The Chemical Composition of the Cell Walls of *Penicillium digitatum* Sacc. and *Penicillium italicum* Whem.

By V. GRISARO, N. SHARON AND RIVKA BARKAI-GOLAN

*Department of Biophysics, The Weizmann Institute of Science, and
Division of Fruit and Vegetable Storage, The Volcani Institute of
Agricultural Research, Rehovoth, Israel*

(Accepted for publication 27 September 1967)

SUMMARY

Purified cell walls were isolated from hyphae of *Penicillium digitatum* Sacc. and *P. italicum* Whem. Electron micrographs showed microfibrils in the walls. The walls are composed chiefly of carbohydrate material, mainly D-glucose, together with D-galactose, glucosamine and mannose. Only small amounts of protein were found. Fractionation with alkali and acid indicated that the walls contain several glycans.

INTRODUCTION

The composition, structure and biosynthesis of fungal cell walls have been investigated only to a limited extent (Aronson, 1965). It is known that the chemical and physical structure of these walls is complex, and that they are composed mainly of carbohydrate material, in the form of polymers of glucose, galactose, mannose and glucosamine (the latter as chitin) (Aronson, 1965; Sharon, 1965), but the various polymeric components have been isolated from the walls and characterized in a few cases only. The walls of two species of the genus *Penicillium* have been studied. Crook & Johnston (1962) analysed qualitatively the wall of *Penicillium notatum* and found glucose to be the major component; small amounts of galactose, glucosamine and mannose were also found. With walls of *P. chrysogenum*, Hamilton & Knight (1962) found mannose, galactose, glucosamine, glucose, xylose and rhamnose in the approximate molar ratios of 1:3:4.5:9:0.5:0.5. Fractionation with alkali and acid indicated that these walls had at least two chemically distinct polysaccharide layers.

Penicillium digitatum and *P. italicum* are the main pathogenic fungi which cause serious spoilage to stored citrus fruit in Israel, and improved methods for the control of these fungi are required. Since several antibacterial substances, notably penicillin, kill bacteria by specifically inhibiting cell-wall biosynthesis (Martin, 1966), it might be possible to develop antifungal agents which act in a similar way. For this purpose, a detailed knowledge of the chemical structure and biosynthesis of fungal walls may be necessary; as a first step we have examined the chemical composition of the walls of *P. digitatum* and *P. italicum*.

METHODS

Culture conditions. Spores of *Penicillium digitatum* Sacc. and of *P. italicum* Whem. were collected from 10-day single-spore cultures on potato glucose agar incubated at 25°. Spore suspensions in distilled water (10⁶ spores/ml.) were used as inocula. Fern-

bach bottles containing 250 ml. potato glucose broth were inoculated with 2 ml. of spore suspension. After 20 hr of incubation at 25° the hyphae were harvested by filtration through cheesecloth and washed four times with cold distilled water. To get sufficient quantities of hyphae, 30 bottles were inoculated with *P. digitatum* spores and 6 bottles with those of *P. italicum*. The same culture media and techniques were used for growth of *P. chrysogenum*.

Preparation of cell walls. In a typical experiment, 1.2 g. of vacuum-dried *Penicillium digitatum* hyphae, 15 g. glass beads (Minnesota Mining and Manuf. Co., Type 130-5005) and 50 ml. water were stirred at top speed in a Servall Omnimixer cooled in an ice-water bath (Hamilton & Knight, 1962; Sharon & Jeanloz, 1964) for 1 hr. Disintegration was complete since no whole cells could be observed in samples examined under the microscope ($\times 1200$). The mixture was then centrifuged in a Clay Adams Safeguard centrifuge at low speed (80 g) for 5 min. and the precipitate (glass beads and intact cells) discarded. The supernatant fluid was centrifuged in the same centrifuge at high speed (800 g) for 20 min. The precipitate was suspended in water (24 ml.) and washed by differential centrifugation, first at low speed and then at high speed. Washing was repeated ten times; in the last few washes, no precipitate formed at low-speed centrifugation, and the supernatant fluid did not absorb at 260 m μ . The washed cell walls were then freeze-dried, yielding 213 mg. of a slightly grey fluffy material (18 % of the dry weight of hyphae used). *P. italicum* gave a similar yield.

