Chemistry of Hyphal Walls of *Phytophthora*

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SUMMARY

Hyphal walls of two phytopathogenic moulds, *Phytophthora cinnamomi* and *P. parasitica*, were isolated essentially free from cytoplasmic contamination. They have a complex chemical structure consisting of polysaccharide, protein and lipid. D-Glucose was the main monosaccharide detected in acid hydrolysates. Chromatographic evidence suggested the presence of small amounts of mannose (0.6%), glucosamine (0.3%) and traces of galactosamine and ribose. Glucans constituted nearly 90% of the wall but only about a maximum 25% of the wall could be regarded as cellulose I on the basis of solubility, resistance to hydrolysis and X-ray diffraction. Most of the wall glucan exhibited chemical and physical properties unlike typical cellulose.

The spectrum of amino acids commonly found in fungal walls was detected; hydrolysates also contained hydroxyproline and two minor unidentified ninhydrin-positive components. Protein comprised 3-5% of the wall. A small amount of lipid (1-3%), mostly of the bound type, was found, and also traces of phosphorus, and compounds with absorption maxima at 263 m μ . Hyphal walls of *Phytophthora cinnamomi* and *P. parasitica* differed only slightly in quantitative composition.

INTRODUCTION

Customarily the cell walls of filamentous fungi are described as chitinous or cellulosic. The grouping of fungi based on the presence of either one or, in some cases, both of these polysaccharides, parallels taxonomic demarcations (Wettstein, 1921; Frey, 1950; Fuller & Barshad, 1960; Aronson & Preston, 1960). With the availability of physical methods for isolating cell walls, the chemical complexity of the walls of fungi has now been clearly realized. In addition to chitin or cellulose, various carbohydrates, proteins, lipids, and other components have been found in the cell walls of moulds (Kreger, 1954; Aronson & Machlis, 1959; Bartnicki-Garcia & Nickerson, 1962; Crook & Johnston, 1962; Hamilton & Knight, 1962; Bartnicki-Garcia & Reyes, 1964; Horikoshi & Iida, 1964; Russell, Sturgeon & Ward, 1964; Potgieter & Alexander, 1965; Wessels, 1965; etc.). The majority of filamentous fungi have chitinous walls. Quantitative studies of wall chemistry in several such fungi have already been reported, but hitherto no comparable studies on cellulosic fungi had been made.

Phytophthora, a notorious genus of plant pathogens, is a prominent example of the relatively few fungi with cellulosic walls. The presence of cellulose in the *phytophthora* mycelium, long claimed (Dastur, 1913), was definitely established through X-ray analysis by Frey (1950). More recently Crook & Johnston (1962)

made a qualitative survey of sugars and amino acids in the cell walls of fungi, including *Phytophthora cactorum*. The present communication, describing the quantitative composition of hyphal walls of two phytophthora species, is an initial study designed to gain insight into the morphogenetic role of cell-wall chemistry in the life-cycle of these plant pathogens. A strain of *Phytophthora cinnamomi* parasitic on avocado, and a strain of *P. parasitica* pathogenic to citrus trees, were selected from among various other phytophthora strains and species tested; the choice was made on the basis of the relative facility with which hyphal walls could be isolated with a minimum of cytoplasmic contamination.

