Chemical Structure of the Insoluble Hyphal Wall Glucan of *Phytophthora cinnamomi**

L. P. T. M. Zevenhuizen† and S. Bartnicki-Garcia

ABSTRACT: The major component of hyphal walls of *Phytophthora cinnamomi* is an extremely insoluble, noncellulosic glucan. This is the residual polysaccharide left after removal of cellulose by exhaustive extraction of the walls with Schweizer's reagent. The structure of this noncellulosic glucan was investigated by methylation and periodate oxidation. Hydrolysis of the methylated glucan yielded the following amounts of *O*-methyl-D-glucoses: 2,3,4,6-tetra-*O*-methyl-D-glucose (15.4 mole %), 2,4,6- (plus some 2,3,6-) tri-*O*-methy-D-glucose (58.6 mole %), 2,4,-di-*O*-methyl-D-glucose (20.7 mole %), and 4,6-di-*O*-methyl-D-glucose (5.2 mole %). The structural significance of the last compound remains uncertain. The glucan consumed 0.41 mole of periodate and produced 0.16 mole of formic acid per mole of anhydroglucose unit. Reduction and hydrolysis of the periodate-oxidized glucan afforded glycerol, erythritol, and glucose in a molar ratio of 23:10:66. The results indicate that the insoluble hyphal wall glucan is a highly branched polysaccharide composed predominantly of β-1→3-linked glucose chains, with about four chain residues per branching point. Glucose residues connected by β links through both C-3 and C-6 serve as branching points. In addition, about 10% of the glucose units appeared joined by 1→4 linkages; these could correspond to cellulose chains firmly bound to the noncellulosic portion of the insoluble hyphal wall glucan.

The possibility was expressed that the noncellulosic glucan of *P. cinnamomi* hyphal walls may be structurally similar to the insoluble cell wall (yeast) glucan of *Saccharomyces cerevisiae*.

Noncellulosic glucose polymers are common components of fungal walls but relatively little is known about their fine structure (for review on fungal walls, see Bartnicki-Garcia, 1968). Most investigations have been made on glucans from yeasts, particularly on the alkali-insoluble glucan of *Saccharomyces cerevisiae*, the so-called yeast glucan (see reviews of early literature by Phaff, 1963, and Clarke and Stone, 1963). These studies, however, have led to conflicting claims on the types of bonds and the existence of branches in the yeast glucan molecule. The first indication that this glucan is a highly branched molecule was obtained by Bell and Northcote (1950). From methylation studies they concluded that the principal glucosidic bond was β-1→3 with interchain linkages of the 1→2 type. A subsequent study based on partial acid hydrolysis (Peat *et al.*, 1958) confirmed the preponderance of β-1→3 links but failed to indicate the presence of either 1→2 links or branchings. The isolation of β-1→6-linked oligosaccharides led Peat and coworkers to conclude that yeast glucan was a linear polymer with random or alternating sequences of β-1→3- and β-1→6-linked units. However, the most recent studies of yeast glucan by methylation analysis and periodate oxidation, agree that this is a highly branched molecule, as Bell and Northcote (1950) proposed, but with the main linkages of the β-1→3 and β-1→6 type as Peat *et al.* had indicated. Misaki and coworkers (1963, 1968) favored the view that the molecule consists of a backbone of β-1→6-linked glucose units with almost every unit carrying a side chain of about eight β-1→3-joined glucose residues. Manners and Patterson (1966) suggested that the degree of substitution of the main chain varied with the glucan sample. Thus, in one of their samples, they found that only about one-half of the β-1→6-linked backbone residues carried lateral β-1→3-linked chains.

Evidence for a highly branched glucan with β-1→6 and β-1→3 linkages was also obtained for another yeast, *Candida albicans*, by Bishop *et al.* (1960) who examined an alkali-soluble glucan believed to be a cell wall component. Brown and Lindberg (1967) afforded evidence for the presence of several glucan fractions in cell walls of *Aureobasidium pullulans*. One was an alkali-soluble glucan made of β-1→3-linked chains with some of the chain residues substituted at C-6 by a single glucosyl unit. The other two were alkali-insoluble glucans: a fibrillar one made of linear β-1→3-linked chains and an amorphous glucan fraction with β-1→3 and β-1→6 links.

Preliminary studies, based chiefly on chromatographic demonstration of gentiobiose and laminaribiose after enzymic or acid hydrolysis, suggest that glucans with β-1→3 and β-1→6 linkages are commonplace in fungi including the Oomycetes where the genus *Phytophthora* belongs. The cell walls of various Oomycetes have been found to consist primarily of two kinds of glucose polymers: the minor component is a poorly crystalline cellulose; the major component is a highly insoluble

* From the Department of Plant Pathology, University of California, Riverside, California. *Received December 9, 1968.* This work was supported by a research grant from the National Institutes of Health (AI-06205).

