The Structure of Cell Walls of Phycomycetes

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SUMMARY

Dilute acid treatment of isolated hyphal walls from *Phytophthora heveae* and *Pythium butleri* separates two fractions: an acid-soluble carbohydrate protein containing glucosamine (about three-fifths of the total glucan weight) and an acid-insoluble pure glucan. Acid and enzymic hydrolyses of the soluble fraction indicate two major glycosidic linkages, β (1 $\rightarrow$ 3) and β (1 $\rightarrow$ 6), and a little of the β (1 $\rightarrow$ 4). Digestion of the residual material by cellulase confirmed the presence of a cellulose-like glucan in these walls. A small amount of laminaribiose and gentibiose confirms the presence of β (1 $\rightarrow$ 3) and β (1 $\rightarrow$ 6) linkages. Comparable analyses indicate that the walls of both fungi possess similar glucan structures, the differences being quantitative. Proteins in the Phycomycetes walls seem to act as binding points between carbohydrate chains.

INTRODUCTION

Novaes-Ledieu, Jiménez-Martínez & Villanueva (1967) reported that cell walls of two oomycetes, *Phytophthora heveae* and *Pythium butleri*, contained 81-90 % of carbohydrates, glucose being the most abundant sugar. Acid yielded a glucan quickly solubilized in N-HCl at 100° and another resistant to hydrolysis. This last glucan resembled a crystalline cellulose, and accounted for 35 and 25 % of the hyphal walls of *P. heveae* and *P. butleri*, respectively. The current investigation reports more information about the structure of cell wall glucans and the possible role of proteins in the wall.

METHODS

Organisms, media and preparation of cell wall material. Hyphal walls of *Phytophthora heveae* and *Pythium butleri* were prepared as described by Novaes-Ledieu *et al.* (1967). Mycelium was disrupted in a Ribi fractionator (Sorvall). Cell walls were freed of cytoplasmic material by washing and differential centrifugation. Lipids were extracted from the cell walls by the method of Ballesta (1966) and extracted materials were lyophilized and stored *in vacuo* at room temperature.

Partial acid digestion of cell walls. About 400 mg. *Phytophthora heveae* walls were hydrolysed in 10 ml. of N-HCl in a boiling water-bath 14 times for 5 min. and finally 5 times for 10 min. *Pythium butleri* walls were treated with the acid 8 times for 10 min. and 5 times for 15 min. After each operation the digested walls were centrifuged and the residues washed twice with N-HCl. The combined washings and HCl extracts were neutralized with NaOH and stored at 4°.

Cellulolytic hydrolysis of the acid-insoluble fraction. About 50 mg. residue in 0.03 M citrate buffer, pH 5.8, were homogenized in a MSE ultrasonic disintegrator for

30 min. and incubated on a reciprocating shaker for 24 hr at 40° with 100 units of crude *Streptomyces* QM 814 cellulase (Reese & Mandels, 1963). The digestion repeated for another 24 hr with fresh cellulase. This enzyme preparation contained a little β (1 $\rightarrow$ 3) glucanase but only negligible glucosidase. Phosphoric acid-swollen, Whatman cellulose, prepared following the method of Walseth as described by Bartnicki-Garcia & Lippman (1967), was used as a control in the enzymic experiments. During incubation the suspensions were protected from bacterial contamination with a layer of toluene. After centrifugation the supernatant liquids were boiled for 5 min. and analysed.

Proteolytic hydrolysis of acid-soluble fraction. Non-dialysable components (3 mg. dry weight) were dissolved in 1 ml. formic-acetic acid buffer, pH 2, and an amount of crystalline pepsin added to obtain a ratio pepsin:protein of 1:40. The mixture was incubated at 37° for 24 hr under toluene and then dialysed against distilled water at 5° for a day using cellophane tubing. Dialysable products were analysed by chromatography and electrophoresis.