Before freeze-drying, the walls were examined with the electron microscope. Electron micrographs were taken with an RCA EMU-2A electron microscope. Specimens were prepared on Formvar and carbon-coated grids. Shadow casting was with Pt at an angle of 1:4.

Fractionation of cell-wall components. The procedure used was based on the work of Hamilton & Knight (1962). Lipids were removed from the cell walls (200 mg.) by extraction with ether (50 ml.) for 2 hr in a Soxhlet extractor. The ether-extracted walls were dried in air at room temperature, suspended in N-NaOH (10 ml.) and the suspension stirred for 3 hr at room temperature. The walls were removed by centrifugation, and extracted again with alkali as before. The alkali extracts were pooled, ethanol (96 %, v/v, in water, 40 ml.) was added, and the mixture allowed to stand overnight at 2-4°. The precipitate which formed was collected by centrifugation, washed with ethanol (96 %, v/v, in water) in the centrifuge until neutral, and dried in vacuum over KOH and CaCl₂.

The residue which remained after alkali extraction was washed with water in the centrifuge until neutral, dried in vacuum, suspended in H₂SO₄ (72 %, v/v, conc. H₂SO₄ in water, 1 ml.) and the suspension allowed to stand at room temperature for 48 hr. The insoluble residue was collected by centrifugation and washed with water till neutral; the first water wash (12 ml.) was added to the 72 % H₂SO₄ extract. The combined extract was diluted to give a final concentration of N-H₂SO₄, and heated at 100° or 6 hr. The hydrolysis mixture was neutralized with solid BaCO₃, the precipitate removed by filtration and washed with a small volume of boiling water. The combined filtrates were freeze-dried.

For analysis of sugars, the ether-extracted walls and the various cell fractions were also hydrolysed as above (N-H₂SO₄, 6 hr, 100°) and neutralized in the same way.

Nitrogen was estimated by the procedure of Dumas (Steyermark, 1961) and *phosphorus* by the method of King (1932). *Ash* was estimated in samples which were oxi-

dized with fuming HNO_3 and then heated at 600° to constant weight. *Neutral sugar* was determined by the phenol method of Dubois *et al.* (1956), and *reducing sugar* by the method of Park & Johnson (1949), with glucose as standard.

Acid hydrolysates were analysed qualitatively by descending *paper chromatography* on Whatman No. 1 paper with the following solvent systems: (a) *n*-butanol + acetic acid + water (25 + 6 + 25 by vol.; upper phase); (b) isoamyl alcohol + pyridine + water (3 + 3 + 0.9 by vol.); (c) *n*-butanol + ethanol + water (4 + 1 + 1 by vol.); (d) *n*-butanol + pyridine + water (6 + 4 + 3 by vol.). Chromatograms were run for 18 hr. *Reducing compounds* were detected by the silver nitrate reagent (Sharon & Jeanloz, 1960) and amino sugars and amino acids by ninhydrin (0.5 % in acetone). The nature of the various sugars present was inferred from experiments with appropriate authentic compounds as markers, and in the case of glucose and galactose this was substantiated by enzymic experiments (see below).

Glucosamine was estimated by the colorimetric Elson-Morgan method as modified by Gardell (1953) with D-glucosamine hydrochloride (Pfanstiehl) as standard. In some of the experiments, the glucosamine was separated from the neutral sugars with the aid of Dowex 50 (Boas, 1953). It was further identified by ion-exchange chromatography on the Beckman-Spinco amino acid analyser (Spackman, Stein & Moore, 1958). D-Glucose and D-galactose were estimated enzymically by the glucose oxidase (Glucostat) and galactose oxidase (Galactostat) reagents (Worthington Biochemical Corporation, Freehold, New Jersey), respectively. *Amino acids* were determined on the Beckman-Spinco amino acid analyser after samples had been hydrolysed for 20 hr in 6 N-HCl at 100° in sealed ampoules (Spackman *et al.* 1958).

