Phytochemistry, 1973, Vol. 12, pp. 2369 to 2373. Pergamon Press. Printed in England.

BIOSYNTHESIS OF SALEP MANNAN

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(*Revised Received* 23 *January* 1973. *Accepted* 16 *March* 1973)

Key Word Index—*Orchis morio*; Orchidaceae; polysaccharide biosynthesis; salep mannan; glucomannan; mucilage.

Abstract—A particulate enzyme system, isolated from growing orchid tubers (*Orchis morio*), was shown to catalyse the transfer of mannose from guanosine-diphosphate-mannose-^{14}C and its incorporation into alkali-insoluble mannan with the same type of linkage [$\beta(1 \rightarrow 4)$-D-mannopyranosyl] as is contained in the naturally-occurring reserve polysaccharide.

INTRODUCTION

THE TUBERS of different orchid species are known to contain a mucilaginous polysaccharide (salep mannan), which is composed of β-1,4 linked glucose and mannose residues in the ratio of *ca.* 1 : 3.[1]

The formation of the salep mucilage takes place during the months of March until July.[2] Analysis of the sugar nucleotides in the growing tubers showed the presence of *inter alia* GDP-mannose[2] which is thought to be a natural substrate for the biosynthesis of the salep mucilage.

RESULTS

The incorporation of mannose from GDP-mannose-^{14}C by the particulate enzyme system is proportional to the protein content in the assay mixture. Enzyme activity increases during the months of April and May and then decreases until the month of August (Fig. 1). The rate of polysaccharide formed *in vivo* during one vegetation period shows a similar trend.[2] The time course of mannan formation is illustrated in Fig. 2. Radioactivity from GDP-mannose-^{14}C is incorporated fairly rapidly during the first 15 min and subsequently levels off.

TABLE 1. EFFECT OF DIFFERENT METAL IONS ON THE RATE OF MANNAN FORMATION

Cation	Radioactivity in mannan-^{14}C (%)	Cation	Radioactivity in mannan-^{14}C (%)
None	32	Ca^{2+}	28
Co^{2+}	100	Fe^{2+}	43
Mn^{2+}	58	Fe^{3+}	45
Mg^{2+}	45		

The conditions were as in the standard assay. Different cations were added to give a final concentration of 5 mmol. Results are expressed as a percentage of the value obtained with Co^{2+}.

[1] BUCHALA, A., FRANZ, G. and MEIER, H. (1973) *Phytochemistry*, submitted for publication.
[2] FRANZ, G. and MEIER, H. (1970) *Planta Med.* **19**, 326.

The reaction showed a sharp pH optimum at 6·5–6·6 in both phosphate and citrate buffers. Mannan formation was stimulated considerably by the addition of Co^{2+}. Other divalent ions either inhibited the reaction or had a less stimulatory effect (Table 1). The optimal Co^{2+} concentration was about 10 mM. The influence of different glycosides on mannan formation was tested (Table 2). Amongst the sugars added only sucrose had a stimulatory effect, which was similar to the effect observed in the case of mannan biosynthesis with enzyme preparations from mung beans.[3] Naturally occurring salep mannan was tested as a possible acceptor molecule but it had no significant influence upon the incorporation of mannose from GDP-mannose-^{14}C. The particles, which served as the enzyme source, after two washings with buffer, still contained 2·7 mg/ml of polysaccharide. The principal sugars released on hydrolysis of the particles were glucose and mannose, demonstrating that these particles contain sufficient endogenous acceptor molecules.

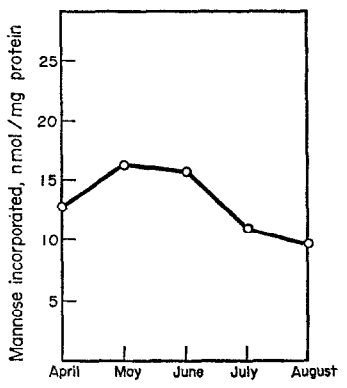

FIG. 1. CHANGES OF ENZYME ACTIVITY DURING GROWTH OF THE ORCHID TUBERS. PLANT MATERIAL WAS HARVESTED AT *ca.* 4-WEEK INTERVALS. REACTION CONDITIONS WERE AS IN THE STANDARD ASSAY.

