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THE COMPOSITION OF THE CELL WALL FRACTION OF THE FUNGUS, *CORDYCEPS MILITARIS*

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SUMMARY

Cell walls of the filamentous form of the fungus, *Cordyceps militaris*, were isolated by repeated centrifugations and washings after disruption of the cells with glass beads in a Sorvall Omnimixer. Chemical analyses showed the walls to be composed of glucose (46%), galactose (7%), mannose (9%), hexosamine (11%), lipid (15%), and protein (6%). The latter consisted of 16 amino acids.

The cell wall composition of *C. militaris* is, both qualitatively and quantitatively, similar to that of the other ascomycetes which have been examined thus far.

INTRODUCTION

The chemical components of the cell walls of fungi differ significantly from those of the true bacteria and the actinomycetes. CUMMINS AND HARRIS[1] and CROOK AND JOHNSTON[2] have made qualitative studies of the cell wall composition of a large number of fungi, including representatives of all the major classes. They concluded that the major carbohydrates are glucose, galactose, mannose, and glucosamine. No muramic acid was detected. Traces of amino acids, typical of protein hydrolysates were also found. The cell walls of the true bacteria and the actinomycetes, in contrast to the fungi, contain glucosamine and muramic acid as their major carbohydrates. A smaller number of amino acids are present in significant quantities.

Quantitative studies on the chemical composition of fungal cell walls are relatively few compared to those of bacterial cell walls. A number of species of Penicillium and Aspergillus have been studied[3–7], and the cell wall components of yeast-like and filamentous forms of *Mucor rouxii* have been compared with those of asexual spores[8,9]. *Pithomyces chartarum* hyphal walls[10] and the polysaccharide components of the hyphal walls of *Neurospora crassa*[11] have also been examined. These studies indicate that, in addition to the components found in the qualitative studies, substantial amounts of lipid are present in fungal cell walls. Protein is also present in far more than trace amounts.

The present study extends the amount of information available on the chemical components of fungal cell walls. In this study we have hydrolyzed the walls of the

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filamentous form of the ascomycete, *Cordyceps militaris*, and determined the monomeric units which were released.

Under certain conditions of growth *Cordyceps militaris* produces 3'-deoxyadenosine (previously referred to as Cordycepin) which it excretes into the medium[12]. This nucleoside inhibits the growth of certain bacteria and also of ascites tumor cells[12–14]. *In vitro* studies on the biosynthesis of Cordycepin have been limited because of the difficulty of extracting enzymes from the cell. This difficulty appears to be due to the fact that the cells can only be disrupted by harsh mechanical methods. In part, we undertook this study of the cell wall structure of Cordyceps with the hope of finding a more gentle means of disrupting the cells for *in vitro* studies.

METHODS

Growth

Cordyceps militaris (Linn.) Link was obtained from Centraalbureau voor Schimmelcultures, Baarn, Holland. The filamentous form used for isolation of cell walls was grown by shaking for 4 days in 1 % casein hydrolysate (Nutritional Biochemical Corporation) and 0.5 % glucose. The filamentous cells were harvested by filtration and stored at −20°.

Isolation of the cell wall fraction

A 10-g portion of frozen cells was mixed in a Sorvall Omnimixer at one-half maximum speed with 30 ml of H_2O for 2.5 min and then for 10-min periods with 40 g of glass beads (Super-brite 130–5005). Throughout this procedure the vessel of the Omnimixer was immersed in ethanol and solid CO_2. When the breakage, as determined by phase contrast microscopy was greater than 80 %, the suspension was centrifuged at 600 × *g* for 5 min at 5°. The pellet was washed 5 times with distilled H_2O. Then it was resuspended in 30 ml H_2O, and the mixing and washing procedures were repeated until the breakage was greater than 95 %.

We separated the walls from the glass beads by repeatedly suspending them in H_2O, allowing the beads to settle and decanting off the cell walls. The walls were collected by centrifugation and then lyophilized and stored at −20°.

Determination of neutral sugars

In order to determine the losses due to the procedures, known amounts of glucose, galactose, and mannose were carried through the following hydrolysis and chromatographic steps. Corrections for the losses were calculated and applied to the values obtained for the cell wall hydrolysates.

Hydrolysis

20 mg of lyophilized cell walls were treated with 0.42 ml of 72 % (w/w) H_2SO_4 at 4° for 18 h. Then the suspension was diluted to 0.5 M H_2SO_4 by the addition of H_2O. The hydrolysis tubes were sealed and placed in a 100° oven for 3 h. The hydrolysate was filtered and then neutralized with $BaCO_3$. After a second filtration to remove $BaSO_4$ and $BaCO_3$, the solution was passed through a column composed of 5 ml (wet-packed volume) of Dowex-1 (OH^- form) underneath 5 ml of Dowex-50 W (H^+ form). The effluent was evaporated to dryness, and the residue was taken up in 2 ml of H_2O.

Glucose and galactose were determined by the "Glucostat (special)" and "Galactostat" reagents, respectively, purchased from Worthington Biochemical Corporation.

