Phytochemistry, Vol. 21, No. 8, pp. 1951–1954, 1982.
Printed in Great Britain.

0031-9422/82/081951-04$03.00/0

BIOSYNTHESIS OF A β-(1→4)-MANNAN IN FENUGREEK SEEDLINGS

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(*Revised received* 4 *December* 1981)

Key Word Index—*Trigonella foenum-graecum*; Leguminosae; polysaccharide biosynthesis; β-mannan.

Abstract—A particulate enzymatic preparation, extracted from fenugreek seedlings (*Trigonella foenum-graecum*) catalyses the transfer of mannose from guanosine diphosphate-[U-^{14}C]mannose and its incorporation into an alkali-soluble polysaccharide. Chemical and enzymatic study of this polysaccharide reveals the presence of only one type of osidic linkage, namely β-(1→4)-D-mannopyranosyl. The influence of some factors on this biosynthesis was studied, as well as the MW of the polysaccharide and the existence of an endogenous acceptor.

INTRODUCTION

The incorporation of mannose from guanosine diphosphate-mannose (GDP-mannose) into mannans or glucomannans was previously described in the seeds of plants in which these polysaccharides may play a role in the cell wall components, but do not represent the reserve polysaccharide of the seeds, for example *Phaseolus aureus* [1–3] or *Pisum sativum* [4]. Similarly, a β-(1→4)-mannan has been synthesized from GDP-mannose with a mannosyltransferase from membrane fractions of Sycamore (*Acer pseudoplatanus*) [5] and the authors pointed out that such a biosynthesis is surprising in cells containing no, or only traces of, mannose in their cell wall. So it seemed interesting to study the biosynthesis of a β-mannan in the seeds of a species for which a mannose-containing polysaccharide is the essential reserve of energy. We have chosen the fenugreek seeds: their reserve polysaccharide is a galactomannan constituted of a β-(1→4)-mannan backbone, substituted by galactosyl residues, branched on the mannan by α-(1→6) linkages, with a ratio, galactose–mannose, very close to one. We report here the results of these experiments.

Franz [6] previously obtained a β-(1→4)-mannan with an enzyme from Salep (*Orchis morio*), but this case is not strictly comparable, since the natural polysaccharide of Salep is actually a glucomannan, with β-(1→4) linkages involving both glucose and mannose.

RESULTS AND DISCUSSION

After incubation of GDP-(U-^{14}C) mannose with a particulate enzymatic preparation of fenugreek seedlings, in a buffered medium (pH 7.5) containing Mg^{2+}, the reaction product is precipitated by addition of ethanol in the presence of a small quantity of a carrier (ivory nut mannan or fenugreek galactomannan). After centrifugation and washing the pellet several times with ethanol–water, the insoluble product is radioactive, and does not contain radioactive GDP-mannose, completely eliminated by repeated washings as was verified by chromatography. This biosynthesis needs no addition of an exogenous acceptor.

Structure of the synthesized radioactive product

The physical properties are those encountered for β-mannans: insolubility in water, ethanol, 0.2 M sodium hydroxide, partial solubility in 2 M sodium hydroxide, and no migration on PC and TLC with several solvent systems.

After total acid hydrolysis, only one radioactive sugar can be identified, namely [^{14}C]mannose. Contrary to the enzymatic preparation from *Phaseolus* [1] that of fenugreek is devoid of an epimerase activity catalysing the conversion of GDP-mannose into GDP-glucose. A mild acid hydrolysis gives a mixture of oligosaccharides, showing the same chromatographic migration as that of a series of β-(1→4)-oligomannosides obtained by partial hydrolysis of ivory nut mannan.

Permethylation experiments give the following results: hydrolysis of the methylated polysaccharides reveals only the presence of 2, 3, 6 - tri - O - methyl - D - mannose and traces of 2, 3, 4, 6 - tetra - O - methyl-D - mannose; after methanolysis of the same product, only methyl - 2, 3, 6 - tri - O - methyl - D - mannoside is obtained. These chemical results correspond to a linear (1→4) polymannosidic chain.

Enzymatic hydrolysis by a β-D-mannannase from *Streptomyces* [7] leads to a series of radioactive oligosaccharides with a chromatographic migration identical to that of reference β-(1→4)-D-oligoman-

nosides, revealing the β-anomeric configuration of the (1→4)-mannosyl linkages. The synthesized polysaccharide has, therefore, the characteristics of a β-(1→4)-mannan.

Factors influencing the mannan biosynthesis

In our experimental conditions, the incorporation of mannose into the radioactive mannan occurs after a latency of *ca* 2 min, after which the process is linear for 10 min. This latency was previously reported for the biosynthesis of the α-mannan of *Micrococcus lysodeikticus* [8].

The need for a divalent cation, a classical feature of the glycosyltransferases, is observed; no transfer of mannose occurs in the absence of Mg^{2+} or Mn^{2+}; Mg^{2+} is more active and its optimal concentration in the medium is 25 mM. The addition of β-mercaptoethanol decreases the incorporation rate.