METHODS

Microbiological techniques. *Phytophthora cinnamomi* Rands strain SB 216-1 and *Phytophthora parasitica* Dastur strain T 89 were isolated and kindly given by Drs G. Zentmyer and P. Tsao from this Department. Stock cultures were maintained on V-8 juice agar slants. Liquid V-8 medium was prepared with the clear supernatant fluid of centrifuged V-8 juice (Campbell Soup Co., Camden, New Jersey), diluted 1/4 with glass-distilled water and its pH adjusted to 6.3 with KOH. V-8 juice agar plates were inoculated and grown for about a week at 27°. Disc plugs were removed from the periphery of growth with a sterile stainless-steel cork borer and inoculated into 350 ml. prescription bottles containing 40 ml. of the following asparagine glucose medium: KH_2PO_4 , 3 g.; CaCl_2 , 3.4 mg.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 mg., with citric acid, 1.4 mg.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.8 mg.; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.3 mg.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.4 mg.; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.3 mg.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; thiamine HCl, 1.0 mg.; L-asparagine, 2 g.; glucose, 20 g.; glass-distilled water to 1000 ml. Glucose in concentrated solution was autoclaved separately and added to the rest of the ingredients which had been previously adjusted to pH 6.0 with KOH and autoclaved for 20 min. at 121°. Inoculum bottles were incubated at 27° for about 5-7 days. Mycelial mats from 5 bottles were placed in a sterile cup of a Servall Omni-mixer and homogenized at medium speed for a few minutes. The homogenized mycelium was centrifuged, washed once with culture medium solution and inoculated into 8 2-l. flasks containing 600 ml. asparagine glucose medium. These flasks were placed on a rotary shaker and agitated 5 days at 27°. The mycelium was filtered through a sintered glass filter, washed with distilled water and lyophilized.

Isolation of cell walls. The mycelium of *Phytophthora cinnamomi* prepared as above was suspended in water (0.5-1.0 g./35 ml.) and blended in a Virtis '45' homogenizer at full speed and centrifuged at 1400 g; the supernatant fluid containing cytoplasmic debris was removed. The residues were combined and suspended in 20 ml. water/g. initial mycelium. Samples (10 ml.) were withdrawn and placed in the cup of a 10 kcyc. Raytheon Sonic Oscillator with 15.5 g. glass beads (150 μ average diameter). The resulting paste was treated at full power for 5 min. Sample temperature was maintained at 0-20° by circulating coolant at -5°. Two to four treatments sufficed to obtain satisfactory breakage. Glass beads were separated by decantation. Cell walls were then spun down at 1400 g for 5 min. and the supernatant fluid, consisting chiefly of cytoplasmic debris, was discarded. The residue containing wall fragments of different size and cytoplasmic

debris was divided into 8 portions, suspended in 30 ml. water each, and homogenized in an Omni-mixer for 30 sec. at half speed. These wall suspensions were fractionated by a series of consecutive 1 min. centrifugations at 50, 210, 500, 900 and 2000 *g*. Each residue was resuspended and centrifuged at the same speed as when first sedimented and the resulting supernatant fluid combined with the residue sedimenting at the next higher speed. The entire procedure was repeated twice. In this way, wall fractionation according to size was achieved; the heavier fractions, sedimenting at 50 and 210 *g*, contained large wall fragments with some hyphal segments partially filled with cytoplasm and glass particles. The lightest pellet (2000 *g*) contained minute wall pieces mixed with fine uncharacterized particles; the fractions sedimenting at 500 and 900 *g* contained the purest walls as revealed by staining with Lugol's iodine solution. Walls were lyophilized and stored in a freezer. The hyphal walls of *P. parasitica* were more difficult to prepare because of a tendency of wall fragments to agglutinate in big clumps after the first washings, making subsequent differential centrifugation impossible. Instead, walls were subjected to repeated treatments in the sonic oscillator (without glass beads) rapidly followed by centrifugation at 210 *g* for 1 min. In this manner, cytoplasmic contaminants were gradually discarded with the supernatant fluids. Treatments were continued until light microscopy revealed practical disappearance of cytoplasmic debris.

Chemical analyses. Three types of hydrolysates were prepared. (a) $N-H_2SO_4$ hydrolysate. Wall suspensions (2 mg./ml.) in $N-H_2SO_4$ were sealed in glass ampoules under N_2 and heated at 105° for various specified times. (b) 22.5 *N*-0.85 *N*- H_2SO_4 hydrolysate. Dry wall samples were soaked with 22.5 *N*- H_2SO_4 and incubated at 30° for 3 hr; the acid was diluted to 0.85 *N* and the suspension heated at 97° for 4 hr. (c) 6*N*-HCl hydrolysate. Walls were suspended in 6*N*-HCl, sealed in glass ampoules under N_2 , and hydrolysed at 105° for 8 hr; excess HCl was removed in a desiccator over NaOH pellets under vacuum.

