Journal of General Microbiology (1975), **91**, 201–206
Printed in Great Britain

Chemical Composition of the Hyphal Wall of a Toxigenic Fungus, *Penicillium rubrum* Stoll

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(*Received* 10 *March* 1975; *revised* 10 *June* 1975)

INTRODUCTION

Quantitative studies on fungal walls are limited; however, the species of Penicillium which have been studied (Applegarth, 1967; Applegarth & Bozoian, 1968; Grisaro, Sharon & Barkai-Golan, 1968) contained, in addition to wall components found in qualitative studies, substantial amounts of lipid and protein. The walls of Penicillium so far examined have contained a glucan, with smaller amounts of galactose and mannose and a glucosamine polymer.

The organism used in this study was a filamentous fungus, *Penicillium rubrum* Stoll, which produces rubratoxin B, an hepatotoxic and teratogenic secondary metabolite (Hayes, 1975). *Penicillium rubrum* has been associated with disease outbreaks among livestock in the U.S.A. (Burnside *et al.* 1957) and with a haemorrhagic syndrome in chicks (Forgacs *et al.* 1958).

The chemical composition of the hyphal wall was of interest because rubratoxin B, a deleterious metabolite which is secreted into the medium, has limited antifungal activity (Reiss, 1972; Wyatt, 1971). The present work reports the chemical composition of wall fractions as a preliminary step towards determining if the hyphal wall of *P. rubrum* is involved in the resistance of the mould to rubratoxin.

METHODS

A rubratoxin-producing strain of *Penicillium rubrum* Stoll, NRRL A11785, from USDA, Peoria, Illinois, U.S.A., was grown as outlined by Hayes & Wilson (1968). Spore suspensions in sterile NaCl solution were prepared from 12-day-old cultures grown on Czapek–Dox agar, incubated at room temperature and standardized by aseptic filtration through glass wool. The extinction of the filtrate at 609 nm was adjusted to 0·6 and 3 ml used to inoculate the growth medium. After 14 days' incubation at room temperature, but before sporulation, hyphae were harvested.

Hyphal walls were prepared by repeated centrifugations and washing with distilled water after disruption of hyphae with glass beads in a Braun MSK mechanical cell homogenizer (Hamilton & Knight, 1962). When this mixture was at least 95 % free of cytoplasm, as ascertained by phase contrast microscopy (×970), the suspension was transferred to centrifuge tubes and allowed to stand for 3 to 4 min in ice, during which time most of the glass beads settled to the bottom. The walls were separated from the glass beads, collected by centrifugation, lyophilized, and stored at −20 °C.

Lyophilized hyphal walls (100 mg) were treated with 3 ml of 13·5 M-H_2SO_4 for 8 h at

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22 °C in a stoppered flask. The acid suspension was then diluted to 1·5 M, the hydrolysis tube sealed, and heated at 100 °C for 18 h. The hydrolysate was filtered and neutralized with $BaCO_3$. The precipitated $BaSO_4$ was removed by centrifugation and washed twice with distilled water. The washes were combined with the neutralized fraction, concentrated to 40 ml, and lyophilized. This fraction was reconstituted with 50 ml of hot 50 % ethanol and filtered; the filtrate was concentrated to 20 ml, lyophilized, and stored at −20 °C until used for identification of monosaccharides.

Total neutral carbohydrate was estimated by the phenol–sulphuric acid method of Dubois *et al.* (1956). Glucose and galactose were estimated using Glucostat and Galactostat reagents, respectively (Worthington Biochemical Corp.) on samples eluted from paper chromatograms. Mannose, the only other hexose detected, was estimated by the tryptophan–borosulphuric acid method of Badin, Jackson & Schubert (1953).

Monosaccharides were separated on Whatman No. 1 paper in *n*-butanol–pyridine–water (6:4:3, by vol.) or *n*-butanol–pyridine–benzene–water (1:5:3:3, by vol.) and further identified on thin layers of cellulose, using ethyl acetate–isopropanol–water (65:23·4:11·6, by vol.). Sugars were visualized on paper chromatograms after spraying with aniline hydrogen phthalate, while thin-layer plates were sprayed with anisaldehyde sulphuric acid (Taylor & Cameron, 1973). Known amounts of glucose, galactose and mannose were hydrolysed and then chromatographed to determine losses incurred during hydrolysis. The experimental values obtained for cell wall hydrolysates were corrected to allow for loss of individual sugars (5 % or less).

