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BIOCHEMICAL ANALYSIS OF THE CELL WALL OF *ASPERGILLUS NIDULANS*

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SUMMARY

1. The chemical composition of the mycelial cell wall of *Aspergillus nidulans* was investigated.

2. The main components of the cell wall are glucose and acetylglucosamine with minor quantities of mannose, galactose, galactosamine, protein and lipid.

3. The polysacharides could be distinguished on the basis of solubility. One polymer is an alkali-soluble glucan and contains only α-1,3-glycosidic linkages, the other component is alkali-resistant and contains chitin (about 50%) and α-1,4- and β-1,3-linked glucan. In addition, minor quantities of galactose and mannose occur in this fraction.

4. *A. nidulans* produces enzymes which hydrolyse the alkali-resistant glucan component.

INTRODUCTION

The biochemistry of morphogenesis is not well known. Microorganisms are highly suitable for this kind of research but higher organisms have a much broader spectrum of morphological structures. Fungi are relatively simple higher organisms that can be handled as microorganisms. For this reason, fungi are chosen more and more often for this kind of work. For a general review, see BARTNICKI-GARCIA[1].

Further study of two strains in the Leiden collection (isolated by Dr. E. Pees) (see ref. 2) recorded as unable to make heterokaryons showed that they cannot produce perithecia either, not even in the rare cases when heterokaryosis could be induced. Once a heterokaryon is established between a perithecial and a non perithecial strain, it grows quite normally but makes only two kinds of perithecia: crossed perithecia and perithecia of the self-fertile strain. Since both heterokaryon formation (anastomosis) and perithecium initiation (clamp connection) depend on changes in the cell wall, it was of interest to analyse the latter's gross chemical composition. Biochemical analysis of the cell wall of *Aspergillus nidulans* is particularly interesting from the point of view of developmental genetics and as a complement to the extensive genetic research on this organism.

MATERIALS AND METHODS

Organism and growth conditions

A biotin-dependent strain of *A. nidulans* (Eidam) (bi A_1) of Glasgow origin was used throughout. The medium and the conditions used were as described by PONTECORVO[3]. For enzyme and cell wall preparations, cultures were grown in aliquots of respectively 0,5 or 5 l of liquid medium supplemented with biotin. *Schizophyllum cummune* strain K8 was grown (for isolating α-1,3 glucan) in 5 l liquid cultures[4].

Preparation of extracellular enzyme extracts

Cultures were grown for 5 days with strong aeration. The filtered medium was mixed with 2 vol. of cold acetone (−18°) and allowed to stand for 2 h at −18°. The precipitate was collected by centrifugation, resuspended in a small volume of water, and dialysed against distilled water at 4°. A precipitate which formed was removed by centrifugation and the supernatant fluid was used for the assay of glucanases.

Preparation of cell walls

Cultures were grown in 5 l of medium under aeration for 2 days. The mycelium was collected by centrifugation, and washed twice with distilled water. It was then suspended in an equal volume of water and homogenized in a Servall Omnimixer for 15 min while immersed in an ice-bath. The homogenate was centrifuged for 5 min at 4000 rev./min and the supernatant fluid discarded. This procedure was repeated until the supernatant fluid became clear. No cytoplasmic contamination could be detected by microscopic observation of preparations stained with cotton blue. The cell wall fraction was then washed with 96% ethanol and dried.

Fractionation of the cell walls

Dried cell wall material (10 g) was first treated with boiling diethyl ether and then with diethyl ether–ethanol–HCl (100:100:1, by vol.) as described by JOHNSON[5] for lipid extraction. The lipid was measured by weight. The cell walls were then heated for 30 min at 75° with 0.5 M acetic acid (200 ml). After washing with acetic acid and water, they were extracted with 5% KOH (200 ml) for 18 h at 37°. After centrifugation, the pellet was washed with 5% KOH. The sediment was boiled with 10% KOH for 30 min, centrifuged, and washed with water. This sediment is called the alkali-insoluble fraction. It contains glucan but also chitin and some galactose and mannose polymers. The hot and cold alkali supernatant fluids combined with the water washings were separately adjusted to pH 7 with glacial acetic acid. The 5% KOH extract gave a copious white flocculent precipitate which was centrifuged, washed 3 times with water and dried. This is called alkali-soluble glucan or S-glucan. Upon neutralization, the hot alkali extract gave some brownish precipitate; this was also washed and dried.