Mannose was separated from glucose and galactose by chromatography on Whatman No. 1 paper in 1-propanol–ethyl acetate–H_2O (7:1:2, by vol.). The spots corresponding to known mannose were cut into small pieces and eluted by shaking in 5 ml of H_2O. The method of SOMOGYI[15] was used to determine the amount of mannose in the solution.

Determination of hexosamine

30 mg of the cell wall fraction were hydrolyzed in 10 ml of 6 M HCl for 6 h at 100° in a sealed tube. HCl was removed with a Buchler Instruments Rotary Evapomix. Hexosamine was determined colorimetrically by the method of REISSIG *et al.*[16]. The sample was first acetylated by the method of WHEAT[17].

Thin-layer chromatography

The sugars in the 3 h 0.5 M H_2SO_4 and the 6 h 6 M HCl hydrolysates were further identified by chromatography on thin layers of cellulose. Three solvent systems were used: ethyl acetate–pyridine–H_2O (12:5:4, by vol.); isopropanol–H_2O (4:1, by vol.); and *n*-butanol–ethanol–H_2O (10:1:2, by vol.). The sugars were visualized by staining with $AgNO_3$, (ref. 18).

Determination of amino acids

30 mg of the cell wall preparation were hydrolyzed in 5 ml of 6 M HCl at 100° for 18 h in sealed, evacuated tubes. The quantity of the individual amino acids present in the hydrolysate was determined with the aid of a Spinco amino acid analyzer.

Lipid

Both readily-extracted and bound lipids were determined by the method of BARTNICKI-GARCIA AND NICKERSON[8].

Phosphate

The method of BARTLETT[19] was used to determine the phosphate content.

Extraction and determination of nucleic acids

DNA and RNA were separated from lipid-extracted cell walls by the method of OGUR AND ROSEN[20]. RNA was determined by the orcinol method of SCHNEIDER[21] and DNA by the method of BURTON[22].

Drying

100 mg of lyophilized walls (or cells) were dried in an oven at 105° to constant weight.

Ash

The dried walls (or cells) were placed in a muffle oven at 950° for 3 h, cooled, weighed, reheated for 1 h, and weighed again. The ash contents of the wall preparations ranged from 6 to 18 % due to a variable amount of minute metal chips which were gouged from the Omnimixer by the glass beads during the cell wall isolation procedure.

Therefore, all values reported in the tables are corrected for ash and also for H_2O content.

Chitin

The method of APPLEGARTH[4] was used to test for chitosan. The residue from this procedure was tested for the presence of chitin by the method of Van Wisselingh[23].

RESULTS

Analytical values for carbohydrate, protein, and lipid (determined in duplicate) for three preparations of cell walls from three separate cell cultures are given in Tables I and II. Carbohydrate, protein, and lipid account for 95 % of the cell wall material. Carbohydrate alone contributes 73 % to the weight of the walls, with protein and lipid contributing 6 and 15 %, respectively.

The major carbohydrate component is glucose (46 %). Galactose, mannose, and hexosamine are present in smaller quantities, 7, 9 and 11 %, respectively. Hydrolysis in 2, 4 and 6 M HCl for 3 h released only one-sixth the amount of glucose released by 0.5 M H_2SO_4.

Thin-layer chromatograms of both the 3 h 0.5 M H_2SO_4 and the 6 h 6 M HCl hydrolysates showed no traces of other sugars even when an aliquot of the hydrolysate corresponding to 100 μg of cell wall was applied at the origin.

No traces of 3'-deoxypentose, the product expected from acid hydrolysis of Cordycepin, were found. Therefore, the Cordycepin which this organism produces in such large quantities is not used to any significant extent in the cell wall structure.

TABLE I

CHEMICAL ANALYSES OF CORDYCEPS CELL WALL FRACTION

The analyses were carried out as described in the text. The results are presented as per cent of the dry cell wall weight corrected for the ash content. The carbohydrate values are those for the anhydro-sugars.

	Preparation					
	1		*2*		*3*	
Glucose	46.9		45.7		46.4	
Galactose	7.6		7.7		6.2	
Mannose	9.0		9.8		8.7	
Hexosamine*	9.6		11.9		11.3	
Protein**	6.9		6.1		5.9	
DNA	—	0.08	—	—	—	—
RNA	—	0.06	—	—	—	—
H_3PO_4	—	1.0	—	—	—	—
Lipid						
Total	13.2		18.1		12.8	
Readily-extracted	—	9.8	—	12.9	—	9.5
Bound	—	3.4	—	3.4	—	3.3
Sum	93.2		99.3		91.3	

* Determined on 6 h 6 M HCl hydrolysates by a method which does not distinguish between the various hexosamines (see METHODS).

** Determined by summation of the amino acid values (Table II).

TABLE II

AMINO ACID ANALYSIS OF THE PROTEIN COMPONENT OF THE CORDYCEPS CELL WALL FRACTION

The analysis was carried out as described in the text. The values are presented as the weight per cent of the dry cell wall corrected for the ash content.

