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Cell Wall Glucans of the Yeast and Mycelial Forms of *Paracoccidioides brasiliensis*

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Glucans were isolated from the cell wall of the yeast (Y) and mycelial (M) forms of *Paracoccidioides brasiliensis*. The alkali-soluble glucan of the Y form had properties of α-1,3-glucan. The alkali-insoluble glucan of the M form was identified as a β-glucan which contains a β-(1 $\rightarrow$ 3)-glycosidic linkage by infrared absorption spectrum, by effect of β-1,3-glucanase, and by partial acid hydrolysis. The alkali-soluble glucans of the M form were a mixture of α- and β-glucans and the ratio of α- to β-glucan was variable, depending on the preparations.

Paracoccidioides brasiliensis shows thermal dimorphism: a yeast form (Y form) at 37 C and a mycelial form (M form) at room temperature (14). The cell wall of the Y form has larger amounts of chitin and smaller amounts of proteins than the M form (12). Glucans account for about 40% of the cell wall of both forms. However, glucan of the Y form is soluble in alkali, whereas those of the M form are divided into alkali-soluble and alkali-insoluble glucans (12). This difference of glucans between the two forms may be important in explaining thermal dimorphism. Although the alkali-soluble glucans are supposed to be α-1,3-glucan (12, 13), the glycosidic linkage of the alkali-insoluble glucan of the M form has not been determined.

In this report, the alkali-insoluble glucan of the M form is identified as β-glucan and the alkali-soluble glucans of the M form are found to be a mixture of α- and β-glucans. Several properties of the glucans of the Y and M forms of *P. brasiliensis* are also compared.

MATERIALS AND METHODS

P. brasiliensis (strain 7193, Instituto Nacional de Tuberculosis, Caracas) was used. The Y and M forms of the fungus were obtained as described before (11) and killed in 1% formaldehyde.

Preparation of glucans. The alkali-soluble glucans of both forms were prepared as follows. The whole cells [80 to 100 g (wet weight)] were washed with water by centrifugation at 8,000 $\times$ *g* for 10 min and treated with acetone, ethyl alcohol, and ether, successively. The defatted cells, suspended in 500 ml of water, were treated 10 times with a Branson Sonifier model S-75 (Heat System Co., N.Y.) (20 kc, 10 min at maximum power). The crude cell walls were collected by centrifugation at 8,000 $\times$ *g* for 10 min and washed with water thoroughly. After treating the crude cell walls of both forms with trypsin (EC 3.4.4.4) and chitinase (EC 3.2.1.14) as described previously (12), glucans were extracted three to four times with 100 ml of 1 N NaOH at room temperature for 1 hr and purified by repeated precipitations (three times) from the alkaline extracts by neutralization with acetic acid. The precipitated glucans were thoroughly washed with water, ethyl alcohol, and ether, successively.

The alkali-insoluble glucan of the M form was prepared as follows. The insoluble materials remaining after extraction of the alkali-soluble glucan were treated with 100 ml of 1 N NaOH by reflux for 2 hr. The insoluble materials were washed thoroughly with water by centrifugation at 12,000 $\times$ *g* for 10 min, and incubated again with chitinase (1 mg/ml) in 50 ml of 0.1 M acetate buffer (*p*H 5.0), containing 10 drops of toluene, for 3 days at 37 C. The insoluble residue was washed with water, ethyl alcohol, and ether, successively.

Analytical methods. Chemical analyses and paper chromatographic studies were performed as described before (12), except for the determinations of total phosphorus (5) and amino acids (20). Infrared spectra of glucans were taken in a Perkin-Elmer 337 grating infrared spectrophotometer, by using the pressed KCl disc technique.

Treatment with glucanases. Samples (5 to 6 mg) were incubated with the *Basidiomycete* enzyme preparation, which was prepared as described later, in 1.7 ml of 0.05 M acetate buffer (*p*H 4.8) at 37 C for 0, 0.5, 1, 3, 8, and 15 hr. At the indicated time, 0.05 ml of the reaction mixture was placed into 2 ml of boiling water for 10 min to inactivate the enzyme. The amount of reducing sugar was determined by Somogyi's colorimetric method (17), with glucose as a standard, and corrected by the control experiment which contained only buffer and enzyme preparation. After 15 hr of incubation, the reaction mixtures were passed through small amounts of Dowex 50 (H^+ form) and Dowex 1 (acetate form) with the use of

water as eluent, concentrated by lyophilization, and used for paper chromatographic studies.