Analytical procedures. Total carbohydrate was determined by the anthrone procedure (Chung & Nickerson, 1954) and reducing sugar by the method of Somogyi (1952), using glucose as standard. Quantitative determination of protein was made by the procedure of Lowry, Rosebrough, Farr, & Randall (1951), with crystalline bovine serum albumin as standard, and by the ninhydrine method (Moore & Stein, 1954). Hexosamine was estimated by the method of Rondle & Morgan (1955) after hydrolysis of the cell wall with 4 N-HCl for 17 hr at 105°.

Paper chromatography. Digestion products were desalted on a mixed resin bed (Dowex-1 $\times$ 4; Dowex-50 $\times$ 2) and chromatographed on Whatman no. 1 paper for biose and triose sugars in *n*-butanol + acetone + water (4 + 5 + 1 by vol.) for 48 hr; ethyl acetate + pyridine + acetic acid + water (5 + 5 + 1 + 3 by vol.) for 36 hr; *n*-butanol + pyridine + water (6 + 4 + 3 by vol.) for 40 hr and detected with aniline hydrogen phthalate in *n*-butanol. Semi-quantitative determination of monosaccharides was made on chromatograms as described by Gottschalk & Ada (1956). For amino acids, glycopeptides and glycoproteins, butanol + formic acid + water (75 + 15 + 10 by vol.) was used for 24 hr. Amino compounds were detected with ninhydrin (0.5 % w/v, in acetone).

Gentibiose was prepared by paper chromatography of partial acid hydrolysates of postulan, a β (1 $\rightarrow$ 6) glucan, extracted from the lichen *Umbilicaria pustulata* (Lindberg & McPherson, 1954). Disaccharide was purified with a modification of the procedure described by Reese, Parrish & Mandels (1962). Laminaribiose and laminaritriose were isolated in a similar way from partial acid hydrolysates of laminarin. The nature of a trisaccharide on a chromatogram was confirmed by elution, followed by second acid hydrolysis and rechromatography of further products. Glucose was assayed with glucose oxidase reagent (Glucostat 'special', Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.).

Zone electrophoresis was carried out on paper (Whatman no. 1) or, for components with a high molecular weight subjected to high voltages, on glass-fibre paper (Whatman GF 81) in pyridine-acetate (pH 6.5), acetic acid-formic acid (pH 2.25) or 0.5 M carbonate (pH 11.5; 750 V; 3 hr) for amino acids. A borate buffer at pH 8.7 was used to separate gentiobiose and laminaritriose (2500 V; 2 hr). The buffers at pH 2.25 or 6.5 and a 0.5 M sodium tetraborate buffer at pH 9.2 separated glycoproteins (1500 to

2500 V; 2 hr). Proteins were revealed with amido black (Smithies, 1955) and polysaccharides on glass-fibre paper with α -naphthol H_2SO_4 .

Electron microscopy. Intact cell walls and acid-insoluble fractions of *Phytophthora heveae* and *Pythium butleri* were shadowed with an alloy of gold and palladium with a Zeiss EM 9 electron microscope.

Sedimentation diagrams. Sedimentation values were obtained at 59,780 rev./min. in a Beckman Spinco Model E analytical ultracentrifuge in a 12 mm. aluminium cell at 20°.

Enzymic attack of walls of living oomycetes. Mycelia were suspended in 0.5 ml. of 0.05 M-citrate buffer, pH 6, containing 0.25 to 0.6 M-MgSO₄. Enzyme preparations (usually 125 units) were added and the mixtures incubated at 27° without shaking. The process was followed by microscopic observations. The β -glucanases preparations tested were: crude cellulase of Basidiomycete QM 806 grown with cellulose possessing a large amount of β (1 $\rightarrow$ 3) exoglucanase; cellulase of *Streptomyces* QM 814 containing a small amount of β (1 $\rightarrow$ 3) glucanase; a β (1 $\rightarrow$ 6) glucanase of *Penicillium brefeldianum* QM 1872 (Reese & Mandels, 1959) also high in β (1 $\rightarrow$ 3) glucanase and β glucosidase but with very little if any β (1 $\rightarrow$ 4) glucanase.