Neutral sugars were separated in descending paper chromatograms (Whatman no. 1) irrigated with *n*-butanol + pyridine + water (6 + 4 + 3, by vol.) for 30 hr or longer. Sugars were revealed (aniline phthalate), eluted and measured (Wilson, 1959). Before chromatography, H_2SO_4 hydrolysates were neutralized with $BaCO_3$ and de-ionized through a double-bed column of Dowex-1 (acetate form) and Dowex-50 (hydrogen form).

Amino sugars were determined in 6*N*-HCl hydrolysates by the Elson-Morgan method as described by Tracey (1955). Hexoses were estimated by the anthrone procedure of Dreywood as described earlier (Bartnicki-Garcia & Nickerson, 1962). Reducing sugars were estimated by the method of Somogyi & Nelson (Hodge & Hofreiter, 1962). Glucose was assayed with a purified glucose oxidase reagent (Glucostat 'special', Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.).

Search for uronic acids was made in H_2SO_4 hydrolysates by adsorption on a 10 ml. column of Dowex-1 (acetate form). A stepwise elution with increasing concentrations of acetic acid up to 4*N* was used. A modified carbazole reagent was used to detect uronic acid in each of the 2 ml. fractions collected (Bitter & Muir, 1962). Protein-N was estimated in 6*N*-HCl hydrolysates by a ninhydrin method described earlier (Bartnicki-Garcia & Nickerson, 1962). Individual amino acids

were resolved on two-dimensional paper chromatograms irrigated first with *n*-butanol+pyridine+water (1+1+1, by vol.); second, with phenol saturated with a 0.2M solution of K_2HPO_4 in water to which 0.01% (w/v) 8-hydroxyquinoline was added. The solvent pair prescribed by Crook & Johnston (1962) was also tested: *n*-butanol+acetic acid+water (100+22+50, by vol.) followed by phenol+water (80+20, by vol.) in the presence of NH_3 but without NaCN. Quantitative amino acid analyses (Spackman, Stein & Moore, 1958) were made in a Spinco 120B Automatic Amino Acid Analyser. Total phosphate was measured colorimetrically (Dryer, Tammes & Routh, 1957). Lipids were extracted in two categories, free and bound, as described earlier (Bartnicki-Garcia & Nickerson, 1962). Hyphal walls were chemically fractionated with acid and alkaline solutions as detailed previously (Bartnicki-Garcia & Nickerson, 1962).

Physical techniques. Infrared spectra were determined in KBr pellets with a Perkin-Elmer Infracord spectrophotometer.

X-ray diagrams of samples spread on a glass microslide were obtained with a Norelco Phillips recording diffractometer using CuK_{α} radiation.

RESULTS

Chemical analyses

Carbohydrates. The anthrone procedure, with glucose as standard, served to assess the total amount of hexosan present in intact walls. To improve sample uniformity, dilute wall suspensions were homogenized by sonic oscillation immediately before pipetting. Nearly 90% of the hyphal wall dry weight of both species of *Phytophthora* was found to be anthrone-positive carbohydrate (Table 1).

Table 1. *Hyphal wall composition of phytophthora*

Component	Method	% wall dry weight	
		<i>P. cinnamomi</i>	<i>P. parasitica</i>
Glucan*	Anthrone	88±2	86±2
Mannose	Paper chromatography	0.6	0.7
Glucosamine	Elson-Morgan	0.8	0.8
Protein	Micro-Kjeldahl N × 6.25	3.6	4.3
Protein	Ninhydrin	3.4	4.1
Free lipids	Direct extraction	0.3	0.2
Bound lipids	Hydrolysis and extraction	2.1	0.9
Phosphorus (as H_2PO_3)	Semidine	0.3	0.4

* Range of values obtained in 8 determinations.