Chemical hydrolysis of cell wall material

1 g of either the alkali-insoluble fraction or complete cell wall material was dissolved in 40% H_2SO_4 (v/v) and stored at 4° for 18 h. The solution was then diluted 8 times with water and boiled for 3 h. The acid hydrolysate was centrifuged to remove insoluble material and neutralized with $BaCO_3$. The mixture was centrifuged, the

sediment washed with water, and this washing combined with the supernatant fluid. The neutralized hydrolysate was de-ionized on a column packed with Dowex 1 (Cl^- form) and Dowex 50 (H^+ form) and then dried *in vacuo*. The foregoing procedure was not sufficient for the hydrolysis of the complete cell walls and was therefore followed by an extraction of the residue of the H_2SO_4 extraction with 10% KOH for 30 min at 100°. This extract was neutralized with glacial acetic acid, after which the precipitate was washed and hydrolysed with 2% HCl for 1 h at 100°. The residue remaining after the alkali extraction contains only chitin, and this was measured by weight. The hydrolysate was dried *in vacuo* and resuspended in a small volume of water. S-glucan was hydrolysed according to JOHNSTON[6].

Enzymatic hydrolysis

For this purpose we used an enzyme preparation of *A. nidulans* prepared as described above and fractionated on a Sephadex G-100 column (1.5 cm × 75 cm). In addition α-amylase (source unknown), β-amylase (Merck), and glucamylase (Merck) were used, untreated or after fractionation. The α-amylase and glucamylase were free of laminarinase but not the β-amylase. A 0.5-ml aliquot (5 mg/ml) of an insoluble substrate or 0.4 ml (1.25 mg/ml) of a soluble substrate were incubated with 0.5 or 0.1 ml, respectively, of the 2-ml fractions of the column, for 3 h at 37°. All enzymes and substrates were dissolved in McIlvaine buffer (0.05 M, pH 6.2). The activity was estimated either as an increase in reducing power with neocuproine[7] or by reacting the supernatant fluid of incubation mixtures with anthrone[8] after sedimenting the remaining insoluble substrate.

Chromatography

For the qualitative sugar determination, thin-layer plates of Silica gel were irrigated with butanol–acetone–water (4:5:1, by vol.) for 1 h, and after drying, with chloroform–actic acid–water (10:7:1, by vol.) in the same direction for 1.5 h. The detecting reagent was aniline hydrogenphtalate.

For the qualitative determination of amino sugars, thin-layer plates of Silica gel were irrigated with propanol–water–ethyl acetate–ammonia (6:3:3:1, by vol.) for 4 h. The detecting reagent was ninhydrin in acetone (0.2%).

For the quantitative determination of mannose and galactose, the hydrolysate was first incubated with glucose oxidase to oxydize the glucose. The mannose was then separated from the galactose by chromatography on Whatman paper (No. 1) in propanol–ethyl acetate–water (7:1:2, by vol.) for 20 h. The spots corresponding to authentic mannose and galactose were cut out and eluted with water.

Amino sugars

After removal of the polysacharides, the chitin was hydrolyzed with 6 M HCl for 16 h at 100° in sealed tubes. The hydrolysate was decolourized with activated carbon, dried *in vacuo*, resuspended in water, and used for chromatography.

Sugar determination

Total sugars were determined with the anthrone reagent with the appropiate sugars as standards[8]. Increase in reducing power was measured with the neocuproine reagent[7]. Glucose and galactose were determined with the Glucostat and the Galac-

tostat reagent (Worthington Biochemical Co., N.J.) Standards and polysacharides were subjected to the same chemical hydrolysis procedure before colorimetric measurements.