† On leave of absence from the Laboratory of Microbiology, Agricultural University, Wageningen, The Netherlands.

noncellulosic glucan with β-1→3 and β-1→6 linkages (Bartnicki-Garcia, 1966; Aronson *et al.*, 1967; Bartnicki-Garcia and Lippman, 1967; Cooper and Aronson, 1967; Novaes-Ledieu *et al.*, 1967).

Earlier findings on *Phytophthora cinnamomi* had revealed that alkali-insoluble glucans constitute nearly 90% of the hyphal wall. By X-ray diffraction and by enzymic and acid hydrolysis, the presence of β-1→4, β-1→3, and β-1→6 linkages was ascertained (Bartnicki-Garcia, 1966; Bartnicki-Garcia and Lippman, 1967). It was estimated that at most 25% of the wall was cellulose, the main component being a Schweizer-insoluble, alkali-insoluble glucan with β-1→3 and β-1→6 linkages. The present study of methylation and periodate oxidation was undertaken to gain a greater insight into the chemical structure of this insoluble, noncellulosic hyphal wall glucan.

Materials and Methods

Cultivation of P. cinnamomi. The organism employed in this study was *P. cinnamomi* strain SB-216-1, isolated by G. A. Zentmyer of this department. Mycelium was obtained by growing the fungus in 350-ml prescription bottles containing 50 ml of asparagine–glucose medium (Bartnicki-Garcia, 1966). Bottles were inoculated with 10 ml of a mycelial suspension from a liquid culture. Incubation was at 25° in the dark for 5–7 days; the bottles were occasionally shaken to disperse the mycelial mats. The mycelium was harvested on a sintered-glass filter and washed several times with distilled water.

Isolation of Hyphal Walls and Preparation of Insoluble Wall Glucan. The mycelial mass (100 g wet weight) was suspended in water (400 ml) and homogenized in an Omni-Mixer operating at 6000 rpm for 1 min. The mycelial suspension was disrupted in a Bronwill MSK cell homogenizer using 0.5-mm glass beads and a single treatment of about 45 sec. Microscopic examination showed complete cell breakage. The suspension was centrifuged at 1000*g* for 5 min; the supernatant was discarded and the cell walls were washed twice with water by centrifugation. To free the mycelial walls of residual cytoplasmic material the walls were suspended in 1% sodium dodecyl sulfate and stirred at 90° for 0.5 hr. Cell walls were sedimented at 500*g* for 5 min and washed twice by resuspending them in the original volume of water and centrifuging at 500*g* for 5 min. Cellulose was removed by extracting the cell walls with Schweizer's reagent (Jayme and Lang, 1963). About 5 g of hyphal walls was suspended in 500 ml of the reagent and stirred for 24 hr under nitrogen at 5°. Unless otherwise stated, the extraction with Schweizer's reagent was repeated twice more. The extracted walls were washed consecutively with water, 1% EDTA solution (to remove Cu^{2+} ions), twice again with water, twice with ethanol, twice with ethyl ether, and finally dried *in vacuo*. This preparation constituted the insoluble or noncellulosic hyphal wall glucan. Cellulose was regenerated by acidifying the chilled cuprammonium hydroxide solution with acetic acid. The precipitated cellulose was then washed and dehydrated as described above for extracted walls.

Methylation of the Insoluble Hyphal Wall Glucan. The insoluble wall glucan was first methylated by the Haworth procedure (Hirst and Percival, 1965). The glucan (1 g) was suspended in 20 ml of water; dimethyl sulfate (40 ml) and sodium hydroxide (80 ml; 30% w/v were added at 15-min intervals in ten equal portions while the mixture was vigorously stirred at 50–60° under a stream of nitrogen. After 3 hr, excess dimethyl sulfate was decomposed by heating the reaction mixture for 0.5 hr at 90°. The insoluble methylated product was separated from the reaction mixture by filtration, and the methylation procedure was repeated using acetone (40 ml) as a solvent. Acetone was distilled by raising the temperature to 90°. The partially methylated glucan (1 g) showed a strong OH band in the 3500-cm^{-1} region of the infrared spectrum; the original morphology of the hyphal walls was retained. This partially methylated glucan remained insoluble in the usual organic solvents (chloroform and acetone), but unlike the original wall glucan, it was almost completely dissolved in 30 ml of dimethyl sulfoxide after stirring for 6 hr at 50°. The resulting viscous solution of partially methylated glucan was subjected to a further methylation by the Hakamori (1964) procedure. The viscous solution was added to a 15-ml solution of methylsulfinylcarbanion in dimethyl sulfoxide prepared according to Sandford and Conrad (1966). The reaction mixture was incubated with continuous stirring for 4–6 hr at 50°. The temperature was then lowered to 20°, and 3 ml of methyl iodide was added dropwise in the course of 15 min. Stirring was continued for 1 hr and a clear, light-colored solution was obtained. This solution was dialyzed for 48 hr against running tap water whereupon the methylated glucan separated as a white solid and was isolated by evaporation of the entire liquid. The Hakamori procedure was repeated once more to yield a methylated glucan with an infrared spectrum showing no significant OH band in the 3500-cm^{-1} region.

