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EXTRACTION AND CHARACTERIZATION OF PROTEINS FROM CELL WALLS OF TWO STRAINS OF *Paracoccidioides brasiliensis.*

(Extracción y caracterización de proteínas de pared celular de dos muestras de Paracoccidioides brasiliensis)

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Palabras clave: ***Paracoccidioides brasiliensis***, proteinas, gp43, pared celular.

SUMMARY

In the present paper we analyzed the proteins from the cell walls of 2 strains of ***Paracoccidioides brasiliensis*** *(PbHC-PE and Pb 18) under yeast-phase. The proteins were extracted by three different methods and were studied by electrophoresis SDS-PAGE. Results of profiles from the two studied strains were different allowing them to be used as chemotaxonomic marker. The transitory secretion of protein gp 43 through the cell wall of both* ***P. brasiliensis*** *strains was observed.*

RESUMEN

En el presente trabajo se analizaron las proteinas de la pared celular de 2 cepas de ***Paracoccidioides brasiliensis*** *en fase levaduriforme (PbHC-PE y Pb 18). Las proteinas fueron extraidas por tres diferentes métodos y estudiadas por electrofóresis SDS-PAGE. Los resultados de los perfiles de las dos cepas fueron diferentes, permitiendo la posibilidad de su uso como marcadores quimiotaxonómicos. Se observó una secreción transitoria de la proteina gp 43 a través de la pared celular de las cepas de* ***P. brasiliensis.***

INTRODUCTION

Paracoccidioides brasiliensis is a thermally dimorphic pathogenic fungus, which is the responsible agent for paracoccidioidomycosis, a deep systemic mycosis occurring in Latin America (8, 19, 24). It develops into a yeast phase at 37° C, while at 23° C grows as a mycelial (6, 7, 8, 22, 28). The temperature induced a differentiation process readily reversible and cell wall polysaccharide composition between the cell types (9, 10). The yeast cell wall polysaccharide is mainly composed of a high content of alpha-1, 3-glucan (95%) and a small amount of alkali-insoluble beta-1, 3-glucan (5%). The opposite is observed for the mycelial phase and the content of chitin is about the same to both dimorphic states (6, 29). The protein content of the ***P. brasiliensis*** cell wall, represents 24-41% and 18-27% of the mycelial and yeast phase, respectively (13, 25).

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This paper reports methods for the solubilization of ***P. brasiliensis*** cell wall proteins by detergents, enzymatic digestion, and their analyses on SDS-PAGE.

MATERIALS AND METHODS

Strains, medium, and growth conditions: *P. brasiliensis* strains Pb 18 and PbHC-PE used in this work were isolated at the Clinical Hospital of the Federal University of Pernambuco, Brazil (PbHC-PE) and obtained from Dr. Aparecida Resende collection, Federal University of Minas Gerais, Brazil. The cells were grown in SABHI (Sabouraud, brain heart infusion) medium, modified by adding 1% of yeast extract and shaked at 200 rpm at 36° C for 10 days.

Extraction and purification of the cell walls: Cells were washed five times with distilled water, twice with 10 mM Tris-HCl buffer (pH 7) containing 1 mM phenyl methyl sulphonyl fluoride (PMSF). Cells were broken with glass beads (425-600 mm) by shaking five times in vortex mixer for 30 s. The cell fragments were collected by centrifugation (1800 g) for 10 min, resuspended in the same buffer and centrifuged once more.

The cells were sonicated 20 KHz for 15 min, in a cold bath, twice, and washed five times with 10 mM Tris-HCl buffer, pH 7, containing 1 mM of PMSF. The walls obtained were purified, the purity was confirmed by the transmission electron microscopy according to the method described by Campos-Takaki (4, 5).

The proteins were solubilized as follows:

Method 1.

The method described by Pastor *et al.* (20) modified as follows:

a) Pre-treatment. Total cells and cell walls were initially pre-treated with 100 mM Tris-HCl, pH 8, containing 5mM DL-dithiotreitol and 5mM EDTA for 30 min at 28° C.

b) SDS treatment. The cells and cell walls were then centrifuged at 1.800 xg for 10 min and treated with 2% SDS prepared in 10 mM Tris-HCl buffer, pH 7, at 100° C for 5 min.

c) Zymolase treatment- The cells and cell walls were again centrifugated at 1.800 xg for 10 min and the residual fraction was treated with 2 mg/ml Zymolase (Sigma Chemical Co.), prepared in 10 mM Tris-HCl buffer, pH 7, for 3h at 28° C. Ammonium sulfate was added to the supernatant of each treatment of the total cells until 60% and 90% of saturation were achieved in order to recover the solubilized proteins.

Method 2.

The method was described by Herrero *et al.* (12) modified as follows: The walls were initially pre-treated with 100 mM Tris-HCl, pH 8, containing 5 mM DL-dithio-treitol and 5 mM EDTA, for 30 min at 28° C. The walls were then centrifuged and treated with zymolase (2 mg/ml in 10 mM Tris-HCl buffer pH 7) for 3h at 36° C.

