The Enzymatic Synthesis of a Glucomannan[1]

Alan D. Elbein and W. Z. Hassid
Department of Biology, Rice University
Houston, Texas
and
Department of Biochemistry, University
of California, Berkeley, California

Received April 1, 1966

Previously, it was shown that extracts of mung bean seedlings incorporated the glucosyl portion of GDP-D-glucose-^{14}C into cellulose (Elbein, et al, 1964). When GDP-D-mannose was added to these incubation mixtures, an increased incorporation of glucose from GDP-D-glucose-^{14}C into a water-insoluble, alkali-insoluble polymer was observed. During that study, it was also found that the mannose moiety of GDP-D-mannose-^{14}C was incorporated into an insoluble polymer (Barber et al, 1964). Evidence is presented in this communication that the mannose component of GDP-D-mannose is incorporated into a glucomannan. The data also shows that an epimerization of GDP-D-mannose to GDP-D-glucose probably occurs, since glucose-^{14}C was also found in the hydrolysis products of the polymer.

Materials and Methods - GDP-D-mannose and GDP-D-glucose were prepared chemically by condensing the corresponding hexose-1-phosphate with GMP-morpholidate (Roseman et al, 1961). D-mannose-1-phosphate was prepared by the method of MacDonald (1962). 4-O-(β-D-mannopyranosyl)-D-mannopyranose, 4-O-(β-D-glucopyranosyl)-D-mannopyranose and O-β-D-mannopyranosyl-(1-4)-O-β-D-mannopyranosyl-(1-4)-D-mannopyranose were generous gifts of Dr. C. T. Bishop, National Research Council, Ottawa. A β-mannanase preparation, which

1 Supported in part by Research Grant GB 3857, and in part by Research Grant G-23763, from the National Science Foundation.

had only slight cellulase activity, was kindly supplied by Dr. E. T. Reese, U.S. Army Quartermaster Corp, Natick, Massachusetts. All other compounds were obtained from commercial sources.

Chromatographic solvent systems were as follows: 1. n-Butanol: pyridine: 0.1 N HCl (5:3:2); 2. Ethyl acetate: acetic acid: water (3:1:3); 3. Ethyl acetate: pyridine: water (12:5:4). Paper chromatography was done using Whatman #1 or 3MM paper or Schleicher and Schuell 589 Blue Ribbon paper. Paper electrophoresis was performed in 0.2 M ammonium formate buffer, pH 3.6, or in 0.05 M sodium tetraborate, pH 9.7.

Total hexose was determined by the anthrone method (Loewus, 1952), glucose with glucose oxidase and reducing sugar by the method of Nelson (1944)

Enzymatic Synthesis of Glucomannan - Mung bean seedlings (Phaseolus aureus) were germinated in the dark and the extract prepared as previously described (Barber et al, 1964) except that 0.1 M phosphate buffer, pH 7.5, was used in place of Tris buffer. The particulate fraction which sedimented at 30,000 x g contained the enzyme activity.

The incubation mixtures contained the following (μmoles in a final volume of 0.2 ml): GDP-D-mannose-^{14}C (500,000 cpm/μmole), 0.016; potassium phosphate buffer, pH 7.5, 5; $MgCl_2$, 5; and 0.1 ml of the particulate enzyme. At the end of the incubation period, 0.5 ml of water was added and the mixture was heated at 100° for 2 minutes. The precipitate was collected by centrifugation and was then extracted with 2 ml of 2% NaOH at 100° for 2 minutes. The precipitate was again obtained by centrifugation, washed several times with water, and its radioactive content determined.

Results - When GDP-D-mannose-^{14}C was incubated with the particulate enzyme, radioactivity was incorporated into a water-insoluble, alkali-insoluble polymer. As shown in Figure 1, this incorporation was linear with time for about 20 minutes and then slowly leveled off during the next hour. The incorporation was also proportional to the protein concentration. When either D-mannose-^{14}C, D-mannose-6-phosphate-^{14}C, D-mannose-1-phosphate-^{14}C,