RESULTS

Partial acid hydrolysis of hyphal walls. The cell walls of *Phytophthora heveae* and *Pythium butleri* are very similar in soluble carbohydrate and in percentage of cell wall solubilized by the acid treatment (Table 1). Most, if not all, of the protein was in the soluble fractions and represented 6 to 11 % of the wall weight. The cell-wall glucosamine was also recovered in the acid-soluble portion.

Table 1. *Analysis of hyphal walls*

Pythium butleri and *Phytophthora heveae* walls were digested with N-HCl as described in the text. Solubilized and residual fractions were analysed.

Organism	Initial weight (mg.)	Soluble carbohydrates*		Soluble protein†		Insoluble residue‡	
		mg.	%	mg.	%	mg.	%
<i>P. heveae</i>	388	200	52	25	6	160	41
<i>P. butleri</i>	361	160	44	40	11	151	42

* Anthrone positive carbohydrate calculated using glucose as standard.

† Protein calculated as serum albumin according to the Lowry procedure.

‡ Dry weight.

Practically all the protein components of the soluble fractions remained in the cellophane bag during dialysis of 100 ml. against 3 l. distilled water for 36 hr at 4°. About 22 and 35 % of the soluble carbohydrate of *P. butleri* and *P. heveae*, respectively, passed through the membrane. These results indicate that the N-HCl treatment hydrolysed a significant portion of the wall-carbohydrates, whereas it had much less effect on the protein. The fact that most protein was recovered in the soluble fraction suggests an association of glucan and protein in the wall of these two oomycetes.

Dialysable soluble fraction. Qualitative analysis of this fraction showed only carbo-

hydrate. Glucose was the dominant single sugar though trace amounts of galactose and mannose were identified (more from *Phytophthora heveae*). Laminaribiose was the principal disaccharide: about 60 % of total disaccharides for *Pythium butleri* and 70 % for *P. heveae*. The *P. butleri* fraction contained considerably more gentiobiose (30 %) than the *P. heveae* fraction. Cellobiose was 6–8 % in *P. butleri* and 12–15 % in the *P. heveae* fraction. A small amount of laminaritriose was found in both.

Non-dialysable soluble fraction. This fraction contained 85 % carbohydrate for *Phytophthora heveae* and 66 % for *Pythium butleri*. Protein was 14 % and 33 % respectively. Glucosamine was found exclusively in this fraction. Acid hydrolysates of the same fraction showed not only a high glucose content but also a significant and unexpected amount of mannose (8 to 10 % of total monosaccharides). A small quantity of galactose (1–2 % of monosaccharides) was also found for *P. heveae*. Acid hydrolysates of the protein revealed all of the common acids of walls in the usual proportions. In order to calculate approximately the proportion of β (1 $\rightarrow$ 3)

Table 2. *Relative amount of di- and tri-saccharides in the various fractions of Oomycetes walls*

The oligosaccharides were estimated on paper chromatograms (Gottschalk & Ada, 1956) after either acid or enzymic hydrolysis (see text).

			<i>P. butleri</i>	<i>P. heveae</i>
First acid soluble fraction	Non-dialysable	Laminaribiose	+++	+++
		Gentiobiose	++++	++++
		Laminaritriose	++	++
		Cellobiose	++	++
	Dialysable	Laminaribiose	++++	++++
		Gentibiose	+++	++
		Laminaritriose	++	++
		Cellobiose	++	+++
	Second acid soluble fraction	Laminaribiose	+	+
		Gentiobiose	++++	++++
Laminaritriose		—	—	
Cellobiose		+	+	
Supernatant from enzymic hydrolysis of residue	Laminaribiose	++	+	
	Gentiobiose	—	—	
	Laminaritriose	+	+	
	Cellobiose	+++++	+++++	
Residual material	Laminaribiose	++	+	
	Gentiobiose	+++	++++	
	Laminaritriose	++	++	
	Cellobiose	+++	+++	

Carbohydrates in percentage: over 80 % of total di-trisaccharides (+++++); 60 to 80 % (++++); 35 to 60 % (+++); 10–35 % (++); 2–10 % (+); less than 2 % (+).

