Chemical Morphology of Glucan and Chitin in the Cell Wall of the Yeast Phase of Paracoccidioides brasiliensis

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Short and thick fibers were observed on the outer surface of the yeast phase of Paracoccidioides brasiliensis, and long and thin fibers were seen on the inner surface. The long fibers disappear with chitinase treatment and are composed of chitin. The short fibers disappear under alkali treatment and are composed of α-glucan. Comparisons with α-(1 → 3)-glucan isolated from Aspergillus niger and Polyergus betulinus and with chitin from fungal origin support our point of view.

Chitin has been chemically identified in the cell walls of different fungi (1, 3, 7, 13). The fine structure of chitin fibrils has been described in Phycomycetes (2) and Polyporus (8). Cell wall glucans of the β-type (5), the α-type (12), or both (4) have been studied in several fungi. The chemistry and ultrastructure of the cell wall of fungi have been reviewed by several authors (5, 9, 15). In this report, we attempt to localize the fibrillar structure of chitin and glucan, the main components of the cell wall of the yeast phase of Paracoccidioides brasiliensis.

MATERIALS AND METHODS

The yeast phase of P. brasiliensis (IVIC-Pb9) was inoculated into a GGY medium (2% glucose, 1% glycine, and 0.2% yeast extract), and was grown at 37 C in 250-ml Erlenmeyer flasks which were placed on a reciprocal shaker at a speed of 100 oscillations/min with a stroke amplitude of about 5 cm. After 3 days of growth, the fungus was harvested by low-speed centrifugation and fixed for 24 hr in 2% glutaraldehyde, 0.1 M phosphate buffer (pH 7.2). After fixation, the fungus was dried by passing it through a series of graded alcohols, ethyl alcohol-ether (1:1), and then allowing the ether to evaporate at 37 C.

In screw-cap tubes, 100 mg (dry weight) of the fungus was mixed with chitinase (EC 2.3.1.14; Calbiochem, Los Angeles Calif.; 1.5 mg/ml) in 6 ml of 0.1 M buffer acetate, pH 5.0, to which three drops of toluene were added to avoid bacterial growth. The tubes were placed in a vertical rotary wheel (10 rev/min) which was immersed in a water bath and incubated at 37 C for 3, 7, and 14 days. After 3 days of incubation, two test tubes were centrifuged at 8,000 X g for 15 min. One of the sediments was used for electron microscopic studies, and the supernatant fractions and other sediment were used for biochemical studies. This procedure was repeated for the 7-day experiment. Four test tubes were used in the 14-day experiment. After 7 days of incubation, the supernatant fractions in all four tubes and one of the sediments were used for glucosamine and glucose determination. Another sediment was used for ultrastructural studies, and the two remaining sediments were mixed again with chitinase and buffer and incubated for 7 days more. After the 14th day, the two supernatant fractions and one of the sediments were used for chemical analyses, and the remaining sediment, for ultrastructural studies.

Two test tubes with 100 mg (dry weight) of yeast were mixed with 6 ml of 1 N NaOH, for 7 hr at 37 C in a vertical rotary wheel. The material for electron microscopic and biochemical studies was taken in the same way as described for 3rd and 7th day experiments with chitinase. Controls with buffer and toluene were run in the same way for 14 days.

After these treatments, the fungus was frozen with liquid nitrogen, ground in a mortar, and suspended in distilled water. The effects of the various treatments were assessed by allowing drops of aqueous suspension of the yeast residue to dry on collodion-coated grids. These grids were shadowed with platinum-vanadium (80:20), and were examined with a Hitachi HU-11B electron microscope. Material to be sectioned was embedded in Maraglas and stained with lead and uranyl acetate.

Yeast previously fixed in 2% glutaraldehyde was frozen with liquid nitrogen, and thick sections were cut with a diamond knife. The sections were received in distilled water, and a drop of the material was suspended on grids, which were allowed to dry and then were shadowed as previously described (freeze-section-shadow). For comparative purposes, shadowing was performed on the α-(1 → 3)-glucan isolated from Aspergillus niger (12), on the α-(1 → 3)-glucan isolated by Duff (8) from Polyergus betulinus, and on chitin (Edward Gurr, Ltd, London, and Sigma Chemical Co., St. Louis, Mo.).

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The sediments obtained after treatment with chitinase or alkali, as well as the controls, were hydrolyzed in sealed tubes with 1 N HCl at 110 C for 5 to 7 hr. The supernatant fluids were used for hexose determination without previous hydrolysis. Hexose was determined by anthrone reaction (16), with glucose as standard.