Measurement of monosaccharide constituents of hyphal-wall polysaccharides was made in two types of H_2SO_4 hydrolysates; in neither case was sugar recovery complete. Direct hydrolysis in $N-H_2SO_4$ was insufficient to dissolve walls completely, even after prolonged digestion for 48 hr; by which time about a quarter of the wall carbohydrate remained insoluble, a quarter had been destroyed, and a half was in solution (Table 2). The soluble carbohydrate was composed almost exclusively of glucose as shown by determination of total and reducing sugar and by paper chromatography.

The 22.5–0.85N-acid hydrolysis was much more efficient (Table 3); respectively, 93 and 87% of the initial polysaccharide of *Phytophthora cinnamomi* and *P. parasitica* was recovered in solution and only 0.1% remained insoluble. Paper chromatograms of this hydrolysate revealed glucose as the chief component. There was also a much smaller amount of two disaccharides subsequently identified as cellobiose and gentiobiose and traces of another one identified as laminaribiose (Bartnicki-Garcia & Lippman, to be published), plus a faint streak of unresolved oligosaccharides.

Table 2. *Hydrolysis of hyphal walls of phytophthora in N-H₂SO₄*

Carbohydrate in hydrolysate	% of initial total carbohydrate			
	<i>P. cinnamomi</i>		<i>P. parasitica</i>	
	18 hr	48 hr	18 hr	48 hr
Total*	97.8	76.1	91.8	74.4
Soluble*	59.0	51.1	61.7	49.0
Insoluble†	38.8	25.0	30.1	25.4
Destroyed‡	2.2	23.9	8.2	25.6

* Determined with anthrone using glucose as standard. Values are % anhydroglucose of initial glucan content in the wall (Table 1).

† Calculated: total – soluble; ‡ Calculated: 100 – total.

Table 3. *Hydrolysis of hyphal walls of phytophthora with 22.5–0.85N-H₂SO₄*

Carbohydrate*	<i>P. cinnamomi</i>		<i>P. parasitica</i>	
	% wall	% initial carbohydrate†	% wall	% initial carbohydrate†
Total soluble (anthrone)	80.7	92.6	75.6	87.5
Glucose (glucostat)	70.9	81.4	64.5	75.6

* As anhydroglucose. † Initial glucan content values are shown in Table 1.

Respectively, 81.4 and 75.6% of the total wall polysaccharide of *Phytophthora cinnamomi* and *P. parasitica* were conclusively identified as D-glucose by the glucose oxidase assay. There are minimum values which do not include glucose di- and oligo-saccharides present in the hydrolysates and do not take into account some glucose inevitably destroyed during hydrolysis. A trace of mannose, 0.6–0.7% of the wall, was also found in both *P. cinnamomi* and *P. parasitica*. Inasmuch as the hydrolysates of wall polysaccharides of *P. cinnamomi* and *P. parasitica* revealed almost exclusively glucose and disaccharides thereof, the determination in intact walls of total carbohydrate by the anthrone method may be regarded as the best approximation to the actual glucan content of the wall (Table 1). In heavily loaded chromatograms (3.1 mg. walls) a vestigial spot appeared which had the colour of a pentose (aniline phthalate spray), the R_f value of ribose, and was visually estimated to be less than 0.1% wall dry wt.

Uronic acids in H₂SO₄ hydrolysates were not detected.

Proteins. The protein content of mycelial walls of phytophthora was estimated by determining amino acid-N in 6N-hydrolysates and by micro-Kjeldahl determination of total nitrogen in intact walls (Table 1). Similar values were obtained

in both determinations; the sum of individual amino acids determined in the automatic amino acid analyser (Table 4) also agreed with the above values. Amino acid quantities (Table 4) are given with the reservation that some destruction took place during hydrolysis as shown by humin formation.