Treatment with the *Trichoderma* enzyme preparation, which was prepared as described later, was performed similarly, except for the use of 0.05 M acetate buffer, *p*H 4.5.

Partial acid hydrolysis. This was performed by the method of Johnston (10). Samples (about 20 mg) were treated with 0.3 ml of 98% formic acid in a boiling water bath for 10 min and, after the addition of 3 ml of 0.4 N H_2SO_4, heating was continued for 50 min. After being allowed to cool, the hydrolysates were passed through Dowex 50 (H^+ form) and Dowex 1 (acetate form) with the use of water as eluent, concentrated by lyophilization, and used for chromatographic studies. Thin-layer chromatography of the hydrolysates on cellulose layers was performed by the method of Aronson et al. (1). Development in a mixture of *n*-propyl alcohol, ethyl acetate, and water (7:1:2, v/v) was repeated three times. Sugars were detected with alkaline silver nitrate (18).

Chemicals. α-1,3-Glucan ($[\alpha]_D$ + 224° in 1 N NaOH) of *Aspergillus niger* was prepared by the method of Johnston (10), and α-1,3-glucan of *Polyporus betulinus* (6) was a gift of R. B. Duff (Macaulay Institute for Soil Research, Aberdeen, Scotland). β-Glucan of *Saccharomyces cerevisiae* was prepared by the method of Northcote and Horne (15). Nigeran (ex *A. niger*) and laminarin (ex *Laminaria hyperborea*) were obtained from the Koch-Light Laboratories, Ltd., Colnbrook, England; chitinase, from Calbiochem, Los Angeles, Calif.; trypsin (type II), β-D(+)-cellobiose, and β-gentiobiose, from the Sigma Chemical Co., St. Louis, Mo.

Crude β-1,3-glucanase (EC 3.2.1.6) of *Basidiomycete* species QM 806 was prepared as follows. The culture filtrate (1 liter) obtained by the method of Reese and Mandels (16) was concentrated to about 20 ml by lyophilization and dialyzed against several changes of water (2 liters) for 24 hr. The nondialyzable fraction was lyophilized and used as the enzyme preparation. Crude α-1,3-glucanase (EC 3.2.1 group) of *Trichoderma viride* was prepared similarly from the culture filtrate (500 ml) obtained by the method of Hasegawa et al. (7), by using α-1,3-glucan of *A. niger* in the culture medium to induce the enzyme.

RESULTS

The glucan of the Y form was free of chitin and proteins and showed high dextrorotation (Table 1). The infrared absorption spectrum (absorption maxima: 820, 845, 925, 1,025, 1,085, 1,148, 1,210, 1,350, 1,410, 1,630, 2,910 and 3,350 cm^{-1}) of this glucan was almost identical to that of α-1,3-glucans of *P. betulinus* and *A. niger* (Fig. 1).

The alkali-soluble glucan of the M form had a measurable but small amount of amino acid and glucosamine (Table 1), and showed some variation in several of its properties.

The alkali-soluble M form glucan (samples no. 1 and 2) showed very low dextrorotation, indicating the existence of β-glycosidic linkages. The infrared absorption spectra (absorption maxima: 880, 1,030, 1,070, 1,154, 1,200, 1,250,

TABLE 1. *Composition and properties of glucans of the yeast and mycelial forms of Paracoccidioides brasiliensis*

Determination	Yeast form, alkali-soluble	Mycelial form					
		Alkali-soluble			Alkali-insoluble		
		1[a]	2	3	1	2	3
Yield[b]	1.65	0.31	0.40	0.33	0.10	0.12	0.12
Total phosphorus[c]	0.00	0.03	0.02	0.02	0.03	0.05	0.03
Total nitrogen[c]	0.02	0.30	0.34	0.25	2.94	1.32	2.50
Hexose as glucose[d]	100.2	99.5	98.7	99.0	78.0	91.0	80.1
Amino sugar as glucosamine-hydrochloride[d]	<0.2	0.5	0.7	0.4	1.1	3.1	2.2
Amino acids as alanine[c]	<0.1	1.9	1.4	1.5	12.9	5.3	10.2
$[\alpha]_D$ in 1 N NaOH	+228° (1.050)[e]	+10° (1.030)[e]	+12° (1.070)[e]	+93° (0.955)[e]			
Periodic acid-Schiff reaction	Weakly positive	Positive	Positive	Positive	Positive	Positive	Positive

[a] Number designates sample number.
[b] Expressed in grams from 100 g (wet weight) of whole cells.
[c] Expressed as per cent.
[d] Expressed as per cent. Paper chromatography studies revealed only glucose and glucosamine as hexose and amino sugar, respectively, in all samples.
[e] Concentration generally used.