Miscellaneous methods

For periodate oxidation[9], 5 ml 0.3 M anhydroglucose was incubated with 5 ml 0.5 M $NaIO_4$ in the dark at 37°. At various times, aliquots of 0.1 ml were taken and diluted in 8.3 ml water and the absorbance read in a spectrophotometer (Unicam SP 500) at 223 nm. Values are corrected for the absorbance of IO_4^-.

Optical rotation was measured with 25 mg S-glucan in 5 ml 1 M NaOH.

Infrared spectra were made by the KBr disc technique.

Protein was measured with the Folin–Ciocalteau reagent with bovine albumin as a standard[10].

RESULTS

Composition of the complete cell wall

Table I gives the amounts of the various components measured. The neutral carbohydrate part consists of 92% glucose, 4.6% galactose, and 3.4% mannose. Where the cell wall was fractionated, the findings are given according to solubility in Table II. Since the alkali-insoluble fraction and S-glucan account for more than half of the weight of the cell wall material, they were investigated further. The bonds of these carbohydrates were of special interest, because they could serve to reveal correlations with the carbohydrate-splitting enzymes.

TABLE I

COMPOSITION OF THE COMPLETE CELL WALL OF *A. nidulans*

Constituents	*Percent dry wt. of complete cell wall*
Neutral carbohydrates	57.6
Aminosugars	19.1
Proteins	10.5
Lipids	4.6
Recovery	91.8

TABLE II

CARBOHYDRATE FRACTIONS IN THE CELL WALL OF *A. nidulans* WITH DIFFERENT SOLUBILITY CHARACTERISTICS AS MEASURED WITH ANTHRONE

Fraction	*Percent dry wt. of complete cell wall*
(I) Alkali-soluble fraction (S-glucan)	22.0
(II) Alkali-resistent fraction	21.0
(III) Hot-alkali-soluble fraction	5.6
(IV) All other carbohydrate fractions pooled	9.1

Structure of the alkali-soluble fraction or S-glucan

S-glucan comprises 22% of the cell wall. It dissolves quickly at room temperature in 5% KOH. S-glucan is not water soluble, even after prolonged heating. Heating at 100° with 2% HCl hydrolyses most of the S-glucan in about 1 h to glucose. Hydrolysis with H_2SO_4 too gives only glucose on the chromatograms. S-glucan and an alkali-soluble fraction of *A. niger* (nigeran) were partially hydrolysed according to JOHNSTON[6]. In both cases nigerose was found on the chromatogram, indicating α-1,3 bonds. The S-glucan and nigeran are not attacked by the following enzymes: α-amylase, glucamylase, and laminarinase. Where the partially hydrolysed fraction was incubated with glucamylase, the nigerose spot disappeared on the chromatogram. This is in agreement with the results of PAZUR AND KLEPPE[11], who found that glucamylase could hydrolyse nigerose. Periodate oxidation of the S-glucan, with nigeran (alternating α-1,3 and α-1,4 bonds) and amylose (α-1,4) as references, gives a low periodate consumption (0.11 mole per mole anhydroglucose) as compared with nigeran (0.48 mole per mole anhydroglucose) and amylose (0.88 mole per mole anhydroglucose), thus indicating that there are few, if any, α-1,4 bonds in S-glucan. Further indications of the presence of α-1,3 bonds in S-glucan are the high dextrorotation $[\alpha]_D$, + 220, in 1 M NaOH and the infrared spectrum (Fig. 1). For comparison there is also an infrared spectrum of S-glucan of *S. commune*, which contains solely α-1,3 bonds[4] and has an $[\alpha]_D$ of + 202 (see ref. 12). In Fig. 1 there is an absorption band at 840 cm^{-1} indicating the presence of an α linkage. The other absorption band, lying near 815 cm^{-1}, and the absence of one near 770 cm^{-1} (found in nigeran and glycogen, see MORENO *et al.*[13]) may be characteristic of an α-1,3 glucan[14].