Both two-dimensional paper chromatography and automatic column chromatography of hyphal wall hydrolysates revealed the common spectrum of protein amino acids, threonine being the most abundant. Hydroxyproline, an amino acid not usually found in fungal wall proteins, was shown in both paper and column chromatograms. Two weak unidentified ninhydrin-violet spots appeared in amino acid paper chromatograms of *Phytophthora cinnamomi* and *P. parasitica*. R_{alanine} values were respectively 1.09 and 0.74 in *n*-butanol+acetic acid+water; 1.64 and 1.23 in phenol+water+ NH_3 . They probably correspond to the unknown amino acid spots of *P. cactorum* reported by Crook & Johnston (1962). (R_{alanine} values in this publication were misprinted; they should be 1.03 and 0.76 in *n*-butanol+acetic acid+water and 1.40 and 1.30 in phenol+water+ NH_3 ; I. R. Johnston, private communication).

Table 4. Overall amino acid composition of protein from hyphal walls of *Phytophthora cinnamomi*

	% of total amino acids*		% of total amino acids*
Threonine	14.8	Glycine	4.5
Glutamic acid	11.1	Arginine	4.2
Serine	9.8	Ammonia	3.7
Aspartic acid	9.0	Tyrosine	2.6
Alanine	9.0	Phenylalanine	2.1
Proline	5.5	Isoleucine	1.8
Hydroxyproline	5.0	Histidine	1.1
Valine	5.0	Cysteine	1.1
Lysine	4.7	Methionine	1.1
Leucine	4.5		

* The sum of amino acids recovered was 3.79% of the initial wall dry weight.

Amino sugars. A glucosamine peak, representing 0.20% of the wall of *Phytophthora cinnamomi*, and a much smaller peak, presumably galactosamine, were detected during the automatic analysis of amino acids. Elson-Morgan determinations on 6 N-hydrolysates confirmed the existence of a small amount of amino sugar in both phytophthora species (Table 1).

Lipids. A small amount of lipid, mainly of the bound type, was detected in the walls of both phytophthora species (Table 1).

Various. Determination of total phosphorus indicated phosphate content of about 0.3% (Table 1). Hot N-KOH extracts of walls showed sharp absorption peaks at 263 μ , suggesting the possible presence of 0.8% nucleic acid components (calculated at an average ϵ of 8800).

Chemical fractionation. Hyphal walls were fractionated by two different methods. The distribution of carbohydrates (Table 5) and protein (Table 6) was investigated by anthrone and ninhydrin assays. Fractionation method I indicated that even at high temperatures the hyphal-wall glucans were alkali-insoluble; likewise, hot acetic acid did not dissolve any appreciable quantity of glucan. In the same

fractionation, over half of the wall protein was dissolved by cold alkali and most of the remainder dissolved in hot alkali. A small proteinaceous residue was left associated with the insoluble glucan. Fractionation method II revealed that a large portion of the wall glucan (61–66%) was soluble in hot dilute acid. Consecutive extraction of hyphal walls with hot acid and hot alkali left only about 20% of insoluble glucan. Recovery of carbohydrate in this method was incomplete, probably due to destruction of glucan by hot alkali after walls had first been treated with hot acid, a situation also observed for the glucan of *Mucor rouxii* spore walls (Bartnicki-Garcia & Reyes, 1964).

Table 5. *Distribution of carbohydrates in hyphal wall fractions of phytophthora*

Method	Fraction	% of initial carbohydrate*	
		<i>P. cinnamomi</i>	<i>P. parasitica</i>
I	Cold N-KOH	2.7	2.1
	Hot N-KOH	3.2	3.3
	Hot N-acetic acid	0.2	0.3
	Residue	93.2	95.6
	Sum	99.3	101.3
II	Cold N-HCl	0.2	0.3
	Hot N-HCl	61.3	65.9
	Hot N-KOH	3.2	3.0
	Residue	21.2	19.7
	Sum	85.9	88.9

* Determined with anthrone reagent and calculated as % anhydroglucose of the initial glucan values shown in Table 1.