For amino sugars, all samples were hydrolyzed in sealed tubes with 6 N HCl at 110 C for 16 hr. After neutralization with NaOH, the amino sugars were determined by the method of Belcher et al. (6), with glucosamine as standard. Correction for losses during hydrolysis was carried out with chitin.

RESULTS

Upon sectioning, the cell wall of the yeast phase exhibited an outer layer composed of fibrillar structures 13 nm thick, and loosely interwoven (Fig. 1). These fibrillar structures give the appearance of a tubule, in that they show two thin, outer electron-dense layers and in between a broad middle layer of low electron density. These fibrils can also be seen attached to a thin, dense, and homogeneous layer or in direct contact with a broad, inner stratum (IL in Fig. 1) which, although devoid of a distinct fibrillar structure, displays dots embedded in a homogeneous substance (Fig. 1). Although the cell wall thickness was difficult to assess precisely because there is no clear limit to the outer surface, the total thickness varies between 0.2 and 0.6 μm.

With the freeze-section-shadow technique, long fibers without a definite arrangement were observed on the cell wall inner surface. Such fibers can be seen in Fig. 2, where the cell wall inner surface is labeled IS. In some cell walls, however, the fibrils tend to accumulate in spots, leaving zones in between where they are less distinct (Fig. 2). The outer surface of the cell wall is amorphous, or shows a coarse network of short fibers (Fig. 3) which sometimes aggregate in bundles.

Yeast cell wall after different treatments. After the 3rd day of incubation with chitinase, the short fibers that are located on the outer surface were more clearly visible and were revealed as bundles (Fig. 4). The bundles of short fibers measure from 20 to 240 nm in width with individual fibers averaging 213 nm in length.

The long fibers observed on the inner surface (Fig. 2) almost disappeared after the 3rd day of incubation with chitinase, and disappeared completely after the 14th day of incubation. Sections of the material showed a clear decrease in cell wall thickness and an increase in electron density. It was difficult to determine whether the diminished thickness of the cell wall was due to extraction of material from the inner or outer surfaces (Fig. 5).

Treatment with sodium hydroxide removed the short fibers completely, leaving the type of fibers described on the inner surface. The shadowed fibers (measuring around 10 nm in width) are loosely interwoven and between them are spaces in which other fibers can be identified. In folded cell walls (Fig. 8), fibers can be seen distinctly on the inner and outer surfaces.

In sections of the material treated with sodium hydroxide, the cell wall is thinner than in the untreated ones.

Fine structure of controls. The α-(1 → 3)-glucan isolated from A. niger shows an interlaced pattern of short fibers, or bundles of fibers, with little of the underlying surface showing. The groups of fibers vary in size from 20 to 200 nm in width, with individual fibers averaging 130 nm in length (Fig. 7).

The α-(1 → 3)-glucan of P. betulinus shows an interlaced pattern of fibers, or bundles of fibers, varying in width from 10 to 108 nm. The length of the fibers varies greatly due to the interlaced pattern (Fig. 6).

Commercial chitin (Fig. 9) appears as a conglomerate of interlaced bundles of large fibers with no underlying surface.

Chemical analyses. Biochemical data from the different treatments performed on the yeast are summarized in Table 1. It can be noted that chitin hydrolysis increased with time under the action of chitinase, with more than 50% being hydrolyzed in the first 72 hr. Material incubated for 21 days did not show significant differences from that incubated for 14 days. Between 10 and 15% of the chitin was not hydrolyzed after 14 days of incubation. The action of alkali removed 86% of the total amount of glucan, leaving from 10 to 15% of alkali-insoluble glucan.

DISCUSSION

The cell wall of the yeast phase of P. brasiliensis shows two types of fibers: a short and thick type of fiber on the outer surface and a long and thin fiber on the inner surface.

Under alkali treatment, the disappearance of the short fibers located on the outer surface of the cell wall, together with a high yield of glucose in the supernatant fluid and a low yield in the residue, demonstrate that this layer is mainly composed of an alkali-soluble glucan.

α-Glucan has been identified as the alkali-soluble glucan of the yeast phase of P. brasiliensis (14). The fact that the short fibers located on the outer surface are alkali-soluble suggests that they are α-glucan. Furthermore, the ultrastructural comparisons of the independently chemically identified alkali-soluble α-(1 → 3)-glucans of A. niger (12) and P. betulinus (8) with the one
FIG. 1. Section of the cell wall of the yeast phase of Paracoccidioides brasiliensis. An outer fibrillar layer loosely interwoven lies on a thin electron-dense layer. The fibrils (F) show two electron-dense layers and a middle layer of low electron density. The inner layer (IL) of the cell wall is broad and translucent and is in direct contact with the cytoplasmic membrane (CM).

