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SHORT COMMUNICATION

INCORPORATION OF RADIOACTIVITY FROM GDP-D-^{14}C-MANNOSE INTO HEMICELLULOSE B

S. S. BRAR* and A. D. ELBEIN†

Department of Biology, Rice University, Houston, Texas 77001, U.S.A.

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Abstract—A particulate enzyme preparation from mung bean seedlings (*Phaseolus aureus*) catalysed the incorporation of mannose from GDP-D-^{14}C-mannose into the Hemicellulose B fraction. Complete acid hydrolysis of this material liberated all of the radioactivity as mannose. Enzymatic hydrolysis of the ^{14}C-polymer with a hemicellulase liberated three neutral, diffusable, ^{14}C-mannose oligosaccharides. One of these, which migrated like a disaccharide, was hydrolysed by β-mannosidase but not by β-glucosidase indicating that mannose was attached by β-linkages. Free glucose was also released by this treatment indicating that the disaccharide was β-mannosyl-glucose. Periodate oxidation of the larger ^{14}C-oligosaccharides followed by reduction and hydrolysis produced ^{14}C-erythritol and ^{14}C-glycerol suggesting that the mannose was attached by 1–4 linkages.

INTRODUCTION

HEMICELLULOSES are components of the cell wall of plants and are typically classified by their solubility in alkali.[1] The hemicelluloses can be subdivided into two classes as follows: neutralization of the alkaline extract results in the precipitation of hemicellulose A while hemicellulose B can be precipitated from the supernatant by the addition of alcohol.[2] Both fractions comprise a mixture of homo- and heteropolysaccharides containing a variety of sugars including arabinose, xylose, glucose, galactose and mannose as well as uronic acids. The mannose containing polysaccharides include glucomannans, galactomannans, glucogalactomannans and mannans.[3–12] In this paper, the incorporation of mannose from GDP-D-^{14}C-mannose into the hemicellulose B fraction was studied with a mung bean particulate enzyme. At least part of the mannose was shown to be attached to glucose by β-(1–4)-linkages.

RESULTS AND DISCUSSION

In order to characterize the hemicellulose B product synthesized from GDP-D-^{14}C-mannose large scale incubation mixtures were prepared and the hemicellulose B was iso-

* Present address: Department of Soils, Punjab Agricultural University, Ludhiana, Punjab, India.

† Present address: Department of Biochemistry, The University of Texas Medical School, San Antonio, Texas.

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TABLE 1. COMPOSITION OF HEMICELLULOSE B AND PARTICULATE ENZYME PREPARATION

Components	Particles (mg/100 mg)	Hemicellulose B (mg/100 mg)
Hexoses	2·81	15·20
Pentoses	0·83	3·74
Uronic Acids	0·54	1·55
Proteins	86 00	66·60

Hemicellulose B was extracted from the particulate enzyme preparation. Protein was estimated by difference using pronase digestion in 0·05 M Tris HCl buffer pH 7·5 at 37° for 48 hr. The insoluble residue after this treatment was isolated by centrifugation and weighed.

lated as described, under materials and methods. Complete hydrolysis of this material released a number of sugars which were identified as galactose, glucose, mannose, arabinose and xylose in 4 different solvents. The presence of uronic acids was confirmed by chromatography in solvent II. The composition of hemicellulose B is given in Table 1. Identical results were obtained from hemicellulose B isolated from particles or delignified shoots. Bailey *et al.*[13] also found similar sugars in the hydrolysate of hemicellulose B prepared from mung bean. All of the radioactivity in the hydrolysates of hemicellulose B was in mannose when GDP-D-^{14}C-mannose was used as substrate in the enzymatic incubations.