and β (1 $\rightarrow$ 6) glucan linkages, we chromatographed a large number of hydrolysates obtained under varying conditions of partial hydrolysis. Pure β (1 $\rightarrow$ 3) and β (1 $\rightarrow$ 6) glucans were submitted to similar hydrolyses, and the degradation products also chromatographed. An approximate ratio of gentiobiose to laminaribiose of 2 was found for walls of both organisms. In all the chromatograms, a small amount of laminaritriose was encountered while traces of cellobiose were detected, chiefly in *P. heveae* fraction. According to chromatographic and electrophoretic assays, acid hydrolysis of phosphoric acid-swollen cellulose did not release disaccharides in

appreciable quantity, so the exact contents of β (1 $\rightarrow$ 4) linkages in glucan complexes were not calculated exactly. Cellulolytic hydrolysis of fraction components confirmed the presence of β (1 $\rightarrow$ 4) linkages and indicated their approximate value. Semi-quantitative estimation of the mono-, di- and trisaccharides of wall fractions is shown in Table 2. Minor amounts of other oligosaccharides, not resolved, appeared in paper chromatograms. It is possible that sophorose exists in *P. butleri* walls.

The degree of polymerization of components of two non-dialysable soluble fractions was indicated by values of reducing power (Somogyi, 1952) in relation to the total anthrone carbohydrate content. The percentages obtained (3.2 % for *P. heveae* and 8.5 % for *P. butleri*) suggested a low degree of polymerization of carbohydrate chains. On the other hand, the sedimentation constants of 2.02 S for *P. heveae* and 1.68 S for *P. butleri* suggested median molecular weights of about 15,000, which agrees with the behaviour on dialysis. The combined data indicate the presence of non-carbohydrate portion in the non-dialysable complexes.

Fig. 1. Ratio of soluble carbohydrate to soluble protein extracted with diluted acid from hyphal walls, at various times of hydrolysis: ●, *P. butleri*; ▲, *P. heveae*.

The ratio of carbohydrate to protein in solubilized components at various times of acid digestion was constant (Fig. 1), which permits one to think that protein and carbohydrate were always extracted in constant proportion. Hence a macromolecular glucan-protein complex may exist in the wall.

Glycoproteins were demonstrated in both *Phytophthora heveae* and *Pythium butleri* fractions by chromatography in butanol-formic acid and, after elution of the spots without spraying, by electrophoresis at various pH values; specific reagents for both proteins and polysaccharides were sprayed after cutting the paper into two equivalent strips.

The *Phytophthora heveae* fraction contained a predominant amido black and α -naphthol positive spot of R_F :0.10. The eluted material was separated at 2500 V, pH 2.25, into two compounds which ran 10 and 15 cm. in 2 hr on Whatman no. 1 paper when

placed up to 7 cm. from the anode. For *Pythium butleri* two main amido black and α -naphthol positive spots were detected with respective R_F values of 0.08 and 0.12. The material with the lower R_F gave two glycoprotein spots running 8 and 28 cm. on similar electrograms, and the material from the second compound gave a single spot running 35 cm. In both the cases additional spots were detected, which corresponded to glycoproteins in lower concentrations. Complete hydrolysates of material removed from each spot always contained glucose and amino acids.

Control acid hydrolyses of laminarin, laminaribiose, cellulose and cellobiose indicate that gentiobiose does not appear to any significant extent on our chromatograms as a reversion product.

Proteolysis of non-dialysable acid fractions. Both *Phytophthora heveae* and *Pythium butleri* yielded similar amino acids and peptides from suspected glucan-protein complexes or protein components, according to the method of Moore & Stein (1954). Dialysable sugars assayed with anthrone were also similar. Peptic digestion released 20 to 25 % of the total protein. In contrast, only 1 to 2 % of carbohydrate passes through the dialysis bag after this treatment.

