant with acetic acid. After standing overnight at 5°, the hemicellulose A was isolated by centrifugation. This was washed two times with dilute acetic acid before counting the radioactivity.

Large scale incubation mixtures were prepared to isolate radioactive hemicellulose A for characterization. The composition of the incubation mixture was as follows: GDP-D-[^{14}C]mannose 15 μmoles; (2×10^6 cpm); $MgCl_2$ 150 μmoles; tris HCl buffer, pH 7·5, 200 μmoles; and 10 ml of enzyme preparation (representing 100 g of sprouts) in a final volume of 12 ml. The incubation was conducted at 37° for 15 min and the reaction was stopped by heating for 5 min at 100°. The reaction mixture was cooled and was then extracted overnight at room temp. with 100 ml $CHCl_3$–MeOH (3:1, v/v) to dissolve lipids. The $CHCl_3$–MeOH and H_2O phases were carefully removed after centrifugation. The insoluble material was resuspended in water and washed 3–4 times by recentrifugation. The water-insoluble residue was extracted for 24 hr with 10% KOH (200 ml) at room temp. and centrifuged. The alkali-soluble supernatant was neutralized with HOAc to precipitate hemicellulose A and was allowed to stand overnight. The hemicellulose A was isolated by centrifugation.

Characterization of hemicellulose A. Portions of hemicellulose A isolated from intact sprouts and from particulate enzyme preparation were hydrolyzed at 100° for 3 hr in 2 or 3 N H_2SO_4 or HCl in order to determine sugar components. H_2SO_4 hydrolysates were neutralized with $BaCO_3$ and the supernatant was desalted with mixed bed ion-exchange resin (equal parts of Dowex 50-H^+ and Dowex-1-CO_3^{2-}). HCl hydrolysates were evaporated under vacuum. Uronic acid compounds were examined in the HCl hydrolysates, after passing through a column of Dowex 1-acetate form and then eluting with 6 N HOAc.

Enzymatically synthesized hemicellulose A (50 mg) was subjected to enzymatic hydrolysis with 10 ml hemicellulase solution (Worthington Co.) at 45°, pH 5·0 in 0·01 M acetate buffer, in a dialysis bag. The enzyme concentration was 10 mg/ml. The diffusates were concentrated *in vacuo*, treated with mixed-bed ion-exchange resin to remove salts and chromatographed.

The periodate oxidation of the larger radioactive-oligosaccharides was performed by a modification of the method of Hay *et al.*[25] as described previously.[10] The isolated radioactive disaccharide was reduced with $NaBH_4$ at room temp. for 12 hr in order to determine the reducing end of the disaccharide. The reaction was stopped by adding HCl dropwise and the contents were hydrolyzed with 2 N HCl for 2 hr at 100°. HCl was removed *in vacuo* at 40° and the residue dissolved in H_2O and passed through a column of Dowex 50-H^+ to remove cations. The solution was evaporated to dryness *in vacuo* and the residue treated with 10 ml of MeOH containing a drop of HCl. MeOH was evaporated under vacuum and this process was repeated about 5–6 times to ensure complete removal of borates. The residue was dissolved in H_2O, treated with mixed-bed ion-exchange resin, concentrated *in vacuo* and a portion was applied to paper-chromatograms. The paper chromatograms were developed in solvent No. II. The remaining portion was tested with glucose oxidase.

The radioactive disaccharides were treated with enzymes at 37° in 0·1 M acetate buffer at pH 5·0. The reaction was stopped on heating for 5 min at 100°. After cooling, the reaction mixture was treated with mixed bed ion-exchange resin, concentrated under vacuum and examined by paper chromatography using solvent No. III.

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Key Word Index—Phaseolus aureus; Leguminosae; mung bean; biosynthesis; hemicellulose; mannose.