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BIOSYNTHESIS OF PURE ARABAN AND XYLAN

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Key Word Index—*Phaseolus mungo*; Leguminosae; biosynthesis; hemicellulose; araban; xylan; plant cell wall.

Abstract—A crude particulate enzyme preparation from mung bean shoots partially freed from sugar transferases synthesized pure araban from UDP-L-^{14}C-arabinose. The preparation thus allowed to study some properties of the UDP-arabinose transferase which was shown to require 7 mM Mn^{2+} and pH 6–6·5 for optimal activity. Pure xylan was synthesized from UDP-D-^{14}C-xylose if a mixture of 0·06% Triton X100 and 35 mM EDTA was added to the crude enzyme preparation. In contrast to the UDP-arabinose transferase the UDP-xylose transferase does not require bivalent metal ions.

INTRODUCTION

THE BIOSYNTHESIS of plant cell wall polysaccharides has been studied so far almost entirely with particulate enzyme preparations[1] as attempts to solubilize and purify the individual enzymes usually result in their inactivation.

In addition to the various sugar transferases, particulate preparations contain enzymes for the interconversion of the parent sugar-nucleotides; one radioactive nucleotide may thus produce a mixture of polysaccharides with different labelled monomers, especially in the biosynthetic pathway derived from UDP-glucuronic acid. For example, arabinose-containing polysaccharides can be found starting with labelled compounds such as UDP-xylose,[2] UDP-glucuronic acid,[3] UDP-galacturonic acid[4] as well as UDP-arabinose. These observations render statements regarding the properties of a certain transferase questionable, unless the reaction products are carefully identified for all experimental conditions.

The aim of the present study was to find conditions which preferentially inactivate or remove interfering transferases, permitting examination of the remaining polymerase activity without enzyme isolation. This report deals mainly with the biosynthesis of arabans because the *in vitro* formation of these polysaccharides has been only poorly investigated. In addition, interference of UDP-xylose transferase was expected to be rather serious as judged from the literature[2,3] and from preliminary experiments.[5]

RESULTS AND DISCUSSION

In order to bypass the difficulties mentioned above, we first tried to extract UDP-xylose-4-epimerase completely by washing the tissue with various buffers and/or detergents, but without success. Other preliminary experiments[5] similar to those reported in Table 1

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TABLE 1. EFFECT OF TRITON X100 ON THE SYNTHESIS OF ARABAN BY MUNG BEAN PREPARATIONS

Enzyme prepn*	Donor nucleotide	Total polysacch (cpm/10 mg protein)	Composition (%) araban	xylan
Crude (control)	UDP-L-^{14}C-arabinose	8268	33	67
	UDP-D-^{14}C-xylose	7599	16	84
After treatment with Triton X100	UDP-L-^{14}C-arabinose	1234	99	1
	UDP-D-^{14}C-xylose	475	51	49

* 5 min incubation with 4× standard mixture containing 4·5 mM Mn^{2+}.

showed that addition of Mn^{2+} to the crude particulate enzyme preparation significantly increased the portion of araban formed from UDP-L-^{14}C-arabinose. The addition of Trition X100 to the assay mixture in concentrations up to 0·12% also raised the portion of araban from about 35 to about 70%[5] although the total amount of polysaccharide formed decreased.

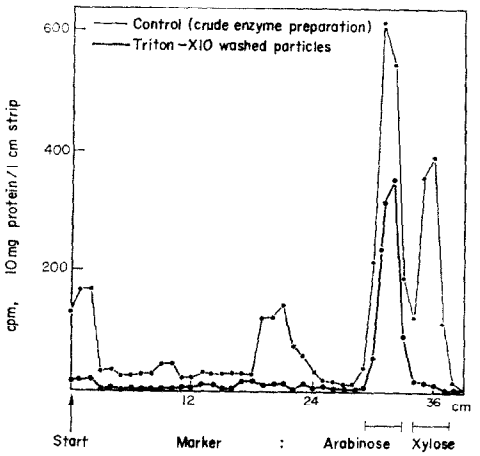

FIG. 1. PATTERN OF DISTRIBUTION OF RADIOACTIVITY ON A PAPER CHROMATOGRAM OF THE ^{14}C-SUGARS DERIVED FROM THE POLYSACCHARIDES FORMED FROM UDP-L-^{14}C-ARABINOSE. Note that the xylose-peak does not represent the total xylan content since part of the reaction product was not hydrolyzed under the conditions employed.