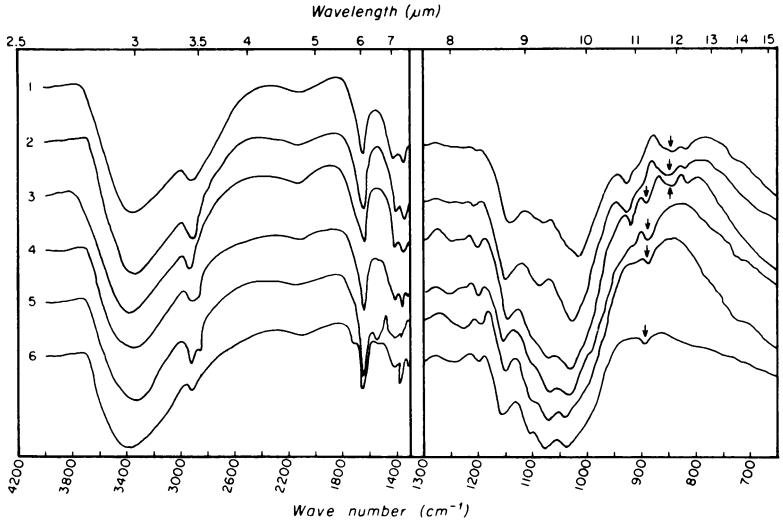

FIG. 1. *Infrared absorption spectra of glucans: 1, α-1,3-glucan of Polyporus betulinus and Aspergillus niger; 2, okali-soluble glucan of the Y form of Paracoccidioides brasiliensis; 3, alkali-soluble glucan of the M form (sample nil. 3) of P. brasiliensis; 4, alkali-soluble glucan of the M form (samples no. 1 and 2) of P. brasiliensis; 5, alkali-anoluble glucan of the M form (samples no. 1, 2, and 3) of P. brasiliensis; 6, laminarin and β-glucan of Saccharomsyces cerevisiae.*

1,360, 1,420, 1,630, 2,890, and 3,380 cm^{-1}) showed an absorption band at 880 cm^{-1} produced by β-glycosidic linkage (3) (Fig. 1, no. 4). However, no absorption bands at 845 and 820 cm^{-1} were observed. Characteristics of the precipitation of β-glucan from the alkaline extract by neutralization were also different from those of the α-glucan of the Y form. Whereas α-glucan was precipitated as white floccules, β-glucan showed as a whole gelatinlike appearance and did not produce white visible floccules.

The alkali-soluble glucan which had $[\alpha]_D$ + 93° was obtained from another lot (sample no. 3) of the culture of the M form. The infrared absorption spectrum of this glucan showed absorption bands at 812, 840, and 890 cm^{-1}, indicating the existence of α- and β-glycosidic linkages (Fig. 1, no. 3).

The alkali-insoluble glucan of the M form was contaminated with appreciable amounts of proteins (Table 1). However, the infrared absorption spectrum (absorption maxima: 883, 1,035, 1,070, 1,150, 1,197, 1,228, 1,360, 1,400, 1,530, 1,625, 2,845, 2,915, and 3,280 cm^{-1}) showed clearly an absorption band at 883 cm^{-1}, indicating a β-glycosidic linkage (Fig. 1, no. 5). There were no absorption bands at 845 and 820 cm^{-1}.

Crude β-1,3-glucanase of *Basidiomycete* species QM 806 hydrolyzed about 76 and 50% of the alkali-soluble and alkali-insoluble β-glucans of the M form, respectively, and effected similar amounts of hydrolysis with other β-glucans (70% of laminarin and 60% of *S. cerevisiae* β-glucan) (Fig. 2). Glucose was the major product of digestion after 15 hr of incubation. The Y form glucan, α-1,3-glucan, and cellulose were not hydrolyzed. Although a crude enzyme preparation was used in this study, the above results strongly suggest the existence of a β-1,3-glycosidic linkage in the β-glucans of the M form.

