Biochemical Studies on the Thermal Dimorphism of *Paracoccidioides brasiliensis*

FUMINORI KANETSUNA, LUIS M. CARBONELL, ICHIRO AZUMA, AND YUICHI YAMAMURA

Center of Microbiology and Cell Biology, Instituto Venezolano de Investigaciones Científicas, Apartado 1827, Caracas, Venezuela, and The Third Department of Internal Medicine, School of Medicine, Osaka University, Osaka, Japan

Received for publication 5 January 1972

The biochemical and morphological changes of the yeastlike (Y) form to the mycelial (M) form of *Paracoccidioides brasiliensis* were examined. The main polysaccharide of hexoses of the Y-form cell wall was α-glucan, whereas the polysaccharides of the M-form cell wall were β-glucan and galactomannan. The α-glucan of the Y form contained mainly α-(1 → 3)-glycosidic linkage. The β-glucan of the M form contained mainly β-(1 → 3)-glycosidic linkage with a few branches at C-6 position. The incorporation of 14C-glucose into the cell wall glucans showed that synthesis of α-glucan decreased rapidly after the temperature of the culture was changed from 37 to 20 C. The synthesis of β-glucan was augmented at an early stage of the morphological change. The M-form cell wall contained 12 times more disulfide linkage than the Y form. The cell-free extracts of the whole cell of the Y form had five times more protein disulfide reductase activity than the M form, whereas extracts of the M form contained five to eight times more β-glucanase activity than the Y form. From these results, a hypothesis for the production of the M form from the Y form is proposed.

*Paracoccidioides brasiliensis* shows thermal dimorphism: a yeastlike (Y) form at 37 C and a mycelial (M) form at 20 C (27). The morphological changes of the fungus were studied extensively by Carbonell et al. (11–14), using the electron microscope. However, the biochemical mechanism of the morphological changes of the fungus is not yet clear, although Nickerson proposed the formation of the M form from the Y form as a result of the selective inhibition of cell division without simultaneous growth inhibition (27, 31).

Both forms of *P. brasiliensis* contain enzymes of glycolysis, the hexose monophosphate shunt, and the citric acid cycle, suggesting that they may utilize glucose through the same metabolic pathways (20). The main cell wall constituents of both forms are lipids, chitin, glucans, and proteins (23), and the main cell wall glucan of the Y form is α-glucan whereas the M-form cell wall contains larger amounts of β-glucan (21). This change of cell wall glucans may play an important role in the dimorphism of the fungus. However, more detailed information on the cell wall components and several enzymatic activities which may concern the cell wall are required for understanding the dimorphism.

In this paper, chemical structure of α- and β-glucans of the cell walls, the time course of the thermally induced change of glucans, and several enzymatic activities of cell-free extracts of both forms are described. A hypothesis for the thermal dimorphism of *P. brasiliensis* is also proposed.

**MATERIALS AND METHODS**

The Y and M forms of *P. brasiliensis* (strain 7193, Instituto Nacional de Tuberculosis, Caracas) were obtained as described previously (20). The cell walls of both forms were prepared by the combined use of a French press and a sonic oscillator as described previously (21, 23).

Fractionation of cell walls. A schematic representation of the various treatments and agents applied to the cell wall of both forms is shown in Fig. 1.

Y-form cell wall. The Y-form cell wall (4 g) was suspended in 200 ml of 1 N NaOH and stirred for 1 hr at 20 C. After centrifugation at 8,000 × g for 10 min, the precipitate was treated with 100 ml of 1 N NaOH three times as described above. The alkali-insoluble residue was washed with water, ethyl alco-

---

1 This report was presented in part at the First Pan American Symposium on Paracoccidioidomycosis, Medellín, Colombia, 25–27 October 1971.
DIMORPHISM IN P. BRASILIENSIS

Yeastlike form
Cell wall (4.0 g)

1N NaOH

Extract

Insoluble residue
(Fr.Y-1; 1.97 g)

Neutralization

Supernatant
(Fr.Y-3; 0.06 g)

Precipitate
(Fr. Y-2; 1.51 g)

Mycelial form
Cell wall (5.0 g)

1N NaOH

Extract

Insoluble residue
(Fr.M-1; 3.50 g)

Neutralization

Supernatant
(Fr.M-3; 0.70 g)

0.5 M Acetic acid (90°C)

Precipitate
(Fr. M-2; 0.016 g)

Insoluble residue

Extract

Chitinase

Insoluble residue

1N NaOH

Extract

Supernatant
(Fr. M-6; 0.10 g)

Neutralization

Precipitate
(Fr. M-4; 0.81 g)

FIG. 1. Fractionation of the cell walls of the yeastlike and mycelial forms of Paracoccidioides brasiliensis. The yield of each fraction is given in parentheses.

hol, and ether, successively (fraction Y-1). From the combined alkaline extracts, glucan was precipitated by neutralization with acetic acid and collected by centrifuging at 12,000 x g for 10 min. Purification of the glucan by precipitation from the alkaline solution (50 ml) by neutralization with acetic acid was repeated two more times. The precipitated glucan was washed with water, ethyl alcohol, and ether, successively (fraction Y-2). The precipitated solutions obtained after removal of glucan from the neutralized alkaline extracts were concentrated by lyophilization, dialyzed against water, and again lyophilized (fraction Y-3).