Hydrolysis of Methylated Glucan. Direct hydrolysis in hot dilute acid was ineffective; therefore, the methylated glucan (100 mg) was first solubilized in 72% H_2SO_4 (1 ml) for 1 hr at room temperature. The acid was diluted to 8% with water and the hydrolysis was completed in a sealed tube at 100° for 18 hr. The reaction mixture was neutralized with a saturated barium hydroxide solution; barium sulfate was removed by centrifugation at 16,000*g* and the clear supernatant was evaporated to dryness at 40° under reduced pressure in a Rotavapor evaporator.

Preparation of Methyl Glucosides. Methylated sugars were converted into their methyl glucosides by heating the hydrolysate (10 mg) in 4% methanolic hydrogen chloride (1 ml) at 100° for 16 hr in a sealed tube. Methanol was removed by distillation and HCl by evaporation in a desiccator over potassium hydroxide under vacuum. The resulting syrup was dissolved in water (5 ml), and samples thereof were used for gas–liquid partition chromatography.

Periodate Oxidation of Hyphal Wall Glucan. A suspension of insoluble wall glucan (1 g) was oxidized in 500 ml of 0.015 M potassium periodate, with stirring,

at room temperature in the dark. Samples of 10 ml were taken at different times for spectrophotometric determination (223 mμ) of periodate consumption and for potentiometric titration of formic acid produced (Hay *et al.*, 1965).

Smith Degradation of Periodate Oxidized Glucan. After oxidation of the glucan as described above, excess periodate was decomposed by the addition of 1 ml of ethylene glycol. The resulting polyaldehyde was isolated by centrifugation, washed with distilled water, resuspended in 200 ml of water, and reduced by stirring the suspension with 500 mg of sodium borohydride at room temperature for 24 hr. Excess sodium borohydride was decomposed by adjusting the pH to 7.0 with acetic acid; the resulting polyalcohol was centrifuged, washed with distilled water, and dried. The yield was 900 mg. For complete hydrolysis, the polyalcohol was solubilized in 72% H_2SO_4 and hydrolyzed, as described above for methylated glucan, to yield a mixture of alditols. The alditols were separated by paper chromatography or converted into acetates for gas–liquid partition chromatographic separation. The alditol mixture (10 mg) was acetylated with 0.1 ml of acetic anhydride in the presence of zinc chloride (1 mg) in a sealed tube at 70° for 20 hr. The reaction mixture was evaporated *in vacuo* at 40° to eliminate the acetic anhydride, and the residue was dissolved in the minimum amount of chloroform and made up to 10 ml with carbon disulfide. For mild hydrolysis (controlled degradation) of the polyalcohol, the procedure of Goldstein *et al.* (1965) was followed.

Separation Techniques. Paper chromatography of methylated sugars was carried out on Whatman No. 3MM paper with 1-butanol–95% ethanol–water–15 N NH_4OH (40:10:49:1, v/v, solvent system A). Preparative chromatography of methylated sugars was achieved on 32 × 2.5 cm cellulose columns (Whatman cellulose powder for · chromatography) eluted with water-saturated butanone (solvent system B). Oligosaccharides were separated on Whatman No. 1 paper using 1-butanol–pyridine–water (6:4:3, v/v, solvent system C). Smith degradation products were separated on Whatman No. 3MM paper with pyridine–ethyl acetate–water (2:5:7, v/v, upper phase, solvent system D). Chromatograms were sprayed with aniline hydrogen phthalate in water-saturated butanol for the detection of reducing sugars and with benzidine–sodium metaperiodate for the detection of alditols. Gas–liquid partition chromatography of methyl glucosides was effected in an Aerograph Model 600D unit, equipped with a flame ionization detector, using a 8 ft × $^1/_8$ in. stainless steel column packed with 10% neopentylglycol–succinate on Aeropak 30, 100–120 mesh; column temperature 175°; N_2 flow rate, 20 cc/min. Alditol acetates from the Smith degradation were separated using the same column but operated at 200°. Peak areas were integrated with a Disc Chart Integrator Model 227 (Disc Instruments, Inc. Cal.) and compared with the values of reference compounds.

The following methylated sugars used as reference compounds were prepared by hydrolysis of various polysaccharides, methylated by the Hakamori technique: 2,3,4-tri-*O*-methyl-D-glucose from dextran, 2,3,6-tri-*O*-methyl-D-glucose from soluble starch, and 2,4,6-tri-*O*-methyl-D-glucose from laminarin. Additionally, 2,4-di-*O*-methyl-D-glucose was prepared from laminarin by the method of Bell and Manners (1954). Laminarin was kindly supplied by the Institute of Seaweed Research, Inveresk, Midlothian, Scotland.