The optimal pH for the transfer reaction is 8.5 and half maximal activity is observed at pH 6.5 and 9.7.

The influence of some nucleotides and GDP-glucose on the transfer of mannosyl is shown in Table 1. GDP and GTP are inhibitors at high concentrations, and GDP-glucose reveals a large inhibitory effect, at the same concentration as GDP-mannose in the medium. This has also been previously observed [5].

The mannosyltransferase activity decreases during storage at +4°: the residual activity is 45% after 24 hr and 33% after 48 hr. At −20°, 45% of the initial activity is observed after several weeks. Addition of β-mercaptoethanol has no protective effect.

Occurrence of a possible acceptor for the mannan biosynthesis

As we have said before, the biosynthesis occurs without the addition of an exogenous acceptor. Moreover, we did not detect mannitol after borohydride reduction of the mannan followed by acid hydrolysis. This leads us to postulate the existence of an endogenous acceptor in the particulate enzymatic preparation. Indeed, it is known that generally the biosynthesis of polysaccharides proceeds via acceptors. In our experiments, the first possibility would be that of glucidic acceptors; traces of galactomannan, of oligomannosides or of polymannosides present in hemicelluloses.

In order to study this possibility, some biosynthesis experiments were carried out with GDP-[U-^{14}C]mannose, in the presence of increasing quantities of these carbohydrate derivatives. The results are given in Table 2 and show no significant modification of the incorporation of mannose in comparison with the standard conditions.

Another possibility would be that of a proteic acceptor, as has been previously suggested [9–11]. But the treatment of the insoluble biosynthesized polymer by pronase, or by a mixture of pronase, trypsin and pepsin do not decrease the MW of the product and no soluble radioactivity appears. It may be possible that the proteases are not able to act because of steric hindrance. These results do not allow us to describe the nature of the acceptor.

MW of the synthesized polymer

This was studied by exclusion chromatography. The polymer only being soluble in 2 M sodium hydroxide, the convenient gel was Sepharose CL 6B (Pharmacia), allowing chromatography in strongly alkaline media (exclusion limit MW 1×10^6 for the polysaccharides and 4×10^6 for proteins). The elution pattern reveals two radioactive peaks: one in the void volume, and one with a K_{av} corresponding to a MW of 90000 for a polysaccharide or 200000 for a protein. The product eluted in the latter peak could correspond to a β-(1→4)-mannan which, by subsequent substitution by galactose residues, would lead to the reserve galactomannan with a MW *ca* 200000, a classical value for the MW for these polysaccharides [12, 13].

The very high MW of the compound eluted in the first peak is not compatible with a β-(1→4)-polymannosidic chain. As we said before, the action of proteases on this product does not decrease the MW, but increases the solubility of the product in 2 M sodium hydroxide. It may be postulated that the radioactive product is associated with a proteic structure which would be dissociated by the proteases. These results are similar to those of Smith *et al.* [5] suggesting that the radioactive polymannoside elaborated by sycamore cells is tightly adsorbed to proteins, but not linked by covalent linkages.

In our biosynthesis experiments, radioactivity was also detected in products extractable by lipid solvents which correspond to mannolipids.

EXPERIMENTAL

Preparation of the particulate enzyme. 10 g of fenugreek seeds, after swelling for 24 hr in H_2O, were germinated for 48 hr at 20°. After the elimination of the integuments, the

Table 1. Influence of some nucleotides and GDP-glucose on mannan biosynthesis

	Percentage of inhibition (molar inhibitor concentration/molar substrate concentration)		
Inhibitors	1	10	100
GMP	13	26	66
cGMP	0	0	1
GDP	14	67	90
GTP	13	77	88
GDP-glucose	72	90	96

Table 2. Influence of possible acceptors on mannan biosynthesis

Possible acceptors	Quantity added (mg)	Radioactivity incorporated (dpm)
Blank (without acceptor)	—	48000
	—	50200
	—	48500
β-(1→4)-Mannotriose	1	49700
	2	51700
	3	52700
Ivory nut mannan	1	48800
	2	50200
	3	47300
Fenugreek galactomannan	1	45000
	2	46000

seedlings were homogenized in a cold mortar with sand, in the presence of 100 ml 0.2 M Tris–HCl buffer (pH 7.5, 4°), containing 1% (w/v) bovine serum albumin and EDTA (0.01 M). The homogenate was strained through gauze, and the material centrifuged at 2000 *g* for 10 min. The supernatant was centrifuged again at 35000 *g* for 45 min and the pellet washed twice by suspension in the same buffer (100 ml), 15 min stirring and centrifugation under the same conditions. The pellet, suspended in 3 ml of the same buffer devoid of EDTA, was used as the enzyme source.