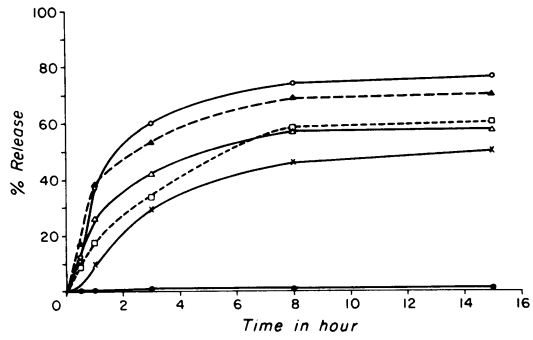

FIG. 2. *Effect of crude β-1,3-glucanase of Basidiomycete species QM 806 on glucans. Per cent release of glucose was estimated by increase of reducing power (17), with glucose as reference, in the reaction mixtures. Symbols: ●, alkali-soluble glucan of the Y form of Paracoccidioides brasiliensis, α-1,3-glucans (Polyporus betulinus and Aspergillus niger) and cellulose; ○, alkali-soluble glucan of the M form (sample no. 1) of P. brasiliensis; △, alkali-soluble glucan of the M form (sample no. 3) of P. brasiliensis; ×, alkali-insoluble glucan of the M form (sample no. 1) of P. brasiliensis; □, β-glucan of Saccharomyces cerevisiae; ▲, laminarin.*

Nonsusceptibility of the Y form glucan to the enzyme preparation indicates that the alkali-soluble glucan of the Y form is practically free of β-glucan.

Crude enzyme preparation of *T. viride* contained high activity of β-glucanases as demonstrated by rapid hydrolysis of laminarin, and hydrolyzed 95 and 73% of alkali-soluble and alkali-insoluble β-glucans of the M form, respectively (Fig. 3). The enzyme preparation also contained α-glucanases. Nigeran, which contains both α-1,3- and α-1,4-linkages (9), was hydrolyzed up to 27% within 15 hr of incubation, and glucose and small amounts of oligosaccharides were liberated. Ninety-eight per cent of glycogen was also hydrolyzed within 3 hr, with liberation of glucose. The enzyme preparation also hydrolyzed about 10% of the Y form glucan of *P. brasiliensis* and α-1,3-glucans of *Polyporus betulinus* and *A. niger*. Glucose was again the major product of digestion. These results suggest that the α-glucan of the Y form of *Paracoccidioides brasiliensis* is similar to the α-1,3-glucans of *P. betulinus* and *A. niger*, although the α-1,3-glucanase activity of the enzyme preparation was low.

The partially acid-hydrolyzed M form glucans show similar chromatographic patterns to those of laminarin and β-glucan of *S. cerevisiae* (Fig. 4). The sugar having R_{Glc} 0.72 may be laminaribiose in agreement with a reported value of R_{Glc} 0.71 (1), although authentic laminaribiose was not available. A sugar having R_{Glc} 0.47 to 0.48 is slightly different from R_{Glc} 0.51 of gentiobiose [reported R_{Glc} for gentiobiose, 0.48 (1)]. However, when authentic gentiobiose was mixed with the acid hydrolysates, there was no separation between gentiobiose and the sugar having R_{Glc} 0.47, suggesting that a slight difference in R_{Glc} may be due to the effects of sulfuric acid or formic acid remaining in the acid hydrolysates. However, the spot with R_{Glc} 0.47 could well be laminaritriose, and the existence of a β-1,6-glycosidic linkage in the M form glucans needs further analysis. Cellobiose was not detected.