M-form cell wall. The M-form cell wall (5 g) was treated with 1N NaOH as described above. From the combined extracts, however, only 16 mg of glucan (fraction M-2) was precipitated by neutralization with acetic acid, leaving large amounts of polysaccharides in the supernatant solution. The supernatant solutions were concentrated by lyophilization, dialyzed against water, and again lyophilized (fraction M-3). The alkali-insoluble residue (fraction M-1) was then extracted four times with 100 ml of 0.5M acetic acid at 90°C for 6-hr periods (2, 25, 26), and a small amount (about 18 mg) of polysaccharide was found in the dialyzed extracts. Since preliminary
treatment of the acetic acid-treated cell wall with 1 N NaOH (at 20 or 75 C) or with dimethyl sulfoxide (26) did not extract glucan, 2.65 g of the acetic acid-treated cell wall was treated with chitinase (1 mg/ml) in 200 ml of 0.05 M acetate buffer, pH 5.0, at 37 C for 1 week. Toluene (1 ml) was added to the reaction mixture to prevent bacterial growth. This treatment hydrolyzed about 90% of chitin in the cell wall. About 20% of glucan was also hydrolyzed by glucanases in the commercial chitinase preparation.

After the treatment with chitinase, the insoluble residue (1.65 g) was treated with 50 ml of 1 N NaOH at 20 C for 2 hr periods. From the combined extracts, glucan was precipitated by neutralization with acetic acid and purified as described above (fraction M-4). The supernatant solutions obtained after removal of glucan from the neutralized alkaline extracts were concentrated by lyophilization, dialyzed against water, and lyophilized (fraction M-5). The final alkal-insoluble residue was washed with water, ethyl alcohol, and ether, successively (fraction M-6).

Methylation of glucans and analysis of methylated glucose. The methylation of glucans was performed essentially by the method of Sandford and Conrad (32). Since the alkali-soluble a-glucan (fraction Y-2) of the Y form is insoluble in dimethyl sulfoxide, 200 mg of the glucan of the Y form was suspended in 20 ml of dimethyl sulfoxide at 40 C under nitrogen gas. After the addition of 3 ml of methanesulfinyl anion prepared by the method of Sandford and Conrad (32), the reaction mixture was incubated with continuous stirring for 5 to 7 hr at 40 C. Then, after the temperature was lowered to 20 C, 1 ml of methyl iodide was added dropwise, and the stirring was continued overnight. The reaction mixture was dialyzed against running tap water for 24 hr and evaporated until dry at 40 C under reduced pressure in a rotary evaporator. The above procedures were repeated four times more, and finally the methylated glucan was extracted with chloroform, and after being washed with water the chloroform extract was evaporated until dry. Yield, 140 mg; [a]D 25° = +255° (C, 0.33) in chloroform.

The methylation of the alkali-soluble b-glucan (fraction M-4) (200 mg) of the M form was performed similarly, except that temperature was 20 C instead of 40 C, because the glucan of the M form is soluble in dimethyl sulfoxide. The procedures were repeated three times. Yield, 217 mg; [a]D 4° = 0 (C, 1.0) in chloroform.

The methylated glucans of the Y and M forms did not show a significant absorption band in the 3,500 cm^-1 regions, indicating that most of the hydroxyl group was substituted by the methoxyl group.

To the methylated glucan (10 mg) we added 0.1 ml of 72% sulfuric acid, and the suspension was kept for 1 hr at 4 C. Then, after the addition of 0.8 ml of water, hydrolysis was continued for 7 hr at 100 C. The reaction mixture was passed through a small column (6 by 0.6 cm) of Amberlite IR4B (carbonate form) to remove sulfonium ions and concentrated to about 1 ml. The methylated glucose was reduced with sodium borohydride (10 mg) at 20 C for 2 hr, and, after treatment with a small amount of Dowex 50 (H^+ form), boric acid was removed by codistillation with methyl alcohol. The dried residue was treated with 2 ml of a mixture of acetic anhydride and pyridine (1:1) at 100 C for 1 hr. The reaction mixture was diluted with water, concentrated until dry, and extracted with chloroform. The partially methylated glucitol acetates dissolved in chloroform were analyzed on a glass column (200 by 0.4 cm) containing 3% of nitrit silicone-polyester copolymer (ECNSS-M) on Chromosorb-W (100-120 mesh) at 180 C at a gas flow rate of 60 ml of nitrogen per min, using a gas chromatograph GC-4BP (Shimadzu Seisakusho Ltd., Kyoto, Japan) (8).