β (1 $\rightarrow$ 3) glucanases digestion of isolated walls and soluble non-dialysable fractions. Two β -glucanase preparations were used to obtain more information about glucan structure of walls: a practically pure β (1 $\rightarrow$ 3) endoglucanase from *Rhizopus arrhizus* and a β (1 $\rightarrow$ 3) exoglucanase from Basidiomycete QM 806, here grown with starch. Both the preparations were relatively free of β (1 $\rightarrow$ 4) glucanase. Cell walls and soluble fractions of two oomycetes were treated with exo and endo β (1 $\rightarrow$ 3) glucanases in 0.05 M citrate buffer, pH 4.8 at 40°, for 4 hr and the products of enzymic hydrolysis studied. Enzymic treatment of each cell wall and its respective soluble non-dialysable fraction with β (1 $\rightarrow$ 3) glucanase from *R. arrhizus* did not show substantial digestion of these components, although the glucan linked in β (1 $\rightarrow$ 3) represents about one-third of the walls. On the contrary, the β (1 $\rightarrow$ 3) glucanase from Basidiomycete QM 806 was able to digest isolated walls and soluble acid fractions, and liberate a large amount of glucose.

Purification of the undigested acid-residue. Dry walls of *Phytophthora heveae* contained 36 % and those of *Pythium butleri* 23 % cellulosic glucan (Novaes-Ledieu *et al.* 1967). The ratios of insoluble residue to dry wall, under the conditions specified above, were somewhat higher, particularly for *P. butleri* (Table 1). The differences might derive from a contaminant glucan that was not digested by acid in the present experiments, so the digested acid residue was subjected to a second acid hydrolysis under more drastic conditions: in 2 N-HCl at 105° for 2½ hr. Only carbohydrates were present in the totally dialysable supernatant. Anthrone analyses indicated that the glucan digested was 10 % dry-wall weight for *P. heveae* and 23 % for *P. butleri*. Glucose was the only monosaccharide, and gentiobiose the most abundant disaccharide, from *P. heveae*; from *P. butleri* a small amount of galactose was also detected. Both hydrolysates contained laminaribiose and cellobiose.

Enzymic attack of cellulosic residue. The cellulase of *Streptomyces* dissolved about 70 % of the glucan in the case of *Phytophthora heveae* and 62 % in *Pythium butleri* to give mainly cellobiose, but also traces of glucose, laminaritriose and laminaribiose, this last sugar being more plentiful in *P. butleri*. Further enzyme treatment of the residue did not liberate any significant amount of carbohydrate. The material remaining after cellulase digestion was hydrolysed for 4 hr, in 2 N-HCl at 105° and chromato-

graphed. Both had a high content of glucose and gentiobiose as well as lesser amounts of cellobiose. *P. heveae* yielded traces of laminaribiose and laminaritriose; in *P. butleri* these amounted to 10 to 15 % of solubilized glucan. A component suspected to be sorbose was also encountered in *P. butleri*. In spite of all the treatments a very small amount of the wall still remained insoluble. This material seems to be a complex carbohydrate.

Electron microscopic observation. Intact cell walls of both fungi showed the mass of crossed fibres embedded in an amorphous matrix typical of hyphal walls of fungi. The acid-insoluble residue conserved the fibrillar structure of the original material.

β -glucanase digestion of living mycelium walls. Nicolas (1965) treated mycelium of two oomycetes, *Pythium ultimum* and *Phytophthora heveae*, with a crude cellulase from *Trichoderma lignorum*, and observed lysis of hyphal walls with release of vacuoles and vacuolated protoplasts. Our studies with *Streptomyces* and basidiomycete cellulases showed considerable digestion of the walls of the mycelium with both preparations, but only the basidiomycete cellulase liberated protoplasts. Failure to obtain protoplasts with other cellulase preparations may be explained by the presence of proteolytic activity in the enzyme preparation. A *Penicillium brefeldianum* enzymic preparation, which does not contain cellulase activity, did not dissolve the walls of the living mycelium of the oomycetes studied while showing a large release of reducing sugars in experiments with both isolated cell walls.