Analytical Techniques. Total carbohydrate was determined by Dreywood's anthrone method (Trevelyan and Harrison, 1952) after first solubilizing the glucan samples in 72% H_2SO_4 at room temperature. Methoxyl and total nitrogen determination were performed by the Schwarzkopf Microanalytical Laboratory, Woodside 77, N. Y. Infrared spectra of methylated glucans, in 5% chloroform solution, were recorded with a Perkin-Elmer Infracord spectrophotometer. The degree of polymerization of glucans was measured by reacting the glucan with sodium cyanide-^{14}C (Isbell, 1965). Optical rotations were measured with a Bendix automatic polarimeter.

Enzymatic Digestion. The insoluble hyphal wall glucan of *P. cinnamomi* (200 mg) was digested with 3 mg of purified exo-β-1→3-glucanase from a Basidiomycete QM 806. The enzyme was obtained through the courtesy of D. E. Eveleigh from the Prairie Regional Laboratory, N. R. C., Saskatoon, Sask., Can. The digestion was carried out in 25 ml of 0.25 M sodium acetate–acetic acid buffer (pH 4.8) at 50° for 72 hr.

Results

Hyphal Wall Glucans of P. cinnamomi. The proportion of cellulosic and noncellulosic glucans was determined in cell wall preparations of *P. cinnamomi* grown in the asparagine–glucose medium for 7 days (Table I). The walls were extracted with Schweizer's reagent and the cellulose thus dissolved was recovered by precipitation from the cuprammonium extract. The ratio of cellulose to insoluble glucan was about 1:10. As seen in Table I, one treatment with Schweizer's reagent was not sufficient to remove all the extractable cellulose; two

TABLE I: Hyphal Wall Glucans in *P. cinnamomi*.[a]

Expt	Mycelium (g wet wt)	Wall Cellulose (g dry wt)	Insoluble Wall Glucan (g dry wt)
I	100	0.5	5.5
II	82	0.335	4.4
		0.100	
		0.023	
		0.458	

[a] Walls were mechanically prepared from the entire mycelium mass and cellulose extracted from these walls by one (expt I) or three consecutive treatments (expt II) with Schweizer's reagent. The final residue constituted the insoluble wall glucan.

TABLE II: Separation of Products of Hydrolysis of the Methylated Insoluble Wall Glucan of *P. cinnamomi* by Gas–Liquid Partition Chromatography.

Methyl Glucosides				
Hyphal Wall Glucan			Mobility[a] R_T	
Component	Yield[b]	Authentic Compds with Similar Mobility	α Anomer	β Anomer
a	15.4 ± 0.7	2,3,4,6-Tetra-*O*-methyl ether	1.00	0.79
b	58.6 ± 0.9	2,4,6-Tri-*O*-methyl ether or 2,3,6-tri-*O*-methyl ether	2.35	1.78
Absent		2,3,4-Tri-*O*-methyl ether	1.92	1.44
c	25.9 ± 0.5	2,4-di-*O*-methyl ether or 4,6-di-*O*-methyl ether	5.30	3.85

[a] Relative to the retention volume of methyl-2,3,4,6-tetra-*O*-methyl α-D-glucoside. [b] In moles per cent; average from four analyses.

subsequent extractions yielded significant amounts of cellulose; after three treatments no more cellulose could be recovered from the extracts. The insoluble non-cellulosic wall glucan of *P. cinnamomi* showed the following properties: anhydroglucose content 90–95%, nitrogen content 0.05%, degree of polymerization 420, and optical rotation of the acetate $[\alpha]_D^{25}$ − 46° (*c* 5.0 in chloroform).

Methylation Analysis of the Insoluble Wall Glucan of P. cinnamomi. Methylation was first carried out by the Haworth procedure. The resulting partially methylated product became soluble in dimethyl sulfoxide and was then subjected to two methylations by the Hakamori method. The final methylated product had no significant OH band at 3500 cm^{-1}. The over-all yield from 1.0 g of glucan was 900 mg of methylated product with $[\alpha]_D^{25}$ − 24° (*c* 1.0, chloroform), methoxyl content 42.46%, and total N 0.00%. The methylated glucan was soluble in dimethyl sulfoxide and chloroform, but only partially soluble in ether.

Hydrolysis of the methylated glucan gave a syrupy mixture of methylated sugars, which were directly separated by paper or column chromatography. The mixture of methyl sugars was also separated by gas–liquid partition chromatography after conversion into their methyl glucosides.