The methylated glucan (10 mg) was also methanolyzed by treatment with 1 ml of 5% methanolic hydrogen chloride in a sealed tube at 100 C for 24 hr. The methanolsate was neutralized with silver carbonate, filtered, and concentrated. The syrup residue dissolved in methyl alcohol was examined on a glass column (200 by 0.4 cm) containing 15% 1,4-butanediol succinate polyester on Shimalite-W (60-80 mesh) at 185 C at a gas flow rate of 75 ml of nitrogen per min in a gas chromatograph GC-4BPF (1).

To isolate tri-O-methyl-α-glucose, 40 mg of methylated α-glucan (fraction M-6) was removed with glacial acetic acid, and the a-glucan (30 mg) was reprecipitated as described above. After removal of sulfonium ions, the concentrated hydrolysate was chromatographed on Toyopak filter paper no. 51A (Toyo Kagaku Sangyo, Osaka, Japan) using ascending development for 24 hr in n-butyl alcohol-ethyl alcohol-water (4:1:5, v/v, upper phase). The main spot (Rf, 0.78) was eluted with 50% methyl alcohol, and the crystalline methylated glucose was obtained by concentration. Yield: 26 mg from α-glucan and 29 mg from β-glucan.

Incorporation of 14C-glucose into glucans. About 16 mg (dry weight) of the Y form was inoculated into each of several 500-ml Erlenmeyer flasks which contained 100 ml of the liquid medium composed of 2% glucose, 1% glycerine, and 0.2% yeast extract (Difco) (20). All the flasks were placed on a reciprocating shaker (3-cm amplitude and 120 strokes per min) at 37 C. After 24 hr of culture, uniformly labeled 14C-glucose was added to four flasks (38.8 μCi per flask) and again after 24 hr of culture at 37 C; 5 ml of formaldehyde (35%) was added to the flasks containing 14C-glucose to kill the fungus. The remaining flasks were transferred to water (10 ml), as a (3-cm amplitude and 80 strokes per min), and culturing was continued at 20 C. At 48, 72, 120, 192, and 288 hr after inoculation, 14C-glucose was added to four flasks, and culture was continued for 24 hr at 20 C as described above.

The fungus was collected by centrifugation, washed with water thoroughly, lyophilized, and weighed. The dried fungus was treated with 30 ml of ether five times. Carrier α-glucan (30 mg) of the Y form was added to the defatted fungus, and treatment of the fungus with 20 ml of 1 N NaOH at 20 C for 2 hr was repeated three times. The alkali extracts were neutralized with acetic acid, and the α-glucan was collected by centrifugation. The α-glucan was purified by reprecipitation from alkaline solution (10 ml) by neutralization, followed by washing with water, ethyl alcohol, and ether, successively. The α-glucan was hydrolyzed with 1 ml of 1 N HCl at 105 C.
for 5 hr. After removal of HCl by repeated evaporation in vacuo over NaOH pellets, the residues were dissolved in 1 ml of water, and 0.5 ml of the hydrolysate was used to determine radioactivity in the a-glucan fraction. The remaining hydrolysate was used for paper chromatography.

The alkali-insoluble residue of the fungus was washed with water and treated with Diazyme (2 mg per ml) in 30 ml of 0.05 M acetate buffer, pH 5.0, at 37 C for 7 hr to remove any remaining glycogen. The insoluble residue was washed with water (30 ml) five times and incubated with a 1,3-α-glucanase preparation (2.4 mg of protein) in 10 ml of 0.05 M acetate buffer, pH 5.0, at 37 C for 24 hr to solubilize β-glucan. Five drops of toluene were added to the reaction mixture to prevent bacterial growth. The supernatant solution was obtained by centrifugation at 12,000 x g for 10 min, and the precipitate was washed with 10 ml of water two times. The supernatant fractions and washings were combined and filtered. The filtrate was concentrated to 1 ml by lyophilization, and 0.5 ml was used to determine radioactivity in the β-glucan fraction.

The radioactivity of the α- and β-glucan fractions (0.5 ml) was determined in a Packard Tri-Carb liquid scintillation spectrometer, model 3320, with 10 ml of solvent [10 g of 2,5-diphenyloxazole (PPO) and 30 mg of p-bis(2,5-phenyloxazolyl)-benzene (POPOP) per liter of p-dioxane]. The identification of a radioactive compound such as glucose was performed by paper chromatography. After 24 hr of descending development in n-butyl alcohol-pyridine-water (30:20:15), the paper was cut into 1 cm segments, and radioactivity was determined in 5 ml of the solvent (4 g of PPO and 50 mg of POPOP per liter of toluene) (36).

Disulfide linkage of the cell walls. The cell walls (10 to 20 mg) were suspended in 1 ml of 8 M urea and incubated at 37 C for 1.5, 4, and 5 hr with or without 50 mg of sodium borohydride. After the destruction of borohydride by dropwise addition of 1 M HCl, the resulting hydrolysate groups in the cell walls were determined by the method of Eilman (16) as follows. To the slightly acidified solution (2.5 ml), 1.0 ml of 0.2 M sodium phosphate buffer (pH 8) and 0.05 ml of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) reagent (4 mg of DTNB per ml of 0.05 M phosphate buffer, pH 7) were added. After centrifugation at 12,000 x g for 20 min, the optical density of the clear supernatant solution was determined at 412 nm, and extinction coefficient ε_m = 13,600 was used to estimate the sulphydryl group.

