BIOSYNTHESIS OF β-1,3- AND β-1,6-LINKED GLUCAN BY PHYTOPHTHORA CINNAMOMI HYPHAL WALLS*

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SUMMARY

Three lines of evidence were obtained to show that uridine diphosphoglucose (UDPG) is the natural precursor for the synthesis of β-1,3- and β-1,6-linked glucan(s) of the hyphal walls of the plant pathogenic fungus Phytophthora cinnamomi: 1) isolation of UDPG from the mycelium; 2) demonstration of UDPG pyrophosphorylase in a soluble fraction of the cytoplasm; 3) enzymic polymerization of glucose from UDP[^{14}C]G into alkali-insoluble glucan by a washed hyphal wall preparation. Most of the glucan synthesizing enzyme(s) was found in the hyphal walls. Seemingly the fungal cell wall has the capability to polymerize one of its components autonomously.

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Relatively little is known on cell wall biosynthesis of fungi. The list of fungal wall polysaccharides, whose synthesis has been obtained in a cell-free system, includes chitin (Glaser and Brown, 1957; Jaworski et al., 1965), mannan (Algranati et al., 1963) cellulose and a glycogen-like polymer (Ward and Wright, 1965), and with this report, β-1,3- and β-1,6-linked glucan.

Whereas the biosynthesis of β-1,3 glucan in vitro has already been studied in higher plants (Feingold et al., 1958), this appears to be the first report on the biosynthesis of a glucan with β-1,6 linkages.

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Glucans comprise nearly 90% dry wt. of the hyphal walls of P. cinnamomi (Bartnicki-Garcia, 1966). About 2/3 consists of glucan(s) with β-1,3 and β-1,6 linkages in an as yet undetermined relationship; (Bartnicki-Garcia and Lippman, 1966); the remainder appears to be a poorly crystalline cellulose.

RESULTS AND DISCUSSION

A study of glucan biosynthesis was initiated with a search for natural precursors (nucleoside diphosphoglucose) accumulated in the mycelium of the fungus grown in a glucose-asparagine medium on a shaker for 2 weeks at 25°. Nucleotides were extracted with cold 5% trichloroacetic acid and separated by ion-exchange chromatography (Hurlbert et al., 1954). A major peak was identified as UDPG by its ultraviolet spectrum; paper cochromatography with authentic UDPG in ethanol-1M ammonium acetate (7:3, pH 7.5) and ethanol-1M ammonium acetate (7:3, pH 3.8); stoichiometric recovery of D-glucose after acid hydrolysis; and enzymic oxidation by UDPG dehydrogenase (Sigma Chemical Co.) according to Strominger et al. (1957).

Cell-free extracts of P. cinnamomi were made in a Hughes Press and fractionated by differential centrifugation. The formation of UDPG was followed spectrophotometrically using UDPG dehydrogenase (Strominger, et al., 1957). An active UDPG pyrophosphorylase which catalyzed the synthesis of UDPG from uridine triphosphate and glucose-1-phosphate was present in the soluble fraction of the fungal cytoplasm; particulate cell fractions had no activity.

These findings suggested that UDPG was the natural precursor for glucan synthesis in P. cinnamomi--an expectation confirmed using uridine diphospho[^{14}C]glucose (Internat. Chemical & Nuclear Corp.) as a substrate in a cell-free system. A preparation of washed fragments of hyphal walls actively incorporated ^{14}C-glucose from UDPG into alkali-insoluble glucan(s) (Table 1). Mg^{++} was required. Addition of soluble cytoplasm to the wall preparation enhanced incorporation greatly; this activation was due to some factor which resisted boiling for 5 min. The incorporation rate of ^{14}C-glucose, under the conditions of Table 1, was about 0.17-0.34 mμmoles per mg wall per hour (5-10%

of the initial radioactivity). Recently, by using a more dilute buffer (Tris-HCl final concn 0.003M) the incorporation rate was doubled. The glucan synthesizing enzyme(s) is specific for UDPG. Adenosine diphospho[^{14}C]glucose, guanosine diphospho[^{14}C]glucose, ^{14}C-glucose-1-phosphate or ^{14}C-D-glucose were not incorporated at all; nor was there any competitive reduction in the incorporation of UDP[^{14}C]G---tested at substrate saturation concentration---by an equimolar amount of nonradioactive thymidine diphosphoglucose, guanosine diphosphoglucose, cytidine diphosphoglucose or adenosine diphosphoglucose.

TABLE 1

Polymerization of glucose from UDP[^{14}C]G into alkali-insoluble glucan

Cellular Fractions**	Incorporation* (dpm/mg wall dry wt.)
Hyphal walls	441
Hyphal walls (boiled)	0
Hyphal walls + soluble cytoplasm (boiled)	3458
Hyphal walls (boiled) + soluble cytoplasm	34

* The incubation mixture (0.5 ml) contained: 3.4 mμmoles UDPG (7 X 10^4 dpm); 5.4 μmoles $MgCl_2$; 0.57 mg hyphal walls; 0.46 mg soluble cytoplasm; 0.2 ml of 0.25M Tris-HCl buffer, pH 7.85. After 1 h incubation the mixture was boiled 5 min and 5 mg of carrier cellulose added. The residue was centrifuged and washed with 2% NaOH at 100°C for 7 min. Radioactivity of the residue was counted by liquid scintillation.

** Hyphal walls were prepared by sedimenting a broken cell preparation (Hughes Press) at 1,000 X g. The residue was washed, blended and centrifuged at 500 X g for 1-2 min. The supernatant, consisting of short wall segments, was used. The soluble cytoplasm was the supernatant after 110,000 X g centrifugation.

TABLE 2

Products of enzymic digestion of ^{14}C-glucan synthesized by hyphal walls*

Component	$R_{glucose}$ BPW	$R_{glucose}$ EAW	Radioactivity %
Glucose	1.00	1.00	37.1
Laminaribiose	0.82	--	12.9
Laminaritriose	0.65	0.32	14.2
Cellobiose	0.65	0.46	0.5
Soluble glucan	0.02	--	35.3

* The deionized digest was first chromatographed on Whatman #3 MM paper irrigated with butanol-pyridine-water, BPW, (6:4:3); radioactive spots were located with a scanner, eluted, and counted by liquid scintillation. A second solvent, ethyl acetate-acetic acid-water, EAW, (9:2:2), was used to resolve laminaritriose from cellobiose, Laminaribiose and laminaritriose were further characterized by the stoichiometric release of glucose upon hydrolysis with purified β-glucosidase or 1N HCl; and by lead tetraacetate oxidation (Charlson & Perlin, 1956) which yielded upon hydrolysis ^{14}C-arabinose.

A crude extracellular preparation* from Streptomyces sp. QMB-814 (Reese et al., 1959) was used to help identify the glucosidic bonds synthesized by the hyphal walls. This enzymic complex which contains mainly endocellulase and some endo-β-1,3-glucanase is known to dissolve the walls of P. cinnamomi mycelium almost completely, yielding, among other products cellobiose, laminaribiose, laminaritriose, and a trace of gentiobiose (Bartnicki-Garcia and Lippman, 1966).

The incubation mixture of Table 1 (with boiled cytoplasm) was scaled up tenfold and the resulting ^{14}C-labeled hyphal walls were washed with hot 2% NaOH and then digested with Streptomyces glucanases at pH 5.7 for 36 h at 50°; essentially all of the radioactivity was recovered in solution. The soluble products were deionized and chromatographed on paper (Table 2). The release of radiolabeled laminaribiose and laminaritriose, identified as described in Table 2, indicated that the biosynthesized glucan contained β-1,3 bonds. Only a trace of radioactivity corresponding chromatographically to cellobiose was detected, hence no conclusion can be made as to the synthesis of β-1,4 glucan. A major portion of the digestion products did not migrate from the origin of paper chromatograms nor dialyzed through a cellophane bag. This soluble glucan fragment was resistant to further digestion by the Streptomyces glucanases but was partly digested by an endo-β-1,6-glucanase from Penicillium digitatum*, thus suggesting the existence of β-1,6 linkages in the biosynthesized glucan. This was confirmed by the isolation of ^{14}C-gentiobiose (6%) as the main oligosaccharide from partial acid hydrolysis (1N HCl at 100° for 1 h) of the soluble glucan. The amount of ^{14}C-gentiobiose was far greater than that obtained from the acid reversion of glucose ($\ll$ 1%), under the same conditions of hydrolysis, therefore excluding the possibility that it was a hydrolysis artifact. Likewise the following experiment ruled out the possibility that the gentiobiose isolated from the soluble glucan was a transglycosylation artifact produced during digestion of the walls with the Streptomyces enzyme complex. Non-

* Kindly supplied by E. T. Reese, U.S. Army Laboratories, Natick, Mass.

radioactive walls were digested with the Streptomyces enzymes in the presence of ^{14}C-oligosaccharides, previously obtained from a similar digestion of ^{14}C-labeled walls; there was no incorporation of radioactivity indicative of transglycosylation into the soluble glucan remaining after digestion for 24 h. The identity of ^{14}C-gentiobiose was confirmed by mixing it with cold authentic gentiobiose. The corresponding octaacetate was prepared (m.p. 194°) and recrystallyzed several times with no loss in specific radioactivity.

Work is now in progress to investigate whether the synthesis of β-1,3- and β-1,6-linked glucose residues corresponds to the formation of a suspected mixed-linkage glucan and, also, to explore the possibility that each type of glucosidic linkage may be formed by a different enzyme. The exact site of hyphal wall glucan biosynthesis in vivo is also under study. Our present information, as illustrated in Table 3, shows that the ability to synthesize glucan is almost exclusively restricted to the hyphal walls. "Mitochondrial"

TABLE 3

Cellular localization of β-glucan synthetase*

Cellular fraction**	Glucan synthesized (dpm/mg cell fraction)	Distribution of glucan synthetase***
Hyphal walls	2229	91%
"Mitochondria"	766	7%
"Ribosomes"	252	2%
Soluble cytoplasm	0	0%

* Incubation conditions as stated in Table 1.

** All incubation mixtures contained activator and primer: Hyphal walls, mitochondria and ribosomes were supplemented with boiled soluble cytoplasm as activator; mitochondria, ribosomes and soluble cytoplasm with boiled hyphal walls as primer. The mitochondrial and ribosomal fractions were the pellets on centrifugation at 10,000 X g and 110,000 X g respectively after hyphal walls had been separated as described in Table 1.

*** Calculated on the basis of the following dry wt percentage composition of a broken cell preparation; hyphal walls 49.3%, mitochondria 11.0%, ribosomes 7.9%, soluble cytoplasm 31.6%.

and "ribosomal" particles had little activity, which may be due to comminution of the hyphal walls during cell rupture.

Although further proof is needed to rule out the possibility that the observed association of enzyme and wall was not an artifact, the present evidence does suggest that the walls contain the primer and enzyme to elaborate one of their polymers *in situ*. Such capacity of the cell wall would lend further credence to previously stated views on fungal morphogenesis (Nickerson and Bartnicki-Garcia, 1964) which postulate a high degree of biosynthetic autonomy for the cell wall.

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