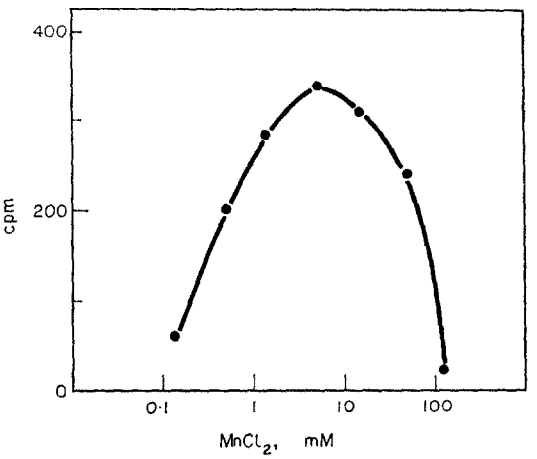

FIG. 2. STIMULATION OF ARABAN BIOSYNTHESIS BY Mn^{2+}. THE STANDARD ASSAY MIXTURE CONTAINING DIFFERENT FINAL CONCENTRATIONS OF $MnCl_2$ WAS INCUBATED FOR 5 min.

The results suggested that the UDP-xylose transferase might be more sensitive to detergent than the UDP-arabinose transferase and led to the development of a satisfactory Triton X100 washing procedure (see Experimental). The particulate preparation obtained produces almost no xylan from UDP-L-^{14}C-arabinose but is still able to synthesize significant amounts of araban from this nucleotide (Fig. 1, Table 1). Independent experiments, in which the UDP-xylose-4-epimerase activity was measured directly provide clear evidence that the enzyme is still remarkably active, after the Triton X100 washing.[5]

Using the washed particulate preparation we were then able to study some properties of the UDP-arabinose transferase. Table 2 demonstrates that the formation of araban is greatly enhanced by the addition of Mn^{2+}, Co^{2+} can substitute only in part, although it was

TABLE 2. EFFECT OF VARIOUS COMPOUNDS ON THE FORMATION OF ARABAN

Addition (1·4 mM)	Araban formed* (cpm)	Addition (1·4 mM)	Araban formed* (cpm)
Control	143	$FeCl_3$	218
$MnSO_4$	1274	$MgCl_2$	217
$MnCl_2$	1054	KCl	173
$CoSO_4$	497	NaCl	155
$FeSO_4$	320	K_2SO_4	144
$FeCl_2$	302	$HgCl_2$	9
$CaCl_2$	224	EDTA	4
$ZnCl_2$	218		

* Incubated 10 min with 2× standard assay.

shown to cause maximum stimulation of the 4-*O*-methylation of the glucuronic acid unit in hemicelluloses.[6] The activation of the transferase by Mn^{2+} is maximal at a concentration of 7 mM, higher concentrations are inhibitory (Fig. 2). When experiments were run in a similar way but with the addition of buffers (Na-cacodylate and K-phosphate) of different pH-values a relative sharp optimum of enzyme activity between pH 6·0 and 6·5 was observed.

TABLE 3. EFFECT OF EDTA AND Mn^{2+} ON THE FORMATION OF ARABAN

Addition to (EDTA 1·2 mM)	Araban formed* (cpm)
No EDTA	204
None	31
$MnCl_2$ (0·12 mM)	44
$MnCl_2$ (1·2 mM)	234
$MnCl_2$ (12 mM)	1503

* Incubated 20 min.

The biosynthesis of araban can be suppressed almost completely (Tables 2 and 3) by the addition of EDTA. Obviously some Mn^{2+} originating from the intact cells was still present in the particulate preparation and is complexed by EDTA leading to the inactivation of the enzyme which can be reactivated by addition of extra Mn^{2+} (Table 3). The transferase responsible for arabinan biosynthesis is also strongly inhibited by heavy metal ions such as Hg^{2+} and Cu^{2+} even in the presence of Mn^{2+}. However, it is fairly stable during storage at low and elevated temperatures and with Triton X100 (Table 4).

The assay procedure already provides some information on the nature of the reaction product. The ethanol–ammonium acetate solvent clearly eliminates any low molecular

[6] H. KAUSS and W. Z. HASSID, *J. Biol. Chem.* **242,** 1680 (1967).

weight material and favors polymers containing labelled arabinose. The proportion of arabinose in the polysaccharide was checked for every experiment reported in this paper by acid hydrolysis followed by paper chromatography of the products and was found to be at least 98% or more. About 20% of the labelled araban was extracted from the paper by boiling in 50 mM Na-phosphate buffer solution containing 50 mM EDTA, pH 6·8, for 30 min[7] or in aqueous 0·5% ammonium oxalate for 20 min.