On paper chromatograms, irrigated with solvent system A for 24 hr, the following components were recognized: (a) 2,3,4,6-tetra-*O*-methyl-D-glucose (R_{tetra} 1.00); (b) two overlapping spots of tri-*O*-methylglucoses, the major one corresponded to 2,4,6-tri-*O*-methyl-D-glucose (R_{tetra} 0.88), and the minor one to 2,3,6-tri-*O*-methyl-D-glucose (R_{tetra} 0.91); and (c) two incompletely separated di-*O*-methylglucoses, the main spot (R_{tetra} 0.69) with the same mobility as 2,4-di-*O*-methyl-D-glucose giving a red-brown color with aniline hydrogen phthalate, and a weaker spot (R_{tetra} 0.63) appearing as a tailing of the main spot and giving a grey-brown color with aniline hydrogen phthalate. This minor component appeared to be 4,6-di-*O*-methyl-D-glucose (see below).

Quantitative determination of the methylated sugars was done by gas–liquid partition chromatography (Table II). Three pairs of peaks appeared (components a, b, and c) corresponding, respectively, to the α- and β-methyl anomers of tetra-, tri-, and di-*O*-methyl glucosides. This method permitted estimation of the over-all proportion of each class of methyl glucosides but failed to separate some of the tri-*O*-methyl and di-*O*-methyl isomers. Thus there was only one pair of methyl tri-*O*-methyl glucoside peaks (component b) corresponding in mobility to the α and β anomers of either methyl-2,3,6- or methyl-2,4,6-tri-*O*-methyl glucoside. These two pairs of isomers, known to be present in the methylated glucan hydrolysate (see below), could not be separated by any of the columns tested (*cf.* Bishop, 1964). However, the tri-*O*-methyl glucoside peaks detected in the hydrolysate were clearly distinct from those of methyl-2,3,4-tri-*O*-methyl glucoside anomers. The latter were absent from the methylated glucan hydrolysate. The two suspected methyl-di-*O*-methyl glucosides (2,4,-; 4,6,-) could not be resolved by gas chromatography; only one pair of peaks was observed (component c).

By cellulose column chromatography three methyl glucose fractions were also obtained which corresponded to the three fractions resolved by gas–liquid partition chromatography. The indicated yields are from 500 mg of methylated sugar mixture. (a) 2,3,4,6-Tetra-*O*-methyl-D-glucose (85 mg) on recrystallization from petroleum ether (bp 30–70°) gave a crystalline product with mp 72–81°, $[\alpha]_D^{25}$ + 81° (*c* 0.1, water). (b) The tri-*O*-methylglucose fraction (220 mg) contained some 2,3,6-tri-*O*-methyl-D-glucose, as revealed by paper chromatography, but the main component was 2,4,6-tri-*O*-methyl-D-glucose. The latter crystallized from ether: mp and mmp 122–124°, $[\alpha]_D^{25}$ + 105° (1 min) → +74° (24 hr), (*c* 0.1, water). (c) The di-*O*-methyl-D-glucose fraction (119 mg) gave only a small crop of crystals which on recrystallization from ethyl acetate yielded a crystalline product (22 mg) with the properties of 4,6-di-*O*-methyl-D-glucose: mp 155–159°, $[\alpha]_D^{25}$ + 109° (1 min) → +67° (24 hr) (*c* 0.1, water). However the chief di-*O*-methylglucose could not be crystallized from the mother liquor. On paper chromatograms, this component appeared essentially pure and showed the same mobility as 2,4-di-*O*-methyl-D-glucose (R_{tetra} 0.69). Moreover, treatment with *p*-nitroaniline

(Van Cleve and Schaefer, 1955) afforded 2,4-di-*O*-methyl-*N*-(*p*-nitrophenyl)-D-glucosylamine with mp and mmp 249–250° after crystallization from ethyl acetate.

Periodate Oxidation of the Insoluble Wall Glucan of P. cinnamomi. The course of periodate consumption and formic acid production, expressed as moles per anhydroglucose unit, is shown in Figure 1. Periodate oxidation was essentially complete in about 24 hr. By extrapolation to zero time, periodate uptake was estimated to be 0.41 mole/glucose unit and formic acid production 0.16. The periodate-oxidized glucan was subjected to Smith degradation by reduction with borohydride to the corresponding polyalcohol followed by either complete hydrolysis (total degradation) or mild hydrolysis (controlled degradation). Prior to hydrolysis the polyalcohol showed the initial microscopic appearance of hyphal walls. Complete hydrolysis yielded (mole %): glycerol, 23.4; erythritol, 10.6; and glucose, 66.0. The controlled degradation was performed by suspending 900 mg of the polyalcohol in 200 ml of water, the pH was adjusted to 1.0 with H_2SO_4, and hydrolysis was allowed to proceed for 48 hr at room temperature with stirring. By this treatment, the turbidity of the suspension decreased considerably yet the remaining insoluble glucan still retained the original microscopic appearance of hyphal wall fragments. This degraded glucan, after centrifugation, washing with water, and drying, amounted to 390 mg. The combined supernatant fluid and washings were neutralized with barium hydroxide; the precipitated barium sulfate was centrifuged at 13,000*g* and the clear supernatant fluid was evaporated *in vacuo*, yielding a residue of 368 mg. This material was redissolved in water and fractionated by methanol precipitation into 100 mg of methanol-insoluble glucan and 222 mg of methanol-soluble products. Paper chromatography of the methanol-soluble products (solvent system D) revealed glycerol as the main component, erythritol, and a trace of glucose.