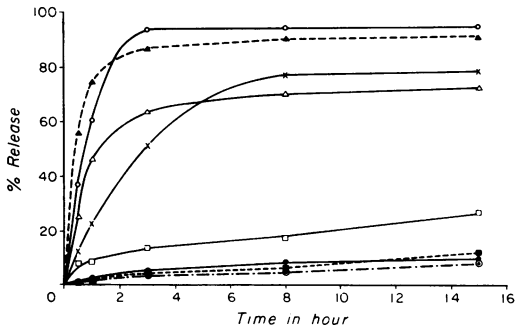

FIG. 3. *Effects of the enzyme preparation of Trichoderma viride on glucans. Symbols:* ●, *alkali-soluble glucan of the Y form of Paracoccidioides brasiliensis;* ■, *α-1,3-glucan of Polyporus betulinus;* ◉, *α-1,3-glucan of Aspergillus niger;* □, *nigeran;* ▲, *laminarin;* ○, *alkali-soluble glucan of the M form (sample no. 1) of P. brasiliensis;* △, *alkali-soluble glucan of the M form (sample no. 3) of P. brasiliensis;* ×, *alkali-insoluble glucan of the M form (sample no. 1) of P. brasiliensis.*

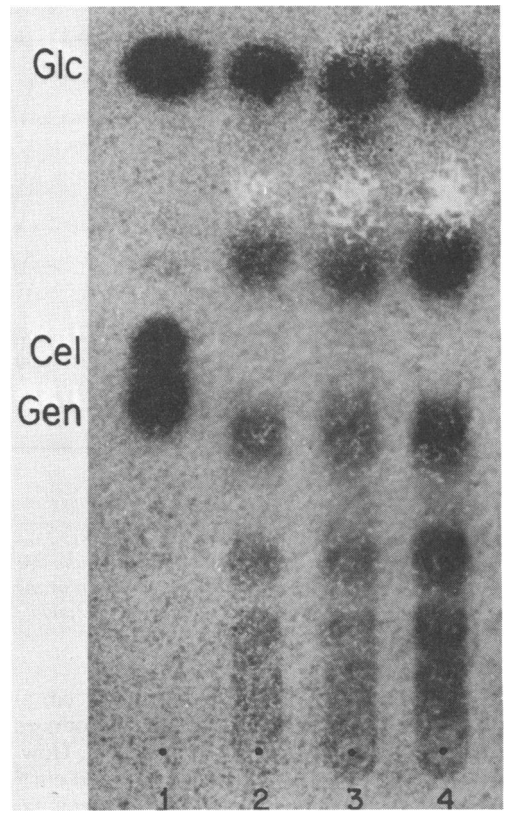

FIG. 4. *Thin-layer chromatograms of partially hydrolyzed β-glucans: 1, glucose (Glc), cellobiose (Cel), and gentiobiose (Gen); 2, alkali-soluble glucan of the M form (sample no. 1) of Paracoccidioides brasiliensis; 3, alkali-insoluble glucan of the M form (sample no. 1) of P. brasiliensis; 4, laminarin. (β-Glucan of Saccharomyces cerevisiae showed identical chromatographic pattern to that of laminarin.)*

DISCUSSION

Almost all of the Y form glucan of *P. brasiliensis* was alkali-soluble, and evidence was previously reported in support of an α-1,3-glycosidic linkage structure (12, 13). In the present study, the infrared absorption spectrum and the effect of crude enzyme preparation of *T. viride* further support evidence of an α-1,3-glycosidic linkage

in the Y form glucan, as compared with those of the known α-1,3-glucans of *P. betulinus* (6) and *A. niger* (10). The infrared absorption spectrum of known α-1,3-glucan is different from those of other α-glucans containing different linkage groups (e.g., nigeran, bacterial dextran, and glycogen) (2). The additional absorption band near 815 cm^{-1} and the absence of the absorption band near 770 cm^{-1} may be characteristic of the α-1,3-glucan.

In contrast to the reported high degree of specificity to α-1,3-glucan of the culture filtrate of *T. viride* (7), glycogen and nigeran, which also contain α-(1 $\rightarrow$ 4)-glycosidic linkage, were also hydrolyzed by our enzyme preparation, and more rapidly than α-1,3-glucan. However, the extent of hydrolysis of the Y form glucan suggests that the Y form glucan is similar to the α-1,3-glucans of *P. betulinus* and *A. niger*.

