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Isolation and characterization of a 30 kD antifungal protein from seeds of *Sorghum bicolor*

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Abstract

An antifungal protein of about 30 000 Da was isolated from seeds of *Sorghum bicolor* L. using chromatographic techniques, including gel filtration, ion exchange, and high-performance liquid chromatography in a reverse-phase column. This protein (termed 30 kD protein) showed a minimal inhibitory concentration of 36 μg/ml for *Candida parapsilosis* and *C. tropicalis*, and 18 μg/ml for *C. albicans*. The 30 kD protein inhibited adherence to the cover glass and formation of a germinative tube of *C. albicans* at concentrations over 300 and 150 μg/ml, respectively. Transmission electron microscopy of yeast forms of *C. albicans* after incubation with 18 μg/ml of the 30 kD protein for 24 h revealed marked ultrastructural changes in the fungus. No toxicity of the 30 kD protein to the culture of Hep2 cells at concentrations equal to or less than 1000 μg/ml was observed.

Keywords: Sorghum seeds; Chromatographic techniques; Antifungal protein; Ultrastructure; Cytotoxicity

1. Introduction

Animals are exposed to millions of potential pathogens daily, through contact, ingestion and inhalation. Their ability to avoid infection depends upon their mechanisms of innate immunity [7]. In recent years, a large number of antimicrobial proteins have been discovered in animals, insects and plants. These molecules, which are either constitutive or inducible, are recognized as important components of the innate defense system [1]. These proteins are termed antimicrobial because they have an unusually broad spectrum of activity. This may include an ability to kill or neutralize Gram-negative and Gram-positive bacteria, fungi (including yeast), parasites, and even enveloped viruses such as HIV and the herpes simplex virus.

The thionins were the first antimicrobial proteins isolated from plants [6]. They have a wide spectrum of activity, but they are toxic to mammalian cells. Most proteins isolated from plants have antifungal activity. There has been a dramatic increase in our knowledge of natural proteins, especially antifungal proteins.

During the last two decades, the incidence of human fungal infections, especially involving immunocompromised patients, has rapidly increased [8]. This results in part from the tremendous advances in medicine that allow us to save patients with neoplastic and immunocompromised diseases who would otherwise not have survived. It is ironic that many of these patients succumb to fungal infections, for which there are few or no drugs available for treatment. The emergence of fungal pathogens resistant to present therapies, the dearth of antifungal compounds and the toxicity of the drugs currently available encourage the use of naturally occurring antifungal proteins and synthetic derivatives with potential promise for clinical use [23].

In the course of screening plants for antifungal proteins, we found that sorghum seeds strongly inhibited the growth of

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species of *Candida*. We describe here the isolation and characterization of an antifungal protein from seeds of *Sorghum bicolor* L.

2. Materials and methods

2.1. Protein extraction

Sorghum seeds were obtained from Embrapa Milho e Sorgo—Sete Lagoas, Minas Gerais, Brazil. The seeds (200 g) were ground in a coffee mill, and the resulting meal was homogenized in 1 l buffer (10 mM sodium dibasic phosphate, 15 mM sodium monobasic phosphate, 100 mM KCl, and 1.5% EDTA) for 2 h at 4 °C. The homogenate was squeezed through cheesecloth and clarified by centrifugation (5 min at 7000 *g*). A protein extract was prepared by the addition of a solution of 50% ethanol/3.3% trifluoroacetic acid (TFA), followed by stirring for 60 min at 4 °C in order to extract the soluble proteins. The preparation was then centrifuged at 30 000 *g* for 60 min at 4 °C and the supernatant lyophilized. The dried material was dissolved in 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid (HEPES) buffer (20 mM), and neutralized with 5 M NaOH before final centrifugation at 30 000 *g* for 30 min at 4 °C; the result was termed the crude extract.