RESULTS

The electron micrographs of the walls (Pl. I, fig. 1, 2) indicate that they are rod-shaped, in accord with the typical morphology of hyphae of *Penicillium digitatum* and *P. italicum*. They exhibit a micro-fibrillar structure (cf. Aronson, 1965) which can clearly be seen in the internal part of the wall (Pl. I, fig. 2). No distinct fibrillar structure was seen on the external surface of the walls. The gross structure of the wall and the fibrils was not affected by prolonged treatment with alkali (N-NaOH, 6 hr, room temperature).

The walls of both organisms were composed mainly of carbohydrates. Glucose, galactose, mannose and glucosamine were identified by paper chromatography of acid hydrolysates of cell walls; trace amounts of rhamnose and xylose were also present. Quantitative analysis showed (Table 1) that the major sugar was D-glucose, which comprised about one half of the weight of the walls. The other neutral sugars, D-galactose and mannose, accounted together for 12–14 % of the weight. The proportions of these sugars, as well as those of glucosamine, did not differ markedly in the two organisms analysed. The main difference was in the quantity of ash. Whereas no ash was found in the walls of *Penicillium italicum*, the ash content of the walls of *P. digitatum* was consistently very high. A small part of this ash was accounted for by the presence of phosphate, but we have no explanation for the source of the bulk of the ash. It was not an artifact of the experimental procedure used, since no ash was found in the walls of *P. italicum*. Furthermore, we did not find any ash in walls of *P. chrysogenum* which we prepared.

The protein content of the walls appeared to be rather low, as could be inferred from the percentage of non-amino sugar nitrogen (total N minus amino sugar N) in the walls. This figure was 0.82 % for *Penicillium digitatum* and 0.20 % for *P. italicum*; using the factor 6.25, this corresponds to 5.1 % and 1.25 % protein in the walls of the two organisms, respectively. Analysis of acid hydrolysates (20 hr, 6N-HCl, 100°) of the walls on the amino acid analyser showed the presence of the typical protein amino acids, which amounted to 3 % of the weight of the walls of *P. digitatum* and to 1.5 % of those of *P. italicum*. A comparison of our results with those of Hamilton & Knight (1962) for walls of *P. chrysogenum* shows that, qualitatively, the composition of the walls of the three *Penicillium* species is similar. There were, however, some quantitative differences, notably in glucose, which was lower in *P. chrysogenum*, and in the content of galactose and glucosamine, which was significantly higher (up to 3 times) in the walls of this organism than in those of *P. digitatum* and *P. italicum*.

Table 1. *Analyses of freeze-dried cell walls of Penicillium digitatum and P. italicum after lipid extraction*

Total reducing sugar, D-glucose, D-galactose and glucosamine were estimated in the walls after hydrolysis (N-H₂SO₄, 6 hr, 100°) and neutralization of the hydrolysate with BaCO₃.

	<i>P. digitatum</i>	<i>P. italicum</i>
	% (w/v) of walls	
Reducing sugar (as glucose)	65.4	72.5
D-Glucose	45.4	51.6
D-Galactose	3.8	3.8
Glucosamine	5.7	9.0
Total nitrogen	1.27	0.90
Amino sugar N (calculated)	0.45	0.70
Phosphorus	1.8	0.13
Ash	29.5	< 0.5

Table 2. *Fractionation of cell-wall material from Penicillium digitatum and P. italicum*

	Wall fraction (%)	% in fraction			
		Neutral sugar	Glucose	Galactose	Glucosamine
<i>P. digitatum</i> (130 mg.)					
Alkali-soluble	22.5	88	61	7	1
Acid-soluble	36	98	60	6.1	7.5
Residue	27	1	—	—	—
Total recovery	86				
<i>P. italicum</i> (69.3 mg.)					
Alkali-soluble	30.3	90	65	4	0
Acid-soluble	53	71	58	5	16.5
Residue	2.9	18	4	—	—
Total recovery	86				

Extraction of the cell walls of *Penicillium digitatum* and of *P. italicum* with ether and evaporation of the solvent from the extract gave a small amount of solid residue (about 4 % of the weight of the walls), indicating a low lipid content. The cell walls were also fractionated with alkali and acid. Table 2 shows that alkali (N, two treatments of 3 hr each at 23°) extracted a fraction which comprised 22 % of the weight of

P. digitatum walls and 30 % of the walls of *P. italicum*. This fraction, composed mainly of glucose, might be a mixture of homo- or hetero-polymers.