Table 6. *Distribution of protein in hyphal walls of phytophthora fractionated according to Method I (Table 5)*

Fraction	% of total protein*	
	<i>P. cinnamomi</i>	<i>P. parasitica</i>
Cold N-KOH	59.5	63.1
Hot N-KOH	27.2	21.4
Hot N-acetic acid	5.1	5.9
Residue	8.2	9.6

* Determined by the ninhydrin method and calculated on the basis of the total protein values (ninhydrin) from Table 1.

Extraction with 17.5% NaOH, under N₂, is commonly used in cellulose technology to determine and separate α -cellulose from low molecular weight celluloses and other carbohydrates (Corbett, 1963). Accordingly, only 3.4% of *Phytophthora cinnamomi* walls dissolved as anthrone-positive carbohydrate. It may not be concluded, however, that the portion of wall glucan insoluble in 17.5% NaOH is α -cellulose since it is known that other glucans, notably yeast cell-wall glucan (Chung & Nickerson, 1954), are highly insoluble in concentrated alkali.

Infrared spectra. Intact walls of *Phytophthora cinnamomi* and *P. parasitica* showed nearly identical spectra, corroborating their chemical similarity. They showed absorption peaks at about 890 cm.⁻¹, indicative of β -linked polysaccharide.

There was no evidence of absorption in the α -glycoside region at 844 cm.^{-1} (Barker, Bourne & Whiffen, 1956). The spectra of isolated walls was compared with that of crystalline cellulose I (Whatman α -cellulose powder for chromatography); there was a general resemblance, but the wall spectra differed noticeably in the blurring of peak sharpness in the $1000\text{--}1500\text{ cm.}^{-1}$ region (Fig. 3). Extraction of walls with cold N-KOH for 1 hr, followed by 0.5 N-KOH at 100° for 30 min., improved the resemblance slightly; minor peaks at $1510\text{--}1540\text{ cm.}^{-1}$ (amines and/or aromatics) disappeared, and other small peaks were enhanced. The treatment removed most of the protein and lipid, leaving a wall composed of glucan almost entirely. Infrared spectra of *Phytophthora* walls showed a much greater similarity to the spectra of amorphous cellulose (cf. O'Connor, DuPre & McCall, 1957) than to that of cellulose I.

Fig. 1. X-ray diagrams. A, Cellulose I; B, insoluble residue after extraction of *Phytophthora cinnamomi* hyphal walls with hot N-HCl ; C, intact hyphal walls of *P. cinnamomi*; D, isolated hyphal walls of *P. parasitica*. Tracings vertically displaced for clarity.

Fig. 2. X-ray diagrams. A, Cellulose II regenerated from Whatman cellulose I; B, material regenerated from *Phytophthora cinnamomi* hyphal walls. Tracings vertically displaced for clarity.

X-ray diagrams. Intact walls showed very weak reflexions suggestive of cellulose I (Fig. 1). Reflexion sharpness did not improve after extraction with KOH as specified above for infrared spectra. Since walls thus treated consist almost entirely of glucan polymers, cellulose crystallinity in the phytophthora walls is either poor or is masked by amorphous glucans. Definite evidence for the presence of cellulose was obtained by twice extracting walls of *Phytophthora cinnamomi*

with Schweizer's cellulose solvent (Jayme & Lang, 1963) for 24 hr under N_2 . Upon acidification of the solvent, a precipitate was obtained which showed X-ray spacings similar to that of cellulose II prepared from Whatman cellulose powder by identical handling (Fig. 2). There was, however, a divergency in the relative intensity of the reflexions. This discrepancy may be an artifact due to lack of randomness in the spatial orientation of cellulose crystallites in the specimen slides.

