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1,3-β-GLUCAN SYNTHASE FROM *CITRUS* PHLOEM

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Key Word Index—*Citrus aurantifolia*; Rutaceae; Mexican lime; polysaccharide biosynthesis; phloem; 1,3-β-glucan synthase.

Abstract—1,3-β-Glucan synthase activity has been demonstrated in particulate fractions of bark extracts from Mexican lime. With respect to substrate, the enzyme kinetics did not conform to the Michaelis–Menten equation. The value of the Hill coefficient was 1.2 and $S_{0.5}$ is 1.1 mM. The enzyme had an optimum pH of 7.5. Maltose, sucrose, and especially cellobiose and glucose, were enzyme activators when tested at physiological concentrations. In the presence of 15 mM $MgCl_2$ the enzymic activity was stimulated at 10 μM UDP-glucose but decreased at 1 mM UDP-glucose, suggesting a minor 1,4-β-glucan synthase activity.

INTRODUCTION

The biosynthesis in higher plants of polymers of glucose residues in which the main linkage is 1,3-β has been described by researchers from different laboratories [1–10]. The most extensive studies have been performed on herbaceous plants such as *Phaseolus aureus* [1–3], *Lupinus albus* [2], *Avena sativa* [4], *Triticum aestivum* [5], *Allium cepa* [6], *Lolium multiflorum* [7], *Lilium longiflorum* [8], *Gossypium hirsutum* [9] and *Petunia hybrida* [10].

Studies on the occurrence of the enzymic synthesis of such polymers are of special interest in the phloem of woody plants where callose, a 1,3-β-glucan, plays an important role in the obliteration of the phloem vessels [11]. 1,3-β-Glucan synthase (EC 2.4.1.34) may be the key enzyme in this process [12] although to our knowledge no reference is found in the literature to the presence of this enzyme in woody plants.

The enzymic synthesis of a glucose polymer with 1,3-β-linkages by particulate fractions of bark extracts from Mexican lime is described in this paper. Some kinetic properties of 1,3-β-glucan synthase are studied. The stability in different extraction media has been previously described [12].

RESULTS AND DISCUSSION

The incubation of bark extracts from Mexican lime in a reaction mixture containing 1 mM UDP-glucose and UDP-glucose-[^{14}C] gave a radioactive product. The value of radioactivity retained in the filters was proportional to the time of incubation (from 0 to 90 min) and to the amount of extract in the assay mixture (from 0 to 100 μl).

The chromatographic pattern of the hydrolysed product was essentially identical to that of hydrolysed laminarin (Table 1). Cellobiose migrated between the dimer and the trimer from both hydrolysates. Although the anomeric configuration has not been directly assayed it can be concluded from this experiment that the product of the enzymic reaction contained mainly 1,3-β-linkages. The formation at 1 mM UDP-glucose of a glucan with 95% of 1,3-β-linkages by an enzyme from oat has been described by Tsai and Hassid [13].

Table 1. Comparison of R_G values of the hydrolysate of the reaction product with other oligosaccharides

	Glucose and cellobiose	Hydrolysate of laminarin	Hydrolysate of the reaction product
Spot 1	1·00 ± 0·07	1·00 ± 0·08	1·00 ± 0·08
Spot 2	—	0·80 ± 0·04	0·82 ± 0·04
Spot 3	0·65 ± 0·03	—	—
Spot 4	—	0·56 ± 0·03	0·58 ± 0·04
Spot 5	—	0·42 ± 0·02	0·43 ± 0·04

Mixtures of glucose and cellobiose with both hydrolysates of laminarin and reaction product were also analysed. The chromatographic patterns of these mixtures were identical to those of laminarin and the hydrolysate of the reaction product with an additional spot with a R_G 0.65.

The activity of 1,3-β-glucan synthase was increased in the presence of different sugars (Table 2). Each sugar was tested at an approximate physiological concentration assuming that sucrose, glucose and fructose constitute the 10% of the dry weight of the phloem tissue [14].

Table 2. Effect of various sugars on 1,3-β-glucan synthase activity

		Sugar concentration	
	None	20 mM	30 mM
		Relative activity	
No sugar	5	—	—
Glucose		53	69
Fructose		6	9
Sucrose		15	20
Cellubiose		81	100
Maltose		21	22

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Cellobiose and glucose were the most effective activators enhancing the activity × 20 and × 14 respectively. A more moderate effect was found with sucrose and maltose but no effect was observed with fructose.

The presence of an enzymic activity catalysing the formation of a 1,3-β-glucan might help in explaining the phenomenon of callose deposition on the sieve areas of the phloem [11]. The enzymic activation might be important in the mechanism of normal callose deposition in citrus phloem as well as in the pathological deposition of that polysaccharide in citrus trees affected by Citrus Tristeza Disease [15]. A more accurate picture about the quality and quantity of the different sugars in citrus phloem is needed to prove this hypothesis.