The cystine-cysteine content of the cell walls was also determined by conversion of cystine and cysteine to cysteic acid by dimethyl sulfoxide, followed by automatic amino acid analysis, according to the method of Spencer and Wold (54).

Preparation of cell-free extracts and enzyme assays. Whole cells of the Y and M forms were washed with 0.05 M potassium phosphate buffer, pH 7.0, and ground with about two volumes of glass powder (5 μm, Heat-Systems Company, Nellville, N.Y.) for 10 min. After suspension of the resulting paste in 0.05 M phosphate buffer, pH 7.0, cell-free extracts were obtained by centrifugation at 500 x g for 10 min.

Protein disulfide reductase (EC 1.6.4.4) activity was determined as follows. Ammonium sulfate (13.3 g) was added to the cell-free extract (25 ml), and the precipitate was collected by centrifugation at 12,000 x g for 20 min. The precipitate was dissolved in 5 ml of water and dialyzed against 2 liters of 0.005 M phosphate buffer, pH 7, overnight. The reaction mixture contained 3 mg of ovalbumin, a dialyzed enzyme preparation (5-9 mg of protein), and 0.4 μmole of reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) in a total volume of 2 ml of 0.05 M phosphate buffer, pH 7.0, in a Thunberg tube. After evaporation, the reaction mixture was incubated at 37 C for 1 hr, and 1.0 ml of 0.2 M sodium phosphate buffer, pH 8.0, and 0.05 ml of DTNB reagent were added to the tube. After centrifugation at 12,000 x g for 20 min, the resulting sulfhydryl group was estimated from the optical density at 412 nm of a clear supernatant solution as described above. The tubes containing the heated enzyme preparation (100 C, 10 min) were used as control.

Glucanase activities were determined as follows. The cell-free extracts were dialyzed overnight against 2 liters of water. The reaction mixture contained a substrate (5-6 mg) and an extract (3-5 mg of protein) in a total volume of 3.4 ml of 0.05 M acetate buffer, pH 5.0, incubated at 37 C for 0, 3, and 5 hr. As substrates, laminarin and the β-glucan of the M form were used for β-glucanase activity and the α-glucan of the Y form was used for α-glucanase activity. At the indicated time, 1.0 ml of the reaction mixture was put into 3 ml of water, heated at 100 C for 10 min, and filtered. The reducing power of the filtrates was determined by Somogyi's method (33), with glucose used as a reference. The reaction mixture without substrate was used as control.

Chitinase activity was estimated by a procedure similar to that described for glucanases, except that chitin was used instead of glucans. Incubation was continued for 10 and 24 hr, and the amino sugar in the filtrates was estimated (7).

Analytical procedures. Hexoses, amino sugars, amino acids, total nitrogen, and phosphorus were estimated as described previously (21, 23). The molar ratio of sugars was determined by the method of Wilson (37). Protein was determined by the method of Lowry et al. (24), using bovine serum albumin as standard. β-1,3-α-Glucanase (EC 3.2.1.6) of Basidiomycete QM 806 was prepared as described previously (21). One milligram of the enzyme preparation hydrolyzed 25 mg of laminarin per hr under the conditions described before. Digestion of glucans with snail digestive juice was performed as described previously (23), and the liberated glucose was estimated by Somogyi's method (33) or by glucose oxidase.

Materials. ECNSS-M on Chromosob-W and 1,4-butanediol succinate polyester on Shimalite-W were obtained from Shimadzu Seikakudo Ltd., Kyoto, Japan; laminarin (ex Laminaria hyperborea), from Koch-Light Laboratories, Ltd., England; DTNB, from K & K Laboratories, Inc., Plainview,
N.Y.; uniformly labeled "C-g-glucose, from International Chemical and Nuclear Corp., Irvine, Calif.; chitinase (EC 3.2.1.14), from Calbiochem, Los Angeles, Calif.; digestive juice of Helix pomatia, from Industrie Biologique Française, Gennevilliers, France; Diazyme [glucoamylase (EC 3.2.1.3)], from Miles Chemical Co., Elkhart, Ind.; glucose oxidase (EC 1.1.3.4) and ovalbumin (grade V), from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Fractionation of cell walls. The chemical composition of the cell wall preparations, which is similar to the cell wall composition reported previously (23), is shown in Table 1. On paper chromatograms, the Y-form cell wall hydrosylate revealed only glucose, whereas the M form contained glucose, galactose, and mannose in a molar ratio of 1:0.3:9.6. The chemical composition of the various fractions of the cell walls is also shown in Table 1. Almost all of the glucan which was extracted with alkali from the Y-form cell wall was precipitated by Fehling's solution (fraction Y-3). About 14% of the cell wall glucans of the Y form remained in the alkali-insoluble residue (fraction Y-1).