The alkali-insoluble fraction

The alkali-insoluble fraction comprises about one-third of the cell wall and contains the components shown in Table III. Thus, the bulk of the neutral carbohy-

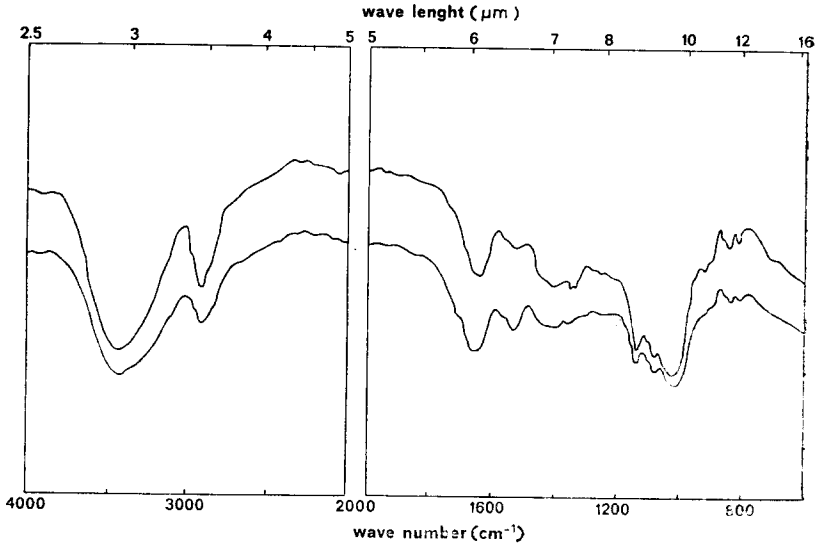

Fig. 1. Infrared spectra of S-glucan of *A. nidulans* (above) and *S. commune*.

drates consist of glucose, as measured with the Glucostat in the hydrolysate. Incubation for 45 min in 2% HCl at 100° dissolves all the neutral carbohydrate of the alkali-insoluble fraction. It makes no difference whether the galactose and mannose contents are measured in the complete cell wall or in the alkali-insoluble fraction. This means that the alkali-resistent component contains all the galactose and mannose of the cell wall.

Several enzymes are capable of attacking the glucan part of the alkali-insoluble fraction as shown by the results in Table IV. It should be noted that α-amylase and glucamylase were free of laminarinase but not β-amylase. Table IV also shows that at least 18% of the polyglucose of the alkali-insoluble fraction has α-1,4 bonds and 15% β-1,3 bonds.

TABLE III

THE COMPOSITION OF THE ALKALI-INSOLUBLE FRACTION OF *A. nidulans*

Constituents	*Percent dry wt. of alkali-insoluble fraction*
Aminosugars	45.0
Glucose	39.1
Galactose	6.8
Mannose	4.9
Protein	0.3

TABLE IV

DISSOLUTION OF THE ALKALI-INSOLUBLE FRACTION WITH DIFFERENT ENZYMES, AS MEASURED WITH ANTHRONE.

Incubation time for the amylase was two 24-h periods, the second with fresh enzyme. Incubation with laminarinase of *A. nidulans* (after the amylase incubation) lasted 24 h.

Enzyme	*Percent of the glucan part dissolved*	*Percent dissolved by laminarinase*
α-Amylase	13.0	14.9
β-Amylase	27.2	6.1
glucamylase	18.5	14.9

Extracellular extracts of *A.nidulans* contain various amylases and laminarinases capable of attacking the alkali-insoluble fraction, as shown by Figs. 2a and 2b for two different enzyme preparations. It can be seen from Figs. 2a and 2b that there are three laminarin-degrading enzymes in *A. nidulans*. Because the second peak also attacks lichenan (the third does not) it is highly probable that the third peak is an exo-laminarinase. This third peak reflects a very clear attack on the alkali-insoluble fraction (Fig. 2a).

Fig. 2b concerns a different enzyme preparation that contains much more amylase. The reason for this is a difference in the number of spores used for inoculation, giving a difference in physiological age.