2.2. Protein purification

The crude extract was applied to a Sephadex G-25 (Amershan Pharmacia, São Paulo) column (30 × 1.5 cm) previously equilibrated with 5 mM Tris–HCl, pH 7.2. The column was eluted with the same buffer at a flow rate of 60 ml/h, and the elution was monitored at 220 nm. The fractions with antifungal activity were pooled and rechromatographed in the same column. After rechromatography, the active fractions were pooled and loaded onto a HiTrap Q (Amershan Pharmacia) ion exchange column (5 × 5 ml) equilibrated with 20 mM Tris–HCl (pH 7.4). The column was eluted with a NaCl gradient from 0 to 500 mM in 20 mM Tris–HCl (pH 7.4) buffer at a flow rate of 180 ml/h. The elution was monitored at 220 nm. The fractions with antifungal activity were pooled and used in an HPLC system (model LC10VP, Shimadzu). The reverse-phase column (Microsorb-MV 100-5 C-18, 250 × 4.6) was equilibrated with 0.1% TFA in water. An elution gradient (0–60% acetonitrile in 0.1% TFA in water from 0–95 min) was employed to elute the protein. The elution was monitored at 220 nm. The active fraction was collected and rechromatographed under the same conditions until a single antifungal activity peak appeared during elution.

2.3. Electrophoresis

Tricine/Tris sodium-dodecyl-sulfate (SDS) electrophoretic analysis of the single peak obtained after the third cycle of HPLC was performed using tricine–SDS gel according to the method of Klafki et al. [10]. The gel was stained using a silver-staining technique described by Nesterenko et al. [21].

2.4. Microbiologic assays

2.4.1. Yeast strains and growth conditions

Antifungal activity was tested against *C. albicans* ATCC 577, *C. parapsilosis* ATCC 22015 and *C. tropicalis* ATCC 28707. The fungi were grown and maintained on Sabouraud-dextrose agar (SDA, Merck SA, São Paulo).

2.4.2. Minimal inhibitory concentration (MIC)

The minimal inhibitory concentrations of the crude extract, fractions, 30 kD protein, and reference antibiotic (Sigma Chemical Co., St. Louis, MO) were determined by microdilution techniques in Sabouraud-dextrose broth (SDB, Merck SA) [17] in a microdilution plate (96 wells). The MIC was defined as the lowest concentration of 30 kD protein which resulted in total inhibition of visual growth. Nystatin (Sigma) was used as the control.

2.4.3. Minimal fungicidal concentration (MFC)

MFCs were determined by subculturing 10 µl of culture from each negative well and from the positive control measured as described above. The MFCs reported in this study correspond to the lowest concentration of samples yielding either negative subcultures or only one colony [20].

2.5. Effect on C. albicans growth

Serial 2-fold dilutions of the 30 kD protein (1200 µg/ml) were done in a microdilution plate (96 wells) containing 100 µl of sterile SDB. Next, 10 µl of a *C. albicans* suspension (1×10^5 colony-forming units (CFU)/ml) was added to each well. The microplate was incubated at 37 °C for 30 min to allow sedimentation of the cells. Absorbance was read in a fluorescent plate reader (model FL-600, Bio-Tek) at 590 nm at different times (0, 2, 4, 6, 8, 24 h). The absorbance values were calculated for each time, subtracting the first determination value (0 h).

2.6. Adherence inhibition assays

C. albicans suspensions (1×10^7 CFU/ml), untreated (control) and previously treated for 2 h with different concentrations of the 30 kD protein (600, 300, 150, 75, 37.5, 18.7, and 9.4 µg/ml) were aliquoted (500 µl) onto a 24-well microtiter plate on which round cover glasses were placed. The microtiter plate was incubated at 37 °C for 1 h. The cover glasses were washed with phosphate buffer saline (PBS) and observed with a phase-contrast microscope.