Amino acids	*Preparation*					
	1		*2*		*3*	
Asp	0.730		0.600		0.641	
Thr	0.730		0.695		0.570	
Ser	0.586		0.547		0.470	
Pro	0.487		0.530		0.413	
Glu	0.770		0.621		0.625	
Gly	0.338		0.299		0.294	
Ala	0.497		0.434		0.413	
Val	0.405		0.310		0.314	
Met	0.102		0.120		0.098	
Ile	0.298		0.216		0.228	
Leu	0.490		0.384		0.412	
Tyr	0.240		0.212		0.204	
Phe	0.286		0.248		0.252	
Glucosamine*	—	6.25	—	6.98	—	6.44
Lys	0.407		0.386		0.400	
His	0.152		0.134		0.144	
Arg	0.380		0.372		0.380	
Sum of amino acids	6.90		6.11		5.85	

* Determined from the glucosamine peak on the amino acid chromatograms (obtained from 18 h 6 M HCl hydrolysates).

The amount of readily-extracted lipid varies to some extent in the three preparations. However, the bound lipids, solubilized by acid treatment of cell walls from which the readily-extracted lipid had been removed, was constant at 3.4 %.

The total amount of protein, determined by summation of the contributions of the individual amino acids, averaged 6.3 % for the three different wall preparations. However, there are some significant differences in the contents of the individual amino acids, as shown in Table II. Since the amount of glucosamine in the hydrolysates is very consistent, the differences between the amino acids are probably real.

One preparation was examined for phosphate content. 1 % (as H_3PO_4) was detected. One quarter of the phosphate appeared in the lipid fraction presumably as phospholipid. The non-lipid portion of the phosphate could, in part, be accounted for by nucleic acid found in small quantities in this wall preparation.

Perchloric acid extracts of this preparation absorbed in the ultraviolet region of the spectrum with a peak at 260 mμ. Colorimetric tests indicated the presence of DNA and RNA. However, the actual quantity of nucleic acid was quite low, 0.14 % of the wall fraction. We have assumed that this small amount of nucleic acid is not an integral part of the cell wall structure, but rather contaminating material from the cytoplasm and nuclei. On this basis we have concluded that the amount of non-cell wall material in our preparation is very low.

The presence of chitin in the cell wall fraction was indicated by the Van Wisselingh test. When the cell walls were extracted with acetic acid, which solubilizes

chitosan, no reaction was obtained with Gram's iodine solution. The fraction insoluble in acetic acid gave a positive Van Wisselingh test. Therefore, the glucosamine in the wall is probably incorporated in polymers of chitin rather than chitosan.

DISCUSSION

The cell wall fraction which we have isolated appears to be reproducible in terms of its chemical components. If contaminants from other fractions of the cell are present, they are present in the same amount in each of our three preparations.

We are beginning a study using enzymes to determine the way in which the sugars are combined in the polymers of the cell wall of *C. militaris*. Treatment of the intact cells with chitinase released *N*-acetylglucosamine and drastically altered the microscopic appearance of the cells. The well-defined wall which outlined the cell disappeared, and the filaments appeared to consist of a string of vacuoles or globules, with no apparent interconnections. However, the cells were still quite rigid. Repeated attempts to disrupt the cells mechanically (sonication, French press, Omnimixer) all failed. Even though the wall had apparently disappeared, the chitinase-treated cells were still as difficult to break as the intact cells.

The total number of amino acids and their distribution on both a molar and a weight basis suggest that a protein is present in the cell wall of this fungus, rather than a small number of amino acids such as is found in bacterial walls. Six amino acids in this protein (aspartic acid, glutamic acid, serine, threonine, glycine, and alanine) account for 52 % of the weight of the protein (59 % on a molar basis). CROOK AND JOHNSTON[2] in a semi-quantitative study of the amino acids in the cell walls of a number of fungi found that five of these amino acids (aspartic acid, glutamic acid, serine, glycine, and alanine) were present to a greater extent than other amino acids. APPLEGARTH[4] in a quantitative study of *Penicillium notatum* found these six amino acids contributed 63 % to the total amount of protein on a molar basis. Other amino acids, in all cases, are present in significant quantities and do not appear to be trace contaminants from the cytoplasm.

The variation in the amount of readily-extracted lipid might be due to the contamination of the walls by membrane lipids or to the presence of a small number of fat globules adhering to the cell wall. Globular bodies may be seen at irregular intervals on the electron micrographs. Since the bound lipid required more vigorous conditions for its extraction, it is probably involved more intimately in the cell wall structure.

The number of fungi for which quantitative studies of the cell wall have been made is still too small to warrant generalization. However, in the few species studied the composition is remarkably similar. Most of these species belong to the class Ascomycetes[3,4,6,7]. We have observed that the cell wall fraction of *C. militaris* is similar to the composition of the other Ascomycetes which have been studied. Glucose is the predominant carbohydrate (36–64 %) in these ascomycete walls. Mannose and galactose are present in smaller quantities (1–10 % and 4.8–11 %, respectively). Chitin varies from 9–20 %, lipid from 1–18 %, and protein from 0.45–9 %. The variations in these quantitative values might be due to differences in the age of the fungal cells which were used or to differences in the method of determination of the quantities.

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