Method 3.

Modified after Kobayashi *et al.* (14). Cell wall proteins were obtained by zymolase treatment of the ***P. brasiliensis*** cells in osmotical stabilized bufffer. The cells were pre-treated with 100 mM Tris-HCl buffer pH 8, containing 5 mM EDTA and 5 mM DL-dithiotreitol for 30 min at 28° C. Subsequently, the cell walls were washed 4 times with 0, 67 mM potassium phosphate buffer, pH 7, 5, containing 1,5 M sorbitol, then incubated in shaker with zymolase (2mg/ml) in the same buffer for 5h at 36°C. Afterwards, the preparation was centrifuged at 10.000 xg, for 10 min, and the supernatant was kept for protein determination.

The electrophoresis of the proteins obtained according to the three procedures described above was carried out in polyacrylamida gel horizontal flatbed system as described by Laemmli (15).

The protein contents were established according to Lowry *et al.* (16), using bovine serum albumin as standard.

RESULTS

The method proposed by Pastor *et al.* (20), modified in this work (Method 1) showed to be the most efficient to extract proteins from the ***P. brasiliensis,*** as it can be seen in Table 1, 2 and 3. The amount of protein extracted from the strain Pbl8 according to Method 1 showed to be similar in all treatments. On the other hand, the SDS treatment did not present the same performance for the protein solubilization of the PbHC-PE strain. Table 1 also shows that the best protein precipitation was achieved with 90% of ammonium sulfate saturation, except for the SDS step. However, the SDS supernatant (Method 1) proteins from the isolated cell walls of both strains was the best (Table 2).

Seven bands were obtained in the electrophoresis in protein profile of both strains using any of the treatments tested from appear molecular weights 48, 42, 34, 29, 21 and 16 (Figures 1 A_1).

Otherwise, the fractions precipitated with ammonium sulfate 60% and 90% showed similar results to PbHC-PE and Pb 18 (Table 1).

The electrophoretic profiles indicated bands from 66 to 12 kDa (Figure 1 A_2). The isolated cell walls proteins from PbHC-PE and Pb 18 showed bands with 66 kDa, 45 kDa, 41 kDa, 37 kDa 26 kDa, 24 kDa, and 14 kDa, except the 43 kDa band which occurred only on the PbHC-PE strain (Tables 2 and Figure 2 A). In the last methodology applied band with molecular weight similar to 21 kDa was identified (Table 3 and Figure 2 C).

Table 1.
Protein concentration of *P. brasiliensis* yeast cell extracts obtained by Method 1.

TREATMENT	Protein content (%)					
	Supernatant		Precipitated with ammonium sulfate			
	PbHC-PE	Pb18	PbHC-PE 60%	PbHC-PE 90%	Pb18 60%	Pb18 90%
Pre-tratment	41.5	34.5	10.32	22.28	13.38	25.52
SDS	9.5	26.4	0.38	13.86	23.95	9.12
Zymolase	49.0	39.1	8.97	27.18	8.68	18.94

Method 1. Pastor et al. (21) modified.

Table 2.
Protein concentration of *P. brasiliensis* cell wall extracts solubilized by Method 1

TREATMENT	Protein content (%) PbHC-PE	Pb 18
Pre-treatment	2.9	2.5
SDS 2%	3.5	3.5
Zymolase	1.4	1.3

Method 1, Pastor et al. (21)

Table 3.

Protein concentration of *P. brasiliensis* extracts solubilized by Method 2 and 3

Strains	Protein content (%) Method 2		Method 3
	Pre-tratment	Zymolase	PbHC-PE
PbHC-PE	4,2	1,4	1,3
Pb 18	4,0	1,0	1,2

Method 2: Herrero et al. 1987 (12), modified.
Method 3: Kobayashi et al. 1984 (14), modified.

The extraction of proteins of cells walls from the whole cell showed low level of protein and bands with 23 kDa, 38 kDa, 45 kDa, and 48 kDa (Table 3, Figure 2 B and C).

DISCUSSION

Recently, there has been an increase in research of the biochemistry, physiology, and mechanism of pathogenicity with the purpose of elucidating the responses of the infected host (10, 17, 18, 21, 23, 24, 25). The cell wall has been recognized in molecular studies as the first step concerning host-parasite relationships (21, 24, 25, 28).

The literature suggested the cell wall structures and their influence in host parasitism process as follows: proteins, glycoproteins, carbohydrates, and lipids (16, 17, 20, 23). On the other hand, the electrophoresis applied to fungal proteins has received much attention in last years (11, 14, 26, 27). The electrophoresis has now been applied to members of every major of the fungi (27), and the technique is an attemp to understand better the taxonomic relationship of organism (1).