2.7. Germ-tube formation assays

Serial dilutions of the 30 kD protein ranging from 600 to 4.4 µg/ml were done in a 96-well microtiter plate containing 100 µl of sterile fetal bovine serum. Next, 10 µl of a *C. albicans* suspension (1×10^4 CFU/ml) was added to each well. After incubation for 3 h at 37 °C, the microtiter plate was evaluated for formation of germ tubes. The germ-tube test cavities were

directly viewed using an inverted microscope. For quantification, cells were considered germinated if they had a germ tube at least twice the length of the cell.

2.8. Transmission electron microscopy

C. albicans treated with 30 kD protein (subinhibitory concentration) were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. Subsequently, they were washed in cacodylate buffer and postfixed in 1% (w/v) OsO_4 in 0.1 M cacodylate buffer (pH 7.2) with 1% potassium ferrocyanide and 5 mM $CaCl_2$ for 30 min at room temperature. The postfixed cells were dehydrated in acetone and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a Zeiss 900 transmission electron microscope.

2.9. Cytotoxicity assay

The cytotoxicity assay was carried out, with some modifications, as previously described [24,25]. Briefly, confluent Hep2 cell monolayers grown in 96-well cell culture plates were incubated with a tenfold serial dilution of the 30 kD protein—starting with a concentration of 1000 µg/ml—for 48 h at 37 °C and 5% CO_2. At that time, cultures fixed with 10% trichloroacetic acid for 1 h at 4 °C were stained for 30 m with 0.4% sulforhodamine B (SRB) in 1% acetic acid and subsequently washed 5 times with deionized water. Bound SRB was solubilized with a 200 µl 10 mM unbuffered Tris–base solution. Absorbance was read in a 96-well plate reader. The cytotoxicity was expressed as a percentage of the optical density of the control.

3. Results

3.1. Purification of 30 kD protein

The starting material for the isolation of antimicrobial protein from *S. bicolor* was the acid-soluble protein extract obtained from the seeds. Purification was carried out by chromatographic procedures, whereby the eluates were monitored by absorbance determination at 220 nm and assayed for growth inhibition of the fungus *C. albicans*. Upon fractionation by gel filtration on Sephadex G-25, the mixture resolved into four peaks, with the antifungal activity coeluting with the first and second peaks (Fig. 1A). These two peaks were pooled and subjected to anion-exchange chromatography on HiTrap Q (Fig. 1B). The proteins not retained by the column contained all the antifungal activity; they were then further rechromatographed under the same conditions.

The active fractions were purified in the final step by reverse-phase chromatography on a C18 silica column (Fig. 2). The unbound fraction obtained from HiTrap Q yielded a well-resolved peak that coeluted exactly with the antifungal activity. After three cycles of reverse-phase chromatography, the elution of a single peak of antifungal activity was achieved (data not shown). Moreover, denaturing gel electrophoresis of this peak showed a single band with an apparent molecular mass of 30 000 Da (Fig. 2, insert).