Treatment of the M-form cell wall with alkali extracted a small amount of glucan which was obtained as an alkali-soluble glucan of the M form. This glucan (fraction M-2) was not analyzed further. The polysaccharide composed mainly of galactose and mannose (glucose:galactose:mannose = 1:12.3:16.5) was found in the supernatant solution (fraction Y-3) after neutralization of the alkali extracts. Sevag's method (35) was applied repeatedly (10 times) to remove proteins from the supernatant solution (680 mg of dry matter containing 427 mg of hexose as mannose), but only 38.2% of polysaccharide was recovered in an aqueous phase. However, since the aqueous phase still contained proteins or peptides, estimated to be as much as 14% from the nitrogen content (2.31%), the polysaccharide fraction (160 mg of hexose as mannose in 20 ml of 0.01 N HCl) was passed through a Dowex 50 column (2.2 by 13 cm, H+ form in water) and the non-adsorbed fraction was collected. After neutralization with NaOH, the nonadsorbed fraction was concentrated by lyophilization, dialyzed against water, and again lyophilized. This purified polysaccharide fraction (144 mg, [α]D25 = +70° in water) contained 97.2% hexose as mannose determined by the anthrone reaction, 0.17% nitrogen, and 0.21% phosphorus. The molar ratio of glucose, galactose, and mannose was 1:8.5:20.6. The polysaccharide was precipitated by Fehling's solution (19), and the molar ratio of glucose, galactose, and mannose of the precipitate ([α]D25 = +75° in water) was 0:1:2.2, which strongly indicates that the polysaccharide is a galactomannan.

Treatment of the alkali-insoluble residue (fraction M-1) of the M-form cell wall with acetic acid extracted small amounts (about 18 mg) of polysaccharides containing glucose, galactose, and mannose in a molar ratio of 1:1.9:3.2. Subsequent treatment of the acetic acid-treated cell wall with chitinase hydrolyzed a part of the glucan. After chitinase treatment, glucan (fraction M-4) was easily extracted with alkali at 20 C and precipitated by neutralization, leaving a small amount of polysaccharide (glucose:galactose:mannose = 1:0.4:0.8) in the supernatant solution (fraction M-5). The final alkali-insoluble residue (fraction M-6) contained a small amount of glucan. The recovery of polysaccharides was about 70% in the

Table 1. Chemical composition of various fractions of the cell walls of yeastlike and mycelial forms of Paracoccidioides brasiliensis

<table>
<thead>
<tr>
<th>Component</th>
<th>Yeastlike form</th>
<th>Mycelial form</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell wall Y-1</td>
<td>Y-2</td>
</tr>
<tr>
<td>Total phosphorus (%)</td>
<td>0.15</td>
<td>0.11</td>
</tr>
<tr>
<td>Total nitrogen (%)</td>
<td>4.25</td>
<td>5.25</td>
</tr>
<tr>
<td>Hexose* (%)</td>
<td>44.4</td>
<td>13.4</td>
</tr>
<tr>
<td>Amino sugar* (%)</td>
<td>32.2</td>
<td>70.8</td>
</tr>
<tr>
<td>Amino acid* (%)</td>
<td>8.6</td>
<td>3.0</td>
</tr>
</tbody>
</table>

*The amounts of hexose, amino sugar, and amino acids are estimated as glucose, glucosamine hydrochloride, and alanine, respectively.
case of the M form, contrasting with 101% in the Y form. This may be due to the partial hydrolysis of polysaccharides in a step of the acetic acid treatment.

These results suggest that (i) the β-1,6-glucan which can be extracted with hot acetic acid (25) does not exist in an appreciable amount in the M form, and (ii) the solubility of the M-form glucan in alkali depends on the previous treatment of the cell wall.

**Structure of glucans.** Table 1 shows the chemical composition of the main cell wall glucans (fractions Y-2 and M-4) which were used for the methylation experiments. The alkali-soluble glucan (fraction Y-2) of the Y form showed high dextrorotation in 25% NaOH and might be pure α-glucan since the snail digestive juice and the β-1,3-glucanase preparation did not liberate glucose from the glucan. The infrared spectrum of the Y-form glucan was identical to that of the α-1,3-glucan (21). The M-form glucan (fraction M-4) was also practically pure β-glucan, as seen from the following facts. The specific optical rotation of the glucan was low ([α]D580 = +7° in 1 N NaOH), and its infrared spectrum showed an absorption band at 885 cm⁻¹, due to β-glycosidic linkages, and no adsorption bands at 840 and 815 cm⁻¹ which do appear in α-glucan (21). Snail digestive juice liberated 96% of glucose estimated by Somogyi’s method. β-1,3-Glucanase also solubilized the M-form glucan completely, although only 48% of hydrolyzed glucan was estimated as free glucose by glucose oxidase.