TABLE 4. EFFECT OF VARIOUS TREATMENTS ON THE FORMATION OF ARABAN

Treatment*	Araban formed (cpm)	(%)
Control	1600	100
$CuSO_4$ ($1{\cdot}2 \times 10^{-3}$ m)	279	18
$HgCl_2$ ($1{\cdot}2 \times 10^{-3}$ m)	64	4
Triton, 0·1%	1411	88
Triton, 0·2%	1260	79
44°, 10 min	1120	70
30°, 1 hr	1459	91
30°, 3 hr	1312	82
−18°, 2 hr	1530	96

* Includes 6 mM Mn^{2+}, run for 20 min.

The material solubilized by this procedure was dialyzed (16 hr, water) immediately or after saponification (4 N NH_4OH, 2 hr) followed by electrophoresis on glass-fibre paper.[8] In every respect it was found to behave like a neutral polysaccharide. Thus it seems to be comprised of neutral arabans rather than araban chains attached to pectic substances. The remaining 80% of the labelled material (insoluble in the above mentioned solvents) was almost completely (96–98%) extracted with 2% NaOH (twice, 100°, 30 min) and thus belongs to the hemicellulose fraction. Further characterization of this polysaccharide was achieved by chromatography on a Sephadex G100 column eluted with 0·1 M NaCl containing some toluene and 1% dimethylformanide.[8] Although 10–15% were eluted together with dextran-blue indicating a molecular weight greater than 100 000 the bulk was eluted shortly after the void volume and seems to have, therefore, a MW between about 80 000 and 100 000. The greater fraction from the Sephadex column did not give clear bands on glass-fibre electrophoresis.[8] The main part of radioactive material moved like a neutral polysaccharide but a significant amount (30–50%) was spread over the weak acid to the stronger acid region. This indicates that at least part of the araban formed from the UDP-L-^{14}C arabinose is linked to polysaccharides containing varying amounts of uronic acids.

A partial acid hydrolysis of the araban led besides arabinose to labelled oligosaccharides. Minor peaks with $R_{arabinose} = 0{\cdot}47$ and 0·09 contained not enough radioactivity for further characterization. A more prominent peak ran with $R_{arabinose} = 0{\cdot}67$ in the same region as xylobiose and maltose and, therefore, most likely represents a disaccharide. On acid hydrolysis it gave only labelled arabinose, and after reduction with sodium borohydride a mixture of arabinose and arabitol in a ratio of 7 : 3. This may indicate that the disaccharide is composed of two labelled arabinose molecules, one of which is not bound at its reducing group. If one assumes that the addition of new arabinose units occurs at the non-reducing

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[8] A. J. BARRETT and D. H. NORTHCOTE, *Biochem. J.* **94,** 617 (1965).

end of polysaccharide chains, the arabinose molecule from the reducing part of the disaccharide must have been formerly located inside the araban chain. As it is partly labelled, it must have been partly formed from the UDP-L-^{14}C-arabinose. This means that not only single arabinose units were transferred but some polysaccharide chains were growing by at least two subsequent additions of arabinose molecules.

It is clear from the data reported so far that the formation of araban is strictly dependent on the presence of Mn^{2+} or other divalent ions. Consequently removing endogenous metal ions from the crude particulate enzyme preparation seemed to offer a simple method for the biosynthesis of xylan without simultaneous production of araban. On the other hand the data listed in Table 5 clearly demonstrate that the addition of EDTA does not solve the

TABLE 5. BIOSYNTHESIS OF PURE XYLAN

Addition*	Total polysaccharide (cpm)	Composition (%) araban	xylan
Control	50 280	13·7	86·3
EDTA	46 550	4·3	95·7
EDTA + Triton	9250	0·5	99·5

* EDTA, 35 mM; Triton X100, 0·06%, incubation for 20 min with 4× standard assay.

problem completely. Even with EDTA concentrations up to 100 mM about 2–3% of the polysaccharides formed were araban. Evidently there are still Mn^{2+} ions present within the particles. They can be liberated to be bound with the EDTA after a mild partial destruction of the membranes by addition of a dilute Triton X100 solution (Table 5). Under these conditions a polysaccharide is synthesized from UDP-D-^{14}C-xylose which is comprised almost entirely of xylose. Although this polysaccharide was not yet studied in detail this method offers a fair possibility for future investigations of the *in vitro* biosynthesis of pure xylans.