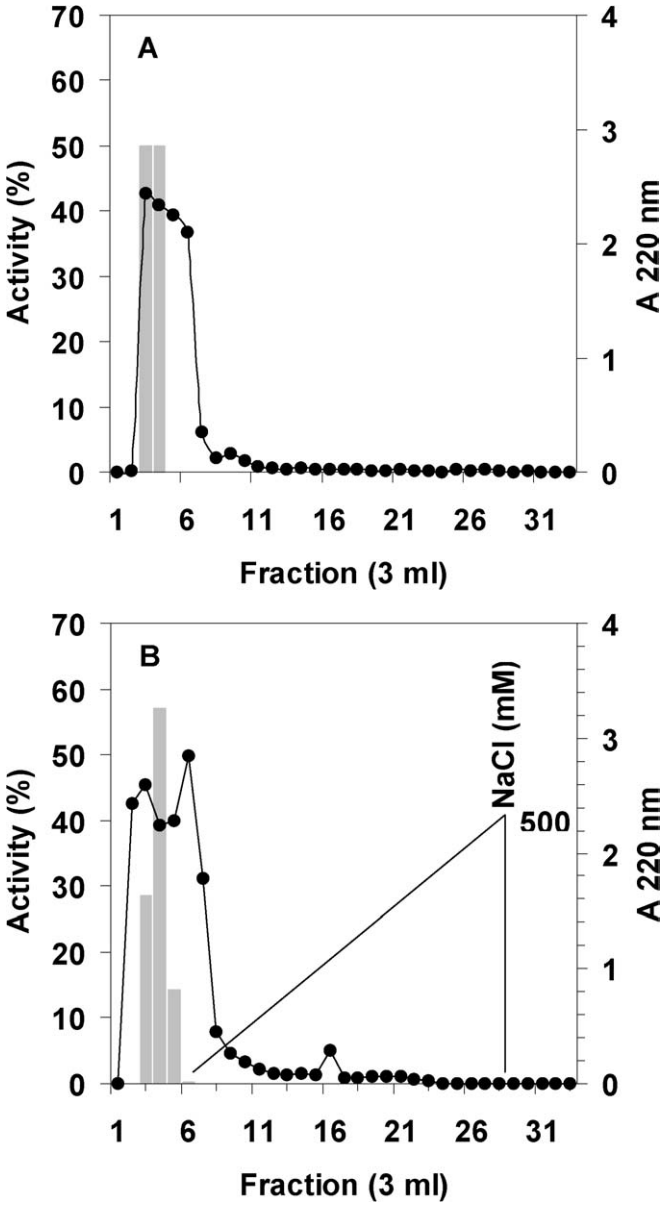

Fig. 1. (A) Gel filtration on Sephadex G-25. The crude extract was applied to a Sephadex G-25 column, previously equilibrated with 5 mM Tris–HCl (pH 7.2), eluted with the same buffer at a flow rate of 60 ml/h, and 3 ml fractions were collected. (B) Ion-exchange chromatography. The active fractions from gel filtration were pooled and loaded onto a HiTrap Q ion-exchange column (5 × 5 ml) equilibrated with 20 mM Tris–HCl (pH 7.4). The column was eluted with a NaCl gradient from 0 to 500 mM in 20 mM Tris–HCl (pH 7.4) buffer at a flow rate of 180 ml/h. The shaded area represents the fractions containing antifungal activity. Data correspond to one representative experiment out of three.

3.2. Minimal inhibitory/fungicidal concentration (MIC/MFC)

The MICs of the crude extract, fractions and the 30 kD protein against *C. albicans* estimated by the microdilution technique are reported in Table 1. It was considered that if the