The values of the 1,3-β-glucan synthase activity obtained with different concentrations of the UDP-glucose showed that the enzyme did not follow simple Michaelis–Menten kinetics. The estimated Hill coefficient was 1.2 and the value of the substrate concentration that provided half-maximum rate was 1.1 mM.

The activity profile showed a maximum at pH 7.5 when tested in a pH range within 6.5 and 8.5. Half maximal activity was obtained at pH 8.5. The activity values were slightly higher when Tris buffer was used instead of phosphate buffer.

Although from the above results, only the 1,3-β-glucan synthase has been unambiguously identified in the bark extracts of Mexican lime, it is not possible to rule out the presence of 1,4-β-glucan synthase activity. In fact its presence is suggested by the differential effect of $MgCl_2$ at low and high UDP-glucose concentration (Table 3).

Table 3. Effect of $MgCl_2$ on relative enzymic activity

[UDP-glucose] mM	[$MgCl_2$] mM 0	15
0.01	100	141
1	100	46

The enzymic activity was increased when the medium contained $MgCl_2$ together with a low UDP-glucose concentration. On the contrary, $MgCl_2$ decreased the enzymic activity in the presence of a higher UDP-glucose concentration. These results are similar to those obtained with extracts from oat [13] where the amount of glucan and the relative proportion of 1,3-β and 1,4-β linkages varied with different concentrations of $MgCl_2$ and UDP-glucose. The $MgCl_2$ produced an activation of a 1,4-β-glucan synthase, detected at low UDP-glucose concentration, and an inhibition of a relatively more active 1,3-β-glucan synthase, predominating at high UDP-glucose concentration.

EXPERIMENTAL

Mexican limes [*Citrus aurantifolia* (Christm.) Swing.] 4-yr-old, were grown in a greenhouse.

Extraction. Bark from 3 to 4-month-old stems was homogenized in a cold mortar with 9 ml/g of 50 mM Tris–HCl pH 7.5 containing 1% BSA and 10 mM 2-mercaptoethanol. Homogenates were centrifuged at 1000 *g* for 5 min. The pellet was discarded and the supernatant centrifuged at 30000 *g* for 15 min. The pellet of the last centrifugation was resuspended in 1 ml of buffer per g of initial bark. All operations were performed at 4°.

Enzyme assays. These were carried out with fresh extracts. 1,3-β-Glucan synthase activity was determined as described before [12]. The routine reaction mixtures contained, in a final vol of 0.4 ml, 50 mM Tris–HCl, 10 mM cellobiose, 1 mM UDP-glucose, *ca.* 80000 cpm UDP-glucose-[^{14}C] and 50 μl of bark extract. The reaction time when studying the effects of pH, $MgCl_2$ or the different sugars was 30 min.

In the determination of optimum pH the buffers were 50 mM Tris–HCl pH 7.5, 8, and 8.5 and 50 mM NaPi pH 6.5, 7, 7.5, and 8.

In the determination of $S_{0.5}$ the concns of UDP-glucose were 0.06, 0.1, 0.15, 0.2, 0.5, 1, 1.5, 2.5, 10, and 15 mM. The reaction was stopped at 0, 5, 10, 15 and 20 min for every concn of the substrate and linearity was always tested. Mean values were obtained from triplicate assays.

Hydrolysis of the enzymically synthesized polymer. 5×10^5 cpm UDP-glucose-[^{14}C] was used in the reaction mixture in this expt. After the reaction was stopped, the mixture was centrifuged at 2800 *g* for 15 min. The supernatant was discarded and the pellet was washed × 4 with 10 ml H_2O and × 2 with 10 ml of 95% EtOH, the supernatant being discarded every time by centrifugation at 2800 *g* for 10 min. The pellet was resuspended in 5 ml of 99% HCO_2H and maintained at 100° for 30 min. The HCO_2H was evaporated *in vacuo* and the sediment resuspended in 5 ml of 0.17 N H_2SO_4 and maintained at 100° for 20 min. The medium was neutralized with $Ba(OH)_2$ and centrifuged at 1000 *g* for 15 min. The sediment was discarded. The supernatant was concentrated *in vacuo* and resuspended in 0.5 ml H_2O and chromatographed.

Chromatography of hydrolysis products. Descending PC was conducted on Whatman No. 1 filter paper [16]. The solvent system was EtOAc–Py–H_2O (10:4:3). The chromatograms were cut into 1 cm^2 pieces and their radioactivity determined with a Nuclear Chicago liquid scintillation counter.

Glucose, cellobiose, and a hydrolysate of laminarin obtained as described above were used as comparative standards and detected on chromatograms with Trevelyan reagent [17].

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