Anthrone determinations showed that only 11% of the wall regenerated as cellulose II and that 63% did not dissolve at all in Schweizer's reagent; the unaccounted glucan (14% of the wall) probably remained in solution.

Fig. 3

Fig. 3. Infrared spectra: A, isolated hyphal walls of *Phytophthora cinnamomi*; B, as A but extracted with KOH; C, isolated hyphal walls of *P. parasitica*; D, as C but extracted with KOH; E, cellulose I. Tracings vertically displaced for clarity.

Fig. 4

Fig. 4. Comparative rate of hydrolysis of isolated hyphal walls of *Phytophthora cinnamomi* (curve A) and Whatman cellulose powder (curve B) in N -HCl at 97° .

The single, broad, X-ray peak of negative walls ($4.04\text{ \AA}$) approximated to the main reflexion of authentic cellulose I ($3.90\text{ \AA}$) and disappeared upon extraction with Schweizer's solvent, leaving an isotropic glucan residue. When, on the other hand, the amorphous glucan was first removed from walls, by N -acid digestion (see below), a residue was left which exhibited a slightly more pronounced X-ray spectrum than did native walls, with its main reflexion ($3.93\text{ \AA}$) closer to that of cellulose I. It may, therefore, be concluded that cellulose I of relative low crystallinity exists in the phytophthora walls.

Acid-extraction of wall glucans. Since hot dilute mineral acid dissolved a major portion of the glucan from hyphal walls of phytophthora (Table 5), the kinetics of hyphal wall solution of *Phytophthora cinnamomi* by N -HCl at 97° was studied. As depicted in Fig. 4, 61.2% of the wall (i.e. 70% of the total polysaccharide) dis-

solved within 30 min. Thereafter the extraction rate decreased sharply to a slow constant rate. The experiment was ended at 7 hr, by which time 69.5 % of the wall had dissolved (i.e. 80 % of the total polysaccharide). On the other hand, only 6.5 % Whatman cellulose dissolved under the same conditions. To calculate the amount of glucan readily extracted by HCl, the upper portion of the curve corresponding to the hydrolysis of a resistant polysaccharide was extrapolated to zero time. Thus, 63 % of the wall consists of a glucan readily soluble in hot dilute mineral acid. Hence, about 25 % of the wall corresponded to a relatively acid-insoluble glucan whose X-ray spectrum (Fig. 1) indicated that it was partly, if not entirely, cellulose I.

The acid-soluble glucan was also rapidly hydrolysed; thus 84 % of the total carbohydrate extracted by N-HCl in 30 min., was dialysable. Under less drastic conditions, using several brief extractions with hot 0.1 N-HCl, depolymerization was considerably decreased and a soluble non-dialysable glucan(s) was isolated. Preliminary measurement of optical rotation in water $[\alpha]_D^{23} - 33^\circ$ suggests it was predominantly a β -glucan.

DISCUSSION

As in the case for the chitinous cell walls of moulds, the cellulosic hyphal walls of *Phytophthora cinnamomi* and *P. parasitica* also consist of polysaccharide, protein, and lipid; they are, however, somewhat simpler than the hyphal walls of a chitinous Phycomycete such as *Mucor rouxii*, which contains a wider variety of building blocks (Bartnicki-Garcia & Nickerson, 1962); in phytophthora, glucose polymers account for nearly 90 % of the hyphal walls. As expected, cellulose was identified in the hyphal walls of *Phytophthora* but its exact content is not known. Qualitative evidence for cellulose includes the following observations. Release of large amounts (about 20 %) of cellobiose during enzymic digestion of *P. cinnamomi* walls with a crude cellulase from *Streptomyces* (Bartnicki-Garcia & Lippman, to be published); very limited solubility of walls in 17.5 % NaOH; partial solubility of hyphal walls in Schweizer's reagent and subsequent regeneration of a polymer with an X-ray pattern similar to cellulose II; X-ray diagrams of intact walls suggestive of cellulose I.