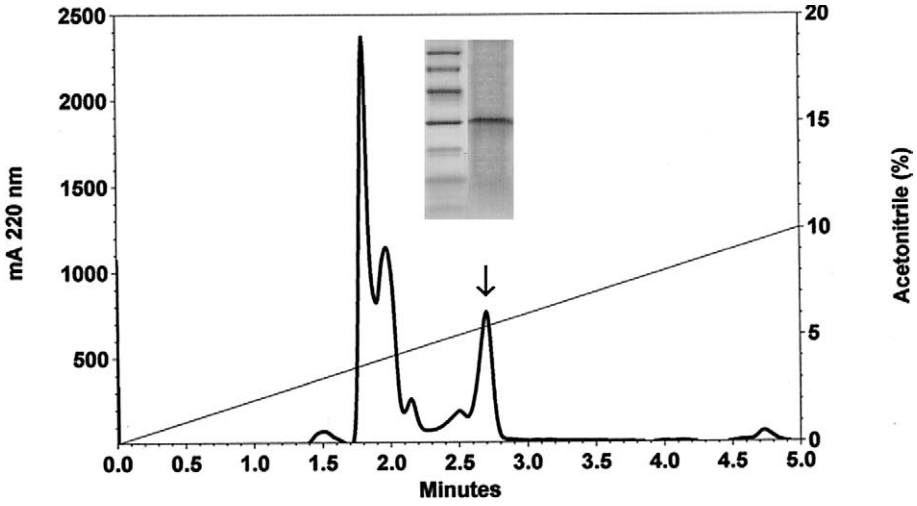

Fig. 2. HPLC on reverse-phase resin Microsorb C-18. The fractions with antifungal activity were pooled and placed in a reverse-phase Microsorb-MV 100-5 C-18 column (250 × 4.6) equilibrated with 0.1% TFA in water. An elution gradient (0–60% acetonitrile in 0.1% TFA in water from 0–95 min) was employed to elute the protein. Three cycles of reverse-phase chromatography of the active fraction (arrow) led to the elution of a single peak containing the antifungal activity (data not shown). (Insert) Sodium-dodecyl-sulfate (SDS) electrophoretic analysis of the single peak obtained after the third cycle of HPLC was performed using tricine–SDS gel as described in Section 2. The standard proteins were phosphorylase b (97 000), albumin (66 000), ovalbumin (45 000), carbonic anhydrase (29 000), trypsin inhibitor (20 000) and α-lactalbumin (14 400). Similar results were obtained in the different analyses.

Table 1
MIC of crude extract, fractions and 30 kD protein obtained from sorghum seeds against *C. albicans*

Material	MIC (μg/ml)
Crude extract	93
Fraction obtained after gel-filtration chromatography	56
Fraction obtained after ion-exchange chromatography	37
30 kD protein	18

The results are reported as the average of three experiments.

extract, fractions, or isolated protein displayed a MIC less than 100 μg/ml, the antifungal activity was strong; from 100 to 500 μg/ml the antifungal activity was moderate; from 500 to 1000 μg/ml the antifungal activity was weak; over 1000 μg/ml they were considered inactive. The 30 kD protein showed strong activity against *C. parapsilosis, C. tropicalis,* and *C. albicans* with MICs of 36, 18 and 18 μg/ml, respectively. No fungicidal effect was observed with any concentration of the crude extract, the fractions or the 30 kD protein (MFC > 1000). The MICs found for nystatin against *C. albicans* were similar to the values for in vitro susceptibility reported in the literature [11].

3.3. *Effect of the 30 kD protein on* C. albicans *growth*

The effect of 30 kD protein on the growth of *C. albicans* is illustrated in Fig. 3. The results indicate that concentrations of 18 μg/ml or higher of 30 kD protein have an inhibitory effect on the growth of this organism. A similar profile of inhibition was achieved with 2 μg/ml of nystatin. Inhibition of growth was not affected by the increasing the 30 kD protein concentration to 600 μg/ml.

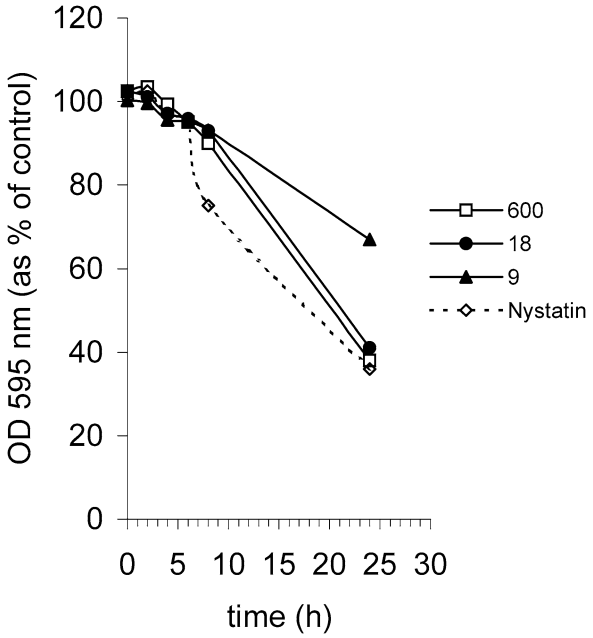

Fig. 3. Effect of different concentrations of the 30 kD protein (in micrograms per milliliter) and nystatin (2 μg/ml) on growth of *C. albicans*. The inhibition is expressed as the relationship between the optical density at 590 nm of each sample and the control (without the protein or nystatin). Data are average of triplicate measurements and correspond to one representative experiment out of three.