Treatment with alkali did not affect the gross structure of the walls as seen with the electron microscope. This may indicate that the soluble polysaccharide(s) are not essential components of the rigid structure of the wall. When the fraction of the wall which was insoluble in alkali was put in 72 % (v/v) conc. H_2SO_4 in water for 2 days another portion went into solution—36 % for *Penicillium digitatum* and 52 % for *P. italicum*. A similar figure was found by Hamilton & Knight (1962) for walls of *P. chrysogenum*. Quantitative analysis of acid hydrolysates of this fraction revealed that it was composed mainly of glucose, with small amounts of galactose. It also contained glucosamine; it is possible that part of this glucosamine was in the form of chitin, since chitin is known to be soluble in concentrated sulphuric acid. This is in agreement with previous findings that *Penicillium* belongs to the group of fungi which contain chitin in their walls (Aronson, 1965). The part of the wall which was insoluble in 72 % (v/v) conc. H_2SO_4 in water did not contain any appreciable amount of carbohydrate; its amount in the case of *P. digitatum* was 27 % of the walls, and this fraction may have contained the bulk of the material corresponding to the ash. Only a small amount of acid-insoluble residue was found in the walls of *P. italicum* in accord with the absence of ash from these walls.

We wish to thank the members of the Section of Biological Ultrastructure for the electron micrographs.

REFERENCES

- ARONSON, J. M. (1965). The cell wall. In *The Fungi*. Ed. by G. C. Ainsworth and A. S. Sussman, vol. 1, p. 49. New York and London: Academic Press.
- BOAS, N. F. (1953). Method for the determination of hexosamines in tissues. *J. biol. Chem.* **204**, 553.
- CROOK, E. M. & JOHNSTON, I. R. (1962). The qualitative analysis of the cell walls of selected species of fungi. *Biochem. J.* **83**, 324.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. *Analyt. Chem.* **28**, 350.
- GARDELL, S. (1953). Separation on Dowex 50 ion exchange resin of glucosamine and galactosamine and their quantitative determination. *Acta chem. scand.* **7**, 207.
- HAMILTON, P. B. & KNIGHT, S. G. (1962). An analysis of the cell walls of *Penicillium chrysogenum*. *Archs Biochem. Biophys.* **99**, 282.
- KING, E. J. (1932). The colorimetric determination of phosphorus. *Biochem. J.* **26**, 292.
- MARTIN, H. H. (1966). Biochemistry of bacterial cell walls. *A. Rev. Biochem.* **35**, 457.
- PARK, J. T. & JOHNSON, M. J. (1949). A submicrodetermination of glucose. *J. biol. Chem.* **181**, 149.
- SHARON, N. (1965). Distribution of amino sugars in microorganisms, plants and invertebrates. In *The Amino Sugars*. Ed. by R. W. Jeanloz and E. A. Balazs, vol. IIA, p. 1. New York and London: Academic Press.
- SHARON, N. & JEANLOZ, R. W. (1960). The diamino-hexose component of a polysaccharide isolated from *Bacillus subtilis*. *J. biol. Chem.* **235**, 1.
- SHARON, N. & JEANLOZ, R. W. (1964). A procedure for the preparation of gram quantities of bacterial cell walls. *Experientia* **20**, 253.
- STEYERMARK, A. (1961). *Quantitative Organic Microanalysis*, p. 177. New York and London: Academic Press.
- SPACKMAN, D. H., STEIN, W. H. & MOORE, S. (1958). Automatic recording apparatus for use in the chromatography of amino acids. *Analyt. Chem.* **30**, 1190.

EXPLANATION OF PLATE

Electron micrographs of cell walls of *Penicillium digitatum* hyphae.

Fig. 1, magnification, $\times 5000$. Fig. 2, magnification $\times 20,000$: note the fibrous structure of the internal part of the cell wall.