The α-glucan of the Y form mainly contains (1→3)-glycosidic linkage (Fig. 2, Table 2). There is also a small amount of (1→4)- or (1→6)-linkage as shown by peak III in Fig. 2. Due to the small amount of peak III, further analysis was not performed. Although the possibility of an artifact of incomplete methylation or demethylation during hydrolysis is not excluded, there may be few branches at the C-2, C-3, or C-6 position.

β-Glucan of the M form contains mainly (1→3)-glycosidic linkage, and there are a few branches at the C-6 position. Since partial acid hydrolysis of β-glucan did not show the existence of cellobiose (21), peak III in Fig. 2 may show the existence of a small amount of (1→6)-linkage in the β-glucan.

To eliminate the possibility of the existence of 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl glucitol in peak II, which is derived from (1→2)-glycosidic linkage, methyl glucosides were also analyzed by gas-liquid chromatography. Two main peaks were obtained corresponding to α- and β-methyl glucosides of 2,4,6-tri-O-methyl-D-glucose. There were no peaks of methyl glucosides of 3,4,6-tri-O-methyl glucose, indicating that there is no appreciable amount of 3,4,6-tri-O-methyl glucose in the hydrolysates of methylated α- and β-glucans.

Further identification of the (1→3)-glycosidic linkage in the α- and β-glucans was performed by the isolation of 2,4,6-tri-O-methyl glucosylamine by preparative paper chromatography. The resulting crystalline methyl glucosylamine from methylated α- and β-glucans showed the same properties: melting point and mixed melting point, 115 to 117 C; [α]D25 = +91° (5 min) → +76° (1 day) (C, 0.575 in water) in the α-glucan; and [α]D25 = +93° (5 min) → +75° (1 day) (C, 0.605 in water) in the β-glucan. After treatment of the tri-O-methyl glucose (5 mg) with 40 mg of aniline in 0.5 ml of ethyl alcohol for 4 hr in a boiling water bath, the crystalline aniline derivative (N-phenyl-2,4,6-O-methyl-D-glucosylamine) was obtained. It had a melting point of 164 to 165 C.
Table 2. Hydrolysis products from methylated α- and β-glucans of Paracoccidioides brasiliensis

<table>
<thead>
<tr>
<th>Peak</th>
<th>Methyl glucose</th>
<th>Molar ratios</th>
<th>α-Glucan</th>
<th>β-Glucan</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2,3,4,6-tetra-O-methyl-β-d-glucose</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2,4,6-tri-O-methyl-β-d-glucose</td>
<td>105.9</td>
<td>30.3</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>2,3,6- or 2,3,4-tri-O-methyl-β-d-glucose</td>
<td>6.2</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>2,6-di-O-methyl-β-d-glucose(?)</td>
<td>0.8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>4,6-di-O-methyl-β-d-glucose(?)</td>
<td>0.6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>2,4-di-O-methyl-β-d-glucose</td>
<td>0.8</td>
<td>0.9</td>
<td></td>
</tr>
</tbody>
</table>

* From Fig. 2.

β-Glucan in the Y-form cell wall. The treatment of the Y-form cell wall with snail digestive juice or β-1,3-glucanase liberated about 10% of total glucan, suggesting the existence of about 4% β-glucan in the Y-form cell wall. The alkali-insoluble residue of the Y-form cell wall contained 13.4% glucose; due to this fact about 14.5% of the cell wall glucans was not extracted with alkali, and this alkali-insoluble glucan was susceptible to snail digestive juice and β-1,3-glucanase. These results suggest that there may be about 4 to 5% β-glucan in the Y-form cell wall.

Incorporation of 14C-β-glucose into glucans. The results of the incorporation of 14C-glucose into cell wall glucans showed the time course of the change of the cell wall glucans with thermally induced morphological change (Fig. 3). After changing the cultivation temperature from 37 to 20 C, synthesis of α-glucan decreased rapidly while the synthesis of β-glucan was augmented at an early stage of the morphological change. The ratio of the radioactivities of the α-glucan fraction to the β-glucan fraction in the Y-form was 7.2. This ratio dropped to 0.52, 0.25, and 0.08 after 2, 4, and 7 days, respectively, of conversion from the Y-form to the M-form. At 20 C β-glucan was synthesized predominantly.

Disulfide linkage in the cell walls. Disulfide linkage in the cell walls was estimated by two methods. The treatment of cell walls with borohydride, followed by the determination of the produced sulphydryl group, showed that the Y- and M-form cell walls contained 0.29 and 1.92 μmoles of sulphydryl group per 100 mg, respectively. The nontreated cell walls of both forms did not show the existence of free sulphydryl groups, indicating that all the sulphydryl groups formed disulfide linkages in the cell wall preparations.

On the other hand, the cystine-cysteine content of the Y- and M-form cell walls, which was determined by the conversion of cystine and cysteine to cysteic acid by dimethyl sulfoxide followed by amino acid analysis, was 0.25 and 3.40 μmoles of half-cystine per 100 mg, respectively. The discrepancy in the amount of the sulphydryl group of the M form between the two methods may result from the incomplete reduction of the disulfide linkage by sodium borohydride due to the more compact nature of the M-form cell wall, although longer treatment of the cell wall with borohydride did not increase significantly the amount of the sulphydryl group.