Eighty to 90% of the radioactivity of labelled hemicellulose B became diffusable in 24 hr by treatment with hemicellulase. Three neutral radioactive oligosaccharides were obtained by this treatment, in addition to mannose. Each of these oligosaccharides was further purified in 3 different solvents (I, II, III) and appeared quite homogenous when rechromatographed for the fourth time in Solvent III. The chromatographic mobilities of the oligosaccharides isolated from hemicellulose B are listed in Table 2. Each of the oligosaccharides was hydrolyzed with 3 N H_2SO_4 or 3 N HCl for 1 hr at 100° and then chromatographed in solvent II and III. Glucose and mannose were identified in the oligosaccharide hydrolysates.

The disaccharide which ran very close to cellobiose was reduced with $NaBH_4$. This reduced disaccharide gave ^{14}C-mannose after acid hydrolysis indicating that mannose was not at the reducing ends. The reduced disaccharide was hydrolysed with acid and tested with glucose oxidase after neutralization. No reaction was observed in this case, whereas the original disaccharide did react with glucose oxidase after hydrolysis suggesting that glucose was at the reducing end. This was confirmed by treatment of the ^{14}C-disaccharide with enzymes such as β-glucosidase and β-mannosidase. β-Glucosidase did not release radioactive mannose while β-mannosidase gave radioactive mannose. β-Mannosidase also released glucose which was identified by glucose oxidase. It appears from these experiments that mannose is attached to glucose through a β-glycosidic linkage.

One of the higher radioactive oligosaccharides which remained at the origin during purification by paper chromatography in three different solvents was subjected to periodate oxidation, followed by reduction with $NaBH_4$ and then acid hydrolysis. The alcohols

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TABLE 2. CHROMATOGRAPHIC MOBILITY OF OLIGOSACCHARIDES ISOLATED FROM HEMICELLULOSE B BY HEMICELLULASE TREATMENT

Compound	R(cellobiose) Solvent II	R(cellobiose) Solvent III
Cellobiose	1·00	1·00
Unknown I	0·00	0·00
Unknown II	0·41	0·45
Unknown III	1·10	1·05

produced were separated by paper chromatography in two solvents (II and III) using standards. ^{14}C-erythritol and ^{14}C-glycerol were produced by this treatment. The radioactivity in erythritol suggested that mannose was attached by 1–4 linkages, and also that at least two mannose residues are present in each chain.

The experiments reported here indicate that a particulate enzyme from mung bean seedlings catalyses the transfer of the mannosyl portion of GDP-mannose into a hemicellulose B component which may be a glucomannan. The evidence indicates that the mannose is attached by β-(1–4) linkages and at least some of it is attached to glucose. However, the possibility exists that mannose may also be attached to other sugars, such as galactose. Glucomannans and galactoglucomannans have previously been isolated from woods and seeds and are usually considered to be part of the hemicelluloses.[3–5,7,14] However, the particulate enzyme from mung bean also catalyses the incorporation of mannose from GDP-mannose into an alkali-insoluble β-(1–4) linked glucomannan.[12] It may be that this insoluble polymer is identical to that in hemicellulose B and that the only difference is in the binding to other components. Thus, the alkali-insoluble glucomannan may be bound to cellulose while that in the hemicellulose B fraction may be free or attached to other components. It is also possible that all of the glucomannan is bound to cellulose and that some of it is released by alkaline extraction. We have found that mannose is also incorporated into the hemicellulose A fraction, but in this case it appears to be attached by β-(1–3) linkages (Brar and Elbein, unpublished work).

EXPERIMENTAL

Materials

GDP-D-^{14}C-mannose was prepared by a modification[15] of the method of Roseman *et al.*[16] GDP-D-^{14}C-mannose was also purchased from New England Nuclear Co. Partially purified β-mannosidase containing slight β-glucosidase activity was kindly donated by Dr. Elwyn Reese, U.S. Quartermaster Corps, Natick, Massachusetts. All other chemicals were obtained from commercial sources.

Analytical Methods

Total hexoses were determined by the anthrone procedure,[17] pentoses by the orcinal method,[18] D-glucose with glucose oxidase (Worthington Co.) and uronic acid by the carbazole method.[19] Radioactivity on paper

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was located with a Packard Radiochromatogram Scanner and was quantitated with a Packard Liquid Scintillation Spectrometer.