EXPERIMENTAL

Material. UDP-L-^{14}C-arabinose (200 mC/mM) and UDP-D-^{14}C-xylose (200 mC/mM) were obtained from NEN and dissolved in H_2O to contain 15 000–25 000 cpm per 5 μl.

Enzyme preparation. A crude particulate enzyme preparation was obtained from dark grown mung bean shoots as previously described[9] but omitting addition of EDTA. For araban synthesis the particles were resuspended in 50 mM K-phosphate buffer, pH 7·3, containing 1% (w/v) bovine serum albumin and 0·1% Triton X100; they were equilibrated in ice for 15 min and then centrifuged at 48 000 *g* for 55 min at 4°. This washing procedure was repeated 3× followed by one washing in the same buffer but without Triton X100. Finally the particulate preparation was resuspended in M K-phosphate buffer, pH 6·8, containing 1% (w/v) albumin and 40 mM sucrose.

Enzyme assay. The standard assay mixture contained 5 μl particulate enzyme suspension, 20 μl 100 mM Na-cacodylate buffer, pH 6·0, (+1% albumin and 40 mM sucrose), 5 μl (approximately 2×10^{-6} M) UDP-L-^{14}C-arabinose (or UDP-D-^{14}C-xylose, see Tables 1 and 5), in a total vol. of 35 μl. The mixture was incubated at 30° for the indicated time, and the reaction was stopped by addition of 50 μl HOAc. The total mixture was applied to a 3·0 × 1·2 cm strip of chromatography paper (SS 2043b). The strip was placed on a plastic sieve dividing a beaker into two compartments, about 200 ml of solvent (99% EtOH 500 mM ammonium acetate, pH 3·6 = 30/70) were added and the strips stirred for 1 hr. The solvent was then renewed and the strip dried after 10 extra min. This treatment resulted in elution of low molecular material. Controls run

[9] H. KAUSS and W. Z. HASSID, *J. Biol. Chem.* **242**, 3449 (1967).

for each experimental condition described here were found to contain 50 cpm or less. The strips were counted in a vial containing 15 ml of a solution of 5 g PPO/l. toluene.

Purity of the product. In order to determine the composition of the polymeric reaction products, strips were washed with toluene after counting, and treated at 95° with about 3 ml of 0·1 N HCl for 30 min, 0·1 N HCl for 1 hr, 1 N HCl for 30 min followed by three times 1 N HCl for 1 hr. This treatment proved sufficient to extract the entire arabinose content. The radioactivity remaining on the paper was due to polymerized xylose, as shown by further hydrolysis with 1 N HCl for 1 hr followed 10 times with 3 N HCl for 30 min. The pooled acid extracts were diluted to contain less than 0·1 N acid and taken to dryness at 30° in a rotary evaporator. Control experiments with ^{14}C-arabinose showed that 95–98% of the arabinose survived the first series of hydrolysis and the evaporation. The residue was applied on Whatman No. 1 paper and chromatographed for 18 hr in *n*-BuOH–pyridine–H_2O–HOAc (60:40:30:3). Marker spots of unlabelled sugars were developed,[10] the chromatogramms cut in 1 cm strips and their radioactivity determined as described before. The portion of xylan and araban was calculated from the sum of radioactivity in the xylose-peak plus xylan remaining on the strips versus the arabinose peak. In the case of crude enzyme preparations (Table 1) the arabinose content was corrected for the arabinose liberated from the oligosaccharides (Fig. 1) by further hydrolysis (1 N HCl, 95°, 1 hr).

If the hydrolysis of the araban was carried out with 1 N HCl for 1 or 3 hr instead of a above, considerable parts of the ^{14}C-arabinose was lost and decomposition products appeared on the chromatogramm.

Partial acid hydrolysis of araban. Paper strips with ^{14}C-araban were covered with HCl (conc. HCl/fuming HCl = 1/1, 0°) and allowed to stand for 15 min at room temp. The strip was removed and the HCl diluted at 0° with 50 vol. H_2O, evaporated and chromatographed as above. The disaccharide was reduced,[11] hydrolyzed with 0·1 N HCl at 95° for 45 min and arabinose and arabitol separated by chromatography for 22 hr in EtOAc–HCOOH–H_2O–HOAc (18:1:4:3).

Protein. The protein content was determined according to Lowry *et al.*[12] using the albumin containing incubation buffer as a blank.

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