In Fig. 2b the second high amylase peak lies at the same place as the activity

towards the alkali-insoluble fraction. Therefore these seem to be attacked by the same enzyme, an amylase. This was confirmed by incubation of the alkali-insoluble fraction with known amylases. Furthermore, other enzyme experiments have provided some indications that there are also β-1,6 linkages in the alkali-insoluble fraction.

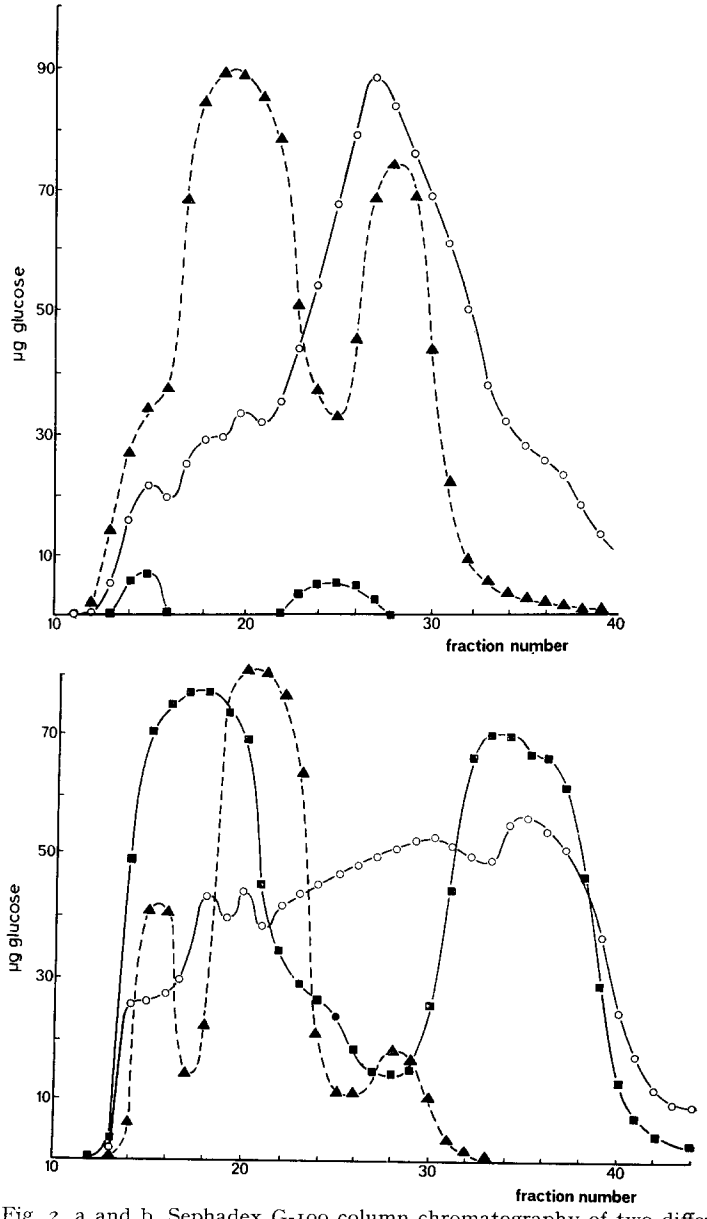

Fig. 2. a and b. Sephadex G-100 column chromatography of two different enzyme preparations from *A. nidulans* incubated with the alkali-insoluble fraction (5 mg/ml, 0.5 ml + 0.5 ml enzyme; ○—○), laminarin (1.25 mg/ml, 0.4 ml + 0.1 ml enzyme; ▲----▲) and amylose (1.25 mg/ml, 0.4 ml + 0.1 ml enzyme; ■—■) for 3 h at 37°. Reducing power measured with the neocuproine reagent and expressed as µg glucose. The preparation of b had a higher amylase content than that of a.

DISCUSSION

The literature contains quantitative data on various chemical components of the cell wall of several Aspergillus species[5,15–19]. One remarkable finding is the high neutral carbohydrate content reported by JOHNSTON[5] for the cell wall of *A. niger* (73–83%). This high level might be explained by the high percentage (3) of carbohydrate he used in his medium as compared with 1% used by RUIZ-HERRERA[15] and the present author.