3.4. *Effect of the 30 kD protein on the adherence of* C. albicans

Differences in the intensity of adhesion of *C. albicans* to the cover glasses are shown for the control (Fig. 4A) and for the 30 kD protein (300 μg/ml) (Fig. 4B). Moderate (⩽50%) and strong (⩾80%) adherence inhibition of *C. albicans* to the cover glass occurred when the yeasts were treated with 150 and

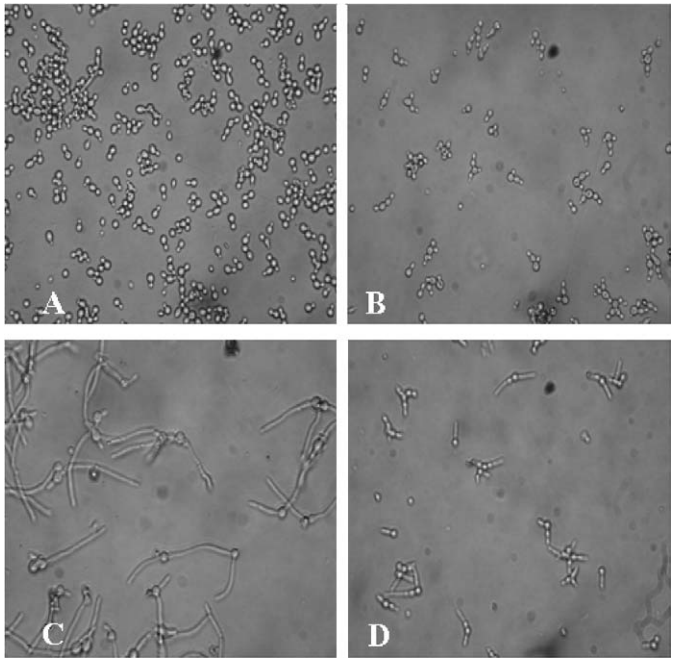

Fig. 4. (A and B) Adherence inhibition assay of *C. albicans* onto the cover glass. (C and D) Effect on *C. albicans* germ-tube formation. (A and C) Untreated yeasts. (B and D) Yeasts treated with the 30 kD protein in concentrations equal to or higher than 300 and 150 μg/ml, respectively. 40x. Similar results were obtained in the different analyses.

300 μg/ml of the 30 kD protein, respectively. Similar results were observed when the adherence of *C. albicans* to the cover glass was assayed with nystatin at 2 μg ml (data not shown).

3.5. Effect of the 30 kD protein on germ-tube formation

The cells were analyzed microscopically in order to determine the minimum concentration of the 30 kD protein which inhibits germ-tube formation of *C. albicans* (Figs. 4C, 4D). The minimum concentration of the 30 kD protein required to completely inhibit (100%) germ-tube formation of *C. albicans* after 2 h incubation was 150 μg/ml.

3.6. Effect of the 30 kD protein on the yeast ultrastructure

In order to investigate the effect of the 30 kD protein on the ultrastructure of *C. albicans* yeast forms, fungal samples were examined by transmission electron microscopy. Analyses of the images showed that control samples had fungal cells surrounded by a specific cell wall composed of dense outer and inner layers separated by a low-density space (Figs. 5A, 5B). Treated cells showed an undefined and thickened cell wall, and a change in the space between the cell wall and the plasma membrane (Figs. 5C, 5E, 5F). The images showed completely deformed cells, with irregular budding sites (Fig. 5D).