The degraded water-insoluble glucan left after the controlled degradation of the polyalcohol (above) was reoxidized with potassium periodate at room temperature in the dark for 40 hr. The periodate consumption was now 0.27 mole and the formic acid production 0.14 mole per anhydroglucose residue.

Digestion of Insoluble Wall Glucan with an Exo-β-1→3-glucanase. The insoluble wall glucan of *P. cinnamomi* was almost completely dissolved by action of an exo-β-1→3-glucanase preparation from Basidiomycete QM 806. The insoluble residue which amounted to 15% of the initial dry weight was centrifuged and the supernatant fluid was deionized by passage through columns of Dowex 50 (H^+) and Dowex 1 (acetate). The deionized solution was evaporated to dryness and dissolved in a small volume of water. Five volumes of methanol was added to precipitate a soluble glucan fraction ("limit dextrin") which represented 20% of the initial dry weight. This limit dextrin consumed 0.86 mole of periodate/mole of anhydroglucose and released 0.40 mole of formic acid. The methanol-soluble fraction was chromatographed on Whatman No. 3MM paper with solvent system C. As expected, glucose was the major single product of digestion (33% of initial dry weight). There were smaller amounts (2–3%) of laminaribiose and gentiobiose, and other slower moving oligosaccharides.

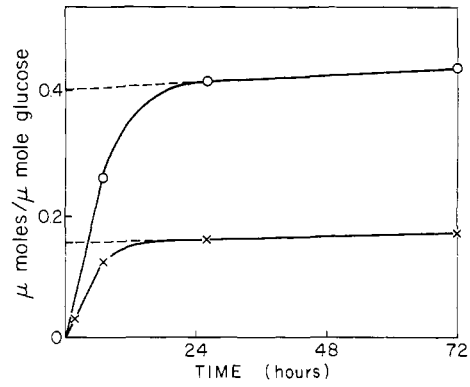

FIGURE 1: Periodate oxidation of insoluble hyphal wall glucan. (○--○) Periodate consumption and (X--X) formic acid production.

Discussion

The data obtained by methylation and periodate oxidation of the insoluble, noncellulosic glucan of hyphal walls of *P. cinnamomi* indicated the presence of the following approximate proportions of differently linked glucosyl residues. The glucosidic linkages are largely, if not entirely, of the β configuration, as deduced from the susceptibility of the wall glucan of β-glucanases, the isolation of β-1→3-, β-1→6-, and β-1→4-linked disaccharides after enzymic or acid hydrolysis (Bartnicki-Garcia and Lippman, 1967), and the negative optical rotation of the soluble derivatives of the wall glucan (methylated glucan and glucan acetate).

Nonreducing end groups (15–20%) were indicated by the mole % of 2,3,4,6-tetra-*O*-methyl-D-glucose detected (15.4) and of formic acid produced (16.0) upon periodate oxidation. The formic acid value constitutes a direct measurement of nonreducing end groups since other glucosyl units that might yield formic acid were not evident. Likewise the molar yield of glycerol after Smith degradation (23.4%) reflects the high proportion of terminal end groups in this glucan. The latter value is probably an overestimate of end groups since it includes glycerol produced by overoxidation of the glucan.

C-3-linked units (45–50%) were computed by deducting from the total amount of tri-*O*-methylglucose (58.6 mole %), the calculated amount corresponding to tri-*O*-methylglucosyl units believed derived from 1→4-linked linear chains (10%, see below). The Smith degradation data showed that a total of 66% glucose units survived periodate oxidation; if 20% are C-3- and C-6-linked branching units (see below), the remainder 46% represents 1→3-linked chains.

C-4-linked units (10%) were estimated from the amount of erythritol obtained after Smith degradation

of the periodate oxidized glucan (10.6 mole %). The periodate consumption by the insoluble wall glucan (0.41 mole/glucose residue) was higher than the value expected solely from oxidation of nonreducing terminal groups, which was calculated to be 0.32 from the amount of formic acid released (0.16 × 2). The excess 0.09 mole of periodate consumed may be interpreted as resulting from the oxidation of about 9% 1→4-linked residues in the wall glucan.

C-3- and C-6-Linked Units (20%). This high proportion of branching units, indicated by the amount of 2,4-di-*O*-methyl-D-glucose recovered, is approximately equal to the proportion of nonreducing end groups, as would be expected if both estimates are correct.