Fig. 3. Time course of the change of incorporation of 14C-glucose into α- and β-glucans of Paracoccidioides brasiliensis due to shift of the temperature of culture from 37 to 20 C. At the time indicated by arrows, 14C-glucose was added to culture medium and incubated for 24 hr. The amount of the fungus grown during 24 hr was calculated from the growth curve. Symbols: ○, growth curve; O, counts per minute of α-glucan fraction per milligram of fungus grown during 24 hr; Δ, counts per minute of β-glucan fraction per milligram of fungus grown during 24 hr; ×, ratio of radioactivities of α-glucan fraction to β-glucan fraction.

Downloaded from http://jb.asm.org/ on January 6, 2018 by guest.
Enzymatic activities of cell-free extracts.

Protein disulfide reductase activity was much higher in the Y form. When NADPH was used as a coenzyme, 27.1 and 4.9 nmoles of the sulphydryl group were produced per hr from disulfide linkages by 1 mg of protein of the cell-free extracts of the Y- and M-forms, respectively. NADH had a lower effect: 18.6 nmoles in the Y form and 3.9 nmoles in the M form. Without coenzyme, the Y and M forms produced 6.9 and 1.6 nmoles of the sulphydryl group, respectively. 

β-Glucanase activity was much higher in the M form than in the Y form. With laminarin used as substrate, the Y and M forms liberated 34 and 277 nmoles of glucose equivalent per mg per hr, respectively. When the β-glucan of the M form was used as substrate, only 7.8 and 39.4 nmoles of glucose equivalent were liberated per mg per hr by the Y and M forms, respectively.

Activities of α-glucanase and chitinase were not found in either form.

DISCUSSION

Several attempts have been made to explain dimorphism (31). For example, Nickerson proposed the formation of the M form from the Y form as the selective inhibition of cell division without simultaneous growth inhibition (27). However, it seems to be difficult to learn the detailed mechanism of the morphological changes of dimorphic fungi from the above assumption.

In the case of P. brasiliensis, the most striking change in thermal dimorphism is the change of the glycosidic linkage in cell wall glucans. The main cell wall glucan of the Y form has long chains of α-(1 → 3)-linked glucose, as suggested from the high ratios of 2, 4, 6-tri-O-methyl glucose to 2, 3, 4, 6-tetra-O-methyl glucose and dimethyl glucose, in contrast to the α-1,3-glucan of Polyporus betulinus which has 12 moles of 2, 4, 6-tri-O-methyl glucose and 4 moles of 4, 6-di-O-methyl glucose per mole of tetra-O-methyl glucose (15). The α-glucan of the Y form contains also small amounts of (1 → 4)- or (1 → 6)-glycosidic linkage. The existence of an α-(1 → 4) linkage in α-1,3-glucan of Aspergillus niger has been reported (18, 29).

The occurrence of α-1,3-glucan has been demonstrated in Aspergillus (18, 29), Schizosaccharomyces (3), Polyporus (3, 15), Cryptococcus (3), and Blastomyces (22), and α-1,3-glucan may be distributed more widely in fungal cell walls than has been suspected.

The β-glucan of the M-form cell wall contains mainly β-1,3-glycosidic linkage, and there are a few branches at the C-6 position. Small amounts of the (1 → 6)-glycosidic linkage were also found in β-glucan. The β-glucan used in the present study may be somewhat degraded by the acetic acid treatment, since the β-glucan is weakly positive in the periodic acid-Schiff reaction, in contrast to β-glucans which are isolated from chitinase-treated M-form cell walls without acetic acid treatment and are positive in the periodic acid-Schiff reaction, as described in a previous report (21). A β-1,3- and β-1,6-linked glucan is distributed widely in fungal cell walls (5). Recently, Bacon et al. (2) studied in detail the solubility in alkali of cell wall β-glucan of Saccharomyces cerevisiae. They found that various chemical, physical, and enzymatic treatments of the cell wall affected the solubility of glucans in alkali. In the case of the M form of P. brasiliensis, without previous treatment with chitinase, no appreciable amount of glucan is extracted with alkali. However, after chitinase treatment a part of β-glucan is easily extracted with 1 N NaOH at 20°C. These results indicate that the solubility of β-glucan depends on the previous treatment of the cell walls. The nonreproducible results of alkali-soluble and alkali-insoluble glucans of the M form reported previously (21, 23) may be explained partially by these facts. In contrast with previous results (21, 23) in the present study, almost all β-glucan of the M form is extracted with alkali. This may be due to the effect of the acetic acid treatment of the cell wall as reported for the Saccharomyces cell wall glucans (2).

It may be confusing to divide β-glucans into alkali-soluble and alkali-insoluble glucans which were described in previous papers (21, 23). Therefore, we will use only the term β-glucan hereafter.