3.7. Cytotoxicity assay

The 30 kD protein was also evaluated for its potential toxic effects on human cells. After 48 h of incubation with 1000 and

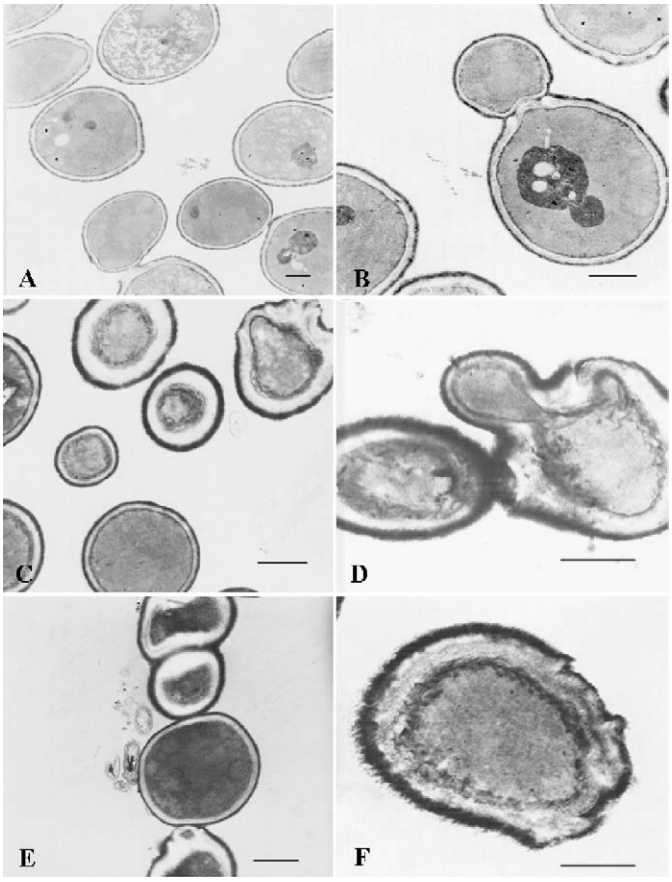

Fig. 5. Transmission electron microscopy of *C. albicans* yeast forms. Normal ultrastructure of untreated yeasts: delimited cell wall composed of dense outer and inner layers separated by a low-density space, and cell with a normal budding profile (A and B) in contrast to the altered morphology of treated (18 μg/ml of the 30 kD protein for 24 h at 37 °C) yeasts: deformed cells, thickening of the cell wall, alteration of the space between the cell wall and the plasma membrane (C, E, F), and irregular budding profile (D). Bars = 1 μm. Similar results were obtained in the different analyses.

100 μg/ml of the protein, 83 and 98% of the cells were still viable, respectively. These results indicate that the protein is selectively toxic to the fungal cells.

4. Discussion

The present paper describes the isolation and characterization of an antifungal protein from seeds of *S. bicolor*. This protein, called 30 kD protein, showed potent antifungal activity against *C. albicans.* Nakamura et al. [19], using assay conditions similar to ours, found MIC values for an essential oil of *Ocimum gratissimum* of about 750 μg/ml on *C. albicans,* whereas Lehrer et al. [16] showed that human α-defensins at 50 μg/ml were lethal to *C. albicans.* The inhibitory activity of the 30 kD protein reported here is comparable to the fluconazole activity described by van Etten et al. [27]. According to these authors, fluconazole (⩾102 μg/ml) did not result in death of *C. albicans*, but simply inhibited fungal growth. Interestingly, a less concentrated inoculum did not result in yeast death.