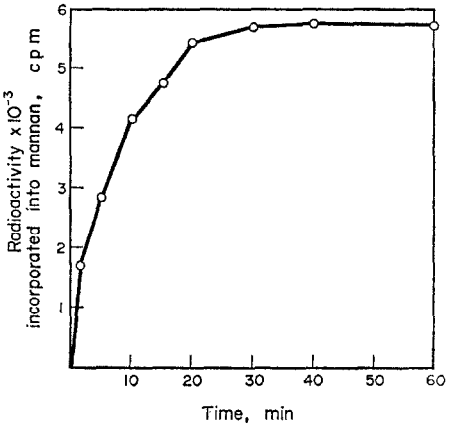

FIG. 2. TIME COURSE OF MANNAM PRODUCTION IN THE STANDARD ASSAY.

The effect of adding different nucleotides to the reaction mixture is shown in Table 3. Unlabelled GDP-glucose and UDP-glucose showed a slight inhibition on the mannose

TABLE 2. EFFECT OF DIFFERENT CARBOHYDRATES ON THE INCORPORATION OF MANNOSE-^{14}C FROM GDP-MANNOSE-^{14}C INTO MANNAN

Carbohydrate	Radioactivity in mannan-^{14}C (%)	Carbohydrate	Radioactivity in mannan-^{14}C (%)
None	62	Glucose	68
Sucrose	100	Cellobiose	48
Mannose	60	Salep mannan	55
Mannobiose (4-*O*-β-D-mannopyranosyl-D-mannose)	53		

10 μl of 0·1 M solutions of the different sugars were added to the standard assay mixtures. Salep mannan (10 μl) was added in a 1% solution. Results are expressed as a percentage of the value obtained with sucrose.

[3] HELLER, J. S. and VILLEMEZ, C. L. (1972) *Biochem. J.* **128**, 243.

transfer from GDP-mannose-^{14}C to the alkali-insoluble material. Partial acid hydrolysis of the reaction products obtained after the addition of unlabelled UDP-glucose or GDP-glucose to the standard reaction mixture showed no alteration on the oligosaccharides formed; i.e. only the β-(1→4)-linked series of mannose containing oligosaccharides was obtained.

In order to characterize the products synthesized from GDP-mannose-^{14}C with particulate enzyme preparations from orchid tubers a large scale assay was carried out and the distribution of radioactivity in lipid-, water-, alkali-soluble and alkali-insoluble fractions was examined (Table 4). A significant amount of radioactivity was incorporated into the $CHCl_3$–MeOH-soluble lipid fraction. The lipid, which was not further investigated, had a chromatographic mobility similar to the mannosyl lipids synthesized by Kauss[4] and Tanner.[5]

TABLE 3. EFFECT OF DIFFERENT NUCLEOTIDES ON THE INCORPORATION OF MANNOSE-^{14}C FROM GDP-MANNOSE-^{14}C INTO MANNAN

Nucleotide (in addition to GDP-mannose-^{14}C)	Radioactivity in mannam-^{14}C (%)	Nucleotide (in addition to GDP-mannose-^{14}C)	Radioactivity in mannan-^{14}C (%)
None	100	UTP	97
GMP	120	ATP	108
GDP	92	GDP-glucose	82
GTP	88	UDP-glucose	87

The conditions were as in the standard assay. Nucleotides were added to a final concentration of 10^{-4} M. Results are expressed as a percentage of the value obtained with the standard mixture.

Acid hydrolysis of the labelled lipid with 1 N HCl gave mannose-^{14}C as the only radioactive product. Most of the radioactivity in the H_2O extract was accounted for by unreacted GDP-mannose-^{14}C and by minor amounts of free mannose-^{14}C. The alkali extract which contained a relatively small amount of radioactivity showed upon hydrolysis mannose-^{14}C as the only radioactive constituent. The insoluble residue remaining after these extractions contained about 6% of the radioactivity initially present as GDP-mannose-^{14}C. Complete hydrolysis of the alkali insoluble residue gave mannose-^{14}C and no other radioactive sugars were detected. Partial acid hydrolysis or acetolysis gave mannose-^{14}C and a series of mannose-^{14}C containing oligosaccharides. β-1,4-Linked mannobiose and mannotriose were identified by PC. The purified oligosaccharides on reduction with $NaBH_4$ and hydrolysis gave mannose and mannitol in the ratios 1 : 1 and 2 : 1 respectively. Periodate oxidation, reduction and hydrolysis of the oligosaccharides gave erythritol and glycerol confirming the presence of (1 → 4) linkages.