In previous papers (21, 23), almost all the glucan of the Y-form cell wall was identified as α-glucan. This may have been due to hydrolysis of β-glucan in the Y-form cell wall by β-glucanases in the commercial chitinase preparation which was used before alkali treatment of the cell wall. The isolation and identification of β-glucan of the Y form were not continued because chitinase preparation free of β-glucanases was not available. However, susceptibility of the glucan in the alkali-insoluble residue of the Y-form cell wall to snail digestive juice and β-1,3-glucanase strongly suggests the existence of about 4 to 5% β-glucan in the Y-form cell wall, because the α-glucan is not hydrolyzed by these enzyme preparations (23).

The localization and function of β-glucan in...
the Y-form cell wall is unknown. However, it is interesting to note that the cell walls of Saccharomyces contain about 1 to 2% chitin located at budding sites (4, 10) and that the Y-form cell wall of Blastomyces dermatitidis contains about 2 to 3% β-glucan (22). We may assume that β-glucan in the Y-form cell walls of P. brasiliensis and B. dermatitidis has a relationship with budding as does chitin in Saccharomyces.

The incorporation of 14C-glucose into glucans shows that the synthesis of α-glucan decreases rapidly when the fungus is transferred from 37 to 20 C, accompanying the concomitant increase of the synthesis of β-glucan. As for the synthesis of α-glucan in the M-form cell wall, the results are not constant. Among five preparations of the M-form cell wall, three preparations did not contain any appreciable amount of α-glucan, and in the other two preparations only about 17% (21) and 60% (23), respectively, of cell wall glucans were of the α-type. In the case of B. dermatitidis, about 60% of the glucans of the M form are α-type (22). The factors responsible for the amount of α-glucan in the M-form cell wall are unknown. Since the existence of α-glucan is not necessary for the production of the M form, the cell wall of hyphae may not contain α-glucan; therefore some segments may have a different composition. For example, the occurrence of chlamydospores (intercalary or terminal) (13) or arthrospores (30) may be one of these factors, because the cell wall of a chlamydospore is much thicker than that of hyphae, suggesting a different chemical composition between them.

The existence of galactose and mannose in the M-form cell wall was reported previously, although these sugars are not detected in the Y-form cell wall (23). The present study shows the existence of very small amounts of galactose and mannose, estimated to be about 1 to 2% of the amount of glucose, in the Y form cell wall also. Such a small amount of galactose and mannose could not be detected on paper chromatograms. The polysaccharide composed mainly of galactose and mannose is extracted with alkali and is soluble in water. The polysaccharide may be a galactomannan, since the precipitation of the polysaccharide with Fehling’s solution does not change significantly the molar ratio of galactose to mannose. The galactomannan may be important for the interpretation of the immunological properties of the fungus as demonstrated in other fungi (17).

The M-form cell wall has more of a disulfide linkage than the Y form. Although the oxidation of the sulphydryl group must occur artifici-
of the deacreases, a-glucan fide reductase be interwoven glucan fibers M-form cell two layers (12).

...Proteins may have no rigidity due to the small amount of disulfide linkage and also due to the active protein disulfide reductase. The chitin strengthens the produced spherical form.

On the other hand, at 20 C, the synthesis of α-glucan decreases at the budding sites and β-glucan fibers grow continuously by the linkage of the glucose unit, one by one, to the nonreducing terminal of β-glucan, forming an apical growth. Proteins may have some degree of rigidity because of the high amount of disulfide linkage and the low activity of protein disulfide reductase and prevent a balloon-like growth of β-glucan. Chitin also may participate in the production of the M form, because β-glucan is not extracted with alkali without previous hydrolysis of chitin by chitinase. In other words, fibers of proteins and chitin may be interwoven with fibers of β-glucan in the M-form cell wall, in contrast to the Y-form cell wall in which α-glucan and chitin form two layers (12). Electron microscope study reveals that in the conversion from the Y form to the M form, the outer layer (α-glucan layer) of the Y form does not continue to the cell wall of the M form, and in general there are no distinct layers in the M-form cell wall (11, 14).

By the above mechanism, all Y-form cells have the capacity to produce the M form as observed in the conversion from the Y form to the M form (11). On the other hand, the conversion from the M form to the Y form occurs in only small parts of the M form (11). Although there may not be sufficient biochemical data to present a hypothesis for the conversion from the M form to the Y form, the information for the synthesis of α-glucan-synthesizing enzymes, protein disulfide reductase, etc., may not be distributed evenly throughout the hyphae, but localized only in some segments. With the transference of the cells from 20 C to 37 C, the synthesis of β-glucan decreases, and the cell wall of some segments may be softened by the combined action of β-glucanase and protein disulfide reductase. Simultaneously, synthesis of α-glucan may occur in all parts of the cell wall of the segments replacing β-glucan, resulting in the Y form.

ACKNOWLEDGMENT

We thank Akira Misaki for valuable suggestions concerning the analysis of the methylated glucans.

LITERATURE CITED


3. Bacon, J. S. D., D. Jones, V. C. Farmer, and D. M.