Amino sugars were determined by a combination of the methods of Boas (1953) and Pearson (1963); this modified method increased the recovery of added glucosamine, presumably by preventing aerial oxidation in partially-dried samples. Results were corrected for losses during hydrolysis by including an internal glucosamine standard. Paper chromatography of amino sugars was in *n*-butanol–acetic acid–water (4:1:5, by vol.) and t-amyl alcohol–formic acid–water (4:1:2, by vol.). The areas corresponding to glucosamine and galactosamine were eluted, and the identity of the hexosamines was confirmed by ninhydrin oxidation (Stoffyn & Jeanloz, 1954), which yielded arabinose or lyxose, and by *n*-acetylation with sodium bicarbonate and acetic anhydride (Crumpton, 1959), which gave products chromatographically identical with *n*-acetylglucosamine and *n*-acetylgalactosamine.

For amino acid analysis, 30 mg samples of wall material were hydrolysed in 6 M-HCl at 110 °C for 72 h in sealed, evacuated tubes. Individual amino acids were determined quantitatively in a Beckman amino acid analyser.

Readily-extracted and bound lipids were determined by the method of Bartnicki-Garcia & Nickerson (1962). Bound lipids were chromatographed on silica gel thin-layer plates, using hexane–ethyl ether–glacial acetic acid (9:1:1, by vol.), sprayed with 50 % H_2SO_4 and charred. A lipid standard containing cholesterol, lecithin, stearic acid, tristearin and a cholesterol ester was included on each plate.

Carbon, hydrogen and nitrogen were determined using a CHN analyser. Total nitrogen was also estimated by the modified micro Kjeldahl method of Folin & Farmer (1912). The amount of nitrogen contributed to glucosamine was calculated from the hexosamine values; the remainder of the nitrogen was multiplied by 6·25 to give an approximate value for the protein content of the fungal wall. Phosphorus was assayed by the method of Taussky & Shorr (1953).

To determine the water content of lyophilized walls, 100 mg samples were dried in an oven at 105 °C to constant weight. To determine the ash content, weighed amounts of lyophilized unhydrolysed wall material were placed in a muffle furnace, heated at 950 °C for 18 h,

Table 1. *Comparisons of the chemical composition of Penicillium hyphal walls*

Composition	*P. chryso-genum*	*P. roque-fortii*	*P. digita-tum*	*P. notatum*	*P. italicum*	*P. rubrum**
Carbohydrate	—	—	—	—	—	67·4†
Neutral Sugars	55·2	56·0	49·2	51·0	55·4	52·0‡
Glucose	36·0	41·0	45·4	43·0	51·6	44·6§
Galactose	11·0	14·0	3·8	7·0	3·8	5·4§
Mannose	4·0	1·0	—	1·0	—	0·6§
Hexosamine	20·1	13·3	5·7	18·5	9·0	15·4‡
Lipid	—	1–2	—	1–2	—	17·0†
Readily extracted	—	—	—	—	—	7·0
Bound	—	—	—	—	—	10·0
Protein	—	9·7	5·1	8·9	1·3	7·2
Ash	—	5·0	29·5	4·0	0·5	4·9†
Phosphate (H_3PO_4)	—	0·9	1·8	1·8	1·13	0·5†
Kjeldahl Nitrogen	—	—	—	—	—	1·6†
Reference	Hamilton & Knight (1962)	Applegarth & Bozoian (1968)	Grisaro *et al.* (1968)	Applegarth (1967)	Grisaro *et al.* (1968)	This paper

* Values given represent six hyphal wall preparations run in duplicate; differences for the separate preparations were less than 5 %

† Analysis was on unhydrolysed cell wall material.

‡ Analysis was on the HCl hydrolysate.

§ Analysis was on the H_2SO_4 hydrolysate.

—, Not done.

cooled, weighed, reheated for 1 h and reweighed. The ashed material was analysed for mineral components by atomic absorption spectrometry.

The procedure of Fuller & Barshad (1960) was followed for chitin. A red–violet colour in the presence of Gram's iodine indicated chitosan formed by alkaline degradation of chitin. Cell wall (500 mg) was also extracted with 10 M-HCl at 4 °C for 10 min. After centrifugation, the supernatant was poured into 300 ml cold 50 % ethanol; the precipitate was collected, dried and finally crushed using an agate pestle and mortar. Lobster chitin (Nutritional Biochemical Corp.) was treated identically and infrared absorption spectra of both were obtained with KBr pellets on a model 521 Perkin–Elmer spectrophotometer.

RESULTS AND DISCUSSION

The mean values for carbohydrate, lipid, protein, ash, phosphorus and nitrogen for duplicate hyphal wall preparations from six cultures are given in Table 1. Differences in values for the cell wall preparations of the six cultures were less than 5 %. Carbohydrate, lipid, protein and ash accounted for 96·5 % of the dry weight of cell wall material. Sixty-seven per cent of the cell wall was carbohydrate with protein and lipid contributing 7·2 and 17 %, respectively.