The Pastor *et al.* (20) method, modified utilizing whole cells of *P. brasiliensis* showed proteins than described by Herrero *et al.* (12) method-SDS, which solubilizes less proteins than pre-treatment and zymolase, though. The electrophoretic patterns showed discreet bands in both samples, after ammonium sulfate precipitation.

On intact cells zymolase treatments released low level of proteins and showed four bands in PbHC-PE and three bands in Pb 18. The presence of a band 43 kDa may be transitory at the location before being integrated into extracellular metabolite. This would explain the observation suggested that 43 kDa band is secreted to the medium (2, 3).

The isolated walls showed less proteins after pre-treatment in both samples by the method modified by Herrero *et al.*, (12) method 2. The yeast form of *P.*

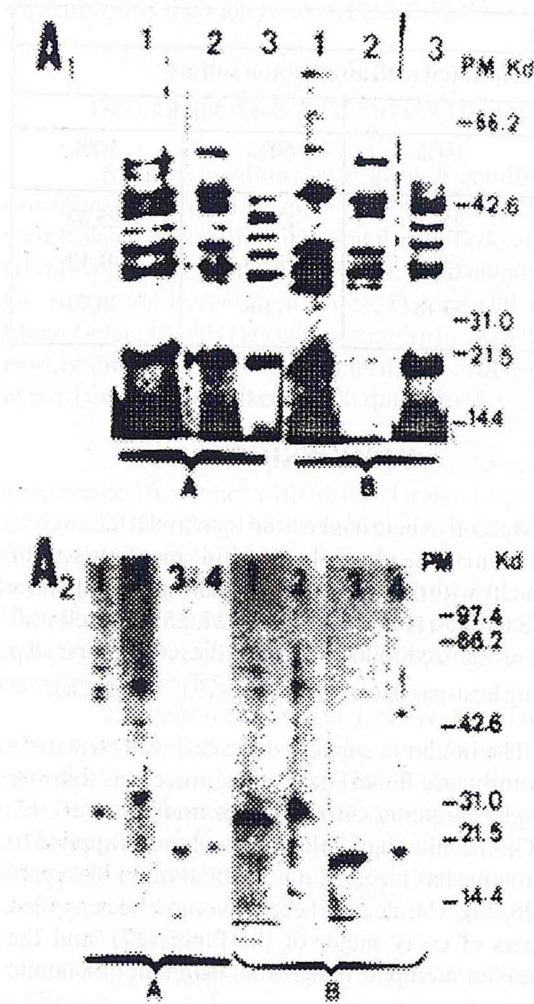

Figure 1: Electrophoresis profile in SDS-PAGE of proteins obtained by Method 1 from cellular extract of ***P. brasiliensis*** PbHC-PE (A) Pb 18 (B) samples. A1: 1 - Fraction obtained after pre-treatment; 2 - Fraction solubilized with 2% SDS; 3 - Fraction solubilized with zymolase. A2: supernatant obtained with 60 and 90% of ammonium sulfate precipitation, 1-2 - Fraction obtained after pre-treatment; 3 - Fraction solubilized by zymolase.

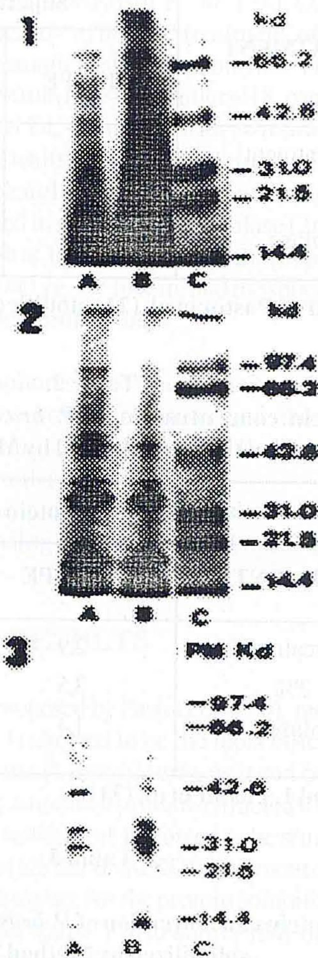

Figure 2. SDS-PAGE profile of proteins solubilized by method 1 (1), Method 2 (2) and Method 3 (3) from cell walls of ***P. brasiliensis*** PbHC-PE (A) and Pb 18 (B), and standards (C).

brasiliensis surface exhibited eight bands in PbHC-PE and six bands in Pb 18. Similar proteins of the 21 kDa were detected in both samples, and the proteins of the 43 kDa occurs in PbHC-PE. This molecule probably corresponds to glycoprotein of the 43 kDa, in its route of secretion, across the cell wall.

The translocation of proteins across the vesicle membranes has been observed in cell-free systems for yeast and higher eukaryotes proteins.

Results described in this paper, therefore, show that the electrophoretic pattern of the proteins in yeast cell wall of *P. brasiliensis* and the gp43 transitory extracellular antigens throw out of the wall

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