A quantitative measurement of cellulose would be arbitrary and largely dependent on which physical or chemical properties were used to define cellulose (Ott & Tennent, 1954). Furthermore, the notion of having true cellulose as a chemical entity separate from the other wall glucans may be artificial, for it is possible that the hyphal walls of *phytophthora* consist of one single glucan species with amorphous regions and a cellulose-like core. More work obviously is needed to clarify this point.

Separation of two glucan fractions from hyphal walls of *Phytophthora cinnamomi* was accomplished by treatment with hot dilute mineral acid. Thus were obtained a readily soluble, rapidly hydrolysable amorphous glucan comprising 63 % of the wall, and a much more acid-resistant polymer representing 25 % of the wall. It is not yet known whether the acid treatment cleaved a single native glucan into these two types. In any event, the acid-resistant glucan showed X-ray reflexions approximating that of cellulose I and may thus be considered to represent that portion of the *phytophthora* wall most closely resembling native crystalline cellulose of higher plants.

Release of gentiobiose and laminaribiose during chemical or enzymic hydrolysis

(Bartnicki-Garcia & Lippman, to be published) indicated the presence of β (1 $\rightarrow$ 6) and β (1 $\rightarrow$ 3) linkages and opens the possibility that two types of glucan exist in the wall—a homogenous β (1 $\rightarrow$ 4) cellulose-like polymer, and an amorphous glucan containing an as yet undetermined number of β (1 $\rightarrow$ 3) and β (1 $\rightarrow$ 6) linkages.

Although *Phytophthora cinnamomi* was examined in greater detail, the data on *P. parasitica* is sufficient to conclude that both species have a similar hyphal-wall composition. Yet, differences in certain properties such as the propensity of *P. parasitica* walls to agglutinate and the more intense staining of *P. cinnamomi* walls with Lugol's solution may correspond to significant chemical differences. Hyphal wall analyses of *P. cinnamomi* and *P. parasitica* agree with the qualitative findings of Crook & Johnston (1962) on the sugars and amino acids present in hydrolysates of *P. cactorum*, except for ribose which was only vaguely detectable in the former species.

Glucosamine, major component of most mould walls, was found in the cellulosic walls of *phytophthora* in such a small quantity as to question its relevance as a structural wall element. This finding agrees with Frey's (1950) failure to obtain X-ray evidence for chitin in various *phytophthora* cultures. Thomas (1943) claimed the presence of chitin in *Phytophthora cinnamomi* on the basis of a positive chitosan staining reaction; this conclusion was challenged by Frey (1950) and seems untenable in view of the present work. Interestingly, much earlier Dastur (1913), who also used cytochemical tests, found staining-reaction evidence for cellulose, but not for chitin, in *P. parasitica*.

Hydroxyproline is not known to occur in the walls of chitinous fungi. Its presence in fungi with cellulosic walls such as *phytophthora* and *saprolegnia* (Crook & Johnston, 1962) may have special significance since a similar concurrence is conspicuously apparent in the cell wall of higher plants, where hydroxyproline is a characteristic component (Lamport & Northcote, 1960). Although assumed to be minimal, the exact extent to which the preparative procedure altered the composition of the hyphal walls of *phytophthora* by addition or deletion of components is not known. Undoubtedly, the physical technique used in this study for cell-wall preparation is superior to the old method of exhaustive chemical extraction of whole organisms which only the most inert substances endured. Nevertheless, upon hyphal breakage, even though the mycelium was coenocytic, cytoplasmic contents did not flow out entirely; solid plugs remained whose dislodgement necessitated the strong action of a sonic oscillator. By this procedure, all visible cytoplasmic debris was essentially, but not entirely, eliminated. Minute residues, whose contribution to the composition of the walls has been assumed to be negligible, were occasionally seen. On the other hand, the extensive action of sonic oscillation may have selectively removed or modified some wall components to an undetermined degree.

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