Enzyme reaction. The standard reaction medium contained: 50 μl GDP-[U^{14}C]mannose (0.50 μCi, 3 nmol) in aq. soln, 150 μl 0.2 M Tris–HCl buffer (pH 7.5) containing $MgCl_2$ (0.05 M) and 150 μl particulate enzyme preparation. The mixture was incubated at 25° for 30 min for the isolation of the radioactive mannan and 5 min for other expts (determination of optimal pH, study of Mg^{2+} and Mn^{2+} activation, nucleotide inhibitions and possible acceptors). In all cases, the reaction was stopped by heating for 2 min at 100°; 2 mg of ivory nut mannan or fenugreek galactomannan were added as carriers and the biosynthesized polymer was precipitated by addition of 0.9 ml EtOH. 10 min later, the mixture was centrifuged, and the pellet washed ×5 (suspension in 300 μl H_2O, addition of 0.9 ml EtOH and centrifugation), in order to remove excess radioactive GDP-mannose. The radioactivity was measured by liquid scintillation (Intertechnique S.L 32), after the addition of 10 ml Instagel (Packard) to 1 ml of the washing supernatant, or to a suspension of the pellet in 5 ml H_2O.

For the optimal pH studies, two series of buffers were used: Tris–maleate, for the pH range 4.65–8.95, and glycine–NaOH for pH 8.25–10.45.

Chemical study of the polymer. Total acid hydrolysis was carried out on washed pellets suspended in 0.5 ml 4 N H_2SO_4 and heated for 4 hr at 100° under reflux. The hydrolysate was neutralized with Amberlite MB_1, concd and submitted to PC (solvent mixtures I and II) and TLC (solvent mixture III).

Partial acid hydrolysis was also carried out on the washed pellets. They were suspended in 0.5 ml 0.1 N H_2SO_4, or in 0.5 ml 1 N oxalic acid and heated for 4 hr at 100° under reflux. The hydrolysates were submitted to the same treatment as above.

For methylation of the synthesized polymer, three washed pellets, after addition of 10 mg of ivory nut mannan, were treated once according to ref. [14] and three times by the method of ref. [15]. After hydrolysis or methanolysis, the products were identified by comparison with reference methylated derivatives of mannose or of methylmannoside respectively by PC (paper: Whatman I impregnated with 10% NH_4OH aq. soln and air-dried, solvent mixture IV), and by TLC (Kieselgel plates impregnated in the same manner, solvent mixture V) The chromatograms were first revealed by autoradiography (labelled products), and then by aniline oxalate reagent (standards).

For reduction expts, the washed pellets were suspended in 1 ml 0.1 M NaOH and excess KBH_4 was added. After 3 days excess hydride and borate were removed and the reduced polymer was hydrolysed with H_2SO_4 and submitted to PC (Schleicher–Schull paper 2043 BMgl, solvent mixture I, and detected by autoradiography).

Enzymatic study of the radioactive polymer. A washed pellet (after removal of EtOH) was suspended in 250 μl 0.05 M acetate buffer (pH 5.7) containing $Ca(OAc)_2$ (2 mM); 125 μl of a β-mannanase preparation from *Streptomyces* was added and the mixture was incubated at 37°. After 4 and 48 hr, the medium was analysed by TLC, with reference samples of β-(1→4)-oligomannosides obtained from ivory nut mannan.

Treatment by proteolytic enzymes. The washed pellets were suspended in 0.1 M Tris–HCl, pH 7.5. After addition of pronase (5 mg) after 0, 18 and 24 hr and incubation at 37°, aliquots of the supernatant were tested for radioactivity at different times. For the assays with a mixture of pronase, trypsin, pepsin (5 mg each), the incubation time was 48 hr and the medium was lyophilized before investigation for radioactivity, in comparison with blanks which were not treated by proteases.

MW study by exclusion chromatography. A column of Sepharose CL 6B (1.6×80 cm) was equilibrated with 2 M NaOH. Void and total vols. were determined with Dextran Blue and $K_2Cr_2O_7$ respectively. Three washed pellets were stirred with 0.7 ml 2 M NaOH (50% of the radioactivity of the pellets was solubilized) after centrifugation. The supernatent was transferred to the Sepharose column and elution carried out with 2 M NaOH, radioactivity being measured in 1.5 ml fractions.

Chromatography. Schleicher–Schull paper 2043 B Mgl

and Whatman paper was used for PC and Kieselgel G 1500 for TLC. Solvent mixtures were: I, *n*-BuOH–pyridine–H_2O (9:5:4) II, *iso*-PrOH–MeCOEt–EtOAc–*n*-BuOH–H_2O (6:5:3:2:5); III, PrOH–EtOAc–EtOH–HOAc–pyridine–H_2O (7:3:3:2:2:4); IV, octane–*iso*-PrOH–NH_4OH [(d = 0.89) 10% aq. soln (50:25:2); V, *iso*-octane–*iso*-PrOH–NH_4OH [(d = 0.89) 10% aq. soln] (65:25:4). Detection of radioactive products was by autoradiography with Kodirex (Kodak). Carbohydrates were revealed on TLC plates with 5% H_2SO_4 in EtOH and on PC by *o*-phenylene–diamine–oxalic acid reagent.

Acknowledgements—We are indebted to Professor B. Fournet (University of Lille) for the gift of reference methylated derivatives of methyl-D-mannoside.

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