The major carbohydrate was glucose (44·6 %). Mannose, galactose and hexosamines were present in smaller quantities (0·6, 5 and 15 %, respectively). The main hexosamine was glucosamine, with traces of galactosamine. Traces of an unidentified reducing substance with a low R_F value, possibly a glucose oligosaccharide or a sugar phosphate resulting from incomplete hydrolysis, were observed on a few chromatograms.

Phosphorus was present in unhydrolysed cell wall material at the level of 0·5 % phosphate (Table 1).

Table 2. *Amino acid analysis of the protein component of the hyphal walls of Penicillium rubrum*

Hyphal wall samples were hydrolysed in 6 M-HCl for 72 h as described in Methods.

Amino acid	Amount of amino acid	
	μM/mg wall	% of total
Lys	0·066	7·2
His	0·023	2·7
Arg	0·043	5·7
Asp	0·087	8·7
Thr	0·081	7·2
Ser	0·054	4·2
Glu	0·089	9·9
Pro	0·707	6·1
Gly	0·907	5·1
Ala	0·104	6·9
Val	0·096	8·5
Met	0·012	1·3
Ile	0·058	5·7
Leu	0·115	11·3
Tyr	0·025	3·4
Phe	0·048	5·9

The lipid fraction loosely associated with the cell wall represented 7 % whereas bound lipids were 10 % (Table 1). The bound lipid fraction contained phospholipid, sterol, fatty acid and triacylglycerol (data not shown).

Results of a typical amino acid analysis are shown in Table 2. Sixteen amino acids were detected. The predominant acidic amino acid was glutamic acid (9·9 %). Lysine was the most abundant basic amino acid (7·2 %) whereas histidine (2·7 %) was the least abundant basic amino acid detected.

The 5 % remaining after ignition represented the mineral components of the hyphal wall. Calcium (0·57 %), magnesium (0·45 %), potassium (0·016 %), manganese (0·013 %) and iron (0·006 %) were the only elements detected. Total nitrogen of the cell wall as determined by the Folin–Farmer procedure was 1·55 %. This figure represented nitrogen contributed by all nitrogen-containing compounds including amino acids and amino sugars. Nitrogen content (1·48 %) obtained from CHN analysis closely paralleled these results. CHN analysis also showed that the cell wall contained 6·45 % hydrogen. The percentage obtained for carbon (39·66 %) agreed with the theoretical carbon value contributed by carbohydrate, amino acids, amino sugars and lipids (35·25 %).

Total protein in walls of *P. rubrum* was 7·2 %. The figure was based on the assumption that, on average, nitrogen is present in a given quantity of protein in the proportion 1 : 6·250.

When hyphal walls which had been digested in 23 M-KOH were treated with Gram's iodine–H_2SO_4, a red colour developed indicating the presence of chitin in the cell wall of *P. rubrum*. Gram's iodine solution, however, did not give a positive reaction when the hyphal walls were extracted with acetic acid which solubilizes chitosan. Infrared spectra of the cell wall extract and the commercial chitin were identical. Therefore, the glucosamine in the wall is probably incorporated in polymers of chitin rather than chitosan.

More cell wall lipid (17 %) was present in *P. rubrum* than in other Penicilium species (Table 1). Among the ascomycetes, however, Ruiz-Herrera (1967) has reported 19 % lipid in the cell wall of a species of Aspergillus. The hyphal walls of *P. rubrum* were estimated to contain 7·2 % protein, which is comparable to protein estimates for cell walls of *P. roque-*

fortii (9·7 %) and *P. notatum* (8·9 %). The chemical components of the cell wall of *P. rubrum* did not deviate significantly either quantitatively or qualitatively from other Penicillium species.

Ninety-seven per cent of the dry weight of *P. rubrum* hyphal wall was accounted for by carbohydrate, lipid, hexosamine, protein and ash. The 3 % of the cell wall unaccounted for may be the result of experimental error and/or handling of cell walls. The oligosaccharide detected on a few thin-layer chromatograms indicated that incomplete hydrolysis of polysaccharides may have occurred. In addition, incomplete degradation would leave an insoluble residue remaining with the precipitate during neutralization of the sulphuric acid hydrolysate. Since anthrone reagent is sensitive to 5-hydroxymethyl furfural, incomplete hydrolysis or incomplete conversion to furfural would, therefore, result in a low figure for total carbohydrate.

Research support was provided by U.S. Public Health Grants ES00464 and ES00674 from the National Institute of Environmental Health Sciences, by a Sigma Xi grant-in-aid, and by grant 670 from The University of Alabama Research Grants Committee. Dr Hayes holds Research Career Development Award 1K04ES 34113, U.S. Public Health Services, National Institutes of Health.

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