In addition, the presence of 5% of 4,6-di-*O*-methyl-D-glucose in the hydrolysate of methylated glucan would indicate that some branching units are connected through C-2 and C-3; however, the validity of this conclusion is uncertain. The relatively small amount of 4,6-di-*O*-methyl-D-glucose isolated may have been an artifact of incomplete methylation or demethylation during hydrolysis. The latter possibility was ruled out by studying the effect of hydrolysis on 2,4,6-tri-*O*-methyl-D-glucose. Under the same conditions used for the hydrolysis of methylated glucan there was no formation of di-*O*-methylglucoses as judged from paper chromatograms. Since the methoxyl content of the methylated glucan (42.46%) was below the theoretical value (45.6%), the possibility remains that the 4,6-di-*O*-methyl-D-glucose resulted from undermethylation. Pertinent to this are the results of various methylation studies on the "yeast" glucan of *S. cerevisiae* in which the quantities of 4,6-di-*O*-methyl-D-glucose reported seemingly reflected the completeness of the methylation. Thus Bell and Northcote (1950) and Manners and Patterson (1966), who reported relatively low methoxyl values of their methylated glucans (41.7 and 41.5%, respectively), found relatively large amounts of this dimethyl sugar (5–10%). Conversely, Misaki *et al.* (1968) who showed a higher methoxyl content (44.4%) reported only "trace" amounts of 4,6-di-*O*-methyl-glucose in their hydrolysates and considered this sugar as an artifact of incomplete methylation.

In conclusion, the above results demonstrate that the insoluble wall glucan of *Phytophthora* is a highly branched polymer containing a predominant proportion of β-1→3-linked chains with branches at glucosyl residues linked by β bonds through C-3 and C-6. The β-1→3 chains are probably very short with an average of four to five units per branching point. The presence of any substantial number of single-unit side branches was ruled out by the finding that the proportion of end groups (formic acid released upon periodate oxidation) in the glucan was not drastically decreased after one cycle of periodate oxidation, reduction, and controlled hydrolysis (degraded glucan). Furthermore, the observation that the degraded glucan retained its initial hyphal morphology supports the contention that the backbone of the insoluble glucan is made of periodate-resistant units, *i.e.*, glucose residues substituted at C-3. This observation argues against the possibility that the detected 1→4 linkages, or any 1→6 linkages from unbranched residues that might have escaped detection in the methylation analysis, play key structural roles in the glucan molecule, except perhaps in binding peripheral glucan fragments solubilized by the controlled Smith degradation. The presence of 1→4-linked glucose residues in the wall glucan, even after exhaustive extraction with Schweizer's reagent, poses several questions: Are they cellulose chains insoluble in Schweizer's reagent? And if so, are these chains covalently bound to the noncellulosic portion? Alternatively, are the 1→4 linkages interspersed in the glucan molecule? No definitive answers are available; however, a structure with interspersed, random or alternating, 1→3 and 1→4 bonds seems unlikely since the diagnostic fragments, after controlled Smith degradation, were not detected, namely 2-*O*-β-glucopyranosyl-D-erythritol and/or the erythritol glucosides of glucose oligosaccharides. Instead, the free erythritol observed suggests that the parent glucan contains sequences of at least two 1→4-linked residues. If cellulose chains occur as an integral part of the Schweizer's insoluble hyphal wall glucan of *Phytophthora* its *over-all* designation as noncellulosic glucan would not be entirely correct.

The results of the digestion with exo-β-1→3-glucanase are consistent with the proposal that the glucan molecule consists of numerous side branches of β-1→3-linked residues, the majority of which would be released as free glucose by the action of the enzyme. The limit dextrin left after enzymic digestion probably represents the core of the glucan molecule. Its high yield of formic acid upon periodate oxidation (0.40 mole/glucose residue) indicates that this limit dextrin contains roughly equal amounts of β-1→3 and β-1→6 linkages. However, we do not have enough information to ascertain whether the main chain of the glucan is made of glucose residues joined by β-1→6, or β-1→3 linkages, or both.

Significantly, except for the relatively high proportion of 1→4 linkages, our findings on the insoluble hyphal wall glucan of *P. cinnamomi* indicate a gross structural similarity with the "yeast" glucan of *S. cerevisiae* (Misaki *et al.*, 1968); both are highly branched glucans with a predominance of β-1→3 linkages and with β-1→6 linkages on branching residues only. A similarity in chemical structure would be in agreement with other outstanding properties of these glucans such as their extreme insolubility and the formation of coarse microfibrillar aggregates upon treatment with hot dilute mineral acid. The latter behavior was demonstrated by Houwink and Kreger (1953) in *S. cerevisiae* and has been also found in *P. cinnamomi* (unpublished results).

References

Aronson, J. M., Cooper, B. A., and Fuller, M. S. (1967), *Science 155*, 332.

Bartnicki-Garcia, S. (1966), *J. Gen. Microbiol. 42*, 57.

Bartnicki-Garcia, S. (1968), *Ann. Rev. Microbiol.* 22, 87.