TABLE 4. DISTRIBUTION OF RADIOACTIVITY IN DIFFERENT FRACTIONS FOLLOWING INCUBATION OF GDP-MANNOSE-^{14}C AS SUBSTRATE WITH PARTICULATE ENZYME PREPARATIONS OF ORCHID TUBERS

Fraction	Distribution of radioactivity (%)	Fraction	Distribution of radioactivity (%)
Chloroform–methanol (3 : 1)	8	Alkali extract	4
Water extract	82	Alkali insoluble residue	6

The sequence of extractions was carried out as described in Experimental.

[4] KAUSS, H. (1969) *FEBS Letters* **5**, 81.
[5] TANNER, W. (1969) *Biochem. Biophys. Res. Commun.* **35**, 144.

DISCUSSION

A particulate enzyme system from young tubers of orchid plants was shown to catalyse the transfer of mannose from GDP-mannose-^{14}C into mannan which has the same type of linkage, i.e. $\beta(1 \rightarrow 4)$,[1] as the naturally occurring salep mucilage. This reserve polysaccharide is now known to contain $\beta(1 \rightarrow 4)$-linked D-mannopyranosyl and D-glucopyranosyl residues in the ratio *ca.* 3 : 1.[1] The donor of the glucose residues in the polymer is not known and there was no significant difference in the alkali-insoluble mannan when the enzyme particles were incubated with UDP-glucose or GDP-glucose in the presence of GDP-mannose-^{14}C. The enzyme particles were not found to have the GDP-mannose-GDP-glucose-epimerase activity present in comparable particulate enzyme preparations isolated from other plants.[6] It is possible that salep mucilages contain mannan and glucomannan which have not yet been separated. There are also differences in the solubilities of the *in vitro* synthesized material and the naturally occurring polysaccharide. The water-solubility of the latter is apparently due to the presence of acetyl groups[1,7] and on deacetylation the polysaccharide becomes water-insoluble and dissolves with difficulty in dilute alkali. The mannan synthesized *in vitro* has similar solubility properties to the mannan isolated from dates[8] which contains no acetyl groups.

Substrate was incorporated into the alkali-insoluble reaction product with rather low efficiency. A similar effect has also been observed with comparable particulate enzymes from mung bean seedlings.[6] The mannan or glucomannans which until now have been synthesized *in vitro* with particulate enzymes from different plant sources are all cell wall polysaccharides. This is the first report of an *in vitro* biosynthesis of a reserve mannan where GDP-mannose is the active substrate. For the formation of various other mannans, GDP-mannose served as a specific substrate in their biosynthesis.[3-6,9-11]

The enzyme system which forms the $\beta(1 \rightarrow 4)$-mannopyranosyl linkages seems to be attached to subcellular particles or membrane fragments. Electron microscopy has shown that the formation of other plant mucilages[12,13] can take place in the golgi vesicles and this possibility is being investigated for orchid tubers.

EXPERIMENTAL

General methods. PC was on Schleicher and Schull No. 2043b paper using the following irrigants: (1) EtOAc–*n*-BuOH–H_2O–HOAc (3 : 4 : 4 : 2·5); (2) EtOAc–pyridine–H_2O (2 : 1 : 2); (3) EtOAc–pyridine–H_2O (8 : 2 : 1); (4) EtOAc–H_2O–HOAc (3 : 1 : 3); and (5) a 0·55% solution of phenylboronic acid in EtOAc–HOAc–H_2O (9 : 2 : 2). Alkaline $AgNO_3$ was used as chromatographic detection reagent. Protein was determined by the method of Lowry *et al.*[14] and the amount of polysaccharide in the enzyme particle suspension was determined by the anthrone method.[15] Guanosine-diphosphate-mannose (mannose-UL-^{14}C) with a specific activity of 52 mCi/mmol was obtained from the International Chemical and Nuclear Corp., Irvine, Calif., U.S.A.

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[7] HUSEMANN, E. (1940) *J. Prakt. Chem.* **155,** 241.
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[9] BRAR, S. S. and ELBEIN, A. D. (1971) *Phytochemistry* **10,** 2099.
[10] ELBEIN, A. D. and HASSID, W. Z. (1966) *Biochem. Biophys. Res. Commun.* **23,** 311.
[11] BEHRENS, N. H. and CABIB, E. (1968) *J. Biol. Chem.* **243,** 502.
[12] HYDE, B. B. (1970) *Am. J. Botany* **57,** 1197.
[13] BONCHET, P. and DEYSSON, G. (1971) *Compt. Rend.* **272,** 819.
[14] LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265.
[15] MORRIS, D. L. (1948) *Science* **107,** 254.