Chromatographic Methods

Descending chromatography was performed on Whatman No. 1 or Whatman 3 mm paper. The following solvents were used: I. *n*-BuOH–pyridine–H_2O (6:4:3, by vol.); II. *n*-BuOH–pyridine–0·1 N HCl (5:3:2, by vol.); III. PrOH–ethylacetate–H_2O (7:1:2, by vol.); IV. ethyl acetate–pyridine–H_2O (8:2:1, by vol.). Papers were usually developed for 48–72 hr for the separation of oligosaccharides. Sugars and alcohols were detected with alkaline $AgNO_3$[20] and pentoses with aniline pthalate.[21]

Enzyme Preparation and Incubation Mixtures

Mung beans (*Phaseoulus aureus*) were grown in a moist chamber in the dark. The particulate enzyme was prepared from 3 to 4-day-old mung bean seedlings as described by Barber *et al.*[22] Large scale incubation mixtures were made to isolate radioactive hemicellulose B for characterization. Incubation mixtures were as follows: GDP-D-^{14}C-mannose 15 μM (2 × 10 counts/min); $MgCl_2$, 150 μM; Tris HCl buffer, pH 7·5, 200 μM and 10 ml of enzyme preparation (representing 100 g sprouts) in a final volume of 12 ml. Final volumes of reaction mixtures and reagents were also varied according to the volume of enzyme preparation. The incubation was run at 37° for 15 min and the reaction was heat killed for 5 min at 100°. The reaction mixture was cooled and extracted overnight at room temp. with 100 ml $CHCl_3$–MeOH (3:1, v/v) to dissolve lipids. $CHCl_3$–MeOH and H_2O phases were separated by centrifugation. The insoluble material which remained at the interface, was resuspended in H_2O and washed several times by recentrifugation. The water-insoluble material was extracted for 24 hr with 200 ml 10% KOH (w/v) at room temp. and then centrifuged. The supernatant after this treatment contained hemicellulose A and B. Hemicellulose A was precipitated by neutralization of the supernatant with acetic acid. After having stood overnight at 5°, the hemicellulose A was isolated by centrifugation and the supernatant was added to 3 vol. EtOH to precipitate hemicellulose B. Hemicellulose B was recovered by centrifutation after standing for several days at 0°.

Characterization

Hemicellulose B was also isolated from particles or delignified sprouts by alkaline extraction and precipitation using the same methods as applied to enzymatic reaction mixtures. Hemicellulose B (50 mg) was hydrolysed at 100° for 3 hr in 2 N 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 desalted with mixed-bed ion-exchange resin (Dowex 50 H^+ plus Dowex-1-CO_3^{2-}), while HCl hydrolysates were evaporated under vacuum. Uronic acids were separated from neutral sugars by means of Dowex-1-acetate column and were eluted with 6 N HOAc from the column. Hemicellulose B (50 mg) was subjected to enzymatic hydrolysis with a hemicellulase (10 mg/ml) (Worthington Co.) at 45° in 0·01 M acetate buffer pH 5·0. The diffusates were changed every 24 hr. The diffusates were concentrated *in vacuo* treated with mixed-bed ion-exchange resin to remove salts and applied to paper chromatograms. The neutral radioactive oligosaccharides were located with a Packard Radiochromatogram Scanner.

The periodate oxidation of radioactive oligosaccharides was performed by a modification of the method of Hay, Lewis and Smith,[23] as described previously.[12] To determine the reducing end of the disaccharide, $NaBH_4$ reduction followed by acid hydrolysis was used. The radioactive disaccharide was also treated with enzymes such as β-glucosidase and β-mannosidase in order to determine the anomeric configuration and the attachment of mannose to other sugars. Treatment with β-glucosidase and β-mannosidase was done at pH 5·0 at 37° in 0 1 M acetate buffer. The reaction was stopped by heating to 100° for 5 min. The reaction mixture was treated with mixed-bed ion-exchange resin, concentrated under reduced pressure and examined by paper chromatography in solvent III.

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