The properties of the alkali-soluble glucans of the M form are variable. In our previous report (12), a lower dextrorotation of the alkali-soluble glucan of the M form was noted, although other properties were similar to those of the Y-form glucan. This suggests the existence of small amounts of β-glucan in the α-glucan. In the present study, practically pure β-glucans (samples no. 1 and 2) and glucans having α- and β-glycosidic linkages (sample no. 3) were obtained as alkali-soluble glucans of the M form. At present, we do not know the factors responsible for the ratio of α- to β-glucan. These may include the period of time after the transformation from the Y form to the M form, age of the cultures (e.g., autolysis of the alkali-insoluble glucan in older hyphae), and also procedures of the preparation of the glucans (e.g., efficient removal of chitin and proteins before the alkali treatments).

The effect of the *Basidiomycete* enzyme preparation and partial acid hydrolysis showed the presence of β-(1 $\rightarrow$ 3)-glycosidic linkage in the alkali-soluble glucan of the M form. The positive periodic acid-Schiff reaction suggests the existence of other glycosidic linkage(s) in addition to β-(1 $\rightarrow$ 3)-linkage.

The alkali-insoluble glucan of the M form was identified as β-glucan by the infrared absorption spectrum and the effect of the *Basidiomycete* enzyme preparation. The presence of β-(1 $\rightarrow$ 3)-glycosidic linkage was also demonstrated by partial acid hydrolysis. The yield of the alkali-insoluble glucan was low, as compared with the alkali-soluble glucan. We tried to remove proteins from the alkali-insoluble fraction of the M form under milder conditions with various proteinases (trypsin, pepsin, Pronase, bromelain, papain, and fungal protease) without success. Reflux in NaOH removed proteins more effectively than the proteinases used. However, this drastic treatment lowered the yield of the alkali-insoluble glucan, and a part of the glucan was recovered from the extracts. This suggests that the alkali-soluble β-glucan of the M form may have a structure similar to that of the alkali-insoluble β-glucan, and both glucans may be maintained together by proteins. However, a possible structural difference between alkali-soluble and alkali-insoluble glucans is not excluded as reported in bakers' yeast (J. S. D. Bacon and V. C. Farmer, Biochem. J., **110**:34 p, 1968).

Since Schweizer's reagent (8) did not extract glucans from either alkali-soluble or alkali-insoluble β-glucans of the M form, there is presumably no possibility for the existence of cellulose. Partial acid hydrolysis of the M form glucans did not reveal cellobiose on a chromatogram.

β-Glucans which have β-(1 $\rightarrow$ 3)- and β-(1 $\rightarrow$ 6)-glycosidic linkages are widely distributed in fungal cell walls (4), and α-1,3-glucans are now found in various fungal cell walls (2). The coexistence of α- and β-glucans in a fungal cell wall has been demonstrated in several fungi (2, 19). In *Schizophyllum commune*, the coexistence of an α-1,3-glucan and a β-1,3- and β-1,6-linked glucan is reported as S-glucan (alkali-soluble) and R-glucan (alkali-insoluble), respectively, and the importance of these glucans in the morphogenesis of the fungus is demonstrated (19). The alkali-soluble S-glucan seems to be exclusively α-1,3-glucan in *S. commune* as well as in the Y form of *P. brasiliensis*, and the alkali-soluble glucan of the M form of *P. brasiliensis* is a mixture of α- and β-glucans, as well as in *Cryptococcus* and *Schizosaccharomyces pombe* (2).

We assumed that glucans play an important role in the thermal dimorphism of *P. brasiliensis* (12). In addition to the changes of the alkali-soluble glucan of the M form, it is sure that with the transformation of the Y form to the M form, synthesis of β-glucan occurs, suggesting a temperature dependency of the biosynthesis of the β-glucan-synthesizing enzymes. Therefore, we assume that the Y and M forms may be determined by the differences of the physical and chemical properties of the glucans and that chitin may only maintain the produced forms. Electron microscopy studies revealed the morphological difference of glucans, i.e., α-glucan as bundles of short and thick fibers and β-glucans as interwoven long and thin fibers (Carbonell et al., *unpublished data*). At present, we have no reasonable explanation of the morphological difference

between α- and β-glucans. Proteins may participate in the formation of the M form with the β-glucan, as suggested by the intimate coexistence of proteins and β-glucan in the alkali-insoluble fraction of the cell wall of the M form.

To elucidate the temperature dependency of the synthesis of glucans, enzymatic studies on α- and β-glucan-synthesizing enzymes of the Y and M forms of *P. brasiliensis* are in progress.

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