Preparation of the particulate enzyme. The particulate enzyme fraction was prepared from freshly harvested (May–August) outdoor-grown young tubers of *Orchis morio*. The tubers were rinsed with cold H_2O and homogenized in a cold mortar with sand in the presence of an equal vol. of 0·05 M phosphate buffer (pH 6·5, 0°) containing 0·01 M mercaptoethanol. The homogenate was filtered through Miracloth (Calbiochem.) and the filtrate centrifuged at 1000 *g* for 10 min. The pellet was discarded and the supernatant centrifuged at 22000 *g* for 40 min. The 22000 *g* pellet was suspended in buffer, centrifuged at the same speed and then resuspended in 0·05 M phosphate buffer (pH 6·5) containing 0·1 M sucrose.

Enzyme reaction. The standard assay mixture contained enzyme (30 μl: *ca.* 6·5 mg protein/ml), GDP-mannose-^{14}C ($6{\cdot}5 \times 10^{-4}$ μM), phosphate buffer (0·05 M, pH 6·5) and other substances, as indicated in the Results, to give a final vol. of 50 μl. The reaction mixture was incubated in a H_2O bath at 37° for 30 min, then HOAc (50 μl) was added and the whole mixture was spotted on a paper chromatogram and developed in irrigant 1. The area at the starting point containing radioactive material (mainly mannan) was cut out, transferred to a counting vial containing 0·5% butyl-PBD in toluene (15 ml) and counted in a Beckman β-Mate liquid scintillation spectrophotometer.[4]

Studies on the biosynthesized polymer (large batch assay). Radioactive mannan was prepared by incubating GDP-mannose-^{14}C (50 μl; $2{\cdot}9 \times 10^{-1}$ μCi) enzyme preparation (100 μl, 0·05 M) phosphate buffer (50 μl; pH 6·5), 0·1 M sucrose (20 μl) and 0·1 M $CoCl_2$ (5 μl) for 60 min. The reaction was terminated by the addition of $CHCl_3$–MeOH (3 : 1; 2 ml) and non-radioactive salep mannan (5 mg) was added as carrier. The precipitate was sedimented by centrifugation and subsequently extracted with $CHCl_3$–MeOH (3 : 1; 2 ml), H_2O (2 ml), KOH 2% (2 ml). The alkali-insoluble residue was suspended in H_2O and freeze-dried. A sample of this material was completely hydrolysed in 4% H_2SO_4, neutralized with $BaCO_3$ and examined by PC (irrigants 2, 3 and 4). Partial acid hydrolysis of the material with 2% H_2SO_4 (100°; 5 hr) gave (PC irrigant 2) oligosaccharides chromatographically indistinguishable from authentic samples of β-1,4-linked mannobiose and mannotriose. The oligosaccharides were purified by preparative PC (irrigants 2 and 4). Acetolysis of the radioactive alkali-insoluble polysaccharide was carried out by the method of Steward *et al.*[16] using a mixture of Ac_2O–HOAc–conc. H_2SO_4 (10 : 10 : 1) by vol. After 12 hr at 40° an excess of pyridine was added to the solution which was then evaporated to dryness. The acetylated sugars were extracted with $CHCl_3$. Deacetylation was carried out with NaOMe in MeOH and the solution was deionized with Dowex 50 H^+. The mannobiose and mannotriose obtained were purified by PC (irrigant 2). Periodate oxidation of the oligosaccharides was carried out by the method of Elbein[6] and the products on hydrolysis were identified by PC (irrigant 3). Samples of the oligosaccharides were reduced with $NaBH_4$ for 18 hr and the excess of $NaBH_4$ was destroyed by the addition of HOAc. The borate produced was removed by codistillation with MeOH and the solutions were deionized (Dowex 50 H^+). Hydrolysis with 4% H_2SO_4 gave mannose and mannitol (PC irrigant 5).

Acknowledgements—The author thanks Professor H. Meier and Dr. A. Buchala for their suggestions on the preparation of the manuscript and for their valuable discussions. The work was carried out with financial support by Swiss National Research Foundation. We also wish to acknowledge the technical assistance of Frl. F. Buser.

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