Although there is some information on the constituent sugars of the cell wall of Aspergillus, little was known about the way in which the different sugars are combined in polymers. While this manuscript was in preparation, however, a publication by BULL[20] on the cell wall of *A. nidulans* appeared. His findings on the gross composition and the way the different sugars are combined in polymers are mostly confirmed by our results, although he used a quite different method to fractionate the cell wall. He did not mention, however, α-1,4 bonds, but it is found by our experiments that these bonds are completely destroyed by the extraction of the alkali-insoluble fraction with hot H_2SO_4 (0.5 M 96° for 16 h according to BULL). Furthermore we used enzymes from the same organism as that from which the cell wall had been isolated. Further HORIKOSHI AND IIDA[16] found in *A. oryzae* a glucan containing β-1,3 and α-1,4 bonds. JOHNSTON[5] distinguished three glucan components in *A. niger*. Two of these were alkali-soluble and one was also soluble in hot water. According to JOHNSTON, the hot-water-soluble component comprises about 8% of the cell wall, has an optical rotation of + 281, and has alternating α-1,3- and α-1,4-glycosidic linkages. The other alkali-soluble component comprises about 25% of the cell wall, has an optical rotation of + 251, and has mostly α-1,3 bonds (2.4 % α-1,4). The alkali-resistant fraction was not characterized further. RUIZ-HERRERA[15] found a glucan in Aspergillus species similar to S-glucan. Our evidence that the S-glucan of *A. nidulans* is an α-1,3-glucan concerns: (1) the high dextro-rotation, (2) the characteristic infrared spectrum, (3) the insusceptibility to amylases and laminarinases, (4) the insolubility in hot water, (5) the presence of nigerose in hydrolysates, and (6) the low periodate consumption.

It is remarkable that so few fungi are capable of lysing this glucan, which occurs not only in Ascomycetes but also in Basidiomycetes[21]. Of the cell wall components, the alkali-insoluble fraction seems to be attacked by the amylases and laminarinases of *A. nidulans*. One of the laminarinases is an endo-laminarinase that attacks laminarin and lichenan, but attacks the alkali-insoluble fraction only slightly or not at all. The question may rise of whether the α-1,4 bonds found are really in the cell wall or are a contamination with, for instance, glycogen. But it seems unlikely that what could not be done by hot alkali (because there would be a wrapper of insoluble material) could be done by amylase, namely dissolving of the glycogen. Besides, α-1,4 bonds are also found in the cell walls of *A. oryzae*[16] and *A. niger*[5].

KUO AND ALEXANDER[22] observed that a combined chitinase–laminarinase enzyme preparation was capable of lysing 96% of the cell wall of *A. nidulans*. But in the light of the results just mentioned it seems probable that other enymes were also present in their preparation. It is also known that glucanases lyse an Aspergillus cell wall much better when used in combination with chitinase[19]. This could explain why only 33% of the alkali-insoluble fraction was hydrolysed (see Table IV). Many

of the controversies about the cell wall composition of certain species may arise from the different growth media used and especially the kind and concentrations of the sugars employed.

It should also be recalled that KANETSUNA AND CARBONELL[14] found that the alkali-soluble glucan fractions of the mycelial and yeast forms of *Paracoccidioides brasiliensis* (grown at 22 and 37°, respectively) were of very different composition. That of the mycelial form contains only α-1,3 bonds, but that of the yeast form contains α-1,3 as well as β-1,3 bonds.

The results seem to indicate that when the processes of perithecium formation are examined, attention should be given to the alkali-insoluble fraction and the enzymes capable of lysing this component. But new data (to be published) indicate that another enzyme is more probably involved in fructification. A profile is now being made of several enzymes that are excreted during various developmental stages in the wild type and in mutants not capable of making perithecia. In *S. commune*, WESSELS[23] also found an enzyme hydrolyzing a fraction of the cell wall and involved in pileus formation. To permit analysis of the influence on fructification, an attempt is now being made to find mutants missing one of the enzymes capable of lysing the cell wall.

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