In order to determine potential action sites in fungal cells which might explain the inhibitory activity of the 30 kD protein,

we studied the effect of the isolated protein on the adherence, germ-tube formation and ultrastructure of the yeast. It has long been recognized that the adherence of the fungi to cells, to the extracellular matrix or to inanimate objects such as plastic catheter surfaces is believed to be important in the pathogenesis of all forms of disease involving *C. albicans,* since this process precedes all infectious diseases [11]. Another important step in the onset of candidiasis is germ-tube formation; the development of the hyphal form has been considered as a potential factor in the infection [5]. Therefore, the development of new molecules that interfere with the adherence process or with germ-tube formation may be very helpful as auxiliary therapy, since fungal infections, particularly in immunocompromised hosts, have posed a major therapeutic challenge. In addition, *Candida* species are the most frequent causes of systemic mycosis in our era [2].

Although many studies have focused on showing the antifungal activity of plant derivatives, few have demonstrated their effects on the morphology and ultrastructure of the fungi. The data obtained in this study show that *C. albicans* underwent remarkable ultrastructural alterations which were visible by electron microscopy, when treated with the 30 kD protein. The ultrastructural changes included thickening of the cell wall, an alteration of the space between the cell wall and the plasma membrane, deformed cells, reduction in cell size and cells with irregular budding sites. Comparison of these results with those induced by imidazole derivatives in *Candida* reveals some similarities. Thickening of the cell wall was observed in yeasts treated with saperconazole and low doses of miconazole and clotrimazole [18]. Treatment with eucalyptol induced similar morphological alterations in species of *Candida* [26].

The mechanism of action is not well understood; however, the deleterious effect of the 30 kD protein on the cell wall of the fungus may be the main reason for the inhibition of germ-tube formation and adherence to the cover glass of treated cells, because the integrity of the cell wall is necessary for cell division and to allow the expression of molecules involved in the adherence process.

There exists great diversity among antifungal proteins, with large variations in molecular mass, N-terminal sequence and antifungal specificity. Thaumatin-like proteins [4,29,31], chitinases [28,32], ribosome-inactivating proteins [14], cyclophilin-like proteins [30] and miraculin-like protein [33] are members of the family of antifungal proteins. In addition, there exist antifungal proteins with other structures such as cysteine protease inhibitor [9] and peptides [15].

Foregoing studies have reported three proteins of 18, 26, and 30 kD isolated from sorghum endosperm, which affected the hyphal growth of *Fusarium moniliforme* [12,13]. The 18 kD antifungal protein removes cell wall polysaccharides, while the 26 and 30 kD protein fraction caused leakage of cytoplamatic contents. More recently, Seetharaman and co-workers [22] identified water-soluble proteins belonging to the permeatin, chitinase, glucanase, and ribosome inactivating protein groups.

Our present studies show that the purified 30 kD protein isolated from sorghum seeds differs clearly from the three proteins reported by Kumari et al. [12,13]. The three antifungal proteins identified in sorghum endosperm act on the filamentous fungus *Fusarium moniliforme*. Our present observations show that the 30 kD protein obtained from sorghum seeds has potent activity against yeast species belonging to the genus *Candida*, but is inactive toward filamentous fungi. On the other hand, 30 kD protein was also inactive toward the 4-methylumbelliferyl derivatives of *N*-acetylglucosamine dimmers and trimers that have been proposed as substrates for the characterization of chitinase (data not shown). Further studies are needed to determine the nature and the specific functions of these proteins.

The 30 kD protein obtained from sorghum seeds could be an alternative to fungal control, since no cytotoxicity was observed when the human cell culture was incubated for 48 h with 1000 μg/ml of the protein. This concentration is 50-fold the minimum necessary to inhibit growth of *C. albicans.* The difficulty of establishing an effective therapy for serious candidiasis is another aspect which encourages the use of the isolated protein described in this paper as an alternative for fungi control. Many fungal pathogens are resistant to current therapies, and the presently available antifungal compounds act on targets also found in mammalian cells [3], which may result in toxicity or an adverse drug interaction. It is therefore imperative to find antifungal compounds that inhibit growth of naturally or acquired resistant fungi without being toxic to host cells.

Acknowledgements

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