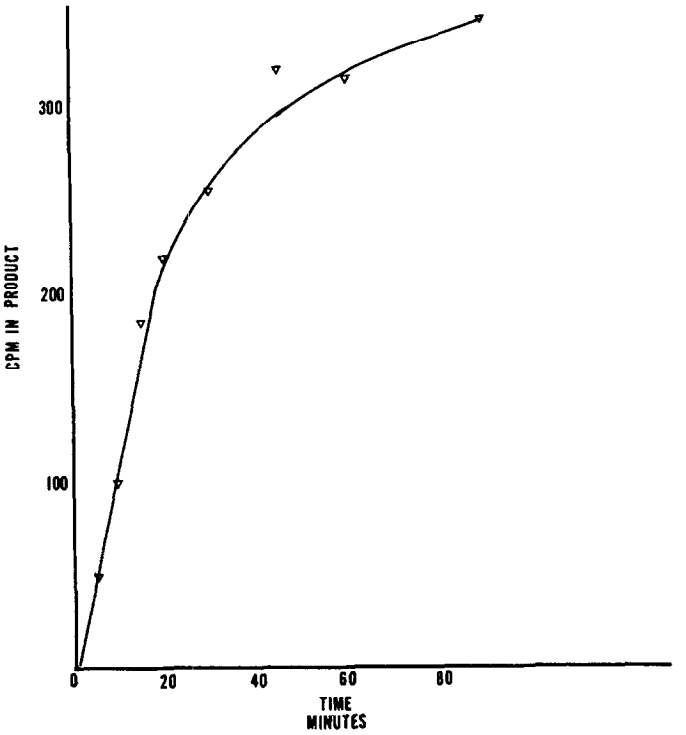

Figure 1. Incorporation of ^{14}C from GDP-mannose-^{14}C into the insoluble polymer as a function of time. Reaction mixtures were as described in the text.

D-glucose-^{14}C, D-glucose-1-phosphate-^{14}C, or D-glucose-6-phosphate-^{14}C were substituted for GDP-D-mannose-^{14}C, little or no incorporation of radioactivity into the polymer occurred (Table 1). The incorporation of mannose was dependent on the presence of Mg^{++}; when Mg^{++} was omitted only 20% as much radioactivity was found in the product. Mg^{++} does not stimulate the incorporation of glucose from GDP-D-glucose-^{14}C into cellulose unless the particles are first treated with an equal volume of saturated $(NH_4)_2SO_4$ (Barber et al, 1964). As shown in Table 1, when GDP-D-glucose was added to reaction mixtures containing GDP-D-mannose-^{14}C, a marked inhibition in the incorporation of

Table 1

Requirements for Glucomannan Synthesis

Omissions	Additions	cpm in product
Experiment 1		
None*	None	583
GDP-D-mannose-^{14}C	D-Mannose-^{14}C	2
GDP-D-mannose-^{14}C	D-Mannose-6-PO_4-^{14}C	0
GDP-D-mannose-^{14}C	D-Mannose-1-PO_4-^{14}C	5
GDP-D-mannose-^{14}C	D-Glucose-^{14}C	22
GDP-D-mannose-^{14}C	D-Glucose-6-PO_4-^{14}C	21
GDP-D-mannose-^{14}C	D-Glucose-1-PO_4-^{14}C	20
Enzyme	Boiled Enzyme	1
Experiment 2		
Mg^{++}	None	135
-	1 µmole Mg^{++}	449
-	5 µmole Mg^{++}	583
Experiment 3		
None	None	508
None	3×10^{-4} µmole GDP-D-glucose	329
None	7×10^{-4} µmole GDP-D-glucose	96
None	7×10^{-4} µmole GDP-D-glucose	82

*Complete reaction mixture is described in the text.

mannose was observed. It was previously shown that the addition of GDP-D-mannose to reaction mixtures containing GDP-D-glucose-^{14}C resulted in a stimulation in the incorporation of radioactivity into an insoluble product. The explanation of these results is not clear at the present time. However, it is possible that the enzyme(s) involved in the synthesis of glucomannan have a greater affinity for GDP-D-glucose than for GDP-D-mannose. This would

explain the inhibition of mannose incorporation by GDP-D-glucose. Since it is known that the D-glucose moiety of GDP-D-glucose is incorporated into cellulose, the stimulation in incorporation observed in the presence of GDP-D-mannose is presumably due to synthesis of another polymer, probably a glucomannan. This hypothesis is based on the fact that both glucose-^{14}C and mannose-^{14}C were isolated from the polymer synthesized in the presence of GDP-D-mannose-^{14}C, indicating that an epimerization of GDP-mannose to GDP-glucose occurs.

Table 2

Distribution of Radioactivity among Various Fractions

Fraction*	Total cpm
Aqueous Phase	260,000
2% NaOH Phase	
70% Ethanol Insoluble	11,500
70% Ethanol Soluble	24,000
Insoluble Product	53,850

* Fractionation procedure is as described in the text.

In order to characterize the product, a large-scale incubation mixture was prepared as follows (μmoles in a final volume of 10 ml); GDP-D-mannose-^{14}C, 0.70 (350,000 cpm); Mg^{++}, 50; potassium phosphate buffer, pH 7.5, 20; and 5 ml of particulate enzyme. After incubation for one hour at 37°, the mixture was treated as previously described. The distribution of radioactivity in the various fractions is shown in Table 2. Most of the activity in

both the aqueous and alkali fractions was in high-molecular weight compounds as indicated by their immobility upon paper electrophoresis or paper chromatography. Although these compounds contain both glucose and mannose, they have not been further characterized as yet.