DISCUSSION

The acid-soluble material studied here was a glucan, or glucans, predominantly linked β (1 $\rightarrow$ 3) and β (1 $\rightarrow$ 6) with an approximate ratio of 1/1, but containing also 5–10 % of β (1 $\rightarrow$ 4) linkages. Since parallel hydrolyses of an acid-swollen cellulose showed only minor degradation, we assumed that the β (1 $\rightarrow$ 4) linkages came from non-cellulosic glucan. The acid-insoluble fraction revealed mainly β (1 $\rightarrow$ 4) glucosidic linkages and also a low content of β (1 $\rightarrow$ 3) and β (1 $\rightarrow$ 6) glucosidic linkages. This acid resistant glucan was not a pure cellulose as was supposed from preliminary experiments. It could be a glucose polymer of mixed linkage, though dominantly β (1 $\rightarrow$ 4), or it could be a mixture of cellulose with another glucan which is very resistant because of a high degree of branching.

The low effectiveness of the β (1 $\rightarrow$ 3) endoglucanase from *Rhizopus arrhizus* on the non-dialysable soluble fraction of two oomycetes walls, containing a considerable percentage of β (1 $\rightarrow$ 3) glucan linkages, may be due to the fact that this polymer is highly branched in such a way as to be inaccessible to the enzyme. Besides the digestion of isolated walls and soluble non-dialysable fractions releasing a large amount of glucose when used, a β (1 $\rightarrow$ 3) exoglucanase from Basidiomycete QM 806 may suggest that β (1 $\rightarrow$ 3) glucan exists in an acid soluble complex as branches, easily attackable by the exoenzyme. On the other hand, the fact that a β (1 $\rightarrow$ 3), β (1 $\rightarrow$ 6) glucanase preparation from *Penicillium brefeldianum* degraded isolated walls, with liberation of sugars but not living cells, indicates the presence of resistant glucan, very insoluble in acid, in the outer layer of the cell wall. Our results differ a little from those obtained by Bartinicki-Garcia & Lippman (1967) with *Phytophthora cinnamomi*. These authors confirmed, in effect, the presence of cellulosic-glucan in cell walls and reported the existence of glucan(s) soluble in acid, but highly insoluble in alkali, with an undetermined proportion of β (1 $\rightarrow$ 3) and β (1 $\rightarrow$ 6) linkages.

Most of the protein of walls was found in the acid-soluble fractions. A chemical extraction of walls with N-KOH showed a very low release of carbohydrates (less than 5 %), whereas the major portion of protein was solubilized. Hence protein is probably not indispensable to cell wall integrity. Possibly protein units are absent from linear carbohydrate chains but serve to bind them together. This hypothesis is in agreement with the fact that pepsin liberated no nitrogenous material from cell walls, whereas it released a large quantity of peptide and amino acids from acid-soluble material. Mild acid treatment presumably solubilized a glucan and the consequent rupture of carbohydrate linkages made the protein units more accessible to proteolytic digestion. As was published previously (Novaes-Ledieu *et al.* 1967), the protein of two oomycetes walls contains a significant proportion of hydroxy amino acids and, in the case of *Pythium butleri*, a high content of aspartic acid. These results could be interpreted as possible binding points between carbohydrate and protein. On the other hand, glucosamine, which has always been found in carbohydrate-protein fractions, could also be a binding point between protein and carbohydrate chains.

The larger protein percentage in *Pythium butleri* could explain the more drastic treatment necessary to solubilize a glucan-protein complex from the wall of this phycomycete and the different behaviour of the digested material on dialysis.

It may be concluded that the major component in cell walls of the two oomycetes is a glucan-protein soluble in dilute acid. The main chain of carbohydrate may contain various β -glycosidic linkages, side chains linked β (1 $\rightarrow$ 3), and protein chains serving to bind together different glucan molecules. This glucan might form the inner layer of the hyphal walls. In addition both phycomycetes contain an acid-resistant glucan with dominant β (1 $\rightarrow$ 4) linkages and a lower content of other kinds of linkages.

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