FIG. 2. Section of a yeast treated with the freeze-section-shadowing technique. Note clearly that the inner surface (IS) of the cell wall is fibrillar.
FIG. 3. Shadowing of the outer surface of the cell wall. Observe the short and thick fibers.

FIG. 4. Shadowing of the cell wall after 3-day incubation in chitinase. Only short and thick fibers revealed as bundles can be seen.

FIG. 5. Section after 7 days of incubation in chitinase. Note the difference in thickness of the two yeast cell walls. The thinner cell wall shows the beginning of a bud (B). Between the two cell walls, abundant fibrils can be seen. The cytoplasmic details (C) are blurred.
Fig. 6. Shadowing of the α-(1 → 3)-glucan of Polyporus betulinus.
Fig. 7. Shadowing of α-(1 → 3)-glucan isolated from Aspergillus niger.
Fig. 8. Shadowing of a folded cell wall treated with alkali. Observe the presence of the same type of fibers on the inner (IS) and outer surface (OS) of the cell wall.
Fig. 9. Shadowing of commercial chitin of fungal origin.
described in *P. brasiliensis* suggest that the short fibers are composed of $\alpha$-($1 \rightarrow 3$)-glucan.

"Rodlets," a type of fibers similar to the short fibers described here, have been observed in the surface of different *Penicillium* conidia (10).

The short alkali-soluble fibers described in *A. niger* (20 to 200 nm) are thicker than the fibers found in *P. betulinus* (10 to 108 nm). These data are relevant, since the former is an *Ascomycete* and the latter is a *Basidiomycete*. Study of the fine structure of the fibrils of the cell wall of fungi could help, as does the study of their chemical constituents (5), to clarify the taxonomy of fungi.

The hydrolysis of the long fibers located on the inner surface of the cell wall by chitinase, together with the high yield of glucosamine in the supernatant fluid and the low yield in the residue, demonstrate that the constituent of these fibers is chitin. Furthermore, this observation correlates with the results of Moreno et al. (14), who identified the presence of chitin in the cell wall of the yeast phase of *P. brasiliensis*.

The action of chitinase on the yeast phase of *P. brasiliensis* seems to be almost the same after the 3rd and 7th days of treatment. Furthermore, on the 14th day there is an increase of chitin hydrolysis which suggests that the change of incubation mixture after the 7th day is responsible for the phenomenon. The high concentration in the supernatant fluid of the products for its action could inhibit the action of chitinase (17). It is worthwhile to point out that there is a residue of chitin remaining even when fibrils identifiable as chitin can no longer be seen. For instance, after 7 days of incubation with chitinase, the cell wall shows almost no fibrils and yet there is 41.25% of the total amount of chitin. After 14 days, there is still 14.4% chitin in the cell wall and no fibrils are detectable. Houwink (11) found 7% chitin in the cell wall of *Saccharomyces cerevisiae*, and no visible changes on electron micrographs after chitin extraction.

Most of the so-called alkali-insoluble glucan in fungi has been identified as $\beta$-glucan (5). After alkali treatment in the present study, about 15% alkali-insoluble glucan remained mixed with the chitin. It was not possible to observe any difference between this glucan and the chitin.

Kanetsuna et al. (13) have demonstrated that the cell wall of the yeast and mycelial phases of *P. brasiliensis* have the same amount of glucan, but that the ratio of alkali-insoluble glucan varies. Comparative ultrastructural studies of these glucans in both phases could produce interesting data about the intrinsic phenomenon of the thermal dimorphism in the fungus under study.

### Table 1. Percentage of hexose and amino sugar which remained in the insoluble material after the action of chitinase and alkali on the cell wall of the yeast phase of *Paracoccidioides brasiliensis*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hexose</th>
<th>Amino sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>92.0</td>
<td>41.6</td>
</tr>
<tr>
<td>7 days</td>
<td>91.5</td>
<td>41.0</td>
</tr>
<tr>
<td>14 days</td>
<td>86.8</td>
<td>14.4</td>
</tr>
<tr>
<td>NaOH, 7 hr</td>
<td>14.0</td>
<td>97.9</td>
</tr>
<tr>
<td>Control, 14 days</td>
<td>99.2</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* The percentage of the insoluble sugars was calculated from the amount of sugar in the supernatant fluids and sediments after treatment. Hexose was determined as glucose and amino sugar as glucosamine.

* Only buffer and toluene were used in the control.

Thermal dimorphism in *P. brasiliensis* is due to complex mechanisms which are related not only to the chemical structure of the different glucans isolated but also to the fine structure and location of glucan in the cell wall.

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**LITERATURE CITED**