The water-insoluble, alkali-insoluble product was not dialyzable. However, after treatment with β-mannanase (0.2%, pH 5.0, 50°) for 24 to 48 hours, 80-90% of the activity became dialyzable. The dialysate was concentrated *in vacuo* and chromatographed in solvent 1. In addition to mannose and glucose (which were identified by paper chromatography and borate electrophoresis), a number of radioactive oligosaccharides were detected. Each of these compounds was then rechromatographed in solvent 2 (Table 3). In both solvents,

Table 3

Properties of Oligossaccharides

Isolated from Polymer

Compound	R cellobiose Solvent 2	Ratio Mannose:Glucose
Cellobiose	1.00	-
4-O-(β-D-mannosyl-mannosyl-mannose)	0.56	-
4-O-(β-D-mannopyranosyl)-D-mannopyranose	1.18	-
4-O-(β-D-glucopyranosyl)-D-mannopyranose	1.08	-
Unknown 1	0.0	2.86
Unknown 2	0.21	3.75
Unknown 3	0.78	1.71
Unknown 4	1.10	1.04
Unknown 5	1.21	No glucose

two of the radioactive compounds ran close to, if not identical with, authentic 4-O-(β-D-mannopyranosyl)-D-mannopyranose and 4-O-(β-D-glucopyranosyl)-D-mannopyranose, respectively. Each of the oligosaccharides appeared homogeneous in solvent 3. Each of the radioactive oligosaccharides was hydrolyzed in 3N HCl at 100° for 30 minutes and then chromatographed in solvent 1 (3 developments of 12 hours each readily separate glucose and mannose). The glucose and mannose areas of the chromatograms were eluted and the amount of sugar in each was determined (mannose by the anthrone procedure, glucose either by anthrone or with glucose oxidase). As shown in Table 3, an oligosaccharide was isolated ($R_{cell.}$ = 1.10) which had a ratio of glucose to mannose of 1.00 to 1.04, and another oligosaccharide ($R_{cell.}$ = 0.78) which exhibited a mobility faster than that of authentic O-β-D-mannopyranosyl-(1-4)-O-β-D-mannopyranosyl-(1-4)-D-mannopyranose and had a ratio of glucose to mannose of 1.00 to 1.71. A slow-moving oligosaccharide was also detected ($R_{cell.}$ = 0.21) which had a ratio of glucose to mannose of 1.00 to 3.75. A faster moving oligosaccharide ($R_{cell.}$ = 1.21) was also detected which contained no glucose.

In each case the glucose and mannose were further identified by recromatography in solvents 2 and 3. The glucose in several of these oligosaccharides was found to be radioactive; the product of its reaction with glucose oxidase was further identified by paper chromatography as gluconic acid (gluconolactone) which was also radioactive.

The linkage in these oligosaccharides has not yet been determined. Presumably the anomeric configuration is β since the polymer was susceptible to hydrolysis by a β-mannanase, and one of the oligosaccharides was tested and found to be hydrolyzed by emulsin. The exact ratio of mannose units to glucose units is not known with certainty but it appears to be about 3 or 4 to 1. However, further experimentation is necessary before these questions can be answered.

It should be mentioned here that Perila and Bishop (1961) isolated and

characterized a β, 1-4 linked glucomannan from Jack Pine. Although a polymer of this type has not been isolated from mung beans, it may be a part of the hemicellulose fraction of these plants.

Summary - The mannose moiety of GDP-D-mannose-^{14}C is transferred by a particulate enzyme from mung bean seedlings into a glucomannan. The product was characterized by isolation of a number of oligosaccharides after treatment of the product with a partially purified β-mannanase. Several of these oligosaccharides contained glucose and mannose in the approximate ratio of 1:1, 1:2 and 1:3 or 4, respectively.

Since glucose-^{14}C was found in the hydrolysis products of the oligosaccharides, it can be assumed that the particulate enzyme contains an epimerase that converts GDP-D-mannose-^{14}C to GDP-D-glucose-^{14}C.

LITERATURE CITED

Elbein, A.D., Barber, G.A., and Hassid, W.Z., J. Am. Chem. Soc. 86, 309 (1964).

Barber, G.A., Elbein, A.D., and Hassid, W.Z., J. Biol. Chem., 239, 4056 (1964).

Roseman, S., Distler, J.T., Moffatt, J.G., and Khorana, H.G., J. Am. Chem. Soc., 83, 659 (1961).

MacDonald, D., J. Org. Chem., 27, 1107 (1962).

Loewus, F.A., Anal. Chem., 24, 219 (1952).

Nelson, N., J. Biol. Chem., 153, 375 (1944).

Perila, O. and Bishop, C.T., Can. J. Chem., 39, 815 (1961).