Bartnicki-Garcia, S., and Lippman, E. (1967), *Biochim. Biophys. Acta 136*, 533.
Bell, D. J., and Manners, D. J. (1954), *J. Chem. Soc.*, 1145.
Bell, D. J., and Northcote, D. H. (1950), *J. Chem. Soc.*, 1944.
Bishop, C. T. (1964), *Advan. Carbohydrate Chem. 19*, 95.
Bishop, C. T., Blank, F., and Gardner, P. E. (1960), *Can. J. Chem. 38*, 869.
Brown, R. G., and Lindberg, B. (1967), *Acta Chem. Scand. 21*, 2379.
Clarke, A. E., and Stone, B. A. (1963), *Rev. Pure Appl. Chem. 13*, 134.
Cooper, B. A., and Aronson, J. M. (1967), *Mycologia 59*, 658.
Goldstein, I. J., Hay, G. W., Lewis, B. A., and Smith, F. (1965), *Methods Carbohydrate Chem. 5*, 361.
Hakamori, S. (1964), *J. Biochem. (Tokyo) 55*, 205.
Hay, G. W., Lewis, B. A., and Smith, F. (1965), *Methods Carbohydrate Chem. 5*, 357.
Hirst, E. L., and Percival, E. (1965), *Methods Carbohydrate Chem. 5*, 287.
Houwink, A. L., and Kreger, D. R. (1953), *Antonie van Leeuwenhoek J. Microbiol. Serol. 19*, 1.
Isbell, H. S. (1965), *Methods Carbohydrate Chem. 5*, 249.
Jayme, G., and Lang, F. (1963), *Methods Carbohydrate Chem. 3*, 75.
Manners, D. J., and Patterson, J. C. (1966), *Biochem. J. 98*, 19c.
Misaki, A., Johnson, J., Kirkwood, S., Scaletti, J. W., and Smith, F. (1968), *Carbohydrate Res. 6*, 150.
Misaki, A., and Smith, F. (1963), 144th National Meeting of the American Chemical Society, April, Los Angeles, Calif., 14c.
Novaes-Ledieu, M., Jimenez-Martinez, A., and Villaneuva, J. R. (1967), *J. Gen. Microbiol. 47*, 237.
Peat, S., Whelan, W. J., and Edwards, T. E. (1958), *J. Chem. Soc.*, 3862.
Phaff, H. J. (1963), *Ann. Rev. Microbiol. 17*, 15.
Sandford, P. A., and Conrad, H. E. (1966), *Biochemistry 5*, 1508.
Trevelyan, W. E., and Harrison, J. S. (1952), *Biochem. J. 50*, 298.
Van Cleve, J. W., and Schaefer, W. C. (1955), *J. Am. Chem. Soc. 77*, 5341.

Purification of Chicken Pancreas Nuclease by Substrate Elution from Phosphorylated Cellulose*

James Eley

ABSTRACT: A nuclease from chicken pancreas adsorbed on phosphorylated cellulose can be specifically eluted by the substrate, ribonucleic acid, and is purified 60–70-fold by this single step. Ribonucleic acid which has been thoroughly hydrolyzed with KOH or with chicken pancreas nuclease will not elute the enzyme. Deoxyribonucleic acid, a poor substrate of the enzyme, and ribonucleic acid which has been thoroughly digested with bovine pancreatic ribonuclease will also elute the enzyme, but the elution is much less sharp than that obtained with ribonucleic acid. The ribonucleic acid can be removed from the phosphorylated cellulose eluent by readsorption of the enzyme onto fresh phosphorylated cellulose and subsequent salt elution. The negatively charged ribonucleic acid is not retained by the cation exchanger.

Whenever a protein interacts specifically and reversibly with another substance the reaction offers a possible means of purifying that protein. Lerman found that certain *p*-azophenol groups covalently bound to cellulose inhibited tyrosinase activity (Lerman, 1953). Such *p*-azophenol celluloses specifically retained tyrosinase while the bulk of the protein in a crude preparation passed through in the wash volume. The tyrosinase could subsequently be eluted by a change in pH of the elution buffer or by sodium benzoate, a strong competitive inhibitor of tyrosinase. A similar procedure has been used more recently for the purification of flavin-requiring enzymes (Arsenis and McCormick, 1964, 1966). These methods, to be useful for purification purposes, require that the substance interacting with the protein (substrate or inhibitor) be irreversibly bound to a stable insoluble medium.

Another approach to the technique is that used by Pogell (1962, 1966) for the purification of fructose-1,6-diphosphatase. Here the phosphatase, adsorbed on CM-cellulose, was specifically eluted from the adsorbent by the substrate fructose 1,6-diphosphate. A considerable purification of the enzyme, much in excess of that obtained by conventional purification techniques, was obtained by this procedure.

* From the Department of Biology, Rensselaer Polytechnic Institute, Troy, New York 12181. *Received October 10, 1968.* This work was supported in part by a grant (